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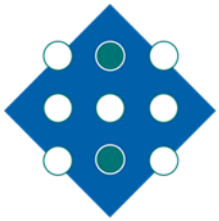

Institut d'Investigació Sanitària Illes Balears

IdISBa

DOCTORAL THESIS

2018

Clonal epidemiology and antimicrobial resistance in *Pseudomonas aeruginosa* chronic respiratory infections: interpatient transmission and resistome evolution of an international cystic fibrosis clone.

Carla López Causapé

DOCTORAL THESIS

2018

Doctoral Degree in Environmental and Biomedical Microbiology

Clonal epidemiology and antimicrobial resistance in *Pseudomonas aeruginosa* chronic respiratory infections: interpatient transmission and resistome evolution of an international cystic fibrosis clone.

Thesis Supervisor and Tutor: Dr Antonio Oliver Palomo

PhD Candidate: Carla López Causapé

To obtain de Degree of Doctor by the Universitat de les Illes Balears

Dr. Antonio Oliver Palomo, Servicio de Microbiología y Unidad de Investigación, Hospital Universitario Son Espases.

DECLARO:

Que la tesis doctoral que lleva por título "Clonal epidemiology and antimicrobial resistance in *Pseudomonas aeruginosa* chronic respiratory infections: interpatient transmission and resistome evolution of an international cystic fibrosis clone", presentada por Carla López Causapé para la obtención del título de doctor, ha sido dirigida bajo mi supervisión y cumple con los requisitos necesarios para optar al título de Doctor Internacional.

Y para que quede constancia de ello firmo el presente documento.

Dr. Antonio Oliver Palomo

En Palma de Mallorca, septiembre de 2018

LIST OF PUBLICATIONS DERIVED FROM THIS THESIS

López-Causapé C, Cabot G, Del Barrio-Tofiño E, Oliver A. The versatile mutational resistome of *Pseudomonas aeruginosa*. *Front Microbiol*. 2018; 9:685.

López-Causapé C, Rubio R, Cabot G, Oliver A. Evolution of *the Pseudomonas aeruginosa* aminoglycoside mutational resistome *in vitro* and in the cystic fibrosis setting. *Antimicrob Agents Chemother*. 2018; 62(4). pii: e02583-17.

López-Causapé C, Oliver A. Insights into the evolution of the mutational resistome of *Pseudomonas aeruginosa* in cystic fibrosis. *Future Microbiol*. 2017; 12:1445-1448.

López-Causapé C, de Dios-Caballero J, Cobo M, Escribano A, Asensio Ó, Oliver A, Del Campo R, Cantón R. Antibiotic resistance and population structure of cystic fibrosis *Pseudomonas aeruginosa* isolates from a Spanish multi-centre study. *Int J Antimicrob Agents*. 2017; 50(3):334-341.

López-Causapé C, Sommer LM, Cabot G, Rubio R, Ocampo-Sosa AA, Johansen HK, Figuerola J, Cantón R, Kidd TJ, Molin S, Oliver A. Evolution of the *Pseudomonas aeruginosa* mutational resistome in an international Cystic Fibrosis clone. *Sci Rep*. 2017; 7(1):5555.

López-Causapé C, Rojo-Molinero E, Macià MD, Oliver A. The problems of antibiotic resistance in cystic fibrosis and solutions. *Expert Rev Respir Med*. 2015; 9(1):73-88.

López-Causapé C, Rojo-Molinero E, Mulet X, Cabot G, Moyà B, Figuerola J, Togores B, Pérez JL, Oliver A. Clonal dissemination, emergence of mutator lineages and antibiotic resistance evolution in *Pseudomonas aeruginosa* cystic fibrosis chronic lung infection. *PLoS One*. 2013; 8(8):e71001.

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I. SUMMARY

Chronic respiratory infection (CRI) by *Pseudomonas aeruginosa* is the main cause of morbidity and mortality in cystic fibrosis (CF). During the progression from early infection to chronic non-eradicable colonization *P. aeruginosa* undergoes a complex evolutionary adaptation and diversification process which eventually leads to a mixed and persistent infecting population in which multidrug resistant variants frequently rise compromising the selection of appropriate antibiotic therapies.

In this work the interplay between three key microbiological aspects of these infections was investigated: the occurrence of transmissible and persistent strains, the emergence of variants with enhanced mutation rates (mutators) and the evolution of resistance to antibiotics. Clonal epidemiology, antibiotic susceptibility profiles, contribution of *P. aeruginosa* classical resistance mechanisms and the role of mutator variants were investigated in two large collections of CF *P. aeruginosa* isolates from the Balearic Islands and Spain. As well, whole genome sequencing (WGS) was used to decipher the phylogeny, interpatient dissemination, within-host evolution, WGS mutator genotypes (*mutome*) and resistome of widespread *P. aeruginosa* clonal complex 274 (CC274), in isolates from two highly-distant countries, Australia and Spain, covering an 18-year period. Finally, due to the relevance of aminoglycosides in the management of CF-CRI, the dynamics of *P. aeruginosa* resistance development to aminoglycosides was also studied *in vitro* by WGS approaches.

Despite discrepancies between molecular genotyping methods, a high degree of genetic diversity was documented among CF isolates from the Balearic Islands and Spain with scarce representation of CF epidemic strains. However, for the first time in Spain, we documented a superinfection with the multidrug resistant Liverpool Epidemic Strain (LES) in a chronically colonized patient. As well, *P. aeruginosa* CC274, previously detected in several CF individuals from Austria, Australia and France, was detected in 5 unrelated chronically colonized patients from the Balearic Islands and, therefore, this clone-type should be added to the growing list of CF epidemic clones. Subsequent analysis of the whole genomes sequences of *P. aeruginosa* isolates from the CC274 *P. aeruginosa* collection provides evidence of interpatient dissemination of mutator sublineages and denotes their potential for unexpected short-term sequence type (ST) evolution and antibiotic resistance spread, illustrating the complexity of *P. aeruginosa* population biology in CF. As well, epidemiological studies demonstrated the coexistence of two divergent lineages but without evident geographical barrier.

Antibiotic resistance significantly accumulated overtime accompanied by hypersusceptibility to certain antibiotics such as aztreonam, which can be explained in terms of collateral susceptibility. Correlation between phenotypes and WGS genotypes of clonal isolates from the CC274 collection allowed us to define the mutational resistome of CF *P. aeruginosa*

which extends beyond the classical mutational resistance mechanisms. Among the new chromosomic resistance determinants encountered, mutations within the penicillin-binding-protein 3 (PBP3), shaping up β-lactam resistance, are noteworthy as well as mutations within the *fusA1* gene, coding for elongation factor G, which along with MexXY overexpresion contribute to high-level aminoglycoside resistance. Strikingly, we encountered that MexXY overexpression is dispensable for *in vitro* resistance development to aminoglycosides which suggests an evolutionary advantage of its overexpression in the CF respiratory tract.

Altogether this work demonstrates that clonal epidemiology and antibiotic resistance evolution in the CF setting results from the complex interplay among mutation-driven resistance mechanisms, within host diversification and interpatient transmission of epidemic strains.

II. RESUMEN EN LENGUA CASTELLANA

La infección respiratoria crónica por *P. aeruginosa* es la principal causa de morbilidad y mortalidad en pacientes con fibrosis quística (FQ). Durante la progresión desde la infección temprana a la colonización crónica, *P. aeruginosa* experimenta un complejo proceso adaptativo y de diversificación que resulta en una población heterogénea y persistente en la que la aparición de resistencias a los antibióticos comprometen la selección de terapias apropiadas.

En este trabajo se investigó la interacción entre tres aspectos microbiológicos clave de estas infecciones: la presencia de cepas transmisibles y persistentes, la aparición de variantes con tasas de mutación incrementadas y la evolución de la resistencia a los antibióticos. La epidemiología clonal, los perfiles de sensibilidad antibiótica, la contribución de los mecanismos clásicos de resistencia de *P. aeruginosa* y el papel de las variantes hipermutadoras se estudiaron en dos grandes colecciones de aislados procedentes de pacientes con fibrosis quística de las Islas Baleares y España. Asimismo, mediante secuenciación de genoma completo, se determinó la filogenia, diseminación interpaciente, evolución intrapaciente, genotipo hipermutador y resistoma de una colección de aislados clonales pertenecientes al complejo clonal 274 (CC274), proviniendo dichos aislados de dos países muy distantes, Australia y España, y cubriendo un período de 18 años. Finalmente, dada la relevancia de los aminoglucósidos en el manejo de estos pacientes, se estudió la dinámica del desarrollo de resistencia a aminoglucósidos *in vitro* mediante secuenciación de genoma completo.

A pesar de encontrarse discrepancias entre los métodos de genotipado molecular, se documentó un alto grado de diversidad genética en las colecciones de las Islas Baleares y España, siendo escasa la representación de cepas epidémicas. No obstante, por primera vez en España, se documentó un caso de sobreinfección con el clon epidémico multirresistente de Liverpool. Además, en 5 pacientes de Baleares, crónicamente colonizados y sin aparente relación epidemiológica, se detectó el CC274. Puesto que este complejo clonal también ha sido detectado en pacientes de países como Austria, Australia y Francia, éste debería incluirse en la creciente lista de cepas epidémicas. El análisis posterior de las secuencias de genoma completo de los aislados del CC274 evidenció la diseminación interpaciente de un sublinaje hipermutador, denotando además el potencial de estas variantes para la inesperada evolución a corto plazo del secuenciotipo y la rápida diseminación de resistencias. Además, los estudios epidemiológicos demostraron la coexistencia de dos linajes divergentes, no evidenciándose barrera geográfica.

Asimismo se documentó una tendencia generalizada a la acumulación de resistencias a los antibióticos en el tiempo, acompañada de hipersensibilidad a ciertos antibióticos como aztreonam, lo cual se puede explicar en términos de sensibilidad colateral. La correlación

entre los fenotipos y genotipos determinados mediante secuenciación del genoma completo de los aislados pertenecientes al CC274 nos permitió definir el resistoma mutacional de *P. aeruginosa* en la FQ, el cual se extiende más allá de los mecanismos mutacionales clásicos. Entre los nuevos determinantes de resistencia cromosómica encontrados caben destacar tanto las mutaciones en la proteína fijadora de penicilina PBP3, que confieren resistencia a betalactámicos, como las mutaciones en *fusA1*, que codifica para el factor de elongación G, y que junto con la hiperexpresión de MexXY contribuyen a la resistencia de alto nivel a aminoglucósidos. Paradójicamente, encontramos que la hiperexpresión de MexXY es prescindible para el desarrollo de resistencia *in vitro* a aminoglucósidos, lo que sugiere que dicha hiperexpresión confiere una ventaja evolutiva *in vivo.*

En conjunto, este trabajo demuestra que, en la FQ, la epidemiología clonal y la evolución de la resistencia a los antibióticos son el resultado de una compleja interacción entre los mecanismos de resistencia mutacionales, la diversificación de la población infectante y la transmisión interpaciente de cepas epidémicas.

III. RESUM EN LLENGUA CATALANA

La infecció respiratòria crònica per *P. aeruginosa* és la principal causa de morbiditat i mortalitat en els pacients amb fibrosi quística (FQ). Durant la progressió des de la infecció primerenca a la colonització crònica, *P. aeruginosa* experimenta un complexe procés adaptatiu i de diversificació que resulta en una població heterogènia i persistent en la qual l'aparició de variants resistents a múltiples antibiòtics comprometen la selecció de teràpies antibiòtiques apropiades.

En aquest treball es va investigar la interacció entre tres aspectes microbiològics clau: la presència de soques transmissibles i persistents, l'aparició de variants amb taxes de mutació incrementades i l'evolució de la resistència als antibiòtics. L'epidemiologia clonal, els perfils de sensibilitat antibiòtica, la contribució dels mecanismes clàssics de resistència i el paper de les variants hipermutadores es van estudiar en dos grans col·leccions d'aïllats procedents de pacients amb FQ de les Illes Balears i Espanya. Així mateix, mitjançant seqüenciació del genoma complet, es va determinar la filogènia, disseminació interpacient, evolució intrapacient, genotip hipermutador i resistoma d'una col·lecció d'aïllats pertanyents al complexe clonal 274 (CC274), provenint de dos països molt distants, Austràlia i Espanya, i cobrint un període de 18 anys. Finalment, donada la rellevància dels aminoglicòsids en el maneig d'aquests pacients, es va estudiar la dinàmica del desenvolupament de resistència a aminoglicòsids *in vitro* mitjançant seqüenciació de genoma complet.

Tot i trobar discrepàncies entre els mètodes de genotipat molecular, es va documentar un alt grau de diversitat genètica en les col·leccions de les Illes Balears i Espanya, sent escassa la representació de soques epidèmiques. No obstant això, per primera vegada a Espanya, es va documentar un cas de sobreinfecció amb el clon epidèmic multiresistent de Liverpool. A més, en 5 pacients de les Illes Balears, crònicament colonitzats i sense aparent relació epidemiològica, es va detectar el CC274. Ja que aquest complexe clonal també ha estat detectat en països com Àustria, Austràlia i França, aquest clon hauria d'incloure a la creixent llista de soques epidèmiques. L'anàlisi posterior de les seqüències de genoma complet dels aïllats pertanyents al CC274, va evidenciar la disseminació interpaciente d'un subllinatge hipermutador, denotant a més el potencial d'aquestes variants per a la inesperada evolució a curt termini del sequenciotip i per a la ràpida disseminació de la resistència antibiòtica. A més, els estudis epidemiològics van demostrar la coexistència de dos llinatges divergents, no existint barrera geogràfica.

Així mateix es va evidenciar una tendència generalitzada a l'acumulació de resistències en el temps, acompanyada d'hipersensibilitat a certs antibiòtics com l'aztreonam, la qual cosa es pot explicar en termes de sensibilitat col·lateral. La correlació entre els fenotips i genotips determinats mitjançant seqüenciació del genoma complet dels aïllats pertanyents al CC274 ens va permetre definir el resistoma mutacional de *P. aeruginosa* en la FQ, el qual s'estén

més enllà dels mecanismes de resistència mutacionals clàssics. Entre els nous determinants de resistència cromosòmica trobats cal destacar tant les mutacions en la proteïna fixadora de penicil·lina PBP3, que confereixen resistència a betalactàmics, així com les mutacions en *fusA1*, que codifica per al factor d'elongació G, i que juntament amb la hiperexpressió de MexXY contribueixen a la resistència d'alt nivell a aminoglucòsids. Paradoxalment, vam trobar a més que la hiperexpressió de MexXY és prescindible per al desenvolupament de resistència *in vitro* a aminoglucòsids, el que suggereix que aquesta hiperexpressió suposa un avantatge evolutiu *in vivo*.

En conjunt, aquest treball demostra que l'epidemiologia clonal i l'evolució de la resistència als antibiòtics en el context de la FQ són el resultat d'una complexa interacció entre els mecanismes de resistència mutacionals, la diversificació de la població infectant i la transmissió interpaciente de ceps epidèmiques.

IV. LIST OF ABBREVIATIONS

AK: amikacin

AMG: aminoglycosides

AT: aztreonam

CC: clonal complex

CF: cystic fibrosis

CFTR: cystic fibrosis transmembrane conductance regulator

CFU: colony forming unit

CI: ciprofloxacin

CO: colistin

COPD: chronic obstructive pulmonary disease

CRI: chronic respiratory infection

DGCs: diguanylate cyclases

DNA: Deoxyribonucleic acid

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FO: fosfomycin

FQ: fluoroquinolones

GM: gentamycin

GlcNAc: N-acetil-glucosamine

ID: identification

IP: imipenem

LB: Luria-Bertani

LE: levofloxacin

LES: Liverpool Epidemic Strain

LPS: lipopolysaccharide

MDR: multidrug resistant

MH: Mueller Hinton

MHA: Mueller Hinton agar

MHB: Mueller Hinton broth

MIC: Minimun Inhibitory Concentrations

min: minutes

MLST: Multilocus Sequence Typing

MMR: Mismatch Repair

MP: meropenem

MST: Minimum Spanning Tree

mRNA: messenger ribonucleic acid

MurNAc: N-acetyl-muramic-acid

nm: nanometre

List of abbreviations

OMP: outer membrane protein
PBP: penicillin-binding protein
PCR: polymerase chain reaction
PDR: pandrug resistant
PFGE: Pulsed Field Gel Electrophoresis
PGN: peptidoglycan
PM: cefepime
PMN: polymorphonuclear phagocyte
PPT: piperacillin/tazobactam
QS: Quorum-sensing
qRT-PCR: real-time quantitative Reverse Transcription-PCR
RIF: rifampicin
RNA: ribonucleic acid
RND: resistance-nodulation-division
ROS: reactive oxygen species
SCV: small colony variants
sec: seconds
SNP: single nucleotide polymorphism
ST: sequence type
TI: ticarcillin
TM: tobramycin
TOL/TAZ: ceftolozane/tazobactam
TZ: ceftazidime
TZ/AVI: ceftazidime/avibactam
WGS: whole genome sequencing
WT: Wild-type
XDR: extensively drug resistant

1. INTRODUCTION

La levedad y el peso

1.1. *Pseudomonas aeruginosa* GENERAL MICROBIOLOGICAL ASPECTS

Pseudomonas aeruginosa is the major pathogenic species in the family *Pseudomonadaceae*. It is a non-spore-forming, Gram-negative straight or slightly curved rod with a length ranging from 1 to 3 μm and a width of 0.5 to 1.0 μm. *P. aeruginosa* produces many cell surface fimbriae or pili and a polar flagellum which confers its motility.

In the laboratory, *P. aeruginosa* is able to grow on a wide variety of media, ranging from minimal to complex. Most isolates are easily recognizable on primary isolation media on the basis of colonial morphology, a grape-like odor and production of hydrosoluble pigments such as pyocyanin (blue), pyorubin (red), pyomelanin (brown-black) and/or pyoverdin (yellow-green or yellow-brown). In fact, the name *aeruginosa* (from Latin *aerūgō* "copper rust or verdigris" plus -*ōsus*, added to a noun to form an adjective indicating an abundance of that noun) stems from the greenish-blue color of bacterial colonies when pyocyanin and pyoverdin pigments are co-produced. Colonies are usually flat and spreading and have a serrated edge, but other morphologies can exist, including, among others, the mucoid or the small colony variants (SCV, section 1.5.).

P. aeruginosa can metabolize a large array of carbon sources. It does not ferment carbohydrates but produces acid from sugars such as glucose, fructose and xylose, but not from lactose or sucrose. Additionally, it is strongly positive in indophenol oxidase, catalase and arginine tests. *P. aeruginosa* grows best aerobically but can also be grown anaerobically in the presence of nitrate as a terminal electron acceptor. As well, although optimal temperature for growing is 37º C, it can also grow at 42ºC, characteristic that differentiates this species from other rarely pathogenic fluorescent *Pseudomonas* such as *P. fluorescens* or *P. putida*.

1.2. NATURAL HABITATS AND CLINICAL SIGNIFICANCE

P. aeruginosa possesses a complex and large genome (5-7 Mb), including a large proportion of regulatory genes (>8%). These features, along with its metabolic versatility, the large number of genes involved in transport and efflux and the documented genome plasticity of individual strains, explain the ability of this opportunistic pathogen to adapt, survive and persist in virtually any environment.

Related to the persistence of *P. aeruginosa* in nature it should be highlighted its ability to form polysaccharide-encased surface-attached communities, known as biofilms (section 1.6.). Moreover, its genome encodes a remarkable repertoire of virulence determinants and outstanding intrinsic antibiotic resistance machinery that confers *P. aeruginosa* an impressive capacity to cause opportunistic infections in humans and evade the activity of antimicrobial treatments [Breidenstein EB *et al*, 2011; Gellatly SL & Hancock RE, 2013; Silby MW *et al*, 2011].

Within the hospital setting, *P. aeruginosa* can be isolated from moist inanimate environments including water in sinks and drains, toilets, showers and hospital equipment that come in contact with water such as mops, respiratory therapy equipment, antiseptics, cleaning solutions, etc. [Pier GB & Ramphal R, 2005]. On the community, its reservoirs include swimming pools, whirlpools and hot tubes, home humidifiers, contact lens solutions, vegetables and soil, among others [Pier GB & Ramphal R, 2005]. Additionally, although not considered part of the resident human microbiota, gastrointestinal, upper respiratory tract or cutaneous colonization may occur, especially in hospitalized and immunocompromised patients [Pier GB & Ramphal R, 2005], and can be an important preliminary step before infection [Taconneli *et al*, 2009]. Representative colonization rates for specific sites in non-hospitalized humans are 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat, and 2.6 to 24% for fecal samples; rates that may exceed 50% during hospitalization, especially among patients who have experienced trauma or a breach in cutaneous or mucosal barriers by mechanical ventilation, tracheostomy, catheters, surgery, or severe burns [Lister *et al*, 2009]. As well, disruption in the normal microbial flora as a result of antimicrobial therapy has also been shown to increase colonization by *P. aeruginosa* [Lister *et al*, 2009].

Thus, *P. aeruginosa* is a ubiquitous microorganism that can be implicated in both, hospital and community acquired infections. Indeed, it is recognized as one of the most frequent and severe causes of acute nosocomial infections, accounting for about 10% of all such infections in most European Union hospitals [de Bentzmann *et al*, 2011] and particularly affecting patients with compromised immune systems (especially neutropenic) or those who are admitted to the Intensive Care Units. *P. aeruginosa* is the number one pathogen causing ventilator associated pneumonia and burn wound infections, being both entities associated

with a very high (>30%) mortality rate [Vincent JL, 2003]. Likewise, it is the most frequent and severe driver of CRI in patients suffering from CF or other chronic underlying respiratory diseases such as bronchiectasis or chronic obstructive pulmonary disease (COPD) [Oliver A *et al*, 2009]. As well, this opportunistic pathogen may also be implicated in bloodstream infections, septic shocks, urinary tract or gastrointestinal infections, keratitis, endophthalmitis, otitis, enterocolitis, osteomyelitis, meningitis or folliculitis, among other types of infections [Pier GB & Ramphal R, 2005].

1.3. INTRINSIC ANTIBIOTIC RESISTANCE

As abovementioned, *P. aeruginosa* is genetically equipped with outstanding intrinsic antibiotic resistance machinery [Breidenstein EB *et al*, 2011; Lister *et al*, 2009; Poole, 2011]. Indeed, *P. aeruginosa* wild-type (WT) susceptible strains exhibit a basal reduced susceptibility to a wide variety of antibiotic classes, including β-lactams, aminoglycosides and fluoroquinolones. Specifically, it is naturally resistant to many β-lactams compounds, such as benzylpenicillin and oxacillin, aminopenicillins (including those with β-lactamase inhibitors), 1st and 2nd generation cephalosporins (e.g. cephalotin, cefoxitin and cefuroxime), several 3rd generation cephalosporins (e.g. cefotaxime) and to the carbapenem ertapenem. As well, it shows natural resistance to the aminoglycoside kanamycin and lower susceptibility to fluoroquinolones.

P. aeruginosa intrinsinc antibiotic resistance has been shown to be combinatorial and results from the interplay of several chromosomally-encoded resistance mechanisms, including the production of a narrow spectrum oxacillinase (PoxB/OXA-50) [Girlich D *et al*, 2004; Kong KF *et al*, 2005] and a more recently described imipenemase (PA5542) [Fajardo A *et al*, 2014], the inducible chromosomal AmpC cephalosporinase [Nordmann P & Guibert M, 1998], the constitutive expression of MexAB-OprM efflux pump [Livermore DM, 2001], the inducible expression of MexXY efflux pump [Aires JR *et al*, 1999] and the reduced permeability of its outer membrane [Livermore DM, 1984]. Whereas its outer membrane acts as a first barrier reducing the penetration of antibiotic compounds into the bacterial cell, its chromosomally-encoded oxacillinase, its imipenemase, its cephalosporinase AmpC and its efflux pumps act removing efficiently the antibiotics that do penetrate into the cell.

Moreover, in addition to the abovementioned resistance mechanisms, recent works have demonstrated that inactivation of a large number of genes, mainly involved in basic functions of the physiology of *P. aeruginosa*, are also involved in antibiotic susceptibility changes [Breidenstein EB *et al*, 2008; Fajardo A *et al*, 2008; Schurek KN *et al*, 2008; Dötsch A *et al*, 2009; Alvarez-Ortega C *et al*, 2010; Khran T *et al*, 2012; Fernandez L *et al*, 2013]. On the whole, these works have demonstrated that intrinsic resistance to antibiotics involves a complex network of elements. Of note, although inactivation of many of these genes just lead to slight decreases in susceptibility (1-2 fold), an overlap between them and genes dysregulated upon antibiotic exposure has been observed, which indicates that *P. aeruginosa* adaptively activates resistance mechanisms to combat the inhibitory effects of antibiotics.

1.3.1. A first barrier to antibiotics: the outer membrane

When compared to other Gram-negative bacteria, *P. aeruginosa* exhibits a lower outer membrane permeability (approximately 8% that of *E. coli* outer membrane) [Nikaido H, 1985]

but a large exclusion limit allowing the entrance of large compounds (3000 molecular weight vs 500 in *E. coli*) [Bellido F *et al*, 1992].

However, in order to survive, *P. aeruginosa* must allow the entrance of nutrients into the cell and this exchange is accomplished through a collection of β-barrel proteins producing water-filled diffusion channels called porins. Up to 163 known or predicted outer membrane proteins (OMPs) have been described within *P. aeruginosa* genomes, of which 64 are found as part of 3 families of porins: the OprD-specific porin family, the TonB-dependent gated porin family, and the OprM efflux/secretion family. Most of these porins have low molecular masses, being the OprF porin the largest one (37.6 kDa). Thus, the low permeability documented for *P. aeruginosa* strains can be explained in terms of a limited number of large general diffusion porins [Hancock RE & Brinkman FS, 2002].

Porins play an important physiological role in the transport of sugars, aminoacids, phosphates, divalent cations and siderophores [Hancock RE & Brinkman FS, 2002] and they have also be implicated in the transport of certain hydrophilic antibiotics such as β-lactams, aminoglycosides, tetracyclines and some fluoroquinolones [Nikaido H *et al*, 1991; Yoshimura F & Nikaido H, 1985]. Therefore, in addition to their contribution to the intrinsic antibiotic resistance, porins can further diminish *P. aeruginosa* susceptibility by regulating their expression or by acquiring mutations with effects onto their structures and functionality (section 1.8).

1.3.2. AmpC-inducible expression

P. aeruginosa possesses an inducible chromosomally-encoded AmpC cephalosporinase which is similar to that found in several members of the *Enterobacteriaceae* [Jacoby GA, 2009]. According to the Bush-Jacoby-Medeiros classification, AmpC is a serine β-lactamase belonging to group I and, based on the Ambler structural classification, to class C β-lactamases. Possibly, AmpC is the most relevant antibiotic resistance mechanism of this opportunistic pathogen.

WT *P. aeruginosa* strains produce only low basal amounts of this enzyme remaining susceptible to antipseudomonal penicillins, penicillin-inhibitor combinations, antipseudomonal cephalosporins (ceftazidime and cefepime) and carbapenems. Nevertheless, AmpC production can significantly be increased under particular circumstances, conferring resistance to all β-lactams. AmpC increased production can occur either, through mutations within its regulatory genes (section 1.8.) or by induction of the *ampC* gene. AmpC induction is a reversible process which occurs under exposure to specific β-lactams and β-lactamase inhibitors such as cefoxitin, imipenem and/or clavulanate [Lister PD *et al*, 2009]. As following detailed, AmpC induction is a complex process intimately linked with peptidoglycan (PGN) recycling (Figure 1.1.).

The PGN of *P. aeruginosa* is built up of chains with n repeats of the disaccharide monomer N-acetyl-glucosamine-N-acetyl-muramic-acid (GlcNAc-MurNAc) connected to other identical chains by stem peptides linked to the MurNac units. The stem peptide from a disaccharide monomer is originally a pentapeptide (L-Alanine-D-Glutamicacid-diaminopimelicacid-D-Alanine-D-Alanine) and connects to a second stem peptide from another disaccharide monomer located on a different chain thanks to the transpeptidase activity of the high molecular mass penicillin-binding proteins (PBP1, PBP2 and PBP3). These PBPs cleave the terminal D-Alanine from the first pentapeptide (carboxypeptidase activity), converting it into a tetrapeptide which eventually binds to the diaminopimelic acid from other pentapeptide (transpeptidation). Thus, these bonds allow for the crosslinking of disaccharide chains which constitute the essential PGN architecture. Once the basic PGN structure is built, some other PBPs, mainly the low molecular mass PBPs (PBP4, PBP5 and PBP6) are thought to finely shape it. These PBPs exert D-carboxypeptidase activities and are known to release the terminal D-Ala from pentapeptides not destined to be cross-linked converting them into tetrapeptides not suitable for transpeptidation [Juan C *et al*, 2017].

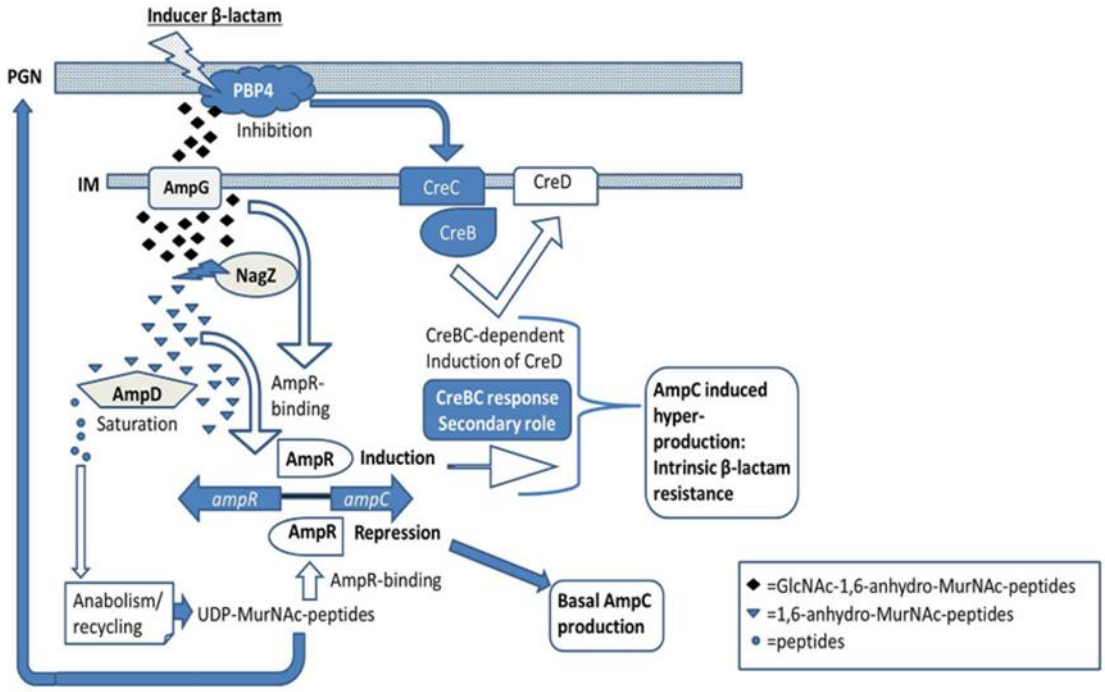


Figure 1.1. Schematic representation of the interplay between PGN recycling, ampC regulation (induction) and intrinsic β-lactam resistance in *P. aeruginosa*. From: Juan C *et al*, 2017.

On each generation *P. aeruginosa* naturally degrades about 50% of its PGN mainly thanks to the action of the periplasmic autolysins (endopeptidases), which break the abovementioned bonds originating not cross-linked peptides, and to the action of the lytic transglycosylases, which break the bonds between the disaccharide units. Up to 90% of the degraded PGN is thought to be recycled, which supposes an outstanding resource-saving strategy. The action of the cited periplasmic enzymes results mainly in GlcNAc-1,6-anhydro-

MurNAc tri-, tetra- and penta- peptides [Vollmer W & Höltje JV, 2001], resulting fragments that are transported through the permease AmpG into the cytosol [Korfmann G & Sanders CC, 1989; Dietz H & Wiedemann B, 1996; Cheng Q & Park JT, 2002]. This is a key step for the downstream AmpC regulation and recycling events, as AmpG is the specific door for the entrance of PGN-derived mediators with AmpC regulator capacity [Zamorano L, 2011]. Once in the cytosol, the cytosolic L, D-carboxypeptidase LdcA cleaves the D-Ala from the tetrapeptides units, avoiding the potential accumulation of UDP-MurNAc tetrapeptides which are thought to be toxic for the bacterial cell [Templin MF *et al*, 1999]. As well, a glycoside hydrolase called NagZ removes the GlcNAc residues [Zamorano L *et al*, 2010] resulting in a pool of cytosolic GlcNAc units plus 1,6-anhydro-MurNAc peptides [Cheng Q *et al*, 2000; Vötsch W & Templin MF, 2000] that, in non-inducer standard conditions, would eventually be recycled into UDP-MurNAc pentapeptides and exported to the nascent PGN.

Classically, it has been believed that the 1,6-anhydro-MurNAc tri- and penta- peptides units [Jacobs C *et al*, 1994; Dietz H *et al*, 1997] are signal molecules that induce ampC transcription and, indeed, the UDP-MurNAc pentapeptide has been identified as a repressor of ampC transcription to basal levels. Thus, these metabolites have been suggested to competitively regulate *ampC* transcription by directly binding to the LysR-type transcriptional regulator AmpR [Jacobs C *et al*, 1994]. AmpR and AmpC coding genes are located next to each other within the genome, divergently codified and with overlapping promoter regions to which AmpR binds to regulate their transcription [Lindquist S *et al*, 1989; Bartowsky E & Normark S, 1993]. Under non-inducer standard conditions, the cytosolic AmpD, through its N-acetyl-muramyl-L-alanine amidase activity, cleaves the stem peptide from both the GlcNAc-1,6-anhydro-MurNAc and the 1,6-anhydro-MurNAc peptides [Höltje JV & Glauner B, 1990; Jacobs C *et al*, 1994], which results in low amounts of activation ligands. On the contrary, the amount of UDP-MurNAc pentapeptides can be increased thanks to the anabolic pathways starting from the pool of AmpD cleaved peptides and, thus, can both, enter into the PGN recycling route and bind to AmpR promoting the formation of an AmpR-deoxyribonucleic acid (DNA) complex that represses ampC transcription to basal levels.

In this sense, it has been proposed that exposure to certain β-lactams known to be AmpC inducers, such as cefoxitin and imipenem, triggers the accumulation of 1,6-anhydro-MurNAc peptides within the cytosol, reaching levels that cannot be efficiently processed by AmpD [Dietz H & Wiedemann B, 1996; Wiedemann B *et al*, 1998; Vollmer W & Höltje JV, 2001]. This accumulation would presumably displace the UDP-MurNAc pentapeptide from AmpR, generating a new complex that would act as an activator of *ampC* transcription and, thus, leading to clinically significant resistance against the inducer and other hydrolysable β-lactams [Jacobs C *et al*, 1994]. The molecular basis for the mentioned increase in the 1,6-anhydro-MurNAc pentapeptides amount during induction is believed to be related with the capacity of the inducer β-lactams to inhibit the DD-carboxypeptidase activity of the low

molecular mass PBPs [Sanders CC *et al*, 1997; Tayler AE *et al*, 2010; Fisher JF & Mobashery S, 2014]. In this sense, *Moyà et al.* showed that the inducer β-lactams can inhibit the non-essential low molecular mass PBP4 (dacB) [Moyà B *et al*, 2009], affecting the PGN composition and favouring the entrance of activation ligands through AmpG. Interestingly, the authors also showed that PBP4 inducer-inhibition additionally triggers the activation of the two-component system CreBC which plays a collateral and minor role during the process. Thus, it has been proposed that PBP4 acts as a sentinel for the cell wall damage caused by the inducers, triggering an AmpR-dependent overproduction of AmpC and activating the CreBC system. The induction mechanism is a reversible process and *ampC* expression returns to basal levels in the absence of the antibiotic inducers [Mark BL *et al*, 2012]. Also it should be highlighted that the hydrolytic effect of AmpC onto a β-lactam will not only depend on the antibiotic inducer capacity but also on the hydrolysing efficiency of the cephalosporinase. Therefore, the inducible expression of AmpC plays a major role in the intrinsic resistance of *P. aeruginosa* to aminopenicillins and most cephalosporins (particularly cephamycins such as cefoxitin) since these molecules are potent inducers of the expression and efficiently hydrolyzed by this enzyme. Likewise, the inducible AmpC plays a major role in the basal reduced susceptibility level of *P. aeruginosa* to the carbapenem imipenem, as the relatively stability of this molecule to the hydrolysis by the cephalosporinase is to some extent compromised by its extremely high potency as inducer [Livermore DM, 1992].

Non-reversible mutational derepression leading to constitutive high-level expression of AmpC will be discussed later in section 1.8.

1.3.3. Efflux-pumps systems: constitutive and inducible expression

Efflux pumps play an important role in antibiotic resistance. These pumps may be specific for a substrate or may extrude a broad range of compounds including dyes, detergents, fatty acids and antibiotics of multiple classes structurally unrelated. Thus, it is probable that efflux pumps were created so that harmful substances could be transported out of the bacterial cell, thus, allowing for survival.

Based primarily on amino acid sequence identity, on the energy source required to drive export and on substrate specificities, efflux pumps have been categorized in five superfamilies including (i) the ATP-binding cassette family, (ii) the small multidrug resistance family, (iii) the major facilitator superfamily, (iv) the resistance-nodulation-division (RND) family, and (v) the multidrug and toxic compound extrusion family. In *P. aeruginosa*, genome sequence analysis has revealed the presence of efflux systems from all five superfamilies, being the RND family the most prevalent with 12 different systems identified.

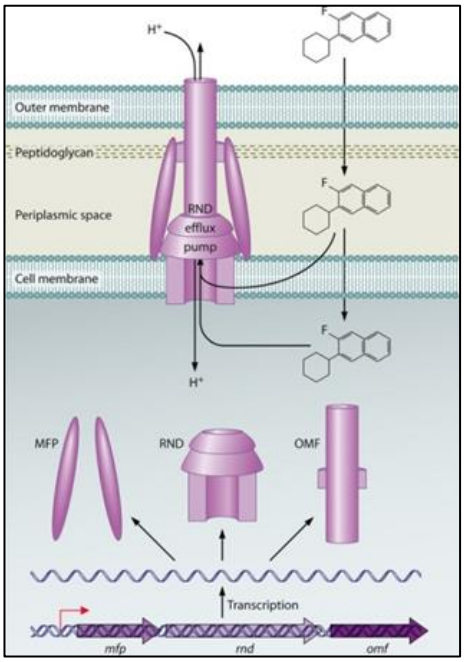


Figure 1.2. Location of RND-type efflux pumps components across the outer and inner membrane in *P. aeruginosa*. MFP: membrane fusion protein, RND: transporter protein, and OMF: outer membrane factor.

The RND-type efflux pumps are secondary active transporters that derive the energy required for compound extrusion by proton motive force and are typically organized as a tripartite consisting of a periplasmic membrane fusion protein, a transporter protein in the inner membrane and an outer membrane factor.

Within this complex, the inner membrane protein captures the substrates from either, the phospholipid bilayer of the inner membrane of the bacterial cell envelope or the cytoplasm, and transports them into the extracellular medium via the OMP, being the cooperation between these proteins mediated by the periplasmic protein (Figure 1.2.) [Lister PD *et al*, 2009; Li XZ *et al*, 2015].

The genes coding for the RND efflux pumps components are organized into operons in the *Pseudomonas aeruginosa* chromosome. Not all of them code for an outer membrane factor and, thus, the tripartite efflux pump is completed by taking this protein from a different efflux pump system (e.g. MexXY). As well, some of them harbor an adjacent regulatory gene transcribed in the same orientation or divergently from the operon and whose products act repressing or activating the operon expression (Figure 1.3. and Table 1.1.) [Lister PD *et al*, 2009].

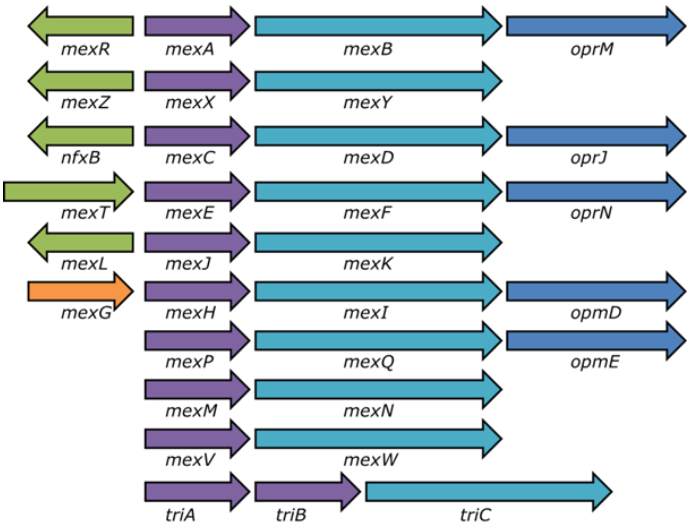


Figure 1.3. RND efflux operons in *P. aeruginosa*. Operons encoding the 10 RND pumps (excluding the 2 metal cation transporters) are represented. Color scheme: green, transcriptional regulator; purple, membrane fusion protein; light blue, RND transporter; dark blue, OMP; and orange, protein with unknown function.

Most RND efflux systems in *P. aeruginosa* exhibit broad substrate specificity and recognize many structurally dissimilar compounds (Table 1.1). Of all systems, MexAB-OprM and MexXY contribute to its intrinsic antibiotic resistance as all the others are not expressed in WT strains.

Table 1.1. Substrates for the RND efflux systems of *P. aeruginosa*

Efflux system	Substrates	
	Antibiotics	Additional compounds
MexAB-OprM	β-lactams (not imipenem), β-lactamase inhibitors, fluoroquinolones (FQ), chloramphenicol, macrolides, novobiocin, tetracyclines, trimethoprim, sulfonamides	Biocides, detergents, dyes, homserin lactones, aromatic hydrocarbons
MexXY-OprM/Opm-[a]	Penicillins (not carbenicillin and sulbenicillin), cephalosporins (not ceftazidime), meropenem, FQ, aminoglycosides (AMG), tetracyclines, macrolides, chloramphenicol	
MexCD-OprJ	Penicillins, cephalosporins (not ceftazidime), meropenem, FQ, chloramphenicol, macrolides, novobiocin tetracyclines, trimethoprim	Biocides, detergents, dyes, aromatic hydrocarbons
MexEF-OprN	FQ, cloranphenicol, trimethoprim	Biocides, aromatic hydrocarbons
MexJK-OprM/OpmH	Tetracyclines, erythromycin	Biocides
MexGHI-OpmD	FQ	Vanadium
MexVW-OprM	FQ, tetracyclines, chloramphenicol, erythromycin	
MexPQ-OpmE	FQ, tetracyclines, chloramphenicol, macrolides	
MexMN-OprM	Chloramphenicol, thiamphenicol	
TriABC-OpmH		Triclosan

[a] MexXY may utilize OpmB, OpmG, OpmH and/or OmpI as OMFs.

1.3.3.1. Constitutive expression of MexAB-OprM

MexAB-OprM was the first RND multidrug efflux system to be described in *P. aeruginosa* [Poole K *et al*, 1993; Li XZ *et al*, 1995]. As shown in Table 1.1., this pump is able to export antibiotic compounds from different families and exhibits the broadest substrate profile for the β-lactam class including carboxypenicillins, aztreonam, cefotaxime, ceftazidime and meropenem.

This system is expressed constitutively in cells grown under standard laboratory conditions [Poole K & Srikumar R, 2001] and laboratory-constructed MexAB-OprM knockout mutants have been shown to be hypersensitive to its substrates [Li XZ *et al*, 1995; Masuda N *et al*, 1999; Morita Y *et al*, 2001]. In WT *P. aeruginosa* strains, MexAB-OprM expression is growth-phase-dependent, reaching its maximum in late log-phase/early stationary phase. This dependency led to the suggestion that MexAB-OprM expression could be regulated by the

quorum sensing (QS) system (cell to cell communication) and, in this sense, it has been demonstrated that N-butyryl-L-homoserin-lactones enhance its expression.

All three components of this efflux pump are encoded within the same operon (Figure 1.3.), which additionally harbors a regulatory protein (MexR) located directly upstream but transcribed divergently from MexA-MexB-OprM coding genes. MexR belongs to the MarR family member and is the major regulator of this efflux pump system. It binds as a stable homodimer to two sites within the *mexR-mexA* intergenic region overlapping the promoters for mexR and mexAB-oprM and, thus, repressing their expression. Recently, it has been demonstrated that MexR repressor capacity depends on its redox state as, within the stable homodimer, MexR-Cys residues form intermonomer disulfide bonds whose oxidation eventually lead to its dissociation from the promoter DNA [Chen H *et al*, 2008; Chen H *et al*, 2010]. MexR activity has been found to be additionally controlled by *armR* encoded product, as it binds to MexR diminishing its repressor activity [Daigle DM *et al*, 2007; Wilke MS *et al*, 2008]. Finally, MexAB-OprM expression is controlled by *nalD*, which encodes a TetR family repressor-like protein that binds to a second promoter upstream of *mexA-mexB-oprM* [Morita Y *et al*, 2006a]. Also of note, it has been shown that *oprM* expression can occur independently of *mexA-mexB*, through an alternative weak promoter within *mexB* [Zhao Q *et al*, 1998], which ensures sufficient levels of this OMP to other *P. aeruginosa* efflux systems (MexXY, MexJK, MexVW and MexMN) even when *mexA-mexB-oprM* expression is compromised.

Mutation-driven overexpression of this efflux system will be discussed later in section 1.8.

1.3.3.2. Inducible expression of MexXY

The MexXY efflux system was discovered several years later, in 1999, being the fourth efflux system to be identified in *P. aeruginosa* PAO1 [Aires JR *et al*, 1999; Mine T *et al*, 1999]. It is able to extrude a wide variety of substrates (Table 1.1.) and, of note, is the only efflux pump encoded in *P. aeruginosa* chromosome with the ability to mediate aminoglycoside resistance.

MexXY expression is induced when bacterial cells are grown in the presence of sub-inhibitory concentrations of some of its antibiotic substrates such as tetracycline, erythromycin or aminoglycosides. Additionally, *P. aeruginosa* PAO1 mutants lacking this efflux system are hypersusceptible to its substrates which suggests that it contributes to the intrinsic antibiotic resistance to these agents [Aires JR *et al*, 1999; Masuda N *et al*, 2000].

Genetically, the operon coding for MexXY lacks an outer membrane factor (Figure 1.3.). Therefore, it takes the OMF protein from other operons to complete the tripartite system. Mainly, OprM completes the tripartite system but other porins such as OpmB, OpmG, OpmH or OpmI can also be implicated (Table 1.1.) [Chuanchuen R *et al*, 2005; Murata T *et al*,

2002]. Located upstream but transcribed divergently from *mexX-mexY*, is encountered *mexZ* which encodes a protein that belongs to the TetR family of transcriptional regulators and negatively regulates its expression (Figure 1.3.). Similar to MexR (section 1.3.3.1.), MexZ binds as a homodimer to an inverted repeated sequence within the intergenic region *mexZ-mexX,* overlapping the putative *mexX-mexY* promoter [Matsuo Y *et al*, 2004] and repressing its expression.

In contrast to other drug-inducible multidrug efflux systems, MexXY inducers do not alter MexZ and *mexZ-mexX* interactions. Instead, induction has been shown to be dependent on drug-ribosome interactions and to occur, although in a lesser extent, even in the *mexZ* mutant [Jeannot K *et al*, 2005]. Therefore, these data suggest an alternative biological role for the MexXY system beyond antibiotics efflux. Multiple pathways participate in the regulation of *mexX-mexY* induction. Although ribosome disruption has been shown to impact the expression of a myriad of genes, by using a transposon insertion mutant library PA5471 was found to be not only drug-inducible but also required for *mexX-mexY* induced expression [Morita Y *et al*, 2006b]. Later on, it was demonstrates that the antimicrobial-inducible PA5471 gene product has interacts with the repressor MexZ and, thus, interfere with its DNA binding activity [Yamamoto M *et al*, 2009].

More recently, it has been also demonstrated the involvement of *parR,* a gene coding for the response regulator of the two-component regulatory system ParR-ParS, in promoting either induced or constitutive *mexX-mexY* upregulation. In addition, this gene was demonstrated to be also implicated in OprD porin downregulation and in lipopolysaccharide (LPS) modification in a MexZ-independent manner [Muller C *et al*, 2011].

Mutation-driven overexpression of this efflux system will be discussed later in section 1.8.

1.4. CHRONIC RESPIRATORY INFECTIONS

On average, about 10,000 L of air are inhaled per person per day and, thus, the respiratory tract is continuously exposed to a wide variety of potential pathogenic microorganisms. However, and due to sophisticated host defence mechanisms at the lung mucosa, infections are rare among healthy individuals. Airway bronchial and alveolar epithelial cells constitute the first line of defense against invading bacteria, providing not only a physical barrier and exhibiting local antimicrobial activity but also acting as sentinels stimulating downstream recruitment and activation of immune cells which clear invading bacteria. As well, resident alveolar macrophages and occasionally dendritic cells are also found in the alveolar epithelium and are key mediators of innate and adaptive immunity [Eisele NA & Anderson DM, 2011]. On the opposite, the immune response within the respiratory airway of patients suffering chronic respiratory underlying diseases such as CF, non-CF bronchiectasias or COPD, is impaired and, therefore, these disorders are characterized by repeated cycles of inflammation, tissue damage and bacterial infections that may eventually lead to the establishment of chronic non-eradicable respiratory infections and a rapid decline of the pulmonary function [Döring G *et al*, 2011]. In fact, *P. aeruginosa* CRI acquired a major relevance within the CF setting, being the most frequent and severe driver of morbidity and mortality.

CF is the most prevalent autosomal recessive hereditary disease affecting Caucasian populations, with approximately 70,000 people affected worldwide and with an estimated incidence of 1 per 2500-5000 newborns in white populations from Europe, Canada and USA [O'Sullivan BP & Freedman SD, 2009]. This chronic respiratory disease is caused by mutations (two-thirds F508Δ) disrupting the function of the CF transmembrane conductance regulator (CFTR) gene, which encodes a chloride channel that is expressed on the apical surface of many epithelial and blood cells. The clinical spectrum of the CF disease is wide; however, pulmonary insufficiency is the first cause of morbidity and mortality among CF patients being approximately 80% of CF deaths related with chronic lung infection [O'Sullivan BP & Freedman SD, 2009]. Fortunately, over the past decades, CF respiratory infections management has considerably been improved and median age of survival of CF patients is now set in more than 40 years in developed countries [McCormick J *et al*, 2010]. As a result, the number of CF adults (age ≥18 years) is larger than the number of children in several EU countries with well-established healthcare systems [McCormick J *et al*, 2010] and forecasts predict a large increase in the number of CF adults by 2025 [Burgel PR *et al*, 2015].

By the time of birth, the respiratory tract of CF children is normal but, soon after, becomes inflamed and infected. Mechanisms underlying the early acquisition of infection and the establishment of *P. aeruginosa* CRI are complex and several factors participate as following described.

One contributing factor is the inability of mutated CFTR to effectively secrete chloride from respiratory epithelial cells into the airway surface liquid which eventually causes excessive water absorption from the airway surface liquid, leading to an impaired mucociliary clearance. Likewise, the viscosity of the secretions may impair the transport of antimicrobial oligopeptides onto the epithelium and, thus, may also negatively affect the migration of neutrophils towards the pathogens. Furthermore, within the highly viscous mucus, a microaerobic/anaerobic milieu prevails due to oxygen consumption by bacterial pathogens or invading neutrophils which abolish the generation of reactive oxygen species (ROS) by neutrophils and other cells impairing bacterial killing. As well, other investigators have demonstrated that the abnormal accumulation of ceramide in the lungs of CF mice and in the epithelial cells from CF patients, results in an increased death rate of respiratory epithelial cells and DNA deposits on the respiratory epithelium, which in turns facilitates bacterial adherence. Finally, *P. aeruginosa* infection may also be facilitated directly by defective CFTR, as in its functional state can bind the pathogen within lipid rafts removing it from the epithelial surface via internalization [Döring G *et al*, 2011]. Of course, *P. aeruginosa* also plays a major role as, thanks to the enormous armamentarium of immunoevasive strategies encoded within its genome, is capable of evading not only host defenses but also repeated courses of antibiotics.

All the above mentioned alterations in the CF airway surface provide an ideal environment for infection/colonization which is not only exploited by *P. aeruginosa*. In fact, CF patients experience multiple bacterial infections throughout their life and whereas, overall, *Staphylococcus aureus* and *Haemophilus influenzae* are typically first cultured in young children, *P. aeruginosa* and other opportunistic multidrug resistant pathogens such as *Achromobacter spp.*, *Stenotrophomonas maltophilia* or *Burkholderia cepacia complex* are first cultured during adolescence and young adulthood (Figure 1.4.) [CFF Patient Registry, 2016 Annual Data Report].

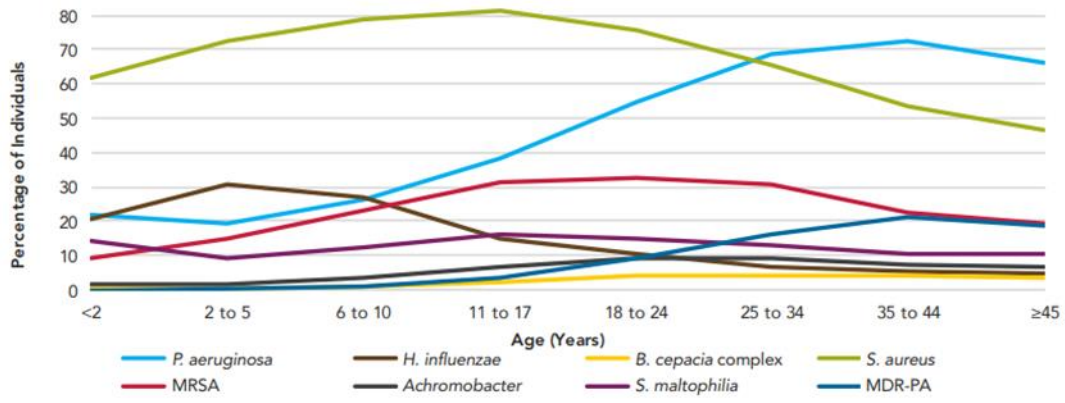


Figure 1.4. Prevalence of respiratory microorganisms by age cohort, 2016. From: Cystic Fibrosis Foundation Patient Registry, 2016 Annual Data Report. Bethesda, Maryland, ©2017 Cystic Fibrosis Foundation.

As shown, *P. aeruginosa* is by far the most significant CF pathogen. Early infection occurs in a large number of patients before the age of 3 years [Speert DP *et al*, 2002], after, and for a variable period of time, *P. aeruginosa* isolation from CF respiratory samples can be intermittent and, usually, involving multiple strains. Eventually, by the age of 25, over 70% of the patients are chronically colonized and a single well adapted strain (or clonal lineage) predominates [Cystic Fibrosis Foundation Patient Registry, 2016 Annual Data Report].

1.5. EVOLUTION AND ADAPTATION TO THE CYSTIC FIBROSIS AIRWAYS

During the progression from early infection to chronic non-eradicable colonization, *P. aeruginosa* undergoes a complex evolutionary adaptation and diversification process that implies both phenotypic and genotypic variations.

Usually, first *P. aeruginosa* CF isolate resembles those from the environment or from acute infections in terms of phenotype and genotype. Thus, during long time, it was extensively accepted that early *P. aeruginosa* acquisition occurs from diverse environmental reservoirs so, generally, each patient harbors its unique non-clonal unrelated strain. However, this classical perception changed in 1986, when an outbreak caused by a *P. aeruginosa* strain resistant to several antibiotics was reported in a CF center in Denmark [Pedersen SS *et al*, 1986]. Since this first description, other strains infecting a large proportion of CF patients have been detected and, in some cases, strongly associated with multidrug resistant profiles. Therefore, nowadays "person-to-person" transmission is also an accepted route of *P. aeruginosa* acquisition among CF patients and certain clones are extensively recognized as epidemic and/or transmissible being worldwide distributed (section 1.8.).

Regardless to the source of infection, if not eradicated, the cell density and the collective growth pattern of *P. aeruginosa* change and a complex diversification process occurs within the bacterial population which, in turns, improve its capacity for survive and persist in the CF airways throughout the lifespan of a CF patient [Renders N *et al*, 2001; Munck A *et al*, 2001].

Overtime, during the course of infection, the genome of *P. aeruginosa* can be modified by either acquiring new mutations or by the acquisition and/or loose of genomic DNA. Whereas few works have focused their attention on DNA acquisition/loose and obtained results are not consistent, different authors have focused on genomic modification based on the acquisition of SNPs and small insertions stablishing mutation rates from 1 to 3 SNPs/year for non-mutator isolates [Marvig RL *et al*, 2015a]. As well, since whole-genome sequencing technologies have become more affordable, many efforts have been put in identify those genes that are recurrent mutated among CF isolates and, therefore, directly implicated in the adaptation to the CF lungs, the so-called pathoadaptative genes. Whereas some genes have been found to be frequently mutated, others have just been found to be mutated in single studies which suggest that different evolutionary pathways exist [Marvig *et al*, 2015a]. Addtionally, the study of longitudinal isolates within single patients have provided evidence that population diversification and stable maintenance of these genetically distinct subpopulations frequently occurs, even including mutator and non-mutator sublineages [Chung JC *et al*, 2012; Marvig RL *et al*, 2013; Feliziani S *et al*, 2014]; diversification procces that have been suggested to be triggered by the spatial heterogeneity of the CF airways [Markussen T *et al*, 2014].

Indeed, in patients in advanced infection-colonization stages, this impressive process of genotypic diversification can be easily recognized in the microbiological cultures of their respiratory samples which are characterized by a mixture of phenotypic varieties following described.

Mucoid variants. One of the most common features of *P. aeruginosa* isolates causing CRI is the frequent conversion to a mucoid phenotype. In fact, the appearance of mucoid colonies within the microbiological culture can be used as a marker of infection chronicity and poor clinical outcomes. This phenotype results from the constitutive production of the extracellular polysaccharide alginate, a polymer of D-mannuronic and L-guluronic acid, which forms a glycocalyx that encapsulates the bacteria, protecting them from adverse environmental stresses such as dessication, oxidizing agents and host defence [Franklin MJ *et al*, 2011]. As well, this extracellular polysaccharide is one of the major components of the biofilms matrix (section 1.6.).

The genetic mechanisms underlying the switch to mucoidity in *P. aeruginosa* have been largely studied and mainly results from the mutational inactivation of the *mucA* gene, which codes for an anti-σ-factor [Govan JR & Deretic V, 1996; Boucher JC *et al*, 1997]. All the enzymes required for alginate production are encoded in the operon *algD-algA* and, in the absence of mutations, the *algD* operon expression is limited by the *mucA* gene product that binds to the alternative ribonucleic acid (RNA) polymerase σ-factor σ22 encoded in *algU* [Folkesson A *et al*, 2012].

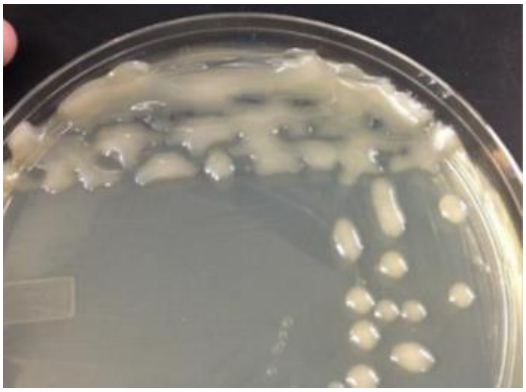


Figure 1.5. Mucoid *P. aeruginosa* on Mueller-Hinton Agar (MHA).

In addition to the *algD* cluster, σ22 is known to regulate, directly and indirectly, a large number of stress response and virulence-associated genes in *P. aeruginosa* which suggests that the importance of the *mucA* mutations goes beyond the conversion to a mucoid phenotype [Folkesson A *et al*, 2012]. Despite the success and theoretical advantages of this variant, the most common situation during late chronic stages is the coexistence of mucoid and non-mucoid variants but with different zonal distribution [Bjarnsholt T *et al*, 2009]; situation that reflects the advantage of diversification for persistence.

Small colony variants (SCV). Another frequent phenotypic variant of chronic stages are the named SCV, which are characterized by their reduced colony size of 1-3 mm. These slow-growing variants have been associated with increased antimicrobial resistance, in particular to aminoglycoside compounds, and a poorer lung function in CF patients [Häussler S *et al*,

1999]. Besides increased aminoglycoside resistance, *P. aeruginosa* SCV can exhibit hyperadherent and autoaggregative behaviors (named rugose SCV, RSCV). These variants can act favoring biofilm formation as showed an increase expression of the *pel* and *psl* exopolysaccharide gene clusters and a decreased expression of flagellum and pilus coding genes [Häussler S *et al*, 2003; Cullen L & McClean S, 2015].

Genetically, the most commonly identified SCV-inducing mutations are loss-of-function mutations in repressor proteins that control the activity of diguanylate cyclases (DGCs) [Malone JG, 2015]. DGCs participate in the production of the ubiquitous bacterial signaling molecule bis-(3',5')-cyclic diguanosine monophosphate which controls a wide range of cellular processes involved in the transition between motile, virulent, and sessile biofilm forming lifestyles [Hengge R, 2009].

Non-motile variants. Early infection of CF airways requires bacterial adhesion to host epithelial cell surfaces, a process that is mediated by flagellum and pilli. By contrast, chronic *P. aeruginosa* isolates are characterized by the lack of twitching and swimming motility due to non-pilation and loss of flagellum, respectively [Mahenthiralingam E *et al*, 1994].

It has been pointed out that this mechanism can enable *P. aeruginosa* to better evade the host immune response, as isolates lacking the flagellum are less effective phagocytosed by alveolar macrophages and polymorphonuclear phagocytes (PMNs). At the genetic level, these variants have been linked to mutations within the *rpoN* gene or to genes participating in flagellum sysnthesis [Mahenthiralingam E *et al*, 1994].

Loss of the Quorum-Sensing (QS) system. In general, *P. aeruginosa* behaves as single cellular organisms in low population densities. However, as cell density increases, bacterial cells can communicate to each other using small signaling molecules inducing changes in gene expression with community purposes. This communication system is known as the QS system and it has been demonstrated to be frequently impaired in late CF isolates [Smith EE *et al*, 2006].

The loss of the QS signaling in *P. aeruginosa* is associated with the presence of mutations in LasR and RhlR. These QS mutants have demonstrated a growth advantage in the presence of low amino acids amount, which is particularly relevant in the CF lungs. Additionally, QS controls the expression of a variety of virulence factors that are generally selected against during CRI [Cullen L & McClean S, 2015]. As well, an increased β-lactamase activity *in vitro* has been documented for these QS mutants, which could be another potential benefit in down regulation of QS mechanisms [D'Argenio DA *et al*, 2007].

Other chronic variants. *P. aeruginosa* variants presenting a modified LPS are also frequent in CF chronic stages of infection [Hancock RE *et al*, 1983; Ernst RK *et al*, 1999]. In Gram-negative bacteria, the LPS is the major component of the outer membrane giving not only

structural integrity but also protecting the bacterial cell from environmental factors and, of course, contributing to cell impermeability. The LPS induces a variety of host immune responses, so its modification may participate in survival and persistance [Hauser AR, 2011]. Within the LPS, three components can be differentiated: (1) the toxic highly acylated lipid A, (2) the central core oligosaccharides and (3) the O-antigen. Structural modification in late CF isolates frequently implies the loss of the O-antigen (as a result of the accumulation of inactivating mutations within the cluster of genes responsible for its production) or an altered lipid A portion (in terms of its pattern of acylation or by the addition of aminoarabinose) [Hauser AR, 2011]. These modifications have important clinical implications as, for instance, it has been demonstrated that the addition of aminoarabinose enhances resistance to antimicrobial peptides and some antibiotics [Ernst RK *et al*, 1999] or that the acylation pattern influences the induced proinflammatory response [Alexander C & Rietschel ET, 2001].

Other adaptive variants that commonly emerge include: auxotrophic variants, pyomelanin hyperproducers, variants which have lost the type III secretion system, variants deficient in pyoverdine and/or pyocianine production, variants resistant to multiple antibiotic compounds and hypermutable variants (sections 1.7. and 1.8.).

1.6. PHYSIOLOGICAL RESISTANCE DURING CYSTIC FIBROSIS CHRONIC RESPIRATORY INFECTIONS

1.6.1. From the planktonic to the biofilm mode of growth

It has been set that the CF lung is a heterogeneous, hostile and stressful environment for invading bacteria. In order to overcome all these challenges, and apart from population diversification, *P. aeruginosa* shifts its mode of growth from free-living cells (planktonic state) to biofilm-forming cells, change that is currently recognized as one of the hallmarks of chronic infections. In fact, both processes are part of the same evolutionary path as all the above described variants (section 1.5.) live together within the biofilm community and contribute to its formation and existence, which constitutes an amazing example of how bacterial populations can enhance its survival and persistence in hostile environments by acting in a cooperative manner.

Biofilms are defined as organized bacterial communities surrounded by an extracellular polymeric matrix that confers resistance against the hostile environment. Biofilm formation classically involves the following stages: attachment, microcolonies formation, biofilm maturation and dispersal or detachment (Figure 1.6.) [O'Toole G *et al*, 2000].

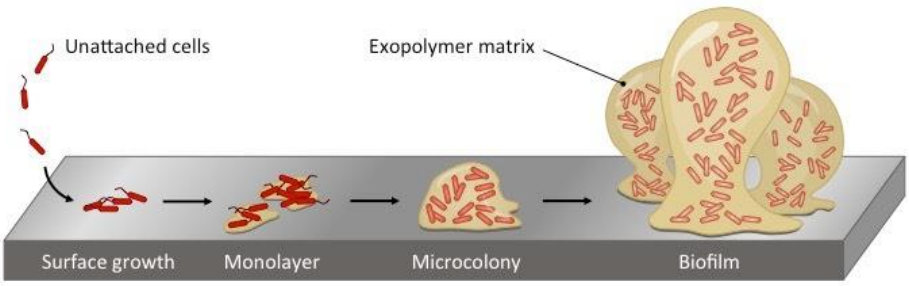


Figure 1.6. Stages of Biofilms formation

As shown, its development starts with the adherence of individual planktonic bacterial cells to a surface with the help of pili and flagella [O'Toole G *et al*, 2000]. Although most biofilm-related infections generally require an attachment to a solid surface, in the case of CF, some studies indicate that the biofilm found in the lung is directly formed on the mucus instead of being in contact with the lung epithelium [Bjarnsholt T *et al*, 2009; Worlitzsch D *et al*, 2002].

Attachment is then followed by bacteria multiplication, thus forming microcolonies, matrix building and eventual biofilm maturation. The extracellular polymeric matrix plays an important role during CRI not only giving cohesion to the structure and acting as a nutrient source but also providing a protective barrier against host defense, desiccation, ROS and antibiotics [Flemming HC & Wingender J, 2010]. This matrix is mainly composed of a conglomerate of exopolyscharides (including alginate provided by mucoid variants), extracellular DNA, proteins, surfactants, lipids, bacterial lytic products and host compounds.

Finally, once matured, biofilm population ensure its persistence in the hostile environment by releasing or dispersing small aggregates or even individual cells to uncolonized sites and reinitiating the biofilm lifecycle during the dispersal stage [O'Toole G *et al*, 2000].

Such a tangled process is known to be closely regulated by intra- and extracellular cues that modulate the levels of diffusible signal molecules, second messengers and small RNAs [Bjarnsholt T, 2013]. QS systems detect these signals as cell density evidences and trigger changes in bacterial gene transcription, including virulence factors and diverse proteins involved in the innate resistance of biofilms to antibiotics and the immune system. In this sense, *P. aeruginosa* biofilms are known to be able to initiate detachment on their own and this process can be mediated by either, alginate lyase overexpression [Boyd A & Chakrabarty AM, 1994] or by up-regulation of motility factors such as the rhamnolipid and type IV pili [Pamp SJ & Tolker-Nielsen T, 2007].

1.6.2. Inherent antimicrobial tolerance of biofilms

One of the most relevant aspects of biofilms is that they determine the persistence of the infection despite long-term antimicrobial treatment. In fact, it is estimated that biofilms can tolerate up to 100–1000 fold higher concentrations of antibiotics than the planktonic cells [Høiby N *et al*, 2010]. As following described, the documented inherent biofilm antimicrobial tolerance is multifactorial (Figure 1.7.).

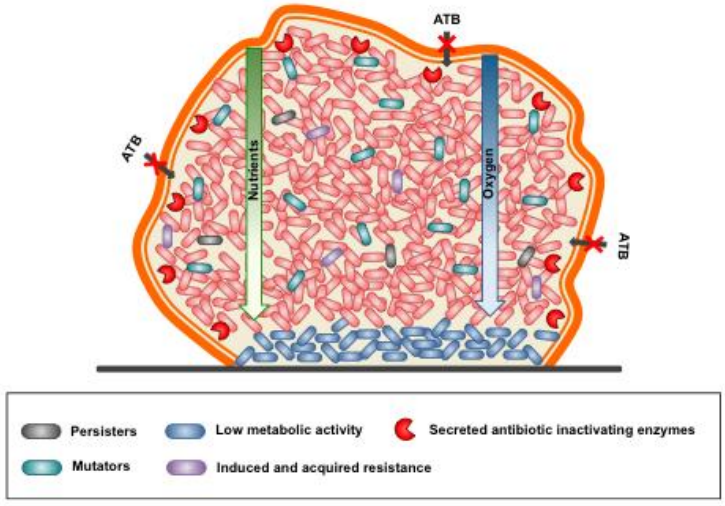


Figure 1.7. Schematic representation of the factors contributing to inherent biofilm antimicrobial resistance. ATB: antibiotics. From: The Problems of antibiotic resistance in CF and soultions. López-Causapé *et al.*, Expert Rev Respir Med 2015

Limited antibiotic penetration. The biofilm matrix acts as a primary barrier preventing the entrance of some compounds such as polar and charged antibiotics [Lewis K, 2008]. This restricted penetration has been linked to some components of the matrix such as alginate or eDNA which have shown antibiotic chelating activity [Alipour M *et al*, 2009] or to the

presence of antibiotic-inactivating enzymes within the matrix. As well, eDNA behaves as an antimicrobial shield and contributes to aminoglycoside tolerance [Chiang WC *et al*, 2013; Mulcahy H *et al*, 2008; Walters MC 3rd *et al*, 2003].

Growth rate and nutrient gradients. Internal gradients of biofilms give rise to anaerobic and nutrient-deficient areas, leading to a slowing down of the metabolism. Indeed, several studies have provided evidence that bacterial metabolic activity is high in the outer part of the biolfilm which compares with the inner parts [Walters MC 3rd *et al*, 2003; Bagge N *et al*, 2004; Werner E *et al*, 2004]. The lack of oxygen and the reduced multiplication rates contribute to fluoroquinolones and aminoglycosides' tolerance as these antibiotics targets processes that occur in growing bacteria [Walters MC 3rd *et al*, 2003]. Furthermore, osmotic stress response may also contribute to antibiotic resistance inducing a change in the proportions of porins [Stewart PS & Costerton JW, 2001].

Persister phenomenon. Persisters are defined as a dormant phenotypic state of bacteria within biofilms, characterized by a high tolerance to antibiotics including compounds that kill non-growing cells. Also, this latent bacterial state behaves as a bumper to host defense and may cause a relapse of infection, being a source of recalcitrant biofilm infection [Lewis K, 2010].

Induction of antimicrobial resistance mechanisms. Induction of resistance mechanisms can significantly differ between biofilm and planktonic growth. Indeed, various studies have found a differential expression of several conventional and biofilm-resistance genes in biofilms [Whiteley M *et al*, 2001; Mulet X *et al*, 2011].

Biofilms and mutation-driven resistance. The antibiotic gradient driven by biofilm physiology favors gradual development of mutational resistance during antimicrobial treatment, which is of particular significance when involving mutator strains (section 1.7.) [Oliver A *et al*, 2000; Macià MD *et al*, 2005; Henrichfreisse B *et al*, 2007]. Also, endogenous oxidative stress [Driffield K *et al*, 2008] and mutagenic ROS released from PMNs are likely to induce mutability in biofilm cells. In fact, recent findings have shown that mutagenesis is intrinsically increased in biofilms [Driffield k *et al*, 2008; Boles BR & Singh PK, 2008].

Horizontal gene transfer. Bacterial proximity within a biofilm allows an effective horizontal gene transfer [Bagge N *et al*, 2004]. Moreover, bacterial eDNA may represent a reservoir for the acquisition of exogenous resistance determinants.

All the above described tolerance mechanisms contribute to the persistence of biofilms, which therefore provide a fertile ground for the emergence and selection of antibiotic-resistant mutants.

1.7. HYPERMUTATION: A MARKER OF CYSTIC FIBROSIS CHRONIC RESPIRATORY INFECTIONS

Hypermutable (or mutator) microorganisms are defined as those that have an increased spontaneous mutation rate as a result of defects in DNA repair or error avoidance systems [Miller JH, 1996]. Although the optimal mutation rate for a bacterial population for a perfectly adapted clonal population is close to zero, in the absence of mutations the population could not adapt to environmental changes. Conversely, a high mutation rate is optimal for populations under strong selective pressure but too many mutations would cause a genetic breakdown (Figure 1.8).

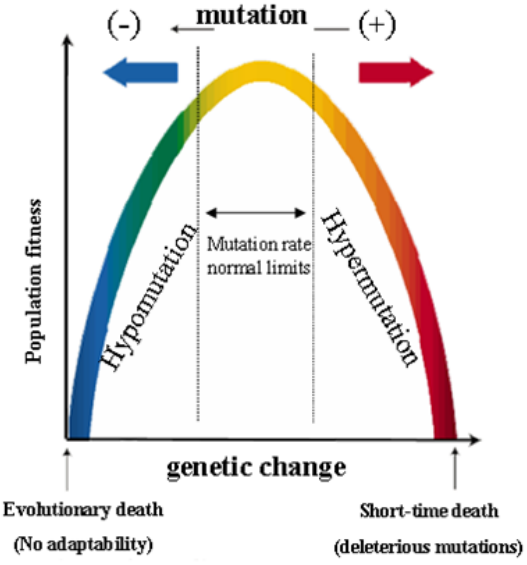


Figure 1.8. Mutation rates and genetic adaptability (fitness). Modified from: Radman 1999.

So, in regular bacterial populations, mutators are present at a rate of the order of 10^5 as a consequence of spontaneous mutations within DNA repair genes or error avoidance systems. Moreover, several investigations have demonstrated that these mutator variants can confer an evolutionary advantage during bacterial adaptation to new or stressful environments as the mutator subpopulation can be dramatically amplified by co-selection (hitchhiking) with other adaptive mutations such as those conferring antibiotic resistance [Cox B & Game J, 1974, Taddei F *et al*, 1997; Mao EF *et al*, 1997; Giraud A *et al*, 2001; Macià MD *et al*, 2006].

1.7.1. Genetic basis for hypermutation

So, in order to adapt and survive in new stressful environments, bacteria can increase their mutation rate and this increase can be either stable or transient.

The stable mutator phenotype is consequence of a defect in one of the several DNA repair or error avoidance systems, the so-called (anti) mutator genes. Several mutator genes with different effects onto the mutation rate have been described (Table 1.2.).

By far, the most frequent cause of hypermutation in natural bacterial populations is the presence of defects on the methyl-directed mismatch repair (MMR) system [Miller JH, 1996; Oliver A, 2010; Oliver A & Mena A, 2010]. The MMR system, which is present in all organisms, detects and repairs DNA replication errors including any kind of mispairs and short insertions or deletions. Besides, this system is the most potent inhibitor of recombination between weekly and moderately diverged sequences. Key components of the

system include the proteins encoded in *mutS* (PA3620), *mutL* (PA4946), *mutH* and *uvrD/mutU* (PA5443) genes. MutS first recognizes and binds to the mismatched DNA initiating the MMR machinery. Then, MutL interacts with MutS and together activate the endonuclease MutH that cleaves the non-methylated strand containing the mismatch. As well, MutL loads the DNA helicase II (UvrD/MutU) onto the DNA which is finally unwinded, excised and repaired by other MMR components. In the particular case of *P. aeruginosa*, mutH homologues do not exist and the recognition of the daughter DNA strain is therefore not based on methylation. Thus, in natural *P. aeruginosa* populations, inactivating mutations within *mutS*, *mutL* and *uvrD* genes can lead to higher mutation rates (from 100- to 1,000-fold) and increased rates of homologous recombination. In the CF setting, up to 60–90% of the mutator variants have a defective MMR system, mainly caused by mutation within *mutS* or *mutL* [Mena A *et al*, 2008; Montanari S *et al*, 2007; Oliver A *et al*, 2002a; Ciofu O *et al*, 2010].

Table 1.2. Principal mutator genes, most functionally characterized in *Escherichia coli*. From: Oliver A & Mena A. Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance. *Clin Microbiol Infect*. 2010; 16(7):798-808.

Gene	Product activity	Mutations produced	Mutator effect
***mutD* (*dnaQ*)**	E subunit of DNA pol III, proofreading activity	All base substitutions, frameshifts	Very strong
MMR system			
mutS	DNA mismatch recognition, binds mismatches	GC → AT, AT → GC, frameshifts	Strong
mutL	Interacts with MutS and MutH		
mutH	Endonuclease, nicks hemi-methylated GATC sequences		
uvrD	DNA Helicase II, strand displacement		
GO system			
mutT	Nucleoside triphosphatase, prevents incorporation of 8-oxoG to DNA	AT → CG	Strong
mutM	DNA glicosylase, removes 8-oxoG from 8-oxoG-C mispairs	G:C → T:A	Weak
mutY	DNA glicosylase, removes A from 8-oxoG-A or A-G mispairs	G:C → T:A	Moderate
Prevention of oxidative damage			
mutA	GlyV, glycyl tRN	AT → TA, GC → TA, AT → CG	Weak-moderate
mutC	GlyW, glycyl tRNA	AT → TA, GC → TA, AT → CG	Weak-moderate
ung	Uracil glicosylase, removes U from U-G mispair	GC → TA	Weak-moderate
sodA*, *sodB	Superoxide dismutase, removes superoxide radicals	AT → TA	Weak
oxyR	Regulates hydrogen peroxide inducible genes	AT → TA	Weak
polA	DNA polymerase I	Frameshifts, deletions	Weak-moderate

As well, although not found in natural populations, mutations in *dnaQ/mutD* (PA1816) can lead to strong mutator phenotypes (up to 10,000 fold) and reduced growth rates [Miller JH *et al*, 1996; Oliver A, 2010; Oliver A & Mena A, 2010].

Other mutator genes in *P. aeruginosa* include those of the GO system, which prevent mutations caused by the oxidative lesion mediated by the 7,8-dihydro-8-oxo-deoxyguanosine (8-oxodG or GO) (Table 1.2.) [Oliver A *et al*, 2002b].

Finally, mutations within those genes involved in the prevention of oxidative damage produced by ROS, such as *oxyR* (PA5344), *sodA/sodM* (PA4468), *sodB* (PA4366), mutator tRNAs (*mutA* and *mutC*), *pfpI* (PA0355), *ung* (PA0750), *mfd* (PA3002), *radA* (PA4609) and *polA* (PA5493) genes have also been linked with mutator phenotypes (Table 1.2.) [Oliver A & Mena A, 2010; Oliver A, 2010].

In addition to the stable mutator phenotype, under particular circumstances, such as when DNA is damaged, a transient mutator phenotype can rise by the induction of the of the error-prone DNA polymerases (IV and V) as part of the SOS response [Friedberg EC & Gerlach VL, 2002; Foster PL, 2007]. Of note, some antibiotics compounds may induce this phenotype which in turns promotes the appearance of antibiotic resistance [Blázquez J *et al*, 2002; Pérez-Capilla T *et al*, 2005].

1.7.2. Prevalence of *P. aeruginosa* mutators in the CF airways

CRI by *P. aeruginosa* in CF patients was the first natural model to reveal a high and unusual prevalence of mutator variants in natural bacterial populations [Oliver A *et al*, 2000].

Prevalence of *P. aeruginosa* hypermutable variants in the CF airways is extremely high, ranging from 30% to 60% [Oliver A, 2010; Montanari S *et al*, 2007; Mena A *et al*, 2008; Ciofu O *et al*, 2005; Marvig RL *et al*, 2013]. Moreover, their proportion significantly increases during the course of CRI as was demonstrated in a 25-year longitudinal study in which the proportion of hypermutable isolates increased from 0% at the onset/early colonization to 65% in late stages [Ciofu O *et al*, 2005].

This unusual high prevalence among *P. aeruginosa* isolates from CF patients compares with the documented prevalence among isolates from environmental sources (6%) or from acute infections (<1%) [Kenna DT *et al*, 2007; Oliver A *et al*, 2000; Gutiérrez O *et al*, 2004; Mulet X *et al*, 2013] and can be explained in terms of hitchhiking as an important role of mutators in adaptive mechanisms [Mena A *et al*, 2008] and in the development of antimicrobial resistance has already been proven [Oliver A *et al*, 2000; Macià MD *et al*, 2005; Henrichfreise B *et al*, 2007].

1.7.3. Hypermutation drivers in the CF airways

Because of the high prevalence of mutator variants encountered in the CF airways, nowadays the chronically infected CF airways are considered to be a mutagenic context, in which both, intrinsic and extrinsic factors are implied [Rodríguez-Rojas A *et al*, 2012; Oliver A, 2010; Oliver A & Mena A, 2010].

In the CF airways, the level of ROS is high mainly due to the increased availability of iron and because the antioxidant mechanisms in CF patients are highly diminished. ROS cause DNA damage and can further increase the inflammatory response, which eventually lead to the establishment of a vicious cycle of inflammation and hypermutation [Rodríguez-Rojas A *et al*, 2012; Oliver A, 2010; Oliver A & Mena A, 2010].

Also the biofilm mode of growth may itself be directly implied in the documented increase mutability. In this sense, *Driffield* and colaborators demonstrated that *P. aeruginosa* antioxidant enzymes coding genes (*katA*, *sodB*, *ahpC* and PA3529) are down-regulated when growing in biofilms compared to planktonic cells [Driffield K *et al*, 2008], which eventually lead to a decrease protection against oxidative mutagenesis. In addition, *Boles* and *Singh* documented that double-strand break mutations tend to occur more frequently within biofilms [Boles BR & Singh PK, 2008]. Besides, through competition experiments it has been demonstrated that *P. aeruginosa* MMRS-deficient variants exhibit enhanced adaptability over WT strains when grown in structured biofilms [Luján AM *et al*, 2011]. As well, Conibear *et al*. demonstrated that the presence of mutator variants can enhance microcolony-based growth initiation and, therefore, the biofilm development [Conibear TC *et al*, 2009]. All these findings suggest a strong and bidirectional link between the biofilm mode of growth and hypermutation.

Finally, the wide use of antibiotics in CF patients also contributes to mutagenesis. Several studies have demonstrated that, when administered at sublethal concentrations, antimicrobials can drive bacterial mutation [Rodríguez-Rojas A *et al*, 2012; Oliver A, 2010; Oliver A & Mena A, 2010]. Within the biofilm sublethal antibiotic concentrations are not rare as the extracellular polymeric matrix acts as a primary barrier preventing the entrance of polar and charged antibiotics [Lewis K, 2008] and, additionally, some of the matrix components such as the alginate or the extracromosomic DNA have shown antibiotic chelating activity [Alipour M *et al*, 2009].

1.7.4. Mutators and antibiotic resistance

Since the first description of hypermutable *P. aeruginosa* strains was made in CF CRI, a strong linkage between mutators and increased antibiotic resistance was noticed as mutators were encountered to be much more resistant than non-mutator CF isolates to each of the eight antipseudomonal agents tested. For instance, the percentage of ceftazidime

resistance reached 80% in hypermutable strains in contrast to a 30% documented for non-mutators; likewise, fluoroquinolone resistance increased from 5% in non-mutators to 40% in mutator variants [Oliver A *et al*, 2000].

Subsequent studies have confirmed and extended this observation, establishing a clear link between mutator phenotypes and multidrug resistant (MDR) profiles [Ciofu O *et al*, 2005; Henrichfreisse B *et al*, 2007; Hogardt M *et al*, 2007; Ferroni A *et al*, 2009]. Current management of CF patients include wide use of antibiotics, so this finding supports that amplification of mutators during CRI occurs along with the selection of antibiotic resistance mutations (adaptive mutations).

Given the high prevalence of P. aeruginosa mutator variants in the CF setting, one of the aims of this work was to define their impact in molecular epidemiology and in antibiotic resistance evolution and spread.

1.8. ACQUIRED ANTIBIOTIC RESISTANCE

In addition to its remarkable intrinsic resistance, *P. aeruginosa* shows an extraordinary capacity for further developing resistance to all available antibiotics. In general, bacteria can increase its intrinsic resistance by either acquiring horizontal resistance determinants and/or through the selection of certain chromosomal mutations that alter their expression and/or function.

1.8.1. Transferable resistance determinants in CF isolates

The CF airway hosts a complex microbiome [Lim YW *et al*, 2014] where genetic exchange could theoretically occur effectively, thus theoretically contributing to the emergence of antibiotic resistance. Most mobile antibiotic resistance genes are encoded on plasmids and transposons, but a recent study have also suggested that phages may also play an important role in the CF setting as the CF virome encodes more antimicrobial resistance sequences than the non-CF virome [Fancello L *et al*, 2011].

Among the transferable resistance determinants, extended spectrum b-lactamases and carbapenemeses are widely distributed worldwide but, with some exceptions, horizontal gene transfer of resistance determinants seems not to be frequent in *P. aeruginosa* [Oliver A *et al*, 2015], especially among CF isolates. Although biofilms are known to provide cell-to-cell contact and stabilise mating pair formation, biofilms theirselves appear to limit the horizontal plasmid spread through a combination of physicochemical and biological factors inherent to the spatial structure and heterogeneity of these structures [Stalder T & Top E, 2016]. However, it should be mentioned that although rare, in late years some authors have reported several cases of CF patients infections with *P. aeruginosa* isolates producing ESBL and/or carbapenemases, including IMP and VIM metallo- β-lactamases [Agarwal G *et al*, 2005; Cardoso O *et al*, 2008; Pollini S *et al*, 2011].

1.8.2. Mutation-driven resistance

In comparison with *P. aeruginosa* isolates causing acute infections, mutation-driven resistance has been shown to be the major contributor to antimicrobial resistance development in CF *P. aeruginosa* isolates [Ferroni A *et al*, 2009], development which is indeed catalyzed by the unusual high prevalence of mutators in the CF airways.

All antibiotics compounds are prone to being compromised by acquiring mutations that eventually lead to alter the expression of chromosomally-encoded resistance mechanisms or that modify the function of its encoded-protein. In *P. aeruginosa*, major mutational resistance mechanisms include overexpression of the chromosomal AmpC cephalosporinase, efflux pumps overexpression, porin loss or altered antibiotic targets (Table 1.3.).

Table 1.3. Mutation-driven resistance mechanisms in *P. aeruginosa.*

Mutation	Resistance mechanisms / Altered target	Antibiotics affected[a]
gyrA, gyrB	DNA gyrase	FQ
parC, parE	DNA topoisomerase IV	FQ
pmrAB	LPS (lipid A)	CO
phoQ	LPS (lipid A)	CO
colRS	LPS (lipid A)	CO
cprS	LPS (lipid A)	CO
parRS	LPS (lipid A)	CO
	OprD downregulation	IP, MP
	MexEF-OprN hyperproduction	FQ
	MexXY-OprM hyperproduction	FQ, AMG, PM
mexR	MexAB–OprM hyperproduction	FQ, TZ, PM, PPT, MP
nalC	MexAB-OprM hyperproduction	FQ, TZ, PM, PPT, MP
nalD	MexAB-OprM hyperproduction	FQ, TZ, PM, PPT, MP
nfxB	MexCD-OprJ Hyperproduction	FQ, PM
mexS	MexEF-OprN hyperproduction	FQ
	OprD downregulation	IP, MP
mexT	MexEF-OprN hyperproduction	FQ
	OprD downregulation	IP, MP
mvaT	MexEF-OprN hyperproduction	FQ
mexZ	MexXY –OprM hyperproduction	FQ, AMG, PM
PA5471.1	MexXY –OprM hyperproduction	FQ, AMG, PM
amgS	MexXY –OprM hyperproduction	FQ, AMG, PM
oprD	OprD porin inactivation	IP, MP
ampC	AmpC structural modification	PPT, TZ, PM, IP, MP
ampD	AmpC hyperproduction	TZ, PM, PPT
ampDh2	AmpC hyperproduction	TZ, PM, PPT
ampDh3	AmpC hyperproduction	TZ, PM, PPT
ampR	AmpC hyperproduction	TZ, PM, PPT
dacB	AmpC hyperproduction	TZ, PM, PPT
glpT	Transporter protein GlpT	FO
rpoB	RNA polymerase β-chain	RIF

β-lactam resistance mechanisms. Development of resistance to antipseudomonal penicillins (ticarcillin and piperacillin), cephalosporins (ceftazidime and cefepime) and monobactams (aztreonam) is the selection of mutations within PGN-recycling genes (*ampD, dacB, ampR*) that eventually leads to the constitutive overexpression of the chromosomal cephalosporinase AmpC [Cabot G *et al*, 2011; Juan C *et al*, 2005; Moyà B *et al*, 2009]. Besides *ampC* overexpression, recent studies have revealed that β-lactam resistance development, including novel β-lactam-β-lactamase inhibitor combinations such as ceftolozane/tazobactam, may also result from mutations leading to the structural modification of AmpC [Cabot G *et al*, 2014; Lahiri SD *et al*, 2014].

Beyond the chromosomal cephalosporinase AmpC, another contributing factor to β-lactam resistance is MexAB-OprM overexpression. This efflux system displays the broadest substrate profile (Table 1.1. and 1.3.) and its mutational overexpression determines reduced susceptibility to all β-lactams with the single exception of imipenem. MexAB-OprM-overproducing mutants can be readily generated *in vitro* in the presence of antibiotic by the selection of any mutational event leading to the inactivation or impairment of the *mexR*, *nalC* or *nalD* regulator genes. As well, these mutants have been shown to be very prevalent among multiresistant non-CF strains, and, of note, rates of MexAB-OprM overproducers of near 50% have been recorded in subpopulations of isolates exhibiting a reduced susceptibility to ticarcillin (≥32 μg/ml) [Li XZ *et al*, 2015].

Likewise, the mutational overexpression of MexXY or MexCD-OprJ can also confer resistance to cefepime. MexCD-OprJ overexpression, which is more frequent among *P. aeruginosa* isolates recovered from CRI not only confers increased cefepime resistance but have also been shown to determine hypersusceptibility to most β-lactams and aminoglycosides [Mulet X *et al*, 2011].

Finally, screenings of transposon mutant libraries have shown that inactivation of *galU*, a gene which code for an enzyme involved in the LPS core, increases ceftazidime and meropenem minimum inhibitory concentrations (MICs) [Dötsch A *et al*, 2009; Álvarez-Ortega C *et al*, 2010].

Carbapenem resistance mechanisms. Mutational inactivation of the porin OprD, together with the inducible expression of AmpC, confers resistance to imipenem and reduced susceptibility to meropenem [Livermore DM *et al*, 1992]. Indeed, the prevalence of imipenem resistant isolates frequently exceeds 20%, and nearly all them are OprD deficient [Cabot G *et al*, 2011; Riera E *et al*, 2011]. As well, MexAB-OprM mutational overexpression determines reduced susceptibility to meropenem and its overexpression plus OprD inactivation is one of the most relevant causes of clinical resistance to this carbapenem [Riera E *et al*, 2011].

Finally, although less frequent, mutation-driven resistance to carbapenems can also result from MexEF-OprN overexpression, as mutations within *mexT, mexS* and/or the ParRS two-component system not only lead to MexEF-OprN overexpression but also to OprD downregulation, which in turns determine a reduced susceptibility to carbapenems [Köhler T *et al*, 1999; Li XZ *et al*, 2015].

Aminoglycoside resistance mechanisms. Resistance to this antibiotic class has been clasically linked to the mutational overexpression of MexXY efflux pump, being its overexpression very frequent among clinical isolates and mainly caused by *mexZ*, *amgS*, or *parRS* mutations [Guénard S *et al*, 2014]. However, recent studies have revealed that the aminoglycoside mutational resistome extends far beyond MexXY overexpression and

several novel resistance determinants have been described; moreover accumulation of mutations within these genes can eventually lead to high-level antibiotic resistance [El'Garch F *et al*, 2007; Schurek KN *et al*, 2008].

Fluoroquinolone resistance mechanisms. Fluoroquinolone resistance in *P. aeruginosa* frequently results from gain-of-function mutations in topoisomerases, including DNA gyrases (GyrA/GyrB) and type IV topoisomerases (ParC/ParE) [Bruchmann S *et al*, 2013].

Besides, overexpression of all 4 major efflux-pumps systems also contributes to fluoroquinolone resistance (Table 1.1. and 1.3.). The overexpression of MexAB-OprM and MexXY-OprM is globally more frequent among clinical strains but its contribution to clinical fluoroquinolone resistance is likely more modest [Bruchmann S *et al*, 2013]. On the other hand, mutational overexpression of MexEF-OprN or MexCD-OprJ efflux pump is associated with high-level fluoroquinolone resistance, and although their prevalence is considered low except in the CF-CRI setting, recent data show that it might be higher than expected [Richardot C *et al*, 2015].

Polymyxin resistance mechanisms. The prevalence of polymyxin (polymyxin B and colistin) resistance is still very low (<5%) among *P. aeruginosa* isolates. Resistance to polymyxins most frequently results from the modification of the LPS caused by the addition of a 4-amino-4-deoxy-l-arabinose moiety in the lipid A structure [Olaitan AO *et al*, 2014] and the underlying mutations are frequently tracked to the PmrAB or PhoPQ two-component regulators, which in turns lead to the activation of the arnBCADTEF operon [Barrow K & Kwon DH, 2009]. More recent studies have also revealed that mutations within the two-component regulator ParRS, in addition to conferring colistin resistance due to the activation of the arnBCADTEF operon, lead to a MDR profile caused by the overprexpression of MexXY, MexEF and the repression of OprD [Muller C *et al*, 2011]. Finally, two additional two-component regulators, ColRS and CprRS, have also been shown to play a role in polymyxin resistance [Gutu AD *et al*, 2013]. Moreover, recent *in vitro* evolution assays have revealed, through WGS, the implication of additional mutations in high level colistin resistance, facilitated by the emergence of mutator (*mutS* deficient) phenotypes [Döβelmann B *et al*, 2017]. Particularly noteworthy among them are those occurring in LptD (essentaial OMP involved in LPS transport), LpxC (UDP-3-O-[hydroxymyristoyl]-N-acetylglucosamine deacetylase involved in lipid A biosynthesis) or MigA (α-1,6-rhamnosyltransferase, involved in the synthesis of the LPS core region [Döβelmann B *et al*, 2017].

P. aeruginosa possesses a complex and large genome, thus, and given the current gaps and the crutial role of mutatrion-driven resistance mechanisms for acquiring antibiotic resistance, one of the aims of this work was to decipher the P. aeruginosa mutational resistome.

1.9. *P. aeruginosa* POPULATION STRUCTURE: CF EPIDEMIC CLONES

Bacterial population structures can range from panmintic or fully sexual, showing random association between loci due to unrestricted recombination (such as *Neisseria gonorrhoeae*), to clonal, characterized by non-random association of alleles and evolving mainly through mutation (such as *Salmonella enterica*) [Smith JM *et al*, 1993].

Early studies suggested a panmintic population structure for *P. aeruginosa* [Denamur E *et al*, 1993; Picard B *et al*, 1994]. Kiewitz and Tümmler later described that *P. aeruginosa* shows a net-like population structure with a high frequency of recombination between isolates [Kiewitz C & Tümmler B, 2000]. Wide consensus was finally reached to conclude that *P. aeruginosa* has a non-clonal epidemic population structure [Curran B *et al*, 2004; Kidd TJ *et al*, 2012; Maâtallah M *et al*, 2011; Pirnay JP *et al*, 2009; Pirnay JP *et al*, 2002]. This means that the population structure of *P. aeruginosa*, similarly to that described in *N. meningitidis*, is composed of a limited number of widespread clones which are selected from a background of a large number of rare and unrelated genotypes that are recombining at high frequency.

Population structure analysis have also revealed that *P. aeruginosa* contains a conserved core and an accessory genome made up of extrachromosomal elements, such as plasmids and blocks of DNA inserted into the chromosome at several loci [Klockgether J *et al*, 2011]. The accessory genome is believed to be acquired through horizontal gene transfer (frequently phage-mediated) from different sources including other species.

Several experimental approaches have been used to define the population structure of *P. aeruginosa,* ranging from single loci to whole genome, mapping or sequencing [Curran B *et al*, 2004; Denamur E *et al*, 1993; Maâtallah M *et al*, 2013; Pirnay JP *et al*, 2009; Wiehlmann L *et al*, 2007]. Strategies based in the combined analysis of up to eight different genomic markers [O-serotype, total genome profile by fluorescent amplified-fragment length polymorphism analysis, nucleotide sequence of the OMP genes (*oprI, oprL*, and *oprD*), pyoverdine receptor gene profile (*fpvA* type and *fpvB* prevalence), prevalence of exoenzyme genes *exoS* and *exoU* and prevalence of group I pilin glycosyltransferase gene *tfpO*] [Pirnay JP *et al*, 2009] or a 58-binary genotypic markers microarray [Cramer N *et al*, 2012; Wiehlmann L *et al*, 2007] have provided very useful and complete information on the core and accessory genomes to define *P. aeruginosa* population structure. *P. aeruginosa* widespread C or PA14 clones, O11/O12 MDR nosocomial clones, or the LES were likely the most notorious successful epidemic lineages identified. Increasing access to WGS data is providing even more detailed information on the population structure and dynamics of epidemic and nonepidemic strains [Cramer N *et al*, 2011; Dettman JR *et al*, 2013; Jeukens J *et al*, 2014; Yang L *et al*, 2011]. However, despite only providing information on the core genome, the likely most popular standardized approach for the analysis of *P. aeruginosa* populations is still the multilocus sequence typing (MLST) scheme developed by *Curran et*

al. in 2004, based in the sequencing of 7 locci evenly distributed in the core genome of *P. aeruginosa*. They include *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*, considered housekeeping genes, and therefore not subjected to positive selection. However, mutation of *mutL*, encoding a component of the DNA MMR system, is a frequent cause of the mutator phenotypes, positively selected in CRI [García-Castillo M *et al*, 2012; Kidd TJ *et al*, 2012; Mena A *et al*, 2008; Oliver A *et al*, 2002a].

The MLST database (http://pubmlst.org/paeruginosa), although biased by the deposition of only a small fraction of the isolates, is a source of very valuable epidemiological information. As shown in Figure 1.9., most of the registered STs (June 4th 2015) are represented by single isolates but up to 18 of them are represented by more than 10 isolates from at least three different countries, thus likely indicating that they are successful clones. Among others they include the wide spread clone C (ST17) and PA14 (ST253) clones, the high-risk clones associated with MDR or extensively drug resistant (XDR) nosocomial infections (such as ST111, ST175, or ST235) or the CF epidemic clone ST146 (LES). Of note, many of these frequent clones are the founder clones of one of the 297 clonal groups or complexes detected by eburst analysis, each including from 2 to 116 STs [Oliver A *et al*, 2015].

As mentioned previously in section 1.5., during long time it was extensively accepted that early *P. aeruginosa* acquisition occurs from environmental sources. However, successful strains infecting a large proportion of CF patients are nowadays recognized and, in some cases, strongly associated with MDR profiles.

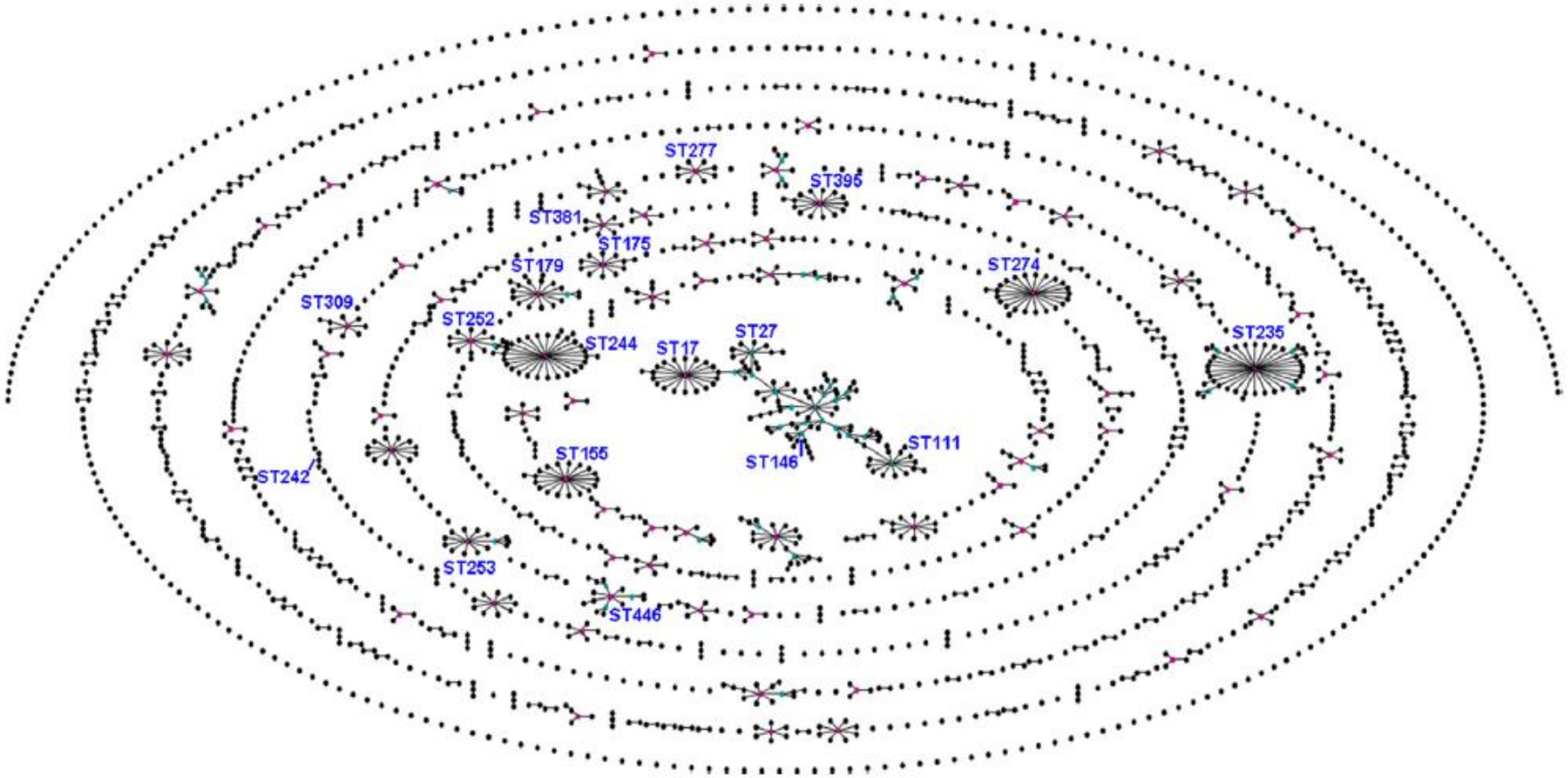


Figure 1.9. Population snapshot of *P. aeruginosa*. The 2106 STs listed on the *P. aeruginosa* PubMLST database (http://pubmlst.org/paeruginosa, 2015/06/03) are displayed in a single eBURST diagram by setting the group definition to zero of seven shared alleles. Each dot representes a ST, and lines connect single-locus variants. In each group of related STs the predictive primary founder is shown in pink, and subgroup founders are shown in blue. STs detected in at least 3 different countries with more than 10 recorded isolates are indicated; note that the ST corresponds to the primary founder or subgroup founder of the CC.

