DEVELOPMENT OF TECHNIQUES FOR THE ANALYSIS OF ACETIC ACID BACTERIA POPULATIONS AND THEIR INTERACTION IN DIFFERENT FOOD ENVIRONMENTS.

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UNIVERSITAT ROVIRA I VIRGILI

Department of Biochemistry and Biotechnology

Development of techniques for the analysis of acetic acid bacteria populations and their interaction in different food environments

DOCTORAL THESIS

Doctoral Thesis presented by

María José Valera Martínez

to receive the Ph.D. degree with International Mention

by the Rovira i Virgili University

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Dr. Albert Mas Baron, professor of the Department of Biochemistry and Biotechnology at the Rovira and Virgili University and Dr. Estibaliz Mateo Alesanco, contract professor of the Department of Immunology, Microbiology and Parasitology at the Basque Country University,

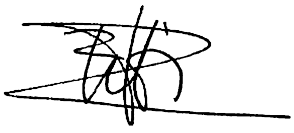
CERTIFY

That the Doctoral Thesis entitled "Development of techniques for the analysis of acetic acid bacteria populations and their interaction in different food environments", presented by María José Valera Martínez to receive the degree of Doctor with International Mention by the Rovira i Virgili University, has been carried out under our supervision in the Department of Biochemistry and Biotechnology of this University. All the results presented in this thesis were obtained in experiments conducted by the above mentioned student.

Tarragona, 5th September 2014

Dr. Albert Mas Baron

Dr. Estibaliz Mateo Alesanco



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Aquí termina un capítulo de mi vida. Siempre recordaré ese momento en que abrí la resolución de la FPU y vi que tenía un lugar para vivir los próximos cuatro años. Todo tiene su fin y esta etapa se termina, me quedo con los buenos momentos, con los menos buenos, que te hacen aprender, y con vosotros. Quizás el tiempo haga que nos volvamos a encontrar, me gusta pensar que así será.

A mis padres y

mi hermano

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JUSTIFICATION AND OBJETIVES

The present work aimed to obtain a Ph.D. degree has been performed from 2009 to 2014 in the research group of Oenological Biotechnology at the Department of Biochemistry and Biotechnology from the Faculty of Oenology of Rovira i Virgili University (URV). During this period, I held a grant from Fundació URV for one year, and then a University Teacher Training (FPU) fellowship from the Spanish Ministry of Education, Culture and Sports (AP2009-0843).

My incorporation in the research group was in the acetic acid bacteria (AAB) research line that was a reference in the field. The group had been developing different molecular methods and applied them for microbiological control of wine and vinegar elaboration. During my thesis, the research group obtained the project "Microbiological Analysis and Control of the Fruit Condiment Production Process" funded by the Spanish Ministry of Education and Science (AGL2007-66417-C02-02), and in the following period (2012–2014), we obtained the project "Microbiological Selection and Control for Transforming Non-commercial Strawberries into New Beverages" funded by the Spanish Ministry of Science and Innovation (AGL2010-22152-C03-02).

In these two projects, a main point was to develop and validate new methodologies for the microbiological control of several food processes, and it was in these points where my thesis was incorporated. At the beginning of this thesis, new molecular techniques had been proposed to analyze the diversity of AAB in niches such as grapes, wine, and vinegar. However, the description of new AAB species and genera in recent years meant the expansion of AAB diversity in these niches, making it necessary to adequate techniques, to understand and determine the actual AAB population, and also to adapt the techniques to the new matrices.

Within this framework, the working hypothesis of the present thesis was as follows: **The control of AAB populations requires the adaptation of molecular techniques and the understanding of the interactions between the different microorganisms**.

In order to demonstrate this hypothesis, the general objective was to design and apply molecular tools for the detection and identification of AAB present in natural samples and for the detection of metabolic activities implied in the interaction among microorganisms. These three specific objectives were developed to achieve the general one:

Objective 1: To apply different techniques for the analysis of AAB populations in new niches and analyze their appropriateness

Objective 2: To develop the techniques for the analysis of the AAB population and production of biofilms

Objective 3: To analyze the interactions between AAB populations by determination of quorum sensing and quorum quenching mechanisms

EXPERIMENTAL DESIGN

To achieve the objectives previously described, the following experimental design was used:

Objective 1: To apply different techniques for the analysis of AAB populations in new niches and analyze their appropriateness

Chapter 1: Diversity of acetic acid bacteria present in healthy grapes from the Canary Islands. Results published in *International Journal of Food Microbiology*, 151, 105–112, 2011

The genetic diversity and identification of AAB species present on sound grapes from the Canary Islands were studied using culture-dependent techniques. The criteria for grape selection were based on the following:

– The island of origin. Samples were collected from vineyards situated in Tenerife, La Palma, and Lanzarote.

– The varieties of grape. Three different varieties were tested: Malvasía and Listan Blanco (white varieties) and Listan Negro (red variety). These varieties are the most common ones in these islands.

Microvinifications were performed to detect AAB population because no direct recovery of bacteria was possible in the most common medium. AAB isolates were characterized using two fingerprint techniques: enterobacterial repetitive intergenic consensus–PCR (ERIC-PCR) and $(GTG)_5$-rep-PCR. Different genotypes were then identified attending to the 16S rRNA gene, first by restriction fragment length polymorphism–PCR (RFLP-PCR) and then by sequencing. The accurate discrimination

of closely related AAB species such as *Acetobacter malorum* and *Acetobacter cerevisiae*, that was not possible by 16S rRNA gene sequencing, was carried out by the 16S–23S rRNA internal transcribed spacer (ITS) region sequence analysis.

Chapter 2: *Acetobacter malorum* and *Acetobacter cerevisiae* identification and quantification by Real-Time PCR with TaqMan-MGB probes. Results published in *Food Microbiology*, 36, 30–39, 2013

Specific primers and TaqMan-MGB probes were designed for the identification and quantification of *Acetobacter malorum* and *Acetobacter cerevisiae* by Real-Time PCR (RT-PCR). As in the previous chapter, we observed the difficulties of differentiating these species; because they share the whole sequence of the 16S rRNA gene except for two base pairs, we had to design primers and probes using the sequences of the 16S–23S rRNA gene ITS region. Although high intraspecific variability in this region exists, there are fragments that correspond to transfer RNA (tRNAIle and tRNAAla) that are highly conserved within strains of the same species and have been described as adequate for the design of primers and probes.

These two species are recently detected on fruits such as strawberry and grapes, in breweries, in fruit vinegars, and in wine or other fermented beverages. Therefore, this technique was applied and evaluated in wine samples from the Canary Island cellars for the identification and quantification of these two AAB species and the other five species of AAB (*Acetobacter aceti*, *Acetobacter pasteurianus*, *Gluconobacter oxydans*, *Komagataeibacter hansenii*, and *Komagataeibacter europaeus*). For these species, we had primers and probes previously designed in our group. To obtain a more accurate quantification of AAB, the application of a PCR enrichment step before the RT-PCR was performed to avoid the possible PCR inhibitors (polyphenols, tannins, and

polysaccharides) usually present in these samples as well as the high amount of nontarget AAB species that can decrease the accuracy of the results. Moreover, the total AAB population was quantified by RT-PCR with SYBR-Green.