1.9.1. The Liverpool Epidemic Strain: a new paradigm in the CF setting

The LES is likely the most prominent epidemic clone infecting and chronically colonizing CF patients. It was originally described in the mid-1990s affecting a unique CF center in Liverpool [Cheng K *et al*, 1996] but it was soon detected in other CF centers across England [Scott FW & Pitt TL, 2004] and Scotland [Edenborough FP *et al*, 2004], and more recently, it has also been detected in CF patients from Canada [Aaron SD *et al*, 2010].

LES isolates show some unique characteristics. For instance, it cannot only infect previously uncolonized patients but also patients already colonized with unique *P. aeruginosa* strains [McCallum SJ *et al*, 2001] as well as non-CF patients [McCallum SJ *et al*, 2002]. Moreover, several studies have demonstrated that patients chronically infected with LES strains have a worse prognosis when compared with patients chronically colonized with unique non-clonal strains [Aaron SD *et al*, 2010; Al-Aloul M *et al*, 2004; Ashish A *et al*, 2012]; however, fortunately, being infected with this clone was not associated with poorer post-transplant outcomes [Srour N *et al*, 2015]. When compared with other CF strains, LES isolates are more frequently resistant to antibiotics and further resistance is also more likely to be developed over time [Ashish A *et al*, 2012; Salunkhe P *et al*, 2005; Tomás M *et al*, 2010].

In order to determine the underlying factors explaining the success of this epidemic clone among CF patients, environmental surveys have been performed but, to date, environmental LES isolates have only been detected in close temporospatial proximity to LES-colonized patients and therefore its high transmissibility cannot be explained by long-term environmental persistence [Panagea S *et al*, 2005]. Therefore, its successful transmission and lung colonization might be due to intrinsic phenotypic and genotypic features.

An unusual phenotype characterized by the overproduction of QS-regulated exoproducts, including pyocyanin and elastase A, is common among LES isolates and can persist within CF patients for several years. As this virulence-related exoproducts have a number of toxic effects directly relevant to CF, this unusual phenotype could explain the greater morbidity and mortality associated LES isolates and may play an important role in the success of this clone [Fothergill JL *et al*, 2007]. Moreover, it has been demonstrated that LESB58 produced more biofilm but was less motile than PAO1 and PA14, properties that can favor its persistence in the CF airway [Kukavica-Ibrulj I *et al*, 2008].

At the genomic level, this epidemic strain also exhibits some unique and relevant features. LESB58 was the first LES isolate sequenced and an accessory genome encoding many large genomic islands and prophages not previously found in other sequenced *P. aeruginosa* strains was revealed [Winstanley C *et al*, 2009]. At first, the presence of this accessory genomic material was thought to be essential for *in vivo* competitiveness [Winstanley C *et al*, 2009] but further comparative genomic studies with other representative LES isolates have

revealed a wide diversity in prophage and genomic islands among isolates [Carter ME *et al*, 2010; Jeukens J *et al*, 2014] even during very short episodes of exacerbations and antibiotic therapy [Fothergill JL *et al*, 2010; Fothergill JL *et al*, 2011]. Recently, it has been also suggested that LES phages may play an important role in host invasions and may confer a large fitness advantage during mixed infections by mediating bacteria-bacteria competition [Burns N *et al*, 2015]. Thus, this accessory genomic material seems to play an important role but further research is needed.

Another interesting point is the common coexistence of distinct LES lineages, exhibiting widely variable phenotypic and genotypic characteristics, within individuals [Williams D *et al*, 2015]. Nevertheless, this characteristic is not exclusive for this lineage and some authors have already demonstrated the coexistence of distinct lineages in the respiratory tract for other successful CF strains [Feliziani S *et al*, 2014, Marvig RL *et al*, 2013]. Thus, it seems that the phenotypic and the genetic diversity observed among LES isolates play a key role in the successful spread of this lineage throughout the CF population, diversity that is indeed enhanced by the frequent presence of mutators in the CF lung [Oliver A *et al*, 2000].

1.9.2. Other successful CF strains

Epidemic strains have also been detected in Australia, the so-called Australian Epidemic Strains AES-1 (also denominated Melbourne Epidemic Strain), AES-2, a cluster of related strains [Anthony M *et al*, 2002; Armstrong D *et al*, 2003; Armstrong DS *et al*, 2002] and, the more recently described, AES-3 [Bradbury R *et al*, 2008]. AES show increased antibiotic resistance, increased virulence gene expression and higher morbidity and mortality during CRI [Armstrong D *et al*, 2003; Griffiths AL *et al*, 2012; Hare NJ *et al*, 2012; Manos J *et al*, 2009; Naughton S *et al*, 2011; Tingpej P *et al*, 2010]. As occurs for LES, AES has not been recovered from any source other than the respiratory secretions of CF patients [Bradbury RS *et al*, 2009; Cramer N *et al*, 2012].

Besides these well-recognized CF epidemic strains, other *P. aeruginosa* MDR transmissible strains have been reported in European countries or Canada [Fluge G *et al*, 2001; Jelsbak L *et al*, 2007; Jones AM *et al*, 2001; Logan C *et al*, 2012; Luna RA *et al*, 2013; Parkins MD *et al*, 2014; Pedersen SS *et al*, 1986; Scott FW & Pitt TL, 2004; van Mansfeld R *et al*, 2009]. Overall, these strains show increased antibiotic resistance, long-term persistence within individuals, and are frequently associated with higher morbidity and mortality. Worldwide distribution of these strains is shown in Figure 1.10.

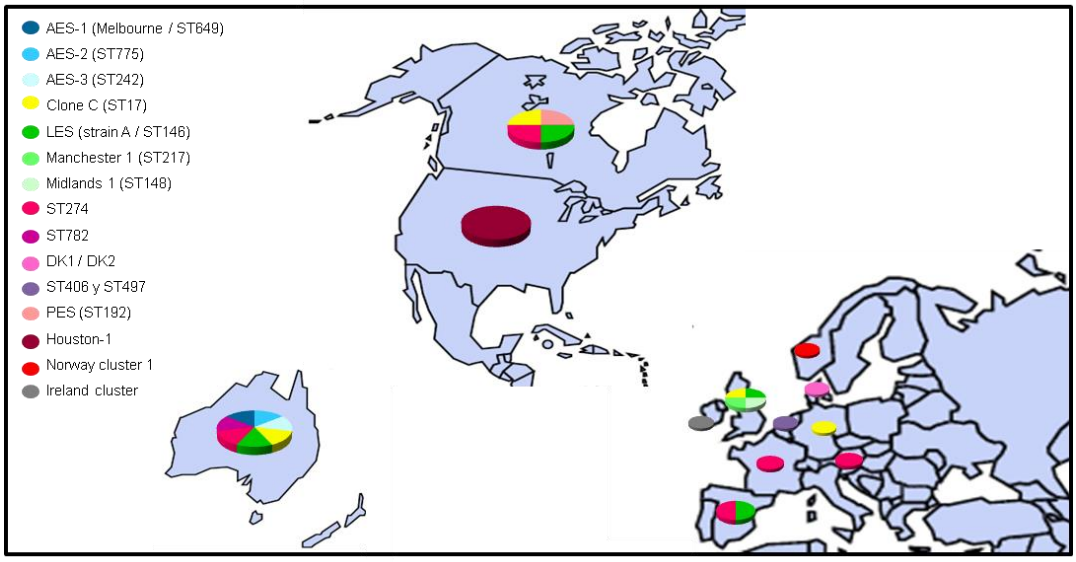


Figure 1.10. Worldwide distribution of *P. aeruginosa* CF epidemic/transmissible strains.

P. aeruginosa CF clonal epidemiology has remained unexplored among CF patients from the Balearic Islands and Spain. Thus, given the relevance of epidemic clones for CF patients outcome and management, one of the aims of this work was to define the P. aeruginosa population structure and to explore the presence of highly transmissible P. aeruginosa clones in our setting.

2. HYPOTHESIS AND OBJECTIVES

El alma y el cuerpo

P. aeruginosa CRI is the main cause of morbidity and mortality in CF individuals. One of the hallmarks of these infections, led by the opportunistic pathogen *P. aeruginosa*, is their long-term (lifelong) persistence despite of the host immune response and intensive antimicrobial therapies. Naturally equipped with a set of chromosomal genes that confer resistance to some antibiotic compounds (intrinsic resistome), *P. aeruginosa* can further develop resistance to virtually all available antimicrobials. Antimicrobial resistance in CF is a multifactorial problem which not only includes bacterial physiological changes, represented by the transition from the planktonic to the biofilm mode of growth, but also the acquisition of multiple chromosomal antibiotic resistance (mutational resistome) and adaptive mutations that eventually lead to a diversified infecting *P. aeruginosa* population. As well, in late years, there is increasing evidence suggesting that adaptation to the CF respiratory tract and antimicrobial resistance development may escape from the scale of the individual patients (epidemic strains).

Besides, recent advances in sequencing technologies have made it possible to obtain the whole genome of bacterial pathogens shaping up a new dimension to explore CF *P. aeruginosa* clonal epidemiology and antimicrobial resistance evolution. Therefore, the objectives of this thesis were:

1. To define the population structure of *P. aeruginosa* isolates infecting CF patients from the Balearic Islands and Spain.

2. To perform a longitudinal and cross-sectional analysis of the antibiotic susceptibility profiles and resistance mechanisms of CF *P. aeruginosa*.

3. To determine the role of mutators onto CF *P. aeruginosa* clonal epidemiology and onto the evolution and spread of antibiotic resistance.

4. To characterize by WGS approaches the phylogeny and evolution of widespread *P. aeruginosa* CC274.

5. To decipher the *P. aeruginosa* CC274 resistome evolution.

6. To assess the evolutionary dynamics and mechanisms of aminoglycoside resistance development *in vitro* and *in vivo*, given their key role in the management of CF patients.

3. MATERIALS AND METHODS

Palabras incomprendidas

3.1. LABORATORY STRAINS, PLASMIDS AND PRIMERS

Table 3.1. Laboratory strains and derived mutants used in this work.

Strain	Genotype / relevant characteristics	Reference
P. aeruginosa		
PAO1	Laboratory reference strain fully sequenced	Stover CK *et al*, 2000
PA14	Laboratory reference strain fully sequenced	He J *et al*, 2004
ATCC®27853™	Laboratory reference strain	
PAOAD	PAO1 Δ*ampD*::lox AmpD is an N-acetyl-anhydromuramyl-L-alanine amidase involved in PGN recycling as well as a negative transcriptional regulator of the *P. aeruginosa* chromosomal β-lactamase AmpC. Its inactivation increases the AmpC expression level.	Juan C *et al*, 2006
PAOMxR	PAO1 Δ*mexR*::lox MexR is a negative transcriptional regulator of *P. aeruginosa* efflux pump MexAB-OprM. Its inactivation results in overexpression.	Mulet X *et al*, 2011
PAOMxZ	PAO1 Δ*mexZ*::lox MexZ is a negative transcriptional regulator of *P. aeruginosa* efflux pump MexXY-(OprM). Its inactivation results in overexpression.	Martínez-Ramos *et al*, 2014
PAONB	PAO1 Δ*nfxB*::lox NfxB is a negative transcriptional regulator of *P. aeruginosa* efflux pump MexCD-OprJ and its own. Its inactivation increases overexpression.	Mulet X *et al*, 2009
PAOD1	PAO1 OprD null spontaneous mutant (W65X) OprD is an OMP related with carbapenems extrusion.	Moyà B *et al*, 2010
PAOMS	PAO1 Δ*mutS*::lox MutS is a component of the DNA MMR system. Its inactivation increases the spontaneous mutation rate by 2-3 log.	Mena A *et al*, 2008

Table 3.2. Plasmids used in this work

Plasmids	Genotype /relevant characteristics	Reference
pUCP24	Gentamycin^R, based in pUC18, *Escherichia-Pseudomonas* shuttle vector.	West SE *et al*, 1994
pUCP*mutS*	Gentamycin^R, pUCP24 harbouring the PAO1 WT *mutS* gene.	Oliver A *et al*, 2004
pUCP*mutL*	Gentamycin^R, pUCP24 harbouring the PAO1 WT *mutL* gene	Mena A *et al*, 2008

Table 3.3. Primers used in this work

Primer	Sequence 5'→3'	Product size (bp)	Use	Reference
acsA-F	ACCTGGTGTACGCCTCGCTGAC	842	MLST amplification	Curran B *et al*, 2004
acsA-R	GACATAGATGCCCTGCCCCTTGAT			
aroE-F	TGGGGCTATGACTGGAAACC	1053	MLST amplification	Curran B *et al*, 2004
aroE-R	TAACCCGGTTTTGTGATTCCTACA			
guaA-F	CGGCCTCGACGTGTGGATGA	940	MLST amplification	Curran B *et al*, 2004
guaA-R	GAACGCCTGGCTGGTCTTGTGGTA			
mutL-F	CCAGATCGCCGCCGGTGAGGTG	940	MLST amplification	Curran B *et al*, 2004
mutL-R	CAGGGTGCCATAGAGGAAGTC			
nuoD-F	ACCGCCACCCGTACTG	1042	MLST amplification	Curran B *et al*, 2004
nuoD-R	TCTCGCCCATCTTGACCA			
ppsA-F	GGTCGCTCGGTCAAGGTAGTGG	989	MLST amplification	Curran B *et al*, 2004
ppsA-R	GGGTTCTCTTCTTCCGGCTCGTAG			
trpE-F	GCGGCCCAGGGTCGTGAG	811	MLST amplification	Curran B *et al*, 2004
trpE-R	CCCGGCGCTTGTTGATGGTT			
acsA-F2	GCCACACCTACATCGTCTAT	390	MLST sequencing	Curran B *et al*, 2004
acsA-R2	AGGTTGCCGAGGTTGTCCAC			
aroE-F2	ATGTCACCGTGCCGTTCAAG	495	MLST sequencing	Curran B *et al*, 2004
aroE-R2	TGAAGGCAGTCGGTTCCTTG			
guaA-F2	AGGTCGGTTCCTCCAAGGTC	372	MLST sequencing	Curran B *et al*, 2004
guaA-R2	GACGTTGTGGTGCGACTTGA			
mutL-F2	AGAAGACCGAGTTCGACCAT	441	MLST sequencing	Curran B *et al*, 2004
mutL-R2	GGTGCCATAGAGGAAGTCAT			
nuoD-F2	ACGGCGAGAACGAGGACTAC	366	MLST sequencing	Curran B *et al*, 2004
nuoD-R2	TGGCGGTCGGTGAAGGTGAA			
ppsA-F2	GGTGACGACGGCAAGCTGTA	369	MLST sequencing	Curran B *et al*, 2004
ppsA-R2	GTATCGCCTTCGGCACAGGA			
trpE-F2	TTCAACTTCGGCGACTTCCA	441	MLST sequencing	Curran B *et al*, 2004
trpE-R2	GGTGTCCATGTTGCCGTTCC			
exoS-F	TCAGGTACCCGGCATTCACTACGCGG	534	*exoS* amplification	Feltman H *et al*, 2001
exoS-R	TCACTGCAGGTTCGTGACGTCTTTCTTTTA			
exoU-F	AGCGTTAGTGACGTGCG	1546	*exoU* amplification	Feltman H *et al*, 2001
exoU-R	GCGCATGGCATCGAGTAATG			
rpsl-1	GCTGCAAAACTGCCCGCAACG	250	mRNA *rpsL* qRT-PCR	Oh H *et al*, 2003
rpsl-2	ACCCGAGGTGTCCAGCGAACC			
acrna-F	GGGCTGGCCTCGAAAGAGGAC	246	mRNA *ampC*qRT-PCR	Juan C *et al*, 2006
acrna-R	GCACCGAGTCGGGGAACTGCA			
mexB-U	CAAGGGCGTCGGTGACTTCCAG	273	mRNA *mexB* qRT-PCR	Oh H *et al*, 2003
mexB-L	ACCTGGGAACCGTCGGGATTGA			
mexD-U	GGAGTTCGGCCAGGTAGTGCTG	236	mRNA *mexD* qRT-PCR	Oh H *et al*, 2003
mexD-L	ACTGCATGTCCTCGGGGAAGAA			
mexF-U	CGCCTGGTCACCGAGGAAGAGT	254	mRNA *mexF* qRT-PCR	Oh H *et al*, 2003
mexF-L	TAGTCCATGGCTTGCGGGAAGC			

Table 3.3. Primers used in this work. (Cont.)

Primer	Sequence 5'→3'	Product size (bp)	Use	Reference
mexY-F	TGGAAGTGCAGAACCGCCTG	270	mRNA *mexY* qRT-PCR	Peña C *et al*, 2009
mexY-R	AGGTCAGCTTGGCCGGGTC			
mexZ-F	ATTGGATGTGCATGGGTG	980	*mexZ* amplification and sequencing	Sobel ML*et al*, 2003
mexZ-R	TGGAGATCGAAGGCAGC			
AD-F	GTACGCCTGCTGGACGATG	910	*ampD* amplification and sequencing	Juan C *et al*, 2006
AD-R	GAGGGCAGATCCTCGACCAG			
dacB-F	CGACCATTCGGCGATATGAC	1400	*dacB* amplification and sequencing	Moyà B *et al*, 2009
dacB-R	CGCGTAATCCGAAGATCCATC			
dacB-IF	GCCAGGGCAGCGTACCGC		*dacB* sequencing	Moyà B*et al*, 2009
dacB-IF2	GTGCTCAACGGCAACCTCTAC			
dacB-IR	GTCGCGCATCAGCAGCCAG			
oprD-F	CGCCGACAAGAAGAACTAG	1413	*oprD* amplification and sequencing	Juan C *et al*, 2010
oprD-R	GTCGATTACAGGATCGACAG			
oprD-F1	ATGCTGAAGTGGGGCGAGATG		*oprD* sequencing	Juan C *et al*, 2010
oprD-F2A	GCAGGCCACTTCACCGAGG			
oprD-F3A	GATTATATCGGCTTCGGC			
oprD-R2	GTCGAGCCCTTCGAATTCGC			
gyrA-1	TTATGCCATGAGCGAGCTGGGCAACGACT	364	*gyrA* QRDR amplification and sequencing	Juan C *et al*, 2010
gyrA-2	AACCGTTGACCAGCAGGTTGGGAATCTT			
gyrB-3	AGCTCGCAGACCAAGGACAAG	600	*gyrB* QRDR amplification and sequencing	Juan C *et al*, 2010
gyrB-4	GGGCTGGGCGATGTAGATGTA			
parC-1	ATGAGCGAACTGGGGCTGGAT	208	*parC* QRDR amplification and sequencing	Juan C *et al*, 2010
parC-2	ATGGCGGCGAAGGACTTGGGA			
parE-1	CGGCGTTCGTCTCGGGCGTGGTGAAGGA	592	*parE* QRDR amplification and sequencing	Juan C *et al*, 2010
parE-2	TCGAGGGCGTAGTAGATGTCCTTGCCGA			
mutS-F1	TTAACATTACCCTCTTTTGCAC	2687	*mutS* amplification and sequencing	Mena A *et al*, 2008
mutS-R1	TCATTTTCTAGTTCTCTCCTCA			
mutS-F4	CGCTCCGCTCCAGGACAGCGC		*mutS* sequencing	Mena A *et al*, 2008
mutS-F5	CGGCTGCCTGCTCGCCTAC			
mutS-F6	CATTCGGCGGAGGGCTACCTG			
mutS-R6	TGGCGGTTTCGCTCATCTCCAC			
mutS-F11	TTCCTGATGGACCTGGAAGCG			

Table 3.3. Primers used in this work. (Cont.)

Primer	Sequence 5'→3'	Product size (bp)	Use	Reference
mutL-F	CGATGATCGCCCAGCGCT	2299	*mutL* amplification and sequencing	Mena A *et al*, 2008
mutL-R	TCCGCCGGGTCGCGGATA			
mutL-F2	TAGCGCGCCTGACCATGA		*mutL* sequencing	Mena A *et al*, 2008
mutL-F3	GCGCATGGTGCGCGACAA			
mutL-F4	GCCTCCGGCGGCTCCTCCG			
mutL-R2	GCAGGTCGGCGATGACAT			

3.2. *Pseudomonas aeruginosa* CYSTIC FIBROSIS ISOLATES

3.2.1. The Balearic Islands *P. aeruginosa* collection.

Since 2003, all *P. aeruginosa* isolates recovered from routine respiratory cultures from CF patients attending the Son Espases University Hospital in Palma de Mallorca (former Son Dureta), reference hospital of the Balearic Islands, have been regularly stored frozen at -80°C. Culture, isolation and identification of *P. aeruginosa* from respiratory samples have always been carried out following the current established microbiological diagnostic procedures and expert recommendations. In this work, different subsets from this huge collection were used as detailed below.

In 2010, with the aim of determine the long-term clonal epidemiology and antibiotic resistance evolution of *P. aeruginosa* CRI in CF patients from the Balearic Islands, 10 sequential isolates from each of 10 chronically colonized CF patients were studied. The 10 included patients were selected based on the fulfilment of the following criteria: (i) wider follow-up period, (ii) higher temporal distribution of isolates and (iii) inclusion of the first *P. aeruginosa* isolate within the 8-year studied period (2003-2010). Likewise, each of the sequential isolates included per patient were separated by at least a 6-month interval.

Occasioned by the results obtained when long-term clonal epidemiology was explored, in 2013 we decided to extend the molecular epidemiology studies to all CF patients, including both children and adults, which had been attended at Son Espases CF Units since 2003. For this purpose, last available *P. aeruginosa* isolate of each CF patient was included. From 2003 to 2013, more than 50 CF patients had been attended at Son Espases University Hospital adult and paediatric CF Units and about the 80% had had a positive sputum culture for *P. aeruginosa* at some time point; thus, a total of 40 isolates were studied. Infection-colonization patterns and basic demographic data were recorded.

3.2.2. The Spanish *P. aeruginosa* collection.

From 2013 to 2014 the first Spanish multicentre study on the microbiology of CF was conducted. The study involved 24 CF Units, 12 paediatric and 12 adult, from 17 different Spanish hospitals. In Spain no national CF patient registry exists and, therefore, the precise number of people suffering from CF is still unknown. Nevertheless, participating hospitals are the reference ones within their geographical areas, attending the majority of the Spanish CF population, thus, a representative patient population from across Spain was included (Figure 3.1.) [de Dios Caballero *et al*, 2016].

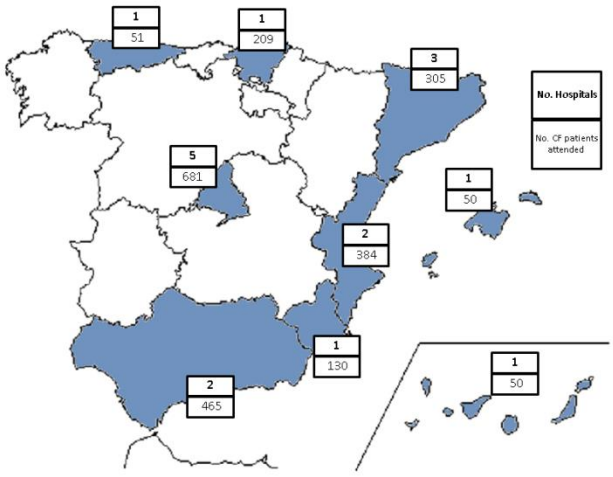


Figure 3.1. Geographical distribution of the participating hospitals and number of CF patients attended.

Fifteen consecutive patients per CF Unit were recruited and a single sputum sample from each was immediately frozen after collection at −80°C and sent to the Ramon y Cajal University Hospital in Madrid for microbiological culture. Samples were plated in appropiate culture media and plates were examined at 24 and 48 h. In order to identify potentially slow-growing bacteria, the incubation time was extended to 5 days [see details in *de Dios Caballero et al*, *2016*]. Colonies with compatible *P. aeruginosa* morphology were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics GmbH, Leipzig, Germany). Finally, all recovered *P. aeruginosa* isolates were collected and frozen for further studies.

From a total of 341 respiratory samples cultured, 79 *P. aeruginosa* were recovered from 75 different CF patients, setting a global colonization rate of 22%, and being higher in the adult population (33%) than in the pediatric population (10%). Infection-colonization patterns, basic demographic data and main patients' characteristics were recorded.

3.2.3. The 274 clonal complex *P. aeruginosa* collection.

The CC274 *P. aeruginosa* collection included 29 isolates, 28 of which had been recovered from 18 different CF patients from two highly-distant countries, Australia and Spain, and 1 blood culture isolate from a Spanish non-CF patient, covering up to an 18-year period from 1995 to 2012.

All isolates had been previously classified within the CC274 (defined as sharing at least 5 alleles with ST274) based on MLST available protocols and databases (http://pubmlst.org/paeruginosa/). All the Australian and 4 CF Spanish isolates were single isolates recovered from different patients attending clinical settings located in different geographical areas, selected randomly from those available. In addition, we included 4 sequential *P. aeruginosa* isolates, each separated by at least 6-month intervals, from each of 4 chronically colonized CF patients attended at Son Espases University Hospital, thus representing intrapatient clone evolution. Sampling time and geographic origin of CC274 *P. aeruginosa* isolates is represented in Figure 3.2.

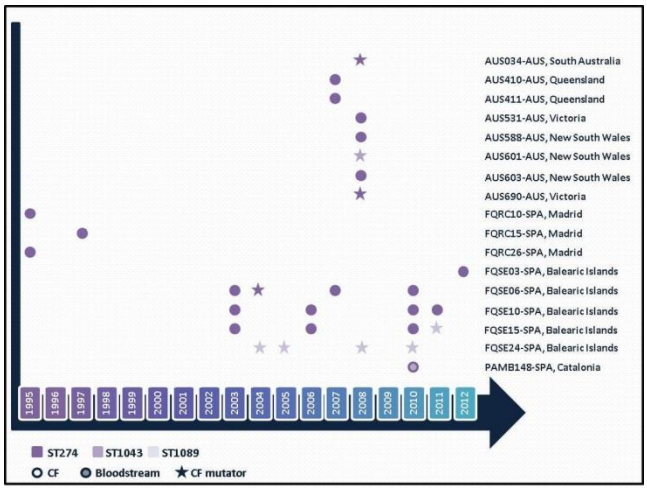


Figure 3.2. CC274 *P. aeruginosa* collection. Sampling time from the 29 studied isolates can be inferred from the X axis. Isolates are labelled according to the following format: Patient identification - Country (AUS: Australia; SPA: Spain), Region

3.2.4. Colony morphotype

Morphotype was assessed plating *P. aeruginosa* isolates onto MHA and by visual examination after 24 and 48 hours of aerobic incubation at 37ºC. Afterwards, isolates were classified as regular, mucoid or SCV morphotype.

Morphotype was investigated in all isolates from the Spanish CF collection and in those from the Balearic Islands collection collected between 2003 and 2010.

3.3. PAO1 *P. aeruginosa IN VITRO* EVOLUTION EXPERIMENT UNDER AMINOGLYCOSIDE PRESSURE

To determine and in-depth study the dynamics of *P. aeruginosa* resistance development to aminoglycosides, 10-ml Mueller-Hinton broth (MHB) tubes (Annex 1) containing 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 mg/l of tobramycin were inoculated with approximately 10^6 CFU/ml of exponentially growing *P. aeruginosa* PAO1 reference strain and incubated for 24 h at 37°C and 180 r.p.m. After incubation, the tubes from the highest antibiotic concentration showing growth were reinoculated, at a 1:1,000 dilution, in fresh 10-ml MHB tubes containing tobramycin concentrations up to 1024 mg/l and incubated again for 24 h at 37ºC and 180 r.p.m. This step was repeated during 14 consecutive days in order to get PAO1-derived tobramycin high-resistant mutants. This procedure was performed in quintuplicate.

At days 1, 7 and 14, two colonies from the highest antibiotic concentration tubes showing growth were purified in antibiotic-free MHA plates (Annex 1) and frozen at -80ºC for further characterization.

3.4. MOLECULAR EPIDEMIOLOGY STUDIES

3.4.1. Pulsed-field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) is a highly discriminatory and reproducible typing method used to determine the genetic relationship between microbial isolates from the same species level which is based on the analysis of the chromosomic DNA. Basically, this genotyping method consists in fragmenting the bacterial chromosomic DNA by using appropriate restriction endonucleases of low cut frequency and then separate the resulted DNA fragments by PFGE in order to obtain a unique macrorestriction pattern for each microbial isolate and, eventually, compare all these unique molecular fingerprints to determine their genetic relationship. In this work, previously defined protocols [Kaufmann ME, 1998] with slight modifications were used, as set forth in detail below.

P. aeruginosa isolates were grown in suspension on 5 ml of Brain Heart Infusion (BHI) broth (Annex 1) under aerobic conditions for 16-20 hours at 37ºC and shaking at 180 r.p.m. Afterwards, a volume of 250 µl (500 µl for mucoid phenotype isolates) was centrifuged at 13000 r.p.m. for 5 minutes and bacterial pellets were washed twice with PIV solution (Annex 1). Then, bacterial pellets were resuspended in 200 µl of this PIV solution, mixed with an equal volume of 1.6% low-melting temperature agarose (Annex 1) at 42ºC and, finally, this molten mixture was used to prepare plugs in adequate molds (Bio-Rad). Once the agarose was set, the plugs were collected and incubated within 1 ml of EC-Lysis solution (Annex 1) at 37ºC for at least 5 hours and, then, in 1 ml of ESP solution (Annex 1) at 50ºC for 16-20 hours in order to release DNA from bacteria embebbed in the agarose plugs. After overnight incubation, ESP solution was removed and plugs were washed in quintuplicate with TE buffer (Annex 1), rinsed with 1 ml of sterile distilled water at 37ºC for at least 10 minutes, transferred to microfuge tubes containing 20 units of *SpeI* restriction enzyme (New England BioLabs) and incubated in appropriate conditions (20 hours, 37ºC). Then, each plug was soaked in 1 ml of TE buffer at 37ºC for 1 hour. In the meantime, a 1% megabase agarose gel (Annex 1) was prepared and, once soaked, plugs were placed and sealed in individual wells of the gel. Right after, fragments were separated in a CHEF- DR III contour-clamped homogeneous-electric-field apparatus (Bio-Rad, La Jolla, CA) in standard 0.5X TBE running buffer (Annex 1) chilled at 14ºC for 26 hours at 6V/cm with pulse times of 5 to 40 s and using a 120º included angle. After electrophoresis, the gel was stained in 0.5X TBE buffer with either a 1 µg/ml solution of ethidium bromide or with an appropriate solution of RedSafe 20.000X and photographed under UV light.

This genotyping method was used to infer clonal relatedness among isolates within the two studied subsets of the Balearic Islands *P. aeruginosa* collection (longitudinal and cross sectional), as well as for isolates from the Spanish and the CC274 collections. For this purpose, the criteria established by *Tenover et al*. [Tenover FC *et al*, 1995] were applied with

the single exception of inferring the genetic relationship within the CC274 *P. aeruginosa* collection in which the Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering method was used [CLIQS 1D Pro, Totallab].

3.4.2. Multilocus sequence typing

Multilocus Sequence Typing (MLST) is a molecular genotyping tool that not only results into highly discriminatory but also electronically portable data between laboratories worldwide. This unambiguous typing method is based in the use and comparison of the nucleotidic sequences of internal fragments of approximately 450-500 bp of seven established house-keeping genes. For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or ST.

In this work the MLST scheme for *P. aeruginosa* proposed by *Curran B et al.*, which is based in the house-keeping genes *acsA* (acetil coenzyme A synthetase), *aroE* (shikimate dehydrogenase), *guaA* (GMP synthase), *mutL* (DNA MMR protein), *nuoD* (NADH dehydrogenase I chain C, D), *ppsA* (phosphoenolpyruvate synthase) and *trpE* (anthralite synthetase component I), was applied [Curran B *et al*, 2004; http://pubmlst.org/paeruginosa/]. Briefly, workflow was as follows.

Genomic DNA was obtained by using a commercially available extraction kit (DNeasy Blood & Tissue kit, Qiagen or High Pure PCR template preparation kit, Roche Diagnostics) and polymerase chain reaction (PCR) amplification of the seven house-keeping genes was performed (primers and PCR Master Mix details in Table 3.3 in section 3.1. and Annex 1, respectively) under the next reaction conditions: initial denaturation at 94ºC for 12 min; 35 cycles of denaturation at 94ºC for 1 min, primer annealing at 59ºC (60ºC for *mutL* amplification) for 1 min, extension at 72ºC for 1 min; followed by a final extension step of 72ºC for 10 min. Hereafter, PCR amplification products were purified and sequenced with the BigDye Terminator kit (PE Applied Biosystems) on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems, Foster City, CA). The *P. aeruginosa* MLST database (https://pubmlst.org/paeruginosa/) was used to assign an allele to each resulting sequence and to assign a ST.

This technique was performed within the two studied subsets of the Balearic Islands collection (see above), as well for the isolates from the Spanish CF *P. aeruginosa* collection. Phylogenetic relationship was assessed by constructing a Minimum Spanning Tree (MST) using the goeBURST algorithm, available at www.phyloviz.net ST were considered to belong to a same CC when sharing at least five of the seven sequenced loci.

3.4.3. Array-tube genotyping

Wiehlmann and collaborators recently developed a species-specific oligonucleotide-microarray for genotyping *P. aeruginosa* isolates [Wiehlmann L *et al*, 2007] based on the Array Tube platform (Alere Technologies GmbH, Jena, Germany) which is currently commercially available. This microarray enables *P. aeruginosa* genotyping by using 13 informative single nucleotide polymorphisms (SNP) at conserved loci, the *fliCa*/*fliCb* multiallelic locus and the presence or absence of the *exoS*/*exoU* marker gene; SNP pattern which is eventually converted into a 4 letter-code that can easily be used and exchanged for the identification of a clone. Additionally, this system also includes 38 genetic markers from the accessory genome for analysis of microevolutionary events within a clone or Array Tube genotype, thus allowing definition of intraclonal diversity. Up to 10 genome islets and 6 genomic islands can be detected, including the ferripyoverdine receptor genes (*fpvA*) type I, IIa, IIb and III; the alternative type-I ferripyoverdine receptor gene *fpvB*; the flagellin glycosylation island; the *P. aeruginosa* Genomic Island type 1 (PAGI-1); the genomic islands of the CLC family PAGI-2/3, the *P. aeruginosa* Pathogenicity Islands type 1 and type 2 (PAPI-1 and PAPI-2) as well as other pKLC102-like islands (Figure 3.3.).

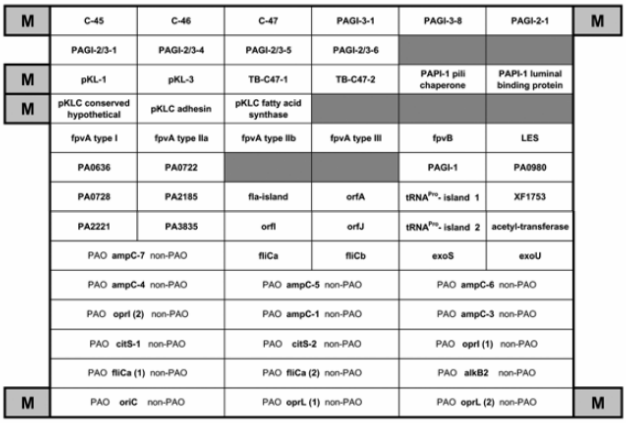


Figure 3.3. Core and accessory genome markers disposition in the Alere™ Array Tube *P. aeruginosa* species-specific microarray.

This microarray was employed for further characterization of the Spanish CF collection. All procedures were performed according to the manufacturer's protocol. Briefly, RNA-free unfragmented genomic DNA from pure and monoclonal *P. aeruginosa* isolates were obtained by using a commercially available extraction kit (High Pure PCR template preparation kit, Roche Diagnostics) and after digestion with 4 µl of RNase A 100 mg/ml solution (Qiagen). After, obtained genomic DNA was amplified approximately 50-fold and internally labelled with biotin-11-dUTP using a linear amplification protocol. Then, a multiplex primer extension reaction was performed with two nested primers per target in each cycle and resulting biotin labelled single-strand DNA (ssDNA) was transferred and hybridised to the DNA oligonucleotide microarrays and red by using the ArrayMate Reader (Alere).

Extended information about probes and primers has been collected in Annex 2. For further details please see literature [Wiehlmann L *et al*, 2007] or the manufacturer's *P. aeruginosa* Genotyping Kit 2 manual available at https://alere-technologies.com/.

The Type 3 Secretion System (T3SS). To further characterize the type 3 Secretion System (T3SS) protein effectors (ExoS, ExoU) independent specific PCR assays were performed. Protocols previously described by *Feltman H* and collaborators were used with slight modifications [Feltman H *et al*, 2001]. Primers and PCR Master Mix details are collected in Table 3.3. and Annex 1, respectively. Reaction conditions were as follow: initial denaturation at 94°C for 12 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec and a final extension step of 10 min at 72°C. Amplified DNA products were resolved by electrophoresis on agarose 1% w/v gels.

3.5. ANTIMICROBIAL SUSCEPTIBILITY PROFILES AND RESISTANCE MECHANISMS

3.5.1. Antimicrobial susceptibility testing

3.5.1.1. *P. aeruginosa* clinical isolates

One of the most important tasks in a Clinical Microbiology Laboratory is to determine the antimicrobial susceptibility profiles of significant bacterial isolates. Currently, there is not a universal standardized method that perfectly reproduces the conditions in which infecting bacteria are grown *in vivo* and different technical approaches can be used. This point is particularly striking in the CF setting, especially at chronic stages in which bacteria change their mode of growth from planktonic to biofilm and in which there is, indeed, a high prevalence of mucoid and hypermutable isolates and, therefore, mutant resistant subpopulations frequently rise.

In this work different susceptibility testing methods were used. Regardless of the technical approach used, it should be highlighted that the following CF expert recommendations were in all cases taken into account: (i) the incubation time was extended up to 24 hours (36-48 hours for slow growing variants) and (ii) a 0.5 or 1 McFarland standard suspension was used for inoculum standardization of non-mucoid or mucoid isolates, respectively.

MICs for all isolates belonging to both, the Balearic Islands and the CC274 *P. aeruginosa* collection, were determined by using commercially available strips containing a gradient of antibiotic concentrations in MHA plates. Standard suspensions were prepared in accordance with morphotypes and MICs were recorded after aerobic incubation of plates at 37°C. The antimicrobials compounds tested included the antipseudomal cephalosporins ceftazidime (TZ) and cefepime (PM), the carbapenems imipenem (IP) and meropenem (MP), the quinolone ciprofloxacin (CI), the aminoglycoside tobramycin (TM) and the polymyxin colistin (CO) (AB bioMèrieux, Solna, Sweden). Besides, for selected subsets of these *P. aeruginosa* collections, MICs for the monobactam aztreonam (AT) (AB bioMèrieux, Solna, Sweden), for amikacin (AK) and for the β-lactam β-lactamase combinations piperacillin/tazobactam (PPT) and ceftolozane/tazobactam (TOL/TAZ) (Liofilchen) were also determined. *P. aeruginosa* PAO1 reference strain was used as control.

In the case of the Spanish *P. aeruginosa* CF collection the disk-difussion method was used to determine the antibiotic susceptibility profiles, except for fosfomycin (FO) for which the agar dilution method was performed [Díez-Aguilar M *et al*, 2013]. The antimicrobials tested included the antipseudomal cephalosporins ceftazidime and cefepime, the monobactam aztreonam, the penicillin- β-lactamase combination piperacillin/tazobactam, the carbapenems imipenem and meropenem, the quinolones ciprofloxacin and levofloxacin (LE), the aminoglycosides tobramycin, gentamicin (GM) and amikacin, and the polymyxin colistin.

Procedures were performed in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org). *P. aeruginosa* PAO1 and ATCC27853 reference strains were used as controls.

Analysis of the data from the Balearic Islands and the Spanish CF *P. aeruginosa* collections was additionally performed taking into account patients' infection-colonization pattern and colony morphotype. Temporal evolution of antibiotic resistance was also explored within the Balearic Islands collection. It should also be mentioned that in the data analysis of the Balearic Islands collection just one isolate per patient, semester, morphotype and antibiotype was included to avoid any bias.

3.5.1.2. *P. aeruginosa* laboratory strains

MICs for *P. aeruginosa* PAO1 reference strain and its evolved derivatives obtained from the *in vitro* experiment under aminoglycoside treatment pressure (section 3.3.) were determined by broth microdilution using customized Sensititre® plates (reference FRCNRP; Thermo Fisher Scientific). These customized plates included the following compounds: ceftolozane/tazobactam, ceftazidime, cefepime, piperacillin/tazobactam, aztreonam, imipenem, meropenem, ciprofloxacin, ticarcillin, tobramycin, amikacin and colistin. Aminoglycosides MICs (GM, AK, TM) were also determined by broth microdilution according to EUCAST (www.eucast.org) and the International Standards Organisation guidelines. *P. aeruginosa* PAO1 and ATCC27853 reference strains were used as controls.

3.5.1.3. Clinical breakpoints and definitions

EUCAST clinical breakpoints for systemic infections were applied (www.eucast.org) for both, MICs and zone diameter inhibition, interpretation. Different versions have been applied in this work, the specific version used in each experiment/analysis is detailed within the results section (Annex 3).

Antibiotic susceptibility profiles were classified in accordance with Magiorakos *et al.* [Magiorakos AP *et al*, 2012] proposed criteria. Subsequently, MDR *P. aeruginosa* was defined as non-susceptibility to at least 1 antibiotic agent in at least 3 antipseudomonal antibiotic classes; XDR *P. aeruginosa* was defined as non-susceptible to at least 1 agent in all but 1 or 2 antipseudomonal antimicrobial categories and, finally, *P. aeruginosa* isolates non-susceptible to all antipseudomonal antibiotics classes were classified as pan-drug resistant (PDR). Likewise, an isolate was considered to be hypersusceptible to an antibiotic compound when the determined MIC was at least two-fold lower than *P. aeruginosa* PAO1 reference strain MIC.

3.5.2. Relative expression of chromosomically encoded *P. aeruginosa* resistance genes by real time qRT-PCR

The expression of the genes encoding the chromosomal β-lactamase AmpC (*ampC*) and four *P. aeruginosa* efflux pumps representative components, MexAB-OprM (*mexB*), MexCD-OprJ (*mexD*), MexXY (*mexY*), and MexEF-OprN (*mexF*), were determined by real-time quantitative Reverse Transcription-PCR (qRT-PCR).

P. aeruginosa isolates were grown in 10 ml of Luria-Bertani (LB) broth (Annex 1) at 37°C and 180 r.p.m. to the late-log-phase (optical density at 600 nm [OD_{600}] of 1) and collected by centrifugation. Total RNA was obtained by using the RNeasy minikit (Qiagen), dissolved in RNAse-free water and treated with 2 units of TURBO DNA-free™ (Ambion) during 30 minutes at 37ºC for remove any contaminating DNA trace. Reaction was then stopped with 5 µl of DNase inactivation reagent (Ambion) and adjusted to a final RNA concentration of 50 ng per µl. A 50 ng sample of purified RNA was then used for one-step reverse transcription and qRT-PCR amplification using the QuantiTect SYBR green RT-PCR kit (Qiagen, Hilden, Germany) on either a SmartCycler II instrument (Cepheid, Sunnyvale, CA) or an Eco real-time PCR system (Illumina). Previously described primers were used for the amplification of *ampC*, *mexB*, *mexY*, *mexD*, *mexF* and *rpsL* (gene used as a reference to normalize the relative amount of mRNA). Primers and PCR Master Mix details are collected in Table 3.3. and Annex 1, respectively. Real time qRT-PCR conditions were as follows: reverse transcription at 50ºC for 20 min, followed by Taq activation at 95ºC for 15 min and 40 cycles of 95°C for 15 sec, 62°C for 30 sec and 72°C for 30 sec, measuring fluorescence emission in the second step of each amplification cycle. As controls, previously characterized knockout mutants and/or clinical strains overexpressing these antibiotic resistance mechanisms were used.

Isolates were considered positive for AmpC, MexCD-OprJ, MexEF-OprN or MexXY(-OprM) overexpression when the corresponding mRNA levels (*ampC*, *mexD*, *mexF* or *mexY*) were at least 10-fold higher than that of *P. aeruginosa* PAO1 reference strain, negative if lower than 5-fold, and borderline if between 5- and 10-fold. Likewise, isolates were considered positive for *mexB* overexpression when the corresponding mRNA level was at least 3-fold higher than that of PAO1, negative if lower than 2-fold and borderline if between 2- and 3-fold. All real time qRT-PCRs were performed in duplicate and mean values (± standard deviations) of mRNA levels were obtained from three independent experiments.

Relative expression of *ampC*, *mexB*, *mexD*, *mexF* and *mexY* was investigated in the subset of 100 CF *P. aeruginosa* isolates (10 sequential isolates from each of 10 chronically colonized patients) of the Balearic Islands and in all isolates from the CC274 collection. As well, *mexY* relative expression was investigated in all PAO1 derivatives isolates obtained in the *in vitro* experiment evolution under aminoglycoside treatment pressure.

3.5.3. Isolation and analysis of the outer membrane protein OprD

The presence or absence of the carbapenem porin OprD was evaluated by analyzing the OMPs profiles. For this purpose, a protocol adapted from those previously described by Filip *et al* was followed [Filip C *et al*, 1973]. First, *P. aeruginosa* isolates were grown in 10 ml of LB broth (Annex 1) at 37°C and 180 r.p.m. to the late-log-phase (optical density at 600 nm [OD_{600}] of 1) and about 3 ml were collected by centrifugation, washed and resuspended in 5ml of cold Tris-Mg buffer pH 7.3 (Annex 1). Cells were then sonicated (4 cycles of 35 sec, ON/OFF pulses of 5/2 sec, amplitude 10%) and centrifuged at 5000 r.p.m. for 2 min to eliminate unbroken cells. Carefully supernatant fluids were transferred and centrifuged at full speed for 30 min at 4ºC to pellet cell envelopes. Afterwards, the obtained pellets were twice resuspended in 1.5 ml of 1% sodium lauryl sarcosinate in Tris-Mg buffer, incubated for 30 min at room temperature and centrifuged again at full speed for 30 min at room temperature. These pellets were finally resuspended in 40 µl of Laemmli's electrophoresis sample buffer (BioRad), boiled for 5 min, centrifuged for remove any insoluble material and stored at -4ºC. Once isolated, OMPs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Coomassie staining. Stacking and separating gel compositions as well as running buffer details are collected in Annex 1. An unstained protein standard ladder (Precision Protein™ Standards, BioRad) was also loaded to detect the presence or absence of OprD (45 kDa). *P. aeruginosa* reference strain PAO1 and its OprD null spontaneous mutant PAOD1 were used as controls (Table 3.1.).

The presence or absence of the carbapenem porin OprD was evaluated within the 100 *P. aeruginosa* CF isolates chronically colonizing 10 CF patients from the Balearic Islands.

3.5.4. DNA sequencing of *P. aeruginosa* antibiotic-resistance related genes

When needed, for isolates showing AmpC or MexXY overexpression, genes encoding their main transcriptional regulators (*ampD dacB*, *mexZ*) were fully sequenced. Briefly, workflow was as follows. Genomic DNA was obtained by using a commercially available extraction kit (DNeasy Blood & Tissue kit, Qiagen or High Pure PCR template preparation kit, Roche Diagnostics) and PCR amplification of the regulatory genes was performed (primers and PCR Master Mix details in Table 3.3. and Annex 1, respectively) under the following reaction conditions: initial denaturation at 94ºC for 12 min; 35 cycles of denaturation at 94ºC for 1 min, primer annealing at 60ºC or 64ºC (for *mexZ* or *ampD*/*dacB*, respectively) for 1 min, extension at 72ºC for 1 min; followed by a final extension step of 72ºC for 10 min. Hereafter, PCR amplification products were purified and sequenced with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA) on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems) using appropiate primers (Table 3.3.).

As well, other antibiotic resistance related genes such as *oprD*, *gyrA*, *gyrB*, *parC* and/or *parE*, were sometimes sequenced in order to explain or confirm the documented antibiotic

susceptibility profiles. The procedure was exactly as it has been described above but using appropriate primers and adjusting PCR conditions (Table 3.3.)

In all cases, obtained DNA sequences were compared with PAO1 DNA and protein sequences using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST.)

3.6. MUTATOR PHENOTYPE AND GENETIC BASIS FOR HYPERMUTATION

3.6.1. Estimation of mutation frequencies

In order to determine which *P. aeruginosa* isolates had an increased spontaneous mutation rate, rifampicin (RIF) resistance mutant frequencies were determined following previously described protocols with slight modifications [Mena A *et al*, 2008; Oliver A *et al*, 2000].

First, a screening was performed in order to detect all *P. aeruginosa* isolates showing an increased spontaneous mutation rate. For this purpose, bacterial suspensions containing approximately $5 \cdot 10^8$ CFU/ml were prepared and a volume of 100 µl was plated on MHA plates supplemented with 300 mg/l of RIF (MHA-RIF) (Annex 1). After 36 h (48 h for slow growing variants) of incubation at 37ºC, plates were carefully examined. In the absence of *P. aeruginosa* colonies, the corresponding isolate was considered to have a normal mutation rate; otherwise, isolates were considered to have an increased spontaneous mutation rate and, therefore, its mutation frequency was estimated as following described.

For each *P. aeruginosa* isolate, 5 independent 10 ml MHB-containing tubes (Annex 1) were inoculated with approximately 10^3 bacterial cells and incubated overnight at 37ºC and 180 r.p.m. in aerobic conditions. Then, cells were recovered by centrifugation (4ºC, 10 min, 3000 r.p.m.) and pellets were resuspended in 1 ml of saline sterile solution. Finally, 1:10 serial dilutions were prepared and plated onto MHA plates and onto RIF-supplemented MHA plates (MHA-RIF) (Annex 1). MHA and MHA-RIF plates were incubated at 37ºC for 24 h and 36h, respectively (36-48 h for slow growing variants). Once incubated, colonies were counted and mutation frecuencies were estimated as the median number of mutant colonies (colonies grown in MHA-RIF/ml) divided by the median number of total cells (colonies grown in MHA/ml). Following previous recommendations [Mena A *et al*, 2008; Oliver A *et al*, 2000], isolates with a RIF resistance mutant frequency higher than $2 \cdot 10^{-7}$ were classified as hypermutators.

In all these procedures, both, *P. aeruginosa* PAO1 reference strain and its derivative hypermutator knockout mutant defective in *mutS*, PAOMS, were used as controls (see Table 3.1. section 3.1.).

Screening was performed in all CF *P. aeruginosa* isolates from the Spanish and the CC274 collection, as well as in the 100 CF isolates chronically infecting the 10 selected patients of the Balearic Islands. Mutation frequencies were determined for all mutator isolates detected in both, the Spanish CF collection and the CC274 collection.

3.6.2. Mismatch repair system deficiency complementation assays

Genetic basis for hypermutation were explored in all *P. aeruginosa* isolates with a demonstrated increased spontaneous mutation rate.

Deficiencies within the MMR system are the most frequent cause of bacterial hypermutation [Mena A *et al*, 2008; Oliver A *et al*, 2000]. Thus, to characterize the mutator phenotype, complementation assays using WT MMR genes *mutS* and *mutL* were performed.

For this end, gentamycin resistant plasmids pUCP*mutS* and pUCP*mutL*, harbouring *P. aeruginosa* PAO1 WT *mutS* and *mutL* genes respectively, were electroporated into the mutator isolates as follows. Plasmid pUCP24 was also electroporated as control cloning vector (Table 3.2.). First, isolates were inoculated in 50ml-tubes containing 5 ml of LB broth (Annex 1) and incubated overnight at 37ºC with shaking at 180 r.p.m. in aerobic conditions. Then, 1 ml of the overnight culture was inoculated into 50 ml of fresh LB broth (Annex 1) and incubated at 37ºC and 180 r.p.m. until the log-phase (optical density at 600 nm [OD_{600}] of 0.5). After, the flask was chilled on ice for 10 min and cells were collected by centrifugation (4ºC, 15 min, 3000 r.p.m.), washed and finally resuspended in 500 µl of sterile chilled Sucrose Magnesium Electroporation Buffer (SMEB) buffer (Annex 1).

Electrocompetent *P. aeruginosa* cells were aliquoted (100 µl), incubated on ice with each of the purified plasmids (5 µl) for 10 min and electroporated on a Gene Pulser Xcell (BioRad) under the following conditions: voltage 2.5 kV, pulse time 2 min, resistance 200 Ω, capacitance 25 µF. Finally, electroporated cells were recovered, incubated for 1h at 37ºC and 180 r.p.m. in 1 ml of Super Optimal broth with Catabolite repression (SOC, Annex 1) and plated onto MHA-GEN plates (Annex 1). After 36-48 h of incubation at 37ºC, independent transformant colonies harbouring pUCP24, pUCP*mutS* and pUCP*mutL* were selected and their mutation frequencies were estimated as previously described (section 3.6.1). A positive complementation result was obtained when the mutation frequency decreased back to basal levels, $1 \cdot 10^{8}$ approximately.

3.6.3. *mutS* and *mutL* sequencing

To complete the genetic characterization of hypermutation and, based on complementation assays results, *mutS* or *mutL* genes were fully sequenced. Briefly, after obtaining genomic DNA by using a commercially available extraction kit (DNeasy Blood & Tissue kit, Qiagen or High Pure PCR template preparation kit, Roche Diagnostics), PCR amplification was performed (see primers and PCR Master Mix details in Table 3.3. and Annex 1, respectively) under the following reaction conditions: initial denaturation at 94ºC for 12 min; 35 cycles of denaturation at 94ºC for 1 min, primer annealing at 60ºC/62ºC for 3 min (*mutS*/*mutL*, respectively), extension at 72ºC for 1 min; followed by a final extension step of 72ºC for 10 min. Then, PCR amplification products were purified and sequenced with the BigDye

Terminator kit (PE Applied Biosystems, Foster City, CA) on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems) and appropiate primers (Table 3.3.).

Obtained DNA and protein sequences were compared with *P. aeruginosa* PAO1 DNA sequence using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST).

All *P. aeruginosa* isolates with a demonstrated increased spontaneous mutation rate were studied.

3.7. WHOLE GENOME SEQUENCING

WGS approaches were used for in-depth study of the CC274 *P. aeruginosa* collection and for the genotypic characterization of PAO1 aminoglycoside-resistant derivatives (variant calling and genome annotation).

3.7.1. Library preparation and sequencing methodology

P. aeruginosa whole genome DNA sequences were obtained as follows. First, genomic DNA was obtained by using a commercially available extraction kit (High Pure PCR template preparation kit, Roche Diagnostics). DNA purity was assessed with a NanoDrop (ThermoScientifics) using the UV absorbance ratios 260/280nm and 260/230nm; DNA samples with ratio values outside ranges 1.8-2.0 and 2.0-2.2, respectively, were excluded. Then, genomic DNA was quantified using a fluorometric-based method (Quant-iT™ PicoGreen ® dsDNA assay kit, LifeTechnologies) and adjusted to a final concentration of 0.1ng/µl.

Therefore, indexed paired-end libraries were prepared with the Nextera® XT DNA library preparation kit (Illumina Inc, USA) according to the manufacturer's protocol with slight modifications. Briefly, genomic DNA (0.5 ng) was first tagmented and, shortafter, amplified and indexed using a limited-cycle PCR program. Libraries were then cleaned-up and normalized by using either, the manual or the bead-based normalization approaches. For manual normalization a fluorometric-based method was used (Quant-iT™ PicoGreen ® dsDNA assay kit, LifeTechnologies), and the conversion factor 1 ng/µl = 1.5 nM was applied to prepare a 4 nM library. The selection of this conversion factor was based on the results obtained on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip. Once normalized, libraries were pooled and appropriately denatured and diluted to result in a 20 pM denatured pooled library that was finally loaded in, either, a MiSeq Reagent Kit v2 or v3 (Illumina inc., USA), and sequenced on an Illumina Miseq ® benchtop sequencer, resulting in 250 bp paired-end reads.

Nextera® XT DNA Library Prep Kit reference guide (#15031942), MiSeq System Denature and Dilute Libraries guide (#15039740) and MiSeq® System User Guide (#15027617) available on Illumina webpage (https://www.illumina.com/index-d.html) can be consulted for further procedures details.

3.7.2. Variant calling

Previously defined and validated protocols were used with slight modifications [Marvig RL *et al*, 2013; Marvig RL *et al*, 2015b]. In short, after mapping obtained 250 bp paired-end reads to the *P. aeruginosa* PAO1 reference genome (GenBank accession number: NC_002516.2) by using Bowtie 2 v2.2.4. (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml), pileup and raw files were generated by using SAMtools v0.1.16

(https://sourceforge.net/projects/samtools/files/samtools/) and PicardTools v1.140 (https://github.com/broadinstitute/picard). Additionally, the Genome Analysis Toolkit (GATK) v3.4-46 (https://www.broadinstitute.org/gatk/) was used for realignment around InDels. Then, from raw files, SNPs were extracted if they met the following criteria: a quality score (Phred-scaled probability of the samples reads being homozygous reference) of at least 50, a root-mean-square (RMS) mapping quality of at least 25 and a coverage depth of at least 3 reads; excluding all ambiguous variants. As well, microInDels were extracted from the totalpileup files applying the following criteria: a quality score of at least 500, an RMS mapping quality of at least 25 and support from at least one-fifth of the covering reads. Pipeline and used scripts are detailed in Annex 4.

For exceptional cases, some positions were indeed manually and individually checked in raw and pileup files without applying any filtering.

3.7.3. De novo assemblies

Sequence reads from each isolate were de novo assembled using Velvet v1.2.10 (https://www.ebi.ac.uk/~zerbino/velvet/) with a k-mer length of 31 and the following parameters: scaffolding = no, ins_length = 500, cov_cutoff = 3, and min_contig_lgth = 500. The script used for this purpose is detailed in Annex 4.

3.7.4. Phylogenetic reconstructions and Beast analysis

Core genome phylogenetic reconstructions were performed using the Parsnp tool from the Harvest Suite package v1.2 with default parameters but forcing the inclusion of all desired genomes (-c) and selecting randomly the reference genome (r!) (http://harvest.readthedocs.io/en/latest/content/parsnp.html).

Bayesian analysis of divergence times was performed using BEAST v2.4.2 (http://beast2.org/). For this purpose, a nexus file including all the curated positions at which at least one of the isolates differed from the reference strain PAO1 was constructed and converted into an.xml file with BEAUTi. Hereafter, BEAST was run with the following user-determined settings; a lognormal relaxed molecular clock model and a general time-reversible substitution model with gamma correction. Divergence times were calculated from a chain length of 50 million steps, sampled every 1,000 steps and discarding the first 5 million steps as a burn-in. Finally, the maximum clade credibility tree was generated using the TreeAnnotator program from the BEAST package and tree parameters were calculated with Tracer v1.6 (http://beast.bio.ed.ac.uk/Tracer). Used scripts are detailed in Annex 4.

Both Phylogenetic reconstructions were displayed using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

3.7.5. Genome annotation: resistome and mutome profiling

SNP and InDels files were annotated by using SnpEff software v4.2 (http://snpeff.sourceforge.net/index.html) with default options.

For the CC274 *P. aeruginosa* collection, annotated SNP and InDels files were then filtered based on an exhaustive literature review that led us to obtain a set of 164 genes known to be related to chromosomal antibiotic resistance in *P. aeruginosa* (see Annex 5). Additionally, the online available tool ResFinder v2.1 (https://cge.cbs.dtu.dk//services/ResFinder/) was used to identify possible horizontally acquired antimicrobial resistance genes. As well, the genetic basis of hypermutation was investigated from WGS data through the analysis of an exhaustive panel of so called mutator genes, thus designated mutome. Genes included within the mutome panel are collected in Annex 5.

4. RESULTS

El alma y el cuerpo

4.1. POPULATION STRUCTURE AND ANTIBIOTIC RESISTANCE OF *Pseudomonas aeruginosa* CYSTIC FIBROSIS RESPIRATORY INFECTIONS

4.1.1. Clonal epidemiology studies

4.1.1.1. Longitudinal analysis of the Balearic Islands collection

A total of 100 *P. aeruginosa* isolates from the Balearic Islands CF collection were studied, including 10 sequential isolates from each of 10 different CF patients covering up an 8-year study period (section 3.2.1.).

Clonal relatedness among isolates was first evaluated by Pulse-field Gel Electrophoresis (PFGE) and the obtained restriction patterns were analysed by using the criteria proposed by *Tenover et al* [Tenover FC *et al*, 1995]. Analysis revealed the presence of 13 different clone types. PFGE clones distribution among the 10 different patients along the 8-year study period is represented in Figure 4.1.

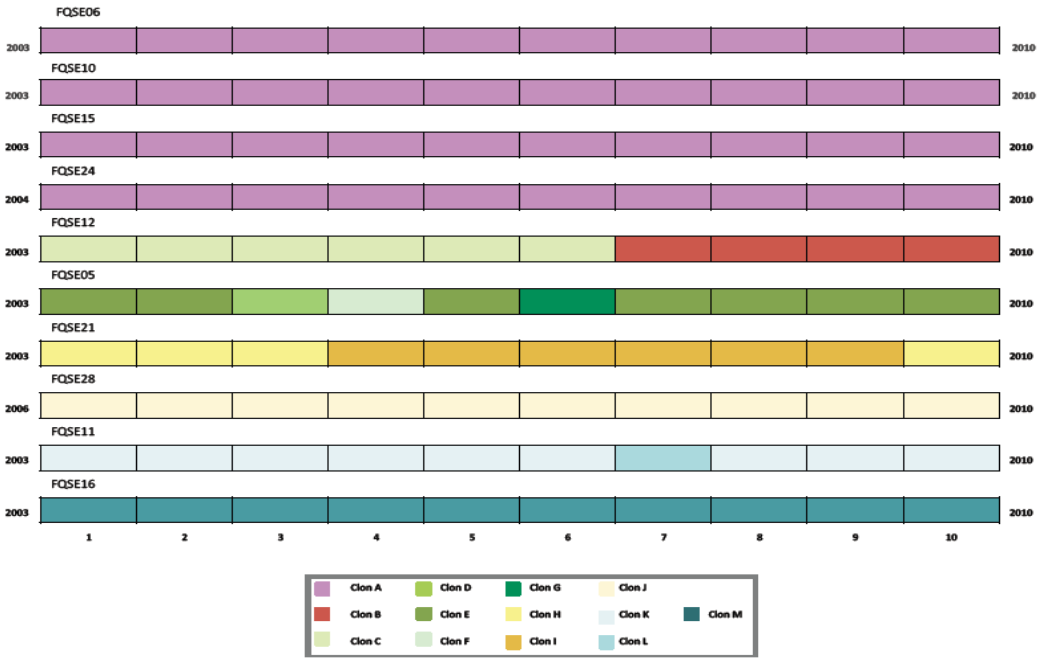


Figure 4.1. PFGE clones distribution. Each bar represents a different patient and each colour represents a different PFGE clone. Isolation years from the first and last isolate included are indicated in the left and right axes, respectively.

As shown, six of the patients showed a single PFGE clone over the whole study period, including those colonized by the designated clone FQSE-A. Conversely, the other four patients showed the coexistence of several clones (2 to 4) or clonal replacements (Figure 4.1.). With the single exception of FQSE-A, all other PFGE clones were detected in unique patients.

First isolate per patient and PFGE clone were further analysed by MLST, yielding 13 different STs not entirely coincident with the clones identified by PFGE (Table 4.1.). Designated FQSE-C and FQSE-D PFGE clones were determined to be the same ST (ST299) and, conversely, the disseminated clone FQSE-A yielded two different ST closely related. Whereas clone FQSE-A isolates from 3 of the 4 colonized patients were identified as ST274, isolates from the fourth patient were identified as a new ST (ST1089). It should also be highlighted the documented superinfection with the LES-1 (ST146) in one of the studied patients (FQSE12, see Figure 4.1. and Table 4.1.).

As highlighted in Table 4.1., up to 8 of the 13 STs encountered had not been previously described (https://pubmlst.org/paeruginosa/), being, indeed, each of them detected in single patients. Of note, just the allelic profile of ST1089 includes new allele sequences, resulting all others from new allele combinations of previously described allele sequences. ST1089 *acsA* and *guaA* non-previously described alleles sequences just differed from those of ST274 by two single point mutations.

Table 4.1. Allelic profiles and associated PFGE clones of the 13 different ST detected.

PFGE clone	ST[a]	Allelic profile[a]						
		acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*
FQSE-A	ST274	23	5	11	7	1	12	7
FQSE-A	**ST1089**	**66**	5	**101**	7	1	12	7
FQSE-B	ST146	6	5	11	3	4	23	1
FQSE-C/D	ST299	17	5	36	3	3	7	3
FQSE-E	**ST1108**	6	3	17	7	3	4	7
FQSE-F	**ST1072**	5	13	25	6	1	7	3
FQSE-G	ST155	28	5	36	3	3	13	7
FQSE-H	**ST1088**	36	27	28	3	4	13	1
FQSE-I	**ST1109**	16	14	3	11	1	15	1
FQSE-J	**ST1071**	5	3	57	6	1	33	42
FQSE-K	**ST701**	29	1	9	13	1	6	23
FQSE-L	ST254	6	5	58	11	3	4	37
FQSE-M	**ST1073**	28	5	36	3	4	10	95

[a]Non-previously described alleles and STs are indicated in bold.

4.1.1.2. Cross-sectional analysis of the Balearic Islands collection

Half of the patients which had had a positive sputum culture for *P. aeruginosa* since 2003 to 2013 were female, being their median age in 2013 of 20.3 years (0-45 years). Most of these patients were chronically colonized by *P. aeruginosa* (n=22) or showed an intermittent infection-colonization pattern (n=12) at the time of the study. Age distribution and associated *P. aeruginosa* infection-colonization patterns have been plotted in Figure 4.2.

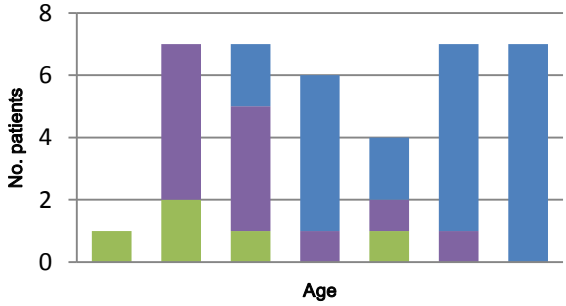


Figure 4.2. Age distribution and infection-colonization pattern of CF patients with a positive sputum culture for *P. aeruginosa* (2003-2013). Colonization patterns are indicated in blue (chronic), purple (intermittent) and green (primocolonization).

Clonal relatedness among isolates was also studied by both, PFGE and MLST. Analysis of PFGE restriction fragments yielded 31 different patterns, collected in Table 4.2.

Table 4.2. PFGE and MLST results summary of the 40 studied isolates.

PFGE Clone	Colonization pattern (No. patients)	ST[a]	Allelic profile[b]						
			acsA	*aroE*	*guaA*	*nuoD*	*mutL*	*ppsA*	*trpE*
FQSE-A	Chronic (4)	ST274	23	5	11	7	1	12	7
	Chronic (1)	**ST1089**	**66**	5	**101**	7	1	12	7
FQSE-C	Chronic (1)	ST299	17	5	36	116	3	7	3
FQSE-F	Chronic (1)	**ST1072**	5	13	25	6	1	7	3
FQSE-I	Chronic (2)	**ST1109**	16	14	3	11	1	15	1
FQSE-K	Chronic (2)	**ST701**	29	1	9	13	1	6	23
FQSE-N	Chronic (1), Intermittent (2)	ST312	5	3	57	6	1	33	47
FQSE-O	Chronic (1), Primocolonization (1)	ST1339	113	5	24	3	1	6	25
FQSE-P	Intermittent(1)	ST277	39	5	9	11	27	5	2
FQSE-Q	Intermittent (1)								
FQSE-R	Intermittent (1)	ST198	11	5	11	11	3	27	7
FQSE-S	Intermittent (1)	ST253	4	4	16	12	1	6	3
FQSE-T	Intermittent (1)	ST279	5	3	57	3	1	33	47
FQSE-U	Chronic (1)	ST285	16	22	6	74	2	41	2
FQSE-V	Intermittent (1)	ST308	13	4	5	5	12	7	15
FQSE-W	Intermittent (1)	ST386	17	5	11	18	4	10	3
FQSE-X	Primocolonization (1)	ST395	6	5	1	1	1	12	1
FQSE-Y	Chronic (1)	ST505	6	20	1	11	4	4	2
FQSE-Z	Intermittent (1)	ST606	23	5	57	30	1	4	3
FQSE-AA	Primocolonization (1)	ST938	15	20	26	13	3	64	2
FQSE-AB	Chronic (1)	ST1527	17	10	129	5	4	112	10
FQSE-AC	Chronic (1)	ST1613	17	5	36	5	4	10	1
FQSE-AD	Primocolonization (1)	ST1637	11	5	3	3	8	1	9
FQSE-AE	Chronic (1)	**ST1837**	28	62	17	3	13	13	7
FQSE-AF	Chronic (1)	**ST1838**	5	1	112	5	1	26	119
FQSE-AG	Intermittent (1)	**ST1839**	17	5	12	9	14	4	7
FQSE-AH	Chronic (1)	**ST1840**	28	5	36	3	2	13	7

[a]Non-previously described alleles and STs are indicated in bold.

Table 4.2. PFGE and MLST results summary of the 40 studied isolates. (Cont.)

PFGE Clone	Colonization pattern (No. patients)	ST[a]	Allelic profile[b]						
			acsA	*aroE*	*guaA*	*nuoD*	*mutL*	*ppsA*	*trpE*
FQSE-AI	Chronic (1)	**ST1841**	15	5	36	17	27	4	2
FQSE-AJ	Primocolonization (1)	**ST1842**	11	5	30	72	4	4	7
FQSE-AK	Primocolonization (1)	**ST1843**	1	5	17	16	3	4	7
FQSE-AL	Intermittent (1)	**ST2188**	101	84	11	3	4	4	7
FQSE-AM	Intermittent (1)	**ST2189**	11	5	3	3	93	1	9

[a]Non-previously described alleles and STs are indicated in bold.

A total of five patients (12.5%) showed clone FQSE-A restriction pattern (section 4.1.1.1.), being indeed all of them chronically colonized. Clone FQSE-N and clones FQSE-I, FQSE-K and FQSE-O, were detected in 3 and 2 different CF patients, respectively (Table 4.2.). Within the 3 patients harbouring clone FQSE-N two were siblings, as well as the 2 patients harbouring clone FQSE-I.

When typed by MLST a total of 31 different STs were also found and, with the single exception of clone FQSE-A, the different PFGE patterns were all related with unique sequences types (Table 4.2.). Conversely, the same ST (ST277) was determined for PFGE clones FQSE-P and FQSE-Q.

Of the 31 STs detected, 13 were first reported in this work (41.9%). Nevertheless, just two alleles sequences had not been previously described and, thus, most new STs resulted from new allele combinations (Table 4.2.). Based on their MLST allelic profile, a MST was constructed to infer relatedness among isolates (Figure 4.3.).

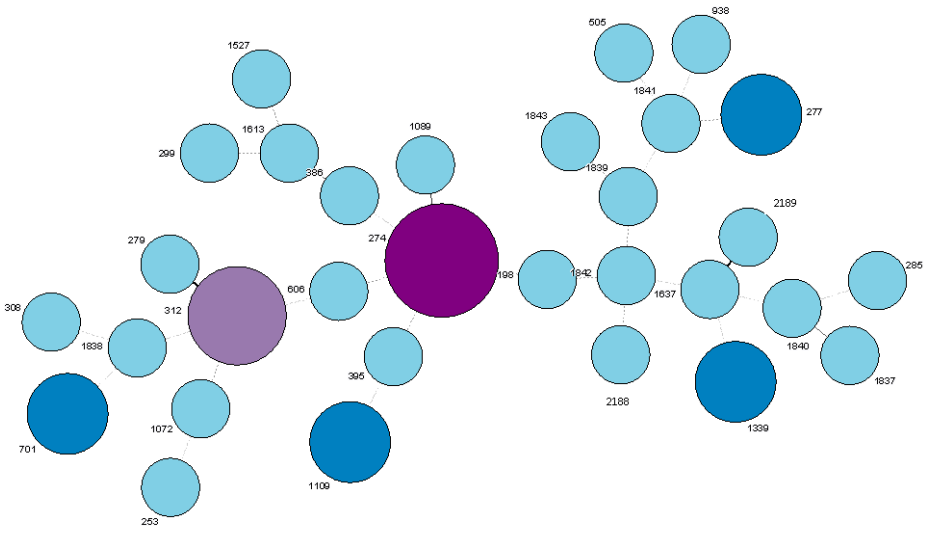


Figure 4.3. MST from the 40 *P. aeruginosa* isolates.

4.1.1.3. Cross-sectional analysis of the Spanish collection

About half of the patients which had had a positive sputum culture for *P. aeruginosa* were female (38/75), being their median age at time of sputum collection of 25.7 (7-51) years. Most of these patients were chronically colonized by *P. aeruginosa* (n=64), whereas just 11 patients showed an intermittent infection-colonization pattern. Primary patients' characteristics are summarized in Table 4.3.

Table 4.3. Primary characteristics of patients colonized by *P. aeruginosa*.

CF Unit	No. patients	Age in years	Colonization pattern (No. patients)	No. Δ508 mutationsa[a]	
				HT	HM
Paediatric	16	15.4 (7-17)	Chronic (15) Intermittent (1)	3	8
Adult	59	28.5 (18-51)	Chronic (49) Intermittent (10)	28	18

[a]HT: heterozygosis, HM: homozygosis.

Considerable genetic diversity was documented among the *P. aeruginosa* Spanish CF isolates. By PFGE, 70 different restriction patterns were observed among the 79 typed isolates. As well, 72 different STs were detected, each grouping 1 to 3 isolates. Up to 48 (67%) had not been previously described (http://pubmlst.org/paeruginosa/) and most resulted from new allele combinations (83.3%), as just 8 new alleles sequences were defined in this work. The Array Tube genotyping technique enabled the detection of 51 new and 14 previously described Array Tube genotypes, containing one to three isolates each (Table 4.4.).

Table 4.4. Genotyping results obtained from the analysis of the Spanish CF collection.

CF Unit	Isolate[a]	PFGE[b]	Array Tube	ST[c]	MLST allelic profile[c]						
					acsA	*aroE*	*guaA*	*nuoD*	*mutL*	*ppsA*	*trpE*
A	1	1	X298	1748	16	5	30	11	4	13	7
B	2	2	6018	**1886**	7	133	36	63	74	15	2
	3	3	6198	**1887**	11	10	1	61	27	4	7
C	4	3	XX98	**1888**	11	10	1	3	4	4	7
	5	4	8782	**1889**	15	121	36	5	10	15	8
D	6	5	F669	**1890**	5	1	11	13	10	7	23
	7	6	4818	**1891**	17	83	1	61	3	4	2
	8	7	7D98	**1866**	1	5	1	98	1	10	10

[a] Different morphotypes in the same CF patient (from 1 to 4).

[b] Isolates exhibiting similar or identical PFGE patterns have been highlighted in grey.

[c] Non-previously described alleles and STs are indicated in bold.

Table 4.4. Genotyping results obtained from the analysis of the Spanish CF collection. (Cont.)

CF Unit	Isolate[a]	PFGE[b]	Array Tube	ST[c]	MLST allelic profile[c]						
					acsA	*aroE*	*guaA*	*nuoD*	*mutL*	*ppsA*	*trpE*
E	9	8	CC0A	1109	16	14	3	11	1	15	1
	10	9	8C2C	**1867**	6	94	**133**	1	1	12	1
	11	10	2C22	**1868**	23	5	11	98	1	12	7
	12	11	6FA8	**1869**	15	24	5	5	50	4	14
F	13	12	F468	**1870**	17	11	3	13	1	2	4
	14	13	AD80	**1892**	17	5	4	66	4	15	19
G	15	14	ED98	**1893**	101	8	78	72	4	13	7
	16	15	882A	270	22	3	17	5	2	10	7
	17	16	6010	116	28	24	22	18	3	15	7
	18	17	0C2E	395	6	5	1	1	1	12	1
	19	18	0C2A	**1894**	17	**187**	1	3	4	15	3
	20	19	2F80	**1895**	17	6	4	14	4	6	2
	21	20	741C	1228	11	5	5	29	4	4	26
	22	21	0C2A	253	4	4	16	12	1	6	3
	23	22	0C2C	395	6	5	1	1	1	12	1
	24	23	F428	**1896**	5	3	5	3	1	33	**189**
H	25	24	0422	**1897**	7	162	12	3	3	4	185
	26	25	2C18	**1871**	28	22	5	43	3	14	19
	27	26	C40A	**1872**	11	5	1	117	9	4	**190**
	28	27	E022	**1873**	1	5	11	3	2	4	3
	29	28	859A	575	11	5	83	2	4	13	7
	30	29	8428	**1898**	15	5	83	11	4	62	7
	31	30	F419	**1899**	39	5	70	28	4	4	63
I	32	30	B01A	**1900**	39	5	68	28	4	4	63
	33	31	2C20	617	16	101	11	97	4	69	88
J	34	32	B01X	**1901**	16	101	11	13	4	69	88
	35	33	F421	560	5	5	57	13	1	40	3
	36	34	262A	**1902**	11	94	7	98	2	7	33
	37	31	2C20	**1903**	23	5	11	13	1	12	137
	38	35	E42A	**1874**	29	**188**	95	13	8	6	11
K	39	35	E428	**1874**	29	**188**	95	13	8	6	11
	40	36	A998	609	16	22	5	11	4	6	10
	41	35	E428	**1875**	29	**188**	95	127	8	6	11
	42	31	2C20	268	23	5	70	7	1	12	7
	43	37	2F88	**1904**	6	103	11	63	4	15	2

[a] Different morphotypes in the same CF patient (from 1 to 4).

[b] Isolates exhibiting similar or identical PFGE patterns have been highlighted in grey.

[c] Non-previously described alleles and STs are indicated in bold.

Table 4.4. Genotyping results obtained from the analysis of the Spanish CF collection. (Cont.)

CF Unit	Isolate[a]	PFGE[b]	Array Tube	ST[c]	MLST allelic profile[c]						
					acsA	*aroE*	*guaA*	*nuoD*	*mutL*	*ppsA*	*trpE*
L	44	38	2418	**1876**	40	181	11	11	3	4	9
	45	39	E418	564	6	10	5	11	2	15	2
	46	40	2C18	**1905**	6	4	42	5	1	4	26
	47	41	4DA8	**1906**	16	3	1	28	1	55	61
	48	42	2708	644	28	3	94	13	1	4	10
	49	43	7C2C	132	6	20	1	3	4	4	2
	50	44	3E18	**1877**	119	10	83	43	3	6	77
M	51[a1]	45	EC18	**1907**	11	5	11	131	3	53	1
	52[a1]	46	2C2C	**1908**	11	6	19	5	4	15	9
	53	47	6A20	**1909**	17	5	5	4	4	4	**191**
	54	48	6FA8	**1910**	15	5	5	85	8	4	14
N	55[a2]	49	AF90	**1911**	11	3	70	3	1	4	60
	56[a2]	50	F420	1251	35	11	25	6	13	6	84
	57	48	6FA8	**1878**	15	100	66	5	50	4	14
	58	51	2610	**1912**	11	48	11	3	1	15	14
O	59	52	4498	**1913**	11	48	98	5	3	10	85
P	60	53	2810	**1914**	6	14	12	7	1	4	20
	61[a3]	54	E020	**1879**	15	5	11	3	67	4	3
	62[a3]	55	E020	508	15	5	11	3	2	4	3
Q	63	56	8E18	189	26	143	1	3	4	4	10
	64	57	E429	**1880**	13	8	9	97	52	**124**	9
R	65	58	D421	253	4	4	16	12	1	6	3
	66	59	2398	**1881**	22	20	11	48	3	3	7
	67	60	XC10	**1882**	16	5	26	124	4	3	26
	68	59	2398	348	22	20	11	3	3	3	7
S	69	61	DF88	**1883**	17	5	11	97	4	12	56
	70	62	EC10	676	28	5	11	77	3	4	92
	71[a4]	63	E020	508	15	5	11	3	2	4	3
	72[a4]	64	E020	508	15	5	11	3	2	4	3
	73	65	A598	575	11	5	83	2	4	13	7
	74	66	F429	313	47	8	7	6	8	11	40
	75	67	0C48	198	11	5	11	11	3	27	7
	76	68	6E10	569	11	5	11	11	3	6	27
	77	69	0810	**1884**	17	5	12	3	**99**	4	7
T	78	70	EA08	27	6	5	6	7	4	6	7
	79	70	EA08	27	6	5	6	7	4	6	7

[a] Different morphotypes in the same CF patient (from 1 to 4).

[b] Isolates exhibiting similar or identical PFGE patterns have been highlighted in grey.

[c] Non-previously described alleles and STs are indicated in bold.

As collected in Table 4.4., identical PFGE band patterns, ST and Array Tube genotypes were detected in *P. aeruginosa* isolates recovered from a pair of siblings (isolates 78 and 79).

The same PFGE band pattern was also observed in unrelated isolates, some of which match with other genotyping methodologies. Isolates from PFGE clone types 3, 30, 35 and 59 were demonstrated to belong to the same CC by MLST (Figure 4.4.) and, with the exception of isolates 31 and 32 (PFGE-30), also to a related Array Tube genotype. The most relevant case concerned a possible intrahospital cross-transmission related to isolates 66 and 68 (PFGE-59), which exhibited an identical PFGE restriction pattern and Array Tube genotype but different MLST, being ascription to different STs due to a single point mutation within *mutL* nucleotide sequence ($nt331_{A\rightarrow C}$) that provokes the switch of allele 48 by allele 3, and consequently the assignation of ST1881 instead of ST348. Similarly, isolates 38, 39 and 41 (PFGE-35) were ascribed to STs 1874 and 1875, just differing in their *mutL* sequence by two point mutations, and to Array Tube genotypes E428 and E42A, just differing in the presence or absence of *exoS*. As shown, discordances within *mutL* nucleotide sequences were frequently involved in ascription to different STs. By contrast, isolates from PFGE clone types 31 and 48 yielded a non-related MLST allelic profile but showed the same Array Tube genotype (Table 4.4. and Figure 4.4.).

Conversely, several isolates exhibiting different PFGE restriction patterns were ascribed to the same ST and/or Array Tube genotype (Table 4.4. and Figure 4.4.).

As above described (section 3.3.3.), the Array Tube genotyping method also includes several probes to explore the accessory genome. In this sense, just isolates 66 and 68 (PFGE-59) exhibited an identical repertoire of accessory genes (data not shown). As well, isolates 38, 39 and 41 (PFGE-35) just differ in the presence or absence of *fpvB* gene, coding for the alternative type-I ferripyoverdine receptor (data not shown).

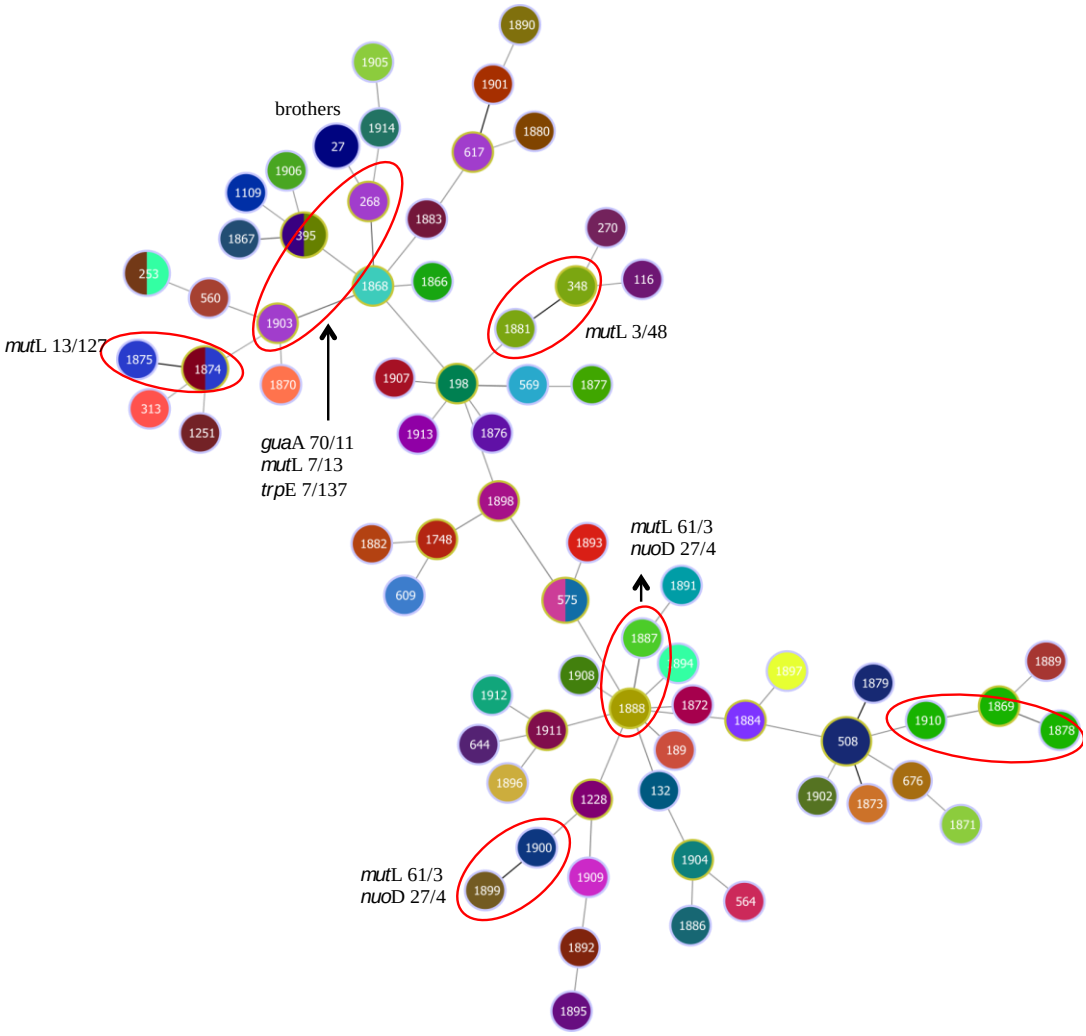


Figure 4.4. MST based on the MLST allelic profiles. Isolates with the same Array Tube genotype are represented with the same colour. As well, isolates ascribed to the same PFGE clone type are indicated with red lines.

Overall, ferripyoverdine receptor genes (*fpvA*) type I, IIa, IIb and III were detected in 23, 22, 6 and 11 isolates respectively, and the alternative type-I ferripyoverdine receptor gene *fpvB* was present only in 45 isolates. Ferripyoverdine receptor genes were not detected in 15 isolates, and all but two were isolates from adult patients. On average, isolates possess 2.3 genome islets and 2.4 genome islands, ranging from 0 to 5. The flagellin glycosylation island was encountered to be the most prevalent one (n=53, 67%). Nevertheless, 2 of these isolates lacked the a-type flagellin and, on the contrary, in 3 isolates expressing the a-type flagellin the flagellin glycosylation island was not detected. PAGI-1 was detected in only 45 isolates (57%) and the genomic islands of the CLC family PAGI-2/3 in 23 isolates (29%). Two isolates harboured both PAPI-1 and PAPI-2, and 43 isolates (54%) harboured only PAPI-2; other pKLC102-like islands were detected in 30 isolates (38%). Statistical differences within the global collection and the different subsets, adult or paediatric population, were not observed (Table 4.5.).

Table 4.5. Prevalence (%) of ferripyoverdine receptor genes, gene islands and genome islets among the Spanish collection.

	fpvA/B	Fla-glyc	PAGI-1/2-3	PAPI-1/2	pKLC102-like	Genome Islets
Global (n=79)	77/57	67	57/29	2/54	38	87
Adult (n=61)	83/59	64	59/26	3/54	36	93
Paediatric (n=18)	72/50	78	50/39	0/56	44	72

The Array Tube genotyping tool also includes probes for detecting T3SS effectors *exoS* and *exoU* genes. An unusual low prevalence, which did not correlate with obtained results by independent specific PCRs (18% vs 81% and 9% vs 10%, respectively), was registered. Coexistence of both genes by specific PCRs was only observed in 3 isolates and for 12 isolates neither *exoS* nor *exoU* was detected.

4.1.2. Antimicrobial resistance

4.1.2.1. Antibiotic susceptibility profiles

Non-susceptibility rates, MIC50 and MIC90 values for the Balearic Islands CF collection (January 2003 to June 2013) are summarized in Table 4.6. Up to 726 *P. aeruginosa* CF isolates were included in the final data analysis.

Colistin was the compound for which a minor percentage of non-susceptibility was registered (5.5%) followed by ceftazidime and meropenem, for which about 20% of the isolates showed *in vitro* resistance. Conversely, aztreonam and ciprofloxacin were the antibiotics exhibiting major resistance rates (44% and 73%, respectively) (Table 4.6.). Overall, the MDR rate was set in 17% and, more worrisome, 1% of the isolates met the PDR criteria.

MIC50 values for all tested compounds but aztreonam were within the susceptibility range, whereas just colistin MIC90 fell within this range (Table 4.6.). Of note, 89% of the isolates exhibited a tobramycin MIC under 64 mg/L, which is the suggested breakpoint for inhaled therapy.

Table 4.6. Non-susceptibility rates, MIC50 and MIC90 obtained for the Balearic Islands collection.

	Antibiotic						
	TZ	**IP**	**MP**	**CI**	**TM**	**CO**	**AT[a]**
% (I+R)[b]	20	33	21	44	28	5	73
MIC50	2	3	0.38	0.5	2	1	3
MIC90	64	32	24	4	96	2	256

a AT was tested just in *P. aeruginosa* isolates obtained in 2012 and 2013 (n=120).

b EUCAST clinical breakpoints version 3.1. was applied (Annex 3).

Results from the antibiotic resistance analysis of the Spanish collection are collected in Table 4.8. Colistin was also the most active compound, and only three isolates (4%) were classified as resistant. Conversely, around the 60% were non-susceptible to both fluoroquinolones tested. Considering co-resistances and excluding aztreonam, 15 isolates

(19%) remained susceptible to all antibiotic compounds and 43 (56%) met the MDR criteria. The XDR rate was set in 16% in the Spanish CF collection.

Antibiotic resistance temporal evolution. When temporal evolution of antibiotic resistance rates was analyzed within the Balearic Islands CF collection, a significant upward trend was registered being of particular concern the increase of the colistin resistance rate, moving from 0% in 2003 to 10% in 2012. As well, the multidrug resistance rate increase from 11% to 37% in the studied period (Figure 4.5.).

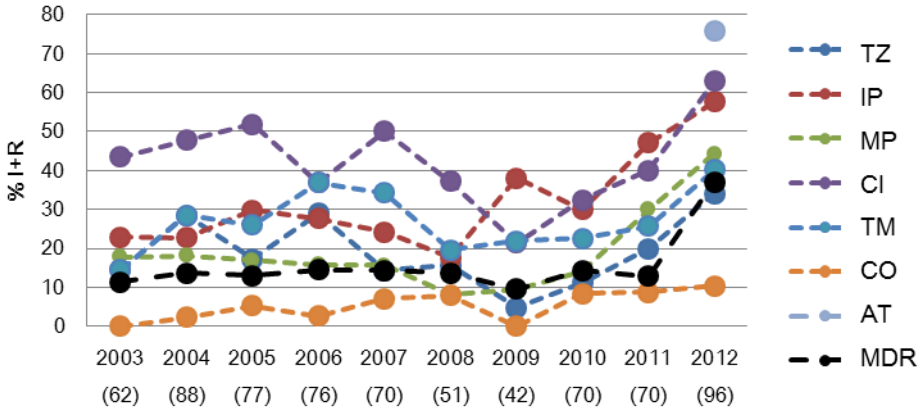


Figure 4.5. Temporal evolution (2003-2012) of antibiotic resistance rates (I+R%) in the Balearic Islands *P. aeruginosa* CF collection. No. of isolates/year is indicated in brackets.

Morphotype and antibiotic susceptibility profiles. Antibiotic resistance depending on colony morphotype was also studied within the Balearic Islands collection, including for this purpose all *P. aeruginosa* isolates obtained from 2003 to 2010. Almost 40% of the isolates showed a mucoid morphotype and about 15% were classified as SCV. Related to patients, up to 61% and 46% did not harbour mucoid or SCV isolate during this period, respectively. In comparison with the entire collection, lower and higher antimicrobial resistance rates for all antimicrobials tested were documented for the mucoid and the SCV subsets, respectively (Table 4.7.).

Table 4.7. Antimicrobial resistance rates (I+R%) determined for the Balearic Islands *P. aeruginosa* CF collection stratified by colonies morphotype.

Morphotype	Antibiotic[a]					
	TZ	IP	MP	CI	TM	CO
Mucoid (n=213)	11	17	9	30	20	2
SCV (n=82)	28	37	18	59	33	8
Global (n=536)	18	26	15	42	26	4

[a] EUCAST clinical breakpoints version 3.1. was applied (Annex 3).

In the Spanish CF collection, the classical CF mucoid morphotype was observed in 17 isolates (21%) and 16 isolates (20%) presented a SCV morphology. Compared to the whole collection, lower and higher antimicrobial resistance rates were also determined for the

mucoid and the SCV subsets, respectively (Table 4.8.). Of note, most SCV (87%) were classified as multi-drug resistant.

Table 4.8. Antimicrobial non-susceptibility rates (I+R%) determined for the whole Spanish *P. aeruginosa* CF collection and stratified by morphotype.

Morphotype	Antibiotic[a]														
	PPT	TZ	PM	AT	IP	MP	CI	LE	TM	GM	AK	CO	FO	MDR	XDR
Mucoid (n=17)	18	23	47	100	18	35	59	59	29	35	41	0	12	18	6
SCV (n=16)	25	50	37	100	44	50	69	69	31	37	37	0	31	87	12
Global (n=79)	18	33	37	100	35	45	59	64	32	37	39	4	19	56	16

[a] EUCAST clinical breakpoints version 6.0. was applied (Annex 3).

Infection-colonization patterns and antibiotic susceptibility profiles. In the analysis of the Balearic Islands collection, isolates obtained from patients chronically colonized exhibited higher resistance rates for all the antibiotics tested. In accordance, MIC90 values for all antibiotics but colistin in the chronically colonized subset fell out the susceptibility range whereas all MIC90 values in the other subset remained within this range. Moreover, all *P. aeruginosa* isolates meeting the MDR criteria had been obtained from respiratory samples of patients chronically colonized. No differences were observed in the MIC50 values for all antibiotics between both subsets, falling all within the susceptibility range (Table 4.9.).

Table 4.9. Non-susceptibility rates, MIC50 and MIC90 obtained for the *P. aeruginosa* CF Balearic Islands collection stratified by patient's colonization pattern.

		Antibiotic					
		TZ	IP	MP	CI	TM	CO
Intermittent and primocolonization (n=47)	% (I+R)[a]	0	4	2	6	4	0
	MIC50	1.5	2	0.25	0.125	1.5	2
	MIC90	4	4	0.5	0.38	3	3
Chronic (n=679)	% (I+R)[a]	22	35	22	47	30	6
	MIC50	2	3	0.38	0.5	2	1
	MIC90	192	32	32	4	256	3

[a] EUCAST clinical breakpoints version 3.1. was applied (Annex 3).

4.1.2.2. Antibiotic resistance mechanisms

Contribution of chromosomically-encoded *P. aeruginosa* efflux pumps, including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY(-OprM), as well as AmpC overexpression and OprD deficiency to the antibiotic resistance profiles during long-term CRI was evaluated. For this purpose, the 100 CF *P. aeruginosa* isolates chronically colonizing the 10 selected patients of the Balearic Islands were in-depth studied.

The antibiotic resistance profiles were variable within and across patients along the study period; however, a significant trend towards the accumulation of resistance was noted in

individual patients and clones, increasing from an average of resistance to 1.1±1.2 antibiotics in the first isolate of each patient to 2.5±0.85 in the last isolate (paired t test, p = 0.016). Overall, within this subset of 100 CF chronic isolates, the lowest susceptibility rate was observed for aztreonam (60% S) and the highest for meropenem (96% S), whereas resistance rates were highest for cefepime (30% R), tobramycin (30% R) and ciprofloxacin (24% R) and lowest for meropenem (1% R), aztreonam (4% R), and colistin (7% R). As EUCAST considers *P. aeruginosa* intrinsically nonsusceptible to aztreonam (mainly due to the basal expression of MexAB-OprM efflux pump), the percentage of susceptible isolates (60%) documented actually reflected the high number of hypersusceptible isolates falling outside of WT MICs distributions (http://www.eucast.org/mic_distributions/). As well, an important number of these isolates showed hypersusceptibility to meropenem with MICs (<0.06 mg/L) falling outside of WT distributions (Figure 4.6.).

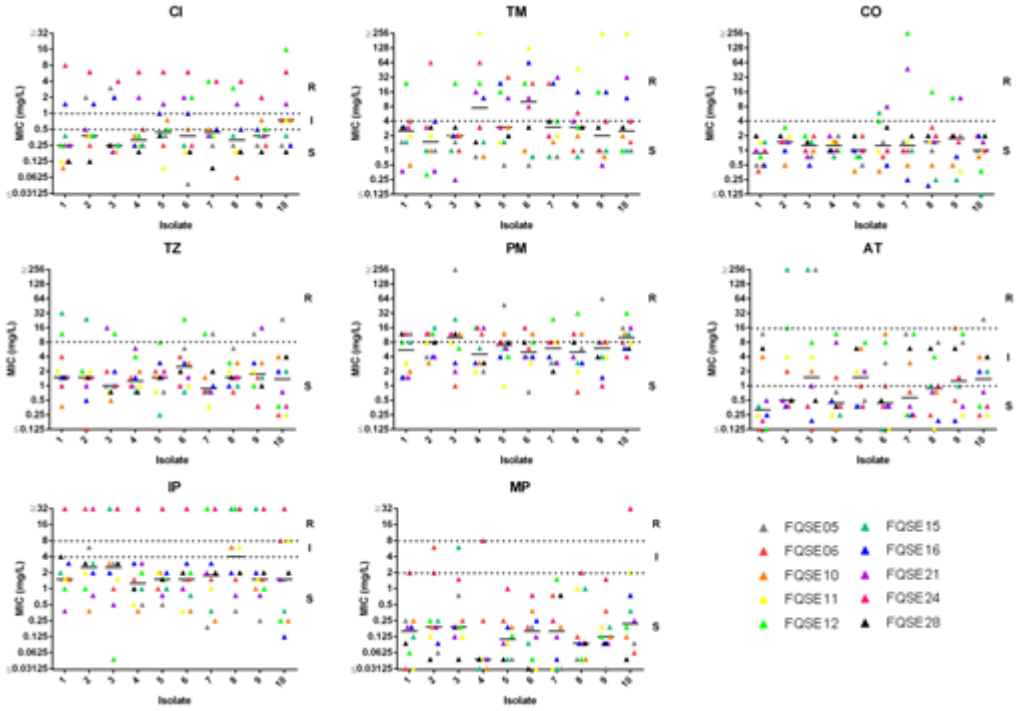


Figure 4.6.. Evolution of minimal inhibitory concentrations (MICs) from the first to the last studied isolate from each patient. Each colour represents a different patient.

Contribution to the documented antibiotic resistance was evaluated within the first and last isolate from each patient and PFGE clone type (Table 4.10.). As for antibiotic resistance, a trend towards accumulation of resistance mechanisms was also noted, moving from 1.4±0.58 in the first to 2.1±0.88 in the last isolates, although differences did not reach statistical significance (p = 0.06).

Table 4.10. MICs and resistance mechanisms detected in first and last isolates from each CF patient and PFGE clone.

Isolate ID	MICs (mg/L)								Resistance mechanisms	*mexZ* mutation[a]
	TZ	PM	AT	IP	MP	CI	TM	CO		
FQSE06-01	0.064	1.5	0.125	1.5	0.016	0.125	4	0.38	*mexY*	S9P
FQSE06-10	1.5	8	0.25	8	0.064	0.75	1.5	2	*mexY*, OprD-	$Nt_{292}\Delta 11$
FQSE10-01	0.38	2	4	1.5	0.25	0.094	0.5	0.5	*mexY*	IS[b]
FQSE10-10	3	12	4	0.25	0.125	0.75	2	0.75	*ampC*, *mexY*	IS[b]
FQSE15-01	1	8	0.38	2	0.125	0.38	1.5	1.5	*mexY*	A144V
FQSE15-10	1.5	12	2	0.38	0.38	0.38	1	0.016	*mexY*	A194P
FQSE24-01	1	12	0.19	>32	2	8	3	1	*mexY*, OprD-	A194P
FQSE24-10	0.38	4	0.38	>32	>32	6	4	1	*mexY*, OprD-	A194P
FQSE12-01	0.75	8	0.125	1	0.064	0.25	24	0.75	*mexY*	R125P
FQSE12-06	8	8	8	2	0.5	2	24	4	*ampC, mexY*	R125P
FQSE12-07	24	24	12	>32	1.5	4	4	>256	*ampC, mexY, mexF*, OprD-	Q164X
FQSE12-10	1	32	0.38	0.25	0.19	16	1	0.38	*mexY, mexF, mexD*, OprD-	Q164X
FQSE05-01	16	12	12	1.5	0.25	0.25	1.5	0.5	*ampC*	ND[c]
FQSE05-03	2	>256	>256	3	0.75	3	1.5	0.75	*ampC, mexY*	ND[c]
FQSE05-04	1	2	0.75	0.5	0.047	0.25	0.75	2	*mexY, mexF*	W158X
FQSE05-06	0.75	0.75	3	1.5	0.19	0.047	0.5	1.5	-	ND[c]
FQSE05-10	12	16	24	1.5	0.094	0.25	1	1	*ampC, mexY*	V43G
FQSE21-01	0.5	1.5	0.5	0.38	0.19	0.25	0.38	1	*ampC, mexY*	$Nt_{61}\Delta 15$
FQSE21-04	16	16	8	3	0.5	2	16	2	*mexB, mexY*	K131R
FQSE21-09	1.5	8	0.38	0.75	0.094	0.38	0.5	12	*mexY*	$Nt_{61}\Delta 15$
FQSE21-10	8	16	0.75	1.5	0.25	1.5	32	1.5	*mexB, mexY*	K131R
FQSE28-01	1.5	12	6	4	0.094	0.125	3	2	*ampC, mexY*	$Nt_{189}\Delta 12$
FQSE28-10	1	6	4	2	0.047	0.19	3	2	*ampC, mexY*	$Nt_{189}\Delta 12$
FQSE11-01	1	3	0.25	1.5	0.032	0.125	2	1.5	*mexY*	WT
FQSE11-07	1	8	0.125	1	0.023	0.38	2	1.5	*mexY*	$Nt_{279}\Delta 12$
FQSE11-10	2	8	0.25	8	2	0.75	>256	1	*mexY*, OprD-	WT
FQSE16-01	1.5	1.5	0.25	3	0.19	1.5	3	0.5	*mexF, mexD*	WT
FQSE16-10	3	6	2	0.125	0.75	0.25	12	2	*ampC, mexY, mexF*	R125P

[a] PAO1 and PA14 were used as reference WT sequences. Mutations are referred to PAO1 sequence.

[b] 1.2 Kb IS located in *mexX-mexZ* intergenic región (nt -72 respect mexZ coding sequence). Encodes a putative transposase identical to that previously reported in *P. pseudoalcaligenes* CECT 5344 (ref ZP_10763279.1)

[c] ND: not done

The most frequent resistance mechanism was MexXY(-OprM) overexpression, which was documented in all 10 patients. Moreover, this mechanism was already present in most patients (8 of 10) in the early isolates (Table 4.10.). Overexpression of the other efflux pumps was much more infrequent, being MexEF-OprJ overexpression documented in 3 patients, MexCD-OprN in 2 and MexAB-OprM just in one of the patients. As well, AmpC overexpression was evidenced in 6 of the patients and lack of OprD production in the 4 patients colonized by imipenem resistant strains. Although a certain correlation was

documented between AmpC overexpression and ceftazidime resistance and between lack of OprD and imipenem resistance, a correlation between phenotype and genotype was not always evident, particularly concerning efflux pumps overexpression.