Objective 2: To develop the techniques for the analysis of the AAB population and production of biofilms

Chapter 3: Acetic acid bacteria diversity from biofilm of strawberry vinegar visualized by microscopy and detected by complementing culture-dependent and culture-independent techniques. Manuscript submitted to *Food Microbiology*.

The biofilm developed by AAB during strawberry vinegar elaboration by a traditional method was analyzed. The cell disposition in this biofilm was visualized and compared with one from grape vinegar by laser confocal microscope and scanning electronic microscope. Furthermore, the AAB diversity was detected by culture-dependent and culture-independent techniques. The isolates recovered from a piece of biofilm inoculated on GY medium were characterized by ERIC-PCR fingerprinting and identified by the 16S rRNA gene and 16S–23S rRNA gene ITS region sequencing. AAB microbiota present in the biofilm by culture-independent techniques was detected after the development of an adequate DNA extraction method. Techniques like denaturing gradient gel electrophoresis (DGGE)–PCR, RT-PCR with TaqMan-MGB probes, and next-generation sequencing were applied, as well as a direct analysis of the 16S rRNA gene by RFLP-PCR and sequencing.

Chapter 4: Cellulose production and cellulose synthase gene detection in acetic acid bacteria. Manuscript submitted to *International Journal of Food Microbiology*.

The effect of different carbon sources (fructose, glucose, and sucrose) and the presence or absence of ethanol on controlled media for the production of cellulose by different AAB strains were evaluated. This phenotype was correlated with the presence/absence of the cellulose synthase gene analyzed by PCR.

Because of the lack of available sequences from AAB in the GenBank databases and the reduced homology between the gene sequences, different sets of degenerate primers were designed based on the DNA sequences, the amino acid sequences, and the combination of both DNA and amino acid sequences.

Cellulose production was also analyzed by epifluorescence microscopy to follow the production of cellulose in strains that produce biofilms. We also monitored the cell viability during the period of pellicle formation by *Komagataeibacter europaeus* strain Gae02.

Objective 3: To analyze the interactions between AAB populations by determination of quorum sensing and quorum quenching mechanisms

Chapter 5: First evidence that a prephenate dehydratase is involved in quorum quenching of acyl homoserine lactone molecules and cellulose-biofilm formation in the strain Gae02. Manuscript in preparation.

A genomic fosmid library construction was carried out from the DNA of *Komagataeibacter europaeus* Gae02 strain in order to analyze its quorum sensing (QS) and quenching (QQ) activity. This strain is a cellulose producer and was recovered from strawberry vinegar.

The clones obtained after the transformation of *Escherichia coli* (*E. coli*) with a genomic library were analyzed in different tests with *Agrobacterium tumefaciens* NTL4 and *Pseudomonas aeruginosa* PAO1 reporter strains to detect both QS and QQ activities of this AAB strain.

The clones obtained from the genome of *Ko. europaeus* strain Gae02 were analyzed in different tests with the reporter strains of *Agrobacterium tumefaciens* NTL4, *Pseudomonas aeruginosa* PAO1 or *Chromobacterium violaceum* Cv026 to detect both QS and QQ activities of this AAB strain.

INTRODUCTION

1. Acetic acid bacteria

1.1. General characteristics

The group of acetic acid bacteria (AAB) is formed by Gram-negative or Gram-variable bacteria and non–spore forming, and attending to their morphology, they can be ellipsoidal to rod shaped. Their size ranges from 0.4 to 1 μm wide and 0.8 to 4.5 μm long and can present peritrichous or polar flagella, which makes them motile microorganisms (Yamada and Yuphkan, 2008). Under the microscope, they are observed as individual cells, in pairs, or in chains. They are able to vary their aggregation state and even their morphology depending on the culture age and the environmental conditions (Park et al., 2003a; Trček et al., 2007).

They have a strict aerobic metabolism with oxygen as the terminal electron acceptor, and they are characterized by being catalase positive and oxidase negative. Although most of the AAB can grow between pH 5.5 and 6.3 (Holt et al., 1994), it has been reported that in some cases, they could grow at a pH lower than 4. Du Toit and Pretorius (2002) reported that in aerated culture containing acetate, AAB were isolated at pH values of 2.0–2.3. Their optimal growth temperature is 25°C–30°C, but some species are able to grow between 38°C and 40°C (Ndoye et al., 2006; Saeki et al., 1997) and weakly at lower temperatures such as 10°C (Joyeux et al., 1984).

AAB can present pigmentation in solid cultures and produce different types of polysaccharides such as cellulose or acetan (De Ley et al., 1984). The exopolysaccharide (EPS) of cellulose is the main constituent of the extracellular matrix produced by AAB and has been widely studied for their industrial applications (Römling, 2002).

These bacteria are ubiquitous organisms, usually found in substrates containing sugar and/or ethanol. Their natural environment includes fruits, flowers, or palm sap, but they are also present in man-made environments such as food and fermented beverages like fruit juices, wine, cider, beer, cocoa, and vinegar (Nielsen et al., 2007; Yamada and Yukphan, 2008). Moreover, they are present in the plant rhizosphere (Fuentes-Ramirez et al., 2001), and more recently, they were also found as symbiotic microorganisms in insects (Crotti et al., 2010).

The members of this group of bacteria are considered *fastidious microorganisms*. Their recovery in laboratory growth media is often difficult, especially when the samples come from extreme environments such as wine or vinegar (Entani et al., 1985). Several authors have described the existence of differences in the quantification of AAB from plate recovery and from microscopy counting (Sokollek et al., 1998; Torija et al., 2010; Trček, 2005). There are several possible explanations for these differences:

– It has been suggested that this low culturability of AAB could be a transition to the viable but nonculturable (VBNC) state (Baena-Ruano et al., 2006; Ilabaca et al., 2008; Mesa et al., 2003; Millet and Lonvaud-Funel, 2000).

– The common laboratory culture media could not be appropriate for their growth, being selective even among strains (Gullo et al., 2006; Sokollek et al., 1998). It has been reported that the cells proceeding from an extreme medium are adapted to these conditions, and it is difficult to recover them on laboratory media; although the cells are alive, they cannot grow under these new conditions (Torija et al., 2010).

– AAB frequently occur in pairs or chains or form aggregates that probably represent a single colony when plated (Ilabaca et al., 2008; Torija et al., 2010).