In relation to PFGE clone type, isolates 07 and 10 from patient FQSE12 (FQSE-B/ST146/LES-1) were associated with a higher number of resistance mechanisms (Table 4.10.). As shown, the initial MDR isolate from this clone already expressed 4 resistance mechanisms [MexXY(-OprM), MexEF-OprN and AmpC overexpression plus OprD deficiency], as well as the last isolate did [MexXY(-OprM), MexCD-OprJ, MexEF-OprN and OprD deficiency]. Nevertheless, a significant reduction of the MDR profile was documented, likely influenced by the modification of the resistance mechanisms expressed: MexCD-OprJ instead of AmpC overexpression.

Additionally, as almost all early and late isolates overexpressed the efflux-pump MexXY(-OprM), its major regulator *mexZ* was sequenced. Most of the strains showed *mexZ* mutations including deletions, premature stop codons, insertion sequences (IS), or nonsynonymous aminoacid substitutions, thus supporting MexXY overexpression (Table 4.10.).

4.1.3. Prevalence of mutators, mutant frequencies and genetic basis for hypermutation

4.1.3.1. Analysis of the Spanish collection

The prevalence of mutators in the Spanish CF *P. aeruginosa* collection was set at 15.2%. A total of 12 isolates, recovered from 8 adults and 4 children, were classified as mutators ranging their rifampicin mutation frequency from $2x10^{-5}$ to $4.5x10^{-7}$. All these mutators belonged to unrelated genetic lineages (Figure 4.4.), and, of note, most of them were ascribed to new STs.

In 8 of the mutator isolates inactivating mutations within *mutS* (n=7) or *mutL* (n=1) genes were encountered and their implication in the mutator phenotype was confirmed by complementation studies. Three additional mutator isolates showed amino acid substitutions in the MutS and/or MutL proteins, and these aminoacid changes were also demonstrated to be involved in the observed phenotype. The remaining isolate showed WT sequences of *mutS* and *mutL* genes (Table 4.11.).

Table 4.11. Rifampicin mutation frequencies and genetic basis for hypermutation of mutator isolates from the Spanish CF collection.

Isolate ID	ST	Mutation frequency	Detected mutation[a]	Complemented with
6	**1890**	$1.38 \cdot 10^{-6}$	*mutL* (nt644Δ21)	*mutL*
9	1109	$8.26 \cdot 10^{-6}$	*mutL* (nt1120Δ5)	*mutS*
13	**1870**	$5.20 \cdot 10^{-6}$	Not detected	-
16	270	$2.93 \cdot 10^{-7}$	*mutS* (G1290A, E431K)	*mutS*
			mutL (G1872A, G632E)	*mutL*
26	**1871**	$2.50 \cdot 10^{-6}$	*mutL* (T1309C, A437T)	*mutL*
27	**1872**	$4.57 \cdot 10^{-7}$	*mutL* (T647G, V216G)	*mutL*
42	268	$2.47 \cdot 10^{-6}$	*mutS* (nt1600Δ13)	*mutS*
47	**1906**	$1.80 \cdot 10^{-6}$	*mutS* (nt1336Δ2)	*mutS*
49	132	$2.00 \cdot 10^{-5}$	*mutS* (nt399Δ12)	*mutS*
53	**1909**	$3.20 \cdot 10^{-6}$	*mutS* (nt1377Δ1)	*mutS*
55	**1911**	$2.71 \cdot 10^{-6}$	*mutS* (nt2577ins9pb)	*mutS*
60	**1914**	$3.07 \cdot 10^{-6}$	*mutS* (nt1198IS-4-like)	*mutS*

[a] PAO1 and PA14 were used as reference WT sequences. Mutations are referred to PAO1 sequence.

[b] Non-previously described STs are indicated in bold.

4.1.3.2. Analysis of the Balearic Island collection

During CF-CRI, an unusual high prevalence of mutators has been previously demonstrated [Oliver A *et al*, 2000]. Thus, their prevalence within the subset of 100 CF *P. aeruginosa* isolates chronically colonizing patients from the Baleric Islands was additionally evaluated. Up to 29% of the isolates were determined to exhibit a mutator phenotype, showing 6 of the 10 patients at least 1 mutator isolate during the study period (Figure 4.7.). In two of the patients (FQSE16 and FQSE24) all isolates were mutators and in other two, mutators emerged at late stages of colonization (FQSE15 and FQSE21). In one more patient a mutator lineage emerged (FQSE06) but it was not fixed and in other one it was replaced by the multi-drug resistant ST146/LES-1 epidemic strain (FQSE12).

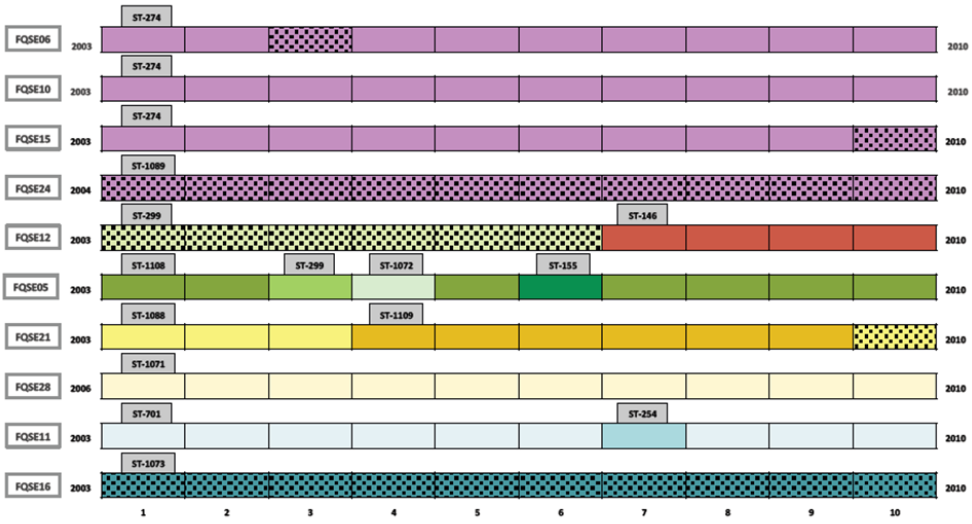


Figure 4.6. Encountered mutator isolates within the 10 sequential isolates from each of the 10 CF patients from the Baleric Islands.
Mutator isolates are marked with a checked pattern.

As shown in Figure 4.6., mutators were detected within 3 of the 4 patients chronically colonized by the widespread clone FQSE-A (ST274/ST1089) being, indeed, all isolates from patient FQSE24 mutators. As initially MLST had been just performed only in the first isolate per PFGE clone type and patient (section 4.1.1.1), and a different ST (ST1089) was detected within this patient we decided to extend the MLST analysis to the last available isolate from each of these patients as well as the two additional sporadic mutator isolates detected in patients FQSE06 and FQSE15. In all cases, the determined ST coincided with that of the first isolate, except for the mutator lineage emerging from one of the patients (FQSE06) that was also identified as ST1089 (Figure 4.6.). So, mutator lineages were detected in 3 of the 4 patients harbouring clone FQSE-A, two belonging to ST1089 and one to ST274. Therefore, available data clearly suggest that ST1089 has recently evolved from ST274 through point mutations linked to the emergence of a mutator lineage. Moreover, when genetic basis of hypermutation was investigated within all these mutator isolates all were demonstrated to be defective in *mutS*. Consequently, MutS encoding gene was sequenced from the three mutator isolates as well as for several representative non-mutator isolates. The three mutator isolates harboured the same inactivating mutation, a 4 bp deletion from nt814; whereas this mutation was absent in the non-mutator isolates.

Hypermutation has been pointed out as a driver of antibiotic resistance. In accordance, a significant trend ($p= 0.009$) towards resistance to a higher number of antibiotics among mutator isolates (2.28±0.22) than among non-mutator isolates (1.49±0.17) was noted in this subset.

4.2. *Pseudomonas aeruginosa* RESISTOME EVOLUTION IN CYSTIC FIBROSIS CHRONIC RESPIRATORY INFECTIONS

4.2.1. Mutational resistome evolution of the international CC274 cystic fibrosis clone

4.2.1.1. Prevalence and genetic basis for hypermutation

Among the CC274 study collection, nine isolates (31%) were mutators, belonging to six (35%) different patients, residing in both Australia (n=3) and Spain (n=3). Data from sequential isolates were available for the Spanish isolates: one was chronically infected with a persistent mutator lineage (FQSE24), whereas the other two harboured a mixed population of mutator and non-mutator isolates (FQSE06 and FQSE15) (Figure 3.2. and section 4.1.3.).

Sequence variation within an exhaustive panel of so called mutator genes (Annex 5) was analyzed in all mutator and non-mutator isolates. The three Australian mutator isolates showed unique mutations in either *mutL* or *mutS*, whereas all mutator isolates from the three Spanish patients were found to share the same inactivating mutation in *mutS*. Complementation studies with plasmids harbouring WT MMR system genes (*mutS* and *mutL*) were performed in these mutator isolates. As shown in Table 4.12., WT rifampicin resistance mutation frequencies were restored in all mutator isolates upon *mutS* or *mutL* complementation, which correlated in all cases with the presence of specific mutations in these genes. Of note, while mutator phenotypes could be explained in all cases by specific mutations in MMR genes, the contrary was not always true, since one of the non-mutator isolates (FQSE03) showed a missense mutation in *mutS*. Moreover, the presence of polymorphisms in other mutator genes was frequent, but showed no association with mutator phenotypes (Table 4.12.).

Table 4.12. Mutator phenotype and genetic basis for hypermutation in the *P. aeruginosa* CC274 collection.

Isolate ID[a]	ST	Mutation frequency	Complement with	Sequence variation in mutator genes (mutome)[b]									
				ung	*mfd*	*mutS*	*sodB*	*mutT*	*sodM*	*mutL*	*mutM*	*oxyR*	*polA*
AUS034	274	$1.59 \cdot 10^{-6}$	*mutL*					E236D		R631C	D61N L132P		D876E
AUS410	274	$2.04 \cdot 10^{-8}$	-				E25V						D876E
AUS411	274	$6.63 \cdot 10^{-8}$	-					E236D			D61N		D876E
AUS531	274	$1.84 \cdot 10^{-8}$	-					E236D			D61N		D876E
AUS588	274	$2.06 \cdot 10^{-8}$	-				E25V						D876E
AUS601	1043	$1.54 \cdot 10^{-6}$	*mutL*	S13R			E25V			P159S H288Y		F106L H219Y	D876E
AUS603	274	$1.23 \cdot 10^{-8}$	-				E25V						D876E
AUS690	274	$3.71 \cdot 10^{-6}$	*mutS*		Q1123H	C224R T287P		E236D			D61N		D876E
FQRC10	274	$2.22 \cdot 10^{-9}$	-					E236D			D61N		D876E
FQRC15	274	$2.39 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQRC26	274	$9.09 \cdot 10^{-9}$	-					E236D			D61N		D876E
FQSE03	274	$1.11 \cdot 10^{-8}$	-			L374V		E236D			D61N		D876E
FQSE06-0403	274	$3.33 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE06-1104	274	$1.04 \cdot 10^{-6}$	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE06-0807	274	$5.03 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE06-0610	274	$6.50 \cdot 10^{-9}$	-					E236D			D61N		D876E
FQSE10-0503	274	$3.49 \cdot 10^{-9}$	-					E236D			D61N		D876E
FQSE10-0106	274	$2.22 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE10-0110	274	$9.80 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE10-0111	274	$4.35 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE15-0803	274	$9.64 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE15-0906	274	$1.18 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE15-0310	274	$3.96 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE15-1110	1089	$3.12 \cdot 10^{-5}$	*mutS*		A868T	Nt814Δ4		E236D			D61N		D876E
FQSE24-0304	1089	$8.46 \cdot 10^{-6}$	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-1005	1089	$1.96 \cdot 10^{-5}$	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-0308	1089	$3.88 \cdot 10^{-6}$	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-1010	1089	$5.95 \cdot 10^{-6}$	*mutS*			Nt814Δ4		E236D			D61N		D876E
PAMB148	274	$5.00 \cdot 10^{-8}$	-					E236D	L202R		D61N		D876E

[a] Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Sequential isolates from a same patient are separated by dashed lines.

[b] Sequence variations respect to those of PAO1. No mutations were found in other genes associated with mutator phenotypes, including *pfpI*, *mutY*, *dnaQ*, *PA2583*, *PA2819.1*, *PA2819.2*, *radA* and *uvrD*.

4.2.1.2. PHYLOGENETIC ANALYSIS

PFGE macrorestriction patterns indicated that all isolates were clonally related, including mutators, which were indistinguishable from non-mutators. When an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram was constructed based on PFGE patterns, all isolates from the Balearic Islands clustered together in the same branch, although patterns from one of the patients (FQSE10) were slightly different. In contrast, Australian isolates were less clonal and clustered in different branches (Figure 4.7.).

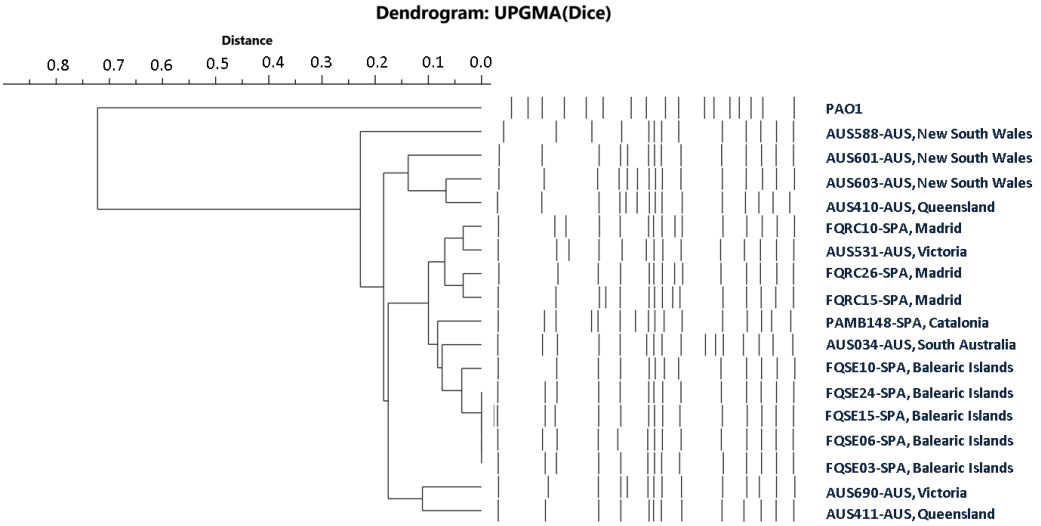


Figure 4.7. UPGMA phylogenetic tree showing the relationship among the CC274 *P. aeruginosa* collection. The tree was constructed based on the DNA macrorestriction fragment patterns obtained by pulsed-field gel electrophoresis (PFGE) using SpeI restriction enzyme Isolates are labelled according to the following format: Patient identification - Country (AUS: Australia; SPA: Spain), Region.

Conversely, by MLST, two new and closely ST274-related STs had been detected. As above described, mutators from patients FQSE15 and FQSE24 differed from ST274 by only two point mutations in two of the MLST alleles (*acsA* and *guaA*) leading to ST1089. Nevertheless, the mutator from patient FQSE06, which indeed shared the same inactivating mutation in *mutS*, still belonged to ST274 (section 4.1.1.1.). On the other hand, the Australian mutator AUS601 was also determined to be a new ST (ST1043), but, in this case, just differing from ST274 by two missense mutations in *mutL* allele.

To better understand the evolutionary trajectory, success and international dissemination of CC274, whole-genome based phylogenetic analysis of all 29 isolates were performed. First, to determine the genetic relationship between CC274 isolates and other well-recognized CF epidemic clones, whole-genome sequence reads of all 29 isolates were *de novo* assembled and a phylogenetic tree based on core genome alignment was constructed with default parameters on Parsnp. CC274 was determined to belong to the phylogenetic cluster

containing strain PAO1, as well as other well-known CF epidemic clones such as LESB58, AES-1 and DK2 (Figure 4.8.-A).

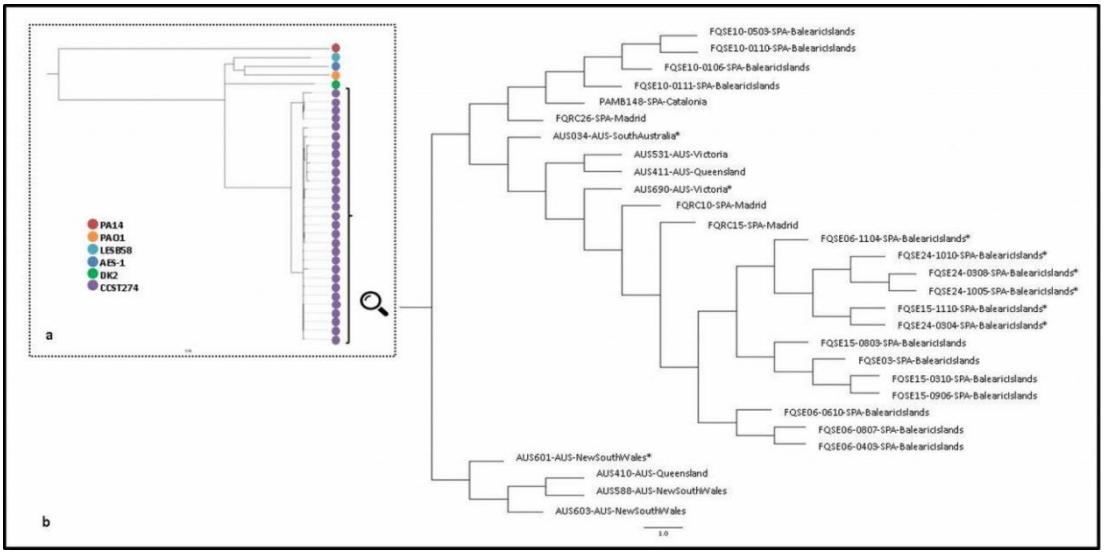


Figure 4.8. Core-genome phylogenetic reconstructions of *P. aeruginosa* CC274 CF clone. (A) Genetic relationship between CC274 and other well-recognized CF epidemic clones. (B) Genetic relationship between the CC274 collection isolates. Both reconstructions were made with Parsnp using default parameters. Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates - Country (AUS: Australia; SPA: Spain) - Region. Mutator isolates are identified with an asterisk.

Up to 16,070 common SNP were found by mapping sequence reads for each isolate against *P. aeruginosa* reference PAO1 strain genome. As well, a total of 5,525 high-quality intraclonal SNP were detected, of which 2,294 were unique and thus detected in single isolates. A high degree of intraclonal diversity was observed, with SNP differences between isolates ranging from 20 to 3,256. To elucidate the phylogenetic relationship among isolates two different approaches were used. In both, core-genome and Bayesian time-based analysis, CC274 isolates grouped into two clusters, one including just four Australian isolates and a second major cluster that included all other Australian and Spanish isolates (Figure 4.8-B and figure 4.9.). SNP differences between isolates from the different clusters ranged from 2396 to 3256 and, according to Bayesian time-based analysis, the common ancestor of CC274 was set, approximately, 380 years ago.

As shown, the major cluster further subdivided and, although both phylogenetic reconstructions did not match exactly with each other, both analyses supported that different lineages are currently coexisting with a worldwide distribution, having evolved from a common antecessor set approximately 275 years ago. SNP differences between isolates from Australia and Spain ranged from 114 to 1204, and similar results were obtained when only the Australian (min-max: 230-826) or the Spanish (min-max: 20-839) were compared, supporting no geographical barrier for lineage evolution.

Within the major cluster, all sequential isolates cultured from an individual patient clustered under the same branch with the single exception of all the Spanish isolates that exhibited a mutator phenotype which clustered together, independently of the patient involved and their ST.

Focusing on the sequential isolates, a unidirectional evolution route could not be stablished. Instead, a diversified intrapatient clone evolution that leads to a mix of genetically different sublineages coexisting in the CF respiratory airways was observed. Within a patient, minimum and maximum SNP differences between isolates ranged from 20 to 676, which overlapped with interpatient SNP differences, ranging from 51 to 3256 (51 to 839 for patients from the same hospital).

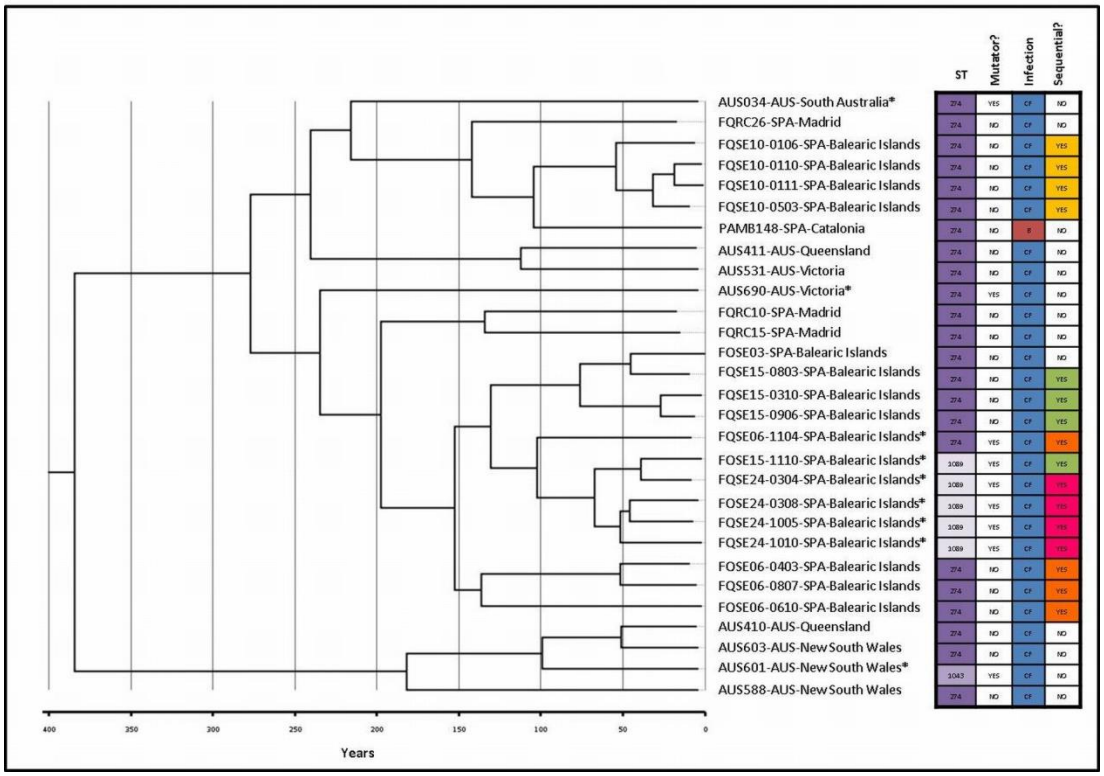


Figure 4.9. Bayesian phylogenetic reconstruction of *P. aeruginosa* CC274 CF clone. The tree was based on 5525 intraclonal variable positions identified by whole-genome sequencing. Divergence times of predicted ancestors and sampling dates can be inferred from the X axis taking into account that time zero corresponds to the most recent isolate (2012). The same labelling of Figure 4.8. was used. Isolates characteristics are summarized at the right board, where: (CF) Cystic Fibrosis CRI, and (B) Bloodstream. Sequential *P. aeruginosa* isolated from a same patient are indicated with the same colour.

4.2.1.3. THE CC274 RESISTOME

MICs determined for a panel of 11 antipseudomonal agents are collected in Table 4.13.

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	TOL/TAZ (≤4)	IP (≤4)	MP (≤2)	TM (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
AUS034*	>256	>256	>256	>256	16	>32	>32	6	>256	1.5	>256	+	-	+	*gyrB* (R441L), *mexR* (R85H), *mexA* (M1X), *mexB* (F178S, M555I), *oprD* (E264X), *phoQ* (E266X), *parR* (M59I), *mexY* (V1000L), *mexZ* (Nt334Δ13), *fusA2* (P329L), PA2489(R12L, A244T), *mexS* (P254Q), *mexT* (L157M), PBP4(W350R), *capD* (I7M, S51G), *gyrA* (T83I), *mexK* (S426G), *mpl* (Nt112ins1, V124G), *fusA1* (V93A, P554L, D588G), *rpoB* (D831G, D964G), *mexW* (A627V, Q771P), PBP3 (P527T, G63S)
AUS410	4	24	1	12	4	>32	>32	64	>256	1	0.38	-	-	+	*gyrB* (S466F), *mexB* (M552T), *oprD* (Nt583Δ1), *lasR* (A50V, D73G), *sucC* (V44G, A384V), *oprF* (Nt574Δ31), *mexY* (V32A), *mexZ* (Q164X), *mexT* (D327Y), *mexE* (F7Y), *mpl* (D168Y), PA2489(A125T, G185S, P260S), *capD*(I7M, S51G), *fusA1*(P618L), *rpoC*(E386K), *mexW*(Q511R), PBP3(G216S), *pagL*(Nt286Δ1), *amgS*(S64L)
AUS411	>256	>256	>256	>256	6	>32	>32	>256	>256	0.38	0.25	-	-	+	*gyrB* (S466F), *mexB* (Q104E, F246C, L376V), *phoQ* (H248P), *lasR* (D73G), *parS* (D381E, T163N), *sucC* (C261G), *mexY* (D201A, G287A), PA2489 (R12L, A244T), *fusA2* (I640L), mexE (V104G), *htpX* (Nt683Δ5), *mexK* (S426G), *capD* (I7M), *fusA1* (K504E), *rpoC* (N690S), *mexW* (A627V,Q771P), PBP3 (Q372P), *pagL* (N159D)
AUS531	3	3	4	12	1	2	0.75	1	6	0.125	1	-	-	-	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexW* (A627V, Q771P)
AUS588	2	8	3	8	1	1	0.75	1	8	0.125	0.75	-	-	-	PA2489 (A125T, G185S, P260S), *mexE* (F7Y, V276M), *capD* (I7M), *mexW* (Q511R)
AUS601*	>256	>256	>256	1	3	>32	>32	24	>256	16	0.25	-	-	+	*mexB* (M552T), *oprD* (Nt1044ins4), *phoQ* (K234N, T315A), *lasR* (A50V), *sucC* (T102I, A384V), *mexY* (V32A), *mexZ* (Q164X), *fusA2* (S445X), *mexT*(D327Y), *mexE*(F7Y), *ftsK* (A152V), PA2489 (A125T, G185S, P260S), *capD* (S51G), *gyrA* (T83I), *mpl* (G113D), *ampC* (V239A), *fusA1* (P618L), *rpoC* (E386K), *mexW* (Q511R), PBP3 (R504C), *pagL* (E163G), *pmrB* (L31P), *amgR* (E204D)
AUS603	6	8	24	2	1.5	>32	8	1	8	0.25	1.5	+	-	+	*mexB* (M552T), *lasR* (A50V, D73G), *sucC* (V44G, A384V), *mexY* (V32A), *mexZ* (Q164X), *mexT* (D327Y), *mexE* (F7Y), PA2489 (A125T, G185S, P260S), PBP4 (S315G), *opmE* (E204D), *capD* (I7M, Nt1438Δ1), *mpl* (Nt112ins1, Nt1317Δ1), *fusA1* (P618L), *mexW* (Q511R)

[a] Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Sequential isolates from a same patient are separated by dashed lines. Mutators isolates are identified with and asterisk.

[b] Clinical susceptibility breakpoints established by EUCAST v7.0 for each antibiotic are shown in brackets.

[c] The main antibiotic resistance related mutations documented for each isolate are shown. For this purpose, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes and/or (iii) mutations in genes found to be under high evolutionary pressure.

Table 4.13. Antibiotic susceptibility profiles and main antibiotic resistance related mutations detected among CC274 isolates. (Cont.)

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	TOL/TAZ (≤4)	IP (≤4)	MP (≤2)	TM (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
AUS690*	6	12	0.75	3	6	4	2	24	>256	12	0.125	-	+	+	*gyrB* (Q467R), *mexR* (H133P), *mexB* (Nt712Δ1), *phoP* (T221I), *lasR* (T178I), *parS* (L10P), *oprF* (K250R), *mexY* (G402S, A850T), *mexZ* (Nt529Δ1), PA2489 (R12L, A244T), *fusA2* (L104P, Nt889Δ1), *htpX* (G187D), *capD* (I7M, S51G), *gyrA* (T83A, T325I), *mexK* (G487E), *mexH* (Nt1086ins1), *fusA1* (Y552C, T671I), *rpoC* (E136G, D616G, V808L), *rpoB* (F1046S), *mexW* (A627V, Q771P), *pagL* (P158L), *pmrB* (F124L), *amgS* (R188C), *parE* (P438S)
FQRC10	2	2	4	12	1	1.5	1	1	8	0.094	0.5	-	-	-	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexH* (D356N), *mexW* (A627V, Q771P)
FQRC15	1	0.75	6	6	1	1.5	1	0.75	8	0.19	1	-	-	-	PA2489 (R12L, A244T), *capD* (I7M), *mexW* (A627V, Q771P)
FQRC26	4	6	24	24	1	0.25	1.5	1	6	1.5	0.38	-	+	-	*mexY* (V875M), *mexT* (R164H), PA2489 (R12L, A244T), *capD* (I7M, S51G), *gyrA*(Q106L), *mexW* (A627V, Q771P)
FQSE03	3	8	0.5	2	1.5	2	0.38	1	6	3	0.25	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), PA2489 (R12L, A244T), *capD* (I7M, S51G), *gyrA* (D87N), *mexW* (A627V, Q771P)
FQSE06-0403	0.75	2	0.25	4	0.38	1	0.5	24	16	0.19	0.19	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexY* (G287A), *mexZ* (S9P), PA2489 (R12L, A244T), *mpl* (S257L), *capD* (I7M, S51G), *fusA1* (Y552C, T671I), *mexW* (A627V, Q771P), PBP3 (P215L), *amgR* (A8V)
FQSE06-1104*	0.38	1	0.094	0.38	0.38	6	0.19	1	24	0.75	2	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A194P), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *capD* (I7M, S51G), *gyrA* (D87G), *mexK* (Q585X), *rpoB* (Y583C), *mexW* (A627V, Q771P), *pmrB* (V185I, G221D, R287Q), PBP1A (E161G), *amgR*(A8V)
FQSE06-0807	4	8	0.75	4	2	1.5	0.75	24	>256	0.5	1	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexY* (G287A), *mexZ* (S9P), *mexT* (P270Q), PA2489 (R12L, A244T), *mpl* (S257L), *capD* (I7M, S51G), *fusA1* (N482S, Y552C, T671I), *mexW* (A627V, Q771P), PBP3 (P215L), *amgR* (A8V)
FQSE06-0610	4	24	0.75	8	1.5	1	0.25	1.5	24	0.75	0.19	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (Nt290Δ11), PA2489 (R12L, A244T), *mexW* (A627V, Q771P), *capD* (I7M, S51G), *amgR* (A8V)

[a] Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Sequential isolates from a same patient are separated by dashed lines. Mutators isolates are identified with and asterisk.

[b] Clinical susceptibility breakpoints established by EUCAST v7.0 for each antibiotic are shown in brackets.

[c] The main antibiotic resistance related mutations documented for each isolate are shown. For this purpose, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes and/or (iii) mutations in genes found to be under high evolutionary pressure.

Table 4.13. Antibiotic susceptibility profiles and main antibiotic resistance related mutations detected among CC274 isolates. (Cont.)

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	TOL/TAZ (≤4)	IP (≤4)	MP (≤2)	TM (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
FQSE10-0503	1.5	12	4	4	1.5	1	0.25	0.75	8	0.25	0.25	-	-	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T), *nalD* (Nt459Δ13), *mexW* (A627V, Q771P), *capD* (I7M, S51G)
FQSE10-0106	0.75	3	0.125	0.75	0.5	0.38	0.032	0.75	4	0.38	1.5	-	-	+	*mexB* (L738P), *mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK*(A38T), *capD* (S51G), *nalD* (Nt396Δ2), *mexW* (A627V, Q771P), *nfxB*(X188ext)
FQSE10-0110	3	8	16	8	2	1	0.125	0.75	4	0.75	0.5	-	+	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T), *rpoB* (D659E, E904K), *mexW* (A627V, Q771P), *pmrB* (R287Q)
FQSE10-0111	3	16	12	12	8	1.5	1	1	12	0.38	0.38	-	-	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T, D54Y), *capD* (S51G), *mexW* (A627V, Q771P)
FQSE15-0803	2	12	0.38	4	1.5	6	1	1	12	0.19	0.25	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), PA2489 (R12L, A244T), *capD* (I7M, S51G), *pmrB* (E213D), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-0906	0.75	6	0.38	2	1	1	0.047	1.5	12	0.38	0.75	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), *mexS* (Nt848Δ2), *mexT* (Nt534Δ17), PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexK* (S426G), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-0310	1	4	1	1	1	12	0.19	1	8	0.38	0.25	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), *mexS* (Nt848Δ2), *mexT* (Nt534Δ17), PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexK* (P834S), *mpl* (Nt1266Δ1), *rpoC* (Nt1181Δ3), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-1110*	8	24	6	4	1	>32	>32	1	16	1	0.25	-	-	+	*gyrB* (S466F), *mexA* (N71S, D235G), *mexB* (L376V), *oprD* (V67X), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), galU (P123L), PA2050 (G90R, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *htpX* (A141T), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgS* (D267N), *amgR* (A8V)

[a] Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Sequential isolates from a same patient are separated by dashed lines. Mutators isolates are identified with and asterisk.

[b] Clinical susceptibility breakpoints established by EUCAST v7.0 for each antibiotic are shown in brackets.

[c] The main antibiotic resistance related mutations documented for each isolate are shown. For this purpose, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes and/or (iii) mutations in genes found to be under high evolutionary pressure.

Table 4.13. Antibiotic susceptibility profiles and main antibiotic resistance related mutations detected among CC274 isolates. (Cont.)

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	TOL/TAZ (≤4)	IP (≤4)	MP (≤2)	TM (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
FQSE24-0304*	2	24	0.38	8	1	>32	>32	2	24	6	0.38	-	-	+	*gyrB* (S466F), *mexA* (L338P), *oprD* (V67X), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *opmE* (D421G), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgR* (A8V)
FQSE24-1005*	1	16	0.38	2	1.5	>32	8	3	16	6	1	-	-	+	*gyrB* (S466F), *oprD* (V67X), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), *fusA2* (N236S, N561S), PA2489 (R12L, A244T), *fusA1* (K430E), *rpoC*(V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G, R407S), *amgR* (A8V)
FQSE24-0308*	1	8	0.25	0.75	1.5	>32	0.25	2	16	4	1	-	-	+	*gyrB* (S466F), *oprD* (V67X), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), *fusA2* (N236S, N561S), PA2489 (R12L, A244T), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgS* (T92A), *amgR*(A8V)
FQSE24-1010*	1	8	1	1	1	>32	4	4	64	4	0.38	-	-	+	*gyrB* (S466F), *mexA* (L338P), *oprD* (V67X), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, P97L, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *opmE* (L400P, D421G), *mexH* (V221I), *capD* (I7M, S51G, A165V), *fusA1* (K430E), *rpoC*(V693A), *mexW* (A627V, Q771P), PBP3 (G216S), *pmrB* (R287Q), PBP1A (E161G), *amgS* (A13V), *amgR* (A8V)
PAMB148	>256	64	>256	>256	6	1.5	0.75	1.5	16	0.064	0.5	+	-	-	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexY* (V875M, N1036S), *mexW* (A627V, Q771P), *ampD* (P41L)
% I + R	13.8	44.8	48.3	13.8	17.2	44.8	27.6	24.1	62.1	48.3	3.4				

[a] Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Sequential isolates from a same patient are separated by dashed lines. Mutators isolates are identified with and asterisk.

[b] Clinical susceptibility breakpoints established by EUCAST v7.0 for each antibiotic are shown in brackets.

[c] The main antibiotic resistance related mutations documented for each isolate are shown. For this purpose, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes and/or (iii) mutations in genes found to be under high evolutionary pressure.

As shown, resistance rates were lowest for colistin (3.4%), distantly followed by ceftazidime and piperacillin/tazobactam (13.8%). In contrast, resistance to cefepime, aztreonam, imipenem, amikacin and ciprofloxacin was observed in 44.8 to 62% of the isolates. Remarkably, 17.2% of the isolates were resistant to the new combination ceftolozane/tazobactam. As well, antibiotic resistance was shown to be more frequent among mutators, and in Australian isolates in comparison with those from Spain. In fact, all 9 mutator isolates were classified as MDR, as compared to only 3 of 20 non-mutators. Moreover, one of the Australian mutator isolates met the pan-drug resistant (PDR) definition.

The presence of horizontally acquired resistance determinants was explored in the whole-genome sequences using the ResFinder tool. None of the 29 isolates harbored any horizontally acquired genes encoding resistance determinants. The complete list of missense and non-sense mutations encountered in the 164 antibiotic resistance related chromosomal genes investigated (n=164, Annex 5) can be downloaded in the following link https://www.nature.com/articles/s41598-017-05621-5; as well, a summary of these mutations by antibiotic class has been collected in Annex 6. Up to 127 (77.4%) of the 164 studied genes showed non-synonymous mutations in at least one of the isolates studied. Moreover, after discarding non-synonymous mutations present in all isolates (and thus considered intrinsic CC274 polymorphisms), this figure only decreased to 106 (64.6%). The number and distribution of mutations among the 164 antibiotic resistance related genes studied in the CC274 collection has been represented in Figure 4.10. Seventy-three (68.9%) of these genes showed no more than two different mutational events being 44 of them mutated in unique isolates. In contrast, 33 (31.1%) genes appeared to be under high evolutionary pressure showing evidence of at least 3 different mutational events. Particularly noteworthy among them were *mexB* or *mexY*, (coding for efflux pumps proteins), *mexZ* (the main MexXY repressor), *gyrA* (which codes for DNA gyrase subunit A) and *fusA1* (coding for the elongation factor G).

The main antibiotic resistance related mutations documented are listed in Table 4.13. For this purpose, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence, (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes (*e.g.* mutations in genes involved in AmpC, efflux or OprD regulation and β-lactam resistance phenotypes are crosslinked by integrating the analysis of the expression of *ampC*, efflux pumps genes and *oprD*) and/or (iii) mutations in genes found to be under high evolutionary pressure (those with at least 3 different mutational events documented). Overall, the number of mutations was much higher (unpaired T test $p<0.0001$) in mutator (19.2±3.1) than in non-mutator isolates (6.7±3.1). Nevertheless, some Australian non-mutator isolates (*e.g.* AUS410 or

AUS411) also presented a high number of mutations. Of note, unique mutations detected in specific genes support phylogeny reconstructions (see above Figures 4.8. and 4.9.).

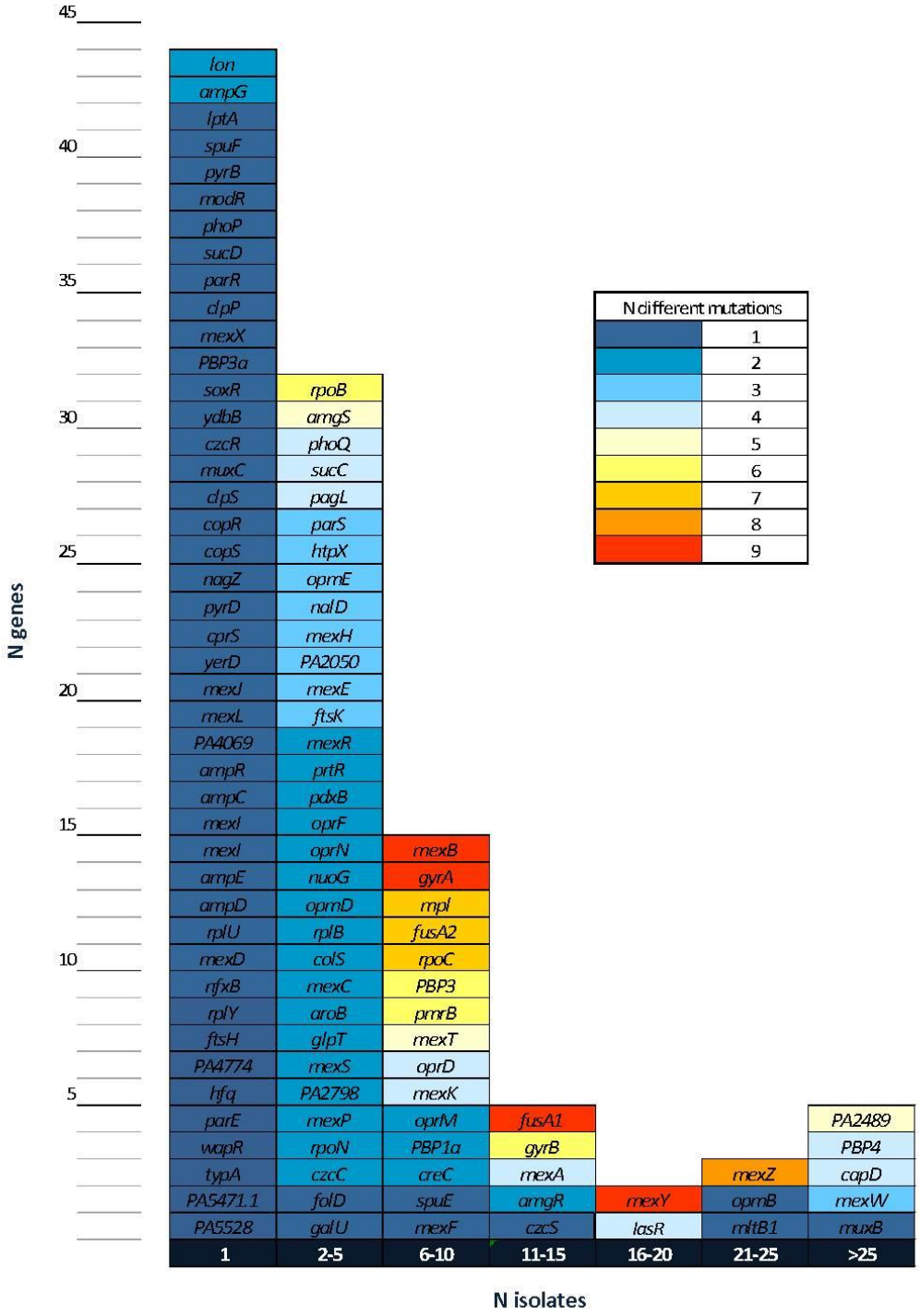
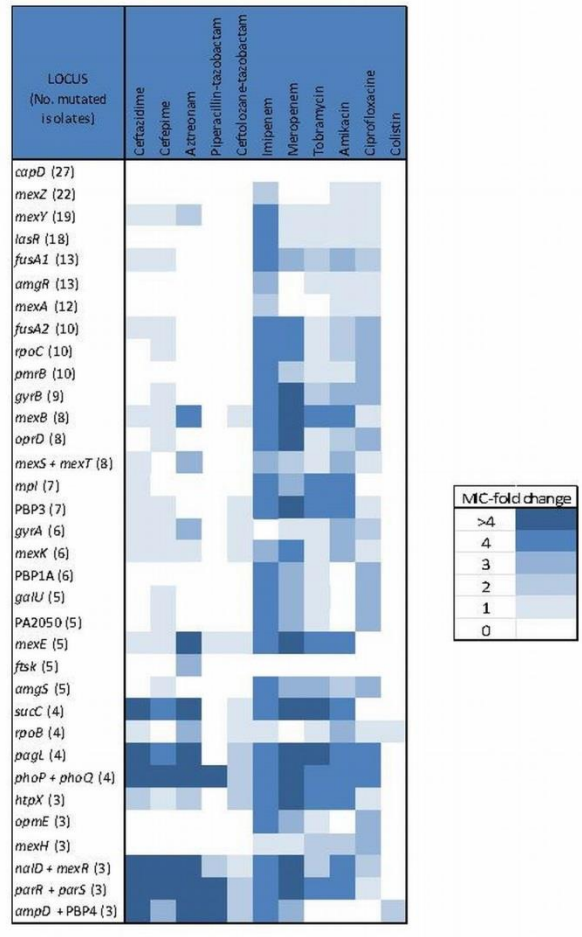


Figure 4.10. Distribution of mutations among the CC274 collection. Mutations encountered among the 164 antibiotic resistance related genes are represented, synonymous and common non-synonymous mutations have been excluded.

To gain insights into the effect on antibiotic resistance profiles of mutations listed in Table 4.13., the median MIC of isolates harboring mutations or not in a specific gene were

compared (Figure 4.11.) Overall, it should be noted that colistin MICs as well as the MICs for the antibiotic combinations piperacillin/tazobactam and ceftolozane/tazobactam were barely affected, whilst carbapenems, aminoglycosides and quinolones MICs are affected by the presence of mutations in many of the selected genes. Apparently, the presence of mutations in some genes such as *capD* (also known as *wbpM*), a gene coding for a protein implicated in O-antigen biosynthesis and previously related with aminoglycoside resistance, or *ftsK,* which codes for a cellular division protein, were not related with an increase in resistance for any antibiotic. Conversely, the presence of mutations in 22 of the genes was shown to produce at least a 2-fold MIC increase for at least 3 different classes of antibiotics. Renowned resistance genes, such as *gyrA*, *gyrB*, *ampD, dacB* (PBP4) or *oprD*, are within this list of 22 genes but, particularly interesting is the presence of not so well-recognized antibiotic resistance related genes such as *fusA1* and *fusA2,* both coding for elongation factor G, or *rpoC,* which codes the β-chain of a DNA-directed RNA polymerase. Mutations in genes coding for two-component regulatory systems, as PhoPQ or ParRS, also require a special mention as mutated isolates showed a strong impact in their MICs for many of the antibiotics tested.



The presence of unique mutations in certain well-known resistance genes, such as *dacB* (PBP4) was observed to increase β-lactam resistance, but mutations within a specific gene did not always correlate or lead to the expected effect on antibiotic resistance (e.g. *pmrB* or *phoP-phoQ* mutated isolates did not exhibit a higher CO MIC). Likewise, several mutations (e.g. *mexZ*, *gyrB* or *oprD*) were associated to extended unexpected antibiotic resistance profiles.

A detailed analysis of the mutational resistome for each class of antibiotics is following provided.

Figure 4.11. MIC-fold change for each antibiotic tested between isolates mutated or not mutated in a specific gene. To evaluate the implication of the presence of mutations in the main genes possibly related with antibiotic resistance the median MIC for both groups were calculated and compared, results are expressed in MIC-fold change. PA2489, *mexW*, *oprF*, *parE* and *nfxB* were excluded since the number of mutated isolates were <3. Some genes were grouped (e.g. *ampD* and *dacB* (PBP4) or *nalD* and *mexR*) according to their well-established effects on resistance (e.g. AmpC or MexAB-OprM overexpression, respectively).

β-lactam resistome. Just three isolates (AUS034, AUS603 and PAMB148) of the CC274 collection were demonstrated to overproduce AmpC. By contrast, at the genomic level, almost all isolates (26/29) contained some variation within *dacB* which codes for the PBP4. Crosslinking phenotypic and genotypic results through *ampC* expression data, suggested that most observed *dacB* allele variations were, in fact, ancestral polymorphisms not involved in antibiotic resistance. AmpC overproduction in the two CF isolates was explained by the presence of specific mutations in *dacB* (S315G or W350R) and by an *ampD* (P41L) mutation in the case of the bloodstream infection isolate PAMB148. Whilst *ampC* overexpression in isolates AUS034 and PAMB148 correlated well with ceftazidime and piperacillin/tazobactam resistance, this was not the case for isolate AUS603 which was documented to be susceptible to these antibiotics. However, unexpected AUS603 β-lactam susceptibility could be explained by the presence of chromosomal mutations whose effects eventually compensate the expected increase in β-lactam resistance. Indeed, this isolate showed an additional non-sense mutation in OprM (Q93X), the OMP of the constitutive MexAB-OprM efflux pump which is well known to play a major role in intrinsic β-lactam resistance. As well, it should also be mentioned that isolate AUS601, exhibiting high-level resistance to ceftazidime, cefepime and aztreonam in the absence of AmpC overproduction, showed an additional mutation (V239A) in AmpC compared to all other CC274 isolates (Table 4.13, Annex 6-β-lactams).

On the other hand, numerous sequence variations were encountered within the essential PBPs coding genes. While some unique mutations were detected in genes coding for PBP1 and PBP3a, the main mutational resistance target among PBPs was found to be PBP3. Up to 7 of 29 isolates presented a mutated PBP3 nucleotide sequence, although β-lactam resistance contribution of each derived *ftsI* (PBP3) allele, if any, depends on the specific point mutation encountered. Missense mutations within the PBP3 (R504C and Q372P) were apparently contributing to resistance in isolates AUS601 and AUS411, since they do not hyperproduce AmpC. Likewise, the P527T mutation of AUS034 likely contributes, together with AmpC overexpression, to the very high-level β-lactam resistance of this isolate, including the new antipseudomonal combination ceftolozane/tazobactam. On the other hand, the P215L and G216S mutations were apparently not linked with phenotypic resistance (Table 4.13. and Annex 6-β-lactams).

Obtained data also demonstrated that the constitutive efflux pump MexAB-OprM is under strong mutational pressure during CF CRI, frequently including inactivating mutations. On the contrary, just 3 isolates showed mutations in regulators leading to MexAB-OprM overexpression (Table 4.13 and Annex 6- β-lactams).

Carbapenem resistome. Imipenem and meropenem resistance correlated in all but two isolates with the presence of non-sense mutations affecting the OMP OprD. High-level meropenem resistance was additionally associated with the presence of PBP3 mutations.

Remarkably, all ST1089 mutator isolates shared the same point mutation in *oprD* (V67X) as well as in *galU* (P123L), also related with carbapenem resistance (Table 4.13. and Annex 6-Carbapenems).

MexEF-OprN overexpression was documented just in isolate FQRC26 and MexAB-OprM in 3 isolates; being all susceptible to both carbapenems tested. On the contrary, resistant isolates AUS411 and AUS603 exhibited WT OprD sequences and no efflux pump overexpression was demonstrated (Table 4.13. and Annex 6-Carbapenems).

Aminoglycoside resistome. Among the CC274 collection, a high proportion of the isolates (23/29) were shown to overexpress MexXY and all but one were mutated in *mexZ,* which codes for the mayor MexXY expression regulator. Remarkably, the same point mutation was detected among different and independent isolates. The single MexXY-overproducing isolate showing no mutations in *mexZ,* presented a unique mutation in *parS*, a gene also involved in the modulation of MexXY expression. Nevertheless, MexXY hyperproduction *per se* cannot explain aminoglycoside resistance in the majority of the isolates. Of note, all high-level resistant isolates hyperproduced MexXY and harboured additional mutations in both genes coding for elongation factor G, *fusA1* and *fusA2* (Table 4.13. and Annex 6-Aminoglycosides).

Fluoroquinolone resistome. Obtained data suggest that contribution of efflux pumps overexpression to high-level resistance to fluoroquinolones is very limited, if any. As shown, just isolate FQSE10-0106 (MIC=0.38 mg/L) was demonstrated to hyperproduce MexCD-OprJ due to a non-sense mutation in *nfxB*. On the other hand, our data shows that high-level fluoroquinolone resistance was associated with the presence of missense mutations in *gyrA*, *gyrB* and/or *parC* quinolone resistance-determining regions (QRDRs). Specifically, up to 9 isolates were mutated in *gyrB* QRDR and all but two harbored the same mutation (S466F), 6 showed mutations in *gyrA* QRDR (T83I, T83A, D87N, D87G and Q106L), and just one isolate was mutated in *parE* (P438S). (Table 4.13. and Annex 6-Fluoroquinolones).

Polymyxin resistome. Many isolates were found to be mutated in genes such as *pagL*, *phoQ* or *pmrB,* but with one exception (i.e. isolate AUS034) phenotypic resistance was not observed. For isolate AUS034, a specific non-sense mutation was detected in the two-component sensor PhoQ, as well as two other specific point mutations within *parR* and *colS*. Five additional isolates were shown to harbour mutations in more than one polymyxin-resistance related genes and showed colistin MICs ranging from 0.125 to 2 mg/L. Up to six different and independent mutational events were registered in PmrB sensor and, strikingly, all Spanish mutators shared the same mutation (Table 4.13. and Annex 6-Aminoglycosides).

4.2.2. EVOLUTIONARY DYNAMICS OF *Pseudomonas aeruginosa* AMINOGLYCOSIDE RESISTANCE DEVELOPMENT

When deciphering the aminoglycoside CC274 *P. aeruginosa* resistome, obtained data demonstrated that for high-level resistance development (in absence of aminoglycoside modifying enzymes) the selection of chromosomal mutations that lead to an enhanced membrane impermeability or MexXY-OprM efflux pump overexpression was not enough. Thus, an *in vitro* evolution experiment was performed in an attempt to elucidate the dynamics and chromosomal mutations involved in aminoglycoside resistance development.

As shown in panels A-E of Figure 4.12., *in vitro* resistance development occurred in a stepwise manner, reaching concentrations ranging from 128 to 512 higher than the initial MIC (0.5 µg/mL). The corresponding tobramycin MICs of the purified colonies at day 14 ranged from 64 to 512 µg/mL, whereas those of gentamycin and amikacin were typically 1 or 2 dilutions higher (Annex 7).

Results obtained from whole-genome sequencing experiments are summarized in Figure 4.12. (detailed in Annex 7). Up to 35 different genes were found to be mutated in at least one of the isolates. Mutants from day 14 showed between 3 and 7 mutations and comparison with those from days 1 and 7 evidenced a stepwise acquisition. However, a few mutations documented at these intermediate stages were not fixed in the population and thus were not seen at day 14.

Among the mutated genes, *fusA1* certainly deserves especial attention since non-synonymous mutations within this gene were detected in all 5 replicate experiments. It should also be noted that the time of detection of *fusA1* mutations varied from day 1 to day 14, and that in 3 of them the same amino acid substitution occurred (I61M). Associated aminoglycoside MICs increments were registered (Figure 4.12. and Annex 7).

Another frequently (3 of 5 replicates) mutated gene was *pmrB*. Emergence of *pmrB* mutations at day seven correlated with increased colistin MICs. However, despite the *pmrB* mutations persisted at day 14, colistin resistance disappeared, likely indicating the acquisition of compensatory mutations. One of these isolates showed an additional mutation in *pagL,* involved in lipid A deacylation and polymyxins resistance (31) was documented (Figure 4.12. and Annex 7).

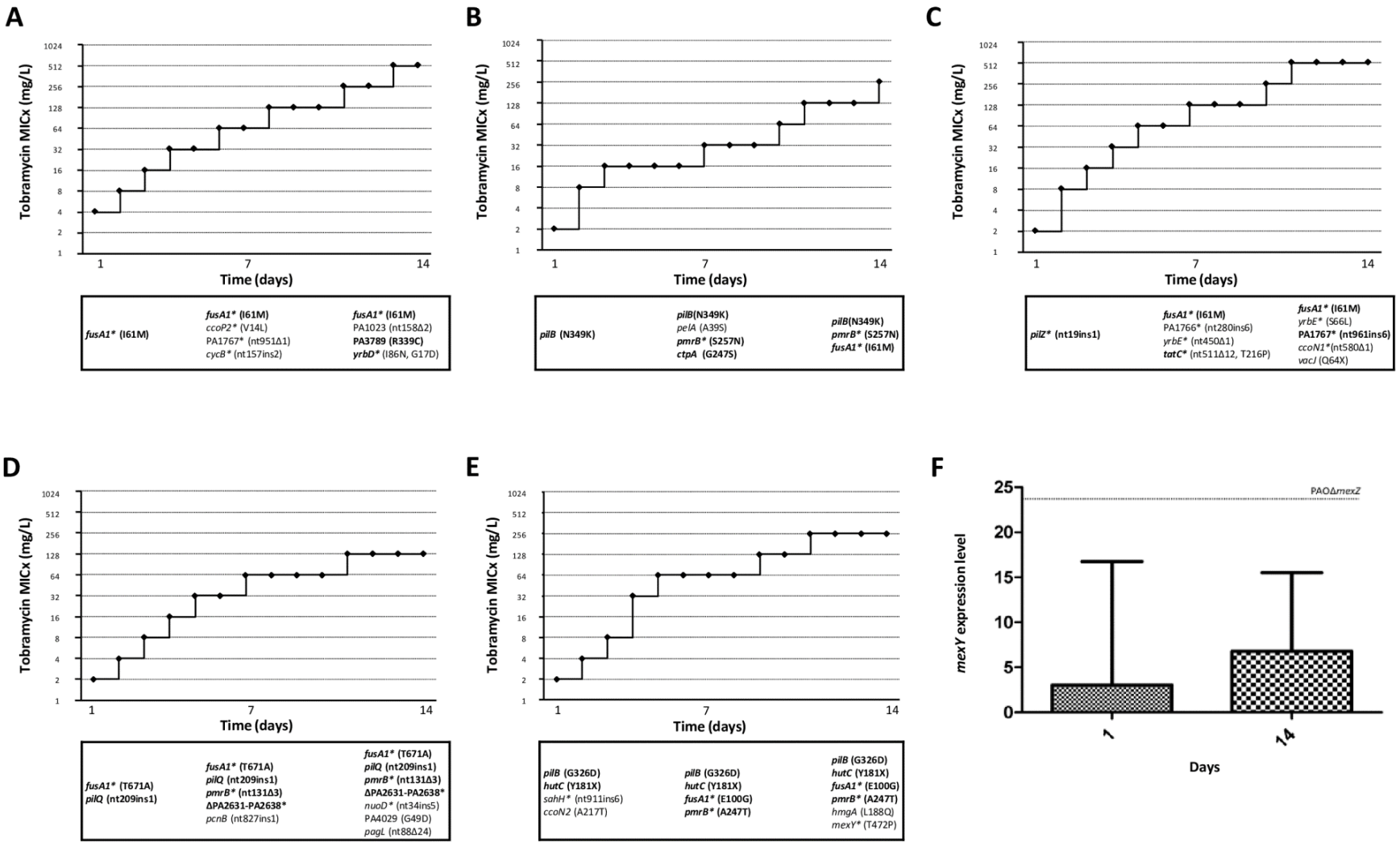


Figure 4.12. Dynamics of resistance development to tobramycin and mutations encountered after 1, 7 and 14 days of tobramycin exposure in the five replicate experiments (A-E). Genetic determinants and specific mutations are highlighted in bold when detected within the two representative colonies studied at each experiment and time point. Genes whose implication in aminoglycoside resistance development has already been demonstrated are indicated with an asterisk. **Median expression level of *mexY* for PAO1-derived resistant mutants after 1 and 14 days of tobramycin exposure (F).**

In relation with other antibiotic classes, a general trend to overtime decreasing MICs particularly for ticarcillin, aztreonam, and ciprofloxacin was noted (Figure 4.13.).

Beyond the mutations actually detected, another relevant aspect to consider are the mutations that were expected but not found in our *in vitro* evolution experiments. No mutations in regulator genes (*mexZ*, PA5471, *parS*) leading to the overexpression of MexXY were seen at any time in any of the 5 replicate experiments. The absence of mutations in these genes was additionally confirmed through Sanger sequencing. Moreover, while *mexY* expression data varied to some extend for the different mutants, values were always below those of a control *mexZ* PAO1 mutant and a statistically significant trend to increased expression at day 14 versus day 1 was not documented (Figure 4.12- panel F).

Among the 3 pairs of isogenic CF *P. aeruginosa* isolates studied, the emergence of *fusA1* mutations was noted in 2 of the 3 tobramycin resistance isolates (Table 4.14. and Annex 7). The resistant isolate not showing *fusA1* mutations was demonstrated to have acquired an exogenous aminoglycoside modifying enzyme (AacA4). As well, in contrast to *in vitro* findings, all 3 CF tobramycin resistant isolates overexpressed *mexY* and showed *mexZ* mutations (Table 4.14. and Annex 7). However, *mexY* overexpression and *mexZ* mutations were also seen in 2 of the 3 susceptible CF isolates.

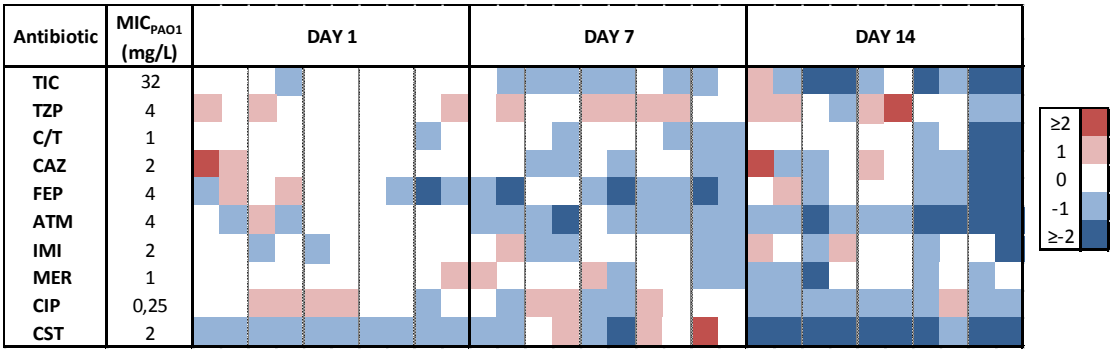


Figure 4.13. MIC-fold changes for each antibiotic tested between the parental strain PAO1 and its derived aminoglycoside resistant mutants. Lower limit for CI and PPT is -1 two-fold dilutions.

Table 4.14. Genomic differences between the three isogenic pairs of tobramycin susceptible-resistant CF isolates.

Locus / Gene[a]	Isolate ID (MIC_{TOB} mg/L)					
	FQSE06-S (1)	FQSE06-R (24)	FQSE11-S (2)	FQSE11-R (>256)	FQSE16-S (4)	FQSE16-R (64)
PA0004/*gyrB*				R138L		
PA0058/*dsbM*			C28R,F206L,R212C			
PA0426/*mexB*			nt772Δ1			Q575R
PA0958/*oprD*				Q424E,S403A		
PA1430/*lasR*				R216Q		
PA2018/*mexY*		G287A				G287S
PA2020/*mexZ*	nt290Δ11	S9P	L138R	L138R		R125P
PA2492/*mexT*						G274D,G300D
PA2639/*nuoD*		G499X				
PA3064/*pelA*	V446I					
PA3141/*capD*					nt512ins1	
PA3168/*gyrA*				Y267N		
PA4020/*mpl*		S257L		Q248X		
PA4266/*fusA1*		Y552C,T671I				Y552C
PA4418/PBP3		P215L				
PA4462/*rpoN*						V473A
PA4568/*rplU*	I74M					
PA4598/*mexD*					P721S, L624P	
PA4600/*nfxB*				E75K		
PA4773/-				A165T		
PA5040/*pilQ*	E676D,E669D					
Resfinder				AacA4		
***mexY* overexpression**	+	+	+	+	-	+

[a]Genes in which mutations were also detected in the resistance evolution experiment are in bold.

5. DISCUSSION

La levedad y el peso

High genetic diversity among CF *P. aeruginosa* isolates. Cross-sectional studies revealed considerable genetic diversity among *P. aeruginosa* isolates infecting CF patients from both Spain and the Balearic Islands. Up to 65% of the CF patients from the Balearic Islands harbored unique PFGE restriction patterns, percentage that increase to 88.6% in the Spanish cohort (65% and 91.1% unique STs, respectively). Moreover, 42% and 67% of the different STs detected within the Balearic Islands and the Spanish collection have not been previously described, finding which supports the extended idea that most CF patients acquire unique *P. aeruginosa* strains from environmental sources.

Globally, the documented genetic diversity is in accordance with those reported from other CF cohorts in which segregation policies applies. In 2001, *Burns et al.* investigated the genetic background of *P. aeruginosa* isolates infecting a cohort of 40 CF paediatric patients from 3 different hospitals of the United States demonstrating a high degree of genotypic variability. Recently, Kidd *et al*. also investigated this issue in a paediatric cohort from Australia and New Zealand, finally concluding that the environment is the most frequent route for *P. aeruginosa* acquisition among CF children. Likewise, a national observational study across Canada including 1,537 isolates from both adult and paediatric CF patients (n=402) has been conducted by *Middleton and collaborators*. In this work, 403 unique STs were detected and, although 39% of STs were shared, most were only detected among a small number of subjects.

With the exception of clone FQSE-A (CC274), shared STs detected among the Balearic Islands and the Spanish collections were also limited to a small number of patients. Indeed, a direct epidemiological relation could be stablished in the majority of the cases as shared STs were mainly detected infecting pairs of siblings. Transmission of *P. aeruginosa* strains between siblings with CF has already been well documented [Kelly NM *et al*, 1982; Thomassen MJ *et al*, 1985; Grothues D *et al*, 1988; Renders NH *et al*, 1997; Tubbs D *et al*, 2001; Abdul Wahab A *et al*, 2014]. In a study performed in Israel, *Picard et al.* showed that when *P. aeruginosa* was isolated from the first-born sibling, up to 91% of the second siblings were also infected; whereas when the first-born was not positive, only 50% of subsequent siblings were infected. Likewise, they also showed that the age of first isolation was significantly earlier in the second sibling compared to the first-born [Picard E *et al*, 2004], finding that other authors have also reported [Slieker MG *et al*, 2010]. Furthermore, worse clinical outcomes (including lower FEV1, faster decline rate of FEV1, more bacterial airway colonization, increased frequency of lung transplants and a trend towards more hospitalizations) have been found in families with multiple CF patients compared to families with only one CF patient, which may reflect the burden and complexity care of this disease [Lavie M *et al*, 2015].

Scarce representation of *P. aeruginosa* CF epidemic strains. Several European and non-European countries have reported the presence of international epidemic *P. aeruginosa*

strains infecting a wide number of CF patients. Likewise, high-risk clone ST175 has been documented to be widely distributed in the Spanish nosocomial setting [Cabot G *et al*, 2012; Cabot G *et al*, 2016a; del Barrio-Tofiño E *et al*, 2017].

In the Spanish collection, the C40A AT-genotype was determined in one of the 79 studied isolates. This genotype has been previously described for *P. aeruginosa* Clone C (ST17) [Hilker R *et al*, 2015; Hall AJ *et al*, 2014] but, curiously, this isolate was ascribed to ST1872 which is a double locus variant of ST17, differing in just two point mutations in *mut*L and *trp*E MLST alleles.

More worrisome, when investigating long-term clonal epidemiology of *P. aeruginosa* colonizing the respiratory tract of CF patients from the Balearic Islands, a clonal replacement of a MDR mutator strain by the MDR LES (ST146) was documented in one of the patients, alerting of the first detection of the likely more world-wide concerning CF epidemic clone in Spain. This unusual and awesome characteristic was also reported by *McCallum et al.* in 4 CF patients infected with unique strains after admission for treatment in a CF center [McCallum SJ *et al*, 2001]. In this case, although the epidemiological driver of LES colonization was not specifically investigated, the fact that the patient has family links with a northern European country could help to explain the acquisition of this CF epidemic clone.

As well, clone FQSE-A was detected in 5 unrelated chronically colonized CF patients from the Balearic Islands, clone that was ascribed to the CC274 by MLST. Moreover, in 4 of them, long-term clonal epidemiology was investigated and this strain was demonstrated to persist during the whole 8-years study period. Therefore, results so far suggested that clone FQSE-A is a CF adapted strain: transmissible and persistent. Furthermore, according to the publicly available MLST database (http://pubmlst.org/paeruginosa/), *P. aeruginosa* ST274 has also been detected infecting multiple CF patients from France, Austria and Australia. Thus, our results add further evidence pointing out that ST274 should be added to the growing list of CF epidemic clones.

Discrepant molecular typing results: role of mutators. PFGE and MLST methods are currently considered the gold-standard tecniques for the establishment of epidemiological links. Compared with MLST, PFGE exhibits a higher discriminatory power (or lower stability) and, conversely, MLST results are more reproducible among different laboratories. Thus, PFGE is the preferred technique for studying local epidemiology and to perform outbreaks investigations whereas MLST has been posed as the golden molecular typing tool for global epidemiological studies and for tracking long-term epidemiological relations.

When exploring the Balearic Islands and the Spanish CF *P. aeruginosa* collections, some discrepancies between these molecular typing methods were detected. Not surprising, due to the overall higher discriminatory power of PFGE, several isolates showing different PFGE

patterns were ascribed to the same ST. Conversely, and much more intriguingly, for some isolates showing identical PFGE patterns different STs were determined.

Clone FQSE-A was detected in 5 CF patients from the Balearic Islands. Whereas isolates from 4 of the patients were ascribed to ST274 by MLST, mutator isolates from the fifth patient were ascribed to ST1089. As ST1089 just differs from ST274 by two point mutations in two of the MLST genes each leading to a non-previously described allele, the available data clearly suggest that mutator ST1089 has recently evolved from ST274. Likewise, recently, García-Castillo and collaborators also reported a ST shift within isolates from a chronically colonized CF patient directly linked to the emergence of a mutator phenotype caused by *mutL* mutations [García-Castillo M *et al*, 2012]. As well, within the CC274 collection one of the Australian mutator isolates was ascribed to a new ST which just differs from ST74 by two missense mutations in *mutL* allele, being one of them (H288Y) responsible for the generation of the new ST.

Although not linked with the emergence of stable mutator phenotypes, similar discrepancies were also documented within the Spanish collection. These discrepancies could be explained in terms of an increase prevalence of transient mutator phenotypes (SOS system) during CF CRI, as the CF lungs are known to be a very stressful environment for bacteria in which mutation supply rate is very high. Of note, although not linked to a stable mutator phenotype, the *mutL* gene was frequently involved in the ascription of clonal isolates (PFGE) to different STs.

As well, other authors have also reported that some *P. aeruginosa* strains are not typable by MLST due to the presence of InDel mutations within the *mutL* fragment analyzed [Kidd TJ *et al*, 2011; del Barrio-Tofiño E *et al*, 2017].

All together these results stress the point that *mutL* lacks the neutrality required for an appropriate MLST marker, especially for epidemiological studies involving isolates causing CF CRI in which not only transient mutator phenotypes frequently rise but also MMR deficient mutators are positively selected [Mena A *et al*, 2008] and, therefore, may determine a lower stability of the MLST profiles than expected (leading to discrepant results) both directly (*mutL* inactivating mutations within the gene fragment evaluated in MLST analysis) and indirectly through the increased spontaneous mutagenesis which can facilitate the emergence of novel alleles through point mutations in any of the 7 house-keeping genes evaluated.

Genomic analysis of the phylogeny, within-host evolution and interpatient transmission of the international CC274 CF *P. aeruginosa* clone. CC274 population structure analysis demonstrated the worldwide coexistence of two separated and divergent clonal lineages, but without evident geographical barrier.

Discussion

Coexistence of distinct evolved CC274 sublineages within a patient was documented. Similar results have been recently reported by Williams *et al.* concerning the LES [Williams D *et al*, 2015]. In that work, they found that multiple coexisting LES lineages are typically infecting CF patients and that genetic divergence between lineages within patients was greater than interpatient diversity, implying acquisition of diverse genetic populations [Williams D *et al*, 2015]. On the opposite, another study focusing on the LES isolated from patients residing the UK and Canada showed less genetic differences, even when transoceanic isolates were compared [Jeukens J *et al*, 2014]. Likewise, *Yang et al*. also documented a lower genetic divergence in the DK2 epidemic clone [Yang L *et al*, 2011] as well as other previous studies with other relevant and/or persistent CF clones which have also reported divergent results [Feliziani S *et al*, 2014; Marvig RL *et al*, 2013; Cramer N *et al*, 2011]. A possible explanation for these observations could be that different routes for adaptation and survival in the CF lung environment are possible and depend on the specific clonal lineages.

The documented within-host diversity may reflect the coexistence of divergent lineages within the infecting inoculum or the occurrence of several independent transmission events during the course of infection. Based on the substantial phenotypic variation previously observed between samples of the LES taken from patients at successive time points [Mowat E *et al*, 2011; Fothergill JL *et al*, 2010], *Williams et al.* finally pointed out recurrent transmissions as the most suitable driver of rapid population genomic flux in LES infections of the CF airway. To gain more insights, *Williams and collaborators* have recently published a work in which they examined the genetic diversity of chronic *P. aeruginosa* LES infections over 13 months among seven chronically infected CF patients attending the same CF center by genome sequencing, documenting rapid and substantial shifts in the relative abundance of lineages and replacement of dominant lineages likely to represent super-infection by repeated transmissions [Williams D *et al*, 2018]. In the case of CC274, and with the exception of mutators, all isolates from an individual patient clustered together in the same branch which makes the acquisition of a mix of genetically different sublineages a more suitable explanation. Nevertheless, whole-genome sequencing of more longitudinal isolates could help to definitely resolve this issue.

By contrast, and more revealing, both phylogenetic reconstructions and mutational resistome analysis based on WGS data allow us to confirm interpatient transmission of mutators (ST274/ST1089). So, compared with classical molecular typing tools, WGS provides detailed genome fingerprints that might be essential for epidemiological studies in which prevalent and ubiquitous clonal lineages are involved. Indeed, WGS closely clustered isolates from four of the patients from the Balearic Islands, likely indicating interpatient transmission or a common source of colonization, whereas isolates from a fifth patient from the same hospital was distantly related.

Insights into the CF *P. aeruginosa* accessory genome. The pangenome of *P. aeruginosa* consists of two different parts: the conserved core genome (90%) and a combinatorial accesory genome (10%), being the accesory genetic elements esential for surviving under certain selective conditions.

A great example of *P. aeruginosa* adaptability is its ability for producing three different types of pyoverdine and four binding-receptors. The major finding in this variable locus was the absence of the alternative receptor for pyoverdine type I (*fpvB*) in 43% of the isolates from the CF Spanish collection, results that do not correlate with previous studies [Pirnay JP *et al*, 2009; De Vos D *et al*, 2001] in which almost all isolates were demonstrated to harbour it. Recently, *Dingemans et al* also found a significant proportion of CF isolates lacking this alternative receptor (22%) and they hypothesized that this receptor may be relieved from selection because *P. aeruginosa* can utilize multiple iron uptake systems in the CF lung to acquire iron in both its ferric and ferrous forms [Dingemans J *et al*, 2014; Hunter RC *et al*, 2013; Konings AF *et al*, 2013]. An alternative hypothesis for the documented absence may be that loss of *fpvB* can be an advantage for evading the immune system and the action of pyocines [Dingemans *et al*, 2014].

As well, with the exception of the flagellin-glycosylation island, other genomic islands included in the Array Tube genotyping tool were underrepresented when compared with other previous studied collections, which maybe reflects the extraordinary ability of *P. aeruginosa* to explote different paths for adaptation and survival in different environments [Liang X *et al*, 2001; Klockgether J *et al*, 2007; Rakhimova E *et al*, 2009]. The high proportion of isolates harbouring this island clearly suggests that glycosylation may confer some advantages in the CF respiratory tract.

Finally, within its genome, *P. aeruginosa* has a large armamentarium of secreted virulence factors that rely on specialized export systems, including the type III secretion system (T3SS) [Frank, Molecular Microbiology 2007]. In accordance with previously published data for CF respiratory isolates, we encountered that up to 81% and 10% of the isolates possess the ExoS and the ExoU encoding genes, respectively, which reflects a diminished virulence during chronic respiratory infections [Feltman H *et al*, 2001; Pirnay J *et al*, 2009].

Antibiotic resistance trends in CF *P. aeruginosa* isolates. Overall, higher non-susceptibility rates to individual agents were documented for the Spanish (2013-2014) collection in comparison with the Balearic Islands (2003-2012) collection. In both collections, aztreonam and ciprofloxacin were the less active antibiotis and colistin the one for which a minor resistance rate was registered. However, it should be mentioned that EUCAST considers *P. aeruginosa* intrinsically resistant to aztreonam (mainly because of the constitutive expression of MexAB-OprM efflux pump), so, all those isolates showing susceptibility deserve spetial mention (discuss later). High resistance rates to individual agents have also been recently

reported by Mustafa *et al*. when studying the antimicrobial susceptibilities of 153 *P. aeruginosa* isolates collected from 2006 to 2012 in 118 CF patients from the United Kingdom, Belgium and Germany [Mustafa MH *et al*, 2016]. Moreover, MDR isolates were also highly prevalent in this Northern European study and within the Spanish collection, finding that compares with the global MDR rate in the Balearic Islands *P. aeruginosa* collection. Of note, a high genetic diversity was documented among the Spanish isolates so maybe the documented higher resistance rates reflect a trend towards increased antibiotic resistance rates as documented in *P. aeruginosa* causing acute infections. Indeed, results from the analysis of antibiotic resistance temporal evolution in the Balearic Islands collection demonstrated a significant upward trend.

Lower non-susceptibility rates were documented for mucoid isolates and, conversely, higher ones were registered for SCV isolates compared with the entire collections. As during CF-CRI an impressive diversification process occur within the infecting population eventually leading to different variants, these results support the importance of perform antibiotic susceptibility testing to at least all different colonies morphotypes encountered within a patient sample.

Non-susceptibility rates values were documented to be higher among chronically colonized CF patients, finding that can be linked to a major antibiotic pressure and, therefore, to an accumulation of resistance mechanisms overtime. Correlation with antibiotics usage was early suggested [Mouton JW *et al*, 1993] but it remains to be demonstrated. In this sense, when we studied long-term CRI, we documented a significant trend towards the accumulation of resistance which was accompanied by a trend towards the accumulation of antibiotic resistance mechanisms.

Mutators as a driver of antibiotic resistance. High proportions of mutator isolates among the CF *P. aeruginosa* population have been demonstrated previously, being frequently associated with antimicrobial resistance [Oliver A, 2010; Montanari S *et al*, 2007; Mena A *et al*, 2008; Ciofu O *et al*, 2005; Marvig RL *et al*, 2013]. Similar rates were documented within the subset of 100 isolates from the Balearic Islands collection and within the CC274 collection, whereas a lower proportion of mutators was found in the Spanish one which maybe reflects earlier stages of chronic colonization.

Defects in the MMR system (*mutS* and *mutL*) were the most frequent cause for hypermutation, which correlate with previous studies [Miller JH, 1996; Oliver A, 2010; Oliver A & Mena A, 2010]. Genetic basis for hypermutation of isolates from the CC274 collection was studied from WGS data (mutome) and, of note, unique missense mutations were encountered in several of the so-called mutator genes in isolates exhibiting a normomutator phenotype, even when located within the MMR system coding-genes.

The obtained antibiotic susceptibility results pointed out mutators as a driver of resistance development in the CF setting, being MDR much more frequent among mutators than in isolates with normal mutation rates. More worrisome, detailed genetic analysis revealed that ST1089 is a *mutS* deficient mutator lineage that have recently evolved from the epidemic strain ST274, which have acquired specific resistance mechanisms and have underwent further interpatient spread.

Altogether these results point out the crutial role of mutators in antibiotic resistance evolution in the CF setting and demonstrate that it can extend beyond intrapatient evolution. Therefore, our results provide evidence of the importance of detecting these hypermutator variants in order to avoid interpatient spread.

Resistome evolution of CF *P. aeruginosa.* Resistome evolution was deeply studied in the CC274 collection by WGS approaches. Whereas horizontally acquired resistance determinants were not encountered, we documented the emergence of mutations in more than 100 genes previously related to antibiotic resistance, which demonstrates the extraordinary capacity of *P. aeruginosa* to develop antibiotic resistance by acquiring chromosomal mutations. While the presence of classical mutational resistance mechanisms was confirmed in several isolates and correlated with resistance phenotypes, our results also provides evidence for a major role of less expected resistance mutations for the majority of antimicrobial classes, including β-lactams, aminoglycosides, fluoroquinolones and polymixins.

β-lactam resistome. The most frequent mutation-driven β-lactam resistance mechanism is likely the overproduction of the chromosomal cephalosporinase AmpC, and it is driven by the selection of mutations in PGN-recycling genes [Juan C *et al*, 2017; Cabot G *et al*, 2011; Moyà B *et al*, 2009]. Among them, the mutational inactivation of *dacB*, encoding the non-essential PBP4, and *ampD*, encoding a *N*-acetyl-muramyl-L-alanine amidase have been found to be the most frequent cause of *ampC* derepression and β-lactam resistance [Juan C *et al*, 2005; Moyà B *et al*, 2009]. The inactivation of PBP4 has also been shown to activate the BlrAB/CreBC regulatory system, further increasing resistance levels [Moyà B *et al*, 2009]. Additionally, specific point mutations leading to a conformation change in the transcriptional regulator AmpR, causing *ampC* upregulation and β-lactam resistance, have been noted among clinical strains. They include the D135N mutation, described in several species besides *P. aeruginosa*, including *Stenotrophomonas maltophilia*, *Citrobacter freundii*, or *Enterobacter cloacae* [Juan C *et al*, 2017] or the R154H mutation, linked to the widespread MDR/XDR ST175 *P. aeruginosa* high-risk clone. Mutation of many other genes, including those encoding other amidases (AmpDh2 and AmpDh3), other PBPs (such as PBP5 and PBP7), lytic transglycosylases (such as SltB1 and MltB), MPL (UDP-*N*-acetylmuramate:Lalanyl-γ-D-glutamyl-meso-diaminopimelate ligase), or NuoN (NADH dehydrogenase I chain N) have been shown to enhance *ampC* expression, either alone or

combined with other mutations, although their impact on β-lactam resistance among clinical strains still needs to be further analyzed [Juan C *et al*, 2017].

Within the CC274 collection, 3 of the isolates overproduced AmpC probably related with the encountered mutations within the PGN recycling genes *dacB* and *ampD*. Moreover, two of them harbored additional inactivating mutations within *mpl,* which may also contribute to AmpC overexpression [Calvopiña K & Avison MB, 2018]. It should be highlighted that one of the isolates (AUS603) did not exhibit phenotypic β-lactam resistance which could be explained in terms of defects in the MexAB-OprM efflux pump system.

Of note, obtained data demonstrated that MexAB-OprM is under strong mutational pressure during CF CRI, including inactivating mutations. This finding correlates with previous investigations that have pointed out that this efflux system is dispensable and, therefore, tends to be lost or inactivated in favour of MexXY-OprM overexpression in CF *P. aeruginosa* subpopulations [Vettoreti L *et al*, 2009]. Results from the CC274 collection support this hypothesis, as while just 3 isolates overexpressed MexAB-OprM, up to 23 overexpressed MexXY. Moreover, many of the isolates showed some degree of hypersusceptibility to aztreonam (substrate of MexAB-OprM) in favour of an increased MIC of cefepime (substrate of MexXY).

Likewise, a susceptibility rate of 60% was documented for aztreonam among the 100 isolates from the 10 chronically colonized patients from the Balearic Islands; percentage that actually reflects the high number of hypersusceptible (MIC ranges 0.125-1 mg/L) isolates falling outside of WT MICs (2-16 mg/L) distributions. As well, in this subset an important number of isolates showed hypersusceptibility to meropenem (substrate of MexAB-OprM) with MICs (<0.06 mg/L) falling outside WT distributions. Although the integrity of MexAB-OprM components was not studied, efflux pumps overexpression was evaluated in the first and last isolate from each patient and clone of this subset and obtained results also support the abovementioned hypothesis.

Nevertheless, results from the *in vitro* experiment under tobramycin pressure and those recently published by *Bolard et al*. [Bolard A *et al*, 2017] suggest that other mechanisms may also be involved in this frequently observed phenotype.

In addition to *ampC* overexpression, recent studies have revealed that β-lactam resistance development, including the novel combinations of β-lactam-β-lactamase inhibitors ceftolozane/tazobactam and ceftazidime/avibactam, may result from mutations leading to the structural modification of AmpC [Cabot G *et al*, 2014; Lahiri SD *et al*, 2014; Fraile-Ribot PA *et al*, 2017; Haidar G *et al*, 2017; MacVane SH *et al*, 2017]. Likewise, recent studies identified diverse AmpC variants associated with high-level cephalosporin resistance, including ceftolozane/tazobactam and ceftazidime/avibactam, in a small proportion (around 1%) of clinical *P. aeruginosa* isolates [Berrazeg M *et al*, 2015]. Currently, over 200

Pseudomonas Derived Cephalosporinase (PDC) variants have been described, including those associated with enhanced ceftolozane/tazobactam and ceftazidime/avibactam resistance. Within the CC274 collection the mutator AUS601, exhibiting high-level resistance to ceftazidime, cefepime and aztreonam in the absence of AmpC overexpression, harbored a mutation within AmpC (V239A) likely contributing to the documented phenotypic resistance. Moreover, in a recent *in vitro* study performed in our group, this specific amino acid substitution was demonstrated to be selected in two of the three β-lactam resistance PAOMS derivatives obtained upon ceftolozane/tazobactam exposure. Of note, contrary to these derivatives, AUS601 remained susceptible to ceftolozane/tazobactam, which illustrates the complexity of mutation-driven resistance within CF isolates.

Besides the chromosomic cephalosporinase AmpC, there is increasing evidence on the role of target modification (essential PBPs) in *P. aeruginosa* β-lactam resistance. Particularly noteworthy are the mutations in *ftsI*, encoding the PBP3, an essential high molecular class B PBP with transpeptidase activity [Chen W *et al*, 2016]. Analysis of the CC274 collection demonstrated that this gene is under strong mutational pressure, as up to 6 different mutations were detected in 7 of the 29 isolates. Although aminoacid substitutions R504C and Q327 are not located in the PBP3 active site, both are very close to two loop regions (residues 332-338 and 526-533) which play an important role in susbstrate recognition [Han S *et al*, 2010]. So, along with the fact that isolates harboring these mutations exhibit β-lactam resistance in the absence of AmpC overexpression, we can conclude that these PBP3 mutations likely contribute to β-lactam resistance. In late years, several other authors have also shown that this PBP is frequently mutated not only among CF *P. aeruginosa* infecting strains [Díaz-Caballero J *et al*, 2015] but also among the so-called high-risk clones [Kos VN *et al*, 2015; Cabot G *et al*, 2016a; del Barrio-Tofiño E *et al*, 2017]. Moreover, PBP3 missense mutations leading to amino acid substitutions in residue 504 (R504C, R504H) have been recently described to occur *in vitro* upon meropenem [Cabot G *et al*, 2016b] and aztreonam [Jorth P *et al*, 2017] exposure as well as among isolates from widespread nosocomial *P. aeruginosa* clones and CF isolates [Cabot G *et al*, 2016a; Kos VN *et al*, 2015; Díaz-Caballero J *et al*, 2015]. Indeed, isolate harboring the Q372P mutation exhibited high-level resistance to carbapenems in the absence of OprD inactivating mutations. As well, mutation P527T may also contribute to β-lactam resistance whereas mutations in residues 215 and 216 apparently not, in agreement with the fact that these residues are not implicated in the formation and stabilization of the inactivating complex β-lactam-PBP3 [Han S *et al*, 2010]. Likewise, several unique mutations were detected in genes coding for PBP1 and PBP3a which role in β-lactam resistance, if any, still needs to be experimentally addressed.

Another apparently relevant mutational β-lactam resistance mechanism is the selection of large (>200 Kb) deletions affecting specific parts of the chromosome upon meropenem exposure [Cabot G *et al*, 2016b]. Although the basis of the conferred resistance phenotype

still needs to be further clarified, these mutants can be recognized by the characteristic brown pigment (pyomelanine) caused by the deletion of one of the affected genes, *hmgA*, coding for a homogentisate-1,2-dioxygenase. This type of deletion has been documented in both, *in vitro* evolved β-lactam resistant mutants and CF isolates [Cabot G *et al*, 2016b; Hocquet D *et al*, 2016]. However, the deletion of *hmgA* is not responsible of the resistance phenotype, which might be linked to the deletion of another of the affected genes, *galU*, coding for a UDP-glucose pyrophosphorylase required for LPS core synthesis; indeed, analysis of transposon mutant libraries have shown that the inactivation of *galU* increases ceftazidime and meropenem MICs [Alvarez-Ortega C *et al*, 2010; Dötsch A *et al*, 2009]. None of the CC274 isolates showed these large deletions neither exhibited brown pigmentation; however, it should be noted that all ST1089 mutator isolates showed the same missense mutation in galU, likely contributing to their carbapenem resistance.

Apart from these emerging β-lactam resistance mechanisms, phenotypic carbapenem resistance has been clasically linked to the mutational inactivation of the carbapenem porin OprD [Lister PD *et al*, 2009; Castanheira M *et al*, 2014]. Overall, our results, from both the CC274 collection and the subset of 100 isolates from the Balearic Islands, confirm that carbapenem resistance is frequently associated with inactivating mutations within OprD. Additionally, Richardot and collaborators recently reported that some amino acid substitutions within OprD can also confer carbapenem resistance, particularly in the CF setting [Richardot C *et al*, 2015]; nevertheless, no amino acid substitutions were detected within the CC274 isolates. As well, carbapenem resistance may also result from *oprD* repression caused by mutations in the MexEF-OprN efflux pump regulators (*mexS/T*) or the ParRS two-component system [Li XZ *et al*, 2015]; however, our results showed that overexpression of MexEF-OprN is not frequent among CF isolates.

Aminoglycoside resistome. Intravenous antimicrobial combinations including an aminoglycoside are frequently used to manage CF exacerbations. Moreover, in the last decade, tobramycin inhalation has become an important contributor to CF treatment as a means to control CRI as well as a first-line treatment for the eradication of early acquisition of *P. aeruginosa* and several aminoglycoside-based inhaled formulations are currently available [Shteinberg M & Elborn JS, 2015].

Whereas resistance to these agents in acute infections are mainly attributed to the production of aminoglycoside modifying enzymes or 16S rRNA methyltransferases, resistance development in the CRI setting has been linked to the selection of chromosomal mutations leading to enhanced membrane impermeability or MexXY overexpression [Pricket MH *et al*, 2017; Guenard S *et al*, 2014; Poole K, 2015; Vogne C *et al*, 2004]. In accordance, most of the CF clinical studied isolates overexpressed this efflux pump system linked to the presence of mutations within *mexZ*, *amgS* and/or *parRS*; moreover, these mutations occur early and were associated with low-level resistance. Of note, mutations leading to

MexXYoverexpression were not seen in any of the five replicate *in vitro* evolution experiments upon tobramycin pressure. Moreover, while mexY expression data varied to some extent for the different mutants, values were always below those of a control *mexZ* PAO1 mutant, and a statistically significant trend toward increased expression at day 14 versus day 1 was no documented. Thus, these results indicate that mutational overexpression of MexXY is not required for the evolution of high-level tobramycin resistance *in vitro*. Altogether these results may indicate that positive selection of mutations leading to the overexpression of MexXY in CF might be driven by factors beyond exposure to aminoglycosides.

Beyond MexXY overexpression, recent studies have revealed that the aminoglycoside mutational resistome extends far, and that high-level resistance may result from the accumulation of multiple mutations, and the involvement of several novel resistance determinants has been recently documented [El'Garch F *et al*, 2007; Schurek KN *et al*, 2008; Feng Y *et al*, 2016]. WGS data revealed that all high-level resistant CC274 isolates not only overexpressed MexXY but also harbored additional mutations in some of these genes, especially highlighting the presence of mutations in both genes coding for elongation factor G, *fusA1* and *fusA2*. Moreover, *Greipel and collaborators* have also recently reported that these genes are under high evolutionary pressure in the CF environment [Greipel L *et al*, 2016], which can be explained in terms of a wide aminoglycoside use in this setting. As well, several works have associated some specific mutations in FusA1 with aminoglycoside resistance *in vitro* [Feng Y *et al*, 2016] and among clinical, particularly CF, strains [Chung JC *et al*, 2012; Markussen T *et al*, 2014], and, more recently Bolard and collaborators have confirmed the implication of such fusA1 mutations in aminoglycoside resistance through site-directed mutagenesis [Bolard A *et al*, 2017].

In accordance, FusA1 mutations were encountered in all 5 replicates from the *in vitro* evolution experiment associated with a 1- to 3-fold increase in MICs of tobramycin, gentamicin and amikacin, which correlates with *Bolard et al.* observations [Bolard A *et al*, 2017]. As well, resistance development was shown to occur in a stepwise manner, reaching MICs at day 14 close to the maximum tobramycin concentrations achieved through inhaled administration and on the range of the breakpoints suggested for inhaled therapy. Morevoer, 22 of the 35 (63%) different mutated genes have also been related to aminoglycoside resistance development by other authors [Bolard A *et al*, 2017; Yen P & Papin JA, 2017; Feng Y *et al*, 2016; Islam S *et al*, 2009; Schurek KN *et al*, 2009; El'Garch F *et al*, 2007], and thus, our data confirm their relevance in this stepwise process. Mutations within pmrB, traditionally linked with polymyxin resistance development [Moskowitz SM *et al*, 2012; Barrow K & Kwon DH, 2009], rise frequently which alerts from a possible mechanism of co-resistance to two relevant antipseudomonal agents.

Finally, it should be mentioned the fact that although mutational resistance is thought to be the rule in CF CRI, horizontally-acquired resistance always needs to be ruled out.

Fluoroquinolone resistome. Major *P. aeruginosa* RND efflux pumps MexAB-OprM, MexXY, MexCD-OprJ and MexEF-OprN modulate fluoroquinolone resistance. Nevertheless, our results showed that, with the exception of MexXY, efflux pump overexpression is infrequent. The low prevalence of MexCD-OprJ overexpression compares with the fact that hyperproducing mutants tend to emerge after both *in vitro* and *in vivo* fluoroquinolone exposure [Cabot G *et al*, 2016b] and to previous data that have pointed out MexCD-OprJ overexpression as an advantage in the CF setting [Mulet X *et al*, 2011].

So, the fluoroquinolone mutational resistome of CF *P. aeruginosa* generally includes specific missense mutations in DNA gyrase (*gyrA* and/or *gyrB*) and topoisomerase IV (*parC* and/or *parE*) Quinolone Resistance-Determining Regions (QRDRs). Of note, our results revealed that QRDR mutations involved in fluoroquinolone resistance in CF might be more variable.

Polymyxin resistome. As it has been previouskly documented by other authors [Moskowitz SM *et al*, 2012; Gutu AD *et al*, 2013; Miller AK *et al*, 2011; Fernández L *et al*, 2010], the analysis of colistin resistance mechanisms is not always straight forward since the presence of mutations in these two-component regulators is not always associated with clinical colistin resistance, which probably denotes partial complementation between the different regulators. In this sense, *Lee and collaborators* showed that individual two-component systems may not be essential for acquisition of colistin (polymyxin E) resistance in *P. aeruginosa* [Lee JY & Ko KS, 2014]. Nevertheless, it should be highlighted that the isolate exhibiting a premature stop codon in *phoQ* exhibited high-level resistance.

On the whole our results have provided new insights into the evolutionary dynamics and mutation-driven mechanisms of *P. aeruginosa* antibiotic resistance, increasing the current knowledge of the mutational resistome of *P. aeruginosa* , summarized in Table 5.1.

Table 5.1. Main genes and mutations known to be involved in increased antibiotic resistance.

Gene	Resistance mechanisms / Altered target	Antibiotics affected[a]	Type of mutation	Relevant examples	References
gyrA	DNA gyrase	FQ	Gain-of-function	G81D, T83A, T83I, Y86N, D87G, D87N, D87Y, Q106L	Bruchmann S *et al*, 2013 Kos VN *et al*, 2015 Cabot G *et al*, 2016a López-Causapé, C *et al*, 2017 del Barrio-Tofiño E *et al*, 2017
gyrB	DNA gyrase	FQ	Gain-of-function	S466F, S466Y, Q467R, E468D	Bruchmann S *et al*, 2013 Kos VN *et al*, 2015 López-Causapé, C *et al*, 2017 del Barrio-Tofiño E *et al*, 2017
parC	DNA topoisomerase IV	FQ	Gain-of-function	S87L , S87W	Bruchmann S *et al*, 2013 Kos VN *et al*, 2015 Cabot G *et al*, 2016a del Barrio-Tofiño E *et al*, 2017
parE	DNA topoisomerase IV	FQ	Gain-of-function	S457G, S457T, E459D, E459K	Bruchmann S *et al*, 2013 Kos VN *et al*, 2015 López-Causapé, C *et al*, 2017 del Barrio-Tofiño E *et al*, 2017
pmrA	LPS (lipid A)	CO	Gain-of-function	L157Q	Lee JY & Ko KS, 2014
pmrB	LPS (lipid A)	CO	Gain-of-function	L14P, A54V, R79H, R135Q, A247T, A248T, A248V,R259H, M292I, M292T	Barrow K & Kwon DH, 2009 Moskowitz SM *et al*, 2012
phoQ	LPS (lipid A)	CO	Loss-of-function		
parR	LPS (lipid A)	CO	Gain-of-function	M59I, E156K	Muller C *et al*, 2011 Guénard S *et al*, 2014
	OprD downregulation	IP, MP			
	MexEF-OprN hyperproduction	FQ			
	MexXY-OprM hyperproduction	FQ, AMG, PM			
parS	LPS (lipid A)	CO	Gain-of-function	L14Q, V101M, L137P, A138T, A168V Q232E, G361R	Muller C *et al*, 2011 Fournier D *et al*, 2013 Guénard S *et al*, 2014
	OprD downregulation	IP, MP			
	MexEF-OprN hyperproduction	FQ			
	MexXY–OprM hyperproduction	FQ, AMG, PM			

Table 5.1. Main genes and mutations known to be involved in increased antibiotic resistance. (Cont.)

Gene	Resistance mechanisms / Altered target	Antibiotics affected[a]	Type of mutation	Relevant examples	References
cprS	LPS (lipid A)	CO	Gain-of-function	R241C	Gutu AD *et al*, 2013
colR	LPS (lipid A)	CO	Gain-of-function	D32N	Gutu AD *et al*, 2013
colS	LPS (lipid A)	CO	Gain-of-function	A106V	Gutu AD *et al*, 2013
mexR	MexAB –OprM hyperproduction	FQ, TZ, PM, PPT, MP, TZ/AVI	Loss-of-function		
nalC	MexAB-OprM hyperproduction	FQ, TZ, PM, PPT, MP, TZ/AVI	Loss-of-function		
nalD	MexAB-OprM hyperproduction	FQ, TZ, PM, PPT, MP, TZ/AVI	Loss-of-function		
nfxB	MexCD-OprJ Hyperproduction	FQ, PM	Loss-of-function		
mexS	MexEF-OprN hyperproduction	FQ	Loss-of-function		
	OprD downregulation	IP, MP			
mexT	MexEF-OprN hyperproduction	FQ	Gain-of-function	G257S, G257T	Juarez P *et al*, 2018
	OprD downregulation	IP, MP			
cmrA	MexEF-OprN hyperproduction	MP, FQ	Gain-of-function	A68V, L89Q, H204L, N214K	Juarez P *et al*, 2017
mvaT	MexEF-OprN hyperproduction	FQ	Loss-of-function		
PA3271	MexEF-OprN hyperproduction	FQ	Loss-of-function		
mexZ	MexXY –OprM hyperproduction	FQ, AMG, PM	Loss-of-function		
PA5471.1	MexXY –OprM hyperproduction	FQ, AMG, PM	Loss-of-function		
amgS	MexXY –OprM hyperproduction	FQ, AMG, PM	Gain of function	V121G, R182C	Lau CH *et al*, 2013
oprD	OprD inactivation	IP, MP	Loss-of-function		
ampC	AmpC structural modification	TZ/AVI, TOL/TAZ	Gain-of-function	T96I, G183D, E247K	Cabot G *et al*, 2014 Fraile-Ribot PA *et al*, 2017
ampD	AmpC hyperproduction	TZ, PM, PPT	Loss-of-function		

Table 5.1. Main genes and mutations known to be involved in increased antibiotic resistance. (Cont.)

Gene	Resistance mechanisms / Altered target	Antibiotics affected[a]	Type of mutation	Relevant examples	References
ampDh2	AmpC hyperproduction	TZ, PM, PPT	Loss-of-function		
ampDh3	AmpC hyperproduction	TZ, PM, PPT	Loss-of-function		
ampR	AmpC hyperproduction	TZ, PM, PPT	Gain-of-function	D135N, G154R	Cabot G *et al*, 2016a Bagge N *et al*, 2002
dacB	AmpC hyperproduction	TZ, PM, PPT	Loss-of-function		
ftsI	Penicillin-binding-protein 3 (PBP3)	TZ, PM, PPT, MP, TZ/AVI, TOL/TAZ	Gain-of-function	R504C, R504H, P527T F533L	Diaz Caballero J *et al*, 2015 Cabot G *et al*, 2016a Cabot G *et al*, 2016b López-Causapé C *et al*, 2017 del Barrio-Tofiño E *et al*, 2017
fusA1	Elongation factor G	AMG	Gain-of-function	V93A, K504E, Y552C, P554L, A555E, N592I, P618L, T671A, T671I	Feng Y *et al*, 2016 López-Causapé C *et al*, 2017 del Barrio-Tofiño E *et al*, 2017 Bolard A *et al*, 2017
glpT	Transporter protein GlpT	FO	Loss-of-function		
rpoB	RNA polymerase β-chain	RI	Gain-of-function	S517F, Q518R, Q518L, D521G, H531Y, H531L, S536F, L538I, S579F, S579Y, N629S, D636Y	Jatsenko T *et al*, 2010

6. CONCLUSIONS

La Gran Marcha

1. The population structure of *P. aeruginosa* isolates infecting CF individuals from the Balearic Islands and Spain is highly diverse, which points out environmental sources as the main route of *P. aeruginosa* acquisition.

2. Epidemic strains were scarce and shared *P. aeruginosa* clones were mainly detected in pairs of siblings involving non-epidemic strains. However, the multidrug resistant Liverpool Epidemic Strain was detected for the first time in Spain.

3. Discrepanices between PFGE and MLST genotyping methods were frequently detected when studying CF isolates mainly due to the lack of neutrality of the *mutL* gene caused by the positive selection of mutator phenotypes. WGS based approaches are a powerful tool that can help in solving this issue.

4. The global trend towards the accumulation of antibiotic resistance during CF-CRI is accompanied by collateral susceptibility to some antibiotics such as aztreonam, which can be explained by the overexpression of MexXY leading to the impairment of MexAB-OprM.

5. Worldwide distributed *P. aeruginosa* CC274 is a well-adapted CF strain, transmissible and persistant and, therefore, it should be added to the list of CF *P. aeruginosa* epidemic clones.

6. Dissemination of evolved mutator lineages, frequently linked to multidrug resistant profiles, between CF patients constitutes a step forward on the spread of antibiotic resistance.

7. Correlation between phenotypes and WGS genotypes of clonal isolates from an epidemic strain allowed us to decipher the *P. aeruginosa* mutational resistome in the CF setting.

8. The β-lactam mutational resistome extends beyond the chromosomic cephalosporinase AmpC. Especial mention deserves gain-of-function mutations within the PBPs, being the PBP3 under high evolutionary pressure in CF isolates.

9. Mutation-driven aminoglycoside resistance development is a stepwise process in which gain-of-function mutations within *fusA1* and loss-of-function mutations within *mexZ* are highly prevalent among CF isolates. The absence of *mexZ* mutations *in vitro* suggests an evolutionary advantage of MexXY overexpression within the respiratory tract of CF patients.

10. Altogether this work demonstrates that clonal epidemiology and antibiotic resistance evolution in the CF setting results from the complex interplay among mutation-driven resistance mechanisms, within host diversification and interpatient transmission of epidemic strains.

7. REFERENCES

La sonrisa de Karenin

Aaron SD, Vandemheen KL, Ramotar K, Giesbrecht-Lewis T, Tullis E, Freitag A, Paterson N, Jackson M, Lougheed MD, Dowson C, Kumar V, Ferris W, Chan F, Doucette S, Fergusson D. Infection with transmissible strains of *Pseudomonas aeruginosa* and clinical outcomes in adults with cystic fibrosis. JAMA. 2010; 304(19):2145-53.

Abdul Wahab A, Taj-Aldeen SJ, Hagen F, Diophode S, Saadoon A, Meis JF, Klaassen CH. Genotypic diversity of *Pseudomonas aeruginosa* in cystic fibrosis siblings in Qatar using AFLP fingerprinting. *Eur J Clin Microbiol Infect Dis*. 2014; 33(2):265-71.

Agarwal G, Kapil A, Kabra SK, Das BK, Dwivedi SN. Characterization of *Pseudomonas aeruginosa* isolated from chronically infected children with cystic fibrosis in India. *BMC Microbiol*. 2005; 5:43.

Aires JR, Köhler T, Nikaido H, Plésiat P. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother*. 1999; 43(11):2624-8.

Al-Aloul M, Crawley J, Winstanley C, Hart CA, Ledson MJ, Walshaw MJ. Increased morbidity associated with chronic infection by an epidemic *Pseudomonas aeruginosa* strain in CF patients. *Thorax*. 2004; 59(4):334-6.

Alexander C, Rietschel ET. Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res*. 2001; 7(3):167-202.

Alipour M, Suntres ZE, Omri A. Importance of DNase and alginate lyase for enhancing free and liposome encapsulated aminoglycoside activity against *Pseudomonas aeruginosa*. *J Antimicrob Chemother*. 2009; 64(2):317-25.

Alvarez-Ortega C, Wiegand I, Olivares J, Hancock RE, Martínez JL. The intrinsic resistome of *Pseudomonas aeruginosa* to β-lactams. *Virulence*. 2011; 2(2):144-6.

Alvarez-Ortega C, Wiegand I, Olivares J, Hancock RE, Martínez JL. Genetic determinants involved in the susceptibility of *Pseudomonas aeruginosa* to beta-lactam antibiotics. *Antimicrob Agents Chemother*. 2010; 54(10):4159-67.

Anthony M, Rose B, Pegler MB, Elkins M, Service H, Thamotharampillai K, Watson J, Robinson M, Bye P, Merlino J, Harbour C. Genetic analysis of *Pseudomonas aeruginosa* isolates from the sputa of Australian adult cystic fibrosis patients. *J Clin Microbiol*. 2002; 40(8):2772-8.

Armstrong D, Bell S, Robinson M, Bye P, Rose B, Harbour C, Lee C, Service H, Nissen M, Syrmis M, Wainwright C. Evidence for spread of a clonal strain of *Pseudomonas aeruginosa* among cystic fibrosis clinics. *J Clin Microbiol*. 2003; 41(5):2266-7.

References

Armstrong DS, Nixon GM, Carzino R, Bigham A, Carlin JB, Robins-Browne RM, Grimwood K. Detection of a widespread clone of *Pseudomonas aeruginosa* in a pediatric cystic fibrosis clinic. *Am J Respir Crit Care Med*. 2002; 166(7):983-7.

Arora SK, Bangera M, Lory S, Ramphal R. A genomic island in *Pseudomonas aeruginosa* carries the determinants of flagellin glycosylation. *Proc Natl Acad Sci U S A.* 2001; 98(16):9342-7.

Ashish A, Shaw M, McShane J, Ledson MJ, Walshaw MJ. Health-related quality of life in Cystic Fibrosis patients infected with transmissible *Pseudomonas aeruginosa* strains: cohort study. *JRSM Short Rep*. 2012; 3(2):12.

Ashish A, Shaw M, Winstanley C, Ledson MJ, Walshaw MJ. Increasing resistance of the Liverpool Epidemic Strain (LES) of *Pseudomonas aeruginosa* (Psa) to antibiotics in cystic fibrosis (CF)-a cause for concern? *J Cyst Fibros*. 2012; 11(3):173-9.

Bagge N, Hentzer M, Andersen JB, Ciofu O, Givskov M, Høiby N. Dynamics and spatial distribution of beta-lactamase expression in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother*. 2004; 48(4):1168-74.

Bagge N, Ciofu O, Hentzer M, Campbell JI, Givskov M, Høiby N. Constitutive high expression of chromosomal beta-lactamase in *Pseudomonas aeruginosa* caused by a new insertion sequence (IS1669) located in *ampD*. *Antimicrob Agents Chemother*. 2002; 46:3406-11.

Barrow K, Kwon DH. Alterations in two-component regulatory systems of phoPQ and pmrAB are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2009; 53(12):5150-4.

Bartowsky E, Normark S. Interactions of wild-type and mutant AmpR of Citrobacter freundii with target DNA. *Mol Microbiol*. 1993; 10:555–65.

Bellido F, Martin NL, Siehnel RJ, Hancock RE. Reevaluation, using intact cells, of the exclusion limit and role of porin OprF in *Pseudomonas aeruginosa* outer membrane permeability. *J Bacteriol*. 1992; 174(16):5196-203.

Sobel ML, McKay GA, Poole K. Contribution of the MexXY multidrug transporter to aminoglycoside resistance in *Pseudomonas aeruginosa* clinical isolates. *Antimicrob Agents Chemother*. 2003; 47(10):3202-7.

Berrazeg M, Jeannot K, Ntsogo Enguéné VY, Broutin I, Loeffert S, Fournier D, Plésiat P. Mutations in β-Lactamase AmpC increase resistance of *Pseudomonas aeruginosa* isolates to antipseudomonal cephalosporins. *Antimicrob Agents Chemother*. 2015; 59(10):6248-55.

Bjarnsholt T. The role of bacterial biofilms in chronic infections. *PMIS Suppl.* 2013; (136):1-51.

Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, Høiby N. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr Pulmonol*. 2009; 44(6):547-58.

Blázquez J, Oliver A, Gómez-Gómez JM. Mutation and evolution of antibiotic resistance: antibiotics as promoters of antibiotic resistance? *Curr Drug Targets*. 2002; 3(4):345-9.

Bolard A, Plésiat P, Jeannot K. Mutations in gene *fusA1* as a novel mechanism of aminoglycoside resistance in clinical strains of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2018; 62(2). pii: e01835-17.

Boles BR, Singh PK. Endogenous oxidative stress produces diversity and adaptability in biofilm communities. *Proc Natl Acad Sci USA*. 2008; 105(34):12503-8.

Boucher JC, Yu H, Mudd MH, Deretic V. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of *muc* mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. *Infect Immun.* 1997; 65(9):3838-46.

Boyd A, Chakrabarty AM. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Appl Environ Microbiol*. 1994; 60(7):2355-9.

Bradbury RS, Roddam LF, Merritt A, Reid DW, Champion AC. Virulence gene distribution in clinical, nosocomial and environmental isolates of *Pseudomonas aeruginosa*. *J Med Microbiol*. 2010; 59(Pt8):881-90.

Bradbury R, Champion A, Reid DW. Poor clinical outcomes associated with a multi-drug resistant clonal strain of *Pseudomonas aeruginosa* in the Tasmanian cystic fibrosis population. *Respirology*. 2008; 13(6):886-92.

Breidenstein EB, de la Fuente-Núñez C, Hancock RE. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol*. 2011; 19(8):419-26.

Breidenstein EB, Khaira BK, Wiegand I, Overhage J, Hancock RE. Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. *Antimicrob Agents Chemother*. 2008; 52(12):4486-91.

Bruchmann S, Dötsch A, Nouri B, Chaberny IF, Häussler S. Quantitative contributions of target alteration and decreased drug accumulation to *Pseudomonas aeruginosa* fluoroquinolone resistance. *Antimicrob Agents Chemother*. 2013; 57(3) :1361-8.

References

Burgel PR, Bellis G, Olesen HV, Viviani L, Zolin A, Blasi F, Elborn JS; ERS/ECFS Task Force on Provision of Care for Adults with Cystic Fibrosis in Europe. Future trends in cystic fibrosis demography in 34 European countries. *Eur Respir J*. 2015; 46(1):133-41.

Burns N, James CE, Harrison E. Polylysogeny magnifies competitiveness of a bacterial pathogen *in vivo*. *Evol Appl*. 2015; 8(4):346-51.

Cabot G, López-Causapé C, Ocampo-Sosa AA, Sommer LM, Domínguez MÁ, Zamorano L, Juan C, Tubau F, Rodríguez C, Moyà B, Peña C, Martínez-Martínez L, Plesiat P, Oliver A. Deciphering the resistome of the widespread *Pseudomonas aeruginosa* Sequence Type 175 international high-risk clone through Whole-Genome Sequencing. *Antimicrob Agents Chemother*. 2016a; 60(12):7415-7423.

Cabot G, Zamorano L, Moyà B, Juan C, Navas A, Blázquez J, Oliver A. Evolution of *Pseudomonas aeruginosa* antimicrobial resistance and fitness under low and high mutation rates. *Antimicrob Agents Chemother*. 2016b; 60(3):1767-78.

Cabot G, Bruchmann S, Mulet X, Zamorano L, Moyà B, Juan C, Haussler S, Oliver A. *Pseudomonas aeruginosa* ceftolozane-tazobactam resistance development requires multiple mutations leading to overexpression and structural modification of AmpC. *Antimicrob Agents Chemother*. 2014; 58(6):3091-9.

Cabot G, Ocampo-Sosa AA, Domínguez MA, Gago JF, Juan C, Tubau F, Rodríguez C, Moyà B, Peña C, Martínez-Martínez L, Oliver A; Spanish Network for Research in Infectious Diseases (REIPI). Genetic markers of widespread extensively drug-resistant *Pseudomonas aeruginosa* high-risk clones. *Antimicrob Agents Chemother*. 2012; 56(12):6349-57.

Cabot G, Ocampo-Sosa AA, Tubau F, Macia MD, Rodríguez C, Moya B, Zamorano L, Suárez C, Peña C, Martínez-Martínez L, Oliver A; Spanish Network for Research in Infectious Diseases (REIPI). Overexpression of AmpC and efflux pumps in *Pseudomonas aeruginosa* isolates from bloodstream infections: prevalence and impact on resistance in a Spanish multicenter study. *Antimicrob Agents Chemother*. 2011; 55(5):1906-11.

Calvopiña K, Avison MB. Disruption of *mpl* activates β-lactamase production in *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* clinical isolates. *Antimicrob Agents Chemother*. 2018. pii: AAC.00638-18.

Cardoso O, Alves AF, Leitão R. Metallo-beta-lactamase VIM-2 in *Pseudomonas aeruginosa* isolates from a cystic fibrosis patient. *Int J Antimicrob Agents*. 2008; 31(4):375-9.

Carter ME, Fothergill JL, Walshaw MJ, Rajakumar K, Kadioglu A, Winstanley C. A subtype of a *Pseudomonas aeruginosa* cystic fibrosis epidemic strain exhibits enhanced virulence in a murine model of acute respiratory infection. *J Infect Dis*. 2010; 202(6):935-42.

Castanheira M, Deshpande LM, Costello A, Davies TA, Jones RN. Epidemiology and carbapenem resistance mechanisms of carbapenem-non-susceptible *Pseudomonas aeruginosa* collected during 2009-11 in 14 European and Mediterranean countries. *J Antimicrob Chemother*. 2014; 69(7):1804-14.

Chen H, Yi C, Zhang J, Zhang W, Ge Z, Yang CG, He C. Structural insight into the oxidation-sensing mechanism of the antibiotic resistance of regulator MexR. *EMBO Rep*. 2010; 11(9):685-90. Erratum in: *EMBO Rep*. 2010; 11(9):717.

Chen H, Hu J, Chen PR, Lan L, Li Z, Hicks LM, Dinner AR, He C. The *Pseudomonas aeruginosa* multidrug efflux regulator MexR uses an oxidation-sensing mechanism. *Proc Natl Acad Sci USA*. 2008; 105(36):13586-91.

Chen W, Zhang YM, Davies C. Penicillin-binding protein 3 is essential for growth of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2016; 61(1). pii: e01651-16.

Cheng K, Smyth RL, Govan JR, Doherty C, Winstanley C, Denning N, Heaf DP, van Saene H, Hart CA. Spread of beta-lactam-resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. *Lancet*. 1996; 348(9028):639-42.

Cheng Q, Park JT. Substrate specificity of the AmpG permease required for recycling of cell wall anhydro-muropeptides. *J Bacteriol.* 2002; 184(23):6434-6.

Cheng Q, Li H, Merdek K, Park JT. Molecular characterization of the beta-N-acetylglucosaminidase of *Escherichia coli* and its role in cell wall recycling. *J Bacteriol*. 2000; 182(17):4836-40.

Chiang WC, Nilsson M, Jensen PØ, Høiby N, Nielsen TE, Givskov M, Tolker-Nielsen T. Extracellular DNA shields against aminoglycosides in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother*. 2013; 57(5):2352-61.

Chuanchuen R, Wannaprasat W, Schweizer HP. Functional characterization of MexXY and OpmG in aminoglycoside efflux in *Pseudomonas aeruginosa*. *Southeast Asian J Trop Med Public Health*. 2008; 39(1):115-22.

Chung JC, Becq J, Fraser L, Schulz-Trieglaff O, Bond NJ, Foweraker J, Bruce KD, Smith GP, Welch M. Genomic variation among contemporary *Pseudomonas aeruginosa* isolates from chronically infected cystic fibrosis patients. *J Bacteriol*. 2012; 194(18):4857-66.

Ciofu O, Mandsberg LF, Bjarnsholt T, Wassermann T, Høiby N. Genetic adaptation of *Pseudomonas aeruginosa* during chronic lung infection of patients with cystic fibrosis: strong and weak mutators with heterogeneous genetic backgrounds emerge in *mucA* and/or *lasR* mutants. *Microbiology*. 2010; 156(Pt4):1108-19.

References

Conibear TC, Collins SL, Webb JS. Role of mutation in *Pseudomonas aeruginosa* biofilm development. *PLoS One*. 2009; 4(7):e6289.

Cox B, Game J. Repair systems in *Saccharomyces*. *Mutat Res*. 1974; 26(4):257-64.

Cramer N, Wiehlmann L, Ciofu O, Tamm S, Høiby N, Tümmler B. Molecular epidemiology of chronic *Pseudomonas aeruginosa* airway infections in cystic fibrosis. *PLoS One*. 2012; 7(11):e50731.

Cramer N, Klockgether J, Wrasman K, Schmidt M, Davenport CF, Tümmler B. Microevolution of the major common *Pseudomonas aeruginosa* clones C and PA14 in cystic fibrosis lungs. *Environ Microbiol*. 2011; 13(7):1690-704.

Cullen L, McClean S. Bacterial Adaptation during Chronic Respiratory Infections. *Pathogens*. 2015; 4(1):66-89.

Curran B, Jonas D, Grundmann H, Pitt T, Dowson CG. Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. *J Clin Microbiol*. 2004; 42(12):5644-9.

Cystic Fibrosis Foundation Patient Registry, 2016 Annual Data Report. Bethesda, Maryland, ©2017 Cystic Fibrosis Foundation.

Daigle DM, Cao L, Fraud S, Wilke MS, Pacey A, Klinoski R, Strynadka NC, Dean CR, Poole K. Protein modulator of multidrug efflux gene expression in *Pseudomonas aeruginosa*. *J Bacteriol*. 2007; 189(15):5441-51.

D'Argenio DA, Wu M, Hoffman LR, Kulasekara HD, Déziel E, Smith EE, Nguyen H, Ernst RK, Larson Freeman TJ, Spencer DH, Brittnacher M, Driffield K, Miller K, Bostock JM, O'Neill AJ, Chopra I. Increased mutability of *Pseudomonas aeruginosa* in biofilms. *J Antimicrob Chemother*. 2008; 61(5):1053-6.

de Bentzmann S, Plésiat P. The *Pseudomonas aeruginosa* opportunistic pathogen and human infections. *Environ Microbiol*. 2011; 13(7):1655-65.

de Champs C, Poirel L, Bonnet R, Sirot D, Chanal C, Sirot J, Nordmann P. Prospective survey of beta-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolated in a French hospital in 2000. *Antimicrob Agents Chemother*. 2002; 46(9):3031-4.

de Chial M, Ghysels B, Beatson SA, Geoffroy V, Meyer JM, Pattery T, Baysse C, Chablain P, Parsons YN, Winstanley C, Cordwell SJ, Cornelis P. Identification of type II and type III pyoverdine receptors from *Pseudomonas aeruginosa*. *Microbiology*. 2003; 149(Pt4):821-31.

de Dios Caballero J, Del Campo R, Royuela A, Solé A, Máiz L, Olveira C, Quintana-Gallego E, de Gracia J, Cobo M, de la Pedrosa EG, Oliver A, Cantón R; GEIFQ (Grupo Español para el Estudio de la Colonización/Infección Broncopulmonar en Fibrosis Quística). Bronchopulmonary infection-colonization patterns in Spanish cystic fibrosis patients: Results from a national multicenter study. *J Cyst Fibros*. 2016; 15:357-65.

del Barrio-Tofiño E, López-Causapé C, Cabot G, Rivera A, Benito N, Segura C, Montero MM, Sorlí L, Tubau F, Gómez-Zorrilla S, Tormo N, Durá-Navarro R, Viedma E, Resino-Foz E, Fernández-Martínez M, González-Rico C, Alejo-Cancho I, Martínez JA, Labayru-Echverria C, Dueñas C, Ayestarán I, Zamorano L, Martinez-Martinez L, Horcajada JP, Oliver A. Genomics and susceptibility profiles of extensively drug-resistant *Pseudomonas aeruginosa* isolates from Spain. *Antimicrob Agents Chemother*. 2017; 61(11). pii: e01589-17. Erratum in: *Antimicrob Agents Chemother*. 2017; 62(1). pii: e02352-17.

Denamur E, Picard B, Decoux G, Denis JB, Elion J. The absence of correlation between allozyme and rrn RFLP analysis indicates a high gene flow rate within human clinical *Pseudomonas aeruginosa* isolates. *FEMS Microbiol Lett*. 1993; 110(3):275-80.

Dettman JR, Rodrigue N, Aaron SD, Kassen R. Evolutionary genomics of epidemic and nonepidemic strains of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA*. 2013; 110(52):21065-70.

De Vos D, De Chial M, Cochez C, Jansen S, Tümmler B, Meyer JM, Cornelis P. Study of pyoverdine type and production by *Pseudomonas aeruginosa* isolated from cystic fibrosis patients: prevalence of type II pyoverdine isolates and accumulation of pyoverdine-negative mutations. *Arch Microbiol*. 2001; 175(5):384-8.

Diaz Caballero J, Clark ST, Coburn B, Zhang Y, Wang PW, Donaldson SL, Tullis DE, Yau YC, Waters VJ, Hwang DM, Guttman DS. Selective sweeps and parallel pathoadaptation drive *Pseudomonas aeruginosa* evolution in the cystic fibrosis lung. *MBio*. 2015; 6(5):e00981-15.

Dietz H, Pfeifle D, Wiedemann B. The signal molecule for beta-lactamase induction in *Enterobacter cloacae* is the anhydromuramyl-pentapeptide. *Antimicrob Agents Chemother*. 1997; 41:2113–20.

Dietz H, Wiedemann B. The role of N-actylglucosaminyl-1,6 anhydro N-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid-D-alanine for the induction of beta-lactamase in *Enterobacter cloacae*. *Zentralbl Bakteriol.* 1996; 284(2-3):207-17.

Diez-Aguilar M, Morosini MI, del Campo R, Garcia-Castillo M, Zamora J, Canton R. *In vitro* activity of fosfomycin against a collection of clinical *Pseudomonas aeruginosa* isolates from

16 Spanish hospitals: establishing the validity of standard broth microdilution as susceptibility testing method. *Antimicrob Agents Chemother* 2013; 57:5701–3.

Dingemans J, Ye L, Hildebrand F, Tontodonati F, Craggs M, Bilocq F, De Vos D, Crabbé A, Van Houdt R, Malfroot A, Cornelis P. The deletion of TonB-dependent receptor genes is part of the genome reduction process that occurs during adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis lung. *Pathog Dis*. 2014; 71(1):26-38.

Dößelmann B, Willmann M, Steglich M, Bunk B, Nübel U, Peter S, Neher RA. Rapid and Consistent Evolution of Colistin Resistance in Extensively Drug-Resistant *Pseudomonas aeruginosa* during Morbidostat Culture. *Antimicrob Agents Chemother*. 2017; 61(9). pii: e00043-17.

Döring G, Parameswaran IG, Murphy TF. Differential adaptation of microbial pathogens to airways of patients with cystic fibrosis and chronic obstructive pulmonary disease. *FEMS Microbiol Rev*. 2011; 35(1):124-46.

Dötsch A, Becker T, Pommerenke C, Magnowska Z, Jänsch L, Häussler S. Genomewide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2009; 53(6):2522-31.

Edenborough FP, Stone HR, Kelly SJ, Zadik P, Doherty CJ, Govan JR. Genotyping of *Pseudomonas aeruginosa* in cystic fibrosis suggests need for segregation. *J Cyst Fibros*. 2004; 3(1):37-44.

Eisele NA, Anderson DM. Host defense and the airway epithelium: frontline responses that protect against bacterial invasion and pneumonia. *J Pathog*. 2011; 2011:249802.

El'Garch F, Jeannot K, Hocquet D, Llanes-Barakat C, Plésiat P. Cumulative effects of several nonenzymatic mechanisms on the resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother*. 2007; 51(3):1016-21.

Ernst RK, Yi EC, Guo L, Lim KB, Burns JL, Hackett M, Miller SI. Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science*. 1999; 286(5444):1561-5.

Fajardo A, Hernando-Amado S, Oliver A, Ball G, Filloux A, Martinez JL. Characterization of a novel Zn^{2+}-dependent intrinsic imipenemase from *Pseudomonas aeruginosa*. *J Antimicrob Chemother*. 2014; 69(11):2972-8.

Fajardo A, Martínez-Martín N, Mercadillo M, Galán JC, Ghysels B, Matthijs S, Cornelis P, Wiehlmann L, Tümmler B, Baquero F, Martínez JL. The neglected intrinsic resistome of bacterial pathogens. *PLoS One*. 2008; 3(2):e1619.

Fancello L, Desnues C, Raoult D, Rolain JM. Bacteriophages and diffusion of genes encoding antimicrobial resistance in cystic fibrosis sputum microbiota. *J Antimicrob Chemother*. 2011; 66(11):2448-54.

Feliziani S, Marvig RL, Luján AM, Moyano AJ, Di Rienzo JA, Krogh Johansen H, Molin S, Smania AM. Coexistence and within-host evolution of diversified lineages of hypermutable *Pseudomonas aeruginosa* in long-term cystic fibrosis infections. PLoS Genet. 2014; 10(10):e1004651.

Feltman H, Schulert G, Khan S, Jain M, Peterson L, Hauser AR. Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. *Microbiology*. 2001; 147(Pt10):2659-69.

Feng Y, Jonker MJ, Moustakas I, Brul S, Ter Kuile BH. Dynamics of mutations during development of resistance by *Pseudomonas aeruginosa* against five antibiotics. *Antimicrob Agents Chemother*. 2016; 60(7):4229-36.

Fernández L, Alvarez-Ortega C, Wiegand I, Olivares J, Kocíncová D, Lam JS, Martínez JL, Hancock RE. Characterization of the polymyxin B resistome of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2013; 57(1):110-9.

Fernández L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock RE. Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrob Agents Chemother*. 2010; 54(8):3372-82.

Ferroni A, Guillemot D, Moumile K, Bernede C, Le Bourgeois M, Waernessyckle S, Descamps P, Sermet-Gaudelus I, Lenoir G, Berche P, Lim YW, Evangelista JS 3rd, Schmieder R, Bailey B, Haynes M, Furlan M, Maughan H, Edwards R, Rohwer F, Conrad D. Clinical insights from metagenomic analysis of sputum samples from patients with cystic fibrosis. *J Clin Microbiol*. 2014; 52(2):425-37.

Filip C, Fletcher G, Wulf JL, Earhart CF. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *J Bacteriol*. 1973; 115:717–722.

Fisher JF, Mobashery S. The sentinel role of peptidoglycan recycling in the β-lactam resistance of the Gram-negative *Enterobacteriaceae* and *Pseudomonas aeruginosa*. *Bioorg Chem.* 2014; 56:41–8.

Flemming HC, Wingender J. The biofilm matrix. *Nat Rev Microbiol*. 2010; 8(9):623-33.

References

Fluge G, Ojeniyi B, Høiby N, Digranes A, Ciofu O, Hunstad E, Haanaes OC, Storrøsten OT. Typing of *Pseudomonas aeruginosa* strains in Norwegian cystic fibrosis patients. *Clin Microbiol Infect*. 2001; 7(5):238-43.

Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N, Molin S. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. *Nat Rev Microbiol*. 2012; 10(12):841-51.

Foster PL. Stress-induced mutagenesis in bacteria. *Crit Rev Biochem Mol Biol*. 2007; 42(5):373-97.

Fothergill JL, Mowat E, Walshaw MJ, Ledson MJ, James CE, Winstanley C. Effect of antibiotic treatment on bacteriophage production by a cystic fibrosis epidemic strain of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2011; 55(1):426-8.

Fothergill JL, Mowat E, Ledson MJ, Walshaw MJ, Winstanley C. Fluctuations in phenotypes and genotypes within populations of *Pseudomonas aeruginosa* in the cystic fibrosis lung during pulmonary exacerbations. *J Med Microbiol*. 2010; 59(Pt4):472-81.

Fothergill JL, Panagea S, Hart CA, Walshaw MJ, Pitt TL, Winstanley C. Widespread pyocyanin over-production among isolates of a cystic fibrosis epidemic strain. *BMC Microbiol*. 2007; 7:45.

Fournier D, Richardot C, Müller E, Robert-Nicoud M, Llanes C, Plésiat P, Jeannot K. Complexity of resistance mechanisms to imipenem in intensive care unit strains of *Pseudomonas aeruginosa*. *J Antimicrob Chemother*. 2013; 68:1772-80.

Fraile-Ribot PA, Cabot G, Mulet X, Periañez L, Martín-Pena ML, Juan C, Pérez JL, Oliver A. Mechanisms leading to *in vivo* ceftolozane/tazobactam resistance development during the treatment of infections caused by MDR *Pseudomonas aeruginosa*. *J Antimicrob Chemother*. 2017.

Franklin MJ, Nivens DE, Weadge JT, Howell PL. Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. *Front Microbiol*. 2011; 2:167.

Friedberg EC, Gerlach VL. Novel DNA polymerases offer clues to the molecular basis of mutagenesis. *Cell*. 1999; 98(4):413-6.

García-Castillo M, Máiz L, Morosini MI, Rodríguez-Baños M, Suarez L, Fernández-Olmos A, Baquero F, Cantón R, del Campo R. Emergence of a *mutL* mutation causing multilocus sequence typing-pulsed-field gel electrophoresis discrepancy among *Pseudomonas aeruginosa* isolates from a cystic fibrosis patient. *J Clin Microbiol*. 2012; 50(5):1777-8.

Gellatly SL, Hancock RE. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. *Pathog Dis*. 2013; 67(3):159-73.

Giraud A, Matic I, Tenaillon O, Clara A, Radman M, Fons M, Taddei F. Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. *Science*. 2001; 291(5513):2606-8. Antimicrob Agents Chemother. 2006 Mar;50(3):975-83.

Girlich D, Naas T, Nordmann P. Biochemical characterization of the naturally occurring oxacillinase OXA-50 of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2004; 48(6):2043-8.

Giske CG, Libisch B, Colinon C, Scoulica E, Pagani L, Füzi M, Kronvall G, Rossolini GM. Establishing clonal relationships between VIM-1-like metallo-beta-lactamase-producing *Pseudomonas aeruginosa* strains from four European countries by multilocus sequence typing. *J Clin Microbiol.* 2006; 44:4309-15.

Govan JR, Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev*. 1996; 60(3):539-74.

Greipel L, Fischer S, Klockgether J, Dorda M, Mielke S, Wiehlmann L, Cramer N, Tümmler B. Molecular epidemiology of mutations in antimicrobial resistance loci of *Pseudomonas aeruginosa* isolates from airways of cystic fibrosis patients. *Antimicrob Agents Chemother*. 2016; 60(11):6726-6734.

Griffiths AL, Wurzel DF, Robinson PJ, Carzino R, Massie J. Australian epidemic strain pseudomonas (AES-1) declines further in a cohort segregated cystic fibrosis clinic. *J Cyst Fibros*. 2012; 11(1):49-52.

Grothues D, Koopmann U, von der Hardt H, Tümmler B. Genome fingerprinting of *Pseudomonas aeruginosa* indicates colonization of cystic fibrosis siblings with closely related strains. *J Clin Microbiol*. 1988; 26(10):1973-7.

Guénard S, Muller C, Monlezun L, Benas P, Broutin I, Jeannot K, Plésiat P. Multiple mutations lead to MexXY-OprM-dependent aminoglycoside resistance in clinical strains of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2014; 58(1):221-8.

Gutiérrez O, Juan C, Pérez JL, Oliver A. Lack of association between hypermutation and antibiotic resistance development in *Pseudomonas aeruginosa* isolates from intensive care unit patients. *Antimicrob Agents Chemother*. 2004; 48(9):3573-5.

Gutu AD, Sgambati N, Strasbourger P, Brannon MK, Jacobs MA, Haugen E, Kaul RK, Johansen HK, Høiby N, Moskowitz SM. Polymyxin resistance of *Pseudomonas aeruginosa* phoQ mutants is dependent on additional two-component regulatory systems. *Antimicrob Agents Chemother*. 2013; 57(5):2204-15.

References

Haidar G, Philips NJ, Shields RK, Snyder D, Cheng S, Potoski B, Doi Y, Hao B, Press EG, Cooper VS, Clancy CJ, Nguyen MH. Ceftolozane-Tazobactam for the treatment of multidrug-resistant *Pseudomonas aeruginosa* infections: clinical effectiveness and evolution of resistance. *Clin Infect Dis*. 2017; 65(1):110-120.

Hall AJ, Fothergill JL, McNamara PS, Southern KW, Winstanley C. Turnover of strains and intraclonal variation amongst *Pseudomonas aeruginosa* isolates from paediatric CF patients. *Diagn Microbiol Infect Dis*. 2014; 80(4):324-6.

Han S, Zaniewski RP, Marr ES, Lacey BM, Tomaras AP, Evdokimov A, Miller JR, Shanmugasundaram V. Structural basis for effectiveness of siderophore-conjugated monocarbams against clinically relevant strains of *Pseudomonas aeruginosa. Proc Natl Acad Sci USA*. 2010; 107(51):22002-7.

Hancock RE, Brinkman FS. Function of *Pseudomonas* porins in uptake and efflux. *Annu Rev Microbiol*. 2002; 56:17-38.

Hancock RE, Mutharia LM, Chan L, Darveau RP, Speert DP, Pier GB. *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. *Infect Immun*. 1983; 42(1):170-7.

Hare NJ, Solis N, Harmer C, Marzook NB, Rose B, Harbour C, Crossett B, Manos J, Cordwell SJ. Proteomic profiling of *Pseudomonas aeruginosa* AES-1R, PAO1 and PA14 reveals potential virulence determinants associated with a transmissible cystic fibrosis-associated strain. *BMC Microbiol*. 2012; 12:16.

Hauser AR. *Pseudomonas aeruginosa*: so many virulence factors, so little time. *Crit Care Med*. 2011; 39(9):2193-4.

Häussler S, Ziegler I, Löttel A, von Götz F, Rohde M, Wehmhöhner D, Saravanamuthu S, Tümmler B, Steinmetz I. Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *J Med Microbiol*. 2003; 52(Pt4):295-301.

Häussler S, Tümmler B, Weissbrodt H, Rohde M, Steinmetz I. Small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis. *Clin Infect Dis*. 1999; 29(3):621-5.

Hayden HS, Selgrade S, Klausen M, Goodlett DR, Burns JL, Ramsey BW, Miller SI. Growth phenotypes of *Pseudomonas aeruginosa* lasR mutants adapted to the airways of cystic fibrosis patients. *Mol Microbiol*. 2007; 64(2):512-33.

He J, Baldini RL, Déziel E, Saucier M, Zhang Q, Liberati NT, Lee D, Urbach J, Goodman HM, Rahme LG. The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc Natl Acad Sci USA*. 2004; 101(8):2530-5.

Hengge R. Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol*. 2009; 7(4):263-73.

Henrichfreise B, Wiegand I, Pfister W, Wiedemann B. Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. *Antimicrob Agents Chemother*. 2007; 51(11):4062-70.

Hilker R, Munder A, Klockgether J, Losada PM, Chouvarine P, Cramer N, Davenport CF, Dethlefsen S, Fischer S, Peng H, Schönfelder T, Türk O, Wiehlmann L, Wölbeling F, Gulbins E, Goesmann A, Tümmler B. Interclonal gradient of virulence in the *Pseudomonas aeruginosa* pangenome from disease and environment. *Environ Microbiol*. 2015; 17(1):29-46.

Hocquet D, Petitjean M, Rohmer L, Valot B, Kulasekara HD, Bedel E, Bertrand X, Plésiat P, Köhler T, Pantel A, Jacobs MA, Hoffman LR, Miller SI. Pyomelanin-producing *Pseudomonas aeruginosa* selected during chronic infections have a large chromosomal deletion which confers resistance to pyocins. *Environ Microbiol.* 2016; 18(10):3482-3493.

Hogardt M, Hoboth C, Schmoldt S, Henke C, Bader L, Heesemann J. Stage-specific adaptation of hypermutable *Pseudomonas aeruginosa* isolates during chronic pulmonary infection in patients with cystic fibrosis. *J Infect Dis*. 2007; 195(1):70-80

Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents.* 2010; 35(4):322-32.

Höltje JV, Glauner B. Structure and metabolism of the murein sacculus. *Res Microbiol*. 1990; 141:75–89.

Hunter RC, Asfour F, Dingemans J, Osuna BL, Samad T, Malfroot A, Cornelis P, Newman DK. Ferrous iron is a significant component of bioavailable iron in cystic fibrosis airways. *MBio*. 2013; 4(4).

Islam S, Oh H, Jalal S, Karpati F, Ciofu O, Høiby N, Wretlind B. Chromosomal mechanisms of aminoglycoside resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Clin Microbiol Infect*. 2009; 15(1):60-6.

Jacobs C, Huang LJ, Bartowsky E, Normark S, Park JT. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for beta-lactamase induction. *EMBO J*. 1994; 13(19):4684-94.

Jacoby GA. AmpC beta-lactamases. *Clin Microbiol Rev*. 2009; 22(1):161-82.

Jatsenko T, Tover A, Tegova R, Kivisaar M. Molecular characterization of Rif(r) mutations in *Pseudomonas aeruginosa* and *Pseudomonas putida*. Mutat Res. 2010; 683(1-2):106-14.

References

Jeannot K, Sobel ML, El Garch F, Poole K, Plésiat P. Induction of the MexXY efflux pump in *Pseudomonas aeruginosa* is dependent on drug-ribosome interaction. *J Bacteriol*. 2005; 187(15):5341-6.

Jelsbak L, Johansen HK, Frost AL, Thøgersen R, Thomsen LE, Ciofu O, Yang L, Haagensen JA, Høiby N, Molin S. Molecular epidemiology and dynamics of *Pseudomonas aeruginosa* populations in lungs of cystic fibrosis patients. *Infect Immun*. 2007; 75(5):2214-24

Jeukens J, Boyle B, Kukavica-Ibrulj I, Ouellet MM, Aaron SD, Charette SJ, Fothergill JL, Tucker NP, Winstanley C, Levesque RC. Comparative genomics of isolates of a *Pseudomonas aeruginosa* epidemic strain associated with chronic lung infections of cystic fibrosis patients. *PLoS One*. 2014; 9(2):e87611.

Jones AM, Govan JR, Doherty CJ, Dodd ME, Isalska BJ, Stanbridge TN, Webb AK. Spread of a multiresistant strain of *Pseudomonas aeruginosa* in an adult cystic fibrosis clinic. *Lancet*. 2001; 358(9281):557-8.

Jorth P, McLean K, Ratjen A, Secor PR, Bautista GE, Ravishankar S, Rezayat A, Garudathri J, Harrison JJ, Harwood RA, Penewit K, Waalkes A, Singh PK, Salipante SJ. Evolved aztreonam resistance is multifactorial and can produce hypervirulence in *Pseudomonas aeruginosa*. *MBio*. 2017; 8(5). pii: e00517-17.

Juan C, Torrens G, González-Nicolau M, Oliver A. Diversity and regulation of intrinsic β-lactamases from non-fermenting and other Gram-negative opportunistic pathogens. *FEMS Microbiol Rev*. 2017; 41(6):781-815.

Juan C, Zamorano L, Pérez JL, Ge Y, Oliver A; Spanish Group for the Study of Pseudomonas; Spanish Network for Research in Infectious Diseases. Activity of a new antipseudomonal cephalosporin, CXA-101 (FR264205), against carbapenem-resistant and multidrug-resistant *Pseudomonas aeruginosa* clinical strains. *Antimicrob Agents Chemother*. 2010; 54(2):846-51.

Juan C, Moyá B, Pérez JL, Oliver A. Stepwise upregulation of the *Pseudomonas aeruginosa* chromosomal cephalosporinase conferring high-level beta-lactam resistance involves three AmpD homologues. *Antimicrob Agents Chemother*. 2006; 50(5):1780-7.

Juan C, Maciá MD, Gutiérrez O, Vidal C, Pérez JL, Oliver A. Molecular mechanisms of beta-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. *Antimicrob Agents Chemother*. 2005; 49(11):4733-8.

Juarez P, Broutin I, Bordi C, Plésiat P, Llanes C. Constitutive activation of MexT by amino acid substitutions results in MexEF-OprN overproduction in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2018; 62(5).

Juarez P, Jeannot K, Plésiat P, Llanes C. Toxic electrophiles induce expression of the multidrug efflux pump MexEF-OprN in *Pseudomonas aeruginosa* through a novel transcriptional regulator, CmrA. *Antimicrob Agents Chemother*. 2017; 61(8).

Kaufmann ME. Pulsed-field gel electrophoresis. *Methods Mol Med*. 1998; 15:33-50.

Kelly NM, Fitzgerald MX, Tempany E, O'Boyle C, Falkiner FR, Keane CT. Does *Pseudomonas* cross-infection occur between cystic-fibrosis patients. *Lancet*. 1982; 2(8300):688-90.

Kenna DT, Doherty CJ, Foweraker J, Macaskill L, Barcus VA, Govan JR. Hypermutability in environmental *Pseudomonas aeruginosa* and in populations causing pulmonary infection in individuals with cystic fibrosis. *Microbiology*. 2007; 153(Pt6):1852-9.

Kidd TJ, Ritchie SR, Ramsay KA, Grimwood K, Bell SC, Rainey PB. *Pseudomonas aeruginosa* exhibits frequent recombination, but only a limited association between genotype and ecological setting. *PLoS One*. 2012; 7(9):e44199.

Kidd TJ, Grimwood K, Ramsay KA, Rainey PB, Bell SC. Comparison of three molecular techniques for typing *Pseudomonas aeruginosa* isolates in sputum samples from patients with cystic fibrosis. *J Clin Microbiol*. 2011; 49(1):263-8.

Kiewitz C, Tümmler B. Sequence diversity of *Pseudomonas aeruginosa*: impact on population structure and genome evolution. *J Bacteriol*. 2000; 182(11):3125-35.

Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tümmler B. *Pseudomonas aeruginosa* Genomic Structure and Diversity. *Front Microbiol*. 2011; 2:150.

Klockgether J, Würdemann D, Reva O, Wiehlmann L, Tümmler B. Diversity of the abundant pKLC102/PAGI-2 family of genomic islands in *Pseudomonas aeruginosa*. *J Bacteriol*. 2007; 189(6):2443-59.

Klockgether J, Reva O, Larbig K, Tümmler B. Sequence analysis of the mobile genome island pKLC102 of *Pseudomonas aeruginosa* C. *J Bacteriol*. 2004; 186(2):518-34.

Köhler T, Epp SF, Curty LK, Pechère JC. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. *J Bacteriol*. 1999; 181(20):6300-5.

References

Konings AF, Martin LW, Sharples KJ, Roddam LF, Latham R, Reid DW, Lamont IL. *Pseudomonas aeruginosa* uses multiple pathways to acquire iron during chronic infection in cystic fibrosis lungs. *Infect Immun*. 2013; 81: 2697–2704.

Kong KF, Jayawardena SR, Del Puerto A, Wiehlmann L, Laabs U, Tümmler B, Mathee K. Characterization of poxB, a chromosomal-encoded *Pseudomonas aeruginosa* oxacillinase. *Gene*. 2005; 358:82-92.

Korfmann G, Sanders CC. ampG is essential for high-level expression of AmpC beta-lactamase in *Enterobacter cloacae*. *Antimicrob Agents Chemother*. 1989; 33(11):1946-51.

Kos VN, Déraspe M, McLaughlin RE, Whiteaker JD, Roy PH, Alm RA, Corbeil J, Gardner H. The resistome of *Pseudomonas aeruginosa* in relationship to phenotypic susceptibility. *Antimicrob Agents Chemother*. 2015; 59(1):427-36.

Krahn T, Gilmour C, Tilak J, Fraud S, Kerr N, Lau CH, Poole K. Determinants of intrinsic aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2012; 56(11):5591-602.

Kukavica-Ibrulj I, Bragonzi A, Paroni M, Winstanley C, Sanschagrin F, O'Toole GA, Levesque RC. In vivo growth of *Pseudomonas aeruginosa* strains PAO1 and PA14 and the hypervirulent strain LESB58 in a rat model of chronic lung infection. *J Bacteriol.* 2008; 190(8):2804-13.

Lahiri SD, Johnstone MR, Ross PL, McLaughlin RE, Olivier NB, Alm RA. Avibactam and class C β-lactamases: mechanism of inhibition, conservation of the binding pocket, and implications for resistance. *Antimicrob Agents Chemother*. 2014; 58(10):5704-13.

Larbig KD, Christmann A, Johann A, Klockgether J, Hartsch T, Merkl R, Wiehlmann L, Fritz HJ, Tümmler B. Gene islands integrated into tRNA(Gly) genes confer genome diversity on a *Pseudomonas aeruginosa* clone. *J Bacteriol*. 2002; 184:6665-80.

Lau CH, Krahn T, Gilmour C, Mullen E, Poole K. AmgRS-mediated envelope stress-inducible expression of the mexXY multidrug efflux operon of *Pseudomonas aeruginosa*. *Microbiology open*. 2015; 4(1):121-35.

Lavie M, Shemer O, Sarouk I, Bar Aluma Be, Dagan A, Efrati O, Vilozni D. Several siblings with Cystic Fibrosis as a risk factor for poor outcome. *Respir Med*. 2015; 109(1):74-8.

Lee DG, Urbach JM, Wu G, Liberati NT, Feinbaum RL, Miyata S, Diggins LT, He J, Saucier M, Déziel E, Friedman L, Li L, Grills G, Montgomery K, Kucherlapati R, Rahme LG, Ausubel FM. Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. *Genome Biol*. 2006; 7:R90.

Lee JY, Ko KS. Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates. *Diagn Microbiol Infect Dis*. 2014; 78(3):271-6.

Lewis K. Persister cells. *Annu Rev Microbiol*. 2010; 64:357-72.

Lewis K. Multidrug tolerance of biofilms and persister cells. *Curr Top Microbiol Immunol*. 2008; 322:107-31.

Li XZ, Plésiat P, Nikaido H. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin Microbiol Rev*. 2015; 28(2):337-418.

Li XZ, Nikaido H, Poole K. Role of mexA-mexB-oprM in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 1995; 39(9):1948-53.

Liang X, Pham XQ, Olson MV, Lory S. Identification of a genomic island present in the majority of pathogenic isolates of *Pseudomonas aeruginosa*. *J Bacteriol.* 2001; 183(3):843-53.

Lindquist S, Lindberg F, Normark S. Binding of the *Citrobacter freundii* AmpR regulator to a single DNA site provides both autoregulation and activation of the inducible ampC beta-lactamase gene. *J Bacteriol.* 1989; 171:3746–53.

Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev*. 2009; 22(4):582-610.

Livermore DM. Of *Pseudomonas*, porins, pumps and carbapenems. *J Antimicrob Chemother*. 2001; 47(3):247-50.

Livermore DM. Interplay of impermeability and chromosomal beta-lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 1992; 36(9):2046-8.

Livermore DM. Penicillin-binding proteins, porins and outer-membrane permeability of carbenicillin-resistant and -susceptible strains of *Pseudomonas aeruginosa*. *J Med Microbiol*. 1984; 18(2):261-70.

Logan C, Habington A, Lennon G, Grogan J, Byrne M, O'Leary J, O'Sullivan N. Genetic relatedness of *Pseudomonas aeruginosa* isolates among a paediatric cystic fibrosis patient cohort in Ireland. *J Med Microbiol*. 2012; 61(Pt1):64-70.

Luján AM, Maciá MD, Yang L, Molin S, Oliver A, Smania AM. Evolution and adaptation in *Pseudomonas aeruginosa* biofilms driven by mismatch repair system-deficient mutators. *PLoS One.* 2011; 6(11):e27842.

Luna RA, Millecker LA, Webb CR, Mason SK, Whaley EM, Starke JR, Hiatt PW, Versalovic J. Molecular epidemiological surveillance of multidrug-resistant *Pseudomonas aeruginosa* isolates in a pediatric population of patients with cystic fibrosis and determination of risk factors for infection with the Houston-1 strain. *J Clin Microbiol.* 2013; 51(4):1237-40.

Maâtallah M, Bakhrouf A, Habeeb MA, Turlej-Rogacka A, Iversen A, Pourcel C, Sioud O, Giske CG. Four genotyping schemes for phylogenetic analysis of *Pseudomonas aeruginosa*: comparison of their congruence with multi-locus sequence typing. *PLoS One*. 2013; 8(12):e82069.

Maatallah M, Cheriaa J, Backhrouf A, Iversen A, Grundmann H, Do T, Lanotte P, Mastouri M, Elghmati MS, Rojo F, Mejdi S, Giske CG. Population structure of *Pseudomonas aeruginosa* from five Mediterranean countries: evidence for frequent recombination and epidemic occurrence of CC235. *PLoS One*. 2011; 6(10):e25617.

Maciá MD, Borrell N, Segura M, Gómez C, Pérez JL, Oliver A. Efficacy and potential for resistance selection of antipseudomonal treatments in a mouse model of lung infection by hypermutable *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2006; 50(3):975-83.

Maciá MD, Blanquer D, Togores B, Sauleda J, Pérez JL, Oliver A. Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. *Antimicrob Agents Chemother*. 2005; 49(8):3382-6.

MacVane SH, Pandey R, Steed LL, Kreiswirth BN, Chen L. Emergence of ceftolozane-tazobactam-resistant *Pseudomonas aeruginosa* during treatment is mediated by a single AmpC structural mutation. *Antimicrob Agents Chemother*. 2017; 61(12). pii: e01183-17.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect*. 2012; 18(3):268-81.

Mahenthiralingam E, Campbell ME, Speert DP. Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect Immun*. 1994; 62(2):596-605.

Malone JG. Role of small colony variants in persistence of *Pseudomonas aeruginosa* infections in cystic fibrosis lungs. *Infect Drug Resist*. 2015; 8:237-47.

Manos J, Arthur J, Rose B, Bell S, Tingpej P, Hu H, Webb J, Kjelleberg S, Gorrell MD, Bye P, Harbour C. Gene expression characteristics of a cystic fibrosis epidemic strain of

Pseudomonas aeruginosa during biofilm and planktonic growth. *FEMS Microbiol Lett*. 2009; 292(1):107-14.

Mao EF, Lane L, Lee J, Miller JH. Proliferation of mutators in A cell population. *J Bacteriol*. 1997; 179(2):417-22.

Mark BL, Vocadlo DJ, Oliver A. Providing β-lactams a helping hand: targeting the AmpC β-lactamase induction pathway. *Future Microbiol*. 2011; 6:1415–27.

Markussen T, Marvig RL, Gómez-Lozano M, Aanæs K, Burleigh AE, Høiby N, Johansen HK, Molin S, Jelsbak L. Environmental heterogeneity drives within-host diversification and evolution of *Pseudomonas aeruginosa*. *MBio*. 2014; 5(5):e01592-14.

Martínez-Ramos I, Mulet X, Moyá B, Barbier M, Oliver A, Albertí S. Overexpression of MexCD-OprJ reduces *Pseudomonas aeruginosa* virulence by increasing its susceptibility to complement-mediated killing. *Antimicrob Agents Chemother*. 2014; 58(4): 2426–2429.

Marvig RL, Sommer LM, Jelsbak L, Molin S, Johansen HK. Evolutionary insight from whole-genome sequencing of P*seudomonas aeruginosa* from cystic fibrosis patients. *Future Microbiol.* 2015a; 10(4):599-611.

Marvig RL, Sommer LM, Molin S, Johansen HK. Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nat Genet.* 2015b; 47: 57-64.

Marvig RL, Johansen HK, Molin S, Jelsbak L. Genome analysis of a transmissible lineage of P*seudomonas aeruginosa* reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. *PLoS Genet*. 2013; 9(9):e1003741.

Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. Contribution of the MexX-MexY-oprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2000; 44(9):2242-6.

Masuda N, Gotoh N, Ishii C, Sakagawa E, Ohya S, Nishino T. Interplay between chromosomal beta-lactamase and the MexAB-OprM efflux system in intrinsic resistance to beta-lactams in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 1999; 43(2):400-2.

Matsuo Y, Eda S, Gotoh N, Yoshihara E, Nakae T. MexZ-mediated regulation of mexXY multidrug efflux pump expression in *Pseudomonas aeruginosa* by binding on the mexZ-mexX intergenic DNA. *FEMS Microbiol Lett*. 2004; 238(1):23-8.

McCallum SJ, Gallagher MJ, Corkill JE, Hart CA, Ledson MJ, Walshaw MJ. Spread of an epidemic *Pseudomonas aeruginosa* strain from a patient with cystic fibrosis (CF) to non-CF relatives. *Thorax*. 2002; 57(6):559-60.

McCallum SJ, Corkill J, Gallagher M, Ledson MJ, Hart CA, Walshaw MJ. Superinfection with a transmissible strain of *Pseudomonas aeruginosa* in adults with cystic fibrosis chronically colonised by *P aeruginosa*. *Lancet*. 2001; 358(9281):558-60.

McCormick J, Mehta G, Olesen HV, Viviani L, Macek M Jr, Mehta A; European Registry Working Group. Comparative demographics of the European cystic fibrosis population: a cross-sectional database analysis. *Lancet*. 2010; 375(9719):1007-13.

Mena A, Smith EE, Burns JL, Speert DP, Moskowitz SM, Perez JL, Oliver A. Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. *J Bacteriol*. 2008; 190(24):7910-7.

Miller AK, Brannon MK, Stevens L, Johansen HK, Selgrade SE, Miller SI, Høiby N, Moskowitz SM. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother*. 2011; 55(12):5761-9.

Miller JH. Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. *Annu Rev Microbiol*. 1996; 50:625-43.

Mine T, Morita Y, Kataoka A, Mizushima T, Tsuchiya T. Expression in *Escherichia coli* of a new multidrug efflux pump, MexXY, from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 1999; 43(2):415-7.

Montanari S, Oliver A, Salerno P, Mena A, Bertoni G, Tümmler B, Cariani L, Conese M, Döring G, Bragonzi A. Biological cost of hypermutation in *Pseudomonas aeruginosa* strains from patients with cystic fibrosis. *Microbiology*. 2007; 153(Pt5):1445-54.

Morales G, Wiehlmann L, Gudowius P, van Delden C, Tümmler B, Martínez JL, Rojo F. Structure of *Pseudomonas aeruginosa* populations analyzed by single nucleotide polymorphism and pulsed-field gel electrophoresis genotyping. *J Bacteriol*. 2004; 186:4228-37.

Morita Y, Cao L, Gould VC, Avison MB, Poole K. *nalD* encodes a second repressor of the mexAB-oprM multidrug efflux operon of *Pseudomonas aeruginosa*. *J Bacteriol*. 2006a; 188(24):8649-54.

Morita Y, Sobel ML, Poole K. Antibiotic inducibility of the MexXY multidrug efflux system of *Pseudomonas aeruginosa*: involvement of the antibiotic-inducible PA5471 gene product. *J Bacteriol*. 2006b; 188(5):1847-55.

Morita Y, Kimura N, Mima T, Mizushima T, Tsuchiya T. Roles of MexXY- and MexAB-multidrug efflux pumps in intrinsic multidrug resistance of *Pseudomonas aeruginosa* PAO1. *J Gen Appl Microbiol.* 2001; 47(1):27-32.

Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SI, Denton M, Conway SP, Johansen HK, Høiby N. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother*. 2012; 56(2):1019-30.

Mouton JW, den Hollander JG, Horrevorts AM. Emergence of antibiotic resistance amongst *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *J Antimicrob Chemother.* 1993; 31(6):919-26.

Mowat E, Paterson S, Fothergill JL, Wright EA, Ledson MJ, Walshaw MJ, Brockhurst MA, Winstanley C. *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. *Am J Respir Crit Care Med*. 2011; 183(12):1674-9.

Moya B, Zamorano L, Juan C, Pérez JL, Ge Y, Oliver A. Activity of a new cephalosporin, CXA-101 (FR264205), against beta-lactam-resistant *Pseudomonas aeruginosa* mutants selected *in vitro* and after antipseudomonal treatment of intensive care unit patients. *Antimicrob Agents Chemother*. 2010; 54(3):1213-7.

Moya B, Dötsch A, Juan C, Blázquez J, Zamorano L, Haussler S, Oliver A. Beta-lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. *PLoS Pathog*. 2009; 5(3):e1000353.

Mulcahy H, Charron-Mazenod L, Lewenza S. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog*. 2008; 4(11):e1000213.

Mulet X, Cabot G, Ocampo-Sosa AA, Domínguez MA, Zamorano L, Juan C, Tubau F, Rodríguez C, Moyà B, Peña C, Martínez-Martínez L, Oliver A; Spanish Network for Research in Infectious Diseases (REIPI). Biological markers of *Pseudomonas aeruginosa* epidemic high-risk clones. *Antimicrob Agents Chemother*. 2013; 57(11):5527-35.

Mulet X, Moyá B, Juan C, Macià MD, Pérez JL, Blázquez J, Oliver A. Antagonistic interactions of *Pseudomonas aeruginosa* antibiotic resistance mechanisms in planktonic but not biofilm growth. *Antimicrob Agents Chemother*. 2011; 55(10):4560-8.

Mulet X, Maciá MD, Mena A, Juan C, Pérez JL, Oliver A. Azithromycin in *Pseudomonas aeruginosa* biofilms: bactericidal activity and selection of *nfxB* mutants. *Antimicrob Agents Chemother*. 2009; 53(4): 1552–1560.

Muller C, Plésiat P, Jeannot K. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and β-lactams in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2011; 55(3):1211-21.

References

Munck A, Bonacorsi S, Mariani-Kurkdjian P, Lebourgeois M, Gérardin M, Brahimi N, Navarro J, Bingen E. Genotypic characterization of *Pseudomonas aeruginosa* strains recovered from patients with cystic fibrosis after initial and subsequent colonization. *Pediatr Pulmonol*. 2001; 32(4):288-92.

Murata T, Gotoh N, Nishino T. Characterization of outer membrane efflux proteins OpmE, OpmD and OpmB of *Pseudomonas aeruginosa*: molecular cloning and development of specific antisera. *FEMS Microbiol Lett*. 2002; 217(1):57-63.

Mustafa MH, Chalhoub H, Denis O, Deplano A, Vergison A, Rodriguez-Villalobos H, Tunney MM, Elborn JS, Kahl BC, Traore H, Vanderbist F, Tulkens PM, Van Bambeke F. Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolated from Cystic Fibrosis patients in Northern Europe. *Antimicrob Agents Chemother*. 2016; 60(11):6735-6741.

Naughton S, Parker D, Seemann T, Thomas T, Turnbull L, Rose B, Bye P, Cordwell S, Whitchurch C, Manos J. *Pseudomonas aeruginosa* AES-1 exhibits increased virulence gene expression during chronic infection of cystic fibrosis lung. *PLoS One*. 2011; 6(9):e24526.

Nikaido H, Nikaido K, Harayama S. Identification and characterization of porins in *Pseudomonas aeruginosa*. *J Biol Chem*. 1991; 266(2):770-9.

Nikaido H. Role of permeability barriers in resistance to beta-lactam antibiotics. *Pharmacol Ther*. 1985; 27(2):197-231.

Nordmann P, Guibert M. Extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*. *J Antimicrob Chemother*. 1998; 42(2):128-31.

Oh H, Stenhoff J, Jalal S, Wretlind B. Role of efflux pumps and mutations in genes for topoisomerases II and IV in fluoroquinolone-resistant *Pseudomonas aeruginosa* strains. *Microb Drug Resist*. 2003; 9(4):323-8.

Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol*. 2014; 5:643.

Oliver A, Mulet X, López-Causapé C, Juan C. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist Updat*. 2015; 21-22:41-59.

Oliver A. Mutators in cystic fibrosis chronic lung infection: Prevalence, mechanisms, and consequences for antimicrobial therapy. *Int J Med Microbiol*. 2010a; 300(8):563-72.

Oliver A, Mena A. Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance. *Clin Microbiol Infect*. 2010b; 16(7):798-808.

Oliver A, Alarcón T, Caballero E, Cantón R. Microbiological diagnosis of bronchopulmonary colonization-infection in cystic fibrosis. *Enferm Infecc Microbiol Clin*. 2009; 27(2):89-104.

Oliver A, Levin BR, Juan C, Baquero F, Blázquez J. Hypermutation and the preexistence of antibiotic-resistant *Pseudomonas aeruginosa* mutants: implications for susceptibility testing and treatment of chronic infections. *Antimicrob Agents Chemother*. 2004; 48(11):4226-33.

Oliver A, Baquero F, Blázquez J. The mismatch repair system (*mutS*, *mutL* and *uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Mol Microbiol*. 2002a; 43(6):1641-50.

Oliver A, Sánchez JM, Blázquez J. Characterization of the GO system of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett*. 2002b; 217(1):31-5.

Oliver A, Cantón R, Campo P, Baquero F, Blázquez J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science*. 2000; 288(5469):1251-4.

Olson MV. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci USA*. 2006; 103(22):8487-92.

O'Sullivan BP, Freedman SD. Cystic fibrosis. *Lancet*. 2009; 373(9678):1891-904.

O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. *Annu Rev Microbiol*. 2000; 54:49-79.

Panagea S, Winstanley C, Walshaw MJ, Ledson MJ, Hart CA. Environmental contamination with an epidemic strain of *Pseudomonas aeruginosa* in a Liverpool cystic fibrosis centre, and study of its survival on dry surfaces. *J Hosp Infect*. 2005; 59(2):102-7.

Pamp SJ, Tolker-Nielsen T. Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*. *J Bacteriol*. 2007; 189(6):2531-9.

Parkins MD, Glezerson BA, Sibley CD, Sibley KA, Duong J, Purighalla S, Mody CH, Workentine ML, Storey DG, Surette MG, Rabin HR. Twenty-five-year outbreak of *Pseudomonas aeruginosa* infecting individuals with cystic fibrosis: identification of the prairie epidemic strain. *J Clin Microbiol.* 2014; 52(4):1127-35.

Pedersen SS, Koch C, Høiby N, Rosendal K. An epidemic spread of multiresistant *Pseudomonas aeruginosa* in a cystic fibrosis centre. *J Antimicrob Chemother*. 1986; 17(4):505-16.

Peña C, Suarez C, Tubau F, Juan C, Moya B, Dominguez MA, Oliver A, Pujol M, Ariza J. Nosocomial outbreak of a non-cefepime-susceptible ceftazidime-susceptible *Pseudomonas aeruginosa* strain overexpressing MexXY-OprM and producing an integron-borne PSE-1 betta-lactamase. *J Clin Microbiol*. 2009; 47(8):2381-7.

References

Pérez-Capilla T, Baquero MR, Gómez-Gómez JM, Ionel A, Martín S, Blázquez J. SOS-independent induction of dinB transcription by beta-lactam-mediated inhibition of cell wall synthesis in *Escherichia coli*. *J Bacteriol*. 2005; 187(4):1515-8.

Picard B, Denamur E, Barakat A, Elion J, Goullet P. Genetic heterogeneity of *Pseudomonas aeruginosa* clinical isolates revealed by esterase electrophoretic polymorphism and restriction fragment length polymorphism of the ribosomal RNA gene region. *J Med Microbiol.* 1994; 40(5):313-22.

Picard E, Aviram M, Yahav Y, Rivlin J, Blau H, Bentur L, Avital A, Villa Y, Schwartz S, Kerem B, Kerem E. Familial concordance of phenotype and microbial variation among siblings with CF. *Pediatr Pulmonol*. 2004; 38(4):292-7.

Pier GB, Ramphal R. *Pseudomonas aeruginosa*. In Mandell GL, Bennet JE, Dolin R (eds). Mandell, Douglas and Bennett's principles and practice of infectious diseases. 6th edition. Elsevier, Churchill, Livingstone; Philadelphia. 2005; 2587-2615.

Pirnay JP, Bilocq F, Pot B, Cornelis P, Zizi M, Van Eldere J, Deschaght P, Vaneechoutte M, Jennes S, Pitt T, De Vos D. *Pseudomonas aeruginosa* population structure revisited. *PLoS One*. 2009; 4(11):e7740.

Pirnay JP, De Vos D, Cochez C, Bilocq F, Vanderkelen A, Zizi M, Ghysels B, Cornelis P. *Pseudomonas aeruginosa* displays an epidemic population structure. *Environ Microbiol*. 2002; 4(12):898-911.

Pollini S, Fiscarelli E, Mugnaioli C, Di Pilato V, Ricciotti G, Neri AS, Rossolini GM. *Pseudomonas aeruginosa* infection in cystic fibrosis caused by an epidemic metallo-β-lactamase-producing clone with a heterogeneous carbapenem resistance phenotype. *Clin Microbiol Infect*. 2011; 17(8):1272-5.

Poole K. *Pseudomonas aeruginosa*: resistance to the max. *Front Microbiol*. 2011; 2:65.

Poole K. Aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2005; 49(2):479-87.

Poole K, Srikumar R. Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. *Curr Top Med Chem*. 2001; 1(1):59-71.

Poole K, Krebes K, McNally C, Neshat S. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *J Bacteriol*. 1993; 175(22):7363-72.

Prickett MH, Hauser AR, McColley SA, Cullina J, Potter E, Powers C, Jain M. Aminoglycoside resistance of *Pseudomonas aeruginosa* in cystic fibrosis results from convergent evolution in the *mexZ* gene. *Thorax*. 2017; 72(1):40-47.

Rakhimova E, Wiehlmann L, Brauer AL, Sethi S, Murphy TF, Tümmler B. *Pseudomonas aeruginosa* population biology in chronic obstructive pulmonary disease. *J Infect Dis*. 2009; 200(12):1928-35.

Renders N, Verbrugh H, Van Belkum A. Dynamics of bacterial colonisation in the respiratory tract of patients with cystic fibrosis. *Infect Genet Evol*. 2001; 1(1):29-39.

Renders NH, Sijmons MA, van Belkum A, Overbeek SE, Mouton JW, Verbrugh HA. Exchange of *Pseudomonas aeruginosa* strains among cystic fibrosis siblings. *Res Microbiol*. 1997; 148(5):447-54.

Richardot C, Plésiat P, Fournier D, Monlezun L, Broutin I, Llanes C. Carbapenem resistance in cystic fibrosis strains of *Pseudomonas aeruginosa* as a result of amino acid substitutions in porin OprD. *Int J Antimicrob Agents*. 2015; 45(5):529-32.

Riera E, Cabot G, Mulet X, García-Castillo M, del Campo R, Juan C, Cantón R, Oliver A. *Pseudomonas aeruginosa* carbapenem resistance mechanisms in Spain: impact on the activity of imipenem, meropenem and doripenem. *J Antimicrob Chemother*. 2011; 66(9):2022-7.

Rodríguez-Rojas A, Oliver A, Blázquez J. Intrinsic and environmental mutagenesis drive diversification and persistence of *Pseudomonas aeruginosa* in chronic lung infections. *J Infect Dis*. 2012; 205(1):121-7.

Sanders CC, Bradford PA, Ehrhardt AF, Bush K, Young KD, Henderson TA, Sanders WE Jr. Penicillin-binding proteins and induction of AmpC beta-lactamase. *Antimicrob Agents Chemother*. 1997; 41(9):2013-5.

Salunkhe P, Smart CH, Morgan JA, Panagea S, Walshaw MJ, Hart CA, Geffers R, Tümmler B, Winstanley C. A cystic fibrosis epidemic strain of *Pseudomonas aeruginosa* displays enhanced virulence and antimicrobial resistance. *J Bacteriol*. 2005; 187(14):4908-20.

Schurek KN, Marr AK, Taylor PK, Wiegand I, Semenec L, Khaira BK, Hancock RE. Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2008; 52(12):4213-9.

Scott FW, Pitt TL. Identification and characterization of transmissible *Pseudomonas aeruginosa* strains in cystic fibrosis patients in England and Wales. J Med Microbiol. 2004; 53(Pt7):609-15.

References

Shteinberg M, Elborn JS. Use of inhaled tobramycin in cystic fibrosis. *Adv Ther*. 2015; 32(1):1-9.

Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW. *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol Rev*. 2011; 35(4):652-80.

Slieker MG, van den Berg JM, Kouwenberg J, van Berkhout FT, Heijerman HG, van der Ent CK. Long-term effects of birth order and age at diagnosis in cystic fibrosis: a sibling cohort study. *Pediatr Pulmonol*. 2010; 45(6):601-7.

Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Sobel ML, McKay GA, Poole K. Contribution of the MexXY multidrug transporter to aminoglycoside resistance in *Pseudomonas aeruginosa* clinical isolates. *Antimicrob Agents Chemother*. 2003; 47(10):3202-7.

Smith JM, Smith NH, O'Rourke M, Spratt BG. How clonal are bacteria? *Proc Natl Acad Sci USA*. 1993; 90(10):4384-8.

Spangenberg C, Montie TC, Tümmler B. Structural and functional implications of sequence diversity of *Pseudomonas aeruginosa* genes *oriC*, *ampC* and *fliC*. *Electrophoresis*. 1998; 19(4):545-50.

Speert DP, Campbell ME, Henry DA, Milner R, Taha F, Gravelle A, Davidson AG, Wong LT, Mahenthiralingam E. Epidemiology of *Pseudomonas aeruginosa* in cystic fibrosis in British Columbia, Canada. *Am J Respir Crit Care Med*. 2002; 166(7):988-93.

Spencer DH, Kas A, Smith EE, Raymond CK, Sims EH, Hastings M, Burns JL, Kaul R, Olson MV. Whole-genome sequence variation among multiple isolates of *Pseudomonas aeruginosa*. *J Bacteriol*. 2003; 185:1316-25.

Srour N, Chaparro C, Vandemheen K, Singer LG, Keshavjee S, Aaron SD. Effect of infection with transmissible strains of *Pseudomonas aeruginosa* on lung transplantation outcomes in patients with cystic fibrosis. *J Heart Lung Transplant.* 2015; 34(4):588-93.

Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet*. 2001; 358(9276):135-8.

Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*. 2000; 406(6799):959-64.

Tacconelli E, De Angelis G, Cataldo MA, Mantengoli E, Spanu T, Pan A, Corti G, Radice A, Stolzuoli L, Antinori S, Paradisi F, Carosi G, Bernabei R, Antonelli M, Fadda G, Rossolini GM, Cauda R. Antibiotic usage and risk of colonization and infection with antibiotic-resistant bacteria: a hospital population-based study. *Antimicrob Agents Chemother*. 2009; 53(10):4264-9.

Taddei F. Effect of mutator *P. aeruginosa* on antibiotic resistance acquisition and respiratory function in cystic fibrosis. *Pediatr Pulmonol*. 2009; 44(8):820-5.

Taddei F, Vulić M, Radman M, Matić I. Genetic variability and adaptation to stress. *EXS*. 1997; 83:271-90.

Tayler AE, Ayala JA, Niumsup P, Westphal K, Baker JA, Zhang L, Walsh TR, Wiedemann B, Bennett PM, Avison MB. Induction of beta-lactamase production in *Aeromonas hydrophila* is responsive to beta-lactam-mediated changes in peptidoglycan composition. *Microbiology*. 2010; 156(Pt8):2327-35.

Templin MF, Ursinus A, Höltje JV. A defect in cell wall recycling triggers autolysis during the stationary growth phase of *Escherichia coli*. *EMBO J*. 1999; 18(15):4108-17.

Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol*. 1995; 33(9):2233-9.

Thibault Stalder, Eva Top. Plasmid transfer in biofilms: a perspective on limitations and opportunities. *NPJ Biofilms Microbiomes*. 2016; 2:16022.

Thomassen MJ, Demko CA, Doershuk CF, Root JM. *Pseudomonas aeruginosa* isolates: comparisons of isolates from campers and from sibling pairs with cystic fibrosis. *Pediatr Pulmonol.* 1985; 1(1):40-5.

Tingpej P, Elkins M, Rose B, Hu H, Moriarty C, Manos J, Barras B, Bye P, Harbour C. Clinical profile of adult cystic fibrosis patients with frequent epidemic clones of *Pseudomonas aeruginosa*. *Respirology*. 2010; 15(6):923-9.

Tomás M, Doumith M, Warner M, Turton JF, Beceiro A, Bou G, Livermore DM, Woodford N. Efflux pumps, OprD porin, AmpC beta-lactamase, and multiresistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob Agents Chemother*. 2010; 54(5):2219-24.

Totten PA, Lory S. *Characterization of the type a flagellin gene from Pseudomonas aeruginosa* PAK. *J Bacteriol*. 1990; 172:7188-99.

References

Tubbs D, Lenney W, Alcock P, Campbell CA, Gray J, Pantin C. *Pseudomonas aeruginosa* in cystic fibrosis: cross-infection and the need for segregation. *Respir Med*. 2001; 95(2):147-52.

van Mansfeld R, Willems R, Brimicombe R, Heijerman H, van Berkhout FT, Wolfs T, van der Ent C, Bonten M. *Pseudomonas aeruginosa* genotype prevalence in Dutch cystic fibrosis patients and age dependency of colonization by various *P. aeruginosa* sequence types. *J Clin Microbiol*. 2009; 47(12):4096-101. Erratum in: *J Clin Microbiol.* 2013; 51(1):386.

Vettoretti L, Plésiat P, Muller C, El Garch F, Phan G, Attrée I, Ducruix A, Llanes C. Efflux unbalance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob Agents Chemother*. 2009; 53(5):1987-97.

Vincent JL. Nosocomial infections in adult intensive-care units. *Lancet*. 2003; 361(9374):2068-77.

Vogne C, Aires JR, Bailly C, Hocquet D, Plésiat P. Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Antimicrob Agents Chemother*. 2004; 48(5):1676-80.

Vollmer W, Höltje JV. Morphogenesis of Escherichia coli. *Curr Opin Microbiol*. 2001; 4(6):625-33.

Vötsch W, Templin MF. Characterization of a beta-N-acetylglucosaminidase of *Escherichia coli* and elucidation of its role in muropeptide recycling and beta-lactamase induction. *J Biol Chem*. 2000;275(50):39032-8.

Walters MC 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother*. 2003; 47(1):317-23.

Werner E, Roe F, Bugnicourt A, Franklin MJ, Heydorn A, Molin S, Pitts B, Stewart PS. Stratified growth in *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol*. 2004; 70(10):6188-96.

West SE, Schweizer HP, Dall C, Sample AK, Runyen-Janecky LJ. Construction of improved Escherichia-Pseudomonas shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene. 1994; 148(1):81-6.

Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, Greenberg EP. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature*. 2001; 413(6858):860-4.

Wiedemann B, Pfeifle D, Wiegand I, Janas E. Beta-lactamase induction and cell wall recycling in gram-negative bacteria. *Drug Resist Updat.* 1998; 1(4):223-6.

Wiehlmann L, Wagner G, Cramer N, Siebert B, Gudowius P, Morales G, Köhler T, van Delden C, Weinel C, Slickers P, Tümmler B. Population structure of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA*. 2007; 104(19):8101-6.

Wilke MS, Heller M, Creagh AL, Haynes CA, McIntosh LP, Poole K, Strynadka NC. The crystal structure of MexR from *Pseudomonas aeruginosa* in complex with its antirepressor ArmR. *Proc Natl Acad Sci USA*. 2008; 105(39):14832-7.

Williams D, Fothergill JL, Evans B, Caples J, Haldenby S, Walshaw MJ, Brockhurst MA, Winstanley C, Paterson S. Transmission and lineage displacement drive rapid population genomic flux in cystic fibrosis airway infections of a *Pseudomonas aeruginosa* epidemic strain. *Microb Genom*. 2018.

Williams D, Evans B, Haldenby S, Walshaw MJ, Brockhurst MA, Winstanley C, Paterson S. Divergent, coexisting *Pseudomonas aeruginosa* lineages in chronic cystic fibrosis lung infections. *Am J Respir Crit Care Med*. 2015; 191(7):775-85.

Winstanley C, Langille MG, Fothergill JL, Kukavica-Ibrulj I, Paradis-Bleau C, Sanschagrin F, Thomson NR, Winsor GL, Quail MA, Lennard N, Bignell A, Clarke L, Seeger K, Saunders D, Harris D, Parkhill J, Hancock RE, Brinkman FS, Levesque RC. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa*. *Genome Res*. 2009; 19(1):12-23.

Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T, Botzenhart K, Yankaskas JR, Randell S, Boucher RC, Döring G. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest*. 2002; 109(3):317-25.

Yamamoto M, Ueda A, Kudo M, Matsuo Y, Fukushima J, Nakae T, Kaneko T, Ishigatsubo Y. Role of MexZ and PA5471 in transcriptional regulation of mexXY in *Pseudomonas aeruginosa*. *Microbiology*. 2009; 155(Pt10):3312-21.

Yang L, Jelsbak L, Marvig RL, Damkiær S, Workman CT, Rau MH, Hansen SK, Folkesson A, Johansen HK, Ciofu O, Høiby N, Sommer MO, Molin S. Evolutionary dynamics of bacteria in a human host environment. *Proc Natl Acad Sci USA*. 2011; 108(18):7481-6.

Yen P, Papin JA. History of antibiotic adaptation influences microbial evolutionary dynamics during subsequent treatment. *PLoS Biol*. 2017; 15(8):e2001586.

Yoshimura F, Nikaido H. Diffusion of beta-lactam antibiotics through the porin channels of Escherichia coli K-12. *Antimicrob Agents Chemother*. 1985; 27(1):84-92.

References

Zamorano L, Reeve TM, Juan C, Moyá B, Cabot G, Vocadlo DJ, Mark BL, Oliver A. AmpG inactivation restores susceptibility of pan-beta-lactam-resistant Pseudomonas aeruginosa clinical strains. *Antimicrob Agents Chemother*. 2011; 55(5):1990-6.

Zamorano L, Reeve TM, Deng L, Juan C, Moyá B, Cabot G, Vocadlo DJ, Mark BL, Oliver A. NagZ inactivation prevents and reverts beta-lactam resistance, driven by AmpD and PBP 4 mutations, in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2010; 54(9):3557-63.

Zhao Q, Li XZ, Srikumar R, Poole K. Contribution of outer membrane efflux protein OprM to antibiotic resistance in *Pseudomonas aeruginosa* independent of MexAB. *Antimicrob Agents Chemother*. 1998; 42(7):1682-8.

8. ANNEX 1

BACTERIAL CULTURE MEDIA AND LABORATORY REAGENTS

8.1. BACTERIAL CULTURE MEDIA

Brain heart infusion (BHI) broth. The Oxoid (ThermoScientific) commercially available dehydrated media was used (CM1135). As recommended, 37 g were dissolved in 1 litre of distilled water, mixed and sterilized by autoclaving at 121°C for 15 minutes.

Luria-Bertani (LB) broth. 5 g NaCl, 5 g yeast extract and 10 g tryptone were dissolved in 1 litre of distilled water, mixed and sterilized by autoclaving at 121°C for 15 minutes.

Mueller-Hinton broth (MHB). The Oxoid (ThermoScientific) commercially available dehydrated media was used (CM0337). As recommended, 38 g were dissolved in 1 litre of distilled water, mixed and sterilized by autoclaving at 121°C for 15 minutes.

Mueller-Hinton agar (MHA). 15 g of commercially available bacteriological agar per litre of MH broth were added, mixed and sterilized by autoclaving at 121°C for 15 minutes. Once at room temperature, media was plated.

Mueller-Hinton Rifampicin agar (MHA-RI). 1l of room-temperature autoclaved MHA was supplemented with 300 mg of rifampicin and plated.

Mueller-Hinton Gentamycin agar (MHA-GM). 1l of room-temperature autoclaved MHA was supplemented with 50 and 250 mg of gentamycin and plated.

8.2. PULSED-FIELD GEL ELECTROPHORESIS STOCK BUFFERS, AGAROSE AND WORKING SOLUTIONS

1X Tris EDTA (TE) Buffer
1 mM EDTA (pH 7.6)
10 mM Tris-HCl (pH 7.6)
Store at room temperature

5X TBE Stock Buffer/Liter
54 g of Tris base
27.5 g of boric acid
20 ml of 0.5 M EDTA (pH 7.6)
Store at room temperature. **To prepare 0.5X TBE dilute 100 ml 5X TBE with 900 ml of sterile distilled water.**

PIV Solution
10 mM Tris-HCl (pH 7.6)
1 M NaCl
Prepare immediately before use.

EC-Lysis Solution
6 mM Tris-HCl (pH 7.6)
1 M NaCl
0.1 M EDTA (pH 7.6)
0.5% Brij®58
0.2% sodium deoxycholate
1% sodium lauroyl sarcosinate
20 µg/mlRNase
100 µg/ml lysozyme
Prepare immediately before use.

ESP Solution
0.5 M EDTA (pH 9.0-9.5)
1% sodium lauroyl sarcosinate
1 mg/ml proteinase K
Prepare immediately before use.

1.6% Low-melt agarose solution
1.6 g low-melt agarose (Bio-Rad)
100 ml 0.5X TBE
Mixed and dissolved well by heating the mixture (60-80 ºC). It can be stored at room temperature and heat before use.

1% Megabase agarose gel
1.5/2 g megabase agarose (Bio-Rad)
150/200 ml 0.5X TBE
Mixed and dissolved well by heating the mixture (60-80 ºC).

8.3. POLYMERASE CHAIN REACTION MASTER MIX

MASTER MIX AmpliTaq Gold™ DNA polymerase (Applied Biosystems™)		
	Reagents	**Volume (µl)**
-	Buffer II (10X)	10
-	DMSO	10
-	$MgCl_2$ (25 mM)	6
-	dNTPs (10 mM)	2
-	Taq polymerase (5 units/µl)	1
-	Forward primer 100 µM	1
-	Reverse primer 100 µM	1
-	PCR grade water	69
-	Chromosomal DNA	1
Total reaction volume = 100 µl		

8.4. REAL-TIME QUANTITATIVE REVERSE TRANSCRIPATSE POLYMERASE CHAIN REACTION (QRT-PCR) MASTER MIX

MASTER MIX QuantiTect SYBR green RT-PCR kit (Qiagen)		
Reagents	SMART CYCLER II Instrument (Cepheid)	Eco real-time PCR System (Illumina)
	Volume (µl)	
- RT-PCR master mix (2X)	12.5	5
- Quantitec RT mix	0.25	0.1
- Forward primer	0.1 (100 µM)	0.4 (10 µM)
- Reverse primer	0.1 (100 µM)	0.4 (10 µM)
- RNAse free water	11.3	3.1
- RNA (50ng/µl)	1	1
Total reaction volume	**50**	**10**

8.5. ISOLATION OF SMALL AMOUNTS OF OUTER MEMBRANE PROTEINS STOCK BUFFERS

Tris-Mg Buffer
- 10mM Tris-HCl (pH=7.3)
- 5mM $MgCl_2$
Store at 4ºC

Laemmli's sample Buffer
- 0.125 M Tris-HCl (pH=6.8)
- 4% SDS
- 20% Glycerol
- 10% β-mercaptoethanol
Store at -4ºC

8.6. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) REAGENTS AND BUFFERS

Separating Gel (10%)	
Reagents	**Volume**
Tris-HCl 1M pH=8.8	1.75 ml
10% ammonium persulfate (*APS*) solution	70 µl
10 % sodium dodecyl sulfate solution	70 µl
TEMED	3 µl
40% polyacrylamide	1.75 ml
Sterile distilled water.	3.36 ml
Total volume = 7 ml	

Stacking Gel (5%)	
Reagents	**Volume**
Tris-HCl 1M pH=6.9	375 µl
10% ammonium persulfate (*APS*) solution	30 µl
10 % sodium dodecyl sulfate solution	30 µl
TEMED	3 µl
40% polyacrylamide	375 µl
Sterile distilled water.	2.2 ml
Total volume = 3 ml	

Running Buffer 1X
3 g Tris_Base
14.4 g glycine
10 ml of 10% sodium dodecyl sulfate solution
Add sterile distilled water up to 1 l and adjust to pH 8.3.
Store at room temperature

8.7. COMPLEMENTATION ASSAYS REAGENTS AND BUFFERS

Sucrose Magnesium Electroporation Buffer (SMEB)		
	Reagents	**Volume**
-	0.5 M HEPES buffer (pH 7)	2 ml
-	1 M $MgCl_2$	1 ml
-	Sucrose	102.7 g
-	Sterile distilled water.	Bring volume to 1000 ml
Autoclave and store at 4ºC until use		

Super Optimal Broth (SOB)		
	Reagents	**Volume**
-	Tryptone	2 g
-	Yeast extract	0.5 g
-	5 M NaCl	0.2 ml
-	1 M KCl	0.25 ml
Add sterile distilled water until 100 ml, adjust to pH 7, autoclave and add…		
-	1M $MgCl_2$	1 ml
-	1 M $MgSO_4$	1 ml

Super Optimal broth with Catabolite repression (SOC)		
	Reagents	**Volume**
-	SOB	20 ml
-	1 M Glucose	0.4 ml

9. ANNEX 2

ARRAY-TUBE GENOTYPING SYSTEM, PROBES AND PRIMERS INFORMATION

PROBES	REFERENCE	PROBE SEQUENCE	ANTISENSE PRIMER 1	ANTISENSE PRIMER 2
oriC PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GAAGCCCAGCAATTGCGTGTTTC	AGCCTCGACACCGGTTCTCG	ACCATCTCGTTCATCCCCAGG
oriC non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	GAAGCCCAGCAACTGCGTGTTTC		
oprL (1) PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GGTGCTGCAGGGTGTTTCGCCGG	TTCTGAGCCCAGGACTGCTCG	TCGACGCGACGGTTCTGAGCC
oprL (1) non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	GGTGCTGCAGGGCGTTTCGCCGG		
oprL (2) PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GTGCTGCAGGGTGTTTCGCCG		
oprL (2) non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	GCTGCAGGGCGTTTCGCCG		
fliCa (1) PAK	PAK, *Totten and Lory* 1990, flagellin type a2, *Giske et al.* 2006	CAAGATCGCCGCAGCGGTCAAC	AGCTGATGGTATCGCCGTCGC	CTAGTGATCGCACCGGAGCC
fliCa (1) non-PAK	ATCC15691, *Spangenberg et al.* 1998, flagellin type a1, *Giske et al.* 2006	CAAGATCGCCGCTGCGGTCAAC		
fliCa (2) PAK	PAK, *Totten and Lory* 1990, flagellin type a2, *Giske et al.* 2006	CAAGATCGCCGCAGCGGTCAACGAC		
fliCa (2) non-PAK	ATCC15691, *Spangenberg et al.* 1998, flagellin type a1, *Giske et al.* 2006	CAAGATCGCCGCTGCGGTCAACGAC		
alkB2 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CCTCGCCCTGTTCCCACCGCTCTGG	TTCCTCGCCGGCATAGTAGGC	
alkB2 non-PAO1	ATCC 15691, *Morales et al.* 2004	CTCGCCCTGTTCCCGCCGCTCTGG		
citS-1 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	TCGAGCAACTGGCAGAGAAATCCG	GCAGGTAGCAGGTTTCCAGG	AACTGTTCCTTCTGCGCGGCG
citS-1 non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	CGAGCAACTGGCGGAGAAATCCG		
citS-2 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GCGGAAAACTTCCTGCACATGATGTT	TGATCGGCTTGGTCTCGCAGG	GCTGATCGGCTTGGTCTCGC
citS-2 non-PAO1	*Kiewitz and Tummler.* 2000	GCGGAAAACTTCCTCCACATGATGTT		

Annex 2

PROBES	REFERENCE	PROBE SEQUENCE	ANTISENSE PRIMER 1	ANTISENSE PRIMER 2
oprI (1) PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	AGCTCAGCAGACTGCTGACGAGG	GCTGGCTTTTTCCAGCATGCG	TTGCGGCTGGCTTTTTCCAGC
oprI (1) non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	AGCTCAGCAGACCGCTGACGAG		
oprI (2) PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GCTCAGCAGACTGCTGACGAGGCTAACG		
oprI (2) non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	GCTCAGCAGACCGCTGACGAGGCTAAC		
ampC-1 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	ACGGCCGCCGGGTGACGCC	CGCATCTTGTCCTGGGTCAGG	TCGTCGAGGCGCATCTTGTCC
ampC-1 non-PAO1	*De Champs et al.* 2002, *Kiewitz and Tummler*, 2000	ACGGCCGCCAGGTGACGCCG		
ampC-3 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CGACCTACGCGCCGGGCAG	GGCGAGATAGCCGAACAGGC	CACTTGCTGCTCCATGAGCC
ampC-3 non-PAO1	*De Champs et al.* 2002, *Kiewitz and Tummler.* 2000	CGACCTATGCGCCGGGCAGC		
ampC-4 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CGTTCGAACGGCTCATGGAGCAG	ACGTCGAGGTGGGTCTGTTCG	GTAGCCTTCGGCATCCAGCG
ampC-4 non-PAO1	*De Champs et al.* 2002, *Kiewitz and Tummler* 2000	CGTTCGAACGACTCATGGAGCAGC		
ampC-5 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	TGGAGCAGCAAGTGTTCCCGGC	amplified with primers of ampC-4	
ampC-5 non-PAO1	*De Champs et al.* 2002, *Kiewitz and Tummler.* 2000	TGGAGCAGCAACTGTTCCCGGC		
ampC-6 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GAACAAGACCGGTTCCACCAACGG	TCGGCATTGGGATAGTTGCGG	
ampC-6 non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	AACAAGACCGGCTCCACCAACGG		
ampC-7 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CGACCTGGGCCTGGTGATCCT	TTGGGATAGTTGCGGTTGGC	TGGCGTAGGCGATCTTCACCC

PROBES	REFERENCE	PROBE SEQUENCE	ANTISENSE PRIMER 1	ANTISENSE PRIMER 2
fliC a	ATCC15691, *Spangenberg et al.* 1998	GTCGCTGAACGGCACCTACTTCA	CGATCGCGATGTCGACGGTGC	TGCCGATCGCGATGTCGACG
fliC b	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GCCGACCAACTGAACTCCAACTCG	TGACGTTCTCGCCGGTAGCG	CAGTAGCGGTACCGGTCTGC
exoS	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CAGCCCAGTCAGGACGCGCA	CAGGGTCGCCAGCTCGCTCGCC	AGGGTCGCCAGCTCGCTCGC
exoU	UCBPP-PA14, complete genome, *Lee et al.* 2006	CGCCAGTTTGAGAACGGAGTCACC	AGTGATCTGCCGCGGCCCTGCC	GTGATCTGCCGCGGCCCTGC
fpvA type I	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CCTGAATCCGACCATTCGCGAGTC	CGTTCAGGTCGTAGACCGCGC	GCGATACCAACTGTCCTGCGGC
fpvA type IIa	*de Chial et al.* 2003	TCGGACTGTACTCCTACGAAGCAGC	TGCCGAAGGTGAATGGCTTGCC	CCTGATGGTCCGATCCCAGC
fpvA type IIb	*Spencer et al.* 2003	CCAATCCCTATCGCTGGAACCGTACC	GCCGAGGGTCAAGAACCACTGG	TCTTGGCCCAGTCATAGCGGC
fpvA type III	*de Chial et al.* 2003	GCTCGGGACTCGCATTTCGTCC	TAACCCCAAGGCCCATTGGAGG	GCCACCGCCTTCGAATAACCCC
fpvB	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GCGTTATTGCTCGGTCTCTCCTCG	AATTGCTCGAGGGATGCGGC	GGTCGAAACGGATGCGCAGG
LES	LES400 (personal communication *C. Winstanley*)	TGCATAGGAGTCATGCCGACAGCA	GCCCCGCGTCATTTTCACGTCG	AATGCTCTGGGCAACGAGCC
PA0636	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GCCAATTGGGTCAGCAAGCAACG	ATGCCATCGTTGAAGGCACCGC	TGCCATCGTTGAAGGCACCG
PA0722	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CGTGTCGCGAACTCGCATGGC	TCTGGCGGAATCAGGTAGGCC	CTTCCGGGGAGAAACCACCG
PA0728	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CTGGAGCCTGCGAAAGTGGCTC	AGCCAAGACGGTTGTTCGCGG	TCAATGACGCCGAGTTGGCGC
PA2185	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	ACGAGGGTGATGGCTGGGAATACG	CTCGGACAGGTTCACGCTGG	GCCATTCGCTGCAACACCTCC
PA2221	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CAGTTGTCGCCAGGTCTGGAGAATCC	TTCCTGGGCCAGAGTTGGACC	AGCTTAAGGCCGTGGCACTCG
PA3835	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CACATCAATGTCAGCCCACGCCA	CCGGAGAATTCGCGTCCACC	TGCTGACGATGAAGCCCCAGC
fla-island	*Arora et al.* 2001	ACCTGTGTCGCTGGAGGGTATGTT	CCCGTGTTTCCGTAGACCTTGC	

Annex 2

PROBES	REFERENCE	PROBE SEQUENCE	ANTISENSE PRIMER 1	ANTISENSE PRIMER 2
orfA	*Arora et al.* 2001	CGCTGGAGGGTATGTTCCGCAAGG	GTTCCACAGGCGCTGCGGCGC	GTTCCACAGGCGCTGCGGCG
orfI	*Arora et al.* 2001	CCTGGACCTCTCCAAGGTTCGCCT	AAACTGCCCCGCCCCCCATCC	GGAAAAACTGCCCCGCCCCCC
orfJ	*Arora et al.* 2001	GCCATTCCGACGACCAAACAAGGC	ACGCTCGCAGCGCCTCACGCG	GGCCTGGCTGCGAACGCTCGC
PA0980	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CGGTATGAAGATGGGTGGTTGGGTCG	ACCTCCAGCACCGACACACC	ATCCGATCCACCTCCAGCACC
XF1753	UCBPP-PA14, complete genome, *Lee et al.* 2006	TGCGAGGACCAGAAACCTTGATGG	GCGCGCGTTCGAGAAACAGG	CGGAGGTTGAAAAGCTGGCCC
acetyltransferase	UCBPP-PA14, complete genome, *Lee et al.* 2006	CGAAGCGTAGGGTCTTCGTAGCC	ACGACGTCACCGTCGAGACCG	ACCGCCTTTCTGGTGAGCTGG
pKL-1	*Klockgether et al.* 2004	CACCATGCAAATGCTCGATGGACTGC	ATCTGAACCGAGGGGATCCGC	CCCGGGAGTCATTGGTCTGG
pKL-3	*Klockgether et al.* 2004	TCTGAACTGCGGCTATCACCTGGA	GACCTACACTCCAACCGCTGG	TTCCCTTGCTGCCGAGAAGC
TB-C47-1	*P. aeruginosa* TB, pKLC102 related gene island integrated in tRNA(Lys) PA4541.1	GCAGGCGTCCAAGTTGGAGCTCTCC	GCCTGTTGGACCCCTTTGACC	TACTCCTGCCTGTTGGACCCC
TB-C47-2	*P. aeruginosa* TB, pKLC102 related gene island integrated in tRNA(Lys) PA4541.1	TCCAACAGGCAGGAGTACAGGGTG	TCTGTCAATCCCCTTTGGGG	AGCCCCTTTCTGTCAATCCCC
PAPI-1 pili chaperone	UCBPP-PA14, complete genome, *Lee et al.* 2006	GGAACACAACGTGGGGCGTGAC	CGCTCAAGCGCTATCCCACC	CGCCATCGGCCTGTACAACG
PAPI-1 luminal binding protein	UCBPP-PA14, complete genome, *Lee et al.* 2006	CCAGTTGGCACCACCATGCTTGC	CGGTAGAGAGCTGGGTTGGC	AACCTGGAGCTAGGGCAGAGC
pKLC conserved hypothetical	*Klockgether et al.* 2004	GCCTGCCTACTTGTTCCCAACGC	CTACCCAGCTTGGGCGTAGC	AAGCGATAGCCGTGCTCCTGC
pKLC adhesin	*Klockgether et al.* 2004	GGCTGTATTGCCCGCCATTCTCC	CCGGCTATATCCGCGGCTACC	ATTGGCGCTGCTGTTTACGCCC
pKLC fatty acid synthase	*Klockgether et al.* 2004	CGACAGACAGAAAGGGTTCTTGCGC	GGTGGCGTCGGGTTTTTCTGC	AGGTCGTAGCGGAAGGTGGTGG
PAGI-2/3-4	*Larbig et al.* 2002	GCGCCTTCTCCTCTTTGCAGATGT	TGTCCCGGCTCAGTTCAACG	GCAACACCTTGGCGTTTGTCC
PAGI-2/3-5	*Larbig et al.* 2002	CAGTATGGTACGGACACGAAGCGC	TCAAGCTCGTTGTGGACCGC	GTTACGACGGCGTGCTGTCGG
PAGI-2/3-6	*Larbig et al.* 2002	CCATGGTCGGAACAGGCACGATATGC	CAACACGCGACTGGCGATCC	TACATCATCCGCAACGGCGGC

PROBES	REFERENCE	PROBE SEQUENCE	ANTISENSE PRIMER 1	ANTISENSE PRIMER 2
C-45	*Larbig et al.* 2002	CGAGGAGTTTCGGACCCGCTTTGA	TCATCCAGCAAGCCATTGCGC	TGGAGTCGCTTTCCGCCATCG
C-46	*Larbig et al.* 2002	CGAAGTCTGAGGTGTGGACCCGC	CGCGGTGCTGGTTGCGCTGC	CGCTGGCAGTTCCGCTGGCC
C-47	*Larbig et al.* 2002	CCACTCGATCATGTTGAGCATCGGCTCC	TATTGACGACCTACCGCGCGCC	CACCAAGAACCCGCTGCTCG
PAGI-2	*Larbig et al.* 2002	GCATCATTGCGCGTCACATCTGGT	ACGCAACGTATTCGGCGACCC	CGCAACGTATTCGGCGACCC
PAGI-2/3-1	*Larbig et al.* 2002	GACCGCAAGCAGAAACGGCATGC	GGTGCTCGACCCAAGCATCG	TCCTTGAGTTCCTTGGCGCGG
PAGI-3-1	*Larbig et al.* 2002	CCCGTTGCTCATAACCCGTTCCTG	GACGAATACCCAGCTGCGTGG	GCAGACGAATACCCAGCTGCG
PAGI-3-8	*Larbig et al.* 2002	GGTTAGTCCCTTCTGCCCGCATCG	ATCGTGGCAGGATGTCCACCG	TAGGCGGGCCTTTTGAAGGTGC
tRNA(Pro)- island 1	*P. aeruginosa* TB, gene island integrated into tRNA(Pro) PA2736.1	GTGTCACGGCCCATGTCTAGCAGC	TCCACGCCGAGGGACGTGCC	GCTCCACGCCGAGGGACGTGCC
tRNA(Pro)- island 2	*P. aeruginosa* TB, gene island integrated into tRNA(Pro) PA2736.1	AGGCCATGGGCTAGCCGGATGC	AGGAGGCCGATGACAACACCC	TGCCGATTCCATGCTCACGCC
PAGI-1	*Liang et al.* 2001	TTCTCGGTGTCGAGGGATTCTCGG	GCATTCGCCACGGAAGGAAGG	GAAGGCATCATGGCATTCGCC

10. ANNEX 3

EUROPEAN COMMITTEE ON ANTIBIOTIC SUSCEPTIBILITY TESTING (EUCAST) CLINICAL BREAKPOINTS FOR *Pseudomonas spp.*

Antibiotic compound[a]	Version 3.1.					Version 6.0.					Version 7.0.				
	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)	
	S≤	R>		S≥	R<	S≤	R>		S≥	R<	S≤	R>		S≥	R<
PPT	16	16	30-6	19	19	16	16	30-6	18	18	16	16	30-6	18	18
TZ	8	8	10	16	16	8	8	10	17	17	8	8	10	17	17
PM	8	8	30	18	18	8	8	30	19	19	8	8	30	19	19
TOL/TAZ	-	-	-	-	-	-	-	-	-	-	4	4	30-10	-	-
IP	4	8	10	20	17	4	8	10	20	17	4	8	10	20	17
MP	2	8	10	24	18	2	8	10	24	18	2	8	10	24	18
AT	1	16	30	50	16	1	16	30	50	16	1	16	30	50	16
CI	0.5	1	5	25	22	0.5	1	5	25	22	0.5	0.5	5	26	26
LE	1	2	5	20	17	1	2	5	20	17	1	1	5	22	22
AK	8	16	30	18	15	8	16	30	18	15	8	16	30	18	15
GM	4	4	10	15	15	4	4	10	15	15	4	4	10	15	15
TM	4	4	10	16	16	4	4	10	16	16	4	4	10	16	16
CO	4	4	-	-	-	4	4	-	-	-	2	2	-	-	-

11. ANNEX 4

USED SCRIPTS IN THE ANALYISIS OF WHOLE-GENOME SEQUENCING DATA

11.1. VARIANT CALLING: PIPELINE

STEP 1: CHECK MiSeq® READS

```
#!/bin/bash

# Use: this script checks all the obtained reads for a sequenced sample (MS). If a single string number is found (no contamination) it will save a new file where the string number would have been replaced with a /1 or /2 depending on the read-file number (forward/1 or reverse/2).

#Input: name of MiSeq sequence file (MS) and if it is "/1" or "/2" (FR)

MS=$1

FR=$2

grep @M ~/$MS |awk '{print $2}' | sort | uniq -c > tmpC

cat tmpC

SEQ_NUM=`cat tmpC | wc -l`

SEQ_ID=`awk '{print $2}' tmpC`

echo $SEQ_ID

if [ $SEQ_NUM -eq 1 ]

then

        echo replace $SEQ_ID of file: $MS with /$FR


        sed "\#@M#s# $SEQ_ID#/$FR#g" ~/$MS > ~/new_$MS

else

        echo error -possible contamination

fi

rm tmpC
```

STEP 2: PAO1 REFERENCE GENOME MAPPING (SAM FILES)

```
#!/bin/bash
```

Use: this script maps all checked reads (forward/reverse) for a sequenced sample (new_MS) to *P. aeruginosa* PAO1 reference genome (previously indexed: "indexed_PAO1_file").

~/bowtie2-2.2.6/bowtie2 --phred33 –x ~/indexed_PAO1_file -q -1 ~/new_MS_L001_R1_001.fastq -2 ~/new_MS_L001_R2_001.fastq -X 1000 -S ~/MS_mapPAO1.sam 2> ~/output_DO_bowtie2_PAO1_MS.txt

STEP 3: GENERATING PILEUP AND RAW FILES FROM SAM FILES

#!/bin/bash

Use: this script will generate the raw and totalpileup files from the sam_file obtained in step 2.

~/samtools-0.1.16/samtools view -b –S ~/MS_mapPAO1.sam > ~/MS_mapPAO1.bam

java -jar ~/picard-tools-1.140/picard.jar SortSam INPUT=~/MS_mapPAO1.bam OUTPUT=~/MS_mapPAO1_sorted.bam SORT_ORDER=coordinate

java -jar ~/picard-tools-1.140/picard.jar MarkDuplicates METRICS_FILE=~/MS_metrics.txt

INPUT=~/MS_mapPAO1_sorted.bam OUTPUT=~/MS_mapPAO1_sorted_dedup.bam

java -jar ~/picard-tools-1.140/picard.jar AddOrReplaceReadGroups INPUT=~/MS_mapPAO1_sorted_dedup.bam OUTPUT=~/MS_mapPAO1_addrg.bam LB=XXX PL=Illumina PU=XXX SM=XXX

java -jar ~/picard-tools-1.140/picard.jar BuildBamIndex INPUT=~/MS_mapPAO1_addrg.bam

java -jar ~/GenomeAnalysisTK-3.4-46/GenomeAnalysisTK.jar -T RealignerTargetCreator -I ~/MS_mapPAO1_addrg.bam -R ~/PAO1complete.fasta -o /MS_mapPAO1.realigned.intervals

java -jar ~/GenomeAnalysisTK-3.4-46/GenomeAnalysisTK.jar -T IndelRealigner –I ~/MS_mapPAO1_addrg.bam –R ~/PAO1complete.fasta --maxConsensuses 60 --maxReadsForConsensuses 240 --maxReadsForRealignment 6000 --targetIntervals ~/MS_mapPAO1.realigned.intervals -o ~/MS_mapPAO1.realigned.bam

~/samtools-0.1.16/samtools sort ~/MS_mapPAO1.realigned.bam /MS_mapPAO1.realigned.sorted

~/samtools-0.1.16/samtools pileup -c -f ~/PAO1complete.fasta ~/MS_mapPAO1.realigned.sorted.bam > ~/MS_mapPAO1.realigned.totalpileup

~/samtools-0.1.16/samtools pileup -vc -f ~/PAO1complete.fasta ~/MS_mapPAO1.realigned.sorted.bam > ~/MS_mapPAO1.realigned.raw

NOTE. When adding or replacing groups additional information (XXX) about the sequencing process should be indicated, where: LB: DNA library preparation identifier; PU:platform unit; SM: sample number.