1.2. AAB taxonomy

The AAB belong to the *Acetobacteraceae* family. They are classified as *α*-proteobacteria (De Ley et al., 1984; Sievers et al., 1994). The classification of this bacteria group has been substantially changed several times, including additions of new genera and species (Malimas et al., 2009; Yamada et al.,2012; Yukphan et al.,2010) and reclassifications of species and strains (Lisdiyanti et al., 2006; Malimas et al., 2008).

Nowadays, AAB are grouped into 14 genera: *Acetobacter (A.), Gluconobacter (G.), Acidomonas (Ac.), Gluconacetobacter (Ga.), Asaia (As.), Kozakia (K.), Swaminathania (Sw.), Saccharibacter (S.), Neoasaia (N.), Granulibacter (Gr.), Tanticharoenia (T.), Ameyamaea (Am.), Neokomagataea (Ne.),* and *Komagataeibacter (Ko.)* (Yamada et al., 2012). The genera with the highest diversity of species were, until recently, *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter* (Yamada and Yukphan, 2008). However, a short time ago, Yamada et al. (2012) proposed the new genus *Komagataeibacter*, which includes 12 species that previously belonged to the *Gluconacetobacter* genus, and with *Ko. xylinus* as the type species of this new genus. Therefore, nowadays, *Acetobacter* continues to be the most diverse genus with 24 species, but now followed by *Komagataeibacter* and *Gluconobacter* with 14 species and *Gluconacetobacter* with 11 species (Table 1).

The first bacteria belonging to the AAB group were observed from naturally fermented vinegar in 1837 by F. T. Kützing; these microorganisms isolated were first classified as a new species and named *Ulvina aceti* (Asai, 1968). A few decades later, in 1868, Louis Pasteur performed the first systematic study of acetic acid fermentation describing the "vinegar mother" for the first time as a mass of live microorganisms, with the ability of producing acetic acid. In his work, he pointed out that this process was not possible when these microorganisms were not in the medium.

Table 1: Species of AAB described to the date classified into their genera

Genus	**Species**		
Acetobacter	*A. aceti*	*A. malorum*	*A. peroxydans*
(24 species)	*A. cerevisiae*	*A. nitrogenifigens*	*A. syzygii*
	A. cibinongensis	*A. oeni*	*A. fabarum*
	A. estunensis	*A. orientalis*	*A. ghanaensis*
	A. indonesiensis	*A. orleanensis*	*A. senegalensis*
	A. lovaniensis	*A. pasteurianus*	*A. farinalis*
	A. pomorum	*A. tropicalis*	*A.papayae*
	A. persici	*A. okinawensis*	*A. lambici*
Gluconobacter	*G. albidus*	*G. oxydans*	*G. kanchanaburiensis*
(14 species)	*G. cerinus*	*G. roseus*	*G. uchimurae*
	G. frateurii	*G. sphaericus*	*G. nephelii*
	G. japonicus	*G. thailandicus*	*G. cerevisiae*
	G. kondonii	*G. wancherniae*	
Komagataeibacter	*Ko. xylinus*	*Ko. europaeus*	*Ko. rhaeticus*
(14 species)	*Ko.kakiaceti*	*Ko. hansenii*	*Ko. saccharivorans*
	Ko.medellinensis	*Ko. sucrofermentans*	*Ko. swingsii*
	Ko.maltaceti	*Ko. intermedius*	*Ko. nataicola*
	Ko. kombuchae	*Ko. oboediens*	
Gluconacetobacter	*Ga. azotocaptans*	*Ga. liquefaciens*	*Ga. asukensis*
(11 species)	*Ga. diazotrophicus*	*Ga. johannae*	*Ga. tumulicola*
	Ga. sacchari	*Ga. entanii* [a]	*Ga. takamatsuzukensis*
	Ga. tumulisoli	*Ga. aggeris*	
Asaia	*As. bogorensis*	*As. siamensis*	*As. krungthepensis*
(8 species)	*As. lannensis*	*As. spathodeae*	*As. platycodi*
	As. prunellae	*As. astilbes*	
Neokomagataea	*Ne. thailandica*	*Ne. tanensis*	
(2 species)			
Acidomonas	*Ac. methanolica*		
Neoasaia	*N. chiangmaiensis*		
Swaminathania	*Sw. salitolerans*		
Kozakia	*K. baliensis*		
Granulibacter	*Gr. bethesdensis*		
Saccharibacter	*S. floricola*		
Tanticharoenia	*T. sakaeratensis*		
Ameyamaea	*Am. chiangmaiensis*		

[a] The 16S sequence is closer to *Komagataeibacter* but the type strain is not available in any culture collection and it was not reclassified by Yamada *et al.*, 2012

Later on, in 1879, Hansen described that the microbiota that converted the alcohol into acetic acid were composed of a mixture of several bacterial species and not only one unique strain. Afterward, Beijerinck proposed *Acetobacter* as the first genus of AAB in 1899.

In the beginning, AAB taxonomy was subjected to study and classification based on morphological, biochemical, and physiological criteria. One of the first classifications was made by Visser't Hooft in 1925. Ten years later, Asai (1935) structured the group of AAB by dividing it into two genera: *Acetobacter* and *Gluconobacter*. Some years later, in 1950, Frateur proposed a new classification based on five physiological criteria: catalase activity, production of gluconic acid from glucose, oxidation of acetic acid to carbon dioxide and water, oxidation of lactic acid to carbon dioxide and water, and oxidation of glycerol into dihydroxyacetone. Attending to these features, the genus *Acetobacter* was composed of four groups: *peroxydans*, *oxydans*, *mexosydans*, and *suboxydans* (reviewed by Barja et al., 2003).

Yamada et al. (1997) used a classification based on the differences in the ubiquinone system. Attending to the use of this protein complex, these authors reclassified the species belonging to *Acetobacter*, and some of them were reordered in the new genus *Gluconacetobacter*. Regarding their criteria, *Acetobacter* genus comprises species that use Q-9 as the main respiratory quinone, and the *Gluconacetobacter* species use Q-10.

In general, the classical microbiological tests used for AAB classification by phenotypic characterization are considered not reliable, laborious, and time-consuming (Cleenwerck and De Vos, 2008). Although morphological, biochemical, and physiological criteria have been commonly used to differentiate AAB genera, they present difficulties in the interpretation of the results obtained. A high variety of tests are commonly performed to phenotypically characterize AAB and to describe new taxa:

determination of colony and cell morphology, Gram staining, tests to determine oxidase and catalase activity, acid production from ethanol (Carr, 1968; Frateur, 1950), oxidation of acetate and lactate to carbon dioxide and water (Asai et al., 1964; Swings, 1992), growth in acetic acid presence (Yamada et al., 2000), growth in nitric acid presence (Loganathan and Nair, 2004), formation of 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, and 2,5-diketo-D-gluconic acid from D-glucose (Gosselé et al., 1980; Swings, 1992), ketogenesis from glycerol (Carr, 1968), growth on different carbon sources and formation of water-soluble brown pigment(s) (Swings, 1992), acid production from sugars and sugar alcohols (Asai et al., 1964), cellulose production, flagellation (Rhodes, 1958; Toda, 1928), and motility. However, although these tests provide features of the bacteria, they are not reliable enough, and the methods used are not correctly standardized (Cleenwerk and de Vos, 2008; Gullo et al., 2012). Moreover, some of the features studied seem to be variable in AAB such as physiological deficiencies caused by the inactivation of enzymes like the membrane-bound alcohol dehydrogenase (ADH) and cellulose synthase (Coucheron, 1991; Kondo and Horinouchi, 1997). This variability deriving from genetic instability, which can affect reliability of phenotypic assays, has been attributed to mobile genetic elements, mainly transposons, widely distributed in the genome (Coucheron, 1991).