STEP 4: GENERATING SNP AND INDEL FILES

#!/bin/bash

Use: this script will extract SNP and InDel positions from the raw and totalpileup files obtained in step 3.

cat ~/MS_mapPAO1.realigned.raw | awk '$6>=50 && $7>=25 && $8>=3' | awk '$4!="M" && $4!="R" && $4!="W" && $4!="S" && $4!="Y" && $4!="K"' > ~/MS.snps

awk '$3=="*"' ~/MS_mapPAO1.realigned.totalpileup | awk -v var1=500 -v var2=25 '$6>=var1 && $7>=var2' | awk '($9=="*" && $12*5>=$8) || ($10=="*" && $11*5>=$8) || ($9!="*" && $10!="*" && ($12*5>=$8 || $11*5>=$8))' > ~/MS.indels

11.2. DE NOVO ASSEMBLIES

RUNNING VELVET

#!/bin/bash

Use: this script will generate de novo assemblies from checked read sequences files.

sh ~/velvet_1.2.10/contrib/shuffleSequences_fasta/shuffleSequences_fasta.sh ~/new_MS_L001_R1_001.fastq ~/new_MS_L001_R2_001.fastq ~/MS_interleaved.fastq

~/velvet_1.2.10/velveth ~/velvet_MS 31 -shortPaired -fastq ~/MS_interleaved.fastq

~/velvet_1.2.10/velvetg ~/velvet_MS -scaffolding no -ins_length 500 -cov_cutoff 3 -min_contig_lgth 500

mv ~/velvet_MS/contigs.fa ~/MS.500.denovoassembly.fasta

11.3. GENERATING THE NEXUS FILES FOR BEAST ANALYSIS

STEP 1: GENERATING COMMON FILES WITHIN A GROUP OF SAMPLES

#!/bin/bash

Annex 4

#Input: Samples is a file in which all samples included within the group/lineage should be listed in 1 column.

```
SAMPLES=$1

LINEAGE=$2

NO=`cat $SAMPLES | wc -l`

rm tmpS_raw.$LINEAGE

rm tmpS_totalpileup.$LINEAGE

rm tmpS_indels.$LINEAGE

while read line

do

cat ~/"$line"_mapPAO1.realigned.raw | awk '$3!="*"' >> tmpS_raw.$LINEAGE

cat ~/"$line"_mapPAO1.realigned.totalpileup | awk '$8>=3' | awk '$3!="*"' >>
tmpS_totalpileup.$LINEAGE

cat ~/"$line"_mapPAO1.realigned.totalpileup | awk '$3=="*"' >> tmpS_indels.$LINEAGE

done < $SAMPLES

echo "Number of files: $NO"

cat tmpS_totalpileup.$LINEAGE | awk '$8>=3' | cut -f1-3 | sort -nk 2 | uniq -c | awk -v
var1=$NO '$1==var1' | awk '{print $2"\t"$3"\t"$4}' > ~/common_totalpileup_$LINEAGE

cat tmpS_raw.$LINEAGE | cut -f1-4 | awk '$3!=$4' | sort -nk 2 | uniq -c | awk -v var1=$NO
'$1==var1' | awk '{print$2"\t"$3"\t"$4"\t"$5}' > ~/common_raw_$LINEAGE

cat tmpS_indels.$LINEAGE | cut -f1-4 | sort -nk 2 | uniq -c | awk -v var1=$NO '$1==var1' |
awk '{print $2"\t"$3"\t"$4"\t"$5}' > ~/common_indels_$LINEAGE

cat tmpS_totalpileup.$LINEAGE | awk '$4=="M" || $4=="R" || $4=="W" || $4=="S" || $4=="Y"
|| $4=="K"' | cut -f1-3 | sort | uniq > ~/common_ambiguous_$LINEAGE

cat tmpS_totalpileup.$LINEAGE | awk '$5==0' | cut -f1-3 | sort | uniq >
~/common_col5_eq_0_$LINEAGE

rm tmpS_raw.$LINEAGE

rm tmpS_totalpileup.$LINEAGE
```

STEP 2: EXTRACTING COMMON SNP POSITIONS FROM SNP FILES

```
#!/bin/bash

LINEAGE=$1

cat ~MS.snps | fgrep –vf ~/common_raw_$LINEAGE | fgrep -vf
~/common_ambiguous_$LINEAGE | fgrep -vf ~/common_col5_eq_0_$LINEAGE  >
~/MS.$LINEAGE.int.snps

awk ‘NR==FNR{c[$1, $2, $3]++;next};c[$1, $2, $3] > 0' ~/common_totalpileup_$LINEAGE
~/MS.$LINEAGE.int.snps > ~/MS.$LINEAGE.snps’
```

STEP 3: GENERATING THE NEXUS FILE

```
#!/bin/bash

#Before an input file called SNPS should be prepared (SNPS), containing all positions that will be used for generate the nexus file. As well, a file (ISO) including all isolates (MS) to be used for generate the nexus file should be prepared.

ISO=$1

SNPS=$2

N_TAX=`cat $ISO | wc -l`

N_CHAR=`cat $SNPS | wc -l`

while read line

do

        echo "Isolate $line is being processed."

        isolate=`echo $line | awk '{print $1}'`

        fgrep -f $SNPS ~/”$isolate”_mapPAO1.realigned.totalpileup > tmpA

        while read pos

        do

                position=`echo $pos | awk '{print $2}'`

                echo "Position $position is being processed."

                echo "$pos" > tmpB
```

```
fgrep -f tmpB tmpA > tmpC

echo "position $position in $line:"

echo `cat tmpB`

count=`cat tmpC| wc -l`

echo $count

if [ $count -lt 1 ]

then

        echo "Position $position is not present"

        echo $position $line >> missing_positions.txt

        echo "?" >> $isolate.tree

else

        if [ $count -eq 1 ]

        then

                echo "Position $position is found"

                awk '{

                if ($6>=50 || $7>=25 && $8>=3)

                        print $4

                else

                        print "?"

                }' tmpC > $isolate.tmp

                awk '{

                if ($1=="*")

                        print "-"

                else

                        print $0
```

```
                                        }' $isolate.tmp >> $isolate.tree

                                fi

                        fi

        done<$SNPS

        sh ~/transpose.sh $isolate.tree | sed 's/[[:blank:]]//g' > $isolate. trans.tmp

        echo "'$isolate'" > name_file_$isolate. tmp

        paste name_file_$isolate. tmp $isolate. trans.tmp > alignment.$isolate. tmp

done<$ISO

echo "#nexus\n\n[file created from SNP positions found with Bowtie2-GATK-Samtools on
`date`]\n\n\

begin data;\n\

\tdimensions ntax = $N_TAX nchar = $N_CHAR;\n\

\tformat datatype = DNA gap = - missing = ?;\n\n\

\tmatrix\n" > head_nex.tmp

echo "\t;\nend;" > tail_nex.tmp

cat head_nex.tmp alignment.*.*.tmp tail_nex.tmp > alignment.nexus.totalpileup

clean

#rm *tmp*

#rm *.*.tree
```

NOTE. The following script shoud be disposable to run the abovementioned (transpose.sh):

```
#!/bin/bash

FILE=$1

awk '

{

        for (i=1; i<=NF; i++)  {

                        a[NR,i] = $i
```

```
        }
}
NF>p { p = NF }
END {
   for(j=1; j<=p; j++) {
                str=a[1,j]
                        for(i=2; i<=NR; i++){
                                str=str"\t"a[i,j];
                        }
                                print str
                        }
                }' $FILE
```

12. ANNEX 5

Pseudomonas aeruginosa ANTIBIOTIC-RESISTANCE AND HYPERMUTATION RELATED CHROMOSOMAL GENES

Table A5.1. Set of 164 genes known to be related with antibiotic resistance in *Pseudomonas aeruginosa* PAO1 reference strain.

LOCUS	GENE	START BP	END BP
PA0004	*gyrB*	4275	6695
PA0005	*lptA*	7018	7791
PA0018	*fmt*	20068	21012
PA0058	*dsbM*	72680	73384
PA0301	*spuE*	339959	341056
PA0302	*spuF*	341111	342265
PA0355	*pfpI*	399493	400032
PA0392	*yggT*	434226	434819
PA0402	*pyrB*	444687	445691
PA0424	*mexR*	471306	471749
PA0425	*mexA*	472024	473175
PA0426	*mexB*	473191	476331
PA0427	*oprM*	476333	477790
PA0463	*creB*	523254	523943
PA0464	*creC*	523943	525367
PA0465	*creD*	525469	526827
PA0486	*yihE*	547432	548406
PA0487	*modR*	548468	549226
PA0610	*prtN*	672777	673091
PA0611	*prtR*	673191	673961
PA0612	*ptrB*	674419	674619
PA0779	*asrA*	845793	848192
PA0807	*ampDh3*	884799	885566
PA0869	PBP6/7	949716	950648
PA0893	*argR*	976410	977399
PA0958	*oprD*	1043983	1045314
PA1178	*oprH*	1277006	1277608
PA1179	*phoP*	1277688	1278365
PA1180	*phoQ*	1278362	1279708
PA1343	*pagP*	1457175	1457633
PA1345	*gshB*	1458707	1460296
PA1375	*pdxB*	1491913	1493055
PA1409	*aphA*	1533238	1534278
PA1430	*lasR*	1558171	1558890
PA1588	*sucC*	1730181	1731347
PA1589	*sucD*	1731347	1732234
PA1777	*oprF*	1921174	1922226
PA1796	*folD*	1946187	1947041
PA1797	-	1948502	1950334
PA1798	*parS*	1950439	1951725
PA1799	*parR*	1951726	1952433
PA1801	*clpP*	1954069	1954710
PA1803	*lon*	1956227	1958623
PA1812	*mltD*	1969635	1971239
PA1886	*polB*	2054911	2057274
PA2006	-	2194058	2195410
PA2018	*mexY*	2208169	2211306
PA2019	*mexX*	2211322	2212512
PA2020	*mexZ*	2212677	2213309
PA2023	*galU*	2215102	2215941
PA2050	-	2244492	2244998
PA2071	*fusA2*	2272460	2274568
PA2227	*vqsM*	2448568	2449545
PA2272	PBP3a	2501720	2503417
PA2273	*soxR*	2503425	2503895
PA2489	-	2805021	2805836
PA2490	*ydbB*	2805917	2806291
PA2491	*mexS*	2806350	2807369
PA2492	*mexT*	2807469	2808512
PA2493	*mexE*	2808743	2809987
PA2494	*mexF*	2810009	2813197
PA2495	*oprN*	2813194	2814612
PA2522	*czcC*	2842019	2843305
PA2523	*czcR*	2843818	2844492
PA2524	*czcS*	2844489	2845907
PA2525	*opmB*	2846283	2847779
PA2526	*muxC*	2847776	2850886
PA2527	*muxB*	2850883	2854014
PA2528	*muxA*	2854011	2855291
PA2615	*ftsK*	2956805	2959240
PA2621	*clpS*	2964607	2964843
PA2642	*nuoG*	2987721	2990438
PA2649	*nuoN*	2996265	2997725
PA2797	-	3154593	3155075
PA2798	-	3155072	3156256
PA2809	*copR*	3162705	3163385
PA2810	*copS*	3163382	3164713
PA2830	*htpX*	3182986	3183861
PA3005	*nagZ*	3365756	3366754
PA3013	*foaB*	3373254	3374429
PA3014	*faoA*	3374460	3376607
PA3047	PBP4	3410264	3411694
PA3050	*pyrD*	3414701	3415729
PA3077	*cprR*	3450838	3451509
PA3078	*cprS*	3451506	3452801
PA3141	*capD*	3524681	3526678
PA3168	*gyrA*	3556427	3559198
PA3521	*opmE*	3938020	3939495
PA3522	*mexQ*	3939492	3942653
PA3523	*mexP*	3942650	3943807
PA3533	*grxD*	3952061	3952387

Table A5.1. Set of 164 genes known to be related with antibiotic resistance in *Pseudomonas aeruginosa* PAO1 reference strain.(Cont.)

LOCUS	GENE	START BP	END BP
PA3574	*nalD*	4006510	4007148
PA3602	*yerD*	4036265	4037875
PA3676	*mexK*	4116188	4119265
PA3677	*mexJ*	4119270	4120373
PA3678	*mexL*	4120469	4121107
PA3719	*armR*	4165719	4165880
PA3721	*nalC*	4166518	4167159
PA3999	PBP5	4478979	4480139
PA4001	*sltB1*	4481230	4482252
PA4003	PBP2	4480139	4485336
PA4020	*mpl*	4498488	4499843
PA4069	-	4546668	4547552
PA4109	*ampR*	4592990	4593880
PA4110	*ampC*	4594029	4595222
PA4119	*aph*	4607578	4608384
PA4205	*mexG*	4705956	4706402
PA4206	*mexH*	4706410	4707522
PA4207	*mexI*	4707535	4710624
PA4208	*opmD*	4710621	4712084
PA4218	*ampP*	4721614	4722858
PA4238	*rpoA*	4754423	4755424
PA4260	*rplB*	4764880	4765701
PA4266	*fusA1*	4769035	4771155
PA4269	*rpoC*	4772279	4776478
PA4270	*rpoB*	4776544	4780617
PA4273	*rplA*	4781985	4782680
PA4315	*mvaT*	4843812	4844186
PA4374	*mexV*	4903466	4904596
PA4375	*mexW*	4904647	4907703
PA4380	*colS*	4910871	4912151
PA4381	*colR*	4912141	4912824
PA4393	*ampG*	4922407	4924191
PA4406	*lpxC*	4938276	4939187
PA4418	PBP3	4952604	4954343
PA4444	*mltB1*	4977869	4978972
PA4462	*rpoN*	4992870	4994363
PA4521	*ampE*	5063941	5064777
PA4522	*ampD*	5064774	5065340
PA4567	*rpmA*	5116032	5116289
PA4568	*rplU*	5116313	5116624
PA4597	*oprJ*	5149633	5151072
PA4598	*mexD*	5151078	5154209
PA4599	*mexC*	5154237	5155400
PA4600	*nfxB*	5155561	5156124
PA4661	*pagL*	5229459	5229980
PA4671	*rplY*	5239466	5240080
PA4700	PBP1b	5277968	5280292
PA4748	*tpiA*	5332746	5333501
PA4751	*ftsH*	5335782	5337701
PA4773	-	5361586	5362068
PA4774	-	5362146	5363195
PA4775	-	5363198	5364058
PA4776	*pmrA*	5364071	5364736
PA4777	*pmrB*	5364760	5366193
PA4878	*brlR*	5473766	5474578
PA4944	*hfq*	5548397	5548645
PA4964	*parC*	5572222	5574486
PA4967	*parE*	5576028	5577917
PA5000	*wapR*	5617534	5618418
PA5038	*aroB*	5674028	5675134
PA5045	PBP1a	5680898	5683366
PA5117	*typA*	5762659	5764476
PA5199	*amgS*	5851239	5852558
PA5200	*amgR*	5852653	5853396
PA5235	*glpT*	5892910	5894256
PA5297	*poxB*	5964859	5966577
PA5332	*crc*	6002121	6002900
PA5366	*pstB*	6033211	6034044
PA5471	*armZ*	6159560	6160699
PA5471.1	-	6160912	6160953
PA5485	*ampDh2*	6176516	6177295
PA5528	-	6219885	6220739
PA5542	-	6234500	6235744

Table A5.2. Set of genes known to be related with hypermutation in *Pseudomonas aeruginosa* PAO1 reference strain.

LOCUS	GENE	START BP	END BP
PA0355	*pfpI*	399493	400032
PA0357	*mutY*	401131	401943
PA0750	*ung*	818003	818698
PA1816	*dnaQ*	1973470	1974210
PA3002	*mfd*	3360875	3364321
PA3620	*mutS*	4054525	4057092
PA4366	*sodB*	4893697	4894278
PA4400	*mutT*	4930748	4931695
PA4468	*sodM*	4997439	4998050
PA4609	*radA*	5167284	5168645
PA4946	*mutL*	5549780	5551681
PA5147	*mutM*	5795954	5797021
PA5344	*oxyR*	6012047	6012979
PA5443	*uvrD*	6131088	6133274
PA5493	*polA*	6183784	6186525

13. ANNEX 6

MAIN MUTATIONS RELATED WITH ANTIBIOTIC RESISTANCE ENCOUNTERED IN THE CLONAL COMPLEX 274 COLLECTION

Table A6.1. Betalactams. MIC values for betalactams and main mutations related with betalactam resistance encountered in the CC274 collection.

Isolate ID[a]	ST	MIC values					Hyperexpression?	AmpC and its regulators[b]			
		TZ	PM	AT	PPT	TOL/TAZ		*ampR*	*ampC*	*ampD*	PBP4
AUS034*	274	>256	>256	>256	>256	16	+		T21A, T105A, G391A	R11L, G148A, D183Y	W350R, A394P
AUS411	274	>256	>256	>256	>256	6	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
AUS601*	1043	>256	>256	>256	1	3	-		T21A, T105A, G391A, V239A	R11L, G148A, D183Y	
PAMB148	274	>256	64	>256	>256	6	+		T21A, T105A, G391A	R11L, P41L, G148A, D183Y	A394P
FQSE15-1110*	1089	8	24	6	4	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
AUS690*	274	6	12	0.75	3	6	-	T275A	T21A, T105A, G391A	R11L, G148A, D183Y	A394P
AUS603	274	6	8	24	2	1.5	+		T21A, T105A, G391A	R11L, G148A, D183Y	S315G
AUS410	274	4	24	1	12	4	-		T21A, T105A, G391A	R11L, G148A, D183Y	
FQSE06-0610	274	4	24	0.75	8	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE06-0807	274	4	8	0.75	4	2	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQRC26	274	4	6	24	24	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE10-0111	274	3	16	12	12	8	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE10-0110	274	3	8	16	8	2	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE03	274	3	8	0.5	2	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
AUS531	274	3	3	4	12	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE24-0304*	1089	2	24	0.38	8	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE15-0803	274	2	12	0.38	4	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
AUS588	274	2	8	3	8	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	
FQRC10	274	2	2	4	12	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE10-0503	274	1.5	12	4	4	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE24-1005*	1089	1	16	0.38	2	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE24-1010*	1089	1	8	1	1	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE24-0308*	1089	1	8	0.25	0.75	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE15-0310	274	1	4	1	1	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQRC15	274	1	0.75	6	6	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE15-0906	274	0.75	6	0.38	2	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE10-0106	274	0.75	3	0.125	0.75	0.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE06-0403	274	0.75	2	0.25	4	0.38	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE06-1104*	274	0.38	1	0.094	0.38	0.38	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P

[a]Isolates have been ordered according to their MIC values and following the subsequent order: TZ, PM, AT, PPT and TOL/TAZ.

[b] No mutations were encountered in AmpDh2 and AmpDh3.)

Table A6.1. Betalactams. MIC values for betalactams and main mutations related with betalactam resistance encountered in the CC274 collection. (Cont.)

solate ID[a]	Other penicillin-binding proteins[b]					Hyperexpression?	MexAB-OprM and its regulators[c]					
	PBP1A	PBP1B	PBP3	PBP3A	PBP6/7		*mexA*	*mexB*	*oprM*	*mexR*	*nalC*	*nalD*
AUS034*		S25G	G63S, P527T	A104P	S250N	-	M1*	F178S, M555I		R85H	G71E, S209R	
AUS411		S25G	Q372P	A104P	S250N	-		Q104E, F246C, L376V			G71E, S209R	
AUS601*		S25G	R504C	A104P	S250N	-		M552T			G71E, S209R	
PAMB148		S25G		A104P	S250N	-					G71E, S209R	
FQSE15-1110*	E161G	S25G		A104P	S250N	-	N71S, D235G	L376V	E456G		G71E, S209R	
AUS690*		S25G		A104P	S250N	+		$Nt_{712}\Delta1$		H133P	G71E, S209R	
AUS603		S25G		A104P	S250N	-		M552T	Q93*		G71E, S209R	
AUS410		S25G	G216S	A104P	S250N	-		M552T			G71E, S209R	
FQSE06-0610		S25G		A104P	S250N	-	L338P				G71E, S209R	
FQSE06-0807		S25G	P215L	A104P	S250N	-	L338P				G71E, S209R	
FQRC26		S25G		A104P	S250N	+					G71E, S209R	$Nt_{459}\Delta13$
FQSE10-0111		S25G		A104P	S250N	-					G71E, S209R	
FQSE10-0110		S25G		A104P	S250N	+					G71E, S209R	$Nt_{396}\Delta2$
FQSE03		S25G		A104P	S250N	-	L338P				G71E, S209R	
AUS531		S25G		A104P	S250N	-					G71E, S209R	
FQSE24-0304*	E161G	S25G		A95V, A104P	S250N	-	L338P		E456G		G71E, S209R	
FQSE15-0803		S25G		A104P	S250N	-	L338P				G71E, S209R	
AUS588		S25G		A104P	S250N	-					G71E, S209R	
FQRC10		S25G		A104P	S250N	-					G71E, S209R	
FQSE10-0503		S25G		A104P	S250N	-					G71E, S209R	
FQSE24-1005*	E161G, R407S	S25G		A104P	S250N	-			E456G		G71E, S209R	
FQSE24-1010*	E161G	S25G	G216S	A104P	S250N	-	L338P		E456G		G71E, S209R	
FQSE24-0308*	E161G	S25G		A104P	S250N	-			E456G		G71E, S209R	
FQSE15-0310		S25G		A104P	S250N	-	L338P				G71E, S209R	
FQRC15		S25G		A104P	S250N	-					G71E, S209R	
FQSE15-0906		S25G		A104P	S250N	-	L338P				G71E, S209R	
FQSE10-0106		S25G		A104P	S250N	-		L738P			G71E, S209R	
FQSE06-0403		S25G	P215L	A104P	S250N	-	L338P				G71E, S209R	
FQSE06-1104*	E161G	S25G		A104P	S250N	-	L338P				G71E, S209R	

[a]Isolates have been ordered according to their MIC values and following the subsequent order:TZ, PM, AT,PPT and TOL/TAZ.

[b]No mutations were encountered in PBP2 and PBP5.

[c]No mutations were encountered in *armR*.

Table A6.1. Betalactams. MIC values for betalactams and main mutations related with betalactam resistance encountered in the CC274 collection. (Cont.)

Isolate ID[a]	Hyperexpression?	MexXY-OprM and its regulators												
		mexY	*mexX*	*oprM*	*fmt*	*mexZ*	*folD*	*parS*	*parR*	*htpX*	*amgS*	*amgR*	*armZ*	PA5528
AUS034*	+	T543A, Q840E,V1000L	K329Q, L331V, D346H, W358R		I181V	$Nt_{334}\Delta13$		A82T, H398R	M59I				L88P, D119E	
AUS411	+	D201A, G287A, T543A, Q840E,	E287D, K329Q, L331V, D346H, W358R		I181V			A82T, T163N, D381E, H398R		$Nt_{683}\Delta5$			L88P, D119E	
AUS601*	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R				E204D	L88P, D119E	
PAMB148	-	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE15-1110*	+	Y355H, T543A, Q840E,	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R		A141T	D267N	A8V	L88P, D119E	
AUS690*	+	G402S, T543A, Q840E, A850T	K329Q, L331V, D346H, W358R		I181V	$Nt_{529}\Delta1$		L10P, A82T, H398R		G187D	R188C		L88P, D119E	
AUS603	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R	Q93*	I181V	Q164*		A82T, H398R					L88P, D119E	
AUS410	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R			S64L		L88P, D119E	$Nt_{208}\Delta7$
FQSE06-0610	+	T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	A194P		A82T, H398R				A8V	L88P, D119E	
FQSE06-0807	+	G287A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
FQRC26	-	T543A, Q840E, V875M	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE10-0111	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE10-0110	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE03	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R					L88P, D119E	
AUS531	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE24-0304*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE15-0803	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
AUS588	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQRC10	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE10-0503	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE24-1005*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			T92A	A8V	L88P, D119E	
FQSE24-1010*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			A13V	A8V	L88P, D119E	
FQSE24-0308*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE15-0310	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQRC15	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE15-0906	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQSE10-0106	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE06-0403	+	G287A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
FQSE06-1104*	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	$Nt_{290}\Delta11$		A82T, H398R				A8V	L88P, D119E	

[a]Isolates have been ordered according to their MIC values and following the subsequent order:TZ, PM, AT,PPT and TOL/TAZ.

Table A6.2. Carbapenems. MIC values for carbapenems and main mutations related with carbapenem resistance encountered in the CC274 collection.

Isolate ID[a]	ST	MIC values		OprD	Hyperexpression?	AmpC and its regulators[b]			Other penicillin-binding proteins[c]					
		IP	MP	*oprD*		*ampR*	*ampC*	*ampD*	PBP4	PBP1A	PBP1B	PBP3	PBP3A	PBP6/7
AUS034*	274	>32	>32	E264*	+		T21A, T105A, G391A	R11L, G148A, D183Y	W350R, A394P		S25G	P527T, G63S	A104P	S250N
AUS410	274	>32	>32	$Nt_{583}\Delta1$	-		T21A, T105A, G391A	R11L, G148A, D183Y			S25G	G216S	A104P	S250N
AUS411	274	>32	>32		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G	Q372P	A104P	S250N
AUS601*	1043	>32	>32	Nt_{1044}ins4	-		T21A, T105A, V239A, G391A	R11L, G148A, D183Y			S25G	R504C	A104P	S250N
FQSE15-1110*	1089	>32	>32	V67*	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G	S25G		A104P	S250N
FQSE24-0304*	1089	>32	>32	V67*	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G	S25G		A95V, A104P	S250N
AUS603	274	>32	8		+		T21A, T105A, G391A	R11L, G148A, D183Y	S315G		S25G		A104P	S250N
FQSE24-0308*	1089	>32	8	V67*	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G	S25G		A104P	S250N
FQSE24-1010*	1089	>32	4	V67*	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G	S25G	G216S	A104P	S250N
FQSE24-1005*	1089	>32	0.25	V67*	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G, R407S	S25G		A104P	S250N
FQSE15-0803	274	12	0.19		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQSE15-0906	274	6	1		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQSE06-0610	274	6	0.19		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
AUS690*	274	4	2		-	T275A	T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
AUS531	274	2	0.75		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
FQSE03	274	2	0.38		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQRC10	274	1.5	1		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQRC15	274	1.5	1		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQSE10-0110	274	1.5	1		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
FQSE06-0807	274	1.5	0.75		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G	P215L	A104P	S250N
PAMB148	274	1.5	0.75		+		T21A, T105A, G391A	R11L, P41L, G148A, D183Y	A394P		S25G		A104P	S250N
AUS588	274	1	0.75		-		T21A, T105A, G391A	R11L, G148A, D183Y			S25G		A104P	S250N
FQSE06-0403	274	1	0.5		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G	P215L	A104P	S250N
FQSE06-1104*	274	1	0.25		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G	S25G		A104P	S250N
FQSE10-0111	274	1	0.25		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
FQSE10-0106	274	1	0.125		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
FQSE15-0310	274	1	0.047		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQSE10-0503	274	0.38	0.032		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
FQRC26	274	0.25	1.5		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N

[a]Isolates have been ordered according to their MIC values and following the subsequent order: IP and MP

[b] No mutations were encountered in AmpDh2 and AmpDh3.

[c] No mutations were encountered in PBP2 and PBP5.

Table A6.2. Carbapenems. MIC values for carbapenems and main mutations related with carbapenem resistance encountered in the CC274 collection. (Cont.)

Isolate ID[a]	Hyperexpression?	MexEF-OprN and its regulators						
		mexE	*mexF*	*oprN*	*mexS*	*mexT*	*parS*	*parR*
AUS034*	-				D249N, P254Q	L157M, F172I	A82T, H398R	M59I
AUS410	-	F7Y			D249N	F172I, D327Y	A82T, H398R	
AUS411	-	V104G			D249N	F172I	A82T, T163N, D381E, H398R	
AUS601*	-	F7Y			D249N	F172I, D327Y	A82T, H398R	
FQSE15-1110*	-		Nt_{1051}ins9	R127Q	D249N	F172I	A82T, H398R	
FQSE24-0304*	-		Nt_{1051}ins9		D249N	F172I	A82T, H398R	
AUS603	-	F7Y			D249N	F172I, D327Y	A82T, H398R	
FQSE24-0308*	-		Nt_{1051}ins9		D249N	F172I	A82T, H398R	
FQSE24-1010*	-		Nt_{1051}ins9		D249N	F172I	A82T, H398R	
FQSE24-1005*	-		Nt_{1051}ins9		D249N	F172I	A82T, H398R	
FQSE15-0803	-			Nt_{415}ins2	Nt848Δ2, D249N	Nt534Δ17, F172I	A82T, H398R	
FQSE15-0906	-				D249N	F172I	A82T, H398R	
FQSE06-0610	-		Nt_{1051}ins9		D249N	F172I	A82T, H398R	
AUS690*	-				D249N	F172I	L10P, A82T, H398R	
AUS531	-				D249N	F172I	A82T, H398R	
FQSE03	-				D249N	F172I	A82T, H398R	
FQRC10	-				D249N	F172I	A82T, H398R	
FQRC15	-				D249N	F172I	A82T, H398R	
FQSE10-0110	-				D249N	F172I	A82T, H398R	
FQSE06-0807	-				D249N	F172I, P270Q	A82T, H398R	
PAMB148	-				D249N	F172I	A82T, H398R	
AUS588	-	F7Y, V276M			D249N	F172I	A82T, H398R	
FQSE06-0403	-				D249N	F172I	A82T, H398R	
FQSE06-1104*	-				D249N	F172I	A82T, H398R	
FQSE10-0111	-				D249N	F172I	A82T, H398R	
FQSE10-0106	-				D249N	F172I	A82T, H398R	
FQSE15-0310	-				Nt848Δ2, D249N	Nt534Δ17, F172I	A82T, H398R	
FQSE10-0503	-				D249N	F172I	A82T, H398R	
FQRC26	+				D249N	R164H, F172I	A82T, H398R	

[a]Isolates have been ordered according to their MIC values and following the subsequent order: IP and MP

[b] No mutations were encountered in *mvaT*.

Table A6.2. Carbapenems. MIC values for carbapenems and main mutations related with carbapenem resistance encountered in the CC274 collection. (Cont.)

Isolate ID[a]	Hyperexpression?	MexAB-OprM and its regulators[b]					
		mexA	*mexB*	*oprM*	*mexR*	*nalC*	*nalD*
AUS034*	-	M1*	F178S, M555I		R85H	G71E, S209R	
AUS410	-		M552T			G71E, S209R	
AUS411	-		Q104E, F246C, L376V			G71E, S209R	
AUS601*	-		M552T			G71E, S209R	
FQSE15-1110*	-	N71S, D235G	L376V	E456G		G71E, S209R	
FQSE24-0304*	-	L338P		E456G		G71E, S209R	
AUS603	-		M552T	Q93*		G71E, S209R	
FQSE24-0308*	-			E456G		G71E, S209R	
FQSE24-1010*	-	L338P		E456G		G71E, S209R	
FQSE24-1005*	-			E456G		G71E, S209R	
FQSE15-0803	-	L338P				G71E, S209R	
FQSE15-0906	-	L338P				G71E, S209R	
FQSE06-0610	-	L338P				G71E, S209R	
AUS690*	+		$Nt_{712}\Delta1$		H133P	G71E, S209R	
AUS531	-					G71E, S209R	
FQSE03	-	L338P				G71E, S209R	
FQRC10	-					G71E, S209R	
FQRC15	-					G71E, S209R	
FQSE10-0110	+					G71E, S209R	$Nt_{396}\Delta2$
FQSE06-0807	-	L338P				G71E, S209R	
PAMB148	-					G71E, S209R	
AUS588	-					G71E, S209R	
FQSE06-0403	-	L338P				G71E, S209R	
FQSE06-1104*	-	L338P				G71E, S209R	
FQSE10-0111	-					G71E, S209R	
FQSE10-0106	-		L738P			G71E, S209R	
FQSE15-0310	-	L338P				G71E, S209R	
FQSE10-0503	-					G71E, S209R	
FQRC26	+					G71E, S209R	$Nt_{459}\Delta13$

[a]Isolates have been ordered according to their MIC values and following the subsequent order: IP and MP

[b] No mutations were encountered in *armR*.

Table A6.2. Carbapenems. MIC values for carbapenems and main mutations related with carbapenem resistance encountered in the CC274 collection. (Cont.)

Isolate ID[a]	Hyperexpression?	MexXY-OprM and its regulators												
		mexY	*mexX*	*oprM*	*fmt*	*mexZ*	*folD*	*parS*	*parR*	*htpX*	*amgS*	*amgR*	*armZ*	PA5528
AUS034*	+	T543A, Q840E,V1000L	K329Q, L331V, D346H, W358R		I181V	$Nt_{334}\Delta13$		A82T, H398R	M59I				L88P, D119E	
AUS410	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R			S64L		L88P, D119E	$Nt_{208}\Delta7$
AUS411	+	T543A, Q840E,V1000L	K329Q, L331V, D346H, W358R		I181V	$Nt_{334}\Delta13$		A82T, T163N, D381E, H398R	M59I				L88P, D119E	
AUS601*	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R				E204D	L88P, D119E	
FQSE15-1110*	+	Y355H, T543A, Q840E,	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R		A141T	D267N	A8V	L88P, D119E	
FQSE24-0304*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
AUS603	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R	Q93*	I181V	Q164*		A82T, H398R					L88P, D119E	
FQSE24-0308*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE24-1010*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			A13V	A8V	L88P, D119E	
FQSE24-1005*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			T92A	A8V	L88P, D119E	
FQSE15-0803	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE15-0906	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQSE06-0610	+	T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	A194P		A82T, H398R				A8V	L88P, D119E	
AUS690*	+	G402S, T543A, Q840E, A850T	K329Q, L331V, D346H, W358R		I181V	$Nt_{529}\Delta1$		L10P, A82T, H398R		G187D	R188C		L88P, D119E	
AUS531	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE03	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R					L88P, D119E	
FQRC10	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQRC15	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE10-0110	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE06-0807	+	G287A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
PAMB148	-	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
AUS588	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE06-0403	+	G287A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
FQSE06-1104*	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	$Nt_{290}\Delta11$		A82T, H398R				A8V	L88P, D119E	
FQSE10-0111	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE10-0106	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE15-0310	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQSE10-0503	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQRC26	-	T543A, Q840E, V875M	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	

[a]Isolates have been ordered according to their MIC values and following the subsequent order: IP and MP

Table A6.3. Aminoglycosides. MIC values for aminoglycosides and main mutations related with aminoglycoside resistance encountered in the CC274 collection.

Isolate ID[a]	ST	MIC values		Hyperexpression?	MexXY-OprM and its regulators												
		TM	AK		*mexY*	*mexX*	*oprM*	*fmt*	*mexZ*	*folD*	*parS*	*parR*	*htpX*	*amgS*	*amgR*	*armZ*	PA5528
AUS411	274	>256	>256	+	T543A, Q840E,V1000L	K329Q, L331V, D346H, W358R		I181V	$Nt_{334}\Delta13$		A82T, T163N, D381E, H398R	M59I				L88P, D119E	
AUS410	274	64	>256	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R			S64L		L88P, D119E	$Nt_{208}\Delta7$
AUS601*	1043	24	>256	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R				E204D	L88P, D119E	
AUS690*	274	24	>256	+	G402S, T543A, Q840E, A850T	K329Q, L331V, D346H, W358R		I181V	$Nt_{529}\Delta1$		L10P, A82T, H398R		G187D	R188C		L88P, D119E	
FQSE06-0807	274	24	>256	+	G287A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
FQSE06-0403	274	24	16	+	G287A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
AUS034*	274	6	>256	+	T543A, Q840E,V1000L	K329Q, L331V, D346H, W358R		I181V	$Nt_{334}\Delta13$		A82T, H398R	M59I				L88P, D119E	
FQSE24-1010*	1089	4	64	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			A13V	A8V	L88P, D119E	
FQSE24-0308*	1089	3	16	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE24-0304*	1089	2	24	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE24-1005*	1089	2	16	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			T92A	A8V	L88P, D119E	
FQSE06-1104*	274	1.5	24	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	$Nt_{290}\Delta11$		A82T, H398R				A8V	L88P, D119E	
PAMB148	274	1.5	16	-	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE15-0310	274	1.5	12	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQSE06-0610	274	1	24	+	T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	A194P		A82T, H398R				A8V	L88P, D119E	
FQSE15-1110*	1089	1	16	+	Y355H, T543A, Q840E,	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R		A141T	D267N	A8V	L88P, D119E	
FQSE10-0110	274	1	12	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE15-0906	274	1	12	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
AUS588	274	1	8	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
AUS603	274	1	8	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R	Q93*	I181V	Q164*		A82T, H398R					L88P, D119E	
FQRC10	274	1	8	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE15-0803	274	1	8	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
AUS531	274	1	6	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQRC26	274	1	6	-	T543A, Q840E, V875M	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE03	274	1	6	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R					L88P, D119E	
FQRC15	274	0.75	8	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE10-0111	274	0.75	8	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE10-0503	274	0.75	4	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE10-0106	274	0.75	4	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	

[a]Isolates have been ordered according to their MIC values and following the subsequent order: TM and AK.

Table A6.3. Aminoglycosides. MIC values for aminoglycosides and main mutations related with aminoglycoside resistance encountered in the CC274 collection. (Cont.)

Isolate ID[a]	Mutationes encountered in other genes related with aminoglycoside resistance						
	galU	*fusA2*	*nuoG*	*capD*	*rplB*	*fusA1*	*rplY*
AUS411		I640L, G695A	T484A	I7M	G138A	K504E	
AUS410		G695A	T484A	I7M, S51G		P618L	
AUS601*		S445*, G695A	T484A	S51G	I144V	P618L	Q81H
AUS690*		L104P, $Nt_{889}\Delta1$, G695A	T484A	I7M, S51G		Y552C, T671I	
FQSE06-0807		G695A	T484A	I7M, S51G		N482S, Y552C, T671I	
FQSE06-0403		G695A	T484A	I7M, S51G		Y552C, T671I	
AUS034*		P329L, G695A	T484A	I7M, S51G		V93A, P554L, D588G	
FQSE24-1010*	P123L	N236S, N561S, G695A	T484A	I7M, S51G, A165V		K430E	
FQSE24-0308*	P123L	N236S, N561S, G695A	T484A			K430E	
FQSE24-0304*	P123L	N236S, N561S, G695A	T484A	I7M, S51G		K430E	
FQSE24-1005*	P123L	N236S, N561S, G695A	T484A	I7M, S51G		K430E	
FQSE06-1104*		N236S, N561S, G695A	T484A	I7M, S51G			
PAMB148		G695A	V360A, T484A	I7M, S51G			
FQSE15-0310		G695A	T484A	I7M, S51G			
FQSE06-0610		G695A	T484A	I7M, S51G			
FQSE15-1110*	P123L	N236S, N561S, G695A	T484A, A638T	I7M, S51G		K430E	
FQSE10-0110		G695A	T484A	S51G			
FQSE15-0906		G695A	T484A	I7M, S51G			
AUS588		G695A	T484A	I7M			
AUS603		G695A	T484A	I7M, nt1438Δ1		P618L	
FQRC10		G695A	T484A	I7M, S51G			
FQSE15-0803		G695A	T484A	I7M, S51G			
AUS531		G695A	T484A	I7M, S51G			
FQRC26		G695A	T484A	I7M, S51G			
FQSE03		G695A	T484A	I7M, S51G			
FQRC15		G695A	T484A	I7M			
FQSE10-0111		G695A	T484A				
FQSE10-0503		G695A	T484A	S51G			
FQSE10-0106		G695A	T484A	I7M, S51G			

[a]Isolates have been ordered according to their MIC values and following the subsequent order: TM and AK.

Table A6.4. Quinolones. MIC values for ciprofloxacin and main mutations related with quinolone resistance encountered in the CC274 collection.

Isolate ID	ST	MIC	QRDRs				Hyperexpression?	MexCD-OprJ and its regulators				
		CI	*gyrB*	*gyrA*	*parC*	*parE*		*mexD*	*mexC*	*oprJ*	*nfxB*	*vqsM*
AUS601*	1043	16	H148N	T83I			-	E257Q, A536S, S845A		D68G, M69V		not present
AUS690*	274	12	H148N, Q467R	T83A		P438S	-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE24-0304*	1089	6	H148N, H148NS466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE24-1005*	1089	6	H148N, S466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE24-0308*	1089	4	H148N, S466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE24-1010*	1089	4	H148N, S466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE03	274	3	H148N	D87N			-	E257Q, A536S, S845A		D68G, M69V		not present
AUS034*	274	1.5	H148N, R441L	T83I			-	L24M, E257Q, A536S, S845A		D68G, M69V		not present
FQRC26	274	1.5	H148N	Q106L			-	E257Q, A536S, S845A		D68G, M69V		not present
AUS410	274	1	H148N, S466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE15-1110*	1089	1	H148N, S466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE06-1104*	274	0.75	H148N	D87G			-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE06-0610	274	0.75	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE10-0110	274	0.75	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE06-0807	274	0.5	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
AUS411	274	0.38	H148N, S466F				-	E257Q, A536S, S845A	E59D	D68G, M69V		not present
FQSE10-0106	274	0.38	H148N				+	E257Q, A536S, S845A		D68G, M69V	*188ext	not present
FQSE10-0111	274	0.38	H148N				-	E257Q, A536S, S845A	F141L	D68G, M69V		not present
FQSE15-0906	274	0.38	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE15-0310	274	0.38	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
AUS603	274	0.25	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE10-0503	274	0.25	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQRC15	274	0.19	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE06-0403	274	0.19	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE15-0803	274	0.19	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
AUS531	274	0.125	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
AUS588	274	0.125	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQRC10	274	0.094	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
PAMB148	274	0.064	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present

[a] Isolates have been ordered according to their MIC value for CI.

Table A6.4. Quinolones. MIC values for ciprofloxacin and main mutations related with quinolone resistance encountered in the CC274 collection.(Cont.)

Isolate ID[a]	Hyperexpression?	MexEF-OprN and its regulators[b]							Hyperexpression?	MexAB-OprM and its regulators[c]					
		mexE	*mexF*	*oprN*	*mexS*	*mexT*	*parS*	*parR*		*mexA*	*mexB*	*oprM*	*mexR*	*nalC*	*nalD*
AUS601*		F7Y			D249N	F172I, D327Y	A82T, H398R				M552T			G71E, S209R	
AUS690*	-				D249N	F172I	L10P, A82T, H398R		-		$Nt_{712}\Delta1$		H133P	G71E, S209R	
FQSE24-0304*	-		Nt_{1051}ins9		D249N	F172I	A82T, H398R		+	L338P		E456G		G71E, S209R	
FQSE24-1005*	-		Nt_{1051}ins9		D249N	F172I	A82T, H398R		-			E456G		G71E, S209R	
FQSE24-0308*	-		Nt_{1051}ins9		D249N	F172I	A82T, H398R		-			E456G		G71E, S209R	
FQSE24-1010*	-		Nt_{1051}ins9		D249N	F172I	A82T, H398R		-	L338P		E456G		G71E, S209R	
FQSE03	-				D249N	F172I	A82T, H398R		-	L338P				G71E, S209R	
AUS034*	-				D249N, P254Q	L157M, F172I	A82T, H398R	M59I	-	M1*	F178S, M555I		R85H	G71E, S209R	
FQRC26	+				D249N	R164H, F172I	A82T, H398R		+					G71E, S209R	$Nt_{459}\Delta13$
AUS410	-	F7Y			D249N	F172I, D327Y	A82T, H398R		-		M552T			G71E, S209R	
FQSE15-1110*	-		Nt_{1051}ins9	R127Q	D249N	F172I	A82T, H398R	M59I	-	N71S, D235G	L376V	E456G		G71E, S209R	
FQSE06-1104*	-				D249N	F172I	A82T, H398R		-	L338P				G71E, S209R	
FQSE06-0610	-		Nt_{1051}ins9		D249N	F172I	A82T, H398R		-	L338P				G71E, S209R	
FQSE10-0110	-				D249N	F172I	A82T, H398R		+					G71E, S209R	$Nt_{396}\Delta2$
FQSE06-0807	-				D249N	F172I, P270Q	A82T, H398R		-	L338P				G71E, S209R	
AUS411	-	V104G			D249N	F172I	A82T, T163N, D381E, H398R		-		Q104E, F246C, L376V			G71E, S209R	
FQSE10-0106	-				D249N	F172I	A82T, H398R		-		L738P			G71E, S209R	
FQSE10-0111	-				D249N	F172I	A82T, H398R		-					G71E, S209R	
FQSE15-0906	-				D249N	F172I	A82T, H398R		-	L338P				G71E, S209R	
FQSE15-0310	-				Nt848Δ2, D249N	Nt534Δ17, F172I	A82T, H398R		-	L338P				G71E, S209R	
AUS603	-	F7Y			D249N	F172I, D327Y	A82T, H398R		-		M552T	Q93*		G71E, S209R	
FQSE10-0503	-				D249N	F172I	A82T, H398R		-					G71E, S209R	
FQRC15	-				D249N	F172I	A82T, H398R		-					G71E, S209R	
FQSE06-0403	-				D249N	F172I	A82T, H398R		-	L338P				G71E, S209R	
FQSE15-0803	-			Nt_{415}ins2	Nt848Δ2, D249N	Nt534Δ17, F172I	A82T, H398R		-	L338P				G71E, S209R	
AUS531	-				D249N	F172I	A82T, H398R		-					G71E, S209R	
AUS588	-	F7Y, V276M			D249N	F172I	A82T, H398R		-					G71E, S209R	
FQRC10	-				D249N	F172I	A82T, H398R		-					G71E, S209R	
PAMB148	-				D249N	F172I	A82T, H398R		-		M552T			G71E, S209R	

[a] Isolates have been ordered according to their MIC value for CI.

[b] No mutations were encountered in *mvaT,* [c] No mutations were encountered in armR.

Table A6.4. Quinolones. MIC values for ciprofloxacin and main mutations related with quinolone resistance encountered in the CC274 collection.(Cont.)

Isolate ID	Hyperexpression?	MexXY-OprM and its regulators												
		mexY	*mexX*	*oprM*	*fmt*	*mexZ*	*folD*	*parS*	*parR*	*htpX*	*amgS*	*amgR*	*armZ*	PA5528
AUS601*	+	V32A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R				E204D	L88P, D119E	
AUS690*	+	G402S, T543A, Q840E, A850T	K329Q, L331V, D346H, W358R		I181V	$Nt_{529}\Delta1$		L10P, A82T, H398R		G187D	R188C		L88P, D119E	
FQSE24-0304*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE24-1005*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			T92A	A8V	L88P, D119E	
FQSE24-0308*	+	Y355H, V	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE24-1010*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			A13V	A8V	L88P, D119E	
FQSE03	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R					L88P, D119E	
AUS034*	+	T543A, Q840E, V1000L	K329Q, L331V, D346H, W358R		I181V	$Nt_{334}\Delta13$		A82T, H398R	M59I				L88P, D119E	
FQRC26	-	T543A, Q840E, V875M	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
AUS410	+	V32A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R			S64L		L88P, D119E	$Nt_{208}\Delta7$
FQSE15-1110*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R		A141T	D267N	A8V	L88P, D119E	
FQSE06-1104*	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	$Nt_{290}\Delta11$		A82T, H398R				A8V	L88P, D119E	
FQSE06-0610	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A194P		A82T, H398R				A8V	L88P, D119E	
FQSE10-0110	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE06-0807	+	G287A , T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
AUS411	+	D201A, G287A, T543A, Q840E	E287D, K329Q, L331V, D346H, W358R		I181V			A82T, T163N, D381E, H398R		$Nt_{683}\Delta5$			L88P, D119E	
FQSE10-0106	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE10-0111	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE15-0906	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQSE15-0310	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
AUS603	+	V32A, T543A, Q840E	K329Q, L331V, D346H, W358R	Q93*	I181V	Q164*		A82T, H398R					L88P, D119E	
FQSE10-0503	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQRC15	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE06-0403	+	G287A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
FQSE15-0803	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
AUS531	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
AUS588	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQRC10	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
PAMB148	-	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	

[a] Isolates have been ordered according to their MIC value for CI.

Table A6.5. Polymyxins. MIC values for colistin and main mutations related with polymyxin resistance encountered in the CC274 collection.

Isolate ID[a]	ST	MIC	Mutationes encountered in other genes related with polymyxins resistance															
		CO	*oprH*	*phoP*	*phoQ*	*parS*	*parR*	*cprR*	*cprS*	*colS*	*colR*	*lpxC*	*pagL*	PA4773	PA4774	PA4775	*pmrA*	*pmrB*
AUS034*	274	>256			E266*	A82T, H398R	M59I			H415Q						R131Q, A270V	L71R	Y345H
FQSE06-1104*	274	2				A82T, H398R									W274*	R131Q, A270V	L71R	V185I, G221D, R287Q, Y345H
AUS603	274	1.5				A82T, H398R			R56C							R131Q, A270V	L71R	Y345H
FQSE10-0106	274	1.5				A82T, H398R										R131Q, A270V	L71R	Y345H
AUS531	274	1				A82T, H398R										R131Q, A270V	L71R	Y345H
FQRC15	274	1				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE06-0807	274	1				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE24-1005*	1089	1				A82T, H398R				E303D						R131Q, A270V	L71R	R287Q, Y345H
FQSE24-0308*	1089	1				A82T, H398R										R131Q, A270V	L71R	R287Q, Y345H
AUS588	274	0.75				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE15-0906	274	0.75				A82T, H398R										R131Q, A270V	L71R	Y345H
FQRC10	274	0.5				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE10-0110	274	0.5				A82T, H398R										R131Q, A270V	L71R	Y345H
PAMB148	274	0.5				A82T, H398R										R131Q, A270V	L71R	Y345H
AUS410	274	0.38				A82T, H398R							Nt286Δ1			R131Q, A270V	L71R	Y345H
FQRC26	274	0.38				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE10-0111	274	0.38				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE24-0304*	1089	0.38				A82T, H398R										R131Q, A270V	L71R	R287Q, Y345H
FQSE24-1010*	1089	0.38				A82T, H398R										R131Q, A270V	L71R	R287Q, Y345H
AUS411	274	0.25			H248P	A82T, T163N, D381E, H398R							N159D			R131Q, A270V	L71R	Y345H
AUS601*	1043	0.25			K234N, T315A	A82T, H398R							E163G			R131Q, A270V	L71R	L31P, Y345H
FQSE03	274	0.25				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE10-0503	274	0.25				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE15-0803	274	0.25				A82T, H398R										R131Q, A270V	L71R	E213D, Y345H
FQSE15-0310	274	0.25				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE15-1110*	274	0.25				A82T, H398R										R131Q, A270V	L71R	R287Q, Y345H
FQSE06-0403	274	0.19				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE06-0610	274	0.19				A82T, H398R										R131Q, A270V	L71R	Y345H
AUS690*	274	0.125		T221I		L10P, A82T, H398R							P158L			R131Q, A270V	L71R	F124L, Y345H

[a] Isolates have been ordered according to their MIC value for CO.

14. ANNEX 7

EVOLUTIONARY DYNAMICS OF *Pseudomonas aeruginosa* AMINOGLYCOSIDE RESISTANCE DEVELOPMENT: EXPANDED RESULTS

Table A7.1. Antibiotic susceptibility profiles and mutations encountered in parental strain PAO1 and its derived aminoglycoside resistant mutants.

ISOLATE			MIC values (mg/L) and susceptibility profiles																									
			TM		GE		AK		TI		PPT		TZ		PM		AT		TOL/TAZ		IP		MP		CI		CO	
			MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI
PAO1			0.5	S	1	S	2	S	32	I	4	S	2	S	4	S	4	S	1	S	2	I	1	S	0.25	S	2	S
SERIE	DAY	COLONY																										
1	1	1	4	S	8	I	32	I	32	I	8	S	16	I	2	S	4	S	1	S	2	S	1	S	0.25	S	1	S
1	1	2	4	S	16	R	32	I	32	I	4	S	4	S	8	S	2	S	1	S	2	S	1	S	0.25	S	1	S
2	1	1	2	S	8	I	16	S	32	I	8	S	2	S	4	S	8	S	1	S	1	S	1	S	0.5	S	1	S
2	1	2	2	S	8	I	16	S	16	S	4	S	2	S	8	S	2	S	1	S	2	S	1	S	0.5	S	1	S
3	1	1	2	S	8	I	16	S	32	I	4	S	2	S	4	S	4	S	1	S	1	S	1	S	0.5	S	1	S
3	1	2	2	S	8	I	16	S	32	I	4	S	2	S	4	S	4	S	1	S	2	S	1	S	0.5	S	1	S
4	1	1	2	S	8	I	16	S	32	I	4	S	2	S	4	S	4	S	1	S	2	S	1	S	0.25	S	1	S
4	1	2	2	S	8	I	16	S	32	I	4	S	2	S	2	S	4	S	1	S	2	S	1	S	0.25	S	1	S
5	1	1	1	S	4	S	4	S	32	I	4	S	2	S	1	S	4	S	0.5	S	2	S	1	S	≤0.125	S	1	S
5	1	2	2	S	4	S	4	S	32	I	8	S	2	S	2	S	4	S	1	S	2	S	2	S	0.25	S	1	S
1	7	1	32	R	64	R	256	R	32	I	4	S	2	S	2	S	2	S	1	S	2	S	2	S	0.25	S	1	S
1	7	2	32	R	64	R	128	R	16	S	8	S	2	S	1	S	2	S	1	S	4	I	1	S	≤0.125	S	1	S
2	7	1	16	R	64	R	64	R	16	S	4	S	1	S	4	S	2	S	1	S	1	S	1	S	0.5	S	2	S
2	7	2	16	R	64	R	64	R	16	S	4	S	1	S	4	S	≤1	S	0.5	S	1	S	1	S	0.5	S	4	R
3	7	1	32	R	64	R	128	R	16	S	8	S	2	S	2	S	4	S	1	S	2	S	2	S	≤0.125	S	1	S
3	7	2	64	R	64	R	128	R	16	S	8	S	1	S	1	S	2	S	1	S	2	S	0,5	S	≤0.125	S	0.5	S
4	7	1	32	R	64	R	64	R	32	I	8	S	2	S	2	S	2	S	1	S	2	S	1	S	0.5	S	4	R
4	7	2	16	R	32	R	64	R	16	S	8	S	2	S	2	S	2	S	0.5	S	2	S	1	S	0.25	S	2	S
5	7	1	32	R	64	R	128	R	16	S	4	S	1	S	1	S	2	S	0.5	S	1	S	0,5	S	0.25	S	>4	R
5	7	2	32	R	64	R	128	R	32	I	4	S	1	S	2	S	2	S	0.5	S	1	S	0,5	S	0.25	S	2	S
1	14	1	512	R	1024	R	512	R	64	I	8	S	8	S	4	S	2	S	1	S	4	I	1	S	≤0.125	S	0.25	S
1	14	2	256	R	1024	R	512	R	16	S	8	S	1	S	8	S	2	S	1	S	2	S	0,5	S	≤0.125	S	0.5	S
2	14	1	64	R	256	R	512	R	8	S	4	S	1	S	2	S	≤1	S	1	S	1	S	≤0.25	S	≤0.125	S	0.5	S
2	14	2	64	R	256	R	512	R	8	S	≤2	S	2	S	4	S	2	S	1	S	4	I	1	S	≤0.125	S	0.25	S
3	14	1	512	R	1024	R	1024	R	16	S	8	S	4	S	4	S	2	S	1	S	2	S	1	S	≤0.125	S	0.25	S
3	14	2	512	R	1024	R	512	R	32	I	16	S	2	S	4	S	2	S	1	S	4	I	1	S	≤0.125	S	0.25	S
4	14	1	64	R	256	R	512	R	8	S	4	S	1	S	2	S	≤1	S	0.5	S	1	S	0,5	S	≤0.125	S	0.5	S
4	14	2	64	R	256	R	512	R	16	S	4	S	1	S	2	S	≤1	S	1	S	2	S	1	S	0.5	S	1	S
5	14	1	128	R	256	R	512	R	8	S	≤2	S	≤0.5	S	1	S	≤1	S	≤0.25	S	2	S	0,5	S	≤0.125	S	0.5	S
5	14	2	128	R	128	R	512	R	8	S	≤2	S	≤0.5	S	1	S	≤1	S	≤0.25	S	≤0.5	S	1	S	≤0.125	S	0.5	S

Table A7.1. Antibiotic susceptibility profiles and mutations encountered in parental strain PAO1 and its derived aminoglycoside resistant mutants. (Cont.)

ISOLATE			ENCOUNTERED MUTATIONS															
			PA0432	PA1023	PA1554	PA1555	PA1557	PA1766	PA1767	PA2009	PA2018	PA2631	PA2632	PA2633	PA2634	PA2635	PA2636	PA2637	PA2638
			sahH	-	*ccoN1*	*ccoP2*	*ccoN2*	-	-	*hmgA*	*mexY*	*yjcF*	-	-	*aceA*	-	-	*nuoA*	*nuoB*
PAO1			WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
SERIE	DAY	COLONY																	
1	1	1																	
1	1	2																	
2	1	1																	
2	1	2																	
3	1	1																	
3	1	2																	
4	1	1																	
4	1	2																	
5	1	1					A217T												
5	1	2	nt910ins6																
1	7	1				V14L													
1	7	2							nt951Δ1										
2	7	1																	
2	7	2																	
3	7	1																	
3	7	2						nt280ins6											
4	7	1										Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ
4	7	2										Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ
5	7	1																	
5	7	2																	
1	14	1																	
1	14	2		nt158Δ2															
2	14	1																	
2	14	2																	
3	14	1							nt961ins6										
3	14	2			nt580Δ1				nt961ins6										
4	14	1										Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ
4	14	2										Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ
5	14	1									T472P								
5	14	2								L188Q									

Table A7.1. Antibiotic susceptibility profiles and mutations encountered in parental strain PAO1 and its derived aminoglycoside resistant mutants. (Cont.)

ISOLATE			ENCOUNTERED MUTATIONS																
			PA2639	PA2800	PA2960	PA3064	PA3789	PA4029	PA4266	PA4454	PA4455	PA4526	PA4661	PA4727	PA4777	PA5040	PA5070	PA5105	PA5134	PA5300
			nuoD	*vacJ*	*pilZ*	*pelA*	-	-	*fusA1*	*yrbD*	*yrbE*	*pilB*	*pagL*	*pcnB*	*pmrB*	*pilQ*	*tatC*	*hutC*	*ctpA*	*ctpA*
PAO1			WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
SERIE	DAY	COLONY																		
1	1	1							I61M											
1	1	2							I61M											
2	1	1										N349K								
2	1	2										N349K								
3	1	1			nt19ins1															
3	1	2			nt19ins1															
4	1	1							T671A							nt209ins1				
4	1	2							T671A							nt209ins1				
5	1	1										G326D						Y181X		
5	1	2										G326D						Y181X		
1	7	1							I61M											
1	7	2							I61M											
2	7	1				A39S						N349K			S257N					G247S
2	7	2										N349K			S257N					G247S
3	7	1							I61M		nt450Δ1						nt511Δ12			
3	7	2							I61M								T216P			
4	7	1							T671A					nt827ins1	nt131Δ3	nt209ins1				
4	7	2							T671A						nt131Δ3	nt209ins1				
5	7	1							E100G			G326D			A247T			Y181X		
5	7	2							E100G			G326D			A247T			Y181X		
1	14	1					R339C		I61M	I86N										
1	14	2					R339C		I61M	G17D										
2	14	1							I61M			N349K			S257N					
2	14	2							I61M			N349K			S257N					
3	14	1							I61M		S66L									
3	14	2		Q64X					I61M											
4	14	1	nt34ins5						T671A				nt88Δ24		nt131Δ3	nt209ins1				
4	14	2						G49D	T671A						nt131Δ3	nt209ins1				
5	14	1							E100G			G326D			A247T			Y181X		
5	14	2							E100G			G326D			A247T			Y181X		

Table A7.2. MIC_{TOB} values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. Differences between paired isolates are highlighted in bold.

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
Sequence Type		274		701		1613	
MIC_{TOB} (mg/L)		1	24	2	>256	4	64
mexY overexpression		+	+	+	+	-	+
AMEs[a]					**aacA4**		
PA0004	*gyrB*	H148N	H148N		**R138L**		
PA0005	*lptA*						
PA0018	*fmt*	I181V	I181V	A308T, L287V, I196V, I181V	A308T, L287V, I196V, I181V	I181V	I181V
PA0058	*dsbM*			**C28R, F206L, R212C**			
PA0301	*spuE*					A301S	A301S
PA0355	*pfpI*			A58T, E57D	A58T, E57D		
PA0392	*yggT*						
PA0402	*pyrB*						
PA0424	*mexR*			V126E	V126E		
PA0425	*mexA*	L338P	L338P			nt472597Δ1	nt472597Δ1
PA0426	*mexB*			**nt772Δ1**			**Q575R**
PA0427	*oprM*						
PA0432	*sahH*					A286T	A286T
PA0463	*creB*	E128G	E128G	A130T	A130T	E128G, A130T	E128G, A130T
PA0464	*creC*	A397V	A397V	G157A	G157A	A397V	A397V
PA0465	*creD*	V394A	V394A	V335I, V394A, F445L, R451K	V335I, V394A, F445L, R451K	V448I	V448I
PA0486	*yihE*	D210E	D210E	D210E	D210E	D258E, D210E	D258E, D210E
PA0487	*modR*			G179E	G179E		
PA0610	*prtN*			S8T, S4T, M1*	S8T, S4T, M1*		
PA0611	*prtR*			T5P, S4N	T5P, S4N		
PA0612	*ptrB*						
PA0779	*asrA*			R778K	R778K		
PA0807	*ampDh3*			A208V	A208V	A219T	A219T
PA0869	PBP6/7	S250N	S250N				
PA0893	*argR*					P42S	P42S
PA0958[a]	*oprD*				**Q424E, S403A**		
PA1023	-	A33V, E209A, E276Q	A33V, E209A, E276Q	A208S	A208S	E209A, E276Q	E209A, E276Q
PA1178	*oprH*						
PA1179	*phoP*						
PA1180	*phoQ*					G370D	G370D

Table A7.2. MIC_{TOB} values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA1343	*pagP*			F129L	F129L		
PA1345	*gshB*	L17P, D65G, L463R	L17P, D65G, L463R	L17P, L463R, V495I	L17P, L463R, V495I	L463R	L463R
PA1375	*pdxB*	Q150R,179H, R247H,Q365R	Q150R, R179H, R247H, Q365R	H183Y, P192T	H183Y, P192T		
PA1409	*aphA*	L62I, R224L, R297K	L62I, R224L, R297K	L62I, I320V	L62I, I320V	L62I, R297K	L62I, R297K
PA1430	*lasR*	P117G	P117G		**R216Q**		
PA1554	*ccoN1*						
PA1555	*ccoP2*			V231I	V231I		
PA1557	*ccoN2*						
PA1588	*sucC*						
PA1589	*sucD*						
PA1766	-						
PA1767	-						
PA1777	*oprF*						
PA1796	*folD*					N171S	N171S
PA1797[a]	-						
PA1798	*parS*	A82T, H398R	A82T, H398R	H398R	H398R	H398R	H398R
PA1799	*parR*			S170N, L153R	S170N, L153R		
PA1801	*clpP*	P11S	P11S				
PA1803	*lon*			A499S	A499S		
PA1812	*mltD*						
PA1886	*polB*	D176G	D176G	D176G, R196C	D176G, R196C	D176G, Q664R	D176G, Q664R
PA2006	-			E232Q	E232Q	E220K	E220K
PA2009	hmgA			P303H, T226A	P303H, T226A		
PA2018	*mexY*	T543A, Q840E	**G287A**, T543A, Q840E	Q840E, N709H, G589A, A586T, T543A, I536V	Q840E, N709H, G589A, A586T, T543A, I536V	Q840E, T543A, E152D	Q840E, T543A, **G287S**, E152D
PA2019	*mexX*	W358R, K329Q, L331V, D346H	W358R, K329Q, L331V, D346H	S382G, W358R, L331V, K329Q, A38P, A30T	S382G, W358R, L331V, K329Q, A38P, A30T	W358R, R351S, K329Q	W358R, R351S, K329Q
PA2020	*mexZ*	**nt290Δ11**	**S9P**	L138R	L138R		**R125P**
PA2023	*galU*						
PA2050	-			C142G	C142G	C142G	C142G

Table A7.2. MIC_{TOB} values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA2071	*fusA2*	G695A	G695A	S176A, A197D, G695A	S176A, A197D, G695A	S176A, G695A	S176A, G695A
PA2227	*vqsM*	deleted	deleted				
PA2272	PBP3a	A104P	A104P	A104P	A104P	A104P	A104P
PA2273	*soxR*						
PA2489	-	R12L, A244T	R12L, A244T	G185S, A244T	G185S, A244T		
PA2490	*ydbB*	T48S, R104C	T48S, R104C	T48S, R104C	T48S, R104C	T48S, R104C	T48S, R104C
PA2491	*mexS*	D249N	D249N	D249N	D249N	D249N, D201G	D249N, D201G
PA2492	*mexT*	F172I	F172I	F172I	F172I	P60S, F172I	P60S, F172I, **G274D, G300D**
PA2493	*mexE*			S8F	S8F		
PA2494	*mexF*			D230A	D230A		
PA2495	*oprN*			S13P, R363H	S13P, R363H		
PA2522	*czcC*			R225K	R225K	T215A	T215A
PA2523	*czcR*						
PA2524	*czcS*	E44K	E44K	G282S, N353S, G470S	G282S, N353S, G470S	G282S, N353S	G282S, N353S
PA2525	*opmB*	V108A	V108A			S147G, N145S, V108A	S147G, N145S, V108A
PA2526	*muxC*						
PA2527	*muxB*	K646N	K646N			I880V	I880V
PA2528	*muxA*	T261A	T261A	T261A	T261A		
PA2615	*ftsK*	S52P, S287P, G729S	S52P, S287P, G729S	S287P, S52P	S287P, S52P	S52P, S287P, G729S	S52P, S287P, G729S
PA2621	*clpS*						
PA2631	*yjcF*						
PA2632	-	T4A, **P33L**, S127N	T4A, S127N	S74T	S74T		
PA2633	-	S222N, R249H	S222N, R249H		A109T, R249H		A109T, R249H
PA2634	*aceA*	S297A	S297A	S297A	S297A	S297A	S297A
PA2635	-			T410A	T410A		
PA2636	-	T40S, N125D	T40S, N125D	N125D, V178D	N125D, V178D	T180I	T180I
PA2637	*nuoA*						
PA2638	*nuoB*						
PA2639	*nuoD*		**G499X**				

Table A7.2. MIC_{TOB} values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA2642	*nuoG*	T484A	T484A	T484A, K585R	T484A, K585R	T484A	T484A
PA2649	*nuoN*					G326D	G326D
PA2797	-						
PA2798	-	G301S	G301S	T317S, G301S, E297D	T317S, G301S, E297D	G301S	G301S
PA2800	*vacJ*			S172G	S172G		
PA2809	*copR*						
PA2810	*copS*			M48L, R320H	M48L, R320H	E359G	E359G
PA2830	*htpX*						
PA2960	*pilZ*					P9S	P9S
PA3005	*nagZ*						
PA3013	*foaB*			I391V, E225Q	I391V, E225Q		
PA3014	*faoA*			V397I	V397I		
PA3047	*PBP4*	A358V, A394P	A358V, A394P				
PA3050	*pyrD*			R96K, K40E	R96K, K40E		
PA3064	*pelA*	**V446I**, A272T, H141Y, I19T	A272T, H141Y, I19T	V946A, V507A, I377V, T41A	V946A, V507A, I377V, T41A	R862H, V825L, A272T, H141Y, C25R, I19T	R862H, V825L, A272T, H141Y, C25R, I19T
PA3077	*cprR*						
PA3078	*cprS*			T16S, E386D	T16S, E386D		
PA3141	*capD*	I7M, S51G	I7M, S51G	A626V, S51G	A626V, S51G	S486L, **nt512ins1**	S486L
PA3168	*gyrA*				**Y267N**	D87N	D87N
PA3521	*opmE*	S175T, A279S, W354R	S175T, A279S, W354R	W354R, S175T	W354R, S175T	W354R, S175T, S46G	W354R, S175T, S46G
PA3522	*mexQ*	I294V, G505D, D602E, G645A, V921M	I294V, G505D, D602E, G645A, V921M	R656K, G505D, V384I, I294V	R656K, G505D, V384I, I294V	R1036H, P428S, V355M	R1036H, P428S, V355M
PA3523	*mexP*	A297E, R366L	A297E, R366L	A297E, R366L	A297E, R366L		
PA3533	*grxD*						
PA3574	*nalD*		A272T, H141Y, C25R, I19T				
PA3602	*yerD*					L21V	L21V
PA3676	*mexK*			K694R, I21L	K694R, I21L		
PA3677	*mexJ*			A314P	A314P		

Table A7.2. MIC_{TOB} values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA3678	*mexL*					S6P	S6P
PA3719	*armR*			S21T	S21T		
PA3721	*nalC*	G71E, S209R	G71E, S209R	G71E, A145V, S209R	G71E, A145V, S209R	S209R	S209R
PA3789	-	G439S, L195V, E191D, V71A	G439S, L195V, E191D, V71A	L205F, L195V, E191D, V71A	L205F, L195V, E191D, V71A		
PA3999	PBP5						
PA4001	*sltB1*						
PA4003	PBP2						
PA4020	*mpl*	M297V	**S257L,** M297V	S306N, M297V	S306N, M297V, **Q248***	M297V	M297V
PA4029	-			A218T	A218T		
PA4069	-	T234I	T234I				
PA4109	*ampR*			M288R, G283E	M288R, G283E		
PA4110	*ampC*	T21A, T105A, G391A	T21A, T105A, G391A	R79Q, T105A, V205L, V356I, G391A	R79Q, T105A, V205L, V356I, G391A	T105A	T105A
PA4119	*aph*	A42V	A42V				
PA4205	*mexG*						
PA4206	*mexH*			P218T, D302E	P218T, D302E	A123T	A123T
PA4207	*mexI*			A782E	A782E	A491T, A782E	A491T, A782E
PA4208	*opmD*			S112N, A270G	S112N, A270G	S112N, A243E	S112N, A243E
PA4218	*ampP*	M87I, T172A	M87I, T172A	L98F, M87I, L74F	L98F, M87I, L74F		
PA4238	*rpoA*						
PA4260	*rplB*						
PA4266	*fusA1*		**Y552C, T671I**			T456A, K187R	**Y552C,** T456A, K187R
PA4269	*rpoC*					R905C, N606S	R905C, N606S
PA4270	*rpoB*	V51I	V51I	V51I	V51I	G158D, V51I	G158D, V51I
PA4273	*rplA*						
PA4315	*mvaT*						
PA4374	*mexV*	A229G, Q321R	A229G, Q321R	A229G, Q321R	A229G, Q321R	Q321R	Q321R
PA4375	*mexW*	A627V, Q771P	A627V, Q771P	Q511R, G758S	Q511R, G758S	Q511R	Q511R
PA4380	*colS*						

Table A7.2. MIC_{TOB} values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA4381	*colR*					D32G	D32G
PA4393	*ampG*	V81I, A583T	V81I, A583T	A583T	A583T	A583T	A583T
PA4406	*lpxC*						
PA4418	PBP3		**P215L**			Q568R	Q568R
PA4444	*mltB1*	H64R, P106Q	H64R, P106Q	A29T, H64R, T139A	A29T, H64R, T139A	H64R, T139A, V147I, S267N	H64R, T139A, V147I, S267N
PA4454	yrbD						
PA4455	yrbE						
PA4462	*rpoN*					W305R	W305R, **V473A**
PA4521	*ampE*	S33G, S69P	S33G, S69P	L190F, E129D, S69P, S33G	L190F, E129D, S69P, S33G	S69P, S33G	S69P, S33G
PA4522	*ampD*	R11L, G148A, D183Y	R11L, G148A, D183Y	G148A	G148A	V90A	V90A
PA4526	pilB	R470K, K482Q	R470K, K482Q			I211T, T266N	I211T, T266N
PA4567	*rpmA*						
PA4568	*rplU*	**I74M**					
PA4597	*oprJ*	D68G, M69V	D68G, M69V			M69V	M69V
PA4598	*mexD*	E257Q, A536S, S845A	E257Q, A536S, S845A	K1031R, S845A, E257Q	K1031R, S845A, E257Q	**P721S, L624P,** E257Q	E257Q
PA4599	*mexC*			A378T, S330A, H310R, R76Q, P47S	A378T, S330A, H310R, R76Q, P47S		
PA4600	*nfxB*			R21H, D56G	R21H, **E75K,** D56G	A170T, Ter188Rext*	A170T, Ter188Rext*
PA4661	*pagL*			T55S	T55S		
PA4671	*rplY*			A123S	A123S		
PA4700	PBP1b	S25G	S25G	L353Q, S25G	L353Q, S25G	L353Q, S25G	L353Q, S25G
PA4727	pcnB						
PA4748	*tpiA*						
PA4751	*ftsH*						
PA4773	-			C161S, V217I, T271A	C161S, **A165T,** V217I, T271A		
PA4774	-						
PA4775	-	R131Q, A270V	R131Q, A270V	G31S, G152D, E153D	G31S, G152D, E153D		

Table A7.2. MIC_{TOB} values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA4776	*pmrA*	L71R	L71R			L71R	L71R
PA4777	*pmrB*	Y345H	Y345H	A4T, V15I, G68S, Y345H	A4T, V15I, G68S, Y345H	S2P, A4T, Y345H	S2P, A4T, Y345H
PA4878	*brlR*	T111L	T111L	Q42R, P61Q	Q42R, P61Q		
PA4944	*hfq*					D9A	D9A
PA4964	*parC*					Q405R	Q405R
PA4967	*parE*			D533E	D533E		
PA5000	*wapR*	T85A	T85A	T85A, R78K	T85A, R78K	T85A	T85A
PA5038	*aroB*	V85A, A200E, I292T	V85A, A200E, I292T	V85A	V85A	A200E, V85A	A200E, V85A
PA5040	*pilQ*	T690S, **E676D, E669D,** T266M, A106T	T690S, T266M, A106T	K139R, A106T	K139R, A106T	L523I, S505N, S481R, A135T, A128T	L523I, S505N, S481R, A135T, A128T
PA5045	PBP1a			5682740insCGC	5682740insCGC	A368T, S631I, 5682740insCGC	A368T, S631I, 5682740insCGC
PA5070	*tatC*	D195E	D195E	D195E	D195E	D195E	D195E
PA5105	*hutC*						
PA5117	*typA*						
PA5134	*ctpA*	D65N	D65N				
PA5199	*amgS*			I260V	I260V		
PA5200	*amgR*	A8V	A8V				
PA5235	*glpT*					A439T	A439T
PA5297	*poxB*			V19I, V193I, I303V, E506D	V19I, V193I, I303V, E506D		
PA5300	*cycB*						
PA5332	*crc*						
PA5366	*pstB*						
PA5471	*armZ*	L88P, D119E	L88P, D119E	V243A, I237V, D119E, S112N, L88P, C40R	V243A, I237V, D119E, S112N, L88P, C40R	D119E, L88P	D119E, L88P
PA5471.1	-						
PA5485	*ampDh2*			P116S	P116S		
PA5528	-			6220278insACG	6220278insACG		
PA5542	-	I106V	I106V	I106V	I106V	I106V, T68S	I106V, T68S

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Clonal Dissemination, Emergence of Mutator Lineages and Antibiotic Resistance Evolution in *Pseudomonas aeruginosa* Cystic Fibrosis Chronic Lung Infection

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Abstract

Chronic respiratory infection by *Pseudomonas aeruginosa* is a major cause of mortality in cystic fibrosis (CF). We investigated the interplay between three key microbiological aspects of these infections: the occurrence of transmissible and persistent strains, the emergence of variants with enhanced mutation rates (mutators) and the evolution of antibiotic resistance. For this purpose, 10 sequential isolates, covering up to an 8-year period, from each of 10 CF patients were studied. As anticipated, resistance significantly accumulated overtime, and occurred more frequently among mutator variants detected in 6 of the patients. Nevertheless, highest resistance was documented for the nonmutator CF epidemic strain LES-1 (ST-146) detected for the first time in Spain. A correlation between resistance profiles and resistance mechanisms evaluated [efflux pump (*mexB, mexD, mexF*, and *mexY*) and *ampC* overexpression and OprD production] was not always obvious and hypersusceptibility to certain antibiotics (such as aztreonam or meropenem) was frequently observed. The analysis of whole genome macrorestriction fragments through Pulsed-Field Gel Electrophoresis (PFGE) revealed that a single genotype (clone FQSE-A) produced persistent infections in 4 of the patients. Multilocus Sequence typing (MLST) identified clone FQSE-A as the CF epidemic clone ST-274, but striking discrepancies between PFGE and MLST profiles were evidenced. While PFGE macrorestriction patterns remained stable, a new sequence type (ST-1089) was detected in two of the patients, differing from ST-274 by only two point mutations in two of the genes, each leading to a nonpreviously described allele. Moreover, detailed genetic analyses revealed that the new ST-1089 is a *mutS* deficient mutator lineage that evolved from the epidemic strain ST-274, acquired specific resistance mechanisms, and underwent further interpatient spread. Thus, presented results provide the first evidence of interpatient dissemination of mutator lineages and denote their potential for unexpected short-term sequence type evolution, illustrating the complexity of *P. aeruginosa* population biology in CF.

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Introduction

Chronic respiratory infection by *Pseudomonas aeruginosa* is a major driver of morbidity and mortality in cystic fibrosis patients [1,2]. Traditionally, initial colonization is considered to be produced by unique strains acquired from environmental sources that undergo an extensive adaptation within the patient's lungs, leading to life-long persistent infections with little patient to patient transmission [3]. The establishment of the characteristic biofilm structures and the acquisition of a plethora of adaptive mutations (leading to enhanced antimicrobial and host defenses resistance, specific metabolic adaptation and an adapted virulence), are the main responsible for the persistence of these infections despite extensive antimicrobial therapy [4–9].

One of the hallmarks of *P. aeruginosa* chronic respiratory infections, in contrast to acute processes, is the high prevalence of hypermutable (or mutator) strains [10–13]. These variants are found in 30 to 60% of CF patients and show up to 1000-fold increased spontaneous mutation rates caused by defective DNA repair pathways. Among them, the mismatch repair (MMR) system is the one most frequently affected, due to mutations in *mutS* or *mutL* genes [14–16]. Indeed, mutator variants are positively selected during the establishment of chronic infections, linked to the acquisition of mutations related to antibiotic resistance, biofilm growth, metabolic adaptation, or acute virulence attenuation [10,16–20].

Additionally, there is growing evidence suggesting that adaptation to the CF lung environment may escape from the scale of the individual patients [21]. Indeed, the existence of concerning epidemic strains, such as the Liverpool Epidemic Strain (LES-1), capable of infecting hundreds of CF patients in different geographical locations, has been well documented for over two

decades [22,23]. Mutator variants have also been detected in a small proportion of isolates from patients infected by the LES-1 epidemic strain [24,25], but interpatient spread of mutator variants has never been demonstrated. Moreover, recent whole genome sequence analyses have revealed that the origination of CF adapted epidemic strains may result from a limited number of specific mutations with pleiotropic effects [26].

Although whole genome sequencing and microarray analysis will soon take the lead, the current gold standard for typing *P. aeruginosa* strains with the purpose of investigating patient to patient transmission is still the analysis of whole genome macrorestriction fragments through Pulsed-Field Gel Electrophoresis (PFGE) [27]. However, the instability of PFGE profiles, mainly consequence of frequent genome rearrangements, makes this procedure unsuitable for long-term and global epidemiology studies [28]. On the other hand, Multilocus Sequence Typing (MLST), based on sequencing of 7 house keeping genes, provides a much more stable genetic signature and is still currently considered the gold standard for global epidemiology and population structure analyses [29].

In this work, we investigated the interplay between the three above described key microbiological aspects of *P. aeruginosa* CF chronic lung infections: the occurrence of transmissible and persistent strains (PFGE-MLST clonal epidemiology), the emergence of variants with enhanced mutation rates (mutators) and the evolution of antibiotic resistance.

Results and Discussion

Long-term Clonal Epidemiology of *P. aeruginosa* in CF: Transmissible and Persistent Strains

A total of 100 *P. aeruginosa* isolates were studied, including 10 sequential isolates from each of 10 CF patients attended at the reference hospital of the Balearic Islands, Spain. Each of the sequential isolates included were separated by at least a 6-month interval, covering up to an 8-year period from 2003 to 2010. PFGE analysis revealed the presence of 13 different clones; one of them (clone FQSE-A) was detected in four patients while the other twelve were detected in single patients. Figure 1 shows the distribution of the different clones among the different patients along the 8-year study period. Six patients, including the 4 colonized by clone FQSE-A, showed a single clone over the study period, while the other 4 showed the coexistence of several (2 to 4) clones or clonal replacements (Figure 1). Therefore, results so far suggested that clone FQSE-A is a CF adapted (transmissible and persistent) strain. The epidemiological setting driving (direct or indirect) interpatient transmission is uncertain, since recommendations on segregation of patients colonized by *P. aeruginosa* from those free of this pathogen are followed in all hospital visits.

Discrepant MLST vs PFGE Results: Role of Mutators

The first isolate per patient and clone were further analyzed by MLST, yielding 13 sequence types (ST) not entirely coincident with the clones identified by PFGE. The allelic profiles and relevant features of the 13 STs identified are shown in Table 1. Not surprisingly due to the overall higher discriminatory power (or lower stability) of PFGE compared to MLST [28–30], two different PFGE clones (FQSE-C and FQSE-D) shared the same ST (ST-299). Much more intriguingly, the disseminated clone FQSE-A yielded two different STs (Table 1, Figure 1). Clone FQSE-A from 3 of the 4 patients was identified as ST-274, previously detected in multiple CF patients from France, Austria and Australia according to the MLST database (http://pubmlst.org/paeruginosa/). Moreover, this clone has been simultaneously detected in several patients from another CF Unit in Madrid [31] and in a few cases of hospital-acquired infections in recent Spanish multicentre studies [32,33]. Thus, our results add further evidence pointing out that ST-274 should be added to the growing list of CF epidemic clones [22,29]. In contrast, clone FQSE-A from the fourth patient was identified as a new ST (ST-1089) differing from ST-274 by only two point mutations in two of the genes (*acsA* and *guaA*) each leading to a nonpreviously described allele (the only two novel alleles found in the complete collection). Additionally, in contrast to ST-274, ST-1089 showed a mutator phenotype (Table 1). Therefore, the available data clearly suggested that ST-1089 has recently evolved from the CF epidemic clone ST-274 through point mutations linked to the emergence of a mutator lineage. As expected, due to the mutational spectra of DNA MMR deficient strains [12], both mutations were G to A transversions. Moreover, both mutations were apparently not neutral, since they lead to nonsynonymous substitutions in the acetyl-coenzyme A synthetase (G435D) and the GMP synthase (G312S), which are key metabolic enzymes. Whether these mutations were positively selected because the play a role in the intense metabolic adaptation to the CF chronic lung infection setting [18] remains to be explored.

Consistently with our findings, while mutators series appeared to be no more variable in their MLST haplotypes than nonmutator series in a previous study, the only novel alleles found were also from patients with mutator strains [34]. Moreover, a recent study has reported discrepant MLST vs PFGE results directly linked to the emergence of a mutator phenotype caused by *mutL* mutations [35], stressing the point that this gene lacks the neutrality required for an appropriate MLST marker, since MMR deficient mutators (*mutS* and *mutL*) are positively selected in CF chronic infection [15]. Likewise, a recent work has reported a strain that was not typable by MLST due to the presence of a deletion in the *mutL* fragment analyzed [30]. All together, these results indicate that frequent mutator variants from chronic infection may determine a lower stability of the MLST profiles than expected (leading to discrepant results compared to PFGE) both directly (*mutL* inactivating mutations within the gene fragment evaluated in MLST analysis) and indirectly through the increased spontaneous mutagenesis (facilitating the emergence of novel alleles through point mutations in any of the 7 genes evaluated in MLST analysis).

Regarding the other STs detected, as expected from the frequent acquisition of unique clones by CF patients from environmental sources [3,36], 8 of the 13 MLST clones detected corresponded to nonpreviously described STs, each found in single patients (Table 1). Nevertheless, in addition to the above described clone FQSE-A/ST-274 CF epidemic strain, clonal replacement (of a MDR mutator strain) by the MDR LES-1 epidemic strain ST-146 [29,37] was documented in one of the patients, alerting of the first detection of the likely more world-wide concerning CF epidemic clone in Spain. Although the epidemiological driver of LES-1 colonization was not specifically investigated, the fact that the patient has family links with a northern European country could help to explain the acquisition of this clone not previously detected in Spain.

Evidence for Interpatient Spread of a Mutator Lineage (ST-1089) Evolved from a CF Epidemic Strain (ST-274)

Since ST-274 and ST-1089 show the same PFGE pattern and MLST was initially performed only in the first isolate per patient and clone, MLST analysis was extended to the last available isolate from each patient colonized by clone FQSE-A as well as the two additional sporadic mutator isolates detected in two of the patients colonized by this clone (Figure 1). In all cases, the MLST

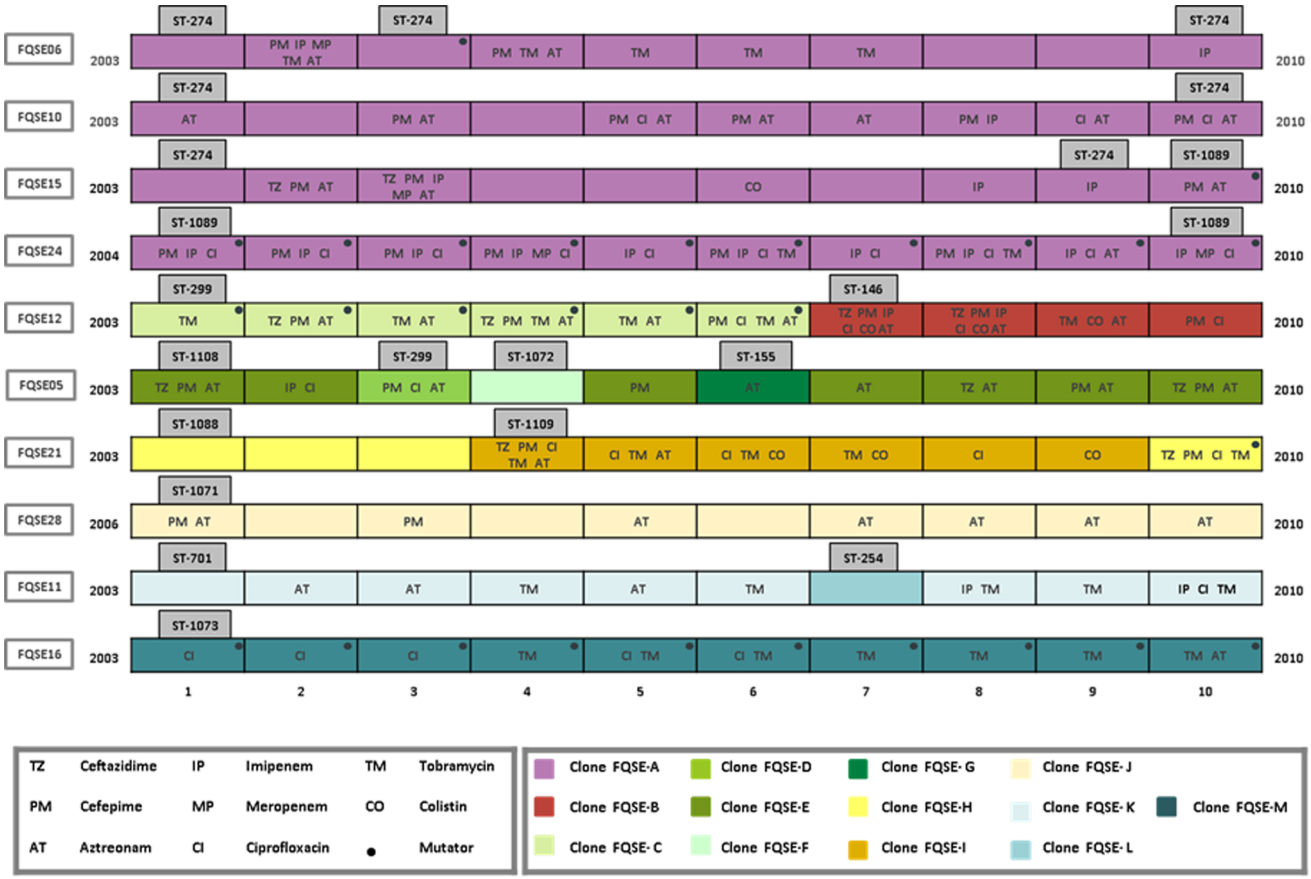

Figure 1. Schematic representation of the 10 sequential isolates from each of the 10 CF patients in the time frame of the study period. Each different clone is represented by a different colour. Resistance profiles and presence mutator phenotypes are indicated for each isolate. Results of MLST analysis are also provided for specific isolates.
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Table 1. Allelic profiles and relevant features of the different ST detected.

Clone	Sequence type	Allelic profile[a]							Relevant features
		acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*	
FQSE-A	ST-274	23	5	11	7	1	12	7	Detected in CF patients in Australia, Austria and France
FQSE-A	ST-1089	66	5	101	7	1	12	7	New Sequence Type, DLV of ST-274 (Mutator)
FQSE-B	ST-146	6	5	11	3	4	23	1	MDR Liverpool Epidemic Strain (LES-1)
FQSE-C/D	ST-299	17	5	36	3	3	7	3	Detected in CF patients in Australia
FQSE-E	ST-1108	6	3	17	7	3	4	7	New Sequence Type
FQSE-F	ST-1072	5	13	25	6	1	7	3	New Sequence Type
FQSE-G	ST-155	28	5	36	3	3	13	7	Detected in CF patients in Australia, Canada and France
FQSE-H	ST-1088	36	27	28	3	4	13	1	New Secuence Type
FQSE-I	ST-1109	16	14	3	11	1	15	1	New Sequence Type
FQSE-J	ST-1071	5	3	57	6	1	33	42	New Sqcuence Type
FQSE-K	ST-701	29	1	9	13	1	6	23	New Sequence Type
FQSE-L	ST-254	6	5	58	11	3	4	37	Detected in CF patients in Australia, Canada
FQSE-M	ST-1073	28	5	36	3	4	10	95	New Sequence Type (mutator)

[a]Nonpreviously described alleles are shown in bold.
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profiles coincided with that of the first isolate, except for the mutator lineage emerging from one of the patients colonized by ST-274 that was also identified as ST-1089 (Figure 1). Thus, the mutator lineage ST-1089 was detected from the first to the last isolate analyzed in one of the patients and only in the last isolate (the only one with mutator phenotype) from a second one. Nevertheless, extended analysis of available isolates of this patient from 2010 to 2012 confirmed the persistence of the ST-1089 mutator lineage. On the other hand, the sporadic mutator lineage detected in the third patient was still ST-274. Therefore, mutator lineages were detected in 3 of the 4 patients with clone FQSE-A, two belonging to ST-1089 and one to ST-274. In order to evaluate the genetic basis of hypermutation, complementation studies with plasmids harboring wild-type MMR genes (*mutS* and *mutL*) were performed in mutator isolates from these three patients. In all cases the isolates were found to be defective in *mutS*. Thus, *mutS* was sequenced from the three mutator isolates and representative nonmutator isolates. Surprisingly, the three mutator isolates had the same inactivating mutation in *mutS* (4 bp deletion from nt 814), obviously absent in the nonmutator isolates. This specific mutation has not been previously noted in dozens of ofmutator variants sequenced so far [13,14,16,38,39], and is not reasonable to believe that it might have emerged independently in three different occasions. Therefore, these results provide evidence for the first time of interpatient spread of mutators. Moreover, they demonstrate the interpatient spread of a mutator lineage (ST-1089) evolved from a CF epidemic strain (ST-274).

Interplay between Clonal Lineages, Mutator Phenotypes, Antimicrobial Susceptibility and Resistance Mechanisms

The overall susceptibility data for the collection of 100 isolates to 8 antipseudomonal agents is summarized in Table 2. Lowest susceptibility was observed for aztreonam (60% S) and highest for meropenem (96% S). However, resistance rates were highest for cefepime (30% R), tobramycin (30% R) and ciprofloxacin (24% R) and lowest for meropenem (1% R), aztreonam (4% R), and colistin (7% R). A significant trend towards increased MICs overtime to individual antibiotics was not noted, although all colistin resistant isolates occurred in the second half of the study (Figure 2). The low percentages of Aztreonam resistance might be of particular interest, considering its recent introduction for the treatment of CF chronic lung infection as inhaled therapy [40]. It should also be noted that EUCAST considers *P. aeruginosa* intrinsically nonsusceptible to this antibiotic (mainly due to the basal expression of MexAB-OprM efflux pump and pharmacokinetic/pharmacodynamic issues) (http://www.eucast.org/clinical_breakpoints/). Therefore, the percentage (60%) of susceptible isolates documented actually reflects the high number of hypersusceptible (MIC ranges 0.125–1 mg/L) isolates falling outside of wild-type MICs (2–16 mg/L) distributions (http://www.eucast.org/mic_distributions/). In addition of aztreonam, an important number of isolates showed hypersusceptibility to meropenem with MICs (<0.06 mg/L) falling outside of wild-type distributions.

Resistance profiles (I+R) to the 8 antipseudomonal agents and mutator phenotypes for the 100 isolates are also indicated in Figure 1. Although the resistance profiles were variable within and across patients along the study period, a significant trend towards the accumulation of resistances was noted, increasing from an average of resistance to 1.1 ± 1.2 antibiotics in the first isolate of each patient to 2.5 ± 0.85 in the last isolate (paired t test, $p = 0.016$).Consistently with previous data [10,11,15], 29% of the isolates showed mutator phenotypes and 6 of the 10 patients showed at least 1 mutator isolate. In two patients all isolates were mutators and in other two, mutators emerged at late stages of colonization. In one more patient a mutator lineage emerged but it was not fixed and in other one it was replaced by the LES-1 epidemic strain (Figure 1). As described above, mutator variants were detected in 3 of the 4 patients colonized by epidemic clon A (ST-274/ST-1089). Mutator variants have also been previously detected in a small proportion of isolates of the LES-1 epidemic strain, but interpatient spread was not previously evidenced [24,25]. In agreement with previous findings [10,11,19,20,41] a significant trend ($p = 0.009$) towards resistance to a higher number of antibiotics among mutator isolates (2.28 ± 0.22) than among nonmutator isolates (1.49 ± 0.17) was also noted, although not all mutator isolates were resistant and some nonmutator isolates, particularly noteworthy the LES-1 epidemic strain ST-146, were resistant to multiple antibiotics (Figure 1).

Resistance mechanisms [efflux pump (*mexB, mexD, mexF,* and *mexY*) and *ampC* overexpression and OprD production] were evaluated in the first and last isolate from each patient and clone and results are shown in Table 3. A trend towards accumulation of resistance mechanisms was noted, from 1.4 ± 0.58 in the first to 2.1 ± 0.88 in the last isolates, although the differences did not reach statistical significance ($p = 0.06$). The most frequent mechanism was *mexY* overexpression, documented in all 10 patients. Moreover, this mechanism was present in most patients (8 of 10) already in the early isolates (Table 3). The overexpression of the other efflux pumps was far more infrequent; *mexF* was documented in 3 patients, *mexD* in 2 and *mexB* only in one. AmpC overexpression was evidenced in 6 of the patients and lack of OprD production in the 4 patients colonized by imipenem resistant strains. Although a certain correlation was documented between *ampC* overexpression and ceftazidime resistance and lack of OprD and imipenem resistance, as previously observed [42,43], a correlation between phenotype and genotype was not always evident, particularly concerning efflux pumps overexpression. Previously described efflux unbalance in CF [44] and antagonistic interactions between certain resistance mechanisms [45] could explain these discrepancies. Particularly, the previously documented frequent inactivation of the constitutive MexAB efflux system could well be responsible of the frequently observed aztreonam and meropenem hypersusceptibily (Table 3, Figure 2). The clone associated with a higher number of resistance mechanisms was ST-146/LES-1 previously associated with MDR phenotypes [43]. The initial MDR isolate from this clone already expressed 4 resistance mechanisms (MexY, MexF, MexD and OprD). The last isolate also expressed 4 resistance mechanisms (MexY, MexD, MexF, and OprD), but showed a significant reduction of the MDR profile, likely influenced by the modification of the mechanisms expressed (MexD instead of AmpC). Although not particularly associated with a high number of resistance mechanisms, all early and late isolates from the epidemic clone FQSE-A (ST-274/ST-1089) overexpressed *mexY*. Thus, *mexZ* was sequenced in all strains that overexpressed *mexY*, in order to determine the underlying genetic mechanism of resistance and to use *mexZ* mutations as epidemiological marker. As expected [46] most of the strains overexpressing *mexY* showed *mexZ* mutations, including deletions, premature stop codons, insertion sequences (IS), or nonsynonymous substitutions (Table 3). Remarkably, clone FQSE-A (ST-274/ST-1089) isolates from the different patients showed different *mexZ* mutations, denoting that interpatient spread precedes resistance development, except for the ST-274/ST-1089 *mutS* deficient mutator lineages (Table 3). Indeed, the ST-274/ST-1089 *mutS* deficient isolates showed the same *mexZ* mutation, demonstrating that the interpatient spread of the mutator lineages occurred after the acquisition of the resistance mechanism.

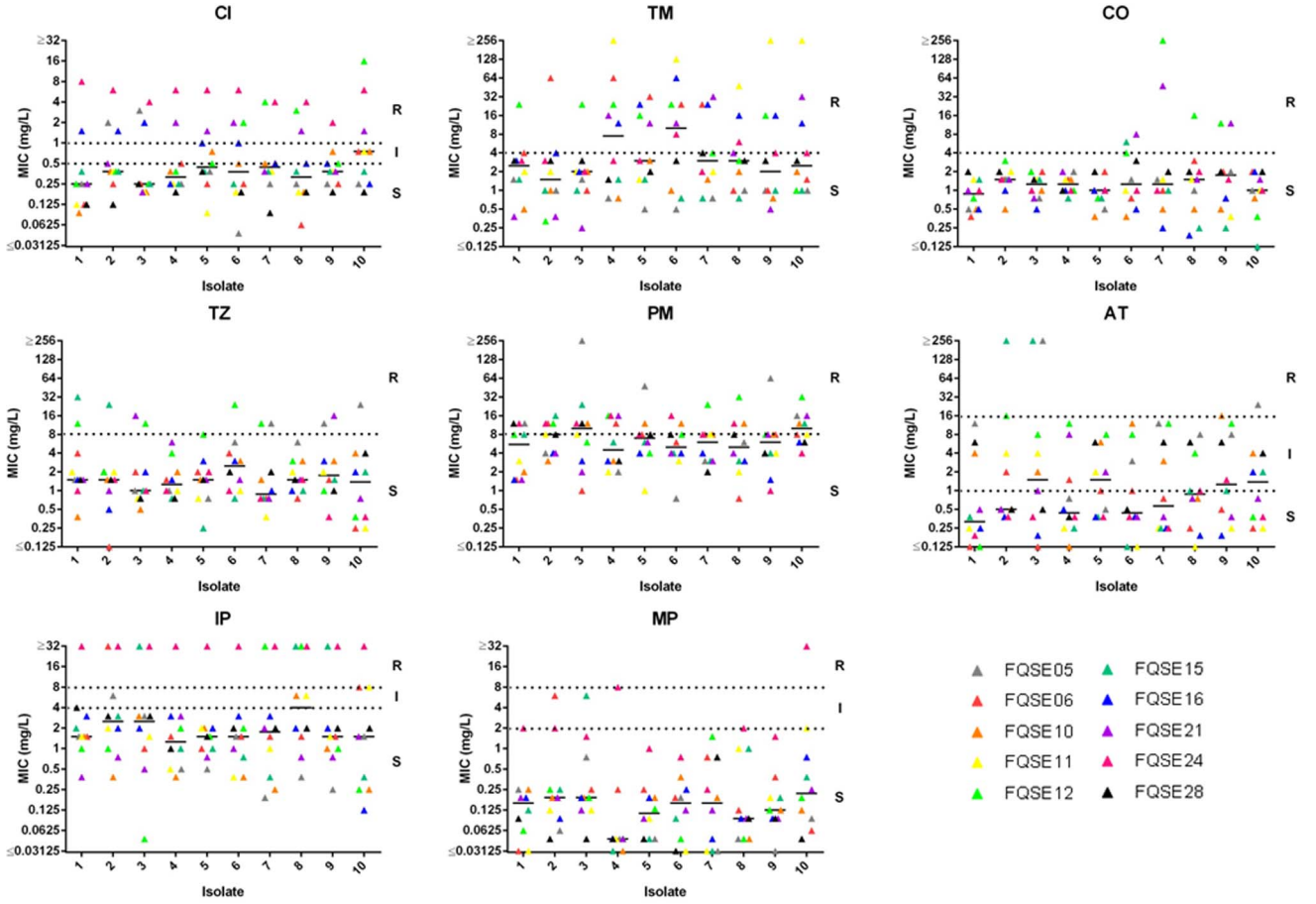

Figure 2. Evolution of minimal inhibitory concentrations (MICs) from the first to the last studied isolate from each patient. Each color represents a different patient. CI, ciprofloxacin; TM, tobramycin; CO, colistin; TZ, ceftazidime; PM, cefepime; AT, aztreonam; IP, impenem; MP, meropenem.
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In summary, the presented results provide evidence for the interpatient spread of the CF epidemic strain ST-274. Much more importantly, they strongly suggest that ST-274 bacterial populations spreading among different patients were not a single genotype, but rather included a *mutS* deficient subpopulation that had already evolved into the new mutator lineage ST-1089 and had acquired specific resistance mutations. In other words, presented results provide the first evidence of interpatient dissemination of mutator lineages and denote their potential for unexpected short-term sequence type evolution (leading to MLST vs PFGE discrepancies), illustrating the complexity of *P. aeruginosa* population biology in CF.

Table 2. Antimicrobial susceptibility of the studied *P. aeruginosa* isolates.

Antibiotic	No. of isolates (n = 100)			No. of patients (n = 10)	
	S[a]	I[a]	R[a]	I+R[a]	R[a]
Ceftazidime	89	NA[b]	11	4	4
Cefepime	70	NA	30	8	8
Imipenem	79	5	16	7	4
Meropenem	96	3	1	3	1
Aztreonam	60	36	4	10	2
Ciprofloxacin	69	7	24	8	5
Tobramycin	70	NA	30	6	6
Colistin	93	NA	7	3	3

doi:10.1371/journal.pone.0071001.t002

Materials and Methods

Ethics Statement

This study was approved by the Research Committee of Hospital Son Espases. All clinical isolates used were obtained from a preexisting collection recovered over years from routine cultures and the study does not include patients' data.

Clinical Isolates and Susceptibility Testing

The collection studied included 10 sequential *P. aeruginosa* isolates from each of 10 CF patients attended at hospital Son Espases, reference hospital of the Balearic Islands, Spain. Each of the sequential isolates included were separated by at least a 6-month interval, covering up to an 8-year period from 2003 to 2010. The first available isolate and the last available isolate (when the project was initiated) from each of the patients was always included in the studied collection. PAO1 strain was used as

Table 3. Antimicrobial susceptibility results and resistant mechanisms detected in first and last studied isolates from each patient and clone.

Patient-Isolate	PFGE Clone	ST	Mutator (Y/N)	MIC (mg/L)								Resistance mechanisms	*mexZ* mutations[a]
				TZ	PM	AT	IP	MP	CI	TO	CO		
FQSE06-0403	FQSE-A	274	N	0.064	1.5	0.125	1.5	0.016	0.125	4	0.38	*mexY*	S9P
FQSE06-1104	FQSE-A	274	Y	0.094	1	0.094	1	0.25	0.25	1	2	*mexY*	A194P
FQSE06-0610	FQSE-A	274	N	1.5	8	0.25	8	0.064	0.75	1.5	2	*mexY*, OprD-	$Nt_{292}\Delta 11$
FQSE10-0503	FQSE-A	274	N	0.38	2	4	1.5	0.25	0.094	0.5	0.5	*mexY*	IS [b]
FQSE10-0111	FQSE-A	274	N	3	12	4	0.25	0.125	0.75	2	0.75	*ampC*, *mexY*	IS
FQSE15-0803	FQSE-A	274	N	1	8	0.38	2	0.125	0.38	1.5	1.5	*mexY*	A144V
FQSE15-0110	FQSE-A	1089	Y	1.5	12	2	0.38	0.38	0.38	1	0.016	*mexY*	A194P
FQSE24-0304	FQSE-A	1089	Y	1	12	0.19	>32	2	8	3	1	*mexY*,OprD-	A194P
FQSE24-1010	FQSE-A	1089	Y	0.38	4	0.38	>32	>32	6	4	1	*mexY*,OprD-	A194P
FQSE12-0603	FQSE-C	299	N	0.75	8	0.125	1	0.064	0.25	24	0.75	*mexY*	R125P
FQSE12-1206	FQSE-C	299	N	8	8	8	2	0.5	2	24	4	*ampC*, *mexY*	R125P
FQSE12-1007	FQSE-B	146	N	24	24	12	>32	1.5	4	4	>256	*ampC*, *mexY*, *mexF*, OprD-	Q164X
FQSE12-1110	FQSE-B	146	N	1	32	0.38	0.25	0.19	16	1	0.38	*mexY*, *mexF*, *mexD*,OprD-	Q164X
FQSE05-0403	FQSE-E	1108	N	16	12	12	1.5	0.25	0.25	1.5	0.5	*ampC*	WT
FQSE05-0704	FQSE-D	299	N	2	>256	>256	3	0.75	3	1.5	0.75	*ampC*, *mexY*	ND
FQSE05-0305	FQSE-F	1072	N	1	2	0.75	0.5	0.047	0.25	0.75	2	*mexY*, *mexF*	W185X
FQSE05-0807	FQSE-G	155	N	0.75	0.75	3	1.5	0.19	0.047	0.5	1.5	–	ND
FQSE05-0111	FQSE-E	1108	N	12	16	24	1.5	0.094	0.25	1	1	*ampC*, *mexY*	V43G
FQSE21-1003	FQSE-H	1088	N	0.5	1.5	0.5	0.38	0.19	0.25	0.38	1	*ampC*, *mexY*	$Nt_{61}\Delta 15$
FQSE21-0505	FQSE-I	1109	N	16	16	8	3	0.5	2	16	2	*mexB*, *mexY*	K131R
FQSE21-0410	FQSE-H	1088	N	1.5	8	0.38	0.75	0.094	0.38	0.5	12	*mexY*	$Nt_{61}\Delta 15$
FQSE21-1110	FQSE-I	1109	Y	8	16	0.75	1.5	0.25	1.5	32	1.5	*mexB*, *mexY*	K131R
FQSE28-1006	FQSE-J	1071	N	1.5	12	6	4	0.094	0.125	3	2	*ampC*, *mexY*	$Nt_{189}\Delta 12$
FQSE28-1110	FQSE-J	1071	N	1	6	4	2	0.047	0.19	3	2	*ampC*, *mexY*	$Nt_{189}\Delta 12$
FQSE11-0603	FQSE-K	701	N	1	3	0.25	1.5	0.032	0.125	2	1.5	*mexY*	WT
FQSE11-0608	FQSE-L	254	N	1	8	0.125	1	0.023	0.38	2	1.5	*mexY*	$Nt_{279}\Delta 12$
FQSE11-1010	FQSE-K	701	N	2	8	0.25	8	2	0.75	>256	1	*mexY*, OprD-	WT
FQSE16-0803	FQSE-M	1073	Y	1.5	1.5	0.25	3	0.19	1.5	3	0.5	*mexF*, *mexD*	WT
FQSE16-0910	FQSE-M	1073	Y	3	6	2	0.125	0.75	0.25	12	2	*ampC*, *mexY*, *mexF*	R125P

[a]PAO1 and PA14 were used as reference wild-types sequences (www.pseudomonas.com).