For all these reasons, the AAB classification using only these phenotypic tests is not adequate (Cleenwerck and de Vos, 2008), and during the last decades, the study of AAB taxonomy has been achieved by polyphasic approaches combining phenotypic, chemotaxonomic, and genotypic data (Mamlouk and Gullo, 2013). The use of phenotypic tests has decreased because of the hurdles that they present. Meanwhile, molecular DNA-based methods have demonstrated to be faster and more reproducible tools for AAB classification and for detection of new species, which have never been

previously described. In fact, the *Bergey's Manual of Systematic Bacteriology* included molecular tests, such as fatty acid composition, electrophoresis of soluble proteins, guanine-cytosine (GC) content, and DNA-DNA hybridization among the taxonomic criteria in the family *Acetobacteraceae* (De Ley et al., 1984).

DNA-DNA hybridization is a powerful molecular technique suggested to discriminate between closely related species of bacteria (McCarthy and Bolton, 1963) due to the comparison of the whole genomes yielding a more accurate molecular homology value than just analyzing gene by gene. However, the most extended sequence of DNA used with taxonomic purposes in AAB is the 16S rRNA genes (Yamada et al., 2012). The drawback of this gene analysis is that it is a highly conserved gene among AAB, and sometimes its study is not resolute enough for the species classification. The analysis of different genetic regions as phylogenetic markers has been proposed to solve the species identification. Cleenwerck et al. (2010) reported the use of housekeeping genes such as *dnaK*, *groEL*, and *rpoB* in obtaining homologies between type strains of different species from the same genera between 95% and 99%, although the lack of sequence available in the databases makes the common use of this technique difficult. González and Mas (2011) proved the utility of the internal transcribed spacer (ITS) sequences of the 16S–23S rDNA genes to differentiate AAB species. Moreover, other protein-coding genes, such as AcOH, have been applied to investigate phylogenetic relationships among *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter* genera (Matsutani et al., 2011). Recently, the utilization of *tuf* gene, which codifies for the elongation factor Tu, has been reported for species discrimination in the *Acetobacter* genus (Huang et al., 2014). It has been demonstrated that the analysis of more than one gene can solve ambiguities that sometimes appear in species identification; however, the increasing availability of full genome sequences, especially of type strains, will provide the

application of new genomic approaches and advances in AAB taxonomy (Mamlouk and Gullo, 2013).

1.3. Molecular techniques

The development of molecular techniques has allowed the detection of new AAB species as well as the identification of AAB species and even the differentiation of strains or genotypes present in different niches, some of them complex matrices. These techniques have been improved during the last decades, and currently, a high number of them have been described for AAB analysis, being polymerase chain reaction (PCR)–based methods preferred because of their rapidity, specificity, reliability, and sensitivity. The molecular study of this bacteria group is carried out, more and more frequently, both by culture-dependent and culture-independent molecular techniques. The culture-dependent techniques require a previous step of cultivation of microorganisms onto agar plates and subsequent DNA isolation and analysis. On the other hand, culture-independent techniques are based on the study of the microorganisms directly from the natural matrix without any previous cultivation step.

The use of both types of technique on the same sample can be a better approach to the real composition of the AAB ecosystems. All of the techniques present advantages and disadvantages that should be taken into account, and they must be applied regarding to the characteristics of the study or the samples analyzed. Both culture-dependent and culture-independent techniques give valuable information that should be considered to get a better comprehension of the microbiota present in complex communities.

1.3.1. Culture-dependent techniques

There are molecular analyses, such as fingerprinting, applied to characterize the genetic variation in AAB, in which the step of culture cannot be bypassed at this moment. AAB recovery onto culture media is essential for the genotyping and also for preserving these

isolates for possible future uses. Furthermore, several molecular techniques have been described for AAB species identification of all these isolates recovered by plate.

Nowadays, several culture media based on different carbon sources such as glucose, mannitol, or ethanol have been tested to improve the recovery of AAB colonies and their later maintenance in pure culture (Entani et al., 1985; Gullo et al., 2006; Vegas et al., 2013). The most commonly used media are compiled in Table 2. The media AE and RAE are specially designed for AAB recovering from vinegar; they are characterized for the presence of acetic acid in their composition (Entani et al., 1985; Sokollek and Hammes, 1997; Sokollek et al., 1998). The GY and GYC media are commonly used for AAB recovery from grape wine or vinegar (Barata et al., 2012; Du Toit and Lambrechts, 2002; González et al., 2004; Prieto et al., 2007; Vegas et al., 2010). The medium GYC is supplemented with calcium carbonate for the detection of the production of acetic acid. The acid can dissolve the calcium carbonate, and the colonies that are able to produce acetic acid present a clear halo on the plate (Vegas et al., 2010). Vegas et al. (2013) performed a comparison between four different media commonly used for AAB recovery (GY, YPM [also called MM], V50, and V50 supplemented with acetic acid); AAB isolated from vinegar samples were able to grow just in the first two media, and most of the isolates were obtained from the GY medium.

After the plate recovery and isolation of AAB, different molecular techniques have been developed to identify the AAB isolates at genera and species level as well as strain characterization.

Table 2. Commonly used media for AAB isolation. Compiled from Carr and Passmore (1979), Entani *et al.* (1985), Gullo and Giudici (2008), Quintero *et al.* (2009), Sokollek and Hammes (1997) and Yamada and Yukphan (2008).