[b]1.2 Kb IS located in *mexX-mexZ* intergenic region (nt -72 respect *mexZ* coding sequence). Encodes a putative transposase identical to that previously reported in *Pseudomonas pseudoalcaligenes* CECT 5344 (ref ZP_10763279.1).

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reference. The antibiotic susceptibility profiles (ceftazidime, cefepime, aztreonam, imipenem, meropenem, ciprofloxacin, tobramycin and colistin) were determined by Etest, using EUCAST breakpoints (http://www.eucast.org/).

Molecular Typing

Clonal relatedness was evaluated in all isolates by PFGE. For this purpose, bacterial DNA embedded in agarose plugs prepared as described previously [47] was digested with SpeI. DNA separation was then performed in a contour-clamped homogeneous-electric-field DRIII apparatus (Bio-Rad, La Jolla, CA) under the following conditions: 6 V/cm^2 for 26 h with pulse times of 5 to 40 s. DNA macrorestriction patterns were interpreted according to the criteria established by Tenover et al. [48]. Representative isolates from each clone and patient were further analyzed by MLST using available protocols and databases (http://pubmlst.org/paeruginosa/).

Characterization of Resistance Mechanisms

The levels of expression of *ampC*, *mexB*,mexD, *mexF* and *mexY* were determined by Real-time reverse transcription (RT)-PCR according to previously described protocols [49,50]. Briefly, strains were grown in 10 ml of LB broth at 37°C and 180 rpm to the late log phase (optical density at 600 nm [OD_{600}] of 1) and collected by centrifugation. Total RNA was isolated by using the RNeasy minikit (Qiagen), dissolved in water, and treated with 2 U of Turbo DNase (Ambion) for 30 min at 37°C to remove contaminating DNA. The reaction was stopped by the addition of 5 µl of DNase inactivation reagent to the mixture. A 50-ng sample of purified RNA was then used for one-step reverse transcription and real-time PCR amplification using the Quanti Tect SYBR green RT-PCR kit (Qiagen) with a SmartCycler II instrument (Cepheid). Previously described primers [49,50] were used for the amplification of *ampC*, *mexB*, *mexD*, *mexF*, *mexY*, and *rpsL* (used as a reference to normalize the relative amount of mRNA). Strains were considered positive for *ampC*, *mexD*, *mexF* or *mexY* overexpression when the corresponding mRNA level was at least 10-fold higher than that of PAO1, negative if lower than 5-fold, and borderline if between 5- and 10-fold. Strains were considered positive for *mexB* overexpression when the corresponding mRNA level was at least 3-fold higher than that of PAO1, negative if lower than 2-fold and borderline if between 2- and 3-fold. All PCRs were performed in duplicate. Mean values (± standard deviations) of mRNA levels obtained in three independent duplicate experiments were considered. Previously characterized strains overexpressing these mechanisms were used as controls [50]. Additionally, the gene encoding the transcriptional regulator of MexXY-OprM efflux pump, *mexZ*, was fully sequenced in representative isolates, from each patient and clone, showing *mexY* overexpression [32]. After duplicate PCR amplification, sequencing reactions were performed with the Big Dye Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were then compared with that of wild-type PAO1 and those available at GenBank. Finally, outer membrane protein (OMP) profiles were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue following previously described protocols [45]. Obtained OprD profiles were compared with those of PAO1 and its OprD-deficient mutant.

Mutant Frequencies and Genetic Basis of Hypermutation

Rifampicin (300 mg/L) resistance mutant frequencies were determined in all strains following previously established procedures [10]. To explore the genetic basis for the mutator phenotypes, complementation studies were performed as described previously [15]. Briefly, plasmid pUCPMSharbouring PAO1 wild-type *mutS*, plasmid pUCPML harbouring PAO1 wild-type *mutL*, and plasmid pUCP24, a control cloning vector, were electroporated into the mutator isolates. Complementation was demonstrated by reversion of the increased rifampicin resistance mutant frequencies in two independent transformant colonies for each strain. Previously described primers and protocols [15] were used for the amplification and sequencing of *mutS* or *mutL* genes according to the results of complementation experiments.

Statistical Analysis

The Graph Pad Prism 5 software was used for graphical representation and statistical analysis. Quantitative variables were compared using the Mann-Whitney U-test or the Student's *t* test as appropriate. Categorical variables were compared using the χ^2 test. A p value of less than 0.05 was considered statistically significant.

Author Contributions

Conceived and designed the experiments: AO. Performed the experiments: CL ER XM GC BM. Analyzed the data: CL ER XM BM JF BT JLP AO. Contributed reagents/materials/analysis tools: JF BT. Wrote the paper: CL ER AO.

References

1. Lyczak JB, Cannon CL, Pier GB (2002) Lung infection associated with cystic fibrosis. Clin Microbiol Rev 15: 194–222.
2. Gibson RL, Burns JL, Rammsey BW (2003) Pathophysiology and management of pulmonary infections in cystic fibrosis. Am J Respir Crit Care Med 168: 918–951.
3. Burns JL, Gibson RL, McNamara S, Yim D, Emerson J, et al. (2001) Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. J Infect Dis 183: 444–52.
4. Costerton J, Stewart P, Greenberg E (1999) Bacterial biofilms: a common cause of persistent infections. Science 284: 1318–1322.
5. Smith EE, Buckley DG, Wu Z, Saenphimmachack C, Hoffman LR, et al. (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci USA 103: 8487–8492.
6. Hogardt M, Heesemann J (2010) Adaptation of *Pseudomonas aeruginosa* during persistence in the CF lung. Int J Med Microbiol 300: 557–532.
7. Hauser AR, Jain M, Bar-Meir M, McColley SA (2011) Clinical significance of microbial infection and adaptation in cystic fibrosis. Clin Microbiol Rev 24: 29–70.
8. Bragonzi A, Paroni M, Nonis A, Cramer N, Montanari S, et al. (2009) *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. Am J Respir Crit Care Med 180: 138–45.
9. Rodríguez-Rojas A, Oliver A, Blázquez J (2012) Intrinsic and environmental mutagenesis drive diversification and persistence of *Pseudomonas aeruginosa* in chronic lung infections. J Infect Dis 205: 121–7.
10. Oliver A, Cantón R, Campo P, Baquero F, Blázquez J (2000) High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science 288: 1251–4.
11. Ciofu O, Riis B, Pressler T, Poulsen HE, Hoiby N (2005) Occurrence of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. Antimicrob Agents Chemother 49: 2276–2282.
12. Oliver A, Mena A (2010) Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance. Clin Microbiol Infect 16: 798–808.
13. Oliver A (2010) Mutators in cystic fibrosis chronic lung infection: Prevalence, mechanisms, and consequences for antimicrobial therapy. Int J Med Microbiol 300: 563–72.
14. Oliver A, Baquero F, Blázquez J (2002) The mismatch repair system (mutS, mutL and uvrD genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. Mol Microbiol 43: 641–50.

15. Mena A, Smith EE, Burns JL, Speert DP, Moskowitz SM, et al. (2008) Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. J Bacteriol 190: 7910–7.
16. Feliziani S, Luján AM, Moyano AJ, Sola C, Bocco JL (2010) Mucoidy, quorum sensing, mismatch repair and antibiotic resistance in *Pseudomonas aeruginosa* from cystic fibrosis chronic airways infections. PLoS One 5: e12669.
17. Luján AM, Maciá MD, Yang L, Molin S, Oliver A, et al. (2011) Evolution and adaptation in *Pseudomonas aeruginosa* biofilms driven by mismatch repair system-deficient mutators. PLoS One 6: e27842.
18. Hoboth C, Hoffmann R, Eichner A, Henke C, Schmoldt S (2009) Dynamics of adaptive microevolution of hypermutable *Pseudomonas aeruginosa* during chronic pulmonary infection in patients with cystic fibrosis. J Infect Dis 200: 118–130.
19. Ferroni A, Guillemot D, Moumile K, Bernede C, Le Bourgeois M, et al. (2009) Effect of mutator*P. aeruginosa* on antibiotic resistance acquisition and respiratory function in cystic fibrosis. Pediatr Pulmonol 44: 820–5.
20. Henrichfreise B, Wiegand I, Pfister W, Wiedemann B (2007) Resistance mechanisms of multiresistant *Pseudomonasaeruginosa* strains from Germany and correlation with hypermutation. Antimicrob Agents Chemother 51: 4062–70.
21. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, et al. (2012) Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat Rev Microbiol 10: 41–51.
22. Cramer N, Wiehlmann L, Tummler B (2010) Clonal epidemiology of *Pseudomonas aeruginosa* in cystic fibrosis. Int J Med Microbiol 300: 526–533.
23. Cramer N, Wielhlmann L, Ciofu O, Tamm S, Hoiby N, et al. (2012) Molecular epidemiology of chronic *Pseudomonas aeruginosa* airway infections in cystic fibrosis. PLoS One 7: e50731.
24. Kenna DT, Doherty CJ, Foweraker J, Macaskill L, Barcus VA, et al. (2007) Hypermutability in environmental *Pseudomonas aeruginosa* and in populations causing pulmonary infection in individuals with cystic fibrosis. Microbiology 153: 1852–9.
25. Mowat E, Paterson S, Fothergill JL, Wright EA, Ledson MJ, et al. (2011) *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. Am J RespirCrit Care Med 183: 1674–9.
26. Yang L, Jelsbak L, Marvig RL, Damkiær S, Workman CT, et al. (2011) Evolutionary dynamics of bacteria in a human host environment. Proc Natl Acad Sci USA 108: 481–6.
27. Römling U, Grothues D, Heuer T, Tümmler B (1992) Physical genome analysis of bacteria. Electrophoresis 13: 626–631.
28. Fothergill JL, White J, Foweraker JE, Walshaw MJ, Ledson MJ, et al. (2010). Impact of *Pseudomonas aeruginosa* genomic instability on the application of typing methods for chronic cystic fibrosis infections. J ClinMicrobiol 48: 2053–9.
29. Curran B, Jonas D, Grundmann H, Pitt T, Dowson CG (2004) Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. J Clin Microbiol 42: 5644–9.
30. Kidd TJ, Grimwood K, Ramsay KA, Rainey P, Bell SC (2011) Comparison of three molecular techniques for typing *Pseudomonas aeruginosa* isolates in sputum samples. J Clin Microbiol 49: 263–268.
31. Férnandez-Olmos A, García-Castillo M, Alba JM, Morosini MI, Lamas A, et al. (2013) Population structure and antimicrobial susceptibility of both non-persistent and persistent *Pseudomonas aeruginosa* isolates recovered in cystic fibrosis patients. J Clin Microbiol. In press.
32. Cabot G, Ocampo-Sosa AA, Domínguez MA, Gago JF, Juan C, et al. (2012) Genetic markers of widespread extensively drug-resistant *Pseudomonas aeruginosa* high-risk clones. Antimicrob Agents Chemother 56: 6349–57.
33. García-Castillo M, Del Campo R, Morosini MI, Riera E, Cabot G, et al. (2011) Wide dispersion of ST175 clone despite high genetic diversity of carbapenem-nonsusceptible *Pseudomonas aeruginosa* clinical strains in 16 Spanish hospitals. J Clin Microbiol 49: 2905–10.
34. Warren AE, Boulianne-Larsen CM, Chandler CB, Chiotti K, Kroll E, et al. (2011) Genotypic and phenotypic variation in *Pseudomonas aeruginosa* reveals signatures of secondary infection and mutator activity in certain cystic fibrosis patients with chronic lung infections. Infect Immun 79: 4802–4818.
35. García-Castillo M, Máiz L, Morosini MI, Rodríguez-Baños M, Suarez L, et al. (2012) Emergence of a *mutL* mutation causing multilocus sequence typing-pulsed-field gel electrophoresis discrepancy among *Pseudomonas aeruginosa* isolates from a cystic fibrosis patient. J Clin Microbiol 50: 1777–8.
36. Kidd TJ, Ritchie SR, Ramsay KA, Grimwood K, Bell SC, et al. (2012) *Pseudomonas aeruginosa* exhibits frequent recombination, but only a limited association between genotype and ecological setting. PLoS One 7: e44199.
37. Salunkhe P, Smart CH, Morgan JA, Panagea S, Walshaw MJ, et al. (2005) A cystic fibrosis epidemic strain of *Pseudomonas aeruginosa* displays enhanced virulence and antimicrobial resistance J Bacteriol 187: 4908–20.
38. Hogardt M, Schubert S, Adler K, Götzfried M, Heesemann J (2006) Sequence variability and functional analysis of MutS of hypermutable *Pseudomonasaeruginosa* cystic fibrosis isolates. Int J Med Microbiol 296: 313–20.
39. Montanari S, Oliver A, Salerno P, Mena A, Bertoni G, et al. (2007) Biological cost of hypermutation in *Pseudomonas aeruginosa* strains from patients with cystic fibrosis. Microbiology 153: 1445–54.
40. Parkins MD, Elborn JS (2010) Aztreonam lysine: a novel inhalational antibiotic for cystic fibrosis. Expert Rev Respir Med 4: 435–44.
41. Macia MD, Blanquer D, Togores B, Sauleda J, Perez JL, et al. (2005) Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. Antimicrob Agents Chemother 49: 3382–3386.
42. Wolter DJ, Black JA, Lister PD, Hanson ND (2009) Multiple genotypic changes in hypersusceptible strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients do not always correlate with the phenotype. J Antimicrob Chemother 64: 294–300.
43. Tomás M, Doumith M, Warner M, Turton JF, Beceiro A, et al. (2010) Efflux pumps, OprD porin, AmpC beta-lactamase, and multiresistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrob Agents Chemother 54: 2219–24.
44. Vettoretti L, Plésiat P, Muller C, El Garch F, Phan G, et al. (2009) Efflux unbalance in *Pseudomonasaeruginosa* isolates from cystic fibrosis patients. Antimicrob Agents Chemother 53: 1987–97.
45. Mulet X, Moyà B, Juan C, Maciá MD, Pérez JL, et al. (2011) Antagonistic interactions of *Pseudomonas aeruginosa* antibiotic resistance mechanisms in planktonic but not biofilm growth. Antimicrob Agents Chemother 55*:* 4560–4568.
46. Vogne C, Aires JR, Bailly C, Hocquet D, Plésiat P (2004) Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Antimicrob Agents Chemother 48: 1676–80.
47. Kaufmann ME (1998) Pulsed-field gel electrophoresis. Methods Mol Med 15: 33–50.
48. Tenover FC, Arbeit RD, Goering RV, Mickelsen A, Murray BE, et al. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol33: 2233–2239.
49. Oh H, Stenhoff J, Jalal S, Wretlind B (2003) Role of efflux pumps and mutations in genes for topoisomerases II and IV in fluoroquinolone-resistant *Pseudomonas aeruginosa* strains. Microb Drug Resist 8: 323–328.
50. Cabot G, Ocampo-Sosa AA, Tubau F, Maciá MD, Rodríguez C, et al. (2011) Overexpression of AmpC and efflux pumps in *Pseudomonas aeruginosa* isolates from bloodstream infections: prevalence and impact on resistance in a Spanish multicenter study. Antimicrob Agents Chemother 55: 1906–11.

EXPERT REVIEWS

The problems of antibiotic resistance in cystic fibrosis and solutions

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Chronic respiratory infection is the main cause of morbidity and mortality in cystic fibrosis (CF) patients. One of the hallmarks of these infections, led by the opportunistic pathogen *Pseudomonas aeruginosa,* is their long-term (lifelong) persistence despite intensive antimicrobial therapy. Antimicrobial resistance in CF is indeed a multifactorial problem, which includes physiological changes, represented by the transition from the planktonic to the biofilm mode of growth and the acquisition of multiple (antibiotic resistance) adaptive mutations catalyzed by frequent mutator phenotypes. Emerging multidrug-resistant CF pathogens, transmissible epidemic strains and transferable genetic elements (such as those encoding class B carbapenemases) also significantly contribute to this concerning scenario. Strategies directed to combat biofilm growth, prevent the emergence of mutational resistance, promote the development of novel antimicrobial agents against multidrug-resistant strains and implement strict infection control measures are thus needed.

Keywords: biofilms • combined treatment • cystic fibrosis • epidemic strains • hypermutation • infection control • multidrug resistance • PK/PD parameters • *Pseudomonas aeruginosa* • sequential treatment

Cystic fibrosis (CF) is the most prevalent autosomal recessive hereditary disease affecting Caucasian populations. Approximately 70,000 people are affected worldwide, but the estimated incidence varies considerably from country to country, within countries and with ethnic background. White populations from Europe, Canada and the USA account for the highest estimated incidences, among whom the disease occurs in 1 in 2500–5000 newborns [1].

CF is caused by mutations disrupting the function of the CF transmembrane conductance regulator (*CFTR*) gene, which encodes a chloride channel that is expressed on the apical surface of many epithelial and blood cells. Since the discovery of the *CFTR* gene, over 1950 different variations have been identified. The most prevalent mutation worldwide is the three-base pair deletion F508del which accounts for approximately two-thirds of all *CFTR* mutations.

The clinical spectrum of CF disease is wide, and depends not only on the *CFTR* genotype but also on other genetic and environmental factors [2]. When CF disease was first recognized in 1938 by Dorothy Hansine Andersen, malnutrition was the leading cause of death among CF patients. The introduction of pancreatic enzyme replacement therapy prompted pulmonary insufficiency to be the first cause of CF morbidity and mortality. Nowadays, approximately 80% of CF-related deaths are associated with chronic lung infection [1].

The respiratory tract of CF children is apparently normal by the time of birth, but soon after, it becomes inflamed and infected. The mechanisms underlying the early acquisition of infection and the establishment of chronic respiratory infection (CRI) are complex. So, several hypotheses have been proposed over the years. Recently, it has been demonstrated that an impaired mucociliary transport is a primary defect in CF, which favors bacterial trapping and persistence in CF lungs [3].

Microorganisms infecting and/or colonizing the CF airway, as well as its frequency vary with CF patients' age. During the initial years, viral pathogens or species such as *Mycoplasma pneumoniae* or *Chlamydophila pneumoniae* are usually involved. Shortly

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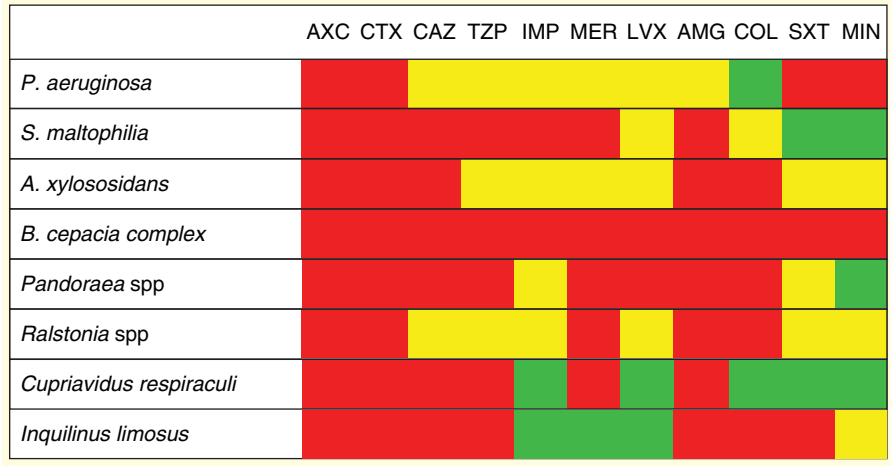

Figure 1. Antimicrobial susceptibility profiles of most frequent non-fermenting Gram-negative rods isolated from cystic fibrosis patients.
Color codes for susceptibility profiles: green: resistance not described or infrequent; yellow: frequently acquired resistance; red: intrinsic resistance or very frequently acquired resistance.
AMG: Aminoglycosides; AXC: Amoxicillin–clavulanic acid; CAZ: Ceftazidime; CTX: Cefotaxime; COL: Colistin; IMP: Imipenem; LVX: Levofloxacin; MER: Meropenem; MIN: Minocycline; SXT: Sulfamethoxazole–trimethoprim; TZP: Piperacillin–tazobactam.

after, common respiratory pediatric pathogens like *Haemophilus influenzae* or *Streptococcus pneumoniae* become predominant; but they are soon replaced by *Staphylococcus aureus* and then by *Pseudomonas aeruginosa*. Eventually, and mainly as a consequence of wide antibiotic use and pulmonary function decline, opportunistic pathogens such as *Achromobacter* spp., *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex (Bcc) or other non-fermenting Gram-negative rods (NFGR) may be isolated [4].

Multidrug-resistant pathogens in CF

Current management of CF respiratory tract infection includes wide use of antibiotics. Obviously, this strategy has reduced CF patients' morbidity and increased their life expectancy [4], but it has also led to the collateral damage by causing an increasing prevalence of multidrug-resistant (MDR) bacteria [5,6]. Methicillin-resistant *S. aureus* (MRSA), MDR *P. aeruginosa* and other intrinsically MDR NFGR are of particular concern (Figure 1).

S. aureus is the pathogen most frequently involved in early respiratory tract infection in CF pediatric patients and its incidence and prevalence has increased over the last years. In 2012, *S. aureus*, including MRSA isolates, was cultured from the respiratory tract samples of 69.0% of CF patients included in the US CF Foundation Patient Registry versus from 55.9% in 2002. Focusing on MRSA the increase is even more worrisome since in 2002 it was only isolated from 9.2% CF patients rising to 26.5% in 2012 [4]. Outside the US, MRSA prevalence rates are considerably lower ranging from 3 to 11% [7]. The mechanism of resistance to methicillin confers resistance to all β-lactams and it is frequently associated with acquired resistance to unrelated antibiotics, such as quinolones and aminoglycosides.

Short after in the course of CF respiratory tract infection, *P. aeruginosa* and other MDR NFGR became the predominant bacterial species infecting or colonizing CF lungs mainly in response to antibiotic pressure.

P. aeruginosa has been the leading cause of respiratory infection in CF patients for decades but it appears that this scenario may be changing and its prevalence may be decreasing. In the USA, the rate of *P. aeruginosa* infection decreased from 57.8% in 2002 to 49.6% in 2012. Nevertheless, MDR *P. aeruginosa* prevalence is increasing and about 10% isolates exhibit resistance to multiple antibiotics [4].

Early infection by *P. aeruginosa* can be intermittent and usually multiple strains with different antibiotic susceptibility profiles are involved. But, eventually, according to the US CF Foundation Patient Registry, by the age of 25, over 70% CF patients are chronically colonized with this pathogen [4] and a single well-adapted strain predominates. *P. aeruginosa* is intrinsically resistant to several antibiotics and has an enormous capability to develop further resistance. So, frequently, this single well-adapted strain exhibits MDR profiles. The estimation of the clinical impact of MDR *P. aeruginosa* is a subject of growing interest and controversy. Some studies reported a significant lung function decline associated with MDR profiles [8]. On the other hand, a recent large multicenter study suggested that MDR is a marker of more severe disease and more intensive antibiotic therapy, but not a primary driver of FEV_1 decline [9]. However, the very loose definition used in this study hinders the estimation of the impact of truly MDR profiles.

Other MDR NFGR frequently isolated from CF lungs include *Achromobacter* spp., Bcc, *Cupriavidus* species, *Inquilinus limosus, Pandoraea* species, *S. maltophilia* and *Ralstonia* species, among others (Figure 1) [6]. The isolation rate of these innately MDR bacteria from the CF respiratory tract is increasing, mainly due to the extensive use of antipseudomonal antibiotics.

Physiological resistance in CF chronic lung infection: role of biofilm

Biofilm growth in CF

Biofilms are defined as organized bacterial communities surrounded by an extracellular polymeric matrix. These structures confer resistance against mechanic clearance, the immune system and antibiotics. In fact, the switch from planktonic to biofilm mode of growth is currently recognized as one of the most relevant drivers of chronic infections, thus playing an important

role in CF [10]. Furthermore, the intrinsic properties of biofilms have significant diagnostic and therapeutic consequences [11].

The architecture of biofilms is complex and is highly influenced by the availability of nutrients and oxygen. The biofilm formation involves three stages: attachment, maturation and dispersal [12]. The biofilm development starts with the adherence of planktonic bacteria to a surface with the help of pili and flagella in Gram-negative bacteria [12] or surface proteins in Gram-positive bacteria [13]. Although most biofilm-related infections generally require an attachment to a solid surface, in the case of CF, some studies indicate that the biofilm found in the lung is directly formed on the mucus instead of being in contact with the lung epithelium [14,15].

Attachment is followed by multiplication of bacteria, thus forming microcolonies and the production of the extracellular polymeric matrix. This matrix plays an important role in the biofilm development not only as a protective barrier against host defense, antibiotics, desiccation or reactive oxygen species (ROS), but also by giving cohesion to the structure and acting as a nutrient source [16]. This physiological barrier is composed of a conglomerate of exopolysaccharides, extracellular DNA (eDNA) proteins, surfactants, lipids, bacterial lytic products and host compounds. In *P. aeruginosa*, one of the most extensively studied exopolysaccharides is the alginate, a polymer of uronic and guluronate, due to its importance in the CF lung. Alginate overproduction is a feature of mucoid strains, a phenotype highly adapted and prevalent in chronic infections. Despite initially considered as a residual material from lysed bacterial and host defense cells [16], currently eDNA has been postulated as an integral part of the matrix [17]. Supporting this theory, it has been observed that DNAse acts by dissolving immature biofilms as well as blocking its initial formation [17].

Finally, some bacteria are released from the biofilm matrix in a dispersal stage. Nonsessile bacteria can thus colonize new return to planktonic phase may responds to biological cues like nutrient limitation and growth rate. Such a tangled process is regulated by intra- and extracellular cues that modulate the levels of diffusible signal molecules, second messengers and small RNAs [18]. Quorum sensing (QS) detects these signals as cell density evidence and triggers changes in bacterial gene transcription, including virulence factors and diverse proteins involved in the innate resistance of biofilms to antibiotics and the immune system. *P. aeruginosa* biofilms are able to initiate detachment on their own; this process can be mediated by alginate lyase overexpression [19] or by the up-regulation of motility factors such as the rhamnolipid and type IV pili [20].

It should be noted that these important insights into the biofilm knowledge have been revealed by *in vitro* models, so it cannot be totally extrapolated to the chronic biofilm infection in CF. The most obvious weakness of *in vitro* models is the absence of the involvement of the immune system. A complex interaction between pathogens and host defense mechanisms determines the altered microenvironment and structure of the *in vivo* biofilm. For instance, NO produced by polymorphonuclear cells (PMNs) leads to oxygen depletion and promotes

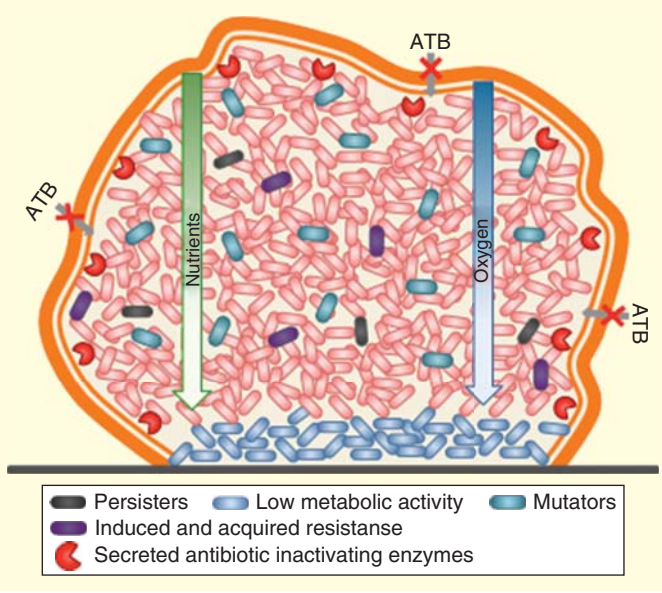

Figure 2. Schematic representation of the factors contributing to inherent biofilm antimicrobial resistance.
ATB: Antibiotics.

growth and persistence driven by denitrification in biofilms [21]. Besides this, *in vivo* biofilm aggregates seem to be smaller and no mushroom structure has been observed on them [22].

Inherent antimicrobial tolerance of biofilms

One of the most relevant aspects of biofilms is that they determine the persistence of the infection despite long-term antimicrobial treatment. Indeed, it is estimated that biofilms can tolerate up to 100–1000 fold higher concentrations of antibiotics than the planktonic cells [11]. Multiple factors contribute to this inherent biofilm antimicrobial resistance (Figure 2).

Antibiotic penetration

The biofilm matrix acts as a primary barrier preventing the entrance of polar and charged antibiotics [23]. Some components of the matrix such as alginate and eDNA show antibiotic chelating activity [24]. Moreover, eDNA also behaves as an antimicrobial shield and contributes to aminoglycoside tolerance [25,26].

Growth rate & nutrient gradients

Internal gradients of biofilms give rise to anaerobic and nutrient-deficient areas, leading to slowing down of the metabolism. The lack of oxygen and the reduced rates of multiplication contribute to the tolerance to fluoroquinolones and aminoglycosides [27]. Moreover, the mucus layers in CF lung are mostly anaerobic, so obligate anaerobes and other pathogens can grow. *P. aeruginosa* can grow in anaerobic or microaerophilic conditions where NO_3^- from PMNs is the final electron acceptor with a lower energetic cost compared to aerobic conditions. Anaerobic biofilms developed in this environment tend to increase alginate production leading to

aminoglycoside tolerance. Furthermore, osmotic stress response may contribute to antibiotic resistance inducing a change in the proportions of porins [10].

Persister phenomenon

Persisters are defined as a dormant phenotypic state of bacteria within biofilms, characterized by a high tolerance to antibiotics. Also, this latent bacterial state behaves as a bumper to host defense and may cause a relapse of infection, being a source of recalcitrant biofilm infection [28].

Induction of resistance mechanisms

Induction of resistance mechanism can significantly differ between biofilm and planktonic growth [29]. Various studies have found a differential expression of several conventional and biofilm-resistance genes in biofilms [30].

Biofilms & mutational resistance

The antibiotic gradient driven by biofilm physiology favors gradual development of mutational resistance during antimicrobial treatment, which is of significance particularly when involving mutator strains which are highly prevalent in CRI [31–33]. Also, endogenous oxidative stress [34] and mutagenic ROS released from PMNs are likely to induce mutability in biofilm cells. In fact, recent findings have shown that mutagenesis is intrinsically increased in biofilms [34,35].

Horizontal gene transfer

Bacterial proximity within a biofilm allows an effective horizontal gene transfer [36]. Moreover, bacterial eDNA may represent a reservoir for the acquisition of exogenous resistance determinants.

Mutational resistance in CF chronic lung infection: role of mutators

Mutational antimicrobial resistance mechanisms in CF pathogens

In addition to intrinsic antibiotic resistance, the antimicrobial susceptibility of bacterial populations can be significantly further compromised by the acquisition of certain chromosomal mutations. This is particularly relevant for the major CF pathogen *P. aeruginosa*, given its extraordinary ability to acquire resistance through mutations that alter the expression and/or function of its chromosomally encoded resistance mechanisms. Although no single mutation can lead to MDR profiles, all antibiotics are prone to being compromised by acquiring mutations that eventually lead to overexpression of efflux pumps, hyperproduction of the chromosomal AmpC cephalosporinase, porin loss or altered antibiotic targets. Table 1 summarizes the most relevant genes involved and the corresponding resistance profiles generated. In addition to these classical resistance mutations, recent whole genome screening mutant libraries reveal a plethora of genes, collectively known as the resistome, which have an impact on antimicrobial susceptibility, including many with central metabolic functions [37].

While it has been shown that mutational resistance can virtually affect all antibiotics, the spontaneous mutation frequencies vary according to the antibiotic agent, the bacterial species and the specific environmental conditions [38]. Moreover, mutational resistance can be significantly enhanced by the presence of mutator phenotypes, which are highly prevalent in CF [39].

Hypermutation and antibiotic resistance in CF

Hypermutable (or mutator) microorganisms are defined as those that have an increased spontaneous mutation rate due to defects in DNA repair or error avoidance systems.

The most frequent cause of hypermutation in natural bacterial populations is the presence of defects on the methyl-directed mismatch repair system. *mutS*, *mutL* and *uvrD* (*mutU*) are the key genes of the methyl-directed mismatch repair system and their inactivation leads to a stable mutator phenotype with an increased rate of mutation from 100- to 1000-fold [40]. Mutations in MutM, MutY and MutT, the three key proteins that compose the GO system, as well as mutations of genes involved in the prevention of oxidative damage produced by ROS, such as *oxyR* and *sodA* (*mutA* and *mutC*) [40] or the recently described *pfpI* [41], also lead to this phenotype.

Under particular circumstances, a transient mutator phenotype can also arise. For instance, bacterial DNA damage induces the SOS response and its error-prone DNA polymerases promote an elevated mutation rate [42] Moreover, some antibiotics can induce a transient mutator phenotype through this mechanism, thus promoting the development of antimicrobial resistance [43–45].

In natural bacterial populations, the presence of the mutator phenotype involves an evolutionary advantage as it can not only enhance mutational resistance but also facilitate bacterial adaptation to new or stressful environments. CRI with *P. aeruginosa* in CF patients represents a major example in nature. Prevalence of mutator *P. aeruginosa* in the CF airways is extremely high, approximately 10–30% of isolates [46], and its presence has been strongly associated with adaptive mechanisms [47] and development of antibiotic resistance [31–33]. The proportion of hypermutable isolates significantly increases during the course of *P. aeruginosa* CRI, as was demonstrated in a 25-year longitudinal study in which the proportion of hypermutable isolates increased from 0% at the onset/early colonization to 65% [48]. Genetic hitch-hiking can explain this observation, which means that mutator alleles reach high frequency by being co-selected with linked beneficial mutations.

Fortunately, CF epidemic strains have not shown an increased prevalence of mutators [49]. Nevertheless, transmission of *P. aeruginosa* hypermutable strains between CF patients has been recently demonstrated [50].

A higher prevalence of mutators in the CF setting has also been noticed for other microorganisms such as *S. pneumoniae*, *H. influenzae*, *S. aureus*, *S. maltophilia* and Bcc [51–56]. Prunier *et al.* found that approximately 14% of CF *S. aureus* isolates were hypermutable in contrast with 1% in non-CF isolates, and that hypermutability was strongly associated with

Table 1. Mutational resistance mechanisms in *Pseudomonas aeruginosa*.

Mutation	Resistance mechanism/altered target	Antibiotics affected							
		TZP	*CAZ*	*CEF*	*IMP*	*MER*	*FQ*	*AMG*	*COL*
gyrA, gyrB	DNA gyrase						•		
parC, parE	DNA topoisomerase IV						•		
pmrAB	Lipopolysaccharide (lipid A)								•
phoPQ	Lipopolysaccharide (lipid A)								•
parRS	Lipopolysaccharide (lipid A) MexXY-OprM hyperproduction OprD porin downregulation			•	•	•	•	•	•
mexR (nalB)	MexAB-OprM hyperproduction	•	•	•		•	•		
nalC	MexAB-OprM hyperproduction	•	•	•		•	•		
nalD	MexAB-OprM hyperproduction	•	•	•		•	•		
nfxB	MexCD-OprJ hyperproduction			•			•		
mexT	MexEF-OprN hyperproduction OprD porin downregulation				•	•	•		
mexS (nfxC)	MexEF-OprN hyperproduction OprD porin downregulation				•	•	•		
mvaT	MexEF-OprN hyperproduction						•		
mexZ	MexXY-OprM hyperproduction			•		•	•	•	
PA5471	MexXY-OprM hyperproduction			•		•	•	•	
ampD	AmpC hyperproduction	•	•	•					
ampD homologues	AmpC hyperproduction	•	•	•					
ampR	AmpC hyperproduction	•	•	•					
dacB	AmpC hyperproduction	•	•	•					
oprD	OprD porin inactivation				•	•			

The bullets indicates which antibiotics are affected by each resistance mechanisms.
AMG: Aminoglycosides; CAZ: Ceftazidime; CEF: Cefepime; COL: Colistin; FQ: Fluoroquinolones; IMP: Imipenem; MER: Meropenem; TZP: Piperacillin–tazobactam.

antibiotic resistance. Similar results were obtained by Román *et al.* for *H. influenzae*. For the emerging CF pathogen *S. maltophilia*, Turrientes *et al.* documented a higher rate of strong mutators in CF patients (17%) versus non-CF patients (3%) and Vidigal *et al.* reported that 31% of CF *S. maltophilia* were strong mutators. Finally, the prevalence of mutators for Bcc isolates has been recently found to be highest among chronically infected CF patients, reaching 40.7% [56].

As evidenced by the unusual high proportion of mutators encountered in chronically colonized CF patients, the CF airway is an ideal environment for mutagenesis. ROS level is increased in CF patients mainly due to an increase in the availability or iron in the CF airways and because the antioxidant mechanisms in CF patients are highly diminished. ROS cause DNA damage and can further increase the inflammatory responses, which eventually lead to the establishment of a vicious cycle of inflammation and hypermutation [57]. The biofilm mode of growth may also increase mutability, as several studies have pointed out [34,35].

Since the first description of hypermutable *P. aeruginosa* strains was made in CF CRI, a strong linkage between mutators and antibiotic resistance has been noticed, as mutators were found to be much more frequently resistant than non-mutators to each of the antipseudomonal agents tested [31]. For instance, the percentage of ceftazidime resistance reached 80% in hypermutable strains in contrast to the 30% observed for non-hypermutable strains and the percentage of fluoroquinolone resistance increased from 5% in non-mutators to 40% in mutators. More recent studies confirmed and extended this observation, establishing a clear link between mutator phenotypes and MDR profiles [33,48,58].

As for *P. aeruginosa*, a strong correlation between hypermutation and mutation-mediated antibiotic resistance has also been observed for *S. aureus* and *H. influenzae* in the CF setting. Prunier *et al.* noted that a high proportion (53%) of the *S. aureus* isolates from CF patients was resistant to erythromycin, and found that more than half of the resistant strains did not contain any acquired macrolide resistance gene but rather

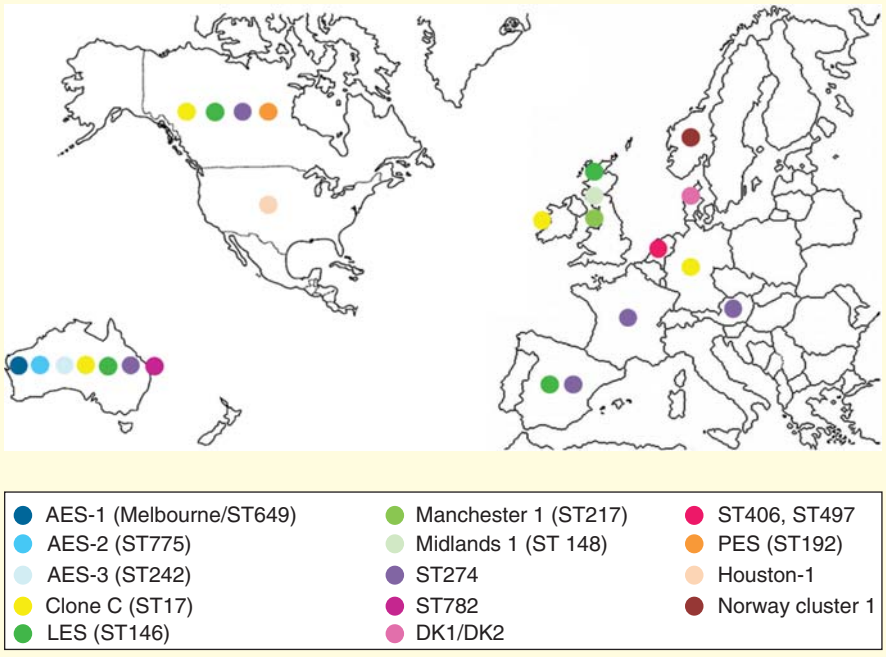

Figure 3. Worldwide distribution of epidemic and transmissible *Pseudomonas aeruginosa* cystic fibrosis strains.
AES: Australian epidemic strain; LES: Liverpool epidemic strain; PES: Prairie epidemic strain.

contained mutations in *rrl* (23S rRNA), *rplD* (L4 protein) or *rplV* (L22 protein); indeed, this high prevalence of mutational macrolide resistance was found to be associated with a high prevalence of hypermutable strains [51]. Recently, it has also been published that linezolid-resistant *S. aureus* can emerge through the accumulation of 23S rRNA mutations linked to the acquisition of a mutator phenotype [59]. Similarly, Román *et al.* found a strong correlation between the high prevalence of hypermutable *H. influenzae* strains in CF patients and the high rates of mutational antibiotic resistance [52].

In addition to the clear statistical link obtained between hypermutation and antibiotic resistance from the analysis of collections of clinical CF isolates, several *in vitro* (planktonic and biofilm) and *in vivo* experiments further highlight the strong linkage between hypermutation and antibiotic resistance [60–65].

Transmissible resistance in CF: role of MDR epidemic strains

MDR epidemic strains in CF

For a long time, it was extensively accepted that acquisition of CF pathogens occurs from the environment, so each patient generally harbors his/her own unrelated strain. Although evidence of *P. aeruginosa* cross-infection among CF siblings existed, before the mid-1980s, there was no proof of spread of epidemic strains. But this classical perception changed in 1986 when an outbreak of *P. aeruginosa* resistant to aminoglycosides, carbenicillin, ureidopenicillins, ceftazidime, cefsulodin and imipenem in a CF center in Denmark was published [66]. Shortly after, ribotype analysis of *B. cepacia* from CF centers evidenced that the transmissible strains not only were those of *P. aeruginosa* [67].

Due to the rapid increase of *Burkholderia cenocepacia* infection (formerly Bcc genomovar III) among CF patients and its poor prognosis, attention was focused on this pathogen. An intercontinental epidemic lineage, the ET-12, was soon identified infecting patients from the UK and Ontario in Canada [68]. Since then, numerous transmissible *B. cenocepacia* lineages have been reported worldwide, and evidence of superinfection with epidemic strains in previously colonized patients has been provided [69]. The global epidemiology of *B. cenocepacia* is now better understood thanks to the introduction of Multi Locus Sequence Typing. Analysis of isolates from the epidemic lineage ET-12 has revealed that they belong to at least five different sequence types (STs), with only ST-28 representing the intercontinental ET-12 clone spread in the UK and Canada [70].

P. aeruginosa epidemic and transmissible strains have been recently reviewed by Fothergill *et al.* [71]. The Liverpool Epidemic Strain was first described affecting a unique CF center, but some time later, this strain was detected in other CF centers across the UK and, eventually, it has also been detected to infect CF patients in Canada and Spain [50]. The Liverpool Epidemic Strain isolates develop antibiotic resistance more frequently than the other CF strains, and resistance is more likely to develop over time. A worse prognosis is predicted for patients colonized with the Liverpool Epidemic Strain, and superinfection of patients already colonized with *P. aeruginosa* strains has also been reported; thus, strict patient segregation policies are to be implemented. In Australia, some transmissible strains have also been detected; these are the so-called Australian epidemic strain-1, Australian epidemic strain-2 and a cluster of related strains. Australian epidemic strain-1 exhibits increased antibiotic resistance and increased virulence gene expression during chronic infection. These and other *P. aeruginosa* MDR transmissible strains have been reported worldwide [72,73] are represented in Figure 3.

To date, few studies have investigated the genetic background and transmissibility of MRSA strains in the CF population. In 2006, a heterogeneous glycopeptide-intermediate phenotype of resistance infecting a long cohort of CF patients was detected in France, which alerts that transmissible strains could also exist among MRSA CF strains [74]. Shortly after, a Spanish study demonstrated the presence of a predominant clone among their CF patients, the hospital-acquired MRSA ST228, suggesting either cross-transmission or a common environmental source. This clone, also prevalent among the circulating clones of MRSA in Spain, exhibited MDR, presented

SCCmec type 1 and was highly persistent [75]. Nevertheless, an increasing prevalence of community-acquired MRSA among CF patients, including panton valentine leukocydin-positive strains, has been observed [76–78]. Recently, a multicenter Italian survey showed a high prevalence (31.4%) of SCCmecIV. Most of these community-acquired MRSA strains (73%) belonged to known epidemic lineages Globally spread being the ST8-MRSA-IV (genetic signature of the American lineages USA500 and USA300) the most frequent [79].

Transferable resistance determinants in CF isolates

The CF airway hosts a complex microbiome [80] where genetic exchange could occur effectively, thus contributing to the emergence of antibiotic resistance. Most mobile antibiotic resistance genes are encoded on plasmids and transposons, but recent studies suggest that phages may also play an important role in the CF airway environment as the CF virome encodes more antimicrobial resistance sequences than the non-CF virome [81]. Phages also appear to be essential for the adaptation of some successful *S. aureus*, *B. cenocepacia* and *P. aeruginosa* CF strains, which supports this idea [74,82,83].

Among the transferable resistance determinants, extended-spectrum β-lactamases and carbapenemeses are widely distributed worldwide. Although this resistance mechanism seems not to be frequent among CF isolates, several reports have been published recently. In 2006, VEB-1 producing *Achromobacter xylosoxidans* was detected in a CF patient in France [84] and previously, the isolation of three non-characterized extended-spectrum β-lactamases–positive *P. aeruginosa* from CF patients in New Delhi had been reported [85]. Transferable carbapenemases have also been detected among CF isolates, including *P. aeruginosa* producing IMP and VIM metallo-β-lactamases [86,87] and *K. pneumoniae* producing KPC-2 carbapenemase [88].

Current & future antimicrobial therapy strategies to combat resistance

As discussed in previous sections, antibiotic resistance due to the increasing prevalence of MDR pathogens and also due to the physiological, mutational and transmissible mechanisms represents one of the major causes of therapeutic failure in CF patients. Depending on the respiratory infection stage and the microorganisms involved, different therapeutic strategies are chosen. Prophylaxis is still controversial and early eradication is generally attempted with aggressive treatment at the first culture of *P. aeruginosa*, *S. aureus* and MRSA, with the objective of preventing CRI [89].

Classical systemic & inhaled therapy in CF

During CRI, CF patients experience a progressive decline of lung function correlating with strain mucoid conversion and phenotypic diversification, biofilm formation and resistance development. In this stage, the bacterial mass increases which leads to periodic flare-ups of respiratory symptoms known as pulmonary exacerbations. Frequently, exacerbations are associated with a poorer quality of life and an increased mortality. CRI eradication is rarely achieved, especially in *P. aeruginosa* chronically colonized patients, and therefore, the aim of antimicrobial treatment is the reduction of the bacterial load and, thus, the inflammatory response. Success in bacterial reduction is microbiologically defined as a decrease of, at least, two logarithms in bacterial counts on comparing two consecutive cultures.

Traditionally, intravenous antibiotics are used to treat exacerbations. The classical strategy consists of combining two agents from different antimicrobial classes to enhance the treatment effect and prevent the emergence of antimicrobial resistance. For instance, for *P. aeruginosa* treatment, a combination of an aminoglycoside or a fluoroquinolone and an antipseudomonal β-lactam at high doses is typically used. Colistin sulphomethate has also shown efficacy on intravenous administration, alone or in combination [90]; however, it is generally reserved for MDR strains or in cases of therapeutic failure. On the other hand, inhaled therapies are the treatment of choice in suppressive or maintenance therapy during CF CRI, in the absence of exacerbations. Administration of antibiotics by inhalation has demonstrated to be safe and effective due to the high concentrations that reach the infection site (pulmonary epithelia) with a very low systemic effect. Since eradication cannot be achieved, these strategies are based on chronic suppressive therapy (colistin) or administered as a 28-day course (on–off) (tobramycin or aztreonam-lysine [AZLI]). Nebulized administration of sodium colistimethate has demonstrated efficacy for the treatment of *P. aeruginosa* CRI, with the normal dose in adults being 0.5–2 millions of international units, two- or three-times a day, administered without off periods. In the case of tobramycin, the recommended dosage by inhalation is 300 mg twice a day, alternating 4 weeks on–off cycles. Several clinical studies have shown that AZLI is a safe and effective treatment for use in CF patients and recommend the use of an on–off 28-day course of AZLI (75 mg, three-times daily) [89].

Pharmacokinetic & pharmacodynamic approach to antimicrobial therapy in CF

Dosing regimens are based on the changes in the concentration of the antibiotic during the course of treatment (pharmacokinetics [PK]) and on the *in vitro* relationship between antibiotic concentration and the growth or death rate of the targeted bacteria (pharmacodynamics [PD]). These factors comprise the PK/PD indices [91], which are used to estimate the potential efficacy of antibiotic treatment regimens.

Aminoglycoside and fluoroquinolone antibiotics exhibit a concentration-dependent activity. The PK/PD parameters that better predict their activity are Cmax/MIC or area under the curve (AUC_{24h})/MIC. These antibiotics are used at high doses and their prolonged post-antibiotic effect, which is defined as the time that the bacteria need to recover their normal growth, allows using them at long dosage intervals. A Cmax/MIC of ≥10–12 for aminoglycosides predicts their efficacy. In the case of fluoroquinolones, an AUC_{24h}/MIC value >125 is thought to

predict therapeutic success and values above 157 have shown to suppress resistance according to mathematical models [92].

Activity of β-lactam antibiotics is time-dependent; thus, to achieve an optimal therapeutic effect, concentrations should be over MIC for a long period of time. The PK/PD parameter T > MIC is required to be at least 40–50% of the dosage interval of administration; this condition is reached with intravenous perfusion for some antibiotics (piperacillin/tazobactam, ceftazidime, cefepime, meropenem or doripenem) or by inhalation three-times a day in the case of aztreonam.

These PK/PD parameters are based on the general susceptible bacterial population. Regarding the selection of resistant mutants, the concentrations of antibiotic that would prevent the selection of single-step resistant mutants are represented by a parameter known as mutant prevention concentration (MPC), which is defined as the MIC of the least susceptible single-step mutant. Actually, the antimicrobial concentration range extending from the MIC of the general population and the MPC is known as the mutant selection window. Antimicrobial concentrations placed inside this window are expected to select the resistant mutant subpopulations, whereas concentrations above this window are expected to restrict selective enrichment.

In this sense, it would be interesting to consider the MPC/MIC index to define the low or high capacity of antibiotics to select resistant mutants. Similarly, as efficacy should refer not only to obtain an optimal clinical response but also to minimize the selection of resistant subpopulations, PK/PD traditional parameters commented above should be also adapted, for example, using AUC_{24h}/MPC for fluoroquinolones or Cmax/MPC for aminoglycosides instead of AUC_{24h}/MIC or Cmax/MIC, respectively.

Current treatment strategies tend to take into account the knowledge of PK/PD. Administration of antibiotics through inhalation minimizes systemic toxicity while reaching high concentrations in the lung epithelia, generally above the MPC values. For instance, in the case of tobramycin, serum concentration after inhalation is under 1 mg/l, whereas it reaches 1200 mg/l in the sputum. Moreover, administration twice a day helps to take advantage of its post-antibiotic effect. On the contrary, inhaled β-lactams, such as AZLI, need to be administered in concentrations over the MIC for long time intervals (T > MIC ≥40–50% dosage interval), with administration of three-times daily being more favorable. Inhaled formulations have also expanded on fluoroquinolones, such as ciprofloxacin and levofloxacin, which are currently in Phase II and III clinical trials, respectively.

Similarly, the on–off 28-day course is based on reaching high concentrations that reduce the bacterial load for a long term (on) and then let susceptible subpopulations grow (off) at the expense of the resistant mutant subpopulations, without selective pressure. Nevertheless, as evidences show that the beneficial effects diminish during off periods, other strategies, such as combination or alternation of inhaled antibiotics, are now being explored.

Combined & sequential treatments

Combinations of antibiotics are routinely used in the treatment of CF pulmonary infection with the aim of preventing or delaying the onset of resistance. Multiple combination bactericidal testing has been shown to help to choose combinations of antimicrobials with higher levels of *in vitro* bactericidal activity, especially in *P. aeruginosa* [93] and Bcc [94]. The impact of multiple combination bactericidal testing on clinical outcome remains, however, controversial and further prospective multicenter studies are required [95,96]. Nevertheless, based on *in vitro* and *in vivo* studies, efficient combinations have been identified [97,98]. An interesting combination of inhaled formulations of a 4:1 (w/w) of fosfomycin/tobramycin was recently under research. This combination has shown to be effective *in vitro* against both Gram-negative and Gram-positive pathogens and has also shown increased activity under anaerobic conditions [99].

Another approach to prevent or delay the onset of resistance may be the use of sequential treatments, for instance, those based on antagonistic resistance mechanisms. Treatment with aminoglycosides often involves the selection of mutants that overexpress the MexXY-OprM efflux pump being frequently related to the inactivation of MexAB-OprM. Taking into account this premise, theoretically, treatment with MexXY-OprM substrates (such as tobramycin) could lead to hypersusceptibility to MexAB-OprM substrates (such as aztreonam). So, sequential treatment with tobramycin followed by aztreonam would entail a clinical benefit by improving the therapeutic efficacy and diminishing the selection of resistant mutants. This was the objective of a recent work [Rojo-Molinero E, Macià MD, Oliver A, unpublished data] where sequential therapies with inhaled antibiotics were found to be superior to individual treatments. Results from this study could support the introduction of sequential regimens with inhaled antibiotics in CF patients' therapy. Furthermore, a double-blind, placebo-controlled multicenter study that used fosfomycin/tobramycin and AZLI suggested that continuous alternating therapy of different inhaled antibiotic therapies could be of benefit in CF patients; unfortunately, this study was lately suspended. Nevertheless, further investigation based on clinical trials is required.

Antimutator strategies

As addressed in the previous sections, hypermutable strains are highly prevalent in CF CRI. Mutation-mediated resistant mechanisms can affect almost all kinds of antipseudomonal antibiotics, including β-lactams, fluoroquinolones and aminoglycosides. However, colistin (as a representative of polymyxins) might apparently be an exception to the strong linkage of mutators with antibiotic resistance in CRI [100], and it is therefore frequently used as last-resource option for the treatment of infections by MDR strains. Similarly, the Phase III antipseudomonal cephalosporin ceftolozane (former CXA-101) is apparently stable to most mutation-driven *P. aeruginosa* β-lactam resistance mechanisms either in planktonic or biofilm growth [64]. Likewise, *in vitro* and *in vivo* studies have shown

that it is possible to suppress resistance due to selection of resistant mutant subpopulations by using appropriate combined regimens [60,61,101].

In addition to methyl-directed mismatch repair–deficient strains, mutator lineages generated by inactivation of the GO system also trigger development of antibiotic resistance, particularly when exposed to conditions promoting oxidative DNA damage as occurs in the CF lungs [102]. Oxidative stress has an important role in the increased mutability of biofilm-growing bacteria, thus contributing to bacterial diversification and development of antibiotic resistance. The addition of antioxidants such as L-proline, *N*-acetylcysteine, β-carotene and L-cysteine has been shown to decrease the resistance of 5-day-old *P. aeruginosa* biofilms to tobramycin *in vitro* [35]. *N*-acetylcysteine has long been used in patients with CF as a mucolytic agent and also as an anti-inflammatory drug. Although clinical trials are required to evaluate its usage as an enhancer of antibiotic activity on biofilms, combination of *N*-acetylcysteine and some antibiotics seems promising.

Old & new therapeutic options directed to treat MDR pathogens

The challenge of MDR has driven to a revival of forgotten antimicrobial agents such as fosfomycin and colistin. Fosfomycin seems to be an effective antibiotic that is used intravenously and in combination with other antibiotics to treat resistant bacteria, including MDR *P. aeruginosa* in CF patients [103]. This antibiotic has a broad-spectrum bactericidal effect by inhibition of the initial step in cell wall synthesis, and also reaches good concentration levels in lungs. Moreover, fosfomycin may have an added benefit, conferring protection against nephrotoxicity [104] and ototoxicity [105]. Colistin shows excellent *in vitro* activity against Gram-negative bacteria and *in vivo* efficacy against MDR carbapenemase-producing microorganisms. The disadvantage of colistin is its toxicity, which is reduced by administering colistimethate sodium via inhalation. In spite of the extensive experience with inhaled colistimethate sodium in CF, more studies are needed to explore the effect in combination with intravenous antibiotics to treat multidrug resistance therapy. In conclusion, these old drugs have significant advantages including a low rate of resistance, good activity both *in vitro* and *in vivo* against MDR pathogens, known toxicity and lower cost compared to new agents.

Despite the efforts taken to overcome MDR with the available drugs, there is an imperative need for discovering new antimicrobial agents. Presently, there are some antimicrobials active against resistant Gram-negative bacteria in advanced stage of clinical development (Phase II or III). Most of these new agents are β-lactam/β-lactamase inhibitor combination products that act by inhibiting the β-lactamases so that the partner antibiotic can interfere with cell wall synthesis. One of them, ceftolozane/tazobactam, currently in Phase III, has demonstrated an excellent activity against *P. aeruginosa*. In addition, the development of high-level resistance to ceftolozane/tazobactam is much slower compared to other antibiotics, and appears to occur efficiently only in mutator background [106]. BAL30072 is a siderophore monosulfactam with an impressive activity against *P. aeruginosa* and *B. cepacia* and has shown a powerful synergistic activity in combination with meropenem [107]. Further, some of these new drugs active against resistant Gram-negative rods have also shown *in vitro* potency versus MRSA, for example, ceftaroline/avibactam and eravacycline, a broad-spectrum fluorocycline. Nevertheless, these achievements will only partial solve the antimicrobial resistance threat, and new drugs with novel mechanisms of action are still needed.

Targeting biofilms

Targeting biofilm arises as an attractive alternative approach to classic therapy since remarkable differences in antimicrobial response have been demonstrated between planktonic and biofilm modes of growth.

Biofilm-guided antibiotic therapy

Antimicrobial susceptibility testing studies performed on biofilm growing bacteria have helped to identify antibiotics that show selective anti-biofilm activity [108]. Such is the case of macrolides against *P. aeruginosa*. Although according to the standard antimicrobial susceptibility testing, azithromycin has no activity against *P. aeruginosa*, this macrolide exhibits bactericidal activity on biofilms [63]. Azithromycin inhibits biofilm growth likely due to its interaction with the QS system implicated in the production of alginate and other virulence factors such as rhamnolipid, elastase, protease and chitinase [109]. Despite this good activity on biofilms, resistant mutants are readily selected, particularly for hypermutable strains. The resistance mechanism selected, the overexpression of MexCD-OprJ, also confers resistance to ciprofloxacin or cefepime and, on the contrary, turns the strains hypersusceptible to aminoglycosides. Then, it is important to optimize the selection of appropriate antipseudomonal therapies in patients undergoing azithromycin maintenance treatment. Finally, macrolides have demonstrated synergy with other antibiotics against multidrug-resistant CF pathogens such as *B. cepacia, A. xylosoxidans* and *S. maltophilia* [110].

Some studies applying PK/PD models that theoretically predict therapeutic success have been developed using *in vitro* and *in vivo* models to mimic the CRI setting and biofilm development. For example, a *P. aeruginosa* flow cell biofilm model that employed a concentration of 2 g/ml of ciprofloxacin, which correlated with the MPC and provided an AUC_{24h}/MIC ratio of 384 that should predict therapeutic success, was used, which demonstrated, nevertheless, that theoretically optimized PK/PD parameters failed to suppress resistance development on biofilms [65]. The results from this study suggested that the increased antibiotic tolerance driven by the special biofilm physiology and architecture probably raised the effective MPC, favoring gradual mutational resistance development, especially in mutator strains. Actually, the exposure of biofilm-grown cells to sub-inhibitory concentrations of antibiotics may not only fail to eradicate the biofilm but may even promote or enhance biofilm formation. Likewise, results of other PK/PD models of *P. aeruginosa* biofilm treatment studies showed an alteration of

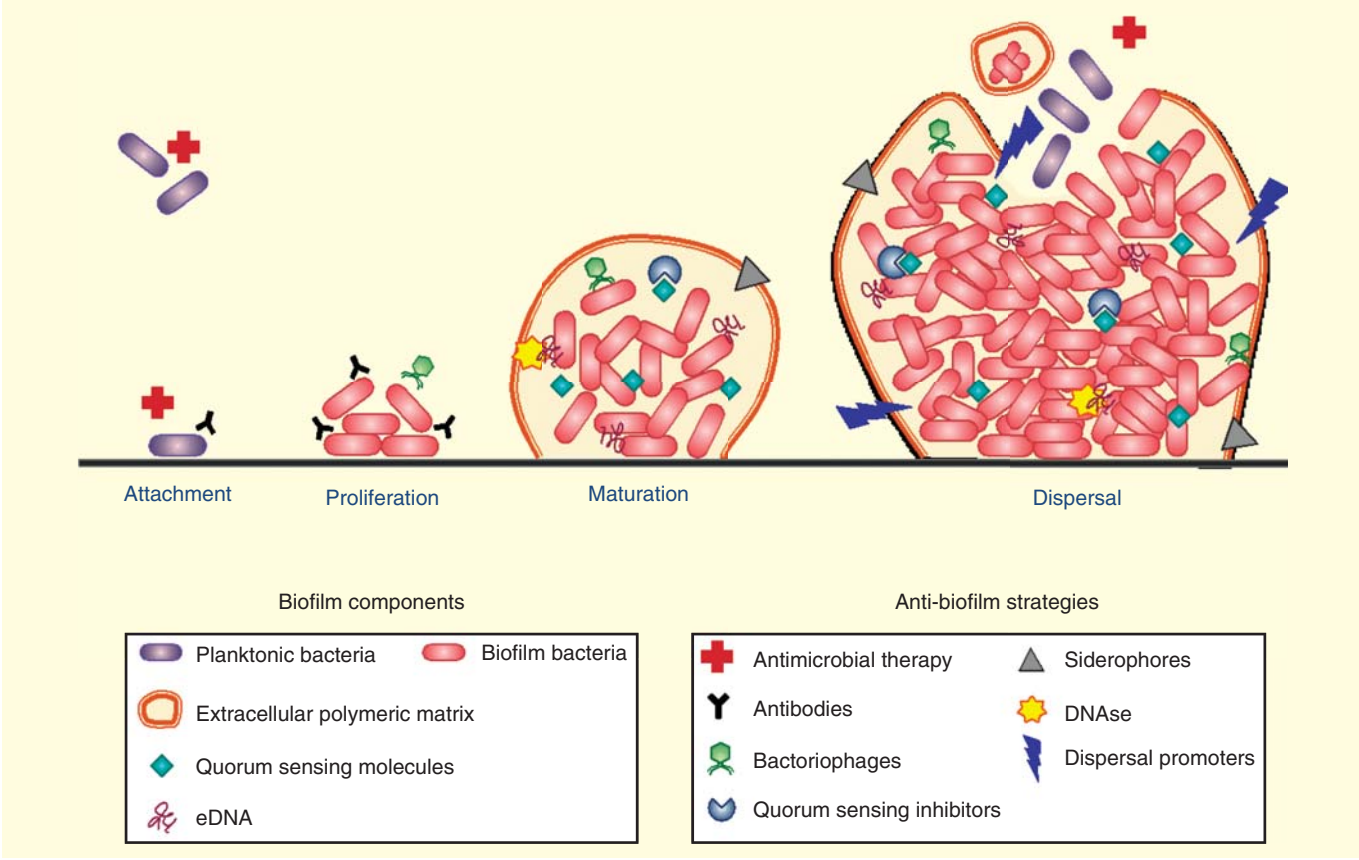

Figure 4. Schematic representation of the different anti-biofilm strategies proposed.
eDNA: Extracellular DNA.

expected PK/PD antibiotic parameters when acting on biofilms. In the study of Hengzhuang *et al.*, β-lactam antibiotics showed time-dependent killing and ciprofloxacin, colistin and tobramycin showed concentration- or dose-dependent killing being then similar to planktonic growth [111]. However, the concentrations of antibiotics needed were, in all cases, very much higher even in the case of time-dependent killing, where on β-lactamase–overproducing strains, the killing pattern of ceftazidime was changed to concentration-dependent killing for biofilm cells. These results alert us of the complexity of mechanisms taking place on *P. aeruginosa* CRI and the difficulty to predict therapeutic efficacy even applying optimal PK/PD parameters.

Anti-biofilm strategies: new alternatives to classic antimicrobial therapy

In recent years, significant efforts have been made trying to elucidate new therapeutic approaches. Some of the different strategies against biofilms are presented below and in Figure 4.

Avoiding biofilm formation

The inhibition of molecules involved in the attachment process seems to be a good approach for this objective. This could be achieved by using specific neutralizing antibodies against flagella, pili, eDNA and exopolysaccharides.

Avoiding biofilm maturation

At this point, the strategies should be directed to weakening of the formed biofilm, mainly targeting the virulence factors, eDNA, QS, small RNAs and iron metabolism. The problem is that during the maturation phase, biofilm loses most of the virulence factors and no drugs have indicated activity in this stage [24].

DNAse

The inhaled recombinant human DNase has shown clinical efficacy in CF patients. This enzyme reduces the viscosity of mucus not only by clearing the DNA released from PMNs but also by dissolving preformed biofilms [17] and facilitating the effects of aminoglycosides [27].

Alginate lyase

The co-administration of inhaled alginate lyase with antibiotics degrades alginate from the extracellular polymeric matrix leading to the elimination of mucoid bacteria from the biofilms [112].

Bacteriophages

Bacteriophage therapy is becoming an attractive co-adjuvant of antibiotics. Phages are able to break through the extracellular

polymeric matrix and reach bacteria within the biofilm, starting a lytic process inside them. The problem is that bacteriophages are species-specific and make the isolation of bacteria necessary to select the suitable phage. Also, bacteria can develop resistance to the lytic activity of phages [113]. Regardless of this, some studies using bacteriophages against *P. aeruginosa* have demonstrated efficacy [114,115].

Iron metabolism

Since iron availability shows up as a critical factor in biofilm formation, chelating agents may be a good option to eliminate the biofilm. The strategy is to take advantage of the iron transport system to introduce inactive metal ions (Sc^{3+}, In^{3+} or Ga^{3+}) or antibiotics conjugated with a siderophore. Several compounds have been shown to interfere with biofilm formation *in vitro*, but most of them have only been effective on abiotic surfaces. Gallium nitrate formulated for inhalation has shown effectiveness in chronic airway infection in animal models [116], although gallium-resistant *P. aeruginosa* mutants have been reported [117]. Also, lactoferrin, a human iron-binding protein, seems to impair biofilm formation in respiratory infections by stimulation of twitching motility [118].

Virulence factors

The development of antibodies against β-lactamase observed in CF [119] supports the development of specific antibodies or substances that bind virulence factors as a good strategy to fight biofilm maturation. The disadvantage of this approach is that only single virulence factors are targeted and they are species- or even strain-specific. Also, there is a risk of inducing immunopathology as a result of increased inflammation owing to an immune complex–mediated reaction.

Quorum sensing inhibitors

Since QS plays a major role in biofilm formation and regulation of the expression of virulence factors, there is an emerging interest in the research of new molecules able to block the QS pathway. The advantage of quorum sensing inhibitors (QSIs) over antibiotics is that the development of resistance is minimized since these molecules target the virulence factor instead of bacterial growth [120]. However, the theoretical benefits of these molecules should be considered with caution since resistance to QSIs has been recently reported in literature [121,122]. Effective QSIs can be found in nature among the secondary metabolites produced by algae, sponges, fungi, food products and higher plants [120]. For example, natural halogenated furanones, solenopsin A, manoalide and its derivate, garlic, patulin and gingseng have shown activity against pathogens implicated in biofilm chronic infections. Nevertheless, activity of these compounds has only been assessed in experimental studies; hence, further clinical studies are needed to establish their efficacy and safety to truly introduce them in the treatment of CF patients.

Driving biofilm dispersal

When the biofilm is mature, some bacteria are released from the biofilm matrix, probably due to the lack of nutrients. Planktonic floating bacteria are more susceptible to antibiotics; taking advantage of this, an adequate antimicrobial treatment with the compounds that promote biofilm disruption could achieve a higher therapeutic success. Studies have investigated the effect of different molecules in the dispersal of biofilms and have obtained promising results. For example, unsaturated fatty acids [123], nitric oxide [124], succinic acid, citrate and compounds that interfere with c-di-GMP levels [125] have shown activity *in vitro*. Curiously, 2-aminoimidazole/triazole may resensitize MDR strains to the effects of conventional antibiotics, apart from its ability to inhibit and disperse biofilms [126].

Infection control in CF

The incorporation of appropriate control measures is one of the most effective available strategies to prevent early infection and transmission of epidemic strains between patients. In the recent past, the use of molecular typing methods has led to an improved understanding of the epidemiology of CF pathogens, highlighting the relevance of epidemic strains among CF patients, described above. In most cases, the initial source of microorganism acquisition is unknown, although the environment seems to be an important reservoir for CF pathogens.

In order to reduce the days of hospitalization and also to improve the quality of life of CF patients, there has been a shift in healthcare delivery from hospitals to ambulatory and home settings. Even though specific measures should be implemented in this new scenario, the lessons learned about transmission of nosocomial pathogens can be applied as infection control strategies for CF.

The American CF Foundation in its last update of infection prevention and control guidelines for CF [127] sets recommendations to prevent transmission, taking into account that all CF patients should be treated as potential transmitters of pathogens.

The most important preventive measure is hand hygiene in healthcare and non-healthcare settings. To prevent droplet transmission, all CF patients, regardless of the respiratory tract culture results, should be separated by at least a distance of 3–6 feet, which has been proposed as the minimal distance to avoid transmission. While the use of mask, gloves and gown is only required for staff in the case of colonization by MDR or epidemic transmissible strains, people with CF should be required to wear a mask in hospitals to reduce the risk of transmission, or even to reduce the acquisition of new pathogens. In particular, CF clinics should schedule and manage patients to avoid contact, minimizing the waiting time in common areas and segregating the patients with MDR or epidemic transmissible strains. In the same way, summer camps and other group activities are not encouraged.

Surfaces and respiratory therapy equipments have to be assiduously cleaned and disinfected to reduce contamination of environmental sources (following institutional policies of control of MDR pathogens). To reduce transmission by medical equipment, the use of single-patient disposable items should be

facilitated. Moreover, it is recommended to clean and disinfect the exam rooms between patients.

Surveillance reports are a valuable tool to document the incidence and prevalence of MDR pathogens, and to review the acquisition of epidemic transmissible strains. The molecular typing of CF pathogens, integrated as a routine in the microbiological diagnosis of respiratory infection, can be used as a marker of success of infection control strategies.

Expert commentary & five-year view

Antimicrobial resistance in CF is a multifactorial problem which includes aspects related to bacterial physiology (development of biofilms), genetic evolution (acquisition of antibiotic resistance mutations linked to mutator phenotypes) and epidemiology (such as the acquisition of infections by MDR pathogens or epidemic strains). Therefore, our present and future strategies should target these key aspects. Among them, the initiatives directed to prevent and/or disrupt biofilms, exemplified by research on QSIs, are particularly encouraging. Likewise, strategies intended to avoid mutational resistance, such as the optimization of PK/PD parameters, innovative combined and sequential regimens, or the use of 'antimutator' adjuvants, are promising as well. Finally, despite being insufficient, novel compounds currently under clinical development will mitigate our needs for the treatment of MDR strains to some extent, but strict infection control measures will remain a key issue.

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Key issues

- The isolation of multidrug resistant (MDR) pathogens from the cystic fibrosis (CF) respiratory tract is increasing, mainly due to the extensive use of antibiotics.
- Biofilm growth is an efficient adaptive strategy for survival and persistence of bacteria in the CF lungs due to its inherent tolerance to the immune system and antibiotics.
- Mutators are highly prevalent in CF chronic respiratory infection and play a major role in resistance development.
- Epidemic and transmissible *Burkholderia cepacia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains have been identified infecting CF patients and frequently show MDR profiles.
- The CF airway hosts a complex microbiome in which genetic exchange can occur contributing to development of resistance.
- Treatments should be based on pharmacokinetic/pharmacodynamic parameters and on the pathogen resistance mechanisms; combined and sequential inhaled antibiotic treatments seem to be a promising alternative.
- Targeting the biofilms and the antimutator strategies arise as innovative approaches to overcome the current lack of effective antimicrobial treatments.
- Strict infection control measures are required to prevent the inter-patient transmission of MDR and epidemic strains.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1. O'Sullivan BP, Freedman SD. Cystic fibrosis. Lancet 2009;373(9678):1891-904
2. Hampton TH, Green DM, Cutting GR, et al. The microbiome in pediatric cystic fibrosis patients: the role of shared environment suggests a window of intervention. Microbiome 2014;28:2-14
3. Hoegger MJ, Fischer AJ, McMenimen JD, et al. Cystic fibrosis. Impaired mucus detachment disrupts mucociliary transport in a piglet model of cystic fibrosis. Science 2014;345(6198):818-22
4. Cystic fibrosis foundation patient registry. 2012 Annual data report. Cystic Fibrosis Foundation; Bethesda, MD
5. Emerson J, McNamara S, Buccat AM, et al. Changes in cystic fibrosis sputum microbiology in the United States between 1995 and 2008. Pediatr Pulmonol 2010; 45(4):363-70

• **Changes in the prevalence of common cystic fibrosis (CF) pathogens and their antibiotic resistance profiles were studied by comparing a contemporary (2008) and an historical cohort (1995) of CF patients.**

6. LiPuma JJ. The changing microbial epidemiology in cystic fibrosis. Clin Microbiol Rev 2010;23(2):299-323
7. Goss CH, Muhlebach MS. Review: staphylococcus aureus and MRSA in cystic fibrosis. J Cystic Fibros 2011;10(5):298-306
8. Lechtzin N, John M, Irizarry R, et al. Outcomes of adults with cystic fibrosis infected with antibiotic-resistant Pseudomonas aeruginosa. Respiration 2006; 73(1):27-33
9. Ren CL, Konstan MW, Yegin A, et al. Multiple antibiotic-resistant Pseudomonas aeruginosa and lung function decline in patients with cystic fibrosis. J Cyst Fibros 2012;11(4):293-9
10. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. Lancet 2001;358(9276):135-8

11. Høiby N, Bjarnsholt T, Givskov M, et al. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents 2010;35(4):322-32

- **An excellent review that focused on the antimicrobial resistance mechanisms of *Pseudomonas aeruginosa* biofilms.**

12. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol 2000;54:49-79
13. Cucarella C, Solano C, Valle J, et al. Bap, a Staphylococcus aureus surface protein involved in biofilm formation. J Bacteriol 2001;183(9):2888-96
14. Bjarnsholt T, Jensen PØ, Fiandaca MJ, et al. Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol 2009;44(6): 547-58
15. Worlitzsch D, Tarran R, Ulrich M, et al. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. J Clin Invest 2002;109(3):317-25
16. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol 2010;8(9): 623-33
17. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science 2002;295(5559):1487
18. Bjarnsholt T, Ciofu O, Molin S, et al. Applying insights from biofilm biology to drug development - can a new approach be developed? Nat Rev Drug Discov 2013; 12(10):791-808
19. Boyd A, Chakrabarty AM. Role of alginate lyase in cell detachment of Pseudomonas aeruginosa. Appl Environ Microbiol 1994; 60(7):2355-9
20. Pamp SJ, Tolker-Nielsen T. Multiple roles of biosurfactants in structural biofilm development by Pseudomonas aeruginosa. J Bacteriol 2007;189(6):2531-9
21. Kolpen M, Bjarnsholt T, Moser C, et al. Nitric oxide production by polymorphonuclear leucocytes in infected cystic fibrosis sputum consumes oxygen. Clin Exp Immunol 2014;177(1):310-19
22. Bjarnsholt T, Alhede M, Alhede M, et al. The in vivo biofilm. Trends Microbiol 2013;21(9):466-74
23. Lewis K. Multidrug tolerance of biofilms and persister cells. Curr Top Microbiol Immunol 2008;322:107-31
24. Alipour M, Suntres ZE, Omri A. Importance of DNase and alginate lyase for enhancing free and liposome encapsulated aminoglycoside activity against Pseudomonas aeruginosa. J Antimicrob Chemother 2009;64(2):317-25
25. Chiang WC, Nilsson M, Jensen PØ, et al. Extracellular DNA shields against aminoglycosides in Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 2013;57(5):2352-61
26. Mulcahy H, Charron-Mazenod L, Lewenza S. Extracellular DNA chelates cations and induces antibiotic resistance in pseudomonas aeruginosa biofilms. PLoS Pathog 2008;4:11
27. Walters MC, Roe F, Bugnicourt A, et al. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of Pseudomonas aeruginosa biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother 2003;47(1):317-23
28. Lewis K. Persister cells. Annu Rev Microbiol 2010;64:357-72
29. Mulet X, Moya B, Juan C, et al. Antagonistic interactions of Pseudomonas aeruginosa antibiotic resistance mechanisms in planktonic but not biofilm growth. Antimicrob Agents Chemother 2011;55(10): 4560-8
30. Whiteley M, Bangera MG, Bumgarner RE, et al. Gene expression in Pseudomonas aeruginosa biofilms. Nature 2001; 413(6858):860-4
31. Oliver A, Canton R, Campo P, et al. High frequency of hypermutable Pseudomonas aeruginosa in cystic fibrosis lung infection. Science 2000;288(5469):1251-4

- **First study demonstrating a high prevalence of mutators in CF chronic respiratory infection and their linkage to antimicrobial resistance.**

32. Macià MD, Blanquer D, Togores B, et al. Hypermutation is a key factor in development of multiple-antimicrobial resistance in Pseudomonas aeruginosa strains causing chronic lung infections. Antimicrob Agents Chemother 2005;49(8):3382-6
33. Henrichfreise B, Wiegand I, Pfister W, Wiedemann B. Resistance mechanisms of multiresistant Pseudomonas aeruginosa strains from Germany and correlation with hypermutation. Antimicrob Agents Chemother 2007;51(11):4062-70

- **Comprehensive analysis of the antimicrobial resistance mechanisms of multidrug-resistant *P. aeruginosa* strains from CF and non-CF patients and their association with mutator phenotypes.**

34. Driffield K, Miller K, Bostock JM, et al. Increased mutability of Pseudomonas aeruginosa in biofilms. J Antimicrob Chemother 2008;61(5):1053-6
35. Boles BR, Singh PK. Endogenous oxidative stress produces diversity and adaptability in biofilm communities. Proc Natl Acad Sci USA 2008;105(34):12503-8
36. Bagge N, Hentzer M, Andersen JB, et al. Dynamics and spatial distribution of beta-lactamase expression in Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 2004;48(4):1168-74
37. Olivares J, Bernardini A, Garcia-Leon G, et al. The intrinsic resistome of bacterial pathogens. Front Microbiol 2013;4:103
38. Breidenstein EB, de la Fuente-Núñez C, Hancock RE. Pseudomonas aeruginosa: all roads lead to resistance. Trends Microbiol 2011;19(8):419-26
39. Blázquez J. Hypermutation as a factor contributing to the acquisition of antimicrobial resistance. Clin Infect Dis 2003;37(9):1201-9
40. Miller JH. Spontaneous mutators in bacteria: insights into pathways of mutagenesis and repair. Annu Rev Microbiol 1996;50:625-43
41. Rodriguez-Rojas A, Blázquez J. The Pseudomonas aeruginosa pfpI gene plays an antimutator role and provides general stress protection. J Bacteriol 2009;191(3):844-50
42. Žgur-Bertok D. DNA damage repair and bacterial pathogens. PLoS Pathog 2013; 9(11):e1003711
43. Blázquez J, Oliver A, Gómez-Gómez JM. Mutation and evolution of antibiotic resistance: antibiotics as promoters of antibiotic resistance? Curr Drug Targets 2002;3(4):345-9
44. Blázquez J, Gómez-Gómez JM, Oliver A, et al. PBP3 inhibition elicits adaptive responses in Pseudomonas aeruginosa. Mol Microbiol 2006;62(1):84-99
45. Pérez-Capilla T, Baquero MR, Gómez-Gómez JM, et al. SOS independent induction of dinB transcription by beta-lactam-mediated inhibition of cell wall synthesis in Escherichia coli. J Bacteriol 2005;187(4):1515-18
46. Oliver A. Mutators in cystic fibrosis chronic lung infection: prevalence, mechanisms, and consequences for antimicrobial therapy. Int J Med Microbiol 2010;300(8):563-72
47. Mena A, Smith EE, Burns JL, et al. Genetic adaptation of Pseudomonas aeruginosa to the airways of cystic fibrosis patients is catalyzed by hypermutation. J Bacteriol 2008;190(24):7910-17

48. Ciofu O, Riis B, Pressler T, et al. Occurence of hypermutable Pseudomonas aeruginosa in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. Antimicrob Agents Chemother 2005;49(6):2276-82

49. Kenna DT, Doherty CJ, Foweraker J, et al. Hypermutability in environmental Pseudomonas aeruginosa and in populations causing pulmonary infection in individuals with cystic fibrosis. Microbiology 2007; 153(Pt 6):1852-9

50. López-Causapé C, Rojo-Molinero E, Mulet X, et al. Clonal dissemination, emergence of mutator lineages and antibiotic resistance evolution in Pseudomonas aeruginosa cystic fibrosis chronic lung infection. PLoS One 2013; 8(8):e71001

51. Prunier AL, Malbruny B, Laurans M, et al. High rate of macrolide resistance in Staphylococcus aureus strains from patients with cystic fibrosis reveals high proportions of hypermutable strains. J Infect Dis 2003; 187(11):1709-16

52. Román F, Cantón R, Pérez-Vázquez M, et al. Dynamics of long-term colonization of respiratory tract by Haemophilus influenzae in cystic fibrosis patients shows a marked increase in hypermutable strains. J Clin Microbiol 2004;42(4):1450-9

53. Del Campo R, Morosini MI, de la Pedrosa EG, et al. Population structure, antimicrobial resistance, and mutation frequencies of Streptococcus pneumoniae isolates from cystic fibrosis patients. J Clin Microbiol 2005;43(5): 2207-14

54. Turrientes MC, Baquero MR, Sánchez MB, et al. Polymorphic mutation frequencies of clinical and environmental Stenotrophomonas maltophilia populations. Appl Environ Microbiol 2010;76(6): 1746-58

55. Vidigal PG, Dittmer S, Steinmann E, et al. Adaptation of Stenotrophomonas maltophilia in cystic fibrosis: molecular diversity, mutation frequency and antibiotic resistance. Int J Med Microbiol 2014; 304(5-6):613-19

56. Martina P, Feliziani S, Juan C, et al. Hypermutation in Burkholderia cepacia complex is mediated by DNA mismatch repair inactivation and is highly prevalent in cystic fibrosis chronic respiratory infection. Int J Med Microbiol 2014;304(8):1182-91

57. Rodríguez-Rojas A, Oliver A, Blázquez J. Intrinsic and environmental mutagenesis drive diversification and persistence of Pseudomonas aeruginosa in chronic lung infections. J Infect Dis 2012;205(1):121-7

58. Ferroni A, Guillemot D, Moumile K, et al. Effect of mutator P. aeruginosa on antibiotic resistance acquisition and respiratory function in cystic fibrosis. Pediatr Pulmonol 2009;44(8):820-5

59. Tazi A, Chapron J, Touak G, et al. Rapid emergence of resistance to linezolid and mutator phenotypes in Staphylococcus aureus isolates from an adult cystic fibrosis patient. Antimicrob Agents Chemother 2013;57(10):5186-8

60. Macià MD, Borrell N, Segura M, et al. Efficacy and potential for resistance selection of antipseudomonal treatments in a mouse model of lung infection by hypermutable Pseudomonas aeruginosa. Antimicrob Agents Chemother 2006;50(3):975-83

61. Plasencia V, Borrell N, Maciá MD, et al. Influence of high mutation rates on the mechanisms and dynamics of in vitro and in vivo resistance development to single or combined antipseudomonal agents. Antimicrob Agents Chemother 2007;51(7): 2574-81

62. Henrichfreise B, Wiegand I, Luhmer-Becker I, Wiedemann B. Development of resistance in wild-type and hypermutable Pseudomonas aeruginosa exposed to clinical pharmacokinetic profiles of meropenem and ceftazidime simulated in vitro. Antimicrob Agents Chemother 2007;51:3642-9

63. Mulet X, Maciá MD, Mena A, et al. Azithromycin in Pseudomonas aeruginosa biofilms: bactericidal activity and selection of nfxB mutants. Antimicrob Agents Chemother 2009;53(4):1552-60

64. Riera E, Macià MD, Mena A, et al. Anti-biofilm and resistance suppression activities of CXA-101 against chronic respiratory infection phenotypes of Pseudomonas aeruginosa strain PAO1. J Antimicrob Chemother 2010;65(7): 1399-404

65. Macià MD, Perez JL, Molin S, Oliver A. Dynamics of mutator and antibiotic-resistant populations in a pharmacokinetic/pharmacodynamic model of Pseudomonas aeruginosa biofilm treatment. Antimicrob Agents Chemother 2011;55(11):5230-7

- **First report demonstrating the amplification of mutator populations under antibiotic treatment in *P. aeruginosa* biofilms.**

66. Pedersen SS, Koch C, Høiby N, Rosendal K. An epidemic spread of multiresistant Pseudomonas aeruginosa in a cystic fibrosis centre. J Antimicrob Chemother 1986;17(4):505-16

67. LiPuma JJ, Mortensen JE, Dasen SE, et al. Ribotype analysis of Pseudomonas cepacia from cystic fibrosis treatment centers. J Pediatr 1988;113(5):859-62

68. Johnson WM, Tyler SD, Rozee KR. Linkage analysis of geographic and clinical clusters in Pseudomonas cepacia infections by multilocus enzyme electrophoresis and ribotyping. J Clin Microbiol 1994;32(4): 924-30

69. Ledson MJ, Gallagher MJ, Corkill JE, et al. Cross infection between cystic fibrosis patients colonised with Burkholderia cepacia. Thorax 1998;53(5):432-6

70. Drevinek P, Mahenthiralingam E. Burkholderia cenocepacia in cystic fibrosis: epidemiology and molecular mechanisms of virulence. Clin Microbiol Infect 2010;16(7): 821-30

71. Fothergill JL, Walshaw MJ, Winstanley C. Transmissible strains of Pseudomonas aeruginosa in cystic fibrosis lung infections. Eur Respir J 2012;40(1):227-38

- **The prevalence, epidemiology, genotypic and phenotypic features, virulence and clinical impact of recently identified *P. aeruginosa* CF epidemic and transmissible strains are reviewed.**

72. Fluge G, Ojeniyi B, Høiby N, et al. Typing of Pseudomonas aeruginosa strains in Norwegian cystic fibrosis patients. Clin Microbiol Infect 2001;7(5):238-43

73. Parkins MD, Glezerson BA, Sibley CD, et al. Twenty-five-year outbreak of Pseudomonas aeruginosa infecting individuals with cystic fibrosis: identification of the prairie epidemic strain. J Clin Microbiol 2014;52(4):1127-35

74. Rolain JM, François P, Hernandez D, et al. Genomic analysis of an emerging multiresistant Staphylococcus aureus strain rapidly spreading in cystic fibrosis patients revealed the presence of an antibiotic inducible bacteriophage. Biol Direct 2009;4:1

75. Molina A, Del Campo R, Máiz L, et al. High prevalence in cystic fibrosis patients of multiresistant hospital-acquired methicillin-resistant Staphylococcus aureus ST228-SCCmecI capable of biofilm formation. J Antimicrob Chemother 2008; 62(5):961-7

76. Elizur A, Orscheln RC, Ferkol TW, et al. Panton-Valentine Leukocidin-positive methicillin-resistant Staphylococcus aureus

lung infection in patients with cystic fibrosis. Chest 2007;131(6):1718-25

77. Campana S, Cocchi P, Döring G, et al. Emergence of an epidemic clone of community-associated methicillin-resistant panton-valentine leucocidin-negative Staphylococcus aureus in cystic fibrosis patient populations. J Clin Microbiol 2007; 45(9):3146

78. Glikman D, Siegel JD, David MZ, et al. Complex molecular epidemiology of methicillin-resistant Staphylococcus aureus isolates from children with cystic fibrosis in the era of epidemic community-associated methicillin-resistant S aureus. Chest 2008; 133(6):381-7

79. Cocchi P, Cariani L, Favari F, et al. Molecular epidemiology of meticillin-resistant Staphylococcus aureus in Italian cystic fibrosis patients: a national overview. J Cyst Fibros 2011;10(6):407-11

80. Lim YW, Evangelista JS 3rd, Schmieder R, et al. Clinical insights from metagenomic analysis of sputum samples from patients with cystic fibrosis. J Clin Microbiol 2014; 52(2):425-37

81. Fancello L, Desnues C, Raoult D, Rolain JM. Bacteriophages and diffusion of genes encoding antimicrobial resistance in cystic fibrosis sputum microbiota. J Antimicrob Chemother 2011;66(11): 2448-54

- **This study shows that phages in the CF sputum microbiota represent a reservoir of mobilizable genes associated with antimicrobial resistance.**

82. Holden MT, Seth-Smith HM, Crossman LC, et al. The genome of Burkholderia cenocepacia J2315, an epidemic pathogen of cystic fibrosis patients. J Bacteriol 2009;191(1):261-77

83. Winstanley C, Langille MG, Fothergill JL, et al. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of Pseudomonas aeruginosa. Genome Res 2009;19(1):12-23

84. Neuwirth C, Freby C, Ogier-Desserrey A, et al. VEB-1 in Achromobacter xylosoxidans from cystic fibrosis patient. Emerg Infect Dis 2006;12(11):1737-9

85. Agarwal G, Kapil A, Kabra SK, et al. Characterization of Pseudomonas aeruginosa isolated from chronically infected children with cystic fibrosis in India. BMC Microbiol 2005;5:43

86. Pollini S, Fiscarelli E, Mugnaioli C, et al. Pseudomonas aeruginosa infection in cystic fibrosis caused by an epidemic metallo-β-lactamase-producing clone with a heterogeneous carbapenem resistance phenotype. Clin Microbiol Infect 2011; 17(8):1272-5

87. Cardoso O, Alves AF, Leitão R. Metallo-beta-lactamase VIM-2 in Pseudomonas aeruginosa isolates from a cystic fibrosis patient. Int J Antimicrob Agents 2008;31(4):375-9

88. Leão RS, Pereira RH, Folescu TW, et al. KPC-2 carbapenemase-producing Klebsiella pneumoniae isolates from patients with Cystic Fibrosis. J Cyst Fibros 2011;10(2): 140-2

89. McCaughey G, Gilpin DF, Elborn JS, Tunney MM. The future of antimicrobial therapy in the era of antibiotic resistance in cystic fibrosis pulmonary infection. Expert Rev Respir Med 2013;7(4):385-96

90. Conway SP, Pond MN, Watson A, et al. Intravenous colistin sulphomethate in acute respiratory exacerbations in adult patients with cystic fibrosis. Thorax 1997;52(11): 987-93

91. Drusano GL. Pharmacokinetics and pharmacodynamics of antimicrobials. Clin Infect Dis 2007;45(Suppl 1):S89-95

92. Jumbe N, Louie A, Leary R, et al. Application of a mathematical model to prevent in vivo amplification of antibiotic-resistant bacterial populations during therapy. J Clin Invest 2003;112(2): 275-85

93. Lang BJ, Aaron SD, Ferris W, et al. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with multiresistant strains of Pseudomonas aeruginosa. Am J Resp Crit Care Med 2000;162(6):2241-5

94. Aaron SD, Ferris W, Henry DA, et al. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with Burkholderia cepacia. Am J Respir Crit Care Med 2000;161(4 Pt 1): 1206-12

95. Aaron SD, Vandemheen KL, Ferris W, et al. Combination antibiotic susceptibility testing to treat exacerbations of cystic fibrosis associated with multiresistant bacteria: a randomised, double-blind, controlled clinical trial. Lancet 2005; 366(9484):463-71

96. Haja Mydin H, Corris PA, Nicholson A, et al. Targeted antibiotic prophylaxis for lung transplantation in cystic fibrosis patients colonised with Pseudomonas aeruginosa using multiple combination bactericidal testing. J Transplant 2012;2012:135738

97. Herrmann G, Yang L, Wu H, et al. Colistin-tobramycin combinations are superior to monotherapy concerning the killing of biofilm Pseudomonas aeruginosa. J Infect Dis 2010;202(10):1585-92

- **Report showing the usefulness of colistin–tobramycin combinations against *P. aeruginosa* biofilms both *in vitro* and *in vivo*.**

98. Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T. Tolerance to the antimicrobial peptide colistin in Pseudomonas aeruginosa biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. Mol Microbiol 2008;68(1):223-40

- **This study reveals the mechanisms of colistin tolerance in *P. aeruginosa* biofilms.**

99. Trapnell BC, McColley SA, Kissner DG, et al. Fosfomycin/tobramycin for inhalation in patients with cystic fibrosis with Pseudomonas airway infection. Am J Respir Crit Care Med 2012;185(2):171-8

100. Maciá MD, Mena A, Borrell N, et al. Increased susceptibility to colistin in hypermutable Pseudomonas aeruginosa strains from chronic respiratory infections. Antimicrob Agents Chemother 2007;51(12): 4531-2

101. Maciá MD, Borrell N, Pérez JL, Oliver A. Detection and susceptibility testing of hypermutable Pseudomonas aeruginosa strains with the Etest and disk diffusion. Antimicrob Agents Chemother 2004;48(7): 2665-72

102. Mandsberg LF, Ciofu O, Kirkby N, et al. Antibiotic resistance in Pseudomonas aeruginosa strains with increased mutation frequency due to inactivation of the DNA oxidative repair system. Antimicrob Agents Chemother 2009;53(6):2483-91

103. Mirakhur A, Gallagher MJ, Ledson MJ, et al. Fosfomycin therapy for multiresistant Pseudomonas aeruginosa in cystic fibrosis. J Cyst Fibros 2003;2(1):19-24

104. Morin JP, Olier B, Voitte G, Fillastre JP. Can fosfomycin reduce the nephrotoxicity of aminoglycosides? Pathol Biol 1984;32(5): 338-42

105. Ohtani I, Ohtsuki K, Aikawa T, et al. Protective effect of fosfomycin against aminoglycoside ototoxicity. ORL J Otorhinolaryngol Relat Spec 1985;47(1): 42-8

106. Cabot G, Bruchmann S, Mulet X, et al. Pseudomonas aeruginosa ceftolozane-tazobactam resistance development requires multiple mutations

leading to overexpression and structural modification of AmpC. Antimicrob Agents Chemother 2014;58(6):3091-9

107. Hornsey M, Phee L, Stubbings W, Wareham DW. In vitro activity of the novel monosulfactam BAL30072 alone and in combination with meropenem versus a diverse collection of important Gram-negative pathogens. Int J Antimicrob Agents 2013;42(4):343-6

108. Moskowitz SM, Foster JM, Emerson J, Burns JL. Clinically feasible biofilm susceptibility assay for isolates of Pseudomonas aeruginosa from patients with cystic fibrosis. J Clin Microbiol 2004;42(5): 1915-22

- **Report documenting the advantages of adapting biofilm susceptibility methods to the clinical microbiology laboratory.**

109. Skindersoe ME, Alhede M, Phipps R, et al. Effects of antibiotics on quorum sensing in Pseudomonas aeruginosa. Antimicrob Agents Chemother 2008;52(10):3648-63

110. Saiman L, Chen Y, Gabriel PS, Knirsch C. Synergistic activities of macrolide antibiotics against Pseudomonas aeruginosa, Burkholderia cepacia, Stenotrophomonas maltophilia, and Alcaligenes xylosoxidans isolated from patients with cystic fibrosis. Antimicrob Agents Chemother 2002;46(4): 1105-7

111. Hengzhuang W, Ciofu O, Yang L, et al. High β-lactamase levels change the pharmacodynamics of β-lactam antibiotics in Pseudomonas aeruginosa biofilms. Antimicrob Agents Chemother 2013;57(1): 196-204

- **First study demonstrating that pharmacokinetic/pharmacodynamic parameters may differ significantly in conventional (planktonic) and biofilm-based therapeutic models.**

112. Alkawash MA, Soothill JS, Schiller NL. Alginate lyase enhances antibiotic killing of mucoid Pseudomonas aeruginosa in biofilms. APMIS 2006;114(2):131-8

113. Moons P, Werckx W, Van Houdt R, et al. Resistance development of bacterial biofilms against bacteriophage attack. Commun Agr Appl Biol Sci 2006;71(1):297-300

114. Morello E, Saussereau E, Maura D, et al. Pulmonary bacteriophage therapy on Pseudomonas aeruginosa cystic fibrosis strains: first steps towards treatment and prevention. PLoS One 2011;6(2):e16963

115. Saussereau E, Vachier I, Chiron R, et al. Effectiveness of bacteriophages in the sputum of cystic fibrosis patients. Clin Microbiol Infect 2014. [Epub ahead of print]

116. Kaneko Y, Thoendel M, Olakanmi O, et al. The transition metal gallium disrupts Pseudomonas aeruginosa iron metabolism and has antimicrobial and antibiofilm activity. J Clin Invest 2007;117(4):877-88

117. García-Contreras R, Lira-Silva E, Jasso-Chávez R, et al. Isolation and characterization of gallium resistant Pseudomonas aeruginosa mutants. Int J Med Microbiol 2013;303(8):574-82

118. Singh PK, Parsek MR, Greenberg EP, Welsh MJ. A component of innate immunity prevents bacterial biofilm development. Nature 2002;417(6888):552-5

119. Ciofu O, Bagge N, Høiby N. Antibodies against beta-lactamase can improve ceftazidime treatment of lung infection with beta-lactam-resistant Pseudomonas aeruginosa in a rat model of chronic lung infection. APMIS 2002;110(12):881-91

120. Jakobsen TH, Bjarnsholt T, Jensen PØ, et al. Targeting quorum sensing in Pseudomonas aeruginosa biofilms: current and emerging inhibitors. Future Microbiol 2013;8(7):901-21

- **An excellent review on the present and future of research on quorum sensing inhibitors.**

121. García-Contreras R, Nuñez-López L, Jasso-Chávez R, et al. Quorum sensing enhancement of the stress response promotes resistance to quorum quenching and prevents social cheating. ISME J 2014. [Epub ahead of print]

122. Kalia VC, Wood TK, Kumar P. Evolution of resistance to quorum-sensing inhibitors. Microb Ecol 2014;68(1):13-23

123. Davies DG, Marques CN. A fatty acid messenger is responsible for inducing dispersion in microbial biofilms. J Bacteriol 2009;191(5):1393-403

124. Barraud N, Hassett DJ, Hwang SH, et al. Involvement of nitric oxide in biofilm dispersal of Pseudomonas aeruginosa. J Bacteriol 2006;188(21):7344-53

125. Gjermansen M, Ragas P, Tolker-Nielsen T. Proteins with GGDEF and EAL domains regulate Pseudomonas putida biofilm formation and dispersal. FEMS Microbiol Lett 2006;265(2):215-24

126. Rogers SA, Huigens RW 3rd, Cavanagh J, Melander C. Synergistic effects between conventional antibiotics and 2-aminoimidazole-derived antibiofilm agents. Antimicrob Agents Chemother 2010;54(5):2112-18

127. Saiman L, Siegel JD, LiPuma JJ, et al. Infection prevention and control guideline for cystic fibrosis: 2013 update. Infect Control Hosp Epidemiol 2014;35(Suppl 1): S1-S67

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Evolution of the *Pseudomonas aeruginosa* mutational resistome in an international Cystic Fibrosis clone

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Emergence of epidemic clones and antibiotic resistance development compromises the management of *Pseudomonas aeruginosa* cystic fibrosis (CF) chronic respiratory infections. Whole genome sequencing (WGS) was used to decipher the phylogeny, interpatient dissemination, WGS mutator genotypes (mutome) and resistome of a widespread clone (CC274), in isolates from two highly-distant countries, Australia and Spain, covering an 18-year period. The coexistence of two divergent CC274 clonal lineages was revealed, but without evident geographical barrier; phylogenetic reconstructions and mutational resistome demonstrated the interpatient transmission of mutators. The extraordinary capacity of *P. aeruginosa* to develop resistance was evidenced by the emergence of mutations in >100 genes related to antibiotic resistance during the evolution of CC274, catalyzed by mutator phenotypes. While the presence of classical mutational resistance mechanisms was confirmed and correlated with resistance phenotypes, results also showed a major role of unexpected mutations. Among them, PBP3 mutations, shaping up β-lactam resistance, were noteworthy. A high selective pressure for *mexZ* mutations was evidenced, but we showed for the first time that high-level aminoglycoside resistance in CF is likely driven by mutations in *fusA1*/*fusA2*, coding for elongation factor G. Altogether, our results provide valuable information for understanding the evolution of the mutational resistome of CF *P. aeruginosa*.

Pseudomonas aeruginosa chronic respiratory infection (CRI) is the main driver of morbidity and mortality in patients suffering from cystic fibrosis (CF). The CF respiratory tract is a dynamic, heterogeneous, hostile, stressful and very challenging scenario for invading bacteria, but *P. aeruginosa* populations can overcome all these challenges and chronically persist in the CF lungs. Mechanisms underlying early acquisition of *P. aeruginosa* infection and the eventual establishment of CRI are complex and, many factors, related to the patient, the environment and the microorganism, are involved[1–3].

The high versatility and adaptability observed for *P. aeruginosa* can be attributed to its complex and large genome (5–7 Mb), which includes an outstanding intrinsic antibiotic resistance machinery and a large proportion of regulatory genes (>8%). In comparison to other Gram-negative pathogens, *P. aeruginosa* exhibits a basal reduced susceptibility to many antibiotics, attributed to the production of an inducible AmpC cephalosporinase, the constitutive (MexAB-OprM) or inducible (MexXY) expression of efflux pumps, and the reduced permeability of its outer membrane. In addition, *P. aeruginosa* intrinsic resistance can be significantly enhanced by the acquisition of multiple mutations that alter the expression and/or function of diverse chromosomal genes[4–6].

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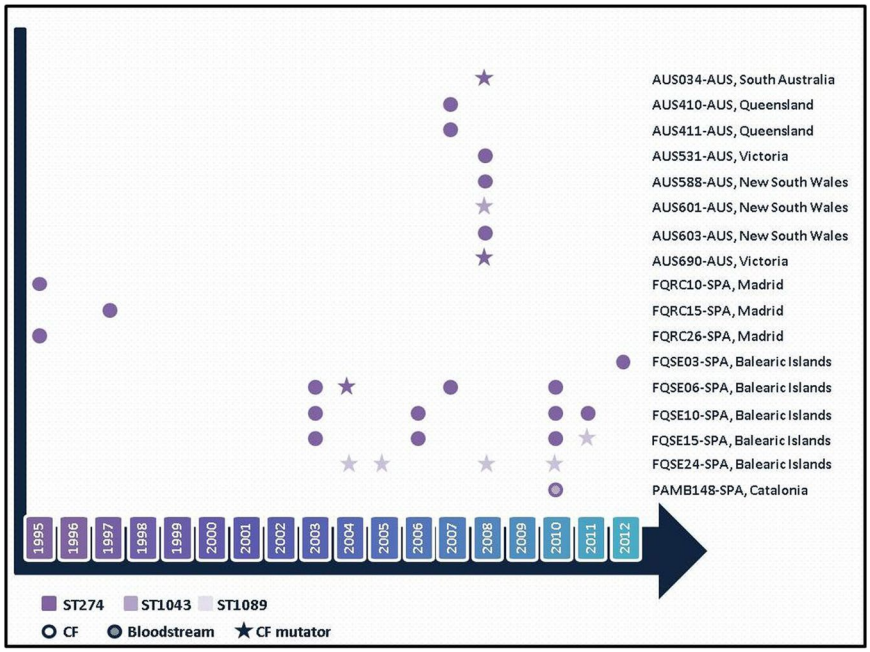

Figure 1. CC274 *P. aeruginosa* collection. Sampling time from the 29 studied isolates can be inferred from the X axis. Isolates are labelled according to the following format: Patient identification - Country (AUS: Australia; SPA: Spain), Region.