Media	**Quantity**	**Media**	**Quantity**
[a]**GYC agar**		[b]**GY Medium**	
D-Glucose	5.0% (w/v)	Glucose	2.0% (w/v)
Yeast extract	1.0% (w/v)	Yeast extract	1.0% (w/v)
Calcium carbonate	0.5% (w/v)	Agar	2.0% (w/v)
Agar	2.0% (w/v)		
GYC Medium		[d]**AE-medium**	
Glucose	10.0% (w/v)	Glucose	0.5% (w/v)
Yeast extract	1.0% (w/v)	Yeast extract	0.3% (w/v)
Calcium carbonate	2.0% (w/v)	Peptone	0.4% (w/v)
Agar	1.5% (w/v)	Agar	0.9% (w/v)
		Absolute ethanol	3 ml (v/v)
		Acetic acid	3 ml (v/v)
[c]**YPM Medium**		[e]**RAE-medium**	
Yeast extract	0.5% (w/v)	Glucose	0.4% (w/v)
Peptone	0.3% (w/v)	Yeast extract	0.1% (w/v)
Mannitol	2.5% (w/v)	Peptone	0.1% (w/v)
Agar	1.2% (w/v)	Absolute ethanol	0 - 4% (v/v)
		Citric acid	0.015% (w/v)
		Na_2HPO_4	0.038% (w/v)
		Agar	0.5-1% (w/v)
V50			
Yeast extract	0.4% (w/v)		
Glycerol	0.2% (w/v)		
Tartaric acid	0.2% (w/v)		
K_2HPO_4	0.05% (w/v)		
$MgSO_4.7H_2O$	0.05% (w/v)		
Na acetate	0.1% (w/v)		
$MnSO_4$	0.02% (w/v)		
$CaCl_2$	0.01% (w/v)		
Ethanol (v/v)	6% (v/v)		
pH 5			

a Glucose yeast extract Calcium carbonate medium

b Glucose yeast extract medium

c Yeast extract peptone mannitol medium

d Acetic acid ethanol medium

e Acetic acid ethanol enriched medium

1.3.1.1. Genotyping

The main difficulty in genotyping studies is finding a rapid and reliable technique for establishing categories that allow to determine the genomic diversity of microorganisms and to distinguish between different strains allowing their characterization. Currently, several techniques have been tested for AAB typing:

Random Amplified Polymorphic DNA-PCR (RAPD-PCR) – This technique is based on the amplification of sequences randomly using just one primer with an arbitrary sequence and the electrophoretical band pattern obtained reflects the genotypic diversity of this isolate. Trček et al. (1997) used this method to study AAB populations in vinegar. They tested six different primers and selected those that were able to generate at least three bands in the pattern. Nanda et al. (2001) used this technique in traditional rice vinegars detecting two different genotypes from a total of 178 AAB isolates analyzed. In later studies, Bartowsky et al. (2003) used this technique to study the diversity of AAB in wines from Australia obtaining six different profiles from 23 isolates, and Prieto et al. (2007) performed a genotyping of isolates from Chilean grape after an enrichment step that allowed the recognition of 57 profiles from just two different species.

Enterobacterial repetitive intergenic consensus–PCR (ERIC-PCR) and repetitive extragenic palindromic–PCR (rep-PCR) – The primers used in this method correspond to inverted palindromic sequences that are highly conserved in enterobacteria, but they are also present in other groups of bacteria. The distribution of these sequences in the genome of AAB makes possible the study of the band pattern created by their amplification discriminating at strain level. These techniques have been used in several studies to characterize AAB isolates from wines (González et al., 2004), fruit vinegars (Hidalgo et al., 2012, 2013a, 2013b), cereal vinegars (Nanda et al., 2001; Wu et al.,

2012), traditional balsamic vinegars (TBVs) (Gullo et al., 2009), wine vinegars (Hidalgo et al., 2010b; Vegas et al., 2010, 2013), submerged vinegars (Fernández-Pérez et al., 2010), and grapes (Mateo et al., 2014; Navarro et al., 2013).

$(GTG)_5$-rep-PCR – The single oligonucleotide with a repetitive sequence $(GTG)_5$ used is able to amplify noncodifying sequences distributed along the genome of mostly all the Gram-negative bacteria and even in some Gram-positive bacteria. Versalovic et al. (1994) proposed the use of this rep-PCR to obtain a genomic fingerprint in individual bacterial strains. This technique has demonstrated its usefulness in AAB species grouping (De Vuyst et al., 2008; Papalexandratou et al., 2009) and AAB typing from several ecological studies (Hidalgo et al., 2010b, 2012, 2013a, 2013b; Mateo et al., 2014; Vegas et al., 2010, 2013).

Amplified fragment length polymorphism (AFLP) – Initially, DNA is digested with a combination of enzymes, and then a specific adapter is linked in the extremes of the generated fragments for the hybridization of specific designed primers and subsequent fragment amplification. It has been considered a technique with good reproducibility and high discriminatory power at species level and various taxa. Actually, this technique has been considered highly reliable compared with RAPD-PCR (Savelkoul et al., 1999). Cleenwerck et al. (2009) carried out studies with culture collection strains of AAB, concluding that it is a better tool for AAB typing than for species identification, allowing the determination of the intraspecies diversity. Papalexandrotou et al. (2009) reported a comparison between $(GTG)_5$-rep-PCR and AFLP-PCR applied for AAB strains classification, and they obtained different clustering results with these techniques, but they concluded that both of them were accurate enough for the phylogenetic classification of AAB.

Multilocus sequence analysis (MLSA) – The taxonomic relation between different strains is evaluated attending to the analysis of housekeeping genes that present highly conserved sequences. All different sequences are collected in a database, and a group name is assigned for each one. These data are used for strains comparison according their group's assignation. Cleenwerck et al. (2010) analyzed ten housekeeping genes; four of them (*atpD*, *glnA*, *gyrA*, and *infB*) were discarded at first because they have several copies in the genome. Of the six genes remaining, three (*recA*, *thrC*, and *gltA*) showed more than one band amplified after specific PCR, or they yielded no product at all. So finally, they used three genes (*dnaK*, *groEL*, and *rpoB*) to reclassify a strain designated formerly as *Ga. xylinus* sbsp. *sucrofermentans* into a new species, nowadays corresponding to *Ko. sucrofermentans*. More recently, Castro et al. (2013) described the new species *Ko. medellensis* and compared the percentage of homology with other strains belonging to several species of *Komagataeibacter* and *Gluconacetobacter* and obtaining between a 92.0% and a 95.6% of similarity. The lack of sequences in the public databases makes its common use in AAB identification difficult at the moment.

Pulsed field gel electrophoresis (PFGE) – The restriction fragments obtained from the digestion of whole bacteria genome immobilized in agarose blocks are visualized after an electrophoresis carried out with electric fields that change their direction at different time intervals. This method was applied by Fernández-Pérez et al. (2010) for typing AAB isolates from several vinegar samples, and they reported that it is a good and highly reliable typing technique, but it is more time-consuming than others like ERIC-PCR.

MALDI-TOF mass spectrometry – Using this technique, Andrés-Barrao et al. (2013) reported the discrimination of different AAB strains attending to their profile of proteins. Although the profile is strain dependent, there is a chromatographic pattern

conserved throughout the species. The dendrogram constructed with the results of this fingerprinting technique allowed the classification of the samples at the species level. However, the results obtained with this method are subjected to the behavior of the strains depending on the media and conditions used, which could change the protein expression. Moreover, it was not tested in environmental samples, but just in pure culture.