Early infection by *P. aeruginosa* in CF patients can be intermittent and usually different strains with distinct antibiotic susceptibility profiles are involved. However, according to the US CF Foundation Patient Registry, over 70% of CF patients are chronically colonized with *P. aeruginosa* by the age of 25 (Annual Report 2015); moreover, in up to 20% of them the involved strain is multidrug resistant (MDR). Although no single mutation can lead to an MDR profile, during CF CRI, *P. aeruginosa* is exposed to numerous and extended antimicrobial therapies which act as selective forces driving to the acquisition of a plethora of adaptive mutations that eventually lead to an enhanced antimicrobial resistance pattern. Moreover, this genetic adaptation is accelerated by the characteristic high prevalence of hypermutable strains (30–60%)[7–10]. Additionally, there is growing evidence suggesting that adaptation to the CF lungs may escape from the scale of the individual patients. Indeed, another remarkable and challenging issue in the CF setting is the existence of concerning *P. aeruginosa* epidemic strains, such as the Liverpool Epidemic Strain (LES-1), the Denmark Epidemic Strain (DK2) or the Australian Epidemic Strains (AES-1, AES-2 and AES-3), as these successful strains are able of infecting hundreds of CF patients even in different geographical locations and, indeed, in many cases exhibiting a MDR profile[11–13]. Therefore, CF CRI by widespread strains may provide a unique and exceptional opportunity to get insight into long-term evolutionary dynamics of *P. aeruginosa* mutational resistome.

Recent advances in sequencing technologies have made it possible to obtain the whole genome of bacterial pathogens. As mentioned above, *P. aeruginosa* CF CRI represent a unique chance to perform evolutionary studies and, accordingly, several works have been performed in this setting; however, most have focused their attention in pathoadaptive mutations[14]. Moreover, *P. aeruginosa* chronic infections are not limited to CF patients, being also frequently implicated in other chronic underlying diseases such as bronchiectasis and chronic obstructive pulmonary disease (COPD)[15]. Thus, an insight into the resistome evolution during CF CRI could be of great benefit not only for individual patients but also for developing new drugs and new treatment strategies.

In a previous study we detected the presence of a transmissible and persistent *P. aeruginosa* lineage chronically infecting up to 4 of 10 selected chronically infected CF patients attended at the reference hospital of the Balearic Islands, Spain[16]. These isolates belonged to the ST274 clonal complex (CC274), which according to the MLST database, appears to colonize CF patients worldwide (http://pubmlst.org/paeruginosa/). In this work, the whole genome of a collection of CC274 strains was obtained in order to characterize the phylogeny, and the mutational resistome evolution of this widespread clonal complex; the CC274 collection included 29 representative isolates recovered from different regions of two highly distant countries, Australia and Spain, covering an 18-year period (1995–2012) and including sequential isolates from several patients (Fig. 1).

Results and Discussion

Prevalence and genetic basis for hypermutation: CC274 mutome. Among the CC274 studied collection, nine isolates (31%) were mutators, belonging to six (35%) different patients, residing in both Australia (n = 3) and Spain (n = 3). Data from sequential isolates were available for the Spanish isolates: one was chronically infected with a persistent mutator lineage (FQSE24), whereas the other two harbored a mixed population of mutator and non-mutator isolates (FQSE06 and FQSE15) (Fig. 1).

Isolate ID[a]	ST	Mutator?	Complement with	Sequence variation in mutator genes (mutome)[b]									
				ung	*mfd*	*mutS*	*sodB*	*mutT*	*sodM*	*mutL*	*mutM*	*oxyR*	*polA*
AUS034	274	Yes	*mutL*					E236D		R631C	D61N L132P		D876E
AUS410	274	No	—				E25V						D876E
AUS411	274	No	—					E236D			D61N		D876E
AUS531	274	No	—					E236D			D61N		D876E
AUS588	274	No	—				E25V						D876E
AUS601	1043	Yes	*mutL*	S13R			E25V			P159S H288Y		F106L H219Y	D876E
AUS603	274	No	—				E25V						D876E
AUS690	274	Yes	*mutS*		Q1123H	C224R T287P		E236D			D61N		D876E
FQRC10	274	No	—					E236D			D61N		D876E
FQRC15	274	No	—					E236D			D61N		D876E
FQRC26	274	No	—					E236D			D61N		D876E
FQSE03	274	No	—			L374V		E236D			D61N		D876E
FQSE06-0403	274	No	—					E236D			D61N		D876E
FQSE06-1104	274	Yes	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE06-0807	274	No	—					E236D			D61N		D876E
FQSE06-0610	274	No	—					E236D			D61N		D876E
FQSE10-0503	274	No	—					E236D			D61N		D876E
FQSE10-0106	274	No	—					E236D			D61N		D876E
FQSE10-0110	274	No	—					E236D			D61N		D876E
FQSE10-0111	274	No	—					E236D			D61N		D876E
FQSE15-0803	274	No	—					E236D			D61N		D876E
FQSE15-0906	274	No	—					E236D			D61N		D876E
FQSE15-0310	274	No	—					E236D			D61N		D876E
FQSE15-1110	1089	Yes	*mutS*		A868T	Nt814Δ4		E236D			D61N		D876E
FQSE24-0304	1089	Yes	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-1005	1089	Yes	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-0308	1089	Yes	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-1010	1089	Yes	*mutS*			Nt814Δ4		E236D			D61N		D876E
PAMB148	274	No	—					E236D	L202R		D61N		D876E

Table 1. Mutator phenotype and genetic basis of hypermutation in CC274. [a]Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. [b]Sequence variations respect to those of PAO1. No mutations were found in other genes associated with mutator phenotypes, including *pfpI*, *mutY*, *dnaQ*, *PA2583*, *PA2819.1*, *PA2819.2*, *radA* and *uvrD*.

In order to evaluate the genetic basis of hypermutation, complementation studies with plasmids harboring wild-type Mismatch Repair system (MMR) genes (*mutS* and *mutL*) were performed in mutator isolates from these six patients. As shown in Table 1, wild-type rifampicin resistance mutation frequencies were restored in all mutator isolates upon *mutS* or *mutL* complementation, which correlated in all cases with the presence of specific mutations in these genes, documented through whole-genome sequencing. The three Australian mutator isolates showed unique mutations in either *mutL* and *mutS*. Interestingly, all mutator isolates from the three Spanish patients were found to share the same inactivating mutation in *mutS*. On the other hand, while mutator phenotypes could be explained in all cases by specific mutations in MMR genes, the contrary was not always true, since one of the non-mutator isolates showed a missense mutation in *mutS*. Moreover, the presence of polymorphisms in other mutator genes was frequent, but showed no association with mutator phenotypes (Table 1). Overall, the prevalence and genetic basis of hypermutation in CC274 was similar to that previously documented for non-clonal CF populations[9,10]; this study is however, to our knowledge, the first investigating the genetic basis of hypermutation from whole genome sequence data, through the analysis of the sequence of an exhaustive panel of so called mutator genes, thus designated mutome.

Phylogeny and interpatient dissemination of the international CC274 CF clone. Pulsed Field Gel Electrophoresis (PFGE) macrorestriction patterns indicated that all isolates were clonally related, including mutators, which were indistinguishable from non-mutators. When an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram was constructed based on PFGE patterns, all isolates from the Balearic Islands clustered together in the same branch, although patterns from one of the patients (FQSE10) were slightly different. In contrast, Australian isolates were less clonal and clustered in different branches (Supplementary Fig. S1).

Conversely, by Multi Locus Sequence Typing (MLST), two new and closely ST274-related sequence types (ST) were detected. Discrepant MLST and PFGE results were linked, directly or indirectly, to the emergence of a mutator phenotype, an event that has already been documented in the CF context[16–18]. Mutators from patients FQSE15 and FQSE24 differed from ST274 by only two point mutations in two of the MLST alleles (*acsA* and *guaA*) leading

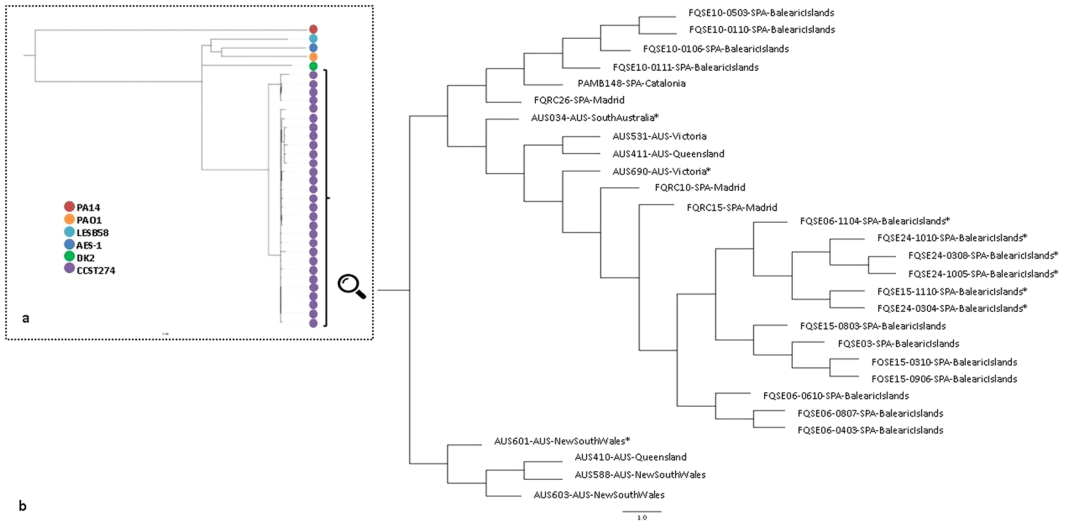

Figure 2. Core-genome phylogenetic reconstructions of *P. aeruginosa* CC274 CF clone. (**a**) Genetic relationship between CC274 and other well-recognized CF epidemic clones. (**b**) Genetic relationship between the CC274 collection isolates. Both reconstructions were made with Parsnp using default parameters. Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates - Country (AUS: Australia; SPA: Spain) - Region. Mutator isolates are identified with an asterisk.

to ST1089, as previously described[16]. Nevertheless, the mutator from patient FQSE06, which indeed shared the same inactivating mutation in *mutS*, still belonged to ST274 (Table 1). On the other hand, the Australian mutator AUS601 was also determined to be a new ST (ST1043), but, in this case, a direct link of the observed PFGE-MLST discrepancy with its MMR system (MutL) deficiency was suggested, since this isolate showed two missense mutations in *mutL* (Table 1), one of them (H288Y) responsible for the generation of the new ST.

To better understand the evolutionary trajectory, success and international dissemination of CC274, whole-genome based phylogenetic analysis of all 29 isolates were performed. Previous studies have already demonstrated that almost all *P. aeruginosa* strains cluster into two major phylogenetic groups, one including PAO1 and the other PA14[19]. In order to determine the genetic relationship between CC274 isolates and other well-recognized CF epidemic clones, whole-genome sequence reads of all 29 isolates were *de novo* assembled and a phylogenetic tree based on core genome alignment was constructed with default parameters on Parsnp[20]. CC274 was determined to belong to the phylogenetic cluster containing strain PAO1, as well as other well-known CF epidemic clones such as LESB58, AES-1 and DK2 (Fig. 2a).

By mapping sequence reads for each isolate against *P. aeruginosa* reference PAO1 strain genome, up to 16,070 common SNPs were found, as well as a total of 5,525 high-quality intraclonal SNPs, of which 2,294 were unique and thus detected in single isolates. A high degree of intraclonal diversity was observed, with SNP differences between isolates ranging from 20 to 3,256. To elucidate the phylogenetic relationship among isolates two different approaches were used. In both, core-genome and Bayesian time-based analysis, CC274 isolates grouped into two clusters, one including just four Australian isolates and a second major cluster that included all other Australian and Spanish isolates (Fig. 2b and Fig. 3). SNP differences between isolates from the different clusters ranged from 2396 to 3256 and, according to Bayesian time-based analysis, the common ancestor of CC274 was set, approximately, 380 years ago.

The major cluster further subdivided and, although both phylogenetic reconstructions did not match exactly with each other, both analyses supported that different lineages are currently coexisting with a worldwide distribution, having evolved from a common antecessor set approximately 275 years ago. SNP differences between isolates from Australia and Spain ranged from 114 to 1204, and similar results were obtained when only the Australian (min-max: 230–826) or the Spanish (min-max: 20–839) were compared, supporting no geographical barrier for lineage evolution.

Within the major cluster, all sequential isolates cultured from an individual patient clustered under the same branch with the single exception of all the Spanish isolates that exhibited a mutator phenotype which clustered together, independently of the patient involved and their ST. Along with the fact that all these mutators shared the same inactivating mutation in *mutS*, as well as many unique antibiotic resistance mutations (Supplementary Data Set S1), phylogenetic analysis clearly demonstrated that ST1089 mutators evolved from a mutator ST274 isolate and that transmission of mutators among the Spanish CF patients occurred at some time point.

Focusing on the sequential isolates, a unidirectional evolution route could not be stablished. Instead, a diversified intrapatient clone evolution that leads to a mix of genetically different sublineages coexisting in the CF respiratory airways was observed. Within a patient, minimum and maximum SNPs differences between isolates ranged from 20 to 676, which overlapped with interpatient SNPs differences, ranging from 51 to 3256 (51 to 839 for patients from the same hospital). Similar results have been reported recently by Williams *et al.* concerning the

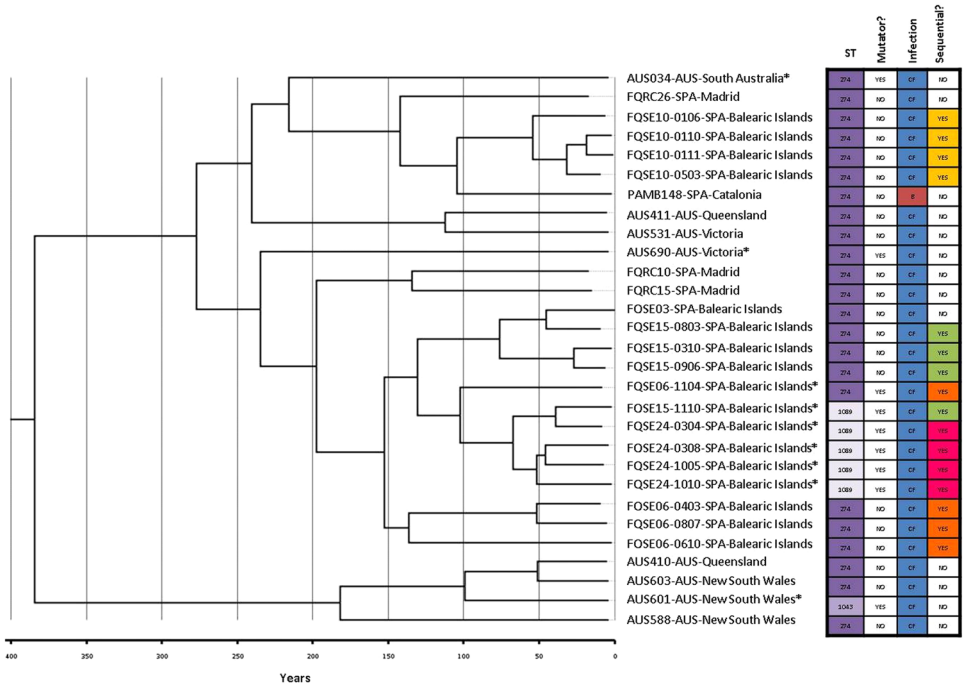

Figure 3. Bayesian phylogenetic reconstruction of *P. aeruginosa* CC274 CF clone. The tree was based on 5525 intraclonal variable positions identified by whole-genome sequencing. Divergence times of predicted ancestors and sampling dates can be inferred from the X axis taking into account that time zero corresponds to the most recent isolate (2012). The same labelling of Fig. 2 was used. Isolates characteristics are summarized at the right board, where: (CF) Cystic Fibrosis CRI and (B) Bloodstream. Sequential *P. aeruginosa* isolated from a same patient are indicated with the same colour.

Liverpool Epidemic Strain, finding that multiple coexisting LES lineages are typically infecting CF patients and that genetic divergence between lineages within patients was greater than interpatient diversity, implying acquisition of diverse genetic populations[21]. However, another study focusing on the LES isolated from patients residing the UK and Canada showed less genetic differences, even when transoceanic isolates were compared[22]. Likewise, Yang *et al.* documented a lower genetic divergence in the DK2 epidemic clone[23]. Moreover, previous studies with other relevant and/or persistent CF clones have also reported divergent results[24–26]. A possible explanation for all these observations could be that different routes for adaptation and survival in the CF lung environment are possible and depend on the specific clonal lineages.

CC274 resistome. Minimum inhibitory concentrations (MICs) determined for a panel of 11 antipseudomonal agents are shown in Table 2. Resistance rates were lowest for colistin (3.4%), distantly followed by ceftazidime and piperacillin-tazobactam (13.8%). In contrast, resistance to cefepime, aztreonam, imipenem, amikacin and ciprofloxacin was observed in 44.8 to 62% of the isolates. Remarkably, 17.2% of the isolates were resistant to the new combination ceftolozane-tazobactam. As shown, antibiotic resistance was more frequent among mutators, and in Australian isolates in comparison with those from Spain. In fact, all 9 mutator isolates were classified as MDR, as compared to only 3 of 20 non-mutators. Moreover, one of the Australian mutator isolates met the pan-drug resistant (PDR) definition[27].

The presence of horizontally acquired resistance determinants was explored in the whole-genome sequences using the ResFinder tool[28]. None of the 29 isolates harbored any horizontally acquired genes encoding resistance determinants, thus indicating that the observed antibiotic resistance profiles reflected the accumulation of mutations within the chromosomal genes. The complete list of antibiotic resistance related genes investigated (n = 164) as well as all missense and non-sense mutations encountered for each of the isolates studied are reported in the Supplementary Data Set S1. Up to 127 (77.4%) of the 164 studied genes showed non-synonymous mutations in at least one of the isolates studied. Moreover, after discarding non-synonymous mutations present in all isolates (and thus considered intrinsic CC274 polymorphisms), this figure only decreased to 106 (64.6%). Figure 4 shows the number and distribution of mutations among the 164 antibiotic resistance related genes studied in the CC274 collection. Seventy-three (68.9%) of these genes showed no more than two different mutational events being 44 of them mutated in unique isolates. In contrast, 33 (31.1%) genes appeared to be under high evolutionary pressure showing evidence of at least 3 different mutational events. Particularly noteworthy among them were *mexB* or *mexY*, (coding for efflux pumps proteins), *mexZ* (the main MexXY repressor), *gyrA* (which codes for DNA gyrase subunit A) and *fusA1* (coding for the elongation factor G).

The main antibiotic resistance related mutations documented are listed in Table 2 along with the susceptibility profiles for each of the isolates. For this purpose, the full list of mutations in the 164 genes studied (available

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	C/T (≤4)	IP (≤4)	MP (≤2)	TO (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
AUS034*	>256	>256	>256	>256	16	>32	>32	6	>256	1.5	>256	+	–	+	*gyrB* (R441L), *mexR* (R85H), *mexA* (M1*), *mexB* (F178S, M555I), *oprD*(E264*), *phoQ*(E266*), *parR* (M59I), *mexY*(V1000L), *mexZ*(Nt334Δ13), *fusA2*(P329L), PA2489(R12L, A244T), *mexS* (P254Q), *mexT*(L157M), PBP4(W350R), *capD* (I7M, S51G), *gyrA*(T83I), *mexK* (S426G), *mpl* (Nt112ins1, V124G), *fusA1* (V93A, P554L, D588G), *rpoB* (D831G, D964G), *mexW* (A627V, Q771P), PBP3 (P527T, G63S)
AUS410	4	24	1	12	4	>32	>32	64	>256	1	0.38	–	–	+	*gyrB* (S466F), *mexB*(M552T), *oprD* (Nt583Δ1), *lasR* (A50V, D73G), *sucC* (V44G, A384V), *oprF* (Nt574Δ31), *mexY* (V32A), *mexZ* (Q164*), *mexT* (D327Y), *mexE* (F7Y), *mpl* (D168Y), PA2489(A125T, G185S, P260S), *capD*(I7M, S51G), *fusA1*(P618L), *rpoC*(E386K), *mexW*(Q511R), PBP3(G216S), *pagL*(Nt286Δ1), *amgS*(S64L)
AUS411	>256	>256	>256	>256	6	>32	>32	>256	>256	0.38	0.25	–	–	+	*gyrB* (S466F), *mexB* (Q104E, F246C, L376V), *phoQ* (H248P), *lasR* (D73G), *parS* (D381E, T163N), *sucC* (C261G), *mexY* (D201A, G287A), PA2489 (R12L, A244T), *fusA2* (I640L), mexE (V104G), *htpX* (Nt683Δ5), *mexK* (S426G), *capD* (I7M), *fusA1* (K504E), *rpoC* (N690S), *mexW* (A627V,Q771P), PBP3 (Q372P), *pagL* (N159D)
AUS531	3	3	4	12	1	2	0.75	1	6	0.125	1	–	–	–	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexW* (A627V, Q771P)
AUS588	2	8	3	8	1	1	0.75	1	8	0.125	0.75	–	–	–	PA2489 (A125T, G185S, P260S), *mexE* (F7Y, V276M), *capD* (I7M), *mexW* (Q511R)
AUS601*	>256	>256	>256	1	3	>32	>32	24	>256	16	0.25	–	–	+	*mexB* (M552T), *oprD* (Nt1044ins4), *phoQ* (K234N, T315A), *lasR* (A50V), *sucC* (T102I, A384V), *mexY* (V32A), *mexZ* (Q164*), *fusA2* (S445*), *mexT*(D327Y), *mexE*(F7Y), *ftsK* (A152V), PA2489 (A125T, G185S, P260S), *capD* (S51G), *gyrA* (T83I), *mpl* (G113D), *fusA1* (P618L), *rpoC* (E386K), *mexW* (Q511R), PBP3 (R504C), *pagL* (E163G), *pmrB* (L31P), *amgR* (E204D)
AUS603	6	8	24	2	1.5	>32	8	1	8	0.25	1.5	+	–	+	*mexB* (M552T), *lasR* (A50V, D73G), *sucC* (V44G, A384V), *mexY* (V32A), *mexZ* (Q164*), *mexT* (D327Y), *mexE* (F7Y), PA2489 (A125T, G185S, P260S), PBP4 (S315G), *opmE* (E204D), *capD* (I7M, Nt1438Δ1), *mpl* (Nt112ins1, Nt1317Δ1), *fusA1* (P618L), *mexW* (Q511R)
AUS690*	6	12	0.75	3	6	4	2	24	>256	12	0.125	–	+	+	*gyrB* (Q467R), *mexR* (H133P), *mexB* (Nt712Δ1), *phoP* (T221I), *lasR* (T178I), *parS* (L10P), *oprF* (K250R), *mexY* (G402S, A850T), *mexZ* (Nt529Δ1), PA2489 (R12L, A244T), *fusA2* (L104P, Nt889Δ1), *htpX* (G187D), *capD* (I7M, S51G), *gyrA* (T83A, T325I), *mexK* (G487E), *mexH* (Nt1086ins1), *fusA1* (Y552C, T671I), *rpoC* (E136G, D616G, V808L), *rpoB* (F1046S), *mexW* (A627V, Q771P), *pagL* (P158L), *pmrB* (F124L), *amgS* (R188C), *parE* (P438S)
FQRC10	2	2	4	12	1	1.5	1	1	8	0.094	0.5	–	–	–	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexH* (D356N), *mexW* (A627V, Q771P)
FQRC15	1	0.75	6	6	1	1.5	1	0.75	8	0.19	1	–	–	–	PA2489 (R12L, A244T), *capD* (I7M), *mexW* (A627V, Q771P)
FQRC26	4	6	24	24	1	0.25	1.5	1	6	1.5	0.38	–	+	–	*mexY* (V875M), *mexT* (R164H), PA2489 (R12L, A244T), *capD* (I7M, S51G), *gyrA*(Q106L), *mexW* (A627V, Q771P)
FQSE03	3	8	0.5	2	1.5	2	0.38	1	6	3	0.25	–	–	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), PA2489 (R12L, A244T), *capD* (I7M, S51G), *gyrA* (D87N), *mexW* (A627V, Q771P)
FQSE06-0403	0.75	2	0.25	4	0.38	1	0.5	24	16	0.19	0.19	–	–	+	*mexA* (L338P), *lasR* (P117G), *mexY* (G287A), *mexZ* (S9P), PA2489 (R12L, A244T), *mpl* (S257L), *capD* (I7M, S51G), *fusA1* (Y552C, T671I), *mexW* (A627V, Q771P), PBP3 (P215L), *amgR* (A8V)
FQSE06-1104*	0.38	1	0.094	0.38	0.38	6	0.19	1	24	0.75	2	–	–	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A194P), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *capD* (I7M, S51G), *gyrA* (D87G), *mexK* (Q585*), *rpoB* (Y583C), *mexW* (A627V, Q771P), *pmrB* (V185I, G221D, R287Q), PBP1A (E161G), *amgR* (A8V)
FQSE06-0807	4	8	0.75	4	2	1.5	0.75	24	>256	0.5	1	–	–	+	*mexA* (L338P), *lasR* (P117G), *mexY* (G287A), *mexZ* (S9P), *mexT* (P270Q), PA2489 (R12L, A244T), *mpl* (S257L), *capD* (I7M, S51G), *fusA1* (N482S, Y552C, T671I), *mexW* (A627V, Q771P), PBP3 (P215L), *amgR* (A8V)
FQSE06-0610	4	24	0.75	8	1.5	1	0.25	1.5	24	0.75	0.19	–	–	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (Nt290Δ11), PA2489 (R12L, A244T), *mexW* (A627V, Q771P), *capD* (I7M, S51G), *amgR* (A8V)
FQSE10-0503	1.5	12	4	4	1.5	1	0.25	0.75	8	0.25	0.25	–	–	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T), *nalD* (Nt459Δ13), *mexW* (A627V, Q771P), *capD* (I7M, S51G)

Continued

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	C/T (≤4)	IP (≤4)	MP (≤2)	TO (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
FQSE10-0106	0.75	3	0.125	0.75	0.5	0.38	0.032	0.75	4	0.38	1.5	−	−	+	*mexB* (L738P), *mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK*(A38T), *capD* (S51G), *nalD* (Nt396Δ2), *mexW* (A627V, Q771P), *nfxB* (*188ext)
FQSE10-0110	3	8	16	8	2	1	0.125	0.75	4	0.75	0.5	−	+	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T), *rpoB* (D659E, E904K), *mexW* (A627V, Q771P), *pmrB* (R287Q)
FQSE10-0111	3	16	12	12	8	1.5	1	1	12	0.38	0.38	−	−	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T, D54Y), *capD* (S51G), *mexW* (A627V, Q771P)
FQSE15-0803	2	12	0.38	4	1.5	6	1	1	12	0.19	0.25	−	−	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), PA2489 (R12L, A244T), *capD* (I7M, S51G), *pmrB* (E213D), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-0906	0.75	6	0.38	2	1	1	0.047	1.5	12	0.38	0.75	−	−	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), *mexS* (Nt848Δ2), *mexT* (Nt534Δ17), PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexK* (S426G), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-0310	1	4	1	1	1	12	0.19	1	8	0.38	0.25	−	−	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), *mexS* (Nt848Δ2), *mexT* (Nt534Δ17), PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexK* (P834S), *mpl* (Nt1266Δ1), *rpoC* (Nt1181Δ3), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-1110*	8	24	6	4	1	>32	>32	1	16	1	0.25	−	−	+	*gyrB* (S466F), *mexA* (N71S, D235G), *mexB* (L376V), *oprD* (V67*), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), galU (P123L), PA2050 (G90R, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *htpX* (A141T), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgS* (D267N), *amgR* (A8V)
FQSE24-0304*	2	24	0.38	8	1	>32	>32	2	24	6	0.38	−	−	+	*gyrB* (S466F), *mexA* (L338P), *oprD* (V67*), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *opmE* (D421G), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgR* (A8V)
FQSE24-1005*	1	16	0.38	2	1.5	>32	8	3	16	6	1	−	−	+	*gyrB* (S466F), *oprD* (V67*), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), *fusA2* (N236S, N561S), PA2489 (R12L, A244T), *fusA1* (K430E), *rpoC*(V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G, R407S), *amgR* (A8V)
FQSE24-0308*	1	8	0.25	0.75	1.5	>32	0.25	2	16	4	1	−	−	+	*gyrB* (S466F), *oprD* (V67*), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), *fusA2* (N236S, N561S), PA2489 (R12L, A244T), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgS* (T92A), *amgR*(A8V)
FQSE24-1010*	1	8	1	1	1	>32	4	4	64	4	0.38	−	−	+	*gyrB* (S466F), *mexA* (L338P), *oprD* (V67*), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, P97L, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *opmE* (L400P, D421G), *mexH* (V221I), *capD* (I7M, S51G, A165V), *fusA1* (K430E), *rpoC*(V693A), *mexW* (A627V, Q771P), PBP3 (G216S), *pmrB* (R287Q), PBP1A (E161G), *amgS* (A13V), *amgR* (A8V)
PAMB148	>256	64	>256	>256	6	1.5	0.75	1.5	16	0.064	0.5	+	−	−	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexY* (V875M, N1036S), *mexW* (A627V, Q771P), *ampD* (P41L)
% I + R	13.8	44.8	48.3	13.8	17.2	44.8	27.6	24.1	62.1	48.3	3.4				

Table 2. Antibiotic susceptibility profile and main antibiotic resistance related mutations detected among CC274 isolates. [a]Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Mutators isolates are identified with and asterisk. [b]Minimal Inhibitory Concentration (MIC) values were determined by grading MIC testing for the following antimicrobial agents: ceftazidime (TZ); cefepime (PM); aztreonam (AT); piperacillin-tazobactam (PPT); cefotolozane-tazobactam (C/T); imipenem (IP); meropenem (MP); tobramycin (TO); amikacin (AK); ciprofloxacin (CI) and colistin (CO). Clinical breakpoints established by EUCAST v7.0 for each antibiotic are shown in brackets. [c]The main antibiotic resistance related mutations documented for each isolate are shown. For this purpose, the full list of mutations in the 164 genes studied (available in Supplementary Data Set S1) was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes (*e.g.* mutations in genes involved in AmpC, efflux or OprD regulation and β-lactam resistance phenotypes are crosslinked by integrating the analysis of the expression of *ampC*, efflux pumps genes and *oprD* and/or (ii) mutations in genes found to be under high evolutionary pressure (those with at least 3 different mutational events documented).

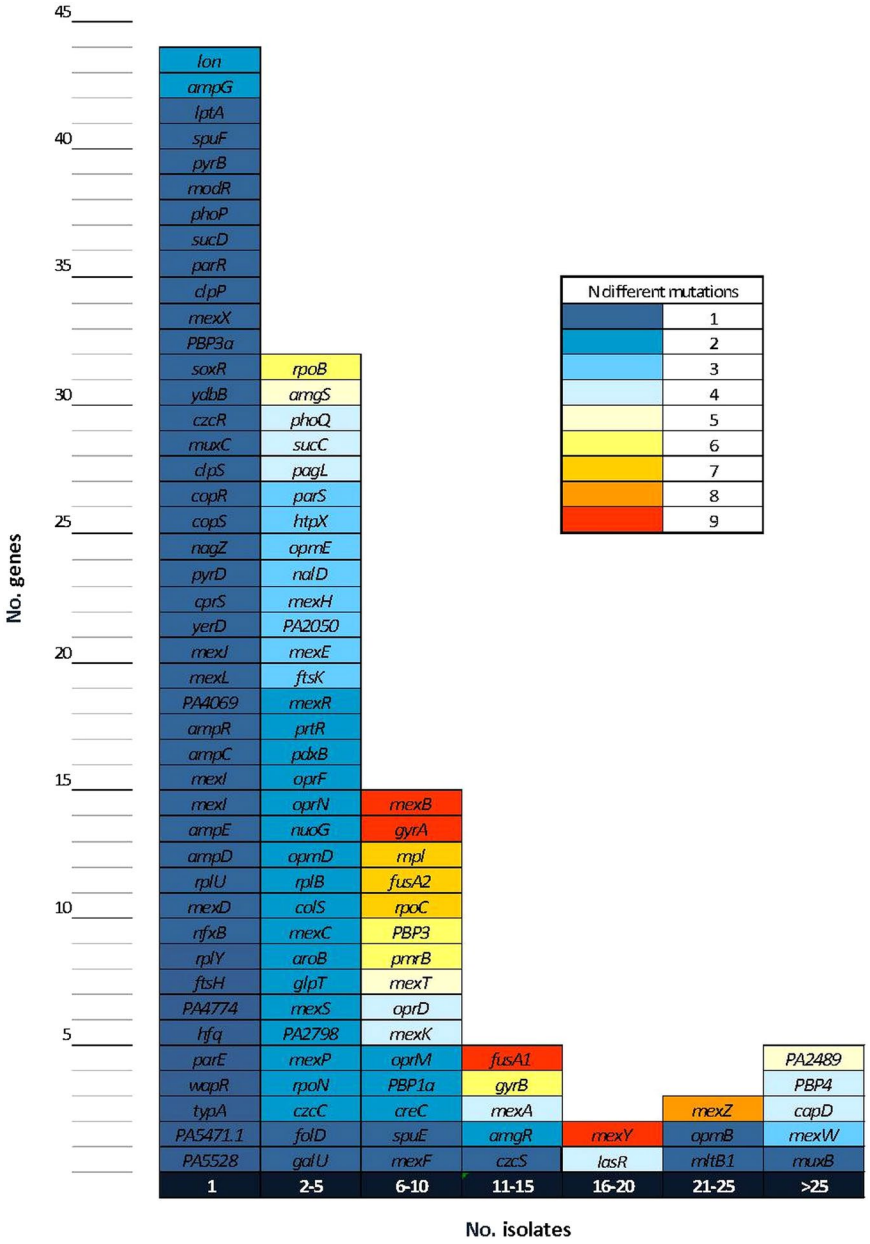

Figure 4. Distribution of mutations among the CC274 collection. Mutations encountered among the 164 antibiotic resistance related genes are represented, synonymous and common non-synonymous mutations have been excluded.

in Supplementary Data Set S1) was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence, (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes (*e.g.* mutations in genes involved in AmpC, efflux or OprD regulation and β-lactam resistance phenotypes are crosslinked by integrating the analysis of the expression of *ampC*, efflux pumps genes and *oprD*) and/or (ii) mutations in genes found to be under high evolutionary pressure (those with at least 3 different mutational events documented). As shown in Table 2, overall, the number of mutations was much higher (unpaired T test $p < 0.0001$) in mutator (19.2 ± 3.1) than in non-mutator isolates (6.7 ± 3.1). This is consistent with the much higher antimicrobial resistance of mutators, documented in this and previous works[8,29]. However, some Australian (*e.g.* AUS410 or AUS411) non-mutator isolates also presented a high number of mutations, perhaps indicating that under a high antibiotic pressure in long-term CRI, MDR profiles may emerge even in the absence of mutator phenotypes. Unique mutations detected in specific genes support phylogeny reconstructions (see above Fig. 2b and Fig. 3). Moreover, they can be very useful to track interpatient transmission, considering that specific mutations detected in multiple isolates, especially within professional antibiotic resistance genes such as *gyrB*, *oprD*, *mexY*, *creC*, *mexZ* or *fusA2*, are unlikely to have occurred independently in different environments. Moreover, the analysis of these mutations

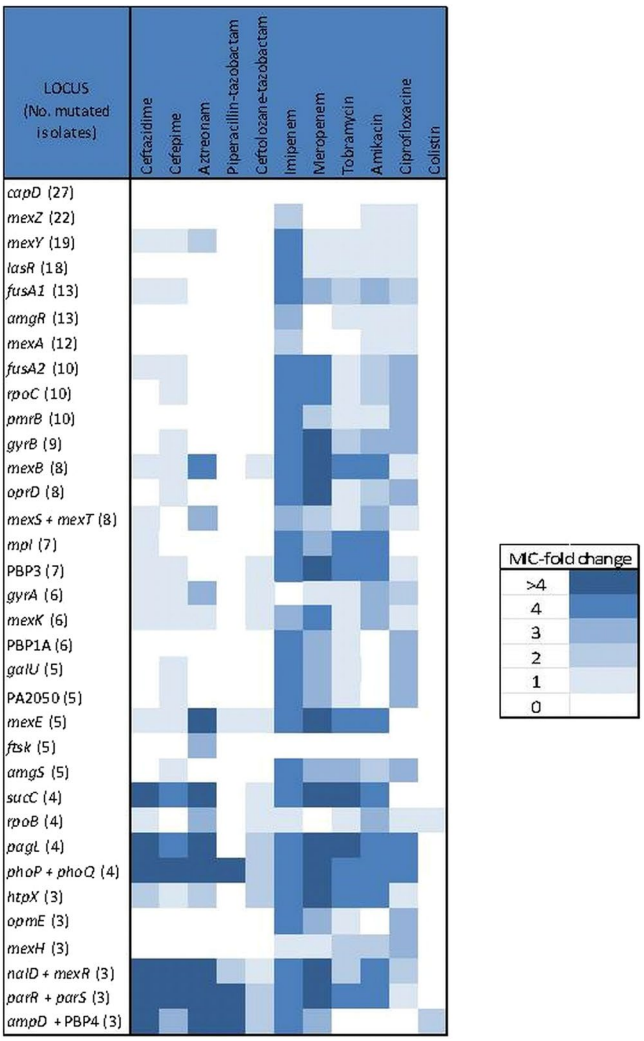

Figure 5. MIC-fold change for each antibiotic tested between isolates mutated or not mutated in a specific gene. To evaluate the implication of the presence of mutations in the main genes possibly related with antibiotic resistance the median MIC for both groups were calculated and compared, results are expressed in MIC-fold change. PA2489, *mexW*, *oprF*, *parE* and *nfxB* were excluded since the number of mutated isolates were < 3. Some genes were grouped (e.g. *ampD* and *dacB* (PBP4) or *nalD* and *mexR*) according to their well-established effects on resistance (e.g. AmpC or MexAB-OprM overexpression, respectively).

can help to understand the basis for the intrapatient diversification and coexistence of multiple lineages in the CF respiratory tract.

To gain insights into the effect on the antibiotic resistance profiles of mutations listed in Table 2, the median MIC of isolates harboring mutations or not in a specific gene were compared and results are summarized in Fig. 5. Overall, it should be noted that colistin MICs as well as the MICs for the antibiotic combinations piperacillin-tazobactam and ceftolozane-tazobactam were barely affected, whilst carbapenems, aminoglycosides and quinolones MICs are affected by the presence of mutations in many of the selected genes. Apparently, the presence of mutations in some genes such as *capD* (also known as *wbpM*), a gene coding for a protein implicated in O-antigen biosynthesis and previously related with aminoglycoside resistance, or *ftsK*, which codes for a cellular division protein, were not related with an increase in resistance for any antibiotic. Conversely, the presence of mutations in 22 of the genes was shown to produce at least a 2-fold MIC increase for at least 3 different classes of antibiotics. Renowned resistance genes, such as *gyrA*, *gyrB*, *ampD*, *dacB* (PBP4) or *oprD*, are within this list of 22 genes but, particularly interesting is the presence of not so well-recognized antibiotic resistance related genes such as *fusA1* and *fusA2*, both coding for elongation factor G, or *rpoC*, which codes the β-chain of a DNA-directed RNA polymerase. Mutations in genes coding for two-component regulatory systems, as PhoPQ or ParRS, also require a special mention as mutated isolates showed a strong impact in their MICs for many of the antibiotics tested.

The presence of unique mutations in certain well-known antibiotic resistance genes, such as *dacB* (PBP4) was observed to increase β-lactam resistance, but it should be noted that mutations within a specific gene did not always correlate or lead to the expected effect on antibiotic resistance (e.g. *pmrB* or *phoP-phoQ* mutated isolates

did not exhibit a higher colistin MIC). Likewise, several mutations (e.g. *mexZ*, *gyrB* or *oprD*) were associated to extended unexpected antibiotic resistance profiles, perhaps suggesting the co-selection of different resistance mutations during *P. aeruginosa* evolution in CF CRI. A detailed analysis of the mutational resistome for each class of antibiotics is provided below and in the Supplementary Data Set S1.

β-lactam resistome. Overproduction of the chromosomally encoded cephalosporinase AmpC is the primary pathway for developing resistance to the antipseudomonal β-lactams, and it is driven by the selection of mutations in peptidoglycan-recycling genes (*ampD*, *dacB* and *ampR*)[30,31]. Just three isolates (AUS034, AUS603 and PAMB148) of the CC274 collection were demonstrated to overproduce AmpC (Table 2). By contrast, at the genomic level, almost all isolates (26/29) contained some variation within *dacB* which codes for the penicillin-binding protein PBP4 (Supplementary Data Set S1-Betalactams). Crosslinking phenotypic and genotypic results through *ampC* expression data, suggested that most observed *dacB* allele variations were, in fact, ancestral polymorphisms not involved in antibiotic resistance. However, AmpC overproduction in the two CF isolates was explained by the presence of specific mutations in *dacB* (S315G or W350R) and by an *ampD* (P41L) mutation in the case of the bloodstream infection isolate PAMB148 (Table 2). Whilst *ampC* overexpression in isolates AUS034 and PAMB148 correlated well with ceftazidime and piperacillin-tazobactam resistance, this was not the case for isolate AUS603 which was documented to be susceptible to these antibiotics. However, unexpected AUS603 β-lactam susceptibility could be explained by the presence of chromosomal mutations whose effects eventually compensate the expected increase in β-lactam resistance. Indeed, this isolate showed a non-sense mutation in OprM (Q93X), the outer membrane protein of the constitutive MexAB efflux pump, which is well known to play a major role in intrinsic β-lactam resistance.

In addition, *P. aeruginosa* may eventually develop β-lactam resistance by acquiring mutations within their macromolecular targets: the essential penicillin-binding proteins (PBPs). While some mutations without apparent effect on resistance were detected in genes coding for PBP1 and PBP3a, the main mutational resistance target among PBPs was found to be PBP3, an essential high molecular class B PBP with transpeptidase activity, in agreement with recent data from CF patients[32] and *in vitro* studies[33]. Indeed, we documented that PBP3 mutations had often occurred (7/29 isolates) among the CC274 collection (Supplementary Data Set S1-Betalactams). Nevertheless, β-lactam resistance contribution of each derived *ftsI* (PBP3) allele, if any, depends on the specific point mutation encountered. Missense mutations within the PBP3 (R504C and Q372P) were apparently the cause of β-lactam resistance in isolates AUS601 and AUS411, since they do not hyperproduce AmpC. Although these mutations are not located in the PBP3 active site, both are very close to two loop regions (residues 332–338 and 526–533) which play an important role in substrate recognition[34]. In fact, PBP3 mutations in residue 504 (R504C, R504H) have been recently described *in vitro*[33] and among isolates from widespread nosocomial *P. aeruginosa* clones[35,36]. Likewise, the P527T mutation of AUS034 likely contributes, together with the overexpression of AmpC, to the very high-level β-lactam resistance of this isolate, including the new antipseudomonal combination ceftolozane-tazobactam. On the other hand, the P215L and G216S mutations were apparently not linked with phenotypic resistance, in agreement with the fact that residues 215 and 216 are not implicated in the formation and stabilization of the inactivating complex β-lactam-PBP3[34].

Obtained data also demonstrated that the constitutive efflux pump MexAB-OprM is under strong mutational pressure during CF CRI, frequently including inactivating mutations, which correlates with previous investigations that pointed out that this efflux pump is dispensable and, therefore, tends to be lost or inactivated in favor of MexXY-OprM hyperproduction in CF *P. aeruginosa* subpopulations[37]. Our data also support this hypothesis, as just 3 isolates showed mutations in regulators leading to MexAB-OprM overexpression, whereas up to 23 isolates hyperproduced the efflux-pump MexXY-OprM (Supplementary Data Set S1 -Betalactams). Moreover, many of the isolates showed some degree of hypersusceptibiltiy to aztreonam (substrate of MexAB-OprM) in favor of an increased MIC of cefepime (substrate of MexXY-OprM) (Supplementary Data Set S1- Betalactams).

Carbapenem resistome. Imipenem and meropenem resistance correlated in all but two isolates with the presence of non-sense mutations affecting the outer membrane protein OprD (Table 2, Supplementary Data Set S1-Carbapenems). High-level meropenem resistance was additionally associated with the presence of PBP3 mutations, in agreement with recent *in vitro* studies showing the selection of PBP3 mutations upon meropenem exposure[33]. Remarkably, all ST1089 mutator isolates shared the same point mutation in *oprD* (V67X) as well as in *galU* (P123L), also related with carbapenem resistance, supporting interpatient transmission of this mutator lineage among CF patients attending the reference hospital of the Balearic Islands.

The expression of OprD is known to be modulated by mutations (*mexS* or *mexT*) leading to the overexpression of the efflux pump MexEF-OprN, and meropenem is a well-known substrate for the efflux pump MexAB-OprM. However, carbapenem resistant isolates AUS411 and AUS603 harboring a wild-type *oprD* allele did not overproduce neither of these two efflux pumps. Thus, the observed phenotype could be related with the presence of specific mutations within the genes coding for PBP3 (*ftsI*) and PBP4 (*dacB*), hypothesis that is current being evaluated in our laboratory.

Aminoglycoside resistome. Intravenous antimicrobial combinations including an aminoglycoside plus a fluroroquinolone or a β-lactam antibiotic are frequently used to manage CF exacerbations. Moreover, in the last decade, tobramycin inhalation has become an important contributor to CF treatment as a means to control chronic infection as well as a first-line treatment for the eradication of early acquisition of *P. aeruginosa* and several aminoglycoside-based inhaled formulations are currently available. Resistance to antipseudomonal aminoglycosides is frequently attributed to the presence of acquired aminoglycoside-modifying enzymes, membrane impermeability or MexXY efflux pump overexpression[38]. Moreover, adaptive resistance, due to MexXY efflux

system overexpression, to this class of antibiotics has been well documented in the CF setting in response to sublethal concentrations[39].

Among the CC274 collection, a high proportion of the isolates (23/29) were shown to overexpress MexXY and all but one were mutated in *mexZ*, which codes for the mayor MexXY expression regulator. In agreement with recent work, which pointed out that mutation of *mexZ* is part of a strongly selected evolutionary pathway[40], several different mutational events were encountered within this regulator. Remarkably, the same point mutation was detected among different and independent isolates, probably indicating interpatient transmission events (Table 2, Supplementary Data Set S1-Aminoglycosides). The single MexXY-overproducing isolate showing no mutations in *mexZ*, presented a unique mutation in *parS*, a gene also involved in the modulation of MexXY expression. Nevertheless, as it has been largely observed in the CF clinical setting[41], MexXY hyperproduction *per se* cannot explain aminoglycoside resistance in the majority of the isolates. In this sense, there is growing evidence that high-level resistance is a stepwise process which arises by the accumulation of several non-enzymatic mechanisms and, moreover, novel genetic resistance determinants have been proposed[42–44]. To our knowledge, no published work has yet investigated the *in vivo* contribution to aminoglycoside resistance of these novel genetic determinants proposed, and many questions remain unresolved. Thus, our work reveals for the first time that all high-level resistant isolates hyperproduced MexXY, but also harbored additional mutations in some of these genes, especially highlighting the presence of mutations in both genes coding for elongation factor G, *fusA1* and *fusA2* (Supplementary Data Set S1 - Aminoglycosides). In fact, *fusA1* and *fusA2* have been recently demonstrated to be under high evolutionary pressure in the CF environment, which can be explained in terms of a wide aminoglycoside use in this setting[45].

Fluoroquinolone resistome. *P. aeruginosa* RND (Resistance-Nodulation-Division) efflux pumps MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN are well-known to extrude fluoroquinolones. Nevertheless, our data suggest that the contribution of the overexpression of these efflux pumps to high-level resistance to fluoroquinolones is very limited, if any (Supplementary Data Set S1 -Fluoroquinoles). Concerning MexCD-OprJ overproduction, it has been shown that, although wild type *P. aeruginosa* strains generally do not express this efflux system[46], hyperproducing mutants tend to emerge after both *in vitro* and *in vivo* fluoroquinolone exposure[33]. Moreover, there is some data suggesting that MexCD-OprJ hyperproduction could be an advantage in the CF environment[47]. Among the CC274 collection, however, just 1 isolate (FQSE10-0106), showing aa ciprofloxacin MIC below the resistance breakpoint (MIC = 0.38 mg/L), was demonstrated to hyperproduce MexCD-OprJ due to a non-sense mutation in *nfxB*.

On the other hand, our data shows that high-level fluoroquinolone resistance was associated with the presence of missense mutations in *gyrA*, *gyrB* and/or *parC* quinolone resistance-determining regions (QRDRs). Specifically, up to 9 isolates were mutated in *gyrB* QRDR and all but two harbored the same mutation (S466F), 6 showed mutations in *gyrA* QRDR (T83I, T83A, D87N, D87G and Q106L), and just one isolate was mutated in *parE* (P438S). Mutations in GyrA residues 83 and 87 are well-known to be relevant in the clinical setting and are frequently encountered in fluoroquinolone-resistant *P. aeruginosa*[35,36], being both residues situated on helix-4. Mutations in residue 106 are in the other hand very infrequent, with only one previous reference of its existence in 1 of 335 quinolone resistant *P. aeruginosa* clinical strains[48].

Polymyxin resistome. Two component-regulatory systems as well as other genes implicated in lipopolysaccharide biosynthesis have been related with polymyxin resistance[49–51]. Several *in vitro* works have addressed the implication of the two-component regulatory systems in polymyxin resistance development, demonstrating that individual alterations in these systems are generally not sufficient to develop high-level resistance[50,52–54] and that individual two-component systems may not be essential for acquisition of colistin (polymyxin E) resistance in *P. aeruginosa*[55]. In agreement with these *in vitro* studies, we found that many isolates were mutated in genes such as *pagL*, *phoQ* or *pmrB*, but with one exception (i.e. isolate AUS034) phenotypic resistance was not observed (Table 2, Supplementary Data Set S1-Polymyxins). For isolate AUS034, a specific non-sense mutation was detected in the two-component sensor PhoQ, as well as two other specific point mutations within *parR* and *colS*. Five additional isolates were shown to harbour mutations in more than one polymyxin-resistance related genes and showing colistin MICs from 0.125 to 2 mg/L (Supplementary Data Set S1- Polymyxins). Remarkably, none of the mutations detected in the two-component regulatory systems PmrAB and PhoPQ have been previously described in the clinical setting, reflecting an individual strain adaptation to the CF lung[36,52,53,56]. Six different and independent mutational events were registered in PmrB sensor, being all but two located near the active site (H249). Moreover, Spanish mutators shared the same mutation, again reflecting the interpatient transmission of a CF-adapted mutator lineage.

Considering that colistin is widely used for the management of CF patients, the frequent documentation of mutations in genes such as *phoQ*, *pmrB* or *pagL* suggests a role in polymyxin resistance, tolerance or adaptation *in vivo*, even when phenotypic resistance is not demonstrated *in vitro*. Thus, further *in vivo* and clinical studies should be performed to decipher the impact of these mutations for the therapeutic management of CF patients.

Concluding remarks. Emergence of international epidemic CF clonal lineages, along with the extraordinary capacity of *P. aeruginosa* to develop resistance to all antibiotic classes, catalyzed by frequent mutator phenotypes, severely compromises the clinical management of *P. aeruginosa* CF CRI. In addition to the assessment of the emerge of mutator phenotypes within an international CF clone, we analyzed for the first time the genetic basis of hypermutation from whole genome sequence data, through the analysis of the sequence of an exhaustive panel of so called mutator genes, thus designated mutome.

CC274 population structure analysis demonstrated the coexistence of two separated and divergent clonal lineages, but without evident geographical barrier. Coexistence of distinct evolved sublineages within a patient

was documented, reflecting coexistence of divergent lineages within the infecting inoculum or alternatively, and less probable, multiple interpatient transmission events. More revealing is the confirmation, by both phylogenetic reconstructions and mutational resistome analysis, of interpatient transmission of mutators. Compared with classical molecular typing tools, WGS provides detailed genome fingerprints that might be essential for epidemiological studies in which prevalent and ubiquitous clonal lineages are involved. Indeed, WGS closely clustered isolates from four of the patients from the Balearic Islands, likely indicating interpatient transmission or a common source of colonization, whereas isolates from a fifth patient from the same hospital was distantly related.

We have documented at whole genome level the extraordinary capacity of *P. aeruginosa* to acquire resistance by mutational events, evidencing the emergence of mutations in over 100 genes related to antibiotic resistance during the evolution of a CF epidemic clone. Moreover, our results confirm that the evolution of *P. aeruginosa* resistome is greatly enhanced when mutator phenotypes are selected. However, the difficulty for correlating genotypic with phenotypic variation (due to random drift mutations among other causes) has been a hallmark of WGS approaches. To minimize this limitation, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes. While the presence of classical mutational mechanisms, such as the overexpression of the β-lactamase AmpC, the inactivation of the carbapenem porin OprD, or QRDR mutations, was confirmed in a number of isolates and correlated with the resistance phenotypes, our results also provided evidence for the existence and important role of less expected resistance mutations and their phenotypes. Among them, PBP3 mutations, shaping up β-lactam resistance are particularly noteworthy. Likewise, our work, as previously others, denote the very high selective pressure for *mexZ* mutations, leading to the overexpression of MexXY, associated with aminoglycoside resistance. However, we show for the first time that high-level aminoglycoside resistance in CF is driven by the acquisition of additional mutations, particularly those in *fusA1* or *fusA2*, coding for elongation factor G. Finally, a complex repertoire of mutations in genes related to polymyxin resistance is evidenced, but with limited correlation with *in vitro* phenotypic resistance. Altogether, our results provide valuable information for understanding the evolution and dynamics of the mutational resistome of *P. aeruginosa* CF clones and it is correlation with resistance phenotypes, which might be useful for guiding new diagnostic tools and therapeutic strategies in CRI.

Material and Methods

P. aeruginosa CC274 collection and susceptibility testing. The CC274 collection included 29 isolates: 28 recovered from 18 CF patients from Australia and Spain and 1 blood culture isolate from a Spanish non-CF patient, covering up to an 18-year period from 1995 to 2012. All isolates had been previously classified within the CC274 (sharing at least 5 alleles with ST274) based on MLST using available protocols and databases (http://pubmlst.org/paeruginosa/). All the Australian and 4 CF Spanish isolates were single isolates recovered from patients attending clinical settings located in different geographical areas, being each area represented by at least 2 independent isolates, selected randomly from those available. In addition, we included 4 sequential *P. aeruginosa*, each separated by at least 6-month intervals, from each of 4 CF patients attended at the reference hospital of the Balearic Islands (Son Espases Hospital, Spain)(Fig. 1), thus representing intrapatient clone evolution. These patients were shown to be chronically colonized with this persistent strain in a previous study[16]. *P. aeruginosa* PAO1 strain was used as reference when needed. Minimal inhibitory concentrations (MICs) of ceftazidime, cefepime, aztreonam, piperacillin-tazobactam, ceftolozane-tazobactam, imipenem, meropenem, tobramycin, amikacin, ciprofloxacin and colistin were determined by Etest and classified according EUCAST clinical breakpoints (http://www.eucast.org/).

Molecular typing. Clonal relatedness among isolates was evaluated by PFGE. For this purpose, bacterial DNA embedded in agarose plugs prepared as described previously was digested with SpeI. DNA separation was then performed in a contour-clamped homogeneous-electric-field DRIII apparatus (Bio-Rad, La Jolla, CA) under the following conditions: 6 V/cm^2 for 26 h with pulse times of 5 to 40 s. DNA macrorestriction patterns were analyzed with UPGMA to infer clonal relatedness (CLIQS 1D Pro, Totallab).

Mutant frequencies and genetic basis of hypermutation. Rifampicin (300 mg/L) resistance mutant frequencies were determined in all strains following previously established procedures[9,10]. To explore the genetic basis for the mutator phenotypes, complementation studies were performed as described previously[9]. Briefly, plasmid pUCPMS harbouring PAO1 wild-type *mutS*, plasmid pUCPML harbouring PAO1 wild-type *mutL*, and plasmid pUCP24, a control cloning vector, were electroporated into the mutator isolates. Complementation was demonstrated by reversion of the increased rifampicin resistance mutant frequencies in two independent transformant colonies for each strain. Additionally, the genetic basis of hypermutation was investigated from whole genome sequence data, through the analysis of an exhaustive panel of so called mutator genes, thus designated mutome. Genes included within the mutome panel, selected according to available informatio[8] were the following: PA0355/*pfpI*, PA0357/*mutY*, PA0750/*ung*, PA1816/*dnaQ*, PA3002/*mfd*, PA3620/*mutS*, PA4366/*sodB*, PA4400/*mutT*, PA4468/*sodM*, PA4609/*radA*, PA4946/*mutL*, PA5147/*mutM*, PA5344/*oxyR*, PA5443/*uvrD* and PA5493/*polA*.

Characterization of resistance mechanisms. The levels of expression of *ampC*, *mexB*, *mexD*, *mexY*, and *mexF* were determined by real-time reverse transcription (RT)-PCR according to previously described protocols[57]. Additionally, for selected isolates, the sequences of resistance genes, such as *oprD* or mexZ was obtained by Sanger sequencing in order to confirm whole-genome sequencing data as needed. Briefly, after duplicate PCR amplification, sequencing reactions were performed with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were then compared with that yielded by WGS technology.

Library preparation and whole-genome sequencing. Genomic DNA was obtained by using a commercially available extraction kit (High Pure PCR template preparation kit; Roche Diagnostics). Indexed paired-end libraries were prepared with Nextera XT DNA library preparation kit (Illumina Inc, USA) and sequenced on an Illumina MiSeq® benchtop sequencer with MiSeq reagent kit v2 (Illumina Inc., USA), resulting in 250 bp paired-end reads.

Variant calling. Previously defined and validated protocols were used with slight modifications[25, 58]. Briefly, paired-ended reads were aligned to the *P. aeruginosa* PAO1 reference genome (GenBank accession: NC_002516.2) with Bowtie 2 v2.2.4 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml)[59] and, eventually, pileup and raw files were obtained by using SAMtools v0.1.16 (https://sourceforge.net/projects/samtools/files/samtools/)[60] and PicardTools v1.140 (https://github.com/broadinstitute/picard). The Genome Analysis Toolkit (GATK) v3.4-46 (https://www.broadinstitute.org/gatk/) was used for realignment around InDels[61]. Median PAO1 coverage was 95.75% (range: 90.4–97.6%). SNPs were extracted from the raw files if they met the following criteria: a quality score (Phred-scaled probability of the samples reads being homozygous reference) of at least 50, a root-mean-square (RMS) mapping quality of at least 25 and a coverage depth of at least 3 reads; excluding all ambiguous variants. MicroInDels were extracted from the totalpileup files applying the following criteria: a quality score of at least 500, an RMS mapping quality of at least 25 and support from at least one-fifth of the covering reads. Finally, all positions in which at least one of the isolates showed some variation were manually and individually checked in all other isolates without applying any filtering.

***De novo* assembly.** Sequence reads from each isolate were *de novo* assembled using Velvet v1.2.10 (https://www.ebi.ac.uk/~zerbino/velvet/)[62] with a k-mer length of 31 and the following parameters: scaffolding = no, ins_length = 500, cov_cutoff = 3, and min_contig_lgth = 500. The median size of the *de novo* assembled obtained genomes was 6.1Mbp, ranging from 5.4 to 6.6Mbp. MUMmer3 v3.23[63] was used to align the obtained genomes against each other in order to confirm that all belong to the same clone type (genomes differing < 10,000 SNPs).

Phylogenetic reconstructions and BEAST analysis. Core genome phylogenetic reconstructions were performed using Parsnp from the Harvest Suite package v1.2 with default parameters forcing the inclusion of all genomes and a randomly selected reference genome (flags: -c / -r!) (http://harvest.readthedocs.io/en/latest/content/parsnp.html)[20]. Bayesian analysis of divergence times was performed using BEAST v2.4.2 (http://beast2.org/)[64]. For this purpose, a nexus file including all the curated positions at which at least one of the isolates differed from the reference strain PAO1 was constructed and converted into an.xml file with BEAUTi. BEAST was run with the following user-determined settings; a lognormal relaxed molecular clock model and a general time-reversible substitution model with gamma correction[25]. Divergence times were calculated from a chain length of 50 million steps, sampled every 1,000 steps and discarding the first 5 million steps as a burn-in. The maximum clade credibility tree was generated using the TreeAnnotator program from the BEAST package and tree parameters were calculated with Tracer v1.6 (http://beast.bio.ed.ac.uk/Tracer). Both Phylogenetic reconstructions were displayed using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Profiling of antibiotic resistance genes. SNPs and InDels for each isolate were annotated by using SnpEff software v4.2 (http://snpeff.sourceforge.net/index.html)[65] with default options. These files were then filtered based on an exhaustive literature review[35] that led us to obtain a set of 164 genes known to be related to chromosomal antibiotic resistance in *P. aeruginosa* (Supplementary Data Set S1). Additionally, we used the online tool ResFinder v2.1 (https://cge.cbs.dtu.dk//services/ResFinder/)[28] to identify possible horizontally acquired antimicrobial resistance genes.

Ethics statement. The study has been approved by the Research Committee from Son Espases University Hospital. All methods were performed in accordance with the relevant guidelines and regulations. Used isolates derived from frozen stocks of laboratory collections obtained from routine cultures. Patient's information or tissue samples were not used in this study.

References

1. Folkesson, A. *et al*. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. *Nat Rev Microbiol* **10**, 841–51 (2012).
2. Rodríguez-Rojas, A., Oliver, A. & Blázquez, J. Intrinsic and environmental mutagenesis drive diversification and persistence of *Pseudomonas aeruginosa* in chronic lung infections. *J Infect Dis.* **05**, 121–7 (2012).
3. Hogardt, M. & Heesemann, J. Adaptation of *Pseudomonas aeruginosa* during persistence in the cystic fibrosis lung. *Int J Med Microbiol.* **300**, 557–62 (2010).
4. Breidenstein, E. B., de la Fuente-Núñez, C. & Hancock, R. E. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol.* **19**, 419–426 (2011).
5. Silby, M. W., Winstanley, C., Godfrey, S. A., Levy, S. B. & Jackson, R. W. *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol. Rev.* **35**, 652–680 (2011).
6. Aghazadeh, M. *et al*. Role of efflux pumps: MexAB-OprM and MexXY(-OprA), AmpC cephalosporinase and OprD porin in non-metallo-β-lactamase producing *Pseudomonas aeruginosa* isolated from cystic fibrosis and burn patients. *Infect Genet Evol.* **24**, 187–92 (2014).
7. Ciofu, O., Riis, B., Pressler, T., Poulsen, H. E. & Høiby, N. Occurrence of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. *Antimicrob Agents Chemother.* **49**, 2276–82 (2005).
8. Oliver, A. Mutators in cystic fibrosis chronic lung infection: Prevalence, mechanisms, and consequences for antimicrobial therapy. *Int J Med Microbiol.* **300**, 563–72 (2010).
9. Mena, A. *et al*. Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. *J Bacteriol.* **190**, 7910–7 (2008).

10. Oliver, A., Cantón, R., Campo, P., Baquero, F. & Blázquez, J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science.* **288**, 1251–4 (2000).
11. Pedersen, S. S., Koch, C., Hoiby, N. & Rosendal, K. An epidemic spread of multiresistant *Pseudomonas aeruginosa* in a cystic fibrosis centre. *J Antimicrob Chemother.* **17**, 505–516 (1986).
12. Cheng, K. *et al*. Spread of beta-lactam-resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. *Lancet.* **348**, 639–642 (1996).
13. Armstrong, D. *et al*. Evidence for spread of a clonal strain of *Pseudomonas aeruginosa* among cystic fibrosis clinics. *J Clin Microbiol.* **41**, 2266–2267 (2003).
14. Winstanley, C., O'Brien, S. & Brockhurst, M. A. *Pseudomonas aeruginosa* evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. *Trends Microbiol.* **24**, 327–37 (2016).
15. Oliver, A., Mena, A., Macià, M. D. Evolution of Pseudomonas aeruginosa pathogenicity: from acute to chronic infections. In: Baquero, F., Nombela, C., Cassell, G. H., Gutiérrez, J. A. (Eds), *Evolutionary Biology of Bacterial and Fungal Pathogens*, ISBN 978-1-55581-414-4, 433–444, (ASM Press, 2008).
16. López-Causapé, C. *et al*. Clonal dissemination, emergence of mutator lineages and antibiotic resistance evolution in *Pseudomonas aeruginosa* cystic fibrosis chronic lung infection. *PLoS One.* **8**, e71001, doi:10.1371/journal.pone.0071001 (2013).
17. García-Castillo, M. *et al*. Emergence of a mutL mutation causing multilocus sequence typing-pulsed-field gel electrophoresis discrepancy among *Pseudomonas aeruginosa* isolates from a cystic fibrosis patient. *J Clin Microbiol.* **50**, 1777–8 (2012).
18. Kidd, T. J., Grimwood, K., Ramsay, K. A., Rainey, P. & Bell, S. C. Comparison of three molecular techniques for typing *Pseudomonas aeruginosa* isolates in sputum samples. *J Clin Microbiol.* **49**, 263–268 (2011).
19. Freschi, L. *et al*. Clinical utilization of genomics data produced by the international *Pseudomonas aeruginosa* consortium. *Front Microbiol.* **6**, 1036 (2015).
20. Treangen, T. J., Ondov, B. D., Koren, S. & Phillippy, A. M. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol.* **15**, 524 (2014).
21. Williams, D. *et al*. Divergent, coexisting *Pseudomonas aeruginosa* lineages in chronic cystic fibrosis lung infections. *Am J Respir Crit Care Med.* **191**, 775–785 (2015).
22. Jeukens, J. *et al*. Comparative genomics of isolates of a *Pseudomonas aeruginosa* epidemic strain associated with chronic lung infections of cystic fibrosis patients. *PLoSOne.* **9**, e87611, doi:10.1371/journal.pone.0087611 (2014).
23. Yang, L. *et al*. Evolutionary dynamics of bacteria in a human host environment. *Proc Natl Acad Sci USA.* **108**, 7481–6 (2011).
24. Feliziani, S. *et al*. Coexistence and within-host evolution of diversified lineages of hypermutable *Pseudomonas aeruginosa* in long-term cystic fibrosis infections. *PLoS Genet.* **10**, e1004651, doi:10.1371/journal.pgen.1004651 (2014).
25. Marvig, R. L., Johansen, H. K., Molin, S. & Jelsbak, L. Genome analysis of a transmissible lineage of *Pseudomonas aeruginosa* reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. *PLoS Genet.* **9**, e1003741, doi:10.1371/journal.pgen.1003741 (2013).
26. Cramer, N. *et al*. Microevolution of the major common *Pseudomonas aeruginosa* clones C and PA14 in cystic fibrosis lungs. *Environ Microbiol.* **13**, 1690–704 (2011).
27. Magiorakos, A. P. *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect.* **18**, 268–81 (2012).
28. Zankari, E. *et al*. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother.* **67**, 2640–2644 (2012).
29. Maciá, M. D. *et al*. Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. *Antimicrob Agents Chemother.* **49**, 3382–6 (2005).
30. Cabot, G. *et al*. Overexpression of AmpC and efflux pumps in *Pseudomonas aeruginosa* isolates from bloodstream infections: prevalence and impact on resistance in a Spanish multicenter study. *Antimicrob. Agents Chemother.* **55**, 1906–1911 (2011).
31. Moyà, B. *et al*. Beta-lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. *PLoS Pathog.* **5**, e1000353, doi:10.1371/journal.ppat.1000353 (2009).
32. Díaz-Caballero, J. *et al*. Selective sweeps and parallel pathoadaptation drive *Pseudomonas aeruginosa* evolution in the cystic fibrosis lung. *MBio.* **6**, e00981–15, doi:10.1128/mBio.00981-15 (2015).
33. Cabot, G. *et al*. Evolution of *Pseudomonas aeruginosa* antimicrobial resistance and fitness under low and high mutation rates. *Antimicrob Agents Chemother.* **60**, 1767–1778 (2016).
34. Han, S. *et al*. Structural basis for effectiveness of siderophore-conjugated monocarbams against clinically relevant strains of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **107**, 22002–22007 (2010).
35. Cabot, G. *et al*. Deciphering the resistome of the widespread *Pseudomonas aeruginosa* sequence type 175 international high-risk clone through whole-genome sequencing. *Antimicrob Agents Chemother.* **60**, 7415–7423 (2016).
36. Kos, V. N. *et al*. The resistome of *Pseudomonas aeruginosa* in relationship to phenotypic susceptibility. *Antimicrob Agents Chemother.* **59**, 427–36 (2015).
37. Vettoretti, L. *et al*. Efflux unbalance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob Agents Chemother.* **53**, 1987–97 (2009).
38. Poole, K. Aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* **49**, 479–87 (2005).
39. Hocquet, D. *et al*. MexXY-OprM efflux pump is necessary for adaptive resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother.* **47**, 1371–5 (2003).
40. Prickett, M. H. *et al*. Aminoglycoside resistance of *Pseudomonas aeruginosa* in cystic fibrosis results from convergent evolution in the mexZ gene. *Thorax.* **72**, 40–47 (2017).
41. Vogne, C., Aires, J. R., Bailly, C., Hocquet, D. & Plésiat, P. Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Antimicrob Agents Chemother.* **48**, 1676–80 (2004).
42. Feng, Y., Jonker, M. J., Moustakas, I., Brul, S. & Ter Kuile, B. H. Dynamics of mutations during development of resistance by *Pseudomonas aeruginosa* against five antibiotics. *Antimicrob Agents Chemother.* **60**, 4229–36 (2016).
43. Schurek, K. N. *et al*. Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **52**, 4213–4219 (2008).
44. El'Garch, F., Jeannot, K., Hocquet, D., Llanes-Barakat, C. & Plésiat, P. Cumulative effects of several nonenzymatic mechanisms on the resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother.* **51**, 1016–21 (2007).
45. Greipel, L. *et al*. Molecular epidemiology of mutations in antimicrobial resistance loci of *Pseudomonas aeruginosa* isolates from airways of cystic fibrosis patients. *Antimicrob Agents Chemother.* **60**, 6726–6734 (2016).
46. Poole, K. *et al*. Overexpression of the mexC-mexD-oprJ efflux operon in nfxB-type multidrug-resistant strains of *Pseudomonas aeruginosa*. *Mol Microbiol.* **21**, 713–724 (1996).
47. Mulet, X. *et al*. Antagonistic interactions of *Pseudomonas aeruginosa* antibiotic resistance mechanisms in planktonic but not biofilm growth. *Antimicrob Agents Chemother.* **55**, 4560–4568 (2011).
48. Takenouchi, T., Sakagawa, E. & Sugawara, M. Detection of gyrA mutations among 335 *Pseudomonas aeruginosa* strains isolated in Japan and their susceptibilities to fluoroquinolones. *Antimicrob Agents Chemother.* **43**, 406–9 (1999).
49. Tomaras, A. P. *et al*. LpxC inhibitors as new antibacterial agents and tools for studying regulation of lipid A biosynthesis in Gram-negative pathogens. *MBio.* **5**, e01551–14, doi:10.1128/mBio.01551-14 (2014).
50. Gutu, A. D. *et al*. Polymyxin resistance of *Pseudomonas aeruginosa* phoQ mutants is dependent on additional two-component regulatory systems. *Antimicrob Agents Chemother.* **57**, 2204–15 (2013).

51. Fernández, L. *et al*. The two-component system CprRS senses cationic peptides and triggers adaptive resistance in *Pseudomonas aeruginosa* independently of ParRS. *Antimicrob Agents Chemother.* **56**, 6212–22 (2012).
52. Moskowitz, S. M. *et al*. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother.* **56**, 1019–30 (2012).
53. Miller, A. K. *et al*. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother.* **55**, 5761–9 (2011).
54. Fernández, L. *et al*. Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrob Agents Chemother.* **54**, 3372–82 (2010).
55. Lee, J. Y. *et al*. Development of colistin resistance in pmrA-, phoP-, parR- and cprR-inactivated mutants of *Pseudomonas aeruginosa*. *J Antimicrob Chemother.* **69**, 2966–71 (2014).
56. Barrow, K. & Kwon, D. H. Alterations in two-component regulatory systems of phoPQ and pmrAB are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* **53**, 5150–4 (2009).
57. Juan, C., Moyá, B., Pérez, J. L. & Oliver, A. Stepwise upregulation of the *Pseudomonas aeruginosa* chromosomal cephalosporinase conferring high-level beta-lactam resistance involves three AmpD homologues. *Antimicrob Agents Chemother.* **50**, 1780–7 (2006).
58. Marvig, R. L., Sommer, L. M., Molin, S. & Johansen, H. K. Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nat Genet.* **47**, 57–64 (2015).
59. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* **9**, 357–9 (2012).
60. Li, H. *et al*. The Sequence Alignment/Map format and SAMtools. *Bioinformatics.* **25**, 2078–9 (2009).
61. DePristo, M. A. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet.* **43**, 491–8 (2011).
62. Zerbino, D. R. & Birney, E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res.* **18**, 821–9 (2008).
63. Kurtz, S. *et al*. Versatile and open software for comparing large genomes. *Genome Biol.* **5**, R12 (2004).
64. Drummond, A. J., Suchard, M. A., Xie, D. & Rambaut, A. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol Biol Evol* **29**, 1969–1973 (2012).
65. Cingolani, P. *et al*. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *Fly (Austin).* **6**, 80–92 (2012).

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Author Contributions

C.L.-C. conceived the study, performed laboratory experiments and bioinformatics analysis, analyzed results and wrote the manuscript. L.M.S. performed bioinformatics analysis. G.C. performed laboratory experiments and analyzed results. R.R. performed laboratory experiments. A.A.O.-S. contributed materials. H.K.J. contributed analysis tools. J.F. contributed materials. R.C. contributed materials and critically reviewed the manuscript. T.J.K. contributed materials and critically reviewed the manuscript. S.M. contributed analysis tools and critically reviewed the manuscript. A.O. conceived the study, analyzed results and wrote the manuscript.

Additional Information

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Antibiotic resistance and population structure of cystic fibrosis *Pseudomonas aeruginosa* isolates from a Spanish multi-centre study

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ABSTRACT

The first Spanish multi-centre study on the microbiology of cystic fibrosis (CF) was conducted from 2013 to 2014. The study involved 24 CF units from 17 hospitals, and recruited 341 patients. The aim of this study was to characterise *Pseudomonas aeruginosa* isolates, 79 of which were recovered from 75 (22%) patients. The study determined the population structure, antibiotic susceptibility profile and genetic background of the strains. Fifty-five percent of the isolates were multi-drug-resistant, and 16% were extensively-drug-resistant. Defective *mut*S and *mut*L genes were observed in mutator isolates (15.2%). Considerable genetic diversity was observed by pulsed-field gel electrophoresis (70 patterns) and multi-locus sequence typing (72 sequence types). International epidemic clones were not detected. Fifty-one new and 14 previously described array tube (AT) genotypes were detected by AT technology. This study found a genetically unrelated and highly diverse CF *P. aeruginosa* population in Spain, not represented by the epidemic clones widely distributed across Europe, with multiple combinations of virulence factors and high antimicrobial resistance rates (except for colistin).

1. Introduction

The lower respiratory tract of patients with cystic fibrosis (CF) is usually chronically colonised by a complex microbial ecosystem. This colonisation triggers an inflammatory response that produces respiratory symptoms and acute exacerbations, and influences the patients' clinical course and outcome. *Pseudomonas aeruginosa* is the most relevant micro-organism in this process. During the first stages of the disease, CF *P. aeruginosa* isolates are almost identical to environmental isolates. The evolved disease is characterised by mucoid colonies and/or multi-drug-resistant isolates [1] that result from the particular CF lung environment, a compartmentalised hostile niche for *P. aeruginosa* that forces the bacteria to an ecological adaptation [2], and frequent mutator phenotypes [3].

Previous epidemiological studies on CF *P. aeruginosa* isolates have been performed using different molecular typing tools. For instance, the use of multi-locus sequence typing (MLST) has allowed the identification of international epidemic CF clones, such as the well-known Liverpool epidemic strain or Clone C. Moreover, several hypertransmissible CF *P. aeruginosa* strains have been described [4], the detection of which should alert clinicians to prevent transmission between patients, including siblings [5] and patients from the same or different centres [6]. By using the array tube (AT) multi-marker array, some genotypes have been found to be most abundant in the global *P. aeruginosa* population, particularly AT genotypes 0C2E, 2C22, C40A, D421 and F429 that have been detected in both clinical and environmental isolates [7–13].

In Spain, the genetic background of *P. aeruginosa* isolates obtained from two different CF units has been reported previously [14,15], with ST274 and ST395 identified as endemic clones at each centre. This study, the first Spanish multi-centre study on the microbiology of CF, was conducted from 2013 to 2014, and included a representative patient population from across Spain [16]. The aim of this study was to characterise *P. aeruginosa* isolates to complete the microbiological description of this micro-organism in patients with CF in Spain.

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2. Materials and methods

2.1. Study design

This study involved 24 CF units (12 paediatric and 12 adult) from 17 hospitals [16]. Fifteen consecutive unselected patients per CF unit were recruited, and a single sputum sample from each patient was frozen immediately after collection at –80°C. The frozen samples were sent to Ramón y Cajal University Hospital and, after slow defrosting, were seeded in plates in the appropriate culture medium (see details in [17]). The plates were examined at 24 and 48 h, and the incubation time was extended to 5 days in order to identify potentially slow-growing bacteria. Colonies with compatible *P. aeruginosa* morphology were identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (Bruker Daltonics GmbH, Leipzig, Germany) and stored for further studies.

2.2. Antibiotic susceptibility

Antibiotic susceptibility was determined by disk diffusion, except for fosfomycin, for which the agar dilution method was performed [17]. The tested compounds included piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, colistin, gentamicin, tobramycin, amikacin, ciprofloxacin, levofloxacin and fosfomycin. The European Committee on Antimicrobial Susceptibility Testing clinical breakpoints for systemic infections were applied (www.eucast.org), except for fosfomycin. PAO1 and ATCC 27853 *P. aeruginosa* reference strains were used as controls. Consensus recommendations [18] were used to evaluate the proportion of multi-drug-resistant (MDR, not susceptible to at least three antimicrobial classes), extensively-drug-resistant (XDR, only susceptible to one or two antimicrobial classes) and pandrug-resistant (PDR; not susceptible to any antibiotics) strains, considering the following seven antimicrobial classes: cephalosporins (ceftazidime and/or cefepime), penicillin-β-lactamase inhibitor combinations (piperacillin-tazobactam), monobactams (aztreonam), carbapenems (imipenem and/or meropenem), fluoroquinolones (ciprofloxacin), aminoglycosides (gentamicin, tobramycin, and/or amikacin) and colistin. For percentages of pseudomonas colonisation and antibiotic resistance, 95% confidence intervals (CI) were calculated using the Exact formula.

2.3. Mutant frequencies and genetic basis for hypermutation

Mutant frequencies for rifampicin (300 mg/L) resistance were determined in triplicate for all strains following previously established procedures [3]. To explore the genetic basis of mutator isolates, previously described primers and protocols were employed to amplify and sequence the *mut*S and *mut*L genes [19]. Briefly, plasmid pUCPMS harbouring PAO1 wild-type *mut*S, plasmid pUCPML harbouring PAO1 wild-type *mut*L, and plasmid pUCP24, a control-cloning vector, were electroporated into the mutator isolates. Complementation was demonstrated by reversion of the increased mutant frequencies for rifampicin resistance in two independent transformant colonies for each mutator isolate. Previously described primers and protocols [19] were used for the amplification and sequencing of *mut*S or *mut*L genes according to the results of complementation experiments.

2.4. Population structure

The genetic diversity of the isolates was explored initially by pulsed-field gel electrophoresis (PFGE) with the macrorestriction enzyme *Spe*I [20]. DNA separation was performed using a contour-clamped homogeneous electric field DRIII apparatus (Bio-Rad, La Jolla, CA, USA) under the following conditions: 6 V/cm^2 for 22 h with pulse times of 5–40 s. Finally, DNA macrorestriction patterns were interpreted according to visual criteria, and after constructing a dendrogram using the Dice coefficient and Phoretix 5.0 software.

All isolates were further genotyped by MLST (http://pubmlst.org/paeruginosa/) using available protocols and databases. MEGA6 software enabled phylogenetic analysis of the MLST alleles and their concatenate sequence. A minimum spanning tree was constructed using the goeBURST algorithm (www.phyloviz.net).

2.5. P. aeruginosa *AT genotyping*

The Clondiag (Alere Technologies GmbH, Jena, Germany) AT species-specific genotyping system was employed according to the manufacturer's protocol [13]. This species-specific micro-array enables the genotyping of *P. aeruginosa* strains using 13 informative single nucleotide polymorphisms at conserved loci, the *fli*Ca/*fli*Cb multi-allelic locus, and the presence or absence of the *exoS*/*exoU* marker gene. The AT system also includes 38 genetic markers from the accessory genome for defining intraclonal diversity.

2.6. exoS and exoU amplification assays

Polymerase chain reaction (PCR) assays for detecting *exoS* and *exoU* genes were performed using previously described primers and protocols [21], with slight modifications. PCR reactions were performed with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) in a DNA thermal cycler (Arktic Thermal Cycler; Thermo Fisher, Waltham, MA, USA) under the following conditions: denaturation for 5 min at 94 °C, followed by 35 cycles at 94 °C for 30 s, at 58 °C for 30 s and at 72 °C for 30 s, and a final extension step of 10 min at 72 °C.

3. Results

3.1. Patients, samples and isolates

From a total of 341 respiratory samples (one per patient), 79 *P. aeruginosa* isolates were recovered from 75 patients (four patients presented colonies with two different morphologies). The global colonisation rate was 22% (95% CI 17–26), and was higher in the adult population (32.7%, 95% CI 25–40) than in the paediatric population (9.9%, 95% CI 5–15) ($P < 0.001$). *P. aeruginosa* colonisation status was defined as intermittent (one patient, 6%) or chronic (15 patients, 94%) for the paediatric population; the corresponding numbers were 10 patients (17%) and 49 patients (83%) for the adult population. The primary characteristics of the patients with CF are shown in Table 1. Clinical data from the entire CF population can be found elsewhere [16].

The classical CF mucoid morphotype was observed in 17 (21.5%, 95% CI 13–32) isolates, whereas the others were classified as metallic (23 isolates, 29.2%, 95% CI 19–40) or Enterobacteriaceae-like (23 isolates, 29.2%, 95% CI 19–40). Sixteen isolates (20%, 95% CI 12–30) presented small colony variant (SCV) morphology. Half of the isolates exhibited brown (16 isolates, 20.2%) or green (25 isolates, 31.6%) pigmentation after 48 h of incubation at 37 °C. In 36 (48%, 95% CI 34–57) of the 75 patients, co-existence of *P. aeruginosa* and *Staphylococcus aureus* was detected by classical culture in agar plates, with seven (9.3%) of the isolates resistant to methicillin.

3.2. Antibiotic susceptibility profiles

Overall (intermediate plus resistant) antibiotic resistance rates are shown in Table 2. Colistin was the most active compound, and only three isolates (4%) were classified as resistant to colistin. Considering co-resistances and excluding aztreonam, 15 isolates

Table 1
Primary characteristics of the 341 patients with cystic fibrosis in the Spanish multi-centre study [16].

Sex	Category	No. of patients	Median value (range)			No. (%) of Δ508 mutations	
			Age (years)	Weight (kg)	FEV_1	Heterozygosis	Homozygosis
Patients colonised by *Pseudomonas aeruginosa*							
Male	Paediatric	10	15 (11–17)	41 (29–57)	65.2 (38–102)	2 (20.0)	4 (80.0)
	Adult	27	28 (18–51)	65 (47–87)	48.6 (20–106)	11 (40.7)	9 (33.3)
Female	Paediatric	6	16 (7–16)	44 (18–58)	78 (30–107)	1 (16.6)	4 (66.6)
	Adult	32	29 (18–40)	52.7 (39–68)	50.5 (18–87)	17 (50.0)	9 (28.1)
Other patients							
Male	Paediatric	65	11 (2–17)	34 (13–71)	86 (44–185)	27 (41.5)	21 (32.3)
	Adult	59	28 (18–56)	68 (41–89)	66 (20–120)	33 (55.9)	15 (25.4)
Female	Paediatric	80	11 (3–17)	35 (11–61)	75 (29–125)	33 (40.7)	35 (43.2)
	Adult	62	26 (18–48)	51.5 (38–91)	57 (17–108)	32 (51.6)	15 (24.1)

FEV_1, forced expiratory volume in 1 s.

(29%) remained susceptible to all antibiotics, 55% were classified as MDR, 16% were classified as XDR, and none corresponded with the PDR phenotype. Isolates displaying a mucoid morphotype were the most susceptible to antibiotics, whereas SCV isolates were the most resistant (Table 2).

3.3. Mutator phenotype

In total, 12 isolates (15.2%) that were recovered from eight adults and four children were considered as mutators (mutation frequency range 2×10^{-5}–4.5×10^{-7}). Eight of these mutator isolates had inactivating mutations in *mut*S ($n = 7$) or *mut*L ($n = 1$) genes. Three additional mutator isolates showed amino acid substitutions in the MutS and/or MutL proteins of uncertain effect, and the remaining isolate showed wild-type sequences of *mut*S and *mut*L genes (Table 3). All mutator isolates belonged to unrelated genetic lineages.

3.4. P. aeruginosa *population structure*

Considering both overall and individual CF unit data, considerable genetic diversity by PFGE (70 patterns) and MLST [72 sequence types (STs)] was observed among the collection (Table 4 and Fig. 1). Identical PFGE band patterns, STs and AT genotypes were detected in the *P. aeruginosa* isolates recovered from a pair of brothers (strains 78 and 79). Additionally, a similar PFGE pattern was detected in unrelated strains, some of which also matched other typing methodologies. The most relevant case concerned a possible intrahospital cross-transmission related to strains 66 and 68 with identical PFGE band patterns and AT genotypes but different MLST. Ascription to different STs was due to a single A331C *mut*S mutation that provokes the switch of allele 48 by allele 3, and consequently the assignation of ST1881 instead of ST348. Other groups of strains with similar PFGE band patterns have been detailed in Table 4 and Fig. 2.

The MLST technique was applied to the entire collection (79 isolates), detecting a total of 72 different STs, 49 of which had not been described previously (six new alleles and non-previously described combinations of known alleles). A high genetically diverse background was observed throughout the MLST results, in which only six of the 49 STs grouped more than one isolate. International epidemic clones were not detected (Fig. 1).

A phylogenetic analysis of the concatenated sequences of the MLST alleles was performed with the study isolates and the entire MLST database to identify particular lineages. The results demonstrated a lack of genetic relationship between the study isolates, rejecting cross-transmission between the various CF units and the predominance of particular lineages (Figs 2 and 3).

3.5. AT genotyping: exploring the accessory genome

The AT multi-marker micro-array enabled the detection of 51 new and 14 previously described AT genotypes containing one to three isolates. The five most abundant AT genotypes (0C2E, 2C22, C40A, D421 and F429) in the global *P. aeruginosa* population [7–9] comprise 10.1% of this Spanish CF collection.

Ferripyoverdine receptor genes (*fpvA*) type I, IIa, IIb and III were detected in 23, 22, six and 11 isolates, respectively, and the alternative type I ferripyoverdine receptor gene *fpvB* was present in 45 isolates. Ferripyoverdine receptor genes were not detected in 15 isolates, and all but two were isolated in adults. These results showed that the isolates possess an average of 2.3 genome islets and 2.4 genome islands (ranging from 0 to 5).

The flagellin glycosylation island was the most prevalent (53 isolates, 67.0%). Among this subset of isolates, two lacked the a-type

Table 2
Percentage of antibiotic resistance, including intermediate plus resistant isolates, of the different *Pseudomonas aeruginosa* morphotypes (95% confidence intervals).

Morphotype	Percentage resistant to													% MDR	% XDR
	P/T	CAZ	CEP	AZT	IMI	MER	COL	GEN	TOB	AMK	CIP	LVX	FOS		
Mucoid ($n = 17$)	17.6 (3–43)	23.5 (6–50)	47.0 (22–72)	100	17.6 (3–43)	35.2 (14–61)	0	35.2 (14–61)	29.4 (10–55)	41.1 (18–67)	58.8 (32–81)	58.8 (32–81)	11.7 (1–36)	17.6 (3–43)	5.8 (0–28)
Enterobacteriaceae ($n = 23$)	17.3 (4–38)	30.4 (13–52)	34.7 (16–57)	100	30.4 (13–52)	34.7 (16–57)	0	39.1 (19–61)	39.1 (19–61)	34.7 (16–57)	43.4 (23–65)	56.5 (34–76)	21.7 (7–43)	56.5 (34–76)	21.7 (7–43)
Metallic ($n = 23$)	13.0 (2–33)	30.4 (13–52)	30.4 (13–52)	100	47.8 (26–69)	60.8 (38–80)	8.7 (1–28)	34.7 (16–57)	26.0 (10–48)	43.4 (23–65)	69.5 (47–86)	73.9 (51–89)	13.0 (2–33)	60.8 (38–80)	21.7 (7–43)
SCV ($n = 16$)	25.0 (7–52)	50.0 (24–75)	37.5 (15–64)	100	43.7 (19–70)	50.0 (24–75)	0	37.5 (15–64)	31.2 (11–50)	37.5 (15–64)	68.75 (41–88)	68.75 (41–88)	31.2 (11–50)	87.5 (61–98)	12.5 (1–38)
Total ($n = 79$)	17.7 (10–27)	32.9 (22–44)	36.7 (26–48)	100	35.4 (25–47)	45.5 (34–57)	2.5 (0–8)	36.7 (26–48)	31.6 (21–43)	39.2 (28–50)	59.4 (47–70)	64.5 (52–75)	18.9 (11–29)	55.7 (44–66)	16.4 (9–26)

P/T, piperacillin/tazobactam; CAZ, ceftazidime; CEP, cefepime; AZT, aztreonam; IMI, imipenem; MER, meropenem; COL, colistin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; CIP, ciprofloxacin; LVX, levofloxacin; FOS, fosfomycin; SCV, small colony variants; MDR, multi-drug-resistant (not susceptible to at least three antimicrobial classes); XDR, extensively-drug-resistant (only susceptible to one or two antimicrobial classes).

Table 3
Mutator isolates detected in the study collection and their molecular basis.

Isolate	ST	Mutation frequency	Complemented with	Detected mutation
49	132	2.00×10^{-5}	*mut*S	$Nt_{399}\Delta 12$
42	268	2.47×10^{-6}	*mut*S	$Nt_{1600}\Delta 13$
16	270	2.93×10^{-7}	*mut*S	G1290A (E431K)
			*mut*L	G1872A (G632E)
9	1109	8.26×10^{-6}	*mut*S	$Nt_{1120}\Delta 5$
13	1870	5.20×10^{-6}	ND	ND
26	1871	2.50×10^{-6}	*mut*L	T1309C (A437T)
27	1872	4.57×10^{-7}	*mut*L	T647G (V216G)
6	1890	1.38×10^{-6}	*mut*L	$Nt_{644}\Delta 21$
47	1906	1.80×10^{-6}	*mut*S	$Nt_{1336}\Delta 2$
53	1909	3.20×10^{-6}	*mut*S	$Nt_{1377}\Delta 1$
55	1911	2.71×10^{-6}	*mut*S	Nt_{2577}ins9pb
60	1914	3.07×10^{-6}	*mut*S	Nt_{1198}IS-4-like

ST, sequence type; ND, not detected.

flagellin, and the three isolates expressing the a-type flagellin lacked the flagellin glycosylation island. PAGI-1 was detected in 45 isolates (56.9%), and the genomic islands of the CLC family PAGI-2/3 were found in 23 isolates (29.1%). Two isolates harboured both PAPI-1 and PAPI-2, and 34 (43%) isolates harboured PAPI-2; other pKLC102-like islands were detected in 30 isolates (37.9%). A significant discrepancy was observed in the prevalence of T3SS effector *exoS* and *exoU* genes detected by this technique (18% and 9% of the isolates, respectively) and by independent specific PCR assays (81% and 10%, respectively). Statistical differences within the overall collection and within the different subsets (mutators, adult population and paediatric population) were not observed.

To investigate potential patient-to-patient and unit-to-unit transmission, the intraclonal diversity of all isolates was analysed, excluding the *exoS* and *exoU* genes as core markers (Table 2 and Fig. 2). This analysis revealed potential patient-to-patient transmission in two cases, including the pair of brothers, and isolates 66 and 68 from unrelated patients. In addition, the same AT genotype was detected in another two patients, each with two morphotypes but with a different repertoire of accessory genes (61, 62, 71 and 72). This finding supports the hypothesis that diversification occurs during adaptation to the CF lung.

4. Discussion

Surveillance and epidemiological studies are useful for detecting and controlling hypertransmissible and hypervirulent strains, and for studying the evolution of antimicrobial resistance trends and the colonisation rates of patients. This study addressed the

Table 4
Results obtained with pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and array tube (AT) techniques. Isolates with identical PGFE, MLST or AT genotype are marked in grey.

Centre	Isolate	PFGE	ST	AT genotype	Centre	Isolate	PFGE	ST	AT genotype
A	1	1	1748	X298	L	44	38	1876	2418
B	2	2	1886	6018		45	39	564	E418
	3	3	1887	6198		46	40	1905	2C18
C	4	3	1888	XX98		47	41	1906	4DA8
	5	4	1889	8782		48	42	644	2708
D	6	5	1890	F669		49	43	132	7C2C
	7	6	1891	4818		50	44	1877	3E18
	8	7	1866	7D98	M	51[a1]	45	1907	EC18
E	9	8	1109	CC0A		52[a1]	46	1908	2C2C
	10	9	1867	8C2C		53	47	1909	6A20
	11	10	1868	2C22		54	48	1910	6FA8
	12	11	1869	6FA8	N	55[a2]	49	1911	AF90
F	13	12	1870	F468		56[a2]	50	1251	F420
	14	13	1892	AD80		57	48	1878	6FA8
G	15	14	1893	ED98		58	51	1912	2610
	16	15	270	882A	O	59	52	1913	4498
	17	16	116	6010	P	60	53	1914	2810
	18	17	395	0C2E		61[a3]	54	1879	E020
	19	18	1894	0C2A		62[a3]	55	508	E020
	20	19	1895	2F80	Q	63	56	189	8E18
	21	20	1228	741C		64	57	1880	E429
	22	21	253	0C2A	R	65	58	253	D421
	23	22	395	0C2C		66	59	1881	2398
	24	23	1896	F428		67	60	1882	XC10
H	25	24	1897	0422		68	59	348	2398
	26	25	1871	2C18	S	69	61	1883	DF88
	27	26	1872	C40A		70	62	676	EC10
	28	27	1873	E022		71[a4]	63	508	E020
	29	28	575	859A		72[a4]	64	508	E020
	30	29	1898	8428		73	65	575	A598
	31	30	1899	F419		74	66	313	F429
I	32	30	1900	B01A		75	67	198	0C48
	33	31	617	2C20		76	68	569	6E10
J	34	32	1901	B01X		77	69	1884	0810
	35	33	560	F421	T	78	70	27	EA08
	36	34	1902	262A		79	70	27	EA08
	37	31	1903	2C20					
	38	35	1874	E42A					
K	39	35	1874	E428					
	40	36	609	A998					
	41	35	1875	E428					
	42	31	268	2C20					
	43	37	1904	2F88					

ST, sequence type.
[a] Different morphotypes in the same patient (from one to four).

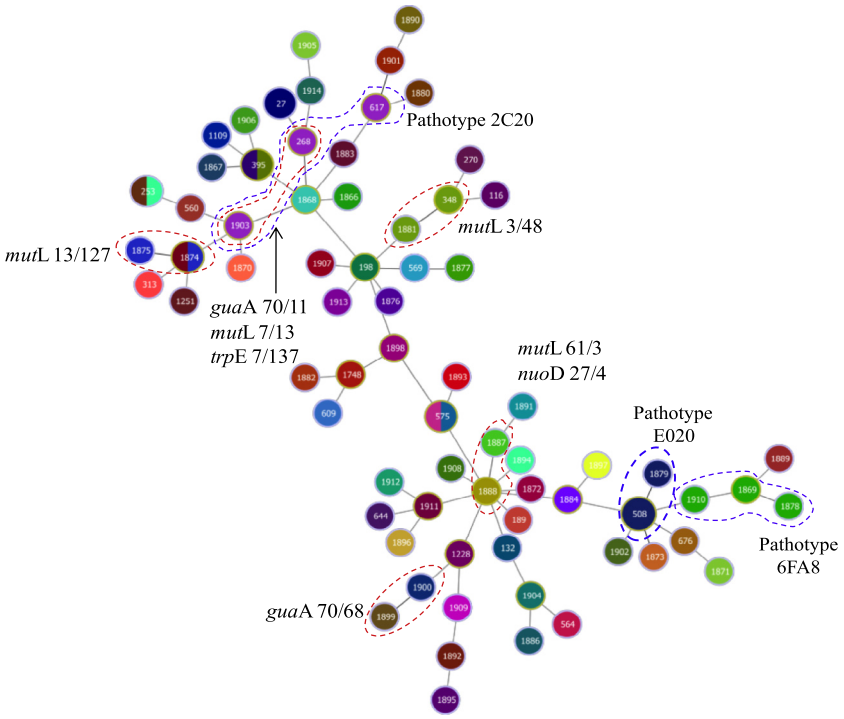

Fig. 1. Minimum spanning tree combining the detected sequence type (numbers inside the circles) and the virulence determinants (colours). The particular cases of strains sharing pulsed-field gel electrophoresis band patterns (red lines) or pathotype (blue lines) are marked with circles, and the primary features are described.

primary phenotypic and genetic features of CF *P. aeruginosa* isolates recovered in the first microbiological multi-centre study performed in Spain [16].

Although this study had a relatively low representation of the total Spanish population with CF (9–10%) [https://www.ecfs.eu/projects/ecfs-patient-registry/annual-reports], the wide geographical distribution of the involved units (17 tertiary care hospitals) assured adequate sampling. To avoid errors related to the patient selection process, the first 15 consecutive patients who agreed to participate in the study were included, without employing any selective criteria for age, colonisation pattern or lung function. Although geographical and seasonal differences in the prevalence of *P. aeruginosa* have been reported [22], no geographical differences were observed in this series. However, the seasonal effect may have been underestimated given that recruitment was conducted from March to October 2013, and the coldest period of the year was not included.

The CF patients in this study had low *P. aeruginosa* colonisation rates (22% overall, 32.7% in adults and 9.9% in children). These data, considered as point prevalence colonisation (data from one sputum sample per patient), are consistent with those of other contemporary studies that revealed a currently decreasing and slowing trend in *P. aeruginosa* lung colonisation in patients with CF. This finding could

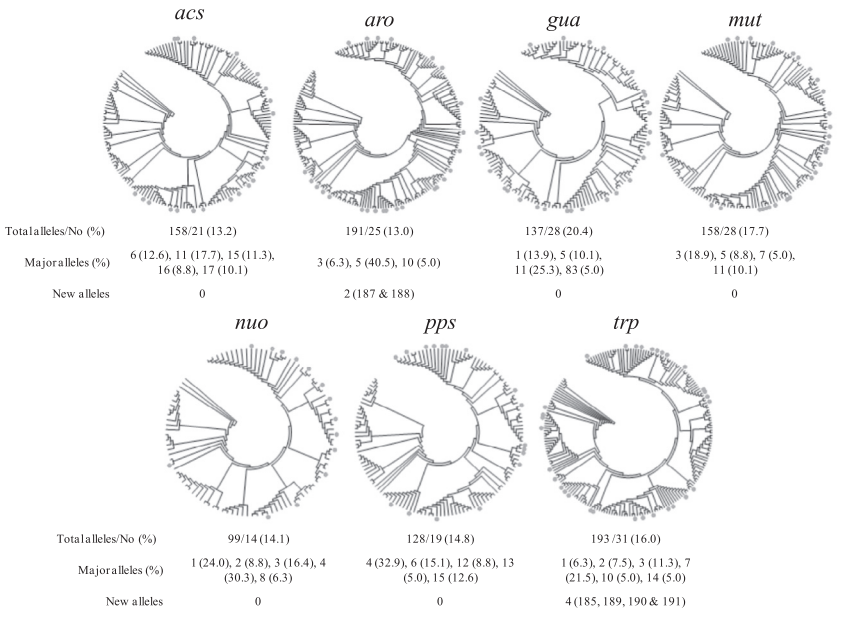

Fig. 2. Phylogenetic analysis of each multi-locus sequence typing allele, comparing those found in the study collection (distribution is marked with a grey circle).

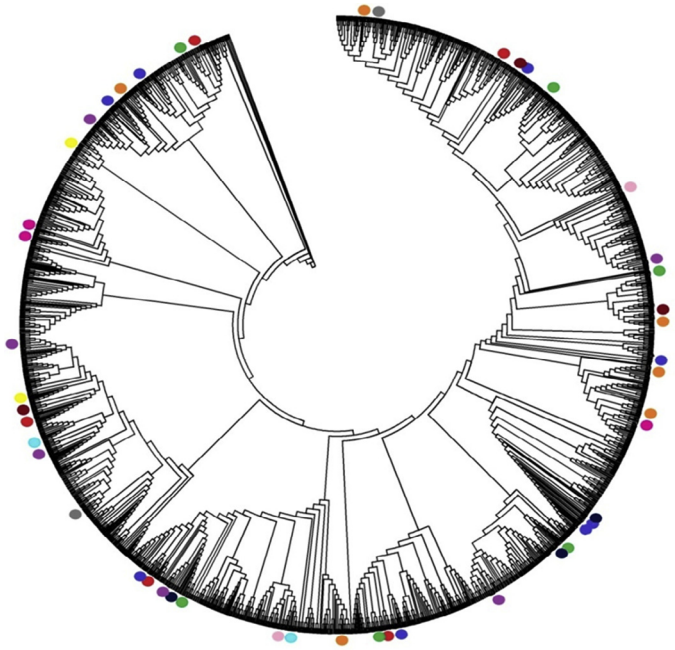

Fig. 3. Phylogenetic analysis of the concatenate sequences of the seven multilocus sequence typing (MLST) alleles, comparing the whole MLST database with the study collection. Circles represent the 79 isolates from the present study, and the colour indicates the hospital in which they were isolated.

be related to better clinical management of patients, newborn screening and bacterial ecosystems [23]. Nevertheless, the possible lack of viable bacterial cells at freezing and defrosting has not been evaluated, and therefore this possibility should not be ruled out. Another important factor to consider is the convenience of a single sputum sample as representative of the total *P. aeruginosa* lung population. It has been demonstrated that different genetic lineages can co-exist in the lung compartments, and the entire population is usually underestimated in a single sputum sample [2,24]. Moreover, a recent report demonstrated the co-existence of several lineages along the respiratory tract in a single CF subject, with evidence of spatial separation between the nasopharynx and the lower lung [25].

Finally, the relatively low number of isolates together with their high genetic variability did not allow specific genotypes to be correlated with patients' clinical status. Nevertheless, despite these potential limitations, one of the most important results of this study is the lack of representation of international CF epidemic clones recognised in several European countries [4]. C40A is the AT genotype previously described for Clone C (ST17) [9,26], and was also detected in isolate 27 of the study collection. The epidemic Clone C belongs to ST17, and, curiously, isolate 27 typed as ST1872 is a double locus variant that differs in just two point mutations in *mut*L and *trp*E alleles. On the other hand, ST175 and ST111 are the most frequent *P. aeruginosa* lineages in the Spanish nosocomial setting [27], and neither lineage was present in the CF collection. These data support the hypothesis that CF isolates are usually acquired from the environment and not from hospital sources.

Overall, the mucoid morphotype was the most typical morphotype observed in the patients with CF. The clinical relevance of the SCV morphotype has been highlighted in recent years [28], and in the present study, SCVs were only detected in adults ($n = 16$) (median age 30 years), whereas the mucoid phenotype was present in adults ($n = 13$) and children ($n = 4$) (median ages 29 and 15 years, respectively). Overall, MDR resistance rates were slightly lower than those encountered in a recent study of *P. aeruginosa* isolates recovered from CF patients in Northern Europe [29]. Moreover, resistance rates to individual agents were also lower than those encountered in Northern Europe.

High proportions of mutator isolates among the CF *P. aeruginosa* population have been demonstrated previously, and are frequently associated with antimicrobial resistance [30]. In this study, a lower proportion of mutators (15%) was found in comparison with another Spanish study (36%) [3], with 55% of these mutator isolates classified as MDR and 16% as XDR. Eight of the 12 mutator isolates were detected in adults, with a clear trend in relation to more evolved stages.