Multilocus enzyme electrophoresis (MLEE) – The bacteria are compared attending to their alloenzymes, which are the enzymes codified by alternative allels from the same locus, and present different electrophoretical mobility. The amino acid sequence of a protein determines its electrophoretic migration rate, and this method analyzed the different electrophoretical mobility of enzymes. Caballero-Mellado and Martínez-Romero (1994) used this technique for studying the genetic diversity of *Ga. diazotrophicus* species present in sugarcane cultivars as endophytic bacterium. Some years later, Fuentes-Ramírez et al. (2001) used this technique for the description of two novel nitrogen-fixing species (*Ga. johannae* and *Ga. azotocaptans*) using the enzymatic profiles to compare different strains that conserved similarities when they belonged to the same species.

1.3.1.2. Genera detection and species identification

The molecular techniques most commonly described for AAB identification are based on the analysis of the loci that codify the rRNA of the ribosomal subunits 16S–23S–5S. These subunits are codified in three genes consecutively disposed (Fig. 1) and separated by spacer sequences, which are posttranscriptionally spliced, named ITS (Internally Transcribed Spacer). Among them, the most commonly used target sequence to design primers and probes in AAB is the 16S rRNA gene, whose sequences are available for practically all known species of AAB; however, other rRNA regions could also be used

and usually applied to complement the AAB identification (Fig. 1). Different molecular analyses have been developed for AAB identification around these gene regions.

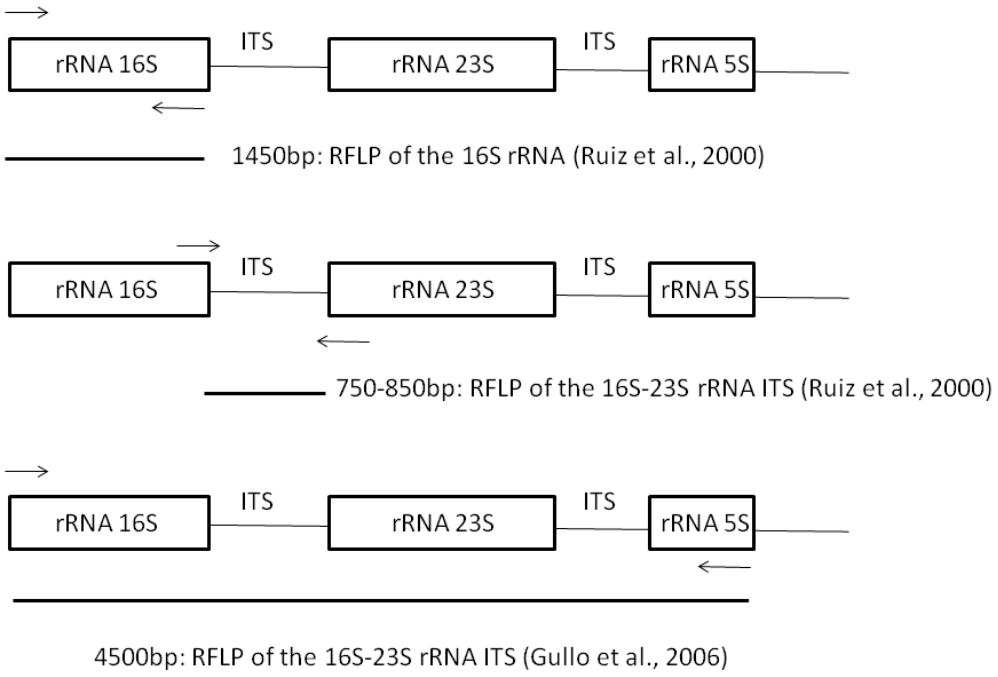

Fig. 1. Structure of the rRNA genes cluster highlighting the regions amplified for common AAB species identification. Modified from Mas et al. (2007).

Restriction fragment length polymorphism (RFLP)–PCR and sequencing of the 16S rRNA gene – The amplification of the 16S rRNA gene and subsequent digestion using restriction enzymes generate several fragments with different lengths depending on the original sequence of the gene that can differentiate the AAB species group. Ruiz et al. (2000) described this method using two endonucleases for rapid identification of AAB species from wine samples. However, the AAB rearrangements and new species description, made that González et al. (2005a) reported the use of at least five enzymes in order to differentiate the species of AAB commonly described in wine or vinegar. Later, other studies described more new enzymes to distinguish between AAB species (Torija et al., 2010; Vegas et al., 2013). Although the 16S rRNA RFLP-PCR analysis

allowed grouping the related species of AAB, the sequencing of this gene is necessary to obtain an accurate identification. Sequence analysis of the 16S rRNA gene has been reported to be a good method of identification for this group of bacteria (Yamada *et al.*, 2012) due to the high degree of conservation of this region in their genome. However, the high sequence identity between different AAB species, that can be up to 99.7%, makes difficult to get a correct identification (Cleenwerck and De Vos, 2008), especially between closely related species. This is emphasized with the description and the reclassification of strains into new species.

RFLP-PCR sequencing of 16S–23S rRNA gene ITS region – The ITS is a sequence with higher variability compared with functional rRNA sequences because it does not codify any ribosomal subunit and can accumulate mutations easier than rRNA genes. Several studies have been performed in amplifying and digesting this DNA fragment with several restriction enzymes (González et al., 2005a; Trček and Teuber, 2002); nevertheless, the results were always not sufficient to identify all the AAB species currently described even in just wine or vinegar. Ruiz et al. (2000) analyzed this region with two enzymes but described this method adequate for type strains identification but not for natural samples. The sequencing of this region and its phylogenetic and molecular evolutionary analysis has been reported as useful to complement the information obtained from 16S rRNA gene and clarify AAB species identification (González and Mas, 2011) even in several ecological studies (Hidalgo et al., 2013a, 2013b; Mateo et al., 2014; Navarro et al., 2013).

RFLP-PCR of the 16S–23S–5S rRNA loci – This technique is similar to the last ones and was described by Gullo et al. (2006). The authors reported that the AAB species differentiation could be performed amplifying the fragment of 4,500 bp and digesting with just one restriction enzyme.

1.3.2. Culture-independent techniques

The main hurdle for AAB analysis is the difficulty in recovering and growth on culture media, especially for samples derived from extreme environments that include conditions involving high ethanol and/or acetic acid concentrations (Millet and Lonvaud-Funel, 2000; Sievers et al., 1992; Sokollek et al., 1998; Trček, 2005). It has been reported that just a low percentage of the total population counted by microscopy are able to grow on common laboratory media (Mesa et al., 2003; Torija et al., 2010; Trček, 2005). There are techniques developed specially for viability detection of bacteria such as LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes) used for microscopy cell visualization and counting. This kit is composed of two fluorescent dyes that stain the cells according to their membrane integrity: SYTO9 fluorochrome is a small molecule that goes through both damaged and intact cellular membranes, and the cells are observed by green color under microscopy. On the other hand, the propidium iodide (PI) fluorochrome is a bigger molecule that can only penetrate cells with damaged membranes, and if SYTO9 is present, PI causes the reduction of its fluorescence, staining the cells with a red color under microscopy. Using this technique, differences have also been reported, up to four orders of magnitude, between viable bacteria counts by microscopy and cultivability recounts by plating (Mesa et al., 2003; Baena-Ruano et al., 2006), evidencing the difficulties of culture recovery of this group of bacteria.

Culture-independent techniques are able to detect, identify, and, in some cases, even enumerate AAB populations directly from complex matrices, including those that have not been recovered by plating. However, the direct analysis of DNA from the original sample cannot ensure the viability of these cells. Furthermore, most of these molecular techniques are based on small sequence amplifications in conserved regions, hampering

some species identification (Andorrà et al., 2008). The difficulties of these techniques in detecting minor species present in complex communities or the preferential annealing of the primers have also been reported (Giraffa and Neviani, 2001). To solve these drawbacks, the performance of an enrichment step using a previous specific PCR has been proposed for AAB identification by several of these techniques (González et al., 2005b; Torija et al., 2010).

One of the main hurdles in culture-independent approaches for a good coverage of the microbial diversity is the efficient recovery of representative and high-quality DNA from microorganisms present in a matrix (Jara et al., 2008; Streit and Smitz, 2004). A suitable DNA extraction protocol is essential for the correct analysis of microbial diversity. Some factors such as incomplete cell lysis, degradation or damage of DNA, or the presence of PCR inhibitors (such as polyphenols, tannins, and polysaccharides) affect the isolation of DNA quantity and quality, resulting in a critical step that directly interferes in the efficiency of molecular techniques (De Vero et al., 2006; Ilabaca et al., 2008; Jara et al., 2008; Mamlouk et al., 2011).

In recent years, a wide variety of culture-independent techniques have been developed, providing a broader view of the AAB populations present in different niches but specially focused on both wine and vinegar:

Denaturing gradient gel electrophoresis–PCR (DGGE-PCR) – The analysis of the 16S rRNA gene is commonly used to determine the AAB biodiversity present in environmental niches using this technique. The differences in the GC composition of the amplicons can be used to detect different AAB species because of the different electrophoretic mobility in denaturing conditions. A polyacrylamide gel with a linear denaturing gradient of urea and formamide is used, and the DNA remains double stranded until it reaches the gel zone at which the denaturing conditions are the same as

the melting temperature (Tm) of the DNA. At this point, the double-stranded DNA is partially denatured, and its motility is reduced, running through the gel based on its decreased electrophoretic mobility (Muyzer and Smalla, 1998). The 5′ end of the forward primer used is special: it has a poly-GC clamp tail of approximately 40 bp, which acts as a high-melting domain and avoids the complete dissociation of the two DNA strands into single strands. A band pattern is obtained that presents as many bands as different species are in the sample. These bands are excised from the polyacrylamide matrix, eluted and reamplified for their sequencing.

This technique is useful for monitoring AAB species succession along the time in processes of wine fermentation (Andorrà et al., 2008; López et al., 2003) or vinegar production (De Vero et al., 2006; Gullo et al., 2009; Haruta et al., 2006). However, the target sequences analyzed are very short, and sometimes the discrimination between closely related AAB species is difficult because of the high homology of the fragment. Moreover, a selective amplification has been detected with this technique, reporting that this method is not the most appropriate for minor species detection (Andorrà et al., 2008).

Temporal temperature gradient electrophoresis–PCR (TTGE-PCR) – It is a variant of the DGGE-PCR that used temperature gradient for denaturalization instead of the use of chemicals compounds, such as urea and formamide. Ilabaca et al. (2008) reported its applicability for AAB diversity analysis in a study about the population dynamics during traditional vinegar production performed in Chile.

Real-time PCR (RT-PCR) – This technique is considered a fast, sensitive, and accurate tool for detecting and enumerating microorganisms. It is based on monitoring the PCR progress, detecting in each cycle the increase in fluorescence produced by a reporter molecule. The fluorescent reporter molecules can be a dye that binds to the double-

stranded DNA, such as SYBR-Green, or a sequence-specific probe, such as TaqMan or TaqMan-MGB probes. The latter ones are very specific probes due to the presence of a nonfluorescence quencher and a minor groove binder (MGB) at the 3′ end. The group MGB increases Tm of the primer, allowing even the detection of single-base mismatches (Kutyavin, 2000; Torija et al., 2010).

Some authors have quantified the total number of AAB cells in wine and vinegar samples using SYBR-Green as fluorescent reporter (Andorrà et al., 2008; González et al., 2005a, Torija et al., 2010). In other studies, several TaqMan-MGB probes were designed to detect and quantify different species of AAB (Torija et al., 2010), and they have been applied for species detection in vinegar samples (Jara et al., 2013; Vegas et al., 2013).

Fluorescence in situ hybridization (FISH) – This technique uses specific oligonucleotide probes that hybridize with the DNA of the bacteria directly inside the cell. The probes are usually designed for 16S rRNA gene and have a fluorochrome that labels cells to be detected and quantified by microscopy or by cytometry. This technique is considered a rapid method, and it has been applied in wine for the identification of lactic acid bacteria (Blasco, 2009). It has been successfully used for the specific detection of *Ga. sacchari* associated with the insect pink sugarcane mealybug (Franke et al., 2000). However, the permeabilization of AAB cells results in complicated probe hybridization aside from the interferences involved from the complex matrix where they are usually embedded (Mas et al., 2007). On the other hand, the intensity of the fluorescence is related with the ribosomal content, and the cells need to be metabolically active for detection by FISH (Franke et al., 2000).

Cloning – This technique is based on the amplification of the 16S rRNA gene followed by the insertion of amplicons in a cloning vector. The resulting construction is

introduced in *E. coli*–competent cells, and the transformed bacteria are properly recovered on differential media plates. After this step, the target sequence is amplified and then is analyzed by RFLP-PCR or sequencing. Ilabaca et al. (2008) applied this technique on vinegar samples obtaining short fragments. Most of them were identified as *A. pasteurianus* by sequence analysis; however, they were not able to classify the 10% of the clones analyzed in a concrete species because their sequences showed the same percentage of homology with *Ko. europaeus*, *Ko. xylinus*, and *Ko. intermedius* attending to the 16S rRNA gene fragment studied.

PCR of the pyrroloquinoline quinone–dependent ADH (PQQ-dependent ADH) – This technique is based on the presence of conserved and nonconserved regions in the *adhA* gene, which makes possible the design of primers specific for each AAB species. Trček (2005) reported the design of primers able to amplify the species *A. aceti*, and it was applied in cider vinegar, detecting the presence of this species in this kind of vinegar.