MLST is the reference technique [31], although the particular features of the CF *P. aeruginosa* isolates might limit the use of this technique [19,32]. In the study collection, MLST demonstrated a highly polyclonal structure with 72 different STs, 49 of which are described for the first time. The acquisition and loss of genetic traits between single lineages, combined with the natural exchange of virulence factors between unrelated isolates, was also suggested after the AT genotyping results. Nevertheless, identical AT genotypes (2C18, 2C22, 6FA8, E42A and 2398) were detected in unrelated strains from independent institutions.

P. aeruginosa possesses remarkable adaptability, primarily due to its large and complex genome. The pan-genome of this opportunistic pathogen consists of the conserved core genome (90%) and a combinatorial accessory genome (10%) essential for adaptation and survival in unfavourable conditions. *P. aeruginosa* might therefore have a large armamentarium of secreted virulence factors that rely on specialised export systems, including the type III secretion system (T3SS) [33]. The authors have investigated the presence of ExoS and ExoU (two T3SS effector proteins), and the results are consistent with previously published data for CF respiratory isolates [21,34].

A major example of *P. aeruginosa* adaptability is its ability to produce three different types of pyoverdine and four binding receptors. The major finding in this variable locus was the absence of the alternative receptor for pyoverdine type I (*fpv*B) in 43% of the isolates; this finding does not correlate with a number of previous studies [34] which found that almost all CF isolates harboured this receptor. Nevertheless, Dingemals et al also found that a significant proportion of CF isolates lacked this alternative receptor (22%) [35]. These authors hypothesised that this receptor might not be under selective pressure, because *P. aeruginosa* can use multiple iron uptake systems in the CF lung to acquire iron in both its ferric and ferrous forms [36,37]. As an alternative hypothesis, the authors suggested that the loss of *fpv*B could be an advantage for evading the immune system and the action of pyocines [35].

The precise role of glycosylation in flagellar function remains unclear, and the high proportion of the Spanish CF isolates harbouring the flagellin glycosylation island suggests that it might confer some advantages for persistence or adaptation. In contrast, other genomic islands (with the exception of PAPI-1) were apparently under-represented in the study collection compared with previous studies on CF isolates [7,8].

The intraclonal diversity analysis revealed two possible patient-to-patient transmissions, one of which was explained by the communal living of the two brothers. Clonal dissemination between CF units was not observed, although the paediatric and adult units were located in the same institution. The complete characterisation of the study isolates does not allow sporadic genetic exchange events between different lineages to be ruled out.

5. Conclusion

This study described the genetic and antimicrobial phenotypes of the Spanish CF *P. aeruginosa* isolates recovered from the first multi-centre study on the microbiology of CF performed in Spain.

This study demonstrated a genetically unrelated and highly diverse population with a total colonisation rate of 22% (32.7% in adults and 9.9% in children), moderate prevalence of mutators, and high antimicrobial resistance rates (except for colistin).

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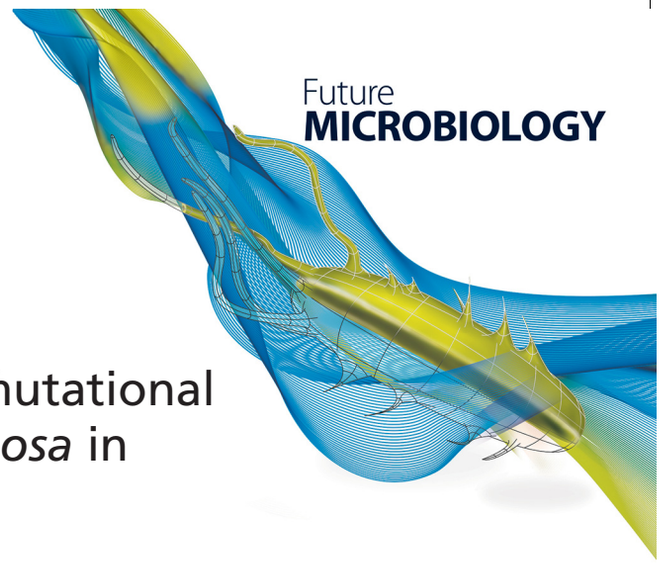
References

[1] Winstanley C, O'Brien S, Brockhurst MA. *Pseudomonas aeruginosa* evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. Trends Microbiol 2016;24:327–37.
[2] Williams D, Evans B, Haldenby S, Walshaw MJ, Brockhurst MA, Winstanley C, et al. Divergent, coexisting *Pseudomonas aeruginosa* lineages in chronic cystic fibrosis lung infections. Am J Respir Crit Care Med 2015;191:775–85.
[3] Oliver A, Cantón R, Campo P, Baquero F, Blázquez J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science 2000;288:1251–84.
[4] Fothergill JL, Walshaw MJ, Winstanley C. Transmissible strains of *Pseudomonas aeruginosa* in cystic fibrosis lung infections. Eur Respir J 2012;40:227–38.
[5] Abdul Wahab A, Taj-Aldeen SJ, Hagen F, Diophode S, Saadoon A, Meis JF, et al. Genotypic diversity of *Pseudomonas aeruginosa* in cystic fibrosis siblings in Qatar using AFLP fingerprinting. Eur J Clin Microbiol Infect Dis 2014;33:265–71.
[6] Scott FW, Pitt TL. Identification and characterization of transmissible *Pseudomonas aeruginosa* strains in cystic fibrosis patients in England and Wales. J Med Microbiol 2004;53:609–15.
[7] Ballarini A, Scalet G, Kos M, Cramer N, Wiehlmann L, Jousson O. Molecular typing and epidemiological investigation of clinical populations of *Pseudomonas aeruginosa* using an oligonucleotide-microarray. BMC Microbiol 2012;12:152.
[8] Cramer N, Wiehlmann L, Ciofu O, Tamm S, Høiby N, Tümmler B. Molecular epidemiology of chronic *Pseudomonas aeruginosa* airway infections in cystic fibrosis. PLoS ONE 2012;7:e50731.
[9] Hilker R, Munder A, Klockgether J, Losada PM, Chouvarine P, Cramer N, et al. Interclonal gradient of virulence in the *Pseudomonas aeruginosa* pangenome from disease and environment. Environ Microbiol 2015;17:29–46.
[10] Rakhimova E, Wiehlmann L, Brauer AL, Sethi S, Murphy TF, Tümmler B. *Pseudomonas aeruginosa* population biology in chronic obstructive pulmonary disease. J Infect Dis 2009;200:1928–35.
[11] Selezska K, Kazmierczak M, Müsken M, Garbe J, Schobert M, Häussler S, et al. *Pseudomonas aeruginosa* population structure revisited under environmental focus: impact of water quality and phage pressure. Environ Microbiol 2012;14:1952–67.
[12] Stewart RM, Wiehlmann L, Ashelford KE, Preston SJ, Frimmersdorf E, Campbell BJ, et al. Genetic characterization indicates that a specific subpopulation of *Pseudomonas aeruginosa* is associated with keratitis infections. J Clin Microbiol 2011;49:993–1003.
[13] Wiehlmann L, Wagner G, Cramer N, Siebert B, Gudowius P, Morales G, et al. Population structure of *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 2007;104:8101–6.
[14] Fernández-Olmos A, García-Castillo M, Alba JM, Morosini MI, Lamas A, Romero B, et al. Population structure and antimicrobial susceptibility of both non-persistent and persistent *Pseudomonas aeruginosa* isolates recovered from cystic fibrosis patients. J Clin Microbiol 2013;51:2761–5.
[15] López-Causapé C, Rojo-Molinero E, Mulet X, Cabot G, Moyà B, Figuerola J, et al. Clonal dissemination, emergence of mutator lineages and antibiotic resistance evolution in *Pseudomonas aeruginosa* cystic fibrosis chronic lung infection. PLoS ONE 2013;8:e71001.
[16] de Dios Caballero J, del Campo R, Royuela A, Solé A, Máiz L, Olveira C, et al. Bronchopulmonary infection-colonization patterns in Spanish cystic fibrosis patients: results from a national multicenter study. J Cyst Fibros 2016;15:357–65.
[17] Díez-Aguilar M, Morosini MI, del Campo R, García-Castillo M, Zamora J, Cantón R. In vitro activity of fosfomycin against a collection of clinical *Pseudomonas aeruginosa* isolates from 16 Spanish hospitals: establishing the validity of standard broth microdilution as susceptibility testing method. Antimicrob Agents Chemother 2013;57:5701–3.
[18] Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012;18:268–81.
[19] Montanari S, Oliver A, Salerno P, Mena A, Bertoni G, Tümmler B, et al. Biological cost of hypermutation in *Pseudomonas aeruginosa* strains from patients with cystic fibrosis. Microbiology 2007;153:1445–54.
[20] García-Castillo M, Máiz L, Morosini MI, Rodríguez-Baños M, Suarez L, Fernández-Olmos A, et al. Emergence of a *mut*L mutation causing multilocus sequence typing-pulsed-field gel electrophoresis discrepancy among *Pseudomonas aeruginosa* isolates from a cystic fibrosis patient. J Clin Microbiol 2012;50:1777–8.
[21] Feltman H, Schulert G, Khan S, Jain M, Peterson L, Hauser AR. Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. Microbiology 2001;147:2659–69.
[22] Psoter KJ, De Roos AJ, Wakefield J, Mayer J, Rosenfeld M. Season is associated with *Pseudomonas aeruginosa* acquisition in young children with cystic fibrosis. Clin Microbiol Infect 2013;19:E483–9.
[23] Salsgiver EL, Fink AK, Knapp EA, LiPuma JJ, Olivier KN, Marshall BC, et al. Changing epidemiology of the respiratory bacteriology of patients with cystic fibrosis. Chest 2016;149:390–400.
[24] Workentine ML, Sibley CD, Glezerson B, Purighalla S, Norgaard-Gron JC, Parkins MD, et al. Phenotypic heterogeneity of *Pseudomonas aeruginosa* populations in a cystic fibrosis patient. PLoS ONE 2013;8:e60225.
[25] Markussen T, Marvig RL, Gómez-Lozano M, Aanæs K, Burleigh AE, Høiby N, et al. Environmental heterogeneity drives within-host diversification and evolution of *Pseudomonas aeruginosa*. MBio 2014;5:e01592-e14.
[26] Hall AJ, Fothergill JL, McNamara PS, Southern KW, Winstanley C. Turnover of strains and intraclonal variation amongst *Pseudomonas aeruginosa* isolates from paediatric CF patients. Diagn Microbiol Infect Dis 2014;80:324–6.
[27] García-Castillo M, del Campo R, Morosini MI, Riera E, Cabot G, Willems R, et al. Wide dispersion of ST175 clone despite high genetic diversity of carbapenem-nonsusceptible *Pseudomonas aeruginosa* clinical strains in 16 Spanish hospitals. J Clin Microbiol 2011;49:2905–10.
[28] Malone JG. Role of small colony variants in persistence of *Pseudomonas aeruginosa* infections in cystic fibrosis lungs. Infect Drug Resist 2015;8:237–47.
[29] Mustafa MH, Chalhoub H, Denis O, Deplano A, Vergison A, Rodriguez-Villalobos H, et al. Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients in Northern Europe. Antimicrob Agents Chemother 2016;60:6735–41.
[30] Dettman JR, Rodrigue N, Aaron SD, Kassen R. Evolutionary genomics of epidemic and nonepidemic strains of *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 2013;110:21065–70.
[31] Waters V, Zlosnik JE, Yau YC, Speert DP, Aaron SD, Guttman DS. Comparison of three typing methods for *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Eur J Clin Microbiol Infect Dis 2012;31:3341–50.
[32] Maatallah M, Cheriaa J, Backhrouf A, Iversen A, Grundmann H, Do T, et al. Population structure of *Pseudomonas aeruginosa* from five Mediterranean

countries: evidence for frequent recombination and epidemic occurrence of CC235. PLoS ONE 2011;6:e25617.
[33] Frank DW. The exoenzyme S regulon of *Pseudomonas aeruginosa*. Mol Microbiol 1997;26:621–9.
[34] Pirnay JP, Bilocq F, Pot B, Cornelis P, Zizi M, Van Eldere J, et al. *Pseudomonas aeruginosa* population structure revisited. PLoS ONE 2009;4:e7740.
[35] Dingemans J, Ye L, Hildebrand F, Tontodonati F, Craggs M, Bilocq F, et al. The deletion of TonB-dependent receptor genes is part of the genome reduction process that occurs during adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis lung. Pathog Dis 2014;71:26–38.
[36] Hunter RC, Asfour F, Dingemans J, Osuna BL, Samad T, Malfroot A, et al. Ferrous iron is a significant component of bioavailable iron in cystic fibrosis airways. MBio 2013;4:e00557–13.
[37] Konings AF, Martin LW, Sharples KJ, Roddam LF, Latham R, Reid DW, et al. *Pseudomonas aeruginosa* uses multiple pathways to acquire iron during chronic infection in cystic fibrosis lungs. Infect Immun 2013;81:2697–704.

Editorial



Future MICROBIOLOGY

Insights into the evolution of the mutational resistome of *Pseudomonas aeruginosa* in cystic fibrosis

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"The analysis of WGS mutational resistomes has proven to be useful for understanding the evolutionary dynamics of classical resistance mechanisms and to depict new ones for the majority of antimicrobial classes"

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Chronic respiratory infection (CRI) by *Pseudomonas aeruginosa* is the main driver of morbidity and mortality in cystic fibrosis (CF) patients [1]. CRI results from an intense adaptation process, where bacterial evolution is tested against host immune responses and years of aggressive antimicrobial treatments [2]. Once established, CRI can seldom be eradicated despite intensive antimicrobial treatments, and therefore our therapeutic goals resignedly move from attempting to cure the infection to minimizing its long-term impact through chronic suppressive therapy [3]. The plasticity of *P. aeruginosa* genome for antimicrobial resistance acquisition, the greatly enhanced mutation supply rate provided by frequent hypermutable variants (mutators) and the highly structured environment determined by the characteristic biofilm growth and the anatomy of the respiratory tract make bacterial evolution and genetic diversification a hallmark of CF CRI [2,4]. While the enhanced evolution of antimicrobial resistance in CF, frequently linked to mutator phenotypes, was noted many years ago [5], it is with the introduction of whole-genome sequencing (WGS) that we are starting to understand its real dimensions [6].

The term resistome was first used to account for the set of primary antibiotic resistance genes that could be eventually transferred to human pathogens [7]. Soon after the concept of intrinsic resistome was introduced to include all chromosomal genes that are involved in intrinsic resistance, and whose presence in strains of a bacterial species is independent of previous antibiotic exposure and is not due to horizontal gene transfer (HGT) [8]. Finally, the term mutational resistome was more recently implemented to account for the set of mutations involved in the modulation of antibiotic resistance levels in the absence of HGT [9]. Recent WGS data obtained from *in vitro* assays on the evolution of antibiotic resistance and clinical isolates, and in particular sequential CF isolates, provide new insights into the evolutionary dynamics and mechanisms of *P. aeruginosa* antibiotic resistance. However, in too many cases, the documented genomic variations fail to provide causative relations in the absence of phenotypic information. The analysis of WGS mutational resistomes has proven to be useful for understanding the evolutionary dynamics of classical resistance mechanisms and to depict new ones for the majority of antimicrobial classes, including β-lactams, aminoglycosides, fluoroquinolones and polymixins.

Regarding β-lactams, the analysis of WGS mutational resistomes has confirmed the major role of classical resistance mutations such as those leading to the overexpression of the chromosomal β-lactamase AmpC (such as DacB [PBP4], AmpD and/or AmpR mutations) or the inactivation of the carbapenem porin OprD. However, the analysis of mutational resistomes of *in vitro* evolved strains and sequential CF isolates have identified other key mutations, such as those occurring in β-lactam targets (essential PBPs), particularly involving mutations in *ftsI* which encodes PBP3, an essential high molecular class B penicillin binding protein (PBP) with transpeptidase

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activity. Indeed, data from CF patients [9,10] as well as from *in vitro* studies [11] have recently demonstrated that PBP3 is under strong mutational pressure, with specific mutations contributing to β-lactam resistance development. Among them are particularly relevant and frequent mutations affecting amino acids R504 or F533, located within the protein domains responsible for the formation and stabilization of the inactivating complex β-lactam–PBP3. Moreover, PBP3 mutations seem to play a role in the emergence of resistance to novel β-lactam–β-lactamase inhibitor combinations, such as ceftolozane/tazobactam [9]. Another relevant mutational β-lactam resistance mechanism is the selection of large (>200 kb) deletions affecting specific parts of the chromosome. Although the basis of the conferred resistance phenotype still needs to be further clarified, these mutants can be recognized by the characteristic brown pigment (pyomelanine) caused by the deletion of one of the affected genes, *hmgA*, coding for a homogentisate-1,2-dioxygenase. This type of deletion has been documented in both, *in vitro* evolved β-lactam-resistant mutants and CF isolates [11,12]. However, the deletion of *hmgA* is not responsible for the resistance phenotype, which may be linked to the deletion of another of the affected genes, *galU*, coding for a UDP-glucose pyrophosphorylase required for lipopolysaccharide core synthesis. Indeed, analysis of transposon mutant libraries has shown that inactivation of galU increases ceftazidime and meropenem minimum inhibitory concentrations [13,14]. Finally, another emerging mutational β-lactam resistance mechanism is the structural modification of AmpC [10,11].

With respect to aminoglycosides, results from analysis of mutational resistomes of CF isolates point to the underlying strong evolutionary pressure of *mexZ* and the relevance of MexXY overexpression for resistance development [15–17]. Moreover, recent *in vitro* studies and findings from CF isolates have revealed that high-level aminoglycoside resistance requires the acquisition of additional mutations; among them, those in FusA1 seem to be particularly frequent and relevant [9,16,18–19]. Likewise, the fluoroquinolone resistome frequently includes mutations in efflux pump regulators, among which *nfxB*, leading to the overexpression of MexCD-OprJ, is particularly noteworthy in the CF setting. However, high-level ciprofloxacin resistance generally involves one or several mutations in the quinolone resistance determining regions of GyrA/B and/or ParC/E [9]. Regarding polymixin resistance, findings from WGS studies of *in vitro* evolved strains and CF isolates have shown that development of high-level colistin resistance requires the acquisition of multiple mutations, including those in the two-component regulators (PmrAB, PhoPQ or ParRS) involved in the addition of 4-amino-4-deoxy-L-arabinose to lipid A from the lipopolysaccharide [9,20]. Finally, in addition to the resistance mechanisms to classical antipseudomonal agents, the CF mutational resistome may also include resistance to other used antibiotics such as the frequent mutations of domain V of 23S rRNA – conferring macrolide resistance [21].

The complexity of the CF isolates resistomes is further enhanced when the intrapatient genetic diversity of CF *P. aeruginosa* populations is introduced. Certainly, to understand the resistance dynamics and evolution, future steps should endeavor to analyze the mutational resistomes in CF at the whole population level, as opposed to the analysis of single isolated colonies. Indeed, full understanding of the evolution of the mutational resistome requires a longitudinal and transversal analysis of *P. aeruginosa* populations in the CF patient. Moreover, recent evidence suggests that interpatient transmission should also be considered when analyzing the evolution of the mutational resistome, especially when introducing mutator lineages of epidemic clones [9].

Beyond addressing a relevant scientific question, the analysis of mutational resistomes would be useful for therapeutic strategy design and monitoring the efficacy of administered antibiotic treatments. Obviously, the evolution of the mutational resistome is a direct consequence of antimicrobial exposure. As such, it is not surprising that exposure to one antibiotic drives evolution of the mutational resistome for that antibiotic. However, the complexity of the actual resistance profile is further increased by the specificity and interactions among different resistance mechanisms. A classic example is cross resistance (or collateral resistance), which implies that exposure to one antibiotic drives also the development of resistance to a different one. Typically this is caused by the developed resistance mechanism (such as efflux pump overexpression) affecting simultaneous different antibiotics. Indeed, potential development of cross resistance is a major issue to consider when using antibiotic combinations [22].

Perhaps less obvious is collateral susceptibility, which implies that exposure to one antibiotic increases the susceptibility to a different one. This might be achieved through two mechanisms. One possible mechanism is that exposure to one antibiotic directly causes increased susceptibility to a different one, for example, mutations in the β-lactamase AmpC increases cephalosporin hydrolysis while reducing that of penicillins or carbapenems [23]. The second possibility is that the development of a resistance mechanism impairs the activity of another existing resistance mechanism, for example, competition between the different efflux pumps, the overexpression of one may impair the expression of another [24]. Indeed, in the CF setting it is very frequent that the overexpression of efflux pump MexXY, involved in aminoglycoside resistance, is linked to the impaired expression of efflux pump MexAB,

involved in the resistance to a broad range of antibiotics including most β-lactams [25]. Thus, the evolution of the mutational resistome for a given antibiotic is not only dependent on the exposure to this antibiotic, but it is also conditioned by the simultaneous or even previous exposures to other antibiotics. An illustrative example is provided in a recent *in vitro* study that demonstrated, for a broad range of antibiotic classes, that the history of exposure and resistance development to a given antibiotic, conditions the dynamics and mechanisms of resistance development when exposed to a second one [18].

Moreover, knowledge of the interactions between resistance mechanisms could be useful in the design of sequential treatments that minimize the risk of resistance development. Such is the case for a recent *in vitro* study showing the effectiveness of aztreonam–tobramycin sequential treatment, based on the antagonism between the resistance mechanisms for each of the antibiotics; overexpression of the efflux pump MexAB (aztreonam) or MexXY (tobramycin) which compete for the same outer membrane channel (OprM) [26].

Finally, it should be noted that a hallmark of *P. aeruginosa* CRI in CF is the biofilm mode of growth. Indeed, biofilm growth, in addition to providing a compact structured environment likely facilitating the evolution of mutational resistance [27], they also add further complexity due to the major differences in the mechanisms involved when compared with conventional planktonic growth that needs to be considered [28]. Likewise, persistence of *P. aeruginosa* in the CF lung despite intensive antimicrobial treatments relays in the acquisition of a vast number of adaptive mutations that extend far beyond classical antibiotic resistance mutations [29].

In summary, the comprehensive analysis of the mutational resistomes of *P. aeruginosa* in CF CRI is expected to become a useful tool for optimizing therapeutic strategies and monitoring the efficacy of administered antibiotic treatments in the near future.

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References

1. Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. *Clin. Microbiol. Rev.* 15(2), 194–222 (2002).
2. Oliver A, Mena A, Macià MD. Evolution of *Pseudomonas aeruginosa* pathogenicity: from acute to chronic infections. In: *Evolutionary Biology of Bacterial and Fungal Pathogens.* Baquero F, Nombela C, Cassell GH, Gutiérrez JA (Eds). ASM Press, WA, USA, 433–444 (2008).
3. López-Causapé C, Rojo-Molinero E, Macià MD, Oliver A. The problems of antibiotic resistance in cystic fibrosis and solutions. *Expert. Rev. Respir. Med.* 9(1), 73–88 (2015).
4. Fernández-Barat L, Ciofu O, Kragh KN *et al.* Phenotypic shift in *Pseudomonas aeruginosa* populations from cystic fibrosis lungs after 2-week antipseudomonal treatment. *J. Cyst. Fibros.* 16(2), 222–229 (2017).
5. Oliver A, Cantón R, Campo P, Baquero F, Blázquez J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288(5469), 1251–1254 (2000).
6. Feliziani S, Marvig RL, Luján AM *et al.* Coexistence and within-host evolution of diversified lineages of hypermutable *Pseudomonas aeruginosa* in long-term cystic fibrosis infections. *PLoS Genet.* 10(10), e1004651 (2014).
7. D'Costa VM, McGrann KM, Hughes DW, Wright GD. Sampling the antibiotic resistome. *Science* 311(5759), 374–377 (2006).
8. Fajardo A, Martínez-Martín N, Mercadillo M *et al.* The neglected intrinsic resistome of bacterial pathogens. *PLoS ONE* 3(2), e1619 (2008).
9. López-Causapé C, Sommer LM, Cabot G *et al.* Evolution of the *Pseudomonas aeruginosa* mutational resistome in an international cystic fibrosis clone. *Sci. Rep.* 7(1), 5555 (2017).
10. Diaz Caballero J, Clark ST, Coburn B *et al.* Selective sweeps and parallel pathoadaptation drive *Pseudomonas aeruginosa* evolution in the cystic fibrosis lung. *MBio.* 6(5), e00981–15 (2015).
11. Cabot G, Zamorano L, Moyà B *et al.* Evolution of *Pseudomonas aeruginosa* antimicrobial resistance and fitness under low and high mutation rates. *Antimicrob. Agents Chemother.* 60(3), 1767–1778 (2016).
12. Hocquet D, Petitjean M, Rohmer L *et al.* Pyomelanin-producing *Pseudomonas aeruginosa* selected during chronic infections have a large chromosomal deletion which confers resistance to pyocins. *Environ. Microbiol.* 18(10), 3482–3493 (2016).

13. Alvarez-Ortega C, Wiegand I, Olivares J, Hancock RE, Martínez JL. Genetic determinants involved in the susceptibility of *Pseudomonas aeruginosa* to beta-lactam antibiotics. *Antimicrob. Agents Chemother.* 54(10), 4159–4167 (2010).
14. Dötsch A, Becker T, Pommerenke C *et al.* Genomewide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53(6), 2522–2531 (2009).
15. Prickett MH, Hauser AR, McColley SA *et al.* Aminoglycoside resistance of *Pseudomonas aeruginosa* in cystic fibrosis results from convergent evolution in the *mexZ* gene. *Thorax* 72(1), 40–47 (2017).
16. Greipel L, Fischer S, Klockgether J *et al.* Molecular epidemiology of mutations in antimicrobial resistance loci of *Pseudomonas aeruginosa* isolates from airways of cystic fibrosis patients. *Antimicrob. Agents Chemother.* 60(11), 6726–6734 (2016).
17. Marvig RL, Sommer LM, Molin S, Johansen HK. Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nat. Genet.* 47(1), 57–64 (2015).
18. Yen P, Papin JA. History of antibiotic adaptation influences microbial evolutionary dynamics during subsequent treatment. *PLoS Biol.* 15(8), e2001586 (2017).
19. Feng Y, Jonker MJ, Moustakas I, Brul S, Ter Kuile BH. Dynamics of mutations during development of resistance by *Pseudomonas aeruginosa* against five antibiotics. *Antimicrob. Agents Chemother.* 60(7), 4229–4236 (2016).
20. Jochumsen N, Marvig RL, Damkiær S *et al.* The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. *Nat. Commun.* 7, 13002 (2016).
21. Mustafa MH, Khandekar S, Tunney MM *et al.* Acquired resistance to macrolides in *Pseudomonas aeruginosa* from cystic fibrosis patients. *Eur. Respir. J.* 49(5), pii:1601847 (2017) (Epub ahead of print).
22. Vestergaard M, Paulander W, Marvig RL *et al.* Antibiotic combination therapy can select for broad-spectrum multidrug resistance in *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents.* 47(1), 48–55 (2016).
23. Cabot G, Bruchmann S, Mulet X *et al. Pseudomonas aeruginosa* ceftolozane-tazobactam resistance development requires multiple mutations leading to overexpression and structural modification of AmpC. *Antimicrob. Agents Chemother.* 58(6), 3091–3099 (2014).
24. Mulet X, Moyá B, Juan C *et al.* Antagonic interactions of *Pseudomonas aeruginosa* antibiotic resistance mechanisms in planktonic but no biofilm growth. *Antimicrob. Agents Chemother.* 55(10), 4560–4568 (2011).
25. Vettoretti L, Plésiat P, Muller C *et al.* Efflux unbalance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob. Agents Chemother.* 53(5), 1987–1997 (2009).
26. Rojo-Molinero E, Macià MD, Rubio R *et al.* Sequential treatment of biofilms with aztreonam and tobramycin is a novel strategy for combating *Pseudomonas aeruginosa* chronic respiratory infections. *Antimicrob. Agents Chemother.* 60(5), 2912–2922 (2016).
27. Macià MD, Pérez JL, Molin S, Oliver A. Dynamics of mutator and antibiotic-resistant populations in a pharmacokinetic/pharmacodynamic model of *Pseudomonas aeruginosa* biofilm treatment. *Antimicrob. Agents Chemother.* 55(11), 5230–5237 (2011).
28. Ciofu O, Rojo-Molinero E, Macià MD, Oliver A. Antibiotic treatment of biofilm infections. *APMIS.* 125(4), 304–319 (2017).
29. Smith EE, Buckley DG, Wu Z *et al.* Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc. Natl Acad. Sci. USA* 103(22), 8487–8492 (2006).

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Evolution of the *Pseudomonas aeruginosa* Aminoglycoside Mutational Resistome *In Vitro* and in the Cystic Fibrosis Setting

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ABSTRACT Inhaled administration of high doses of aminoglycosides is a key maintenance treatment of *Pseudomonas aeruginosa* chronic respiratory infections in cystic fibrosis (CF). We analyzed the dynamics and mechanisms of stepwise high-level tobramycin resistance development *in vitro* and compared the results with those of isogenic pairs of susceptible and resistant clinical isolates. Resistance development correlated with *fusA1* mutations *in vitro* and *in vivo*. *pmrB* mutations, conferring polymyxin resistance, were also frequently selected *in vitro*. In contrast, mutational overexpression of MexXY, a hallmark of aminoglycoside resistance in CF, was not observed in *in vitro* evolution experiments.

KEYWORDS *Pseudomonas aeruginosa*, aminoglycosides, antibiotic resistance, drug resistance mechanisms, mutational resistome, whole-genome sequencing

Pseudomonas aeruginosa is one of the most frequent and severe causes of acute nosocomial infections, as well as the main driver of morbidity and mortality in patients suffering from cystic fibrosis (CF) or other chronic respiratory diseases (1, 2). Compared with other Gram-negative pathogens, *P. aeruginosa* exhibits a basal reduced susceptibility to many antibiotics, and this intrinsic resistance can be much further enhanced by the acquisition of transferable resistance determinants and by the selection of mutations that alter the expression and/or function of diverse chromosomal genes (3–6). This outstanding ability has led to an increasing prevalence of chronic and nosocomial infections produced by multidrug-resistant (MDR) or extensively drug-resistant (XDR) *P. aeruginosa* strains that sharply compromises the selection of appropriate treatments (7, 8). Together with polymyxins, aminoglycosides are often among the few therapeutic options in this scenario (9, 10). Moreover, provision of high local concentrations of tobramycin through inhaled administration has been the basis of the treatment for *P. aeruginosa* chronic respiratory infections in CF patients for many years (11). Whereas resistance to these agents in acute infections is mainly attributed to the production of aminoglycoside-modifying enzymes or 16S rRNA methyltransferases, resistance development in the chronic infection setting has been linked to the selection of chromosomal mutations that lead to enhanced membrane impermeability or MexXY-OprM efflux pump overexpression (12–14). However, high-level resistance development likely requires the accumulation of different resistance mechanisms, and, although recent reports suggest the involvement of additional chromosomal mutations (15–20), there are still important knowledge gaps in this field. Thus, the aim of this work was to analyze the *in vitro* evolution of the aminoglycoside mutational resistome of *P. aeruginosa* and to correlate the documented mutations with those observed *in vivo* during the course of CF chronic respiratory infection.

To determine the dynamics of aminoglycoside resistance development, ~10^6 CFU/ml of exponentially growing *P. aeruginosa* PAO1 reference strain isolates were inoculated into

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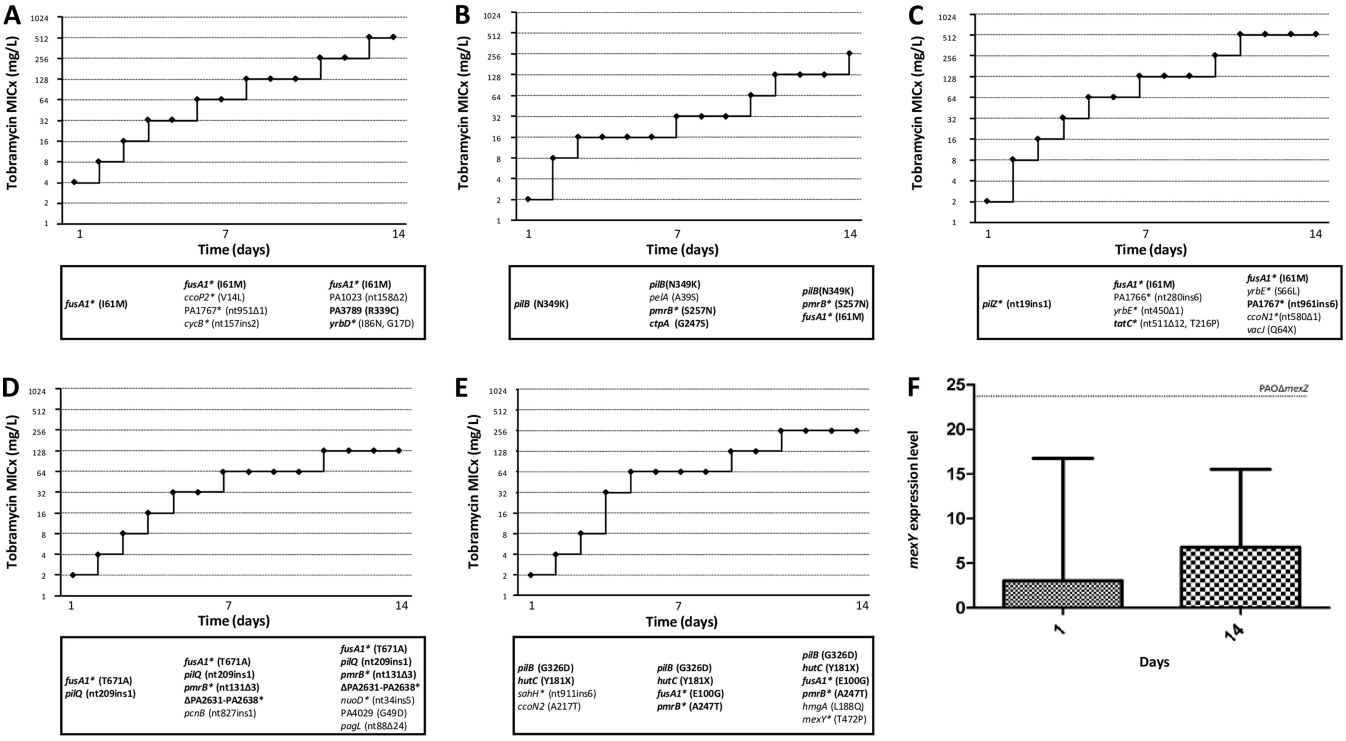

FIG 1 (A to E) Dynamics of resistance development to tobramycin and mutations encountered after 1, 7, and 14 days of tobramycin exposure in five replicate experiments. Genetic determinants and specific mutations are boldfaced when detected within the two representative colonies studied at each experiment and time point. Genes whose implications in aminoglycoside resistance development have already been demonstrated are indicated with an asterisk. (F) Median expression level of *mexY* for PAO1-derived resistant mutants after 1 and 14 days of tobramycin exposure.

10-ml Mueller-Hinton tubes containing 0.5×, 1×, 2×, 4×, 8×, 16×, 32×, 64×, 128×, 256×, 512×, and 1,024× MIC (0.5 μg/ml) values and incubated for 24 h at 37°C and 180 rpm. Tubes from the highest antibiotic concentration showing growth were reinoculated (at a 1:1,000 dilution) in fresh medium containing concentrations up to 1,024× MIC and incubated under the same conditions. This evolution experiment was performed for 14 consecutive days in quintuplicate. At days 1, 7, and 14, two colonies from each of the five replicate experiments were purified in antibiotic-free LB agar plates, and MIC values of tobramycin, gentamicin, amikacin, ticarcillin, piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, ceftolozane-tazobactam, imipenem, meropenem, ciprofloxacin, and colistin were determined by broth microdilution and interpreted according to CLSI 2017 clinical breakpoints (21). Whole-genome sequences (WGS) of all the mutants were obtained and analyzed following previously described protocols (9, 22, 23). Likewise, to correlate *in vitro* with *in vivo* findings, three isogenic pairs (confirmed by pulsed-field gel electrophoresis) of tobramycin-susceptible and -resistant isolates obtained from respiratory samples of three different chronically infected CF patients treated with tobramycin at Son Espases University Hospital were also fully sequenced, and variations in 164 genes related to antibiotic resistance were analyzed (22).

As shown in Fig. 1 panels A to E, *in vitro* resistance development occurred in a stepwise manner, reaching concentrations ranging from 128 to 512 higher than the initial MIC (0.5 μg/ml). The corresponding tobramycin MICs of the purified colonies at day 14 ranged from 64 to 512 μg/ml, whereas those of gentamicin and amikacin were typically 1 or 2 dilutions higher (see Data Set S1 in the supplemental material). These concentrations are close to the maximum tobramycin concentrations achieved through inhaled administration and in the range of the breakpoints suggested for inhaled therapy (24).

Results obtained from WGS experiments are summarized in Fig. 1 and detailed in Data Set S1. Up to 35 different genes were found to be mutated in at least one of the

TABLE 1 Genomic differences between the three isogenic pairs of tobramycin-susceptible and -resistant CF isolates

	Isolate ID (MIC_{TOB} mg/liter)					
Locus/gene[a]	**FQSE06-S (1)**	**FQSE06-R (24)**	**FQSE11-S (2)**	**FQSE11-R (>256)**	**FQSE16-S (4)**	**FQSE16-R (64)**
PA0004/*gyrB*				R138L		
PA0058/*dsbM*			C28R, F206L, R212C			
PA0426/*mexB*			nt772Δ1			Q575R
PA0958/*oprD*				Q424E, S403A		
PA1430/*lasR*				R216Q		
PA2018/*mexY*		G287A				G287S
PA2020/*mexZ*	nt290Δ11	S9P	L138R	L138R		R125P
PA2492/*mexT*						G274D, G300D
PA2639/*nuoD*		G499X				
PA3064/*pelA*	V446I					
PA3141/*capD*					nt512ins1	
PA3168/*gyrA*				Y267N		
PA4020/*mpl*		S257L		Q248X		
PA4266/*fusA1*		Y552C, T671I				Y552C
PA4418/PBP3		P215L				
PA4462/*rpoN*						V473A
PA4568/*rplU*	I74 M					
PA4598/*mexD*					P721S, L624P	
PA4600/*nfxB*				E75K		
PA4773/-				A165T		
PA5040/*pilQ*	E676D, E669D					
ResFinder				AacA4		
mexY overexpression	+	+	+	+	−	+

[a]Genes in which mutations were also detected in the resistance evolution experiment are boldfaced.

isolates. Of these, 22 isolates (63%) were already related to aminoglycoside resistance development in previous studies (15–20, 22), so our data confirm their relevance in this stepwise process; mutations in other genes are evidenced for the first time in this work, and their relevance will need to be confirmed in further studies. Mutants from day 14 showed between 3 and 7 mutations, and comparison with those from days 1 and 7 showed a stepwise acquisition. However, a few mutations documented at these intermediate stages were not fixed in the population and thus were not seen at day 14 (Fig. 1).

Among the mutated genes, *fusA1*, which codes for elongation factor G, deserves special attention because nonsynonymous mutations within this gene were detected in all five replicate experiments. Note that the time of detection of *fusA1* mutations varied from day 1 to day 14 and that the same amino acid substitution (I61M) occurred in 3 of them. This novel mechanism was recently confirmed through site-directed mutagenesis by Bolard et al. (15), being associated with a 1- to 3-fold increase in the MICs of tobramycin, gentamicin, and amikacin, which correlates with our observations (Data Set S1). Mutations in *fusA1* were recently noted to be frequent among CF patients (22, 25–27). Moreover, the emergence of *fusA1* mutations was noted in two of the three tobramycin-resistant CF isolates studied in this work (Table 1; see Data Set S2 in the supplemental material). Interestingly, using ResFinder, the resistant isolate not showing *fusA1* mutations was shown to have acquired an exogenous aminoglycoside-modifying enzyme (AacA4). This surprising finding highlights the fact that, although mutational resistance is thought to be the rule in CF chronic infections, horizontally acquired resistance must be ruled out as well.

Another frequently mutated gene (3 of 5 replicates) was, intriguingly, *pmrB*, which codes for the sensor kinase of the two-component regulatory system PmrA-PmrB (28). *pmrB* mutations have traditionally been linked to polymyxin resistance development (29, 30), so this finding alerts from a possible mechanism of coresistance to two relevant antipseudomonal agents (polymyxins and aminoglycosides). Indeed, the emergence of *pmrB* mutations at day 7 correlated with increased colistin MICs (Data Set S1). However, despite the *pmrB* mutations persisting at day 14, colistin resistance disappeared, likely indicating the acquisition of compensatory mutations. In one of these isolates from day

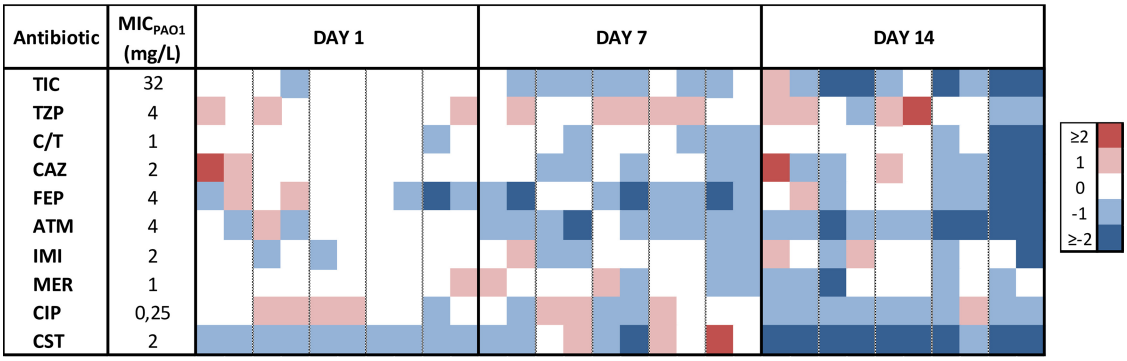

FIG 2 MIC fold changes for each antibiotic tested between parental strain PAO1 and its derived aminoglycoside-resistant mutants. TIC, ticarcillin; TZP, piperacillin-tazobactam; C/T, ceftolozane-tazobactam; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IMI, imipenem; MER, meropenem; CIP, ciprofloxacin; CST, colistin. Lower limit for CIP and TZP is −1 2-fold dilutions.

14, an additional mutation in *pagL* involved in lipid A deacylation and polymyxins resistance was documented (Fig. 1; Data Set S1) (31). In relation to other antibiotic classes, we observed a general trend over time toward decreasing MICs, particularly for ticarcillin, aztreonam, and ciprofloxacin (Fig. 2). This phenomenon is globally known as collateral susceptibility (32). It has been largely observed among *P. aeruginosa* clinical isolates (33), and it has traditionally been linked to a mutational or functional loss of the multidrug efflux system MexAB-OprM (34). Moreover, this phenotype has been proposed to result from an efflux imbalance between the MexAB-OprM and MexXY-OprM systems, as both compete for the recruitment of OprM (35). Nevertheless, our results and those recently published by Bolard et al. (15) suggest that other mechanisms may be involved in this frequently observed phenotype.

Beyond the mutations actually detected, another relevant aspect to consider is the mutations that were expected but not found in our *in vitro* evolution experiments. Intriguingly, mutations leading to the overexpression of MexXY (*mexZ*, PA5471, *parS*), which are a hallmark of aminoglycoside resistance development in the CF setting, were not seen at any time in any of the five replicate experiments. The absence of mutations in these genes was additionally confirmed through Sanger sequencing. Moreover, while *mexY* expression data varied to some extent for the different mutants (real-time reverse transcription-PCR [22, 23]), values were always well below those of a control *mexZ* PAO1 mutant, and a statistically significant trend toward increased expression at day 14 versus day 1 was not documented (Fig. 1F). In contrast to *in vitro* findings, all three CF tobramycin-resistant isolates overexpressed *mexY* and showed *mexZ* mutations (Table 1; Data Set S2). However, *mexY* overexpression and *mexZ* mutations were also seen in two of the three susceptible CF isolates. Thus, our results indicate that mutational overexpression of MexXY is not required for the evolution of high-level tobramycin resistance *in vitro*. On the other hand, findings from CF isolates reveal that mutations leading to the overexpression of MexXY are frequent, occur early, and are associated with low-level resistance. These results may indicate that the positive selection of mutations leading to the overexpression of MexXY in CF might be driven by factors beyond exposure to aminoglycosides.

Altogether, this work provides relevant insights into the evolution of the aminoglycoside resistome, balancing understanding of the role of novel (e.g., *fusA1* mutations) and classic (e.g., mutational MexXY overexpression) resistance mechanisms *in vitro* and in CF chronic respiratory infections. However, further studies are needed to decipher the precise role of some of the mutations detected, such as those in PmrB, in the evolution of aminoglycoside resistance in *P. aeruginosa*.

Accession number(s). Sequence files have been deposited in the European Nucleotide Archive under study PRJEB24151 (accession numbers ERS2060747, ERS2060748, ERS2060749, ERS2060750, ERS2077566, and ERS2077567).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02583-17.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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REFERENCES

1. Gellatly SL, Hancock RE. 2013. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog Dis 67:159–173. https://doi.org/10.1111/2049-632X.12033.
2. Oliver A, Mena A, Macià MD. 2008. Evolution of *Pseudomonas aeruginosa* pathogenicity: from acute to chronic infections, p 433–444. *In* Baquero F, Nombela C, Cassell GH, Gutíerrez JA (ed), Evolutionary biology of bacterial and fungal pathogens. ASM Press, Washington, DC.
3. Breidenstein EB, de la Fuente-Núñez C, Hancock RE. 2011. *Pseudomonas aeruginosa*: all roads lead to resistance. Trends Microbiol 19:419–426. https://doi.org/10.1016/j.tim.2011.04.005.
4. Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW. 2011. *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiol Rev 35:652–680. https://doi.org/10.1111/j.1574-6976.2011.00269.x.
5. Poole K. 2011. *Pseudomonas aeruginosa*: resistance to the max. Front Microbiol 2:65.
6. Lister PD, Wolter DJ, Hanson ND. 2009. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev 22:582–610. https://doi.org/10.1128/CMR.00040-09.
7. Livermore DM. 2009. Has the era of untreatable infections arrived? J Antimicrob Chemother 64(Suppl)1:i29–i36. https://doi.org/10.1093/jac/dkp255.
8. Mesaros N, Nordmann P, Plésiat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, Van Laethem Y, Jacobs F, Lebecque P, Malfroot A, Tulkens PM, Van Bambeke F. 2007. *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. Clin Microbiol Infect 13:560–578. https://doi.org/10.1111/j.1469-0691.2007.01681.x.
9. Del Barrio-Tofiño E, López-Causapé C, Cabot G, Rivera A, Benito N, Segura C, Montero MM, Sorlí L, Tubau F, Gómez-Zorrilla S, Tormo N, Durá-Navarro R, Viedma E, Resino-Foz E, Fernández-Martínez M, González-Rico C, Alejo-Cancho I, Martínez JA, Labayru-Echverria C, Dueñas C, Ayestarán I, Zamorano L, Martinez-Martinez L, Horcajada JP, Oliver A. 2017. Genomics and susceptibility profiles of extensively drug-resistant *Pseudomonas aeruginosa* isolates from Spain. Antimicrob Agents Chemother 61:e01589-17. https://doi.org/10.1128/AAC.01589-17.
10. Cabot G, Ocampo-Sosa AA, Domínguez MA, Gago JF, Juan C, Tubau F, Rodríguez C, Moyà B, Peña C, Martínez-Martínez L, Oliver A, Spanish Network for Research in Infectious Diseases (REIPI). 2012. Genetic markers of widespread extensively drug-resistant *Pseudomonas aeruginosa* high-risk clones. Antimicrob Agents Chemother 56:6349–6357. https://doi.org/10.1128/AAC.01388-12.
11. Shteinberg M, Elborn JS. 2015. Use of inhaled tobramycin in cystic fibrosis. Adv Ther 32:1–9. https://doi.org/10.1007/s12325-015-0179-3.
12. Guénard S, Muller C, Monlezun L, Benas P, Broutin I, Jeannot K, Plésiat P. 2014. Multiple mutations lead to MexXY-OprM-dependent aminoglycoside resistance in clinical strains of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 58:221–228. https://doi.org/10.1128/AAC.01252-13.
13. Poole K. 2005. Aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 49:479–487. https://doi.org/10.1128/AAC.49.2.479-487.2005.
14. Vogne C, Aires JR, Bailly C, Hocquet D, Plésiat P. 2004. Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Antimicrob Agents Chemother 48:1676–1680. https://doi.org/10.1128/AAC.48.5.1676-1680.2004.
15. Bolard A, Plesiat P, Jeannot K. 2018. Mutations in gene *fusA1* as a novel mechanism of aminoglycoside resistance in clinical strains of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 62: e01835-17. https://doi.org/10.1128/AAC.01835-17.
16. Yen P, Papin JA. 2017. History of antibiotic adaptation influences microbial evolutionary dynamics during subsequent treatment. PLoS Biol 15:e2001586. https://doi.org/10.1371/journal.pbio.2001586.
17. Feng Y, Jonker MJ, Moustakas I, Brul S, Ter Kuile BH. 2016. Dynamics of mutations during development of resistance by *Pseudomonas aeruginosa* against five antibiotics. Antimicrob Agents Chemother 60:4229–4236. https://doi.org/10.1128/AAC.00434-16.
18. Islam S, Oh H, Jalal S, Karpati F, Ciofu O, Høiby N, Wretlind B. 2009. Chromosomal mechanisms of aminoglycoside resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Clin Microbiol Infect 15:60–66. https://doi.org/10.1111/j.1469-0691.2008.02097.x.
19. Schurek KN, Marr AK, Taylor PK, Wiegand I, Semenec L, Khaira BK, Hancock RE. 2008. Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 52:4213–4219. https://doi.org/10.1128/AAC.00507-08.
20. El'Garch F, Jeannot K, Hocquet D, Llanes-Barakat C, Plésiat P. 2007. Cumulative effects of several nonenzymatic mechanisms on the resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob Agents Chemother 51:1016–1021. https://doi.org/10.1128/AAC.00704-06.
21. Clinical and Laboratory Standards Institute. 2017. Performance standards for antimicrobial susceptibility testing—27th ed, vol 37. CLSI document M100. Clinical and Laboratory Standards Institute, Wayne, PA.
22. López-Causapé C, Sommer LM, Cabot G, Rubio R, Ocampo-Sosa AA, Johansen HK, Figuerola J, Cantón R, Kidd TJ, Molin S, Oliver A. 2017. Evolution of the *Pseudomonas aeruginosa* mutational resistome in an international cystic fibrosis clone. Sci Rep 7:5555. https://doi.org/10.1038/s41598-017-05621-5.
23. Cabot G, López-Causapé C, Ocampo-Sosa AA, Sommer LM, Domínguez MÁ, Zamorano L, Juan C, Tubau F, Rodríguez C, Moyà B, Peña C, Martínez-Martínez L, Plesiat P, Oliver A. 2016. Deciphering the resistome of the widespread *Pseudomonas aeruginosa* sequence type 175 international high-risk clone through whole-genome sequencing. Antimicrob Agents Chemother 60:7415–7423.
24. Morosini MI, García-Castillo M, Loza E, Pérez-Vázquez M, Baquero F, Cantón R. 2005. Breakpoints for predicting *Pseudomonas aeruginosa* susceptibility to inhaled tobramycin in cystic fibrosis patients: use of high-range Etest strips. J Clin Microbiol 43:4480–4485. https://doi.org/10.1128/JCM.43.9.4480-4485.2005.
25. Greipel L, Fischer S, Klockgether J, Dorda M, Mielke S, Wiehlmann L, Cramer N, Tümmler B. 2016. Molecular epidemiology of mutations in antimicrobial resistance loci of *Pseudomonas aeruginosa* isolates from airways of cystic fibrosis patients. Antimicrob Agents Chemother 60:6726–6734. https://doi.org/10.1128/AAC.00724-16.
26. Markussen T, Marvig RL, Gómez-Lozano M, Aanæs K, Burleigh AE, Høiby N, Johansen HK, Molin S, Jelsbak L. 2014. Environmental heterogeneity drives within-host diversification and evolution of *Pseudomonas aeruginosa*. mBio 5:e01592-14. https://doi.org/10.1128/mBio.01592-14.
27. Chung JC, Becq J, Fraser L, Schulz-Trieglaff O, Bond NJ, Foweraker J,

Bruce KD, Smith GP, Welch M. 2012. Genomic variation among contemporary *Pseudomonas aeruginosa* isolates from chronically infected cystic fibrosis patients. J Bacteriol 194:4857–4866. https://doi.org/10.1128/JB.01050-12.
28. Gooderham WJ, Hancock RE. 2009. Regulation of virulence and antibiotic resistance by two-component regulatory systems in Pseudomonas aeruginosa. FEMS Microbiol Rev 33:279–294. https://doi.org/10.1111/j.1574-6976.2008.00135.x.
29. Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SI, Denton M, Conway SP, Johansen HK, Høiby N. 2012. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother 56:1019–1030. https://doi.org/10.1128/AAC.05829-11.
30. Barrow K, Kwon DH. 2009. Alterations in two-component regulatory systems of phoPQ and pmrAB are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 53:5150–5154. https://doi.org/10.1128/AAC.00893-09.
31. Han ML, Velkov T, Zhu Y, Roberts KD, Le Brun AP, Chow SH, Gutu AD, Moskowitz SM, Shen HH, Li J. 2018. Polymyxin-induced lipid A deacylation in Pseudomonas aeruginosa perturbs polymyxin penetration and confers high-level resistance. ACS Chem Biol 13:121–130. https://doi.org/10.1021/acschembio.7b00836.
32. Pál C, Papp B, Lázár V. 2015. Collateral sensitivity of antibiotic-resistant microbes. Trends Microbiol 23:401–407. https://doi.org/10.1016/j.tim.2015.02.009.
33. Irvin RT, Govan JW, Fyfe JA, Costerton JW. 1981. Heterogeneity of antibiotic resistance in mucoid isolates of *Pseudomonas aeruginosa* obtained from cystic fibrosis patients: role of outer membrane proteins. Antimicrob Agents Chemother 19:1056–1063. https://doi.org/10.1128/AAC.19.6.1056.
34. Li XZ, Nikaido H, Poole K. 1995. Role of mexA-mexB-oprM in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 39:1948–1953. https://doi.org/10.1128/AAC.39.9.1948.
35. Vettoretti L, Plésiat P, Muller C, El Garch F, Phan G, Attrée I, Ducruix A, Llanes C. 2009. Efflux unbalance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrob Agents Chemother 53:1987–1997. https://doi.org/10.1128/AAC.01024-08.

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The Versatile Mutational Resistome of *Pseudomonas aeruginosa*

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One of the most striking features of *Pseudomonas aeruginosa* is its outstanding capacity for developing antimicrobial resistance to nearly all available antipseudomonal agents through the selection of chromosomal mutations, leading to the failure of the treatment of severe hospital-acquired or chronic infections. Recent whole-genome sequencing (WGS) data obtained from *in vitro* assays on the evolution of antibiotic resistance, *in vivo* monitoring of antimicrobial resistance development, analysis of sequential cystic fibrosis isolates, and characterization of widespread epidemic high-risk clones have provided new insights into the evolutionary dynamics and mechanisms of *P. aeruginosa* antibiotic resistance, thus motivating this review. Indeed, the analysis of the WGS mutational resistome has proven to be useful for understanding the evolutionary dynamics of classical resistance pathways and to describe new mechanisms for the majority of antipseudomonal classes, including β-lactams, aminoglycosides, fluoroquinolones, or polymixins. Beyond addressing a relevant scientific question, the analysis of the *P. aeruginosa* mutational resistome is expected to be useful, together with the analysis of the horizontally-acquired resistance determinants, for establishing the antibiotic resistance genotype, which should correlate with the antibiotic resistance phenotype and as such, it should be useful for the design of therapeutic strategies and for monitoring the efficacy of administered antibiotic treatments. However, further experimental research and new bioinformatics tools are still needed to overcome the interpretation limitations imposed by the complex interactions (including those leading to collateral resistance or susceptibility) between the 100s of genes involved in the mutational resistome, as well as the frequent difficulties for differentiating relevant mutations from simple natural polymorphisms.

Keywords: antibiotic resistance, resistome, *Pseudomonas aeruginosa*, multidrug resistance, evolution, resistance development, mutation

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INTRODUCTION

Pseudomonas aeruginosa is one of the most frequent and severe causes of hospital-acquired infections, particularly affecting immunocompromised (especially neutropenic) and Intensive Care Unit (ICU) patients. Indeed, *P. aeruginosa* is the first cause of ventilator associated pneumonia (VAP) and burn wound infections, both associated with a very high mortality rate (Vincent, 2003; Bassetti et al., 2012). Likewise, *P. aeruginosa* is the most frequent driver of chronic respiratory infections in cystic fibrosis (CF) patients or other chronic underlying diseases (Döring et al., 2011).

One of the most striking features of *P. aeruginosa* is its outstanding capacity for developing antimicrobial resistance to nearly all available antipseudomonal agents through the selection of chromosomal mutations. Indeed, treatment failure caused by the development of antimicrobial resistance is a too frequent outcome of *P. aeruginosa* infections. The problem of mutation-mediated antibiotic resistance is further amplified in the chronic infection setting, due to the very high prevalence of hypermutable strains, showing greatly enhanced spontaneous mutation rates caused by defective DNA repair or error avoidance systems (Oliver et al., 2000; Maciá et al., 2005).

Beyond the obvious negative impact of resistance development for the treated patient, the accumulation of several of these chromosomal mutations leads to the emergence of multidrug resistant (MDR), extensively drug-resistant (XDR) or even pan-antibiotic-resistant (PDR) strains, which can be responsible for notable epidemics in the hospital setting (Deplano et al., 2005; Suarez et al., 2011). Moreover, recent studies have evidenced the existence of MDR/XDR global clones disseminated in different hospitals worldwide, and for that reason they have been denominated high-risk clones (Woodford et al., 2011; Oliver et al., 2015). Although high-risk clones are frequently associated with transferable antimicrobial resistance determinants, they also typically show a wide range of chromosomal mutations playing a major role in the resistance phenotype (Oliver et al., 2015). Likewise, recent reports have evidenced the interpatient spread of antimicrobial resistance mutations linked to the transmission of epidemic CF strains (López-Causapé et al., 2017).

Along with growing information from mechanistic studies on chromosomal resistance mechanisms and their complex regulatory pathways, involved in adaptive resistance (Lister et al., 2009; Muller et al., 2011; Skiada et al., 2011; Juan et al., 2017), the introduction of whole-genome sequencing (WGS) approaches is shaping up a new dimension for the mutational resistance landscape. The term resistome was first used to account for the set of primary antibiotic resistance genes that could be eventually transferred to human pathogens (D'Costa et al., 2006). Soon after the concept of intrinsic resistome was introduced to include all chromosomal genes that are involved in intrinsic resistance, and whose presence in strains of a bacterial species is independent of previous antibiotic exposure and is not due to horizontal gene transfer (HGT) (Fajardo et al., 2008). Finally, the term mutational resistome was more recently implemented to account for the set of mutations involved in the modulation of antibiotic resistance levels in the absence of HGT (Cabot et al., 2016b; López-Causapé et al., 2017). Recent WGS data obtained from *in vitro* assays on the evolution of antibiotic resistance, *in vivo* monitoring of antimicrobial resistance development, analysis of sequential CF isolates, and characterization of wide spread epidemic high-risk clones provide new insights into the evolutionary dynamics and mechanisms of *P. aeruginosa* antibiotic resistance (Cabot et al., 2016a; Feng et al., 2016; Del Barrio-Tofiño et al., 2017; Jaillard et al., 2017; López-Causapé et al., 2017). Indeed, the analysis of WGS mutational resistomes has proven to be useful for understanding the evolutionary dynamics of classical resistance mechanisms and to depict new ones for the majority of antimicrobial classes, including β-lactams, aminoglycosides, fluoroquinolones, polymixins and others, as reviewed in the following sections. **Table 1** summarizes the main genes and mutations known to increase resistance levels and therefore shaping up the *P. aeruginosa* mutational resistome.

β-LACTAM MUTATIONAL RESISTOME

The most frequent mutation-driven β-lactam resistance mechanism is likely the overproduction of the chromosomal cephalosporinase AmpC, involving a wide range of genes belonging to complex regulatory pathways of cell-wall recycling (Juan et al., 2017). Among them, the mutational inactivation of *dacB*, encoding the non-essential penicillin-binding protein (PBP) PBP4, and *ampD*, encoding a *N*-acetyl-muramyl-L-alanine amidase have been found to be the most frequent cause of *ampC* derepression and β-lactam resistance (Juan et al., 2005; Moya et al., 2009). The inactivation of PBP4 has also been shown to activate the BlrAB/CreBC regulatory system, further increasing resistance levels (Moya et al., 2009). Additionally, specific point mutations leading to a conformation change in the transcriptional regulator AmpR, causing *ampC* upregulation and β-lactam resistance, have been noted among clinical strains. They include the D135N mutation, described in several species besides *P. aeruginosa*, including *Stenotrophomonas maltophilia*, *Citrobacter freundii,* or *Enterobacter cloacae* (Juan et al., 2017) or the R154H mutation, linked to the widespread MDR/XDR ST175 high-risk clone. Mutation of many other genes, including those encoding other amidases (AmpDh2 and AmpDh3), other PBPs (such as PBP5 and PBP7), lytic transglycosylases (such as SltB1 and mltB), MPL (UDP-*N*-acetylmuramate:Lalanyl-γ-D-glutamyl-meso-diaminopimelate ligase), or NuoN (NADH dehydrogenase I chain N) have been shown to enhance *ampC* expression, either alone or combined with other mutations, although their impact on β-lactam resistance among clinical strains still needs to be further analyzed (Juan et al., 2017).

In addition to *ampC* overexpression, recent studies have revealed that β-lactam resistance development, including the novel combinations of β-lactam-β-lactamase inhibitors ceftolozane/tazobactam and ceftazidime/avibactam, may result from mutations leading to the structural modification of AmpC (Cabot et al., 2014; Lahiri et al., 2014; Fraile-Ribot et al., 2017a; Haidar et al., 2017). Likewise, recent studies identified diverse AmpC variants associated with high-level cephalosporin resistance, including ceftolozane/tazobactam and ceftazidime/avibactam, in a small proportion (around 1%) of clinical *P. aeruginosa* isolates (Berrazeg et al., 2015). Over 200 Pseudomonas Derived Cephalosporinase (PDC) variants have been described so far, including those associated with enhanced ceftolozane/tazobactam and ceftazidime/avibactam resistance (**Table 1**). An update database of PDC variants is maintained in our laboratory and is freely available at https://arpbigidisba.com. Additionally, resistance development to ceftolozane/tazobactam and/or ceftazidime/avibactam may involve mutations leading to the structural modification of narrow spectrum OXA-2 and OXA-10 acquired oxacillinases (Fraile-Ribot et al., 2017a,b).

TABLE 1 | Main genes and mutations known to be involved in increased *P. aeruginosa* antibiotic resistance.

Gene	Resistance mechanisms/ altered target	Antibiotics affected[a]	Type of mutation	Relevant examples of gain-of-function mutations	Reference
gyrA	DNA gyrase	FQ	Gain-of-function	G81D, T83A, T83I, Y86N, D87G, D87N, D87Y, Q106L	Bruchmann et al., 2013; Kos et al., 2015; Cabot et al., 2016b; Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017
gyrB	DNA gyrase	FQ	Gain-of-function	S466F, S466Y, Q467R, E468D	Bruchmann et al., 2013; Kos et al., 2015; Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017
parC	DNA topoisomerase IV	FQ	Gain-of-function	S87L, S87W	Bruchmann et al., 2013; Kos et al., 2015; Cabot et al., 2016b; Del Barrio-Tofiño et al., 2017
parE	DNA topoisomerase IV	FQ	Gain-of-function	S457G, S457T, E459D, E459K	Bruchmann et al., 2013; Kos et al., 2015; Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017
pmrA	Lipopolysaccharide (lipid A)	COL	Gain-of-function	L157Q	Lee and Ko, 2014
pmrB	Lipopolysaccharide (lipid A)	COL	Gain-of-function	L14P, A54V, R79H, R135Q, A247T, A248T, A248V, R259H, M292I, M292T	Barrow and Kwon, 2009; Moskowitz et al., 2012
phoQ	Lipopolysaccharide (lipid A)	COL	Loss-of-function		
parR	Lipopolysaccharide (lipid A)	COL	Gain-of-function	M59I, E156K	Muller et al., 2011; Guénard et al., 2014
	OprD downregulation	IMP, MER			
	MexEF-OprN hyperproduction	FQ			
	MexXY-OprM hyperproduction	FQ, AMG, CEF			
parS	Lipopolysaccharide (lipid A)	COL	Gain-of-function	L14Q, V101M, L137P, A138T, A168V Q232E, G361R	Muller et al., 2011; Fournier et al., 2013; Guénard et al., 2014
	OprD downregulation	IMP, MER			
	MexEF-OprN hyperproduction	FQ			
	MexXY-OprM hyperproduction	FQ, AMG, CEF			
cprS	Lipopolysaccharide (lipid A)	COL	Gain-of-function	R241C	Gutu et al., 2013
colR	Lipopolysaccharide (lipid A)	COL	Gain-of-function	D32N	Gutu et al., 2013
colS	Lipopolysaccharide (lipid A)	COL	Gain-of-function	A106V	Gutu et al., 2013
mexR	MexAB-OprM hyperproduction	FQ, CAZ, CEF, PPT, MER, CAZ/AVI	Loss-of-function		
nalC	MexAB-OprM hyperproduction	FQ, CAZ, CEF, PPT, MER, CAZ/AVI	Loss-of-function		
nalD	MexAB-OprM hyperproduction	FQ, CAZ, CEF, PPT, MER, CAZ/AVI	Loss-of-function		
nfxB	MexCD-OprJ hyperproduction	FQ, CEF	Loss-of-function		
mexS	MexEF-OprN hyperproduction	FQ	Loss-of-function		
	OprD downregulation	IMP, MER			
mexT	MexEF-OprN hyperproduction	FQ	Gain-of-function	G257S, G257A	Juarez et al., 2018
	OprD downregulation	IMP, MER			

(Continued)

TABLE 1 | Continued

Gene	Resistance mechanisms/altered target	Antibiotics affected[a]	Type of mutation	Relevant examples of gain-of-function mutations	Reference
cmrA	MexEF-OprN hyperproduction	MER, FQ	Gain-of-function	A68V, L89Q, H204L, N214K	Juarez et al., 2017
mvaT	MexEF-OprN hyperproduction	FQ	Loss-of-function		
PA3271	MexEF-OprN hyperproduction	FQ	Loss-of-function		
mexZ	MexXY-OprM hyperproduction	FQ, AMG, CEF	Loss-of-function		
PA5471.1	MexXY-OprM hyperproduction	FQ, AMG, CEF	Loss-of-function		
amgS	MexXY-OprM hyperproduction	FQ, AMG, CEF	Gain-of-function	V121G, R182C	Lau et al., 2015
oprD	OprD inactivation	IMP, MER	Loss-of-function		
ampC	AmpC structural modification	CAZ/AVI, C/T	Gain-of-function	T96I, G183D, E247K	Cabot et al., 2014; Fraile-Ribot et al., 2017a
ampD	AmpC hyperproduction	CAZ, CEF, PPT	Loss-of-function		
ampDh2	AmpC hyperproduction	CAZ, CEF, PPT	Loss-of-function		
ampDh3	AmpC hyperproduction	CAZ, CEF, PPT	Loss-of-function		
ampR	AmpC hyperproduction	CAZ, CEF, PPT	Gain-of-function	D135N, G154R	Bagge et al., 2002; Cabot et al., 2016b
dacB	AmpC hyperproduction	CAZ, CEF, PPT	Loss-of-function		
ftsI	Penicillin-binding-protein 3 (PBP3)	CAZ, CEF, PPT, MER, CAZ/AVI, C/T	Gain-of-function	R504C, R504H, P527T, F533L	Diaz Caballero et al., 2015; Cabot et al., 2016a,b; Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017
fusA1	Elongation factor G	AMG	Gain-of-function	I61M, V93A, E100G, K504E, Y552C, P554L, A555E, N592I, P618L, T671A, T671I	Feng et al., 2016; Bolard et al., 2017; Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017, 2018
glpT	Transporter protein GlpT	FOS	Loss-of-function		
rpoB	RNA polymerase β-chain	RIF	Gain-of-function	S517F, Q518R, Q518L, D521G, H531Y, H531L, S536F, L538I, S579F, S579Y, N629S, D636Y	Jatsenko et al., 2010

[a] *FQs, fluoroquinolones; COL, colistin; AMGs, aminoglycosides; CAZ, ceftazidime; CEF, cefepime; PPT, piperacillin-tazobactam; IMP, imipenem; MER, meropenem; CAZ/AVI, ceftazidime/avibactam; C/T, ceftolozane/tazobactam; FOS, fosfomycin; RIF, rifampicin.*

Besides β-lactamases, there is increasing evidence on the role of target modification in *P. aeruginosa* β-lactam resistance. Particularly noteworthy are the mutations in *ftsI*, encoding PBP3, an essential high molecular class B PBP with transpeptidase activity (Chen et al., 2016). Indeed, data from CF patients (Diaz Caballero et al., 2015; López-Causapé et al., 2017), epidemic high-risk clones (Cabot et al., 2016b; Del Barrio-Tofiño et al., 2017) as well as from *in vitro* studies (Cabot et al., 2016a) have recently shown that PBP3 is under strong mutational pressure, and that specific mutations in this PBP contribute to β-lactam resistance development. R504C/H and F533L mutations are likely those most commonly reported, and are located within the protein domains implicated in the formation and stabilization of the inactivating complex β-lactam-PBP3 (Han et al., 2010). Moreover, these specific mutations have been documented to emerge *in vivo* during chronic respiratory infection in CF patients (Diaz Caballero et al., 2015; López-Causapé et al., 2017) and upon meropenem (Cabot et al., 2016a) and aztreonam (Jorth et al., 2017) exposure *in vitro*. However, the precise contribution of PBP3 mutations to β-lactam resistance phenotypes needs to be further investigated using isogenic strains. Likewise, despite unique polymorphisms have also been detected in some clinical strains for other PBPs, their role in β-lactam resistance, if any, still needs to be experimentally addressed.

Other relevant components of the β-lactam mutational resistome are the porins and RND efflux pumps. Mutational inactivation of OprD is well-known to be the primary carbapenem resistance mechanisms in *P. aeruginosa* (Lister et al., 2009; Castanheira et al., 2014). OprD inactivation typically results from indels or nonsense mutations, including the Q142X mutation, characteristic of the widespread ST175 high-risk clone (Cabot et al., 2016b). Additionally, some amino acid substitutions have also been recently associated with OprD-driven resistance, particularly in the CF setting (Richardot et al., 2015). Finally, carbapenem resistance may also result from *oprD* repression caused by mutations in the MexEF-OprN efflux pump regulators

(*mexS/T*) or the ParRS two-component system (Li et al., 2015). Overexpression of MexAB-OprM, caused by mutation of several genes involved in its regulation (*mexR*, *nalC,* or *nalD*) increases MICs of most β-lactams except imipenem, whereas overexpression of MexXY (*mexZ*, *parSR*, *amgS* mutations) is particularly involved in cefepime resistance (Li et al., 2015). Additionally, sequence variations in unique residues are detected in the genes coding for the efflux pump (Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017); however, their contribution to resistance profiles, if any, still needs to be further explored.

Finally, another potentially relevant mutational β-lactam resistance mechanism is the selection of large (>200 kb) deletions affecting specific parts of the chromosome (Cabot et al., 2016a). Although the basis of the conferred resistance phenotype still needs to be further clarified, these mutants can be recognized by the characteristic brown pigment (pyomelanine) caused by the deletion of one of the affected genes, *hmgA*, coding for a homogentisate-1,2- dioxygenase. This type of deletion has been documented in both, *in vitro* evolved β-lactam-resistant mutants and CF isolates (Cabot et al., 2016a; Hocquet et al., 2016). However, the deletion of *hmgA* is not responsible for the resistance phenotype, which could be linked to the deletion of another of the affected genes, *galU*. This gene codes for a UDP-glucose pyrophosphorylase involved in the synthesis of the lipopolysaccharide (LPS) core. Indeed, analysis of transposon mutant libraries has shown that inactivation of *galU* increases ceftazidime and meropenem MICs (Dötsch et al., 2009; Alvarez-Ortega et al., 2010).

AMINOGLYCOSIDE MUTATIONAL RESISTOME

In the absence of horizontally-acquired aminoglycoside modifying enzymes, resistance to this antibiotic class has been particularly linked to the mutational overexpression of MexXY-OprM. Indeed, mutational overexpression of this pump, mainly caused by *mexZ*, *amgS*, or *parRS* mutations (**Table 1**), is very frequent among clinical isolates, from both, CF patients and nosocomial infections (Guénard et al., 2014; Prickett et al., 2017). Moreover, recent studies show that the epidemic high-risk clone ST175 overexpresses MexXY due to a specific mutation in *mexZ* (G195E) (Cabot et al., 2016b). However, recent studies have revealed that the aminoglycoside mutational resistome extends far beyond MexXY overexpression, and that high-level resistance may result from the accumulation of multiple mutations, and the involvement of several novel resistance determinants has been recently documented (El'Garch et al., 2007; Schurek et al., 2008; Feng et al., 2016). Among them is particularly noteworthy *fusA1*, coding for the elongation factor G. Indeed, specific FusA1 mutations have been associated with aminoglycoside resistance *in vitro* (Feng et al., 2016; López-Causapé et al., 2018) and among clinical, particularly CF, strains (Chung et al., 2012; Markussen et al., 2014; Greipel et al., 2016; López-Causapé et al., 2017, 2018). Moreover, the implication of *fusA1* mutations in aminoglycoside resistance has been recently confirmed through site-directed mutagenesis (Bolard et al., 2017).

FLUOROQUINOLONE MUTATIONAL RESISTOME

The fluoroquinolone mutational resistome generally includes specific missense mutations in DNA gyrase (*gyrA* and/or *gyrB*) and topisomerase IV (*parC* and/or *parE*) Quinolone Resistance-Determining Regions (QRDRs) (**Table 1**) (Bruchmann et al., 2013; Kos et al., 2015). High-level fluoroquinolone resistance in *P. aeruginosa* high-risk clones is nearly universal, and typically involves combinations of mutations in GyrA-T83 and ParC-S87 (Del Barrio-Tofiño et al., 2017). QRDR mutations involved in fluoroquinolone resistance in CF might be more variable (López-Causapé et al., 2017). It is also well-known that the mutational overexpression of efflux pumps modulate fluoroquinolone resistance (**Table 1**). While the overexpression of MexAB-OprM and MexXY-OprM is globally more frequent among clinical strains, its contribution to clinical fluoroquinolone resistance is likely more modest (Bruchmann et al., 2013). On the other hand, the mutational overexpression of MexEF-OprN or MexCD-OprJ is associated with high-level (clinical) fluoroquinolone resistance, and although their prevalence is considered low except in the CF chronic infection setting, recent data show that it might be higher than expected (Richardot et al., 2015).

POLYMIXIN MUTATIONAL RESISTOME

Whereas the prevalence of polymyxin (colistin and polymyxin B) resistance is still globally low (<5%), it has increased in the last years because of the frequent use of these last-resource antibiotics for the treatment of MDR/XDR nosocomial and CF strains. Polymyxin resistance results most frequently from the modification of the LPS caused by the addition of a 4-amino-4-deoxy-L-arabinose moiety in the lipid A structure (Olaitan et al., 2014; Jeannot et al., 2017). The involved mutations are frequently located in the PmrAB or PhoPQ two-component regulators, which lead to the activation of the *arnBCADTEF* operon (Barrow and Kwon, 2009). More recent studies have revealed that mutations in the ParRS two-component regulator, not only produce polymyxin resistance due to the activation of the *arnBCADTEF* operon, but also lead to a MDR phenotype determined by the overprexpression of MexXY and the repression of OprD (Muller et al., 2011). Moreover, two additional two-component regulators, ColRS and CprRS, have been recently found to be involved in polymyxin resistance (Gutu et al., 2013). The analysis of colistin resistance mechanisms among clinical strains is not always straight forward, since the presence of mutations in these two-component regulators is not always associated with clinical colistin resistance, probably denoting partial complementation between the different regulators (Moskowitz et al., 2012; Gutu et al., 2013; López-Causapé et al., 2017). Moreover, recent *in vitro* evolution assays have revealed the implication of additional mutations in high level colistin resistance, facilitated by the emergence of mutator (*mutS* deficient) phenotypes (Döβelmann et al., 2017). Particularly noteworthy among them are those occurring in LptD, LpxC, or MigA.

OTHER ANTIBIOTICS

Even if not considered a classical antipseudomonal agent, fosfomycin has emerged in the last decade as a potentially useful antibiotic in urinary tract infections and combined therapy for MDR/XDR *P. aeruginosa* (Michalopoulos et al., 2011). However, fosfomycin resistance spontaneous mutation rates are high and the mechanism involved is typically the mutational inactivation of *glpT*, coding for a glycerol-3-phospate permease required for fosfomycin uptake (Castañeda-García et al., 2009; Rodríguez-Rojas et al., 2010). *glpT* mutations, conferring high-level fosfomycin resistance are also frequently found among MDR/XDR high-risk clones (Del Barrio-Tofiño et al., 2017), and some specific mutations, such as T211P, have been fixed in some widespread lineages as described for ST175 (Cabot et al., 2016b). Another potentially useful antimicrobial for combined therapy against MDR/XDR *P. aeruginosa* is rifampicin (Cai et al., 2017). However, rifampicin resistance may emerge at high frequency due to the selection of specific missense mutations in *rpoB*, coding for the beta subunit of the RNA polymerase (Jatsenko et al., 2010). Another example of newer antibiotic families with antipseudomonal activity are the pacidamycins, uridyl peptide antibiotics, targeting translocase I, an essential enzyme in peptidoglycan biosynthesis (Mistry et al., 2013). Emergence of high-level resistance to this antibiotic class has been shown to involve the selection of mutations in the Opp transporter, a binding protein-dependent ABC transporter used for oligopeptide import. Finally, the *P. aeruginosa* mutational resistome, particularly in the CF setting, may also include resistance to other used antibiotics such as the frequent mutations of domain V of 23S rRNA, conferring macrolide resistance (Mustafa et al., 2017).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The analysis of the *P. aeruginosa* mutational resistome, together with the analysis of the horizontally-acquired resistance determinants, should be useful for establishing the antibiotic resistance genotype, which should correlate with the antibiotic resistance phenotype and as such, it should permit the design of therapeutic strategies and for monitoring the efficacy of administered antibiotic treatments. However, the current applicability of the analysis of the mutational resistome is still limited by the large number of genes involved and the complexity of the resistance phenotypes generated, and, particularly, by the difficulties, in many cases, for differentiating relevant mutations from simple natural polymorphisms. Obviously, the evolution of the mutational resistome is a direct consequence of antimicrobial exposure and as such, it is not surprising that exposure to one antibiotic drives evolution of the mutational resistome for that antibiotic. However, the complexity of the actual resistance profile is further increased by the specificity and interactions among different resistance mechanisms. Indeed, a resistance mutation selected by one antibiotic may have a variable effect among the different agents within the same antibiotic class or family. Likewise, cross resistance (or collateral resistance) implies that exposure to one antibiotic drives also the development of resistance to a different one from the same or other classes. Typically, this is caused by the developed resistance mechanism (such as efflux pump overexpression) affecting simultaneously different antibiotics. Indeed, potential development of cross resistance is a major issue to consider when using antibiotic combinations (Vestergaard et al., 2016). Moreover, cross resistance between antibiotics and antiseptics and other biocides may also occur (Li et al., 2015). Perhaps less obvious is collateral susceptibility, which implies that exposure to one antibiotic increases the susceptibility to a different one (Pál et al., 2015; Imamovic et al., 2017). This might be achieved through two mechanisms. One possible mechanism is that exposure to one antibiotic directly causes increased susceptibility to a different one, for example, mutations in the β-lactamase AmpC increases cephalosporin hydrolysis while reducing that of penicillins or carbapenems (Cabot et al., 2014). The second possibility is that the development of a resistance mechanism impairs the activity of another existing resistance mechanism. An example is competition between the different efflux pumps, since the overexpression of one may impair the expression of another (Mulet et al., 2011). Thus, the evolution of the mutational resistome for a given antibiotic is not only dependent on the exposure to this antibiotic, but it is also conditioned by the simultaneous or even previous exposures to other antibiotics. An illustrative example is provided in a recent *in vitro* study that demonstrated, for a broad range of antibiotic classes, that the history of exposure and resistance development to a given antibiotic, conditions the dynamics and mechanisms of resistance development when exposed to a second one (Yen and Papin, 2017). In summary, the comprehensive analysis of the mutational resistome of *P. aeruginosa* in CF and nosocomial infections is expected to become a useful tool for optimizing therapeutic strategies and monitoring the efficacy of administered antibiotic treatments in the near future.

AUTHOR CONTRIBUTIONS

CL-C and AO wrote the manuscript. CL-C, GC, EdB-T, and AO reviewed the literature.

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REFERENCES

Alvarez-Ortega, C., Wiegand, I., Olivares, J., Hancock, R. E., and Martínez, J. L. (2010). Genetic determinants involved in the susceptibility of *Pseudomonas aeruginosa* to beta-lactam antibiotics. *Antimicrob. Agents Chemother.* 54, 4159–4167. doi: 10.1128/AAC.00257-10

Bagge, N., Ciofu, O., Hentzer, M., Campbell, J. I., Givskov, M., and Høiby, N. (2002). Constitutive high expression of chromosomal beta-lactamase in *Pseudomonas aeruginosa* caused by a new insertion sequence (IS1669) located in ampD. *Antimicrob. Agents Chemother.* 46, 3406–3411. doi: 10.1128/AAC.46.11.3406-3411.2002

Barrow, K., and Kwon, D. H. (2009). Alterations in two-component regulatory systems of phoPQ and pmrAB are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53, 5150–5154. doi: 10.1128/AAC.00893-09

Bassetti, M., Taramasso, L., Giacobbe, D. R., and Pelosi, P. (2012). Management of ventilator-associated pneumonia: epidemiology, diagnosis and antimicrobial therapy. *Expert Rev. Anti. Infect. Ther.* 10, 585–596. doi: 10.1586/eri.12.36

Berrazeg, M., Jeannot, K., Ntsogo Enguéné, V. Y., Broutin, I., Loeffert, S., Fournier, D., et al. (2015). Mutations in β-Lactamase AmpC increase resistance of *Pseudomonas aeruginosa* isolates to antipseudomonal cephalosporins. *Antimicrob. Agents Chemother.* 59, 6248–6255. doi: 10.1128/AAC.00825-15

Bolard, A., Plesiat, P., and Jeannot, K. (2017). Mutations in gene *fusA1* as a novel mechanism of aminoglycoside resistance in clinical strains of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 62, e01835-17. doi: 10.1128/AAC.01835-17

Bruchmann, S., Dötsch, A., Nouri, B., Chaberny, I. F., and Häussler, S. (2013). Quantitative contributions of target alteration and decreased drug accumulation to *Pseudomonas aeruginosa* fluoroquinolone resistance. *Antimicrob. Agents Chemother.* 57, 1361–1368. doi: 10.1128/AAC.01581-12

Cabot, G., Bruchmann, S., Mulet, X., Zamorano, L., Moyà, B., Juan, C., et al. (2014). *Pseudomonas aeruginosa* ceftolozane-tazobactam resistance development requires multiple mutations leading to overexpression and structural modification of AmpC. *Antimicrob. Agents Chemother.* 58, 3091–3099. doi: 10.1128/AAC.02462-13

Cabot, G., Zamorano, L., Moyà, B., Juan, C., Navas, A., Blázquez, J., et al. (2016a). Evolution of *Pseudomonas aeruginosa* antimicrobial resistance and fitness under low and high mutation rates. *Antimicrob. Agents Chemother.* 60, 1767–1778. doi: 10.1128/AAC.02676-15

Cabot, G., López-Causapé, C., Ocampo-Sosa, A. A., Sommer, L. M., Domínguez, M. Á, Zamorano, L., et al. (2016b). Deciphering the resistome of the widespread *Pseudomonas aeruginosa* Sequence Type 175 international high-risk clone through whole-genome sequencing. *Antimicrob. Agents Chemother.* 60, 7415–7423.

Cai, Y., Yang, D., Wang, J., and Wang, R. (2017). Activity of colistin alone or in combination with rifampicin or meropenem in a carbapenem-resistant bioluminescent *Pseudomonas aeruginosa* intraperitoneal murine infection model. *J. Antimicrob. Chemother.* 73, 456–461. doi: 10.1093/jac/dkx399

Castañeda-García, A., Rodríguez-Rojas, A., Guelfo, J. R., and Blázquez, J. (2009). The glycerol-3-phosphate permease GlpT is the only fosfomycin transporter in *Pseudomonas aeruginosa*. *J. Bacteriol.* 191, 6968–6974. doi: 10.1128/JB.00748-09

Castanheira, M., Deshpande, L. M., Costello, A., Davies, T. A., and Jones, R. N. (2014). Epidemiology and carbapenem resistance mechanisms of carbapenem-non-susceptible *Pseudomonas aeruginosa* collected during 2009-11 in 14 European and Mediterranean countries. *J. Antimicrob. Chemother.* 69, 1804–1814. doi: 10.1093/jac/dku048

Chen, W., Zhang, Y. M., and Davies, C. (2016). Penicillin-Binding Protein 3 is essential for growth of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 61, e01651-16. doi: 10.1128/AAC.01651-16

Chung, J. C., Becq, J., Fraser, L., Schulz-Trieglaff, O., Bond, N. J., Foweraker, J., et al. (2012). Genomic variation among contemporary *Pseudomonas aeruginosa* isolates from chronically infected cystic fibrosis patients. *J. Bacteriol.* 194, 4857–4866. doi: 10.1128/JB.01050-12

D'Costa, V. M., McGrann, K. M., Hughes, D. W., and Wright, G. D. (2006). Sampling the antibiotic resistome. *Science* 311, 374–377. doi: 10.1126/science.1120800

Del Barrio-Tofiño, E., López-Causapé, C., Cabot, G., Rivera, A., Benito, N., Segura, C., et al. (2017). Genomics and susceptibility profiles of extensively drug-resistant *Pseudomonas aeruginosa* isolates from Spain. *Antimicrob. Agents Chemother.* 61, e01589-17. doi: 10.1128/AAC.01589-17

Deplano, A., Denis, O., Poirel, L., Hocquet, D., Nonhoff, C., Byl, B., et al. (2005). Molecular characterization of an epidemic clone of panantibiotic-resistant *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 43, 1198–1204. doi: 10.1128/JCM.43.3.1198-1204.2005

Diaz Caballero, J., Clark, S. T., Coburn, B., Zhang, Y., Wang, P. W., Donaldson, S. L., et al. (2015). Selective sweeps and parallel pathoadaptation drive *Pseudomonas aeruginosa* evolution in the cystic fibrosis lung. *mBio* 6, e00981-15. doi: 10.1128/mBio.00981-15

Döring, G., Parameswaran, I. G., and Murphy, T. F. (2011). Differential adaptation of microbial pathogens to airways of patients with cystic fibrosis and chronic obstructive pulmonary disease. *FEMS Microbiol. Rev.* 35, 124–146. doi: 10.1111/j.1574-6976.2010.00237.x

Dötsch, A., Becker, T., Pommerenke, C., Magnowska, Z., Jänsch, L., and Häussler, S. (2009). Genomewide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 53, 2522–2531. doi: 10.1128/AAC.00035-09

Dößelmann, B., Willmann, M., Steglich, M., Bunk, B., Nübel, U., Peter, S., et al. (2017). Rapid and consistent evolution of colistin resistance in extensively drug-resistant *Pseudomonas aeruginosa* during morbidostat culture. *Antimicrob. Agents Chemother.* 61, e00043-17. doi: 10.1128/AAC.00043-17

El'Garch, F., Jeannot, K., Hocquet, D., Llanes-Barakat, C., and Plésiat, P. (2007). Cumulative effects of several nonenzymatic mechanisms on the resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob. Agents Chemother.* 51, 1016–1021. doi: 10.1128/AAC.00704-06

Fajardo, A., Martínez-Martín, N., Mercadillo, M., Galán, J. C., Ghysels, B., Matthijs, S., et al. (2008). The neglected intrinsic resistome of bacterial pathogens. *PLoS One* 3:e1619. doi: 10.1371/journal.pone.0001619

Feng, Y., Jonker, M. J., Moustakas, I., Brul, S., and Ter Kuile, B. H. (2016). Dynamics of mutations during development of resistance by *Pseudomonas aeruginosa* against five antibiotics. *Antimicrob. Agents Chemother.* 60, 4229–4236. doi: 10.1128/AAC.00434-16

Fournier, D., Richardot, C., Müller, E., Robert-Nicoud, M., Llanes, C., Plésiat, P., et al. (2013). Complexity of resistance mechanisms to imipenem in intensive care unit strains of *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 68, 1772–1780. doi: 10.1093/jac/dkt098

Fraile-Ribot, P. A., Cabot, G., Mulet, X., Periañez, L., Martín-Pena, M. L., Juan, C., et al. (2017a). Mechanisms leading to in vivo ceftolozane/tazobactam resistance development during the treatment of infections caused by MDR *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* doi: 10.1093/jac/dkx424 [Epub ahead of print].

Fraile-Ribot, P. A., Mulet, X., Cabot, G., Del Barrio-Tofiño, E., Juan, C., Pérez, J. L., et al. (2017b). In vivo emergence of resistance to novel cephalosporin-β-Lactamase inhibitor combinations through the duplication of amino acid D149 from OXA-2 β-Lactamase (OXA-539) in Sequence Type 235 *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 61, e01117-17. doi: 10.1128/AAC.01117-17

Greipel, L., Fischer, S., Klockgether, J., Dorda, M., Mielke, S., Wiehlmann, L., et al. (2016). Molecular epidemiology of mutations in antimicrobial resistance loci of *Pseudomonas aeruginosa* isolates from airways of Cystic Fibrosis patients. *Antimicrob. Agents Chemother.* 60, 6726–6734. doi: 10.1128/AAC.00724-16

Guénard, S., Muller, C., Monlezun, L., Benas, P., Broutin, I., Jeannot, K., et al. (2014). Multiple mutations lead to MexXY-OprM-dependent aminoglycoside resistance in clinical strains of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 58, 221–228. doi: 10.1128/AAC.01252-13

Gutu, A. D., Sgambati, N., Strasbourger, P., Brannon, M. K., Jacobs, M. A., Haugen, E., et al. (2013). Polymyxin resistance of *Pseudomonas aeruginosa* phoQ mutants is dependent on additional two-component regulatory systems. *Antimicrob. Agents Chemother.* 57, 2204–2215. doi: 10.1128/AAC.02353-12

Haidar, G., Philips, N. J., Shields, R. K., Snyder, D., Cheng, S., Potoski, B. A., et al. (2017). Ceftolozane-Tazobactam for the treatment of multidrug-resistant *Pseudomonas aeruginosa* infections: clinical effectiveness and evolution of resistance. *Clin. Infect. Dis.* 65, 110–120. doi: 10.1093/cid/cix182

Han, S., Zaniewski, R. P., Marr, E. S., Lacey, B. M., Tomaras, A. P., Evdokimov, A., et al. (2010). Structural basis for effectiveness of siderophore-conjugated monocarbams against clinically relevant strains of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 22002–22007. doi: 10.1073/pnas.1013092107

Hocquet, D., Petitjean, M., Rohmer, L., Valot, B., Kulasekara, H. D., Bedel, E., et al. (2016). Pyomelanin-producing *Pseudomonas aeruginosa* selected during chronic infections have a large chromosomal deletion which confers resistance to pyocins. *Environ. Microbiol.* 18, 3482–3493. doi: 10.1111/1462-2920.13336

Imamovic, L., Ellabaan, M. M. H., Dantas Machado, A. M., Citterio, L., Wulff, T., Molin, S., et al. (2017). Drug-driven phenotypic convergence supports rational treatment strategies of chronic infections. *Cell* 172, 121–134.e14. doi: 10.1016/j.cell.2017.12.012

Jaillard, M., van Belkum, A., Cady, K. C., Creely, D., Shortridge, D., Blanc, B., et al. (2017). Correlation between phenotypic antibiotic susceptibility and the resistome in *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 50, 210–218.

Jatsenko, T., Tover, A., Tegova, R., and Kivisaar, M. (2010). Molecular characterization of Rif(r) mutations in *Pseudomonas aeruginosa* and *Pseudomonas putida*. *Mutat. Res.* 683, 106–114. doi: 10.1016/j.mrfmmm.2009.10.015

Jeannot, K., Bolard, A., and Plésiat, P. (2017). Resistance to polymyxins in Gram-negative organisms. *Int. J. Antimicrob. Agents* 49, 526–535. doi: 10.1016/j.ijantimicag.2016.11.029

Jorth, P., McLean, K., Ratjen, A., Secor, P. R., Bautista, G. E., Ravishankar, S., et al. (2017). Evolved aztreonam resistance is multifactorial and can produce hypervirulence in *Pseudomonas aeruginosa*. *mBio* 8, e00517-17. doi: 10.1128/mBio.00517-17

Juan, C., Maciá, M. D., Gutiérrez, O., Vidal, C., Pérez, J. L., and Oliver, A. (2005). Molecular mechanisms of beta-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. *Antimicrob. Agents Chemother.* 49, 4733–4738. doi: 10.1128/AAC.49.11.4733-4738.2005

Juan, C., Torrens, G., González-Nicolau, M., and Oliver, A. (2017). Diversity and regulation of intrinsic β-lactamases from non-fermenting and other Gram-negative opportunistic pathogens. *FEMS Microbiol. Rev.* 41, 781–815. doi: 10.1093/femsre/fux043

Juarez, P., Broutin, I., Bordi, C., Plésiat, P., and Llanes, C. (2018). Constitutive activation of MexT by amino acid substitutions results in MexEF-OprN overproduction in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* doi: 10.1128/AAC.02445-17 [Epub ahead of print].

Juarez, P., Jeannot, K., Plésiat, P., and Llanes, C. (2017). Toxic electrophiles induce expression of the multidrug efflux pump MexEF-OprN in *Pseudomonas aeruginosa* through a novel transcriptional regulator, CmrA. *Antimicrob. Agents Chemother.* 61, e00585-17. doi: 10.1128/AAC.00585-17

Kos, V. N., Déraspe, M., McLaughlin, R. E., Whiteaker, J. D., Roy, P. H., Alm, R. A., et al. (2015). The resistome of *Pseudomonas aeruginosa* in relationship to phenotypic susceptibility. *Antimicrob. Agents Chemother.* 59, 427–436. doi: 10.1128/AAC.03954-14

Lahiri, S. D., Johnstone, M. R., Ross, P. L., McLaughlin, R. E., Olivier, N. B., and Alm, R. A. (2014). Avibactam and class C β-lactamases: mechanism of inhibition, conservation of the binding pocket, and implications for resistance. *Antimicrob. Agents Chemother.* 58, 5704–5713. doi: 10.1128/AAC.03057-14

Lau, C. H., Krahn, T., Gilmour, C., Mullen, E., and Poole, K. (2015). AmgRS-mediated envelope stress-inducible expression of the mexXY multidrug efflux operon of *Pseudomonas aeruginosa*. *Microbiol. Open* 4, 121–135. doi: 10.1002/mbo3.226

Lee, J. Y., and Ko, K. S. (2014). Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates. *Diagn. Microbiol. Infect. Dis.* 78, 271–276. doi: 10.1016/j.diagmicrobio.2013.11.027

Li, X. Z., Plésiat, P., and Nikaido, H. (2015). The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin. Microbiol. Rev.* 28, 337–418. doi: 10.1128/CMR.00117-14

Lister, P. D., Wolter, D. J., and Hanson, N. D. (2009). Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin. Microbiol. Rev.* 22, 582–610. doi: 10.1128/CMR.00040-09

López-Causapé, C., Rubio, R., Cabot, G., and Oliver, A. (2018). Evolution of the *Pseudomonas aeruginosa* aminoglycoside mutational resistome in vitro and in the cystic fibrosis setting. *Antimicrob. Agents Chemother.* doi: 10.1128/AAC.02583-17 [Epub ahead of print].

López-Causapé, C., Sommer, L. M., Cabot, G., Rubio, R., Ocampo-Sosa, A. A., Johansen, H. K., et al. (2017). Evolution of the *Pseudomonas aeruginosa* mutational resistome in an international Cystic Fibrosis clone. *Sci. Rep.* 7:5555. doi: 10.1038/s41598-017-05621-5

Maciá, M. D., Blanquer, D., Togores, B., Sauleda, J., Pérez, J. L., and Oliver, A. (2005). Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. *Antimicrob. Agents Chemother.* 49, 3382–3386. doi: 10.1128/AAC.49.8.3382-3386.2005

Markussen, T., Marvig, R. L., Gómez-Lozano, M., Aanæs, K., Burleigh, A. E., Høiby, N., et al. (2014). Environmental heterogeneity drives within-host diversification and evolution of *Pseudomonas aeruginosa*. *mBio.* 5, e01592-14. doi: 10.1128/mBio.01592-14

Michalopoulos, A. S., Livaditis, I. G., and Gougoutas, V. (2011). The revival of fosfomycin. *Int. J. Infect. Dis.* 15, e732-39. doi: 10.1016/j.ijid.2011.07.007

Mistry, A., Warren, M. S., Cusick, J. K., Karkhoff-Schweizer, R. R., Lomovskaya, O., and Schweizer, H. P. (2013). High-level pacidamycin resistance in *Pseudomonas aeruginosa* is mediated by an opp oligopeptide permease encoded by the opp-fabI operon. *Antimicrob. Agents Chemother.* 57, 5565–5571. doi: 10.1128/AAC.01198-13

Moskowitz, S. M., Brannon, M. K., Dasgupta, N., Pier, M., Sgambati, N., Miller, A. K., et al. (2012). PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob. Agents Chemother.* 56, 1019–1030. doi: 10.1128/AAC.05829-11

Moya, B., Dötsch, A., Juan, C., Blázquez, J., Zamorano, L., Haussler, S., et al. (2009). Beta-lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. *PLoS Pathog.* 5:e1000353. doi: 10.1371/journal.ppat.1000353

Mulet, X., Moyá, B., Juan, C., Macià, M. D., Pérez, J. L., Blázquez, J., et al. (2011). Antagonistic interactions of *Pseudomonas aeruginosa* antibiotic resistance mechanisms in planktonic but not biofilm growth. *Antimicrob. Agents Chemother.* 55, 4560–4568. doi: 10.1128/AAC.00519-11

Muller, C., Plésiat, P., and Jeannot, K. (2011). A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and β-lactams in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* 55, 1211–1221. doi: 10.1128/AAC.01252-10

Mustafa, M. H., Khandekar, S., Tunney, M. M., Elborn, J. S., Kahl, B. C., Denis, O., et al. (2017). Acquired resistance to macrolides in *Pseudomonas aeruginosa* from cystic fibrosis patients. *Eur. Respir. J.* 49:1601847. doi: 10.1183/13993003.01847-2016

Olaitan, A. O., Morand, S., and Rolain, J. M. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front. Microbiol.* 5:643. doi: 10.3389/fmicb.2014.00643

Oliver, A., Cantón, R., Campo, P., Baquero, F., and Blázquez, J. (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* 288, 1251–1254. doi: 10.1126/science.288.5469.1251

Oliver, A., Mulet, X., López-Causapé, C., and Juan, C. (2015). The increasing threat of *Pseudomonas aeruginosa* high-risk clones. *Drug Resist. Updat.* 2, 41–59. doi: 10.1016/j.drup.2015.08.002

Pál, C., Papp, B., and Lázár, V. (2015). Collateral sensitivity of antibiotic-resistant microbes. *Trends Microbiol.* 23, 401–407. doi: 10.1016/j.tim.2015.02.009

Prickett, M. H., Hauser, A. R., McColley, S. A., Cullina, J., Potter, E., Powers, C., et al. (2017). Aminoglycoside resistance of *Pseudomonas aeruginosa* in cystic fibrosis results from convergent evolution in the *mexZ* gene. *Thorax* 72, 40–47. doi: 10.1136/thoraxjnl-2015-208027

Richardot, C., Plésiat, P., Fournier, D., Monlezun, L., Broutin, I., and Llanes, C. (2015). Carbapenem resistance in cystic fibrosis strains of *Pseudomonas aeruginosa* as a result of amino acid substitutions in porin OprD. *Int. J. Antimicrob. Agents* 45, 529–532. doi: 10.1016/j.ijantimicag.2014.12.029

Rodríguez-Rojas, A., Maciá, M. D., Couce, A., Gómez, C., Castañeda-García, A., Oliver, A., et al. (2010). Assessing the emergence of resistance: the absence of biological cost in vivo may compromise fosfomycin treatments for *P. aeruginosa* infections. *PLoS One* 5:e10193. doi: 10.1371/journal.pone.0010193

Schurek, K. N., Marr, A. K., Taylor, P. K., Wiegand, I., Semenec, L., Khaira, B. K., et al. (2008). Novel genetic determinants of low-level aminoglycoside resistance

in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 52, 4213–4219. doi: 10.1128/AAC.00507-08

Skiada, A., Markogiannakis, A., Plachouras, D., and Daikos, G. L. (2011). Adaptive resistance to cationic compounds in *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 37, 187–193. doi: 10.1016/j.ijantimicag.2010.11.019

Suarez, C., Peña, C., Arch, O., Dominguez, M. A., Tubau, F., Juan, C., et al. (2011). A large sustained endemic outbreak of multiresistant *Pseudomonas aeruginosa*: a new epidemiological scenario for nosocomial acquisition. *BMC Infect. Dis.* 11:272. doi: 10.1186/1471-2334-11-272

Vestergaard, M., Paulander, W., Marvig, R. L., Clasen, J., Jochumsen, N., Molin, S., et al. (2016). Antibiotic combination therapy can select for broad-spectrum multidrug resistance in *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 47, 48–55. doi: 10.1016/j.ijantimicag.2015.09.014

Vincent, J. L. (2003). Nosocomial infections in adult intensive-care units. *Lancet* 361, 2068–2077. doi: 10.1016/S0140-6736(03)13644-6

Woodford, N., Turton, J. F., and Livermore, D. M. (2011). Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiol. Rev.* 35, 736–755. doi: 10.1111/j.1574-6976.2011.00268.x

Yen, P., and Papin, J. A. (2017). History of antibiotic adaptation influences microbial evolutionary dynamics during subsequent treatment. *PLoS Biol.* 15:e2001586. doi: 10.1371/journal.pbio.2001586

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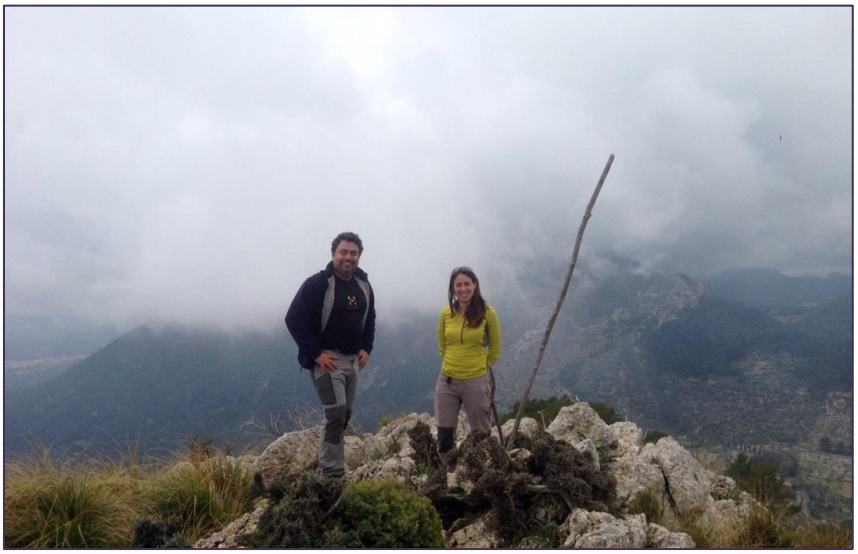

¡Quién busque el infinito, que cierre los ojos!

Milan Kundera, 1984