Next-generation sequencing – This recently applied technique is also named massive sequencing because it allows to process millions of sequences in parallel. There are different platforms used for this kind of massive sequencing, depending on the technology developed. Currently, there are three main technologies commonly used for metagenomic analysis of microbial communities. The first one is the platform Roche (454) GS FLX sequencer, based on "pyrosequencing" using an emulsion PCR on the surfaces of agarose beads with oligomers attached to them. The conditions are set in a way that allows the separation in micelles of each bead to perform the PCR of the same DNA fragment attached to the oligomers and for their subsequent sequencing in a 454 picotiter plate. The sequencing is performed analyzing the light production for the incorporation of each nucleotide, after a downstream set of reactions, catalyzed by luciferase.

Another well-known platform is Illumina Genome Analyzer, which is based on "sequencing by synthesis" to produce sequence reads from amplified DNA fragments by bridge PCR simultaneously in a flow cell, using alternatively the oligomers attached to the surface of the cell. The sequencing reaction is supplied with four different label nucleotides with their 3′-OH chemically inactivated to ensure just a single base incorporation in each cycle; the incorporation of every single nucleotide is detected by imaging in each cycle of PCR.

More recently, the platform Ion Torrent (Life Technologies) was designed, which is based on the measuring of the proton liberation during the DNA polymerization reaction for sequencing. The sample DNA is fragmented, and all the fragments obtained are labeled and attached, in this case, on beads that are placed in a microchip that converts the signal produced by the DNA polymerization into voltage. In every different cycle, the beads are exposed to one nucleotide, the binding of each nucleotide is detected by the changes in voltage in every cell of the microchip.

The next-generation sequencing methodologies are normally based on the previous construction of libraries, which are not subjected to previous cloning in *E. coli*, avoiding the bias issues generated by this approach (Mardis, 2008). These methods allow the detection of a huge number of microorganisms present in mixed communities. The detection is based on the sequencing of conserved genes present in the whole microbiota, the most commonly used region is the 16S rRNA gene. The detection can be performed after a previous amplification of the target genes, as an enrichment step, or directly without any previous enrichment step eliminating the drawbacks of PCR-based techniques (Jung et al., 2011).

The main hurdle of these methods could be the short length of the fragment that can be sequenced. This fact complicates the identification that can be done just at the level of

genera because of the sequences obtained (Bohorquez et al., 2012; Nie et al., 2013). Each platform, and even model of sequencer, allows different fragment lengths of sequencing. On the other hand, next-generation sequencing provides a lot of information about the real composition of the community and allows making inferences about the relative abundance of microorganisms because the number of rough reads correlates directly with the frequency of each microbe (Mardis, 2008). After sequencing, it is necessary to perform a clustering step with these sequences. It could be carried out in two ways: either assigning an identity with reference strains homologous to those resulting from next-generation sequencing (this option is called phylotyping) or assigning operational taxonomic units (OTUs) attending to the similarity with other sequences in the community. The OTUs are groups of sequences obtained from the reads by next-generation sequencing that share a percentage of homology between them of 95% or more, and their clustering is not defined by taxonomic criteria (Schloss and Westcott, 2011).

Next-generation sequencing has been applied in several fermented products to analyze biodiversity such as fermented seafood, kimchi, wine (David et al., 2014; Jung et al., 2011; Roh et al., 2010; Takahashi et al., 2014). In environments where AAB are commonly present, this metagenomic approach has been used in few studies for the microbiota description. For instance, Illeghems et al. (2012) performed a phylogenetic analysis during spontaneous fermentation of cocoa bean, and using the GS FLX system, they detected only the species *A. pasteurianus* and *G. oxydans.* However, using the technique of DGGE-PCR, besides these two species, they also detected *Ko. saccharivorans* and two other species of *Acetobacter*. Moreover, Nie et al. (2013) carried out a study of the microbial succession during the production of traditional Chinese vinegar to study the bacteria present during the process with the Illumina

system. They detected the presence of *Acetobacter* genus that was confirmed by DGGE-PCR, which allowed the identification of *A. pasteurianus*.

1.4. Metabolism of AAB

AAB are characterized by their wide metabolic potential, most of them share some features (Table 3) even though they present some peculiarities at the species or even at the strain level.

AAB are obligate aerobes; their growth is highly dependent upon the availability of molecular oxygen. The oxidation reactions in the metabolism of AAB are accompanied by transfer of electrons from pyrroloquinoline quinone (PQQ) to cytochromes of the respiratory chain. It leads to the formation of a proton motive force and synthesis of ATP via oxidative phosphorylation where the oxygen is the final acceptor. The different genera of AAB are characterized by their major ubiquinone in the respiratory chain, in the case of *Acetobacter* genus, it is coenzyme Q9, but in the other genera of AAB, it is coenzyme Q10 (Table 3). The oxidative reactions occur mainly within the periplasmic space and the cytoplasmic membrane.

AAB are capable of oxidizing a huge range of sugars such as glucose, arabinose, fructose, galactose, mannose, ribose, sorbose, and xylose (De Ley et al., 1984) by the pentose phosphate pathway. Alternatively, cellulose synthesizing strains of AAB deviate their metabolism by the Entner-Doudoroff route more than by pentose phosphate pathway.

Table 3-Resume of the main characteristics of the AAB genera. Adapted from Mamlouk and Gullo, 2013 and Yamada *et al.*, 2012. G., *Gluconobacter*; A., *Acetobacter*; Ga., *Gluconacetobacter*; Ko., *Komagataeibacter*; Ac., *Acidomonas*; As., *Asaia*; K., *Kozakia*; Sw., *Swaminathania*; S., *Saccharibacter*; N., *Neoasaia*; Gr.,*Granulibacter*; Am.; *Ameyamaea*; T., *Tanticharoenia*; Ne., *Neokomagataea*; + positive; - negative; w weak; v variable; nd not determined; nm non-motile; po polar; pe peritrichous

Characteristic	G.	A.	Ga.	Ko.	Ac.	As.	K.	Sw.	S.	N.	Gr.	Am.	T.	Ne.
Production of acetic acid	+	+	+	+	+	-	+	+	v(w/-)	+	v(w/-)	+	-	-
Oxidation of acetate	-	+	+	v	+	w	w	w	-	-	w	+	-	-
Oxidation of lactate	-	+	+	v	v(w/-)	w	w	w	-	-	+	w	-	-
Production of dihydroxyacetone from glycerol	+	v	v	+/nd	-	V	+	+	-	w	-	w	+	-
Production of γ-pyrone compound	v	-	+	-	nd	v(+/w)	v	nd	nd	nd	nd	nd	nd	nd
Production of 2-keto-D-gluconate	+	v	+	v	-	+	+	nd	+	+	nd	+	+	nd
Production of 5-keto-D-gluconate	+	v	v	v	-	+	+	nd	+	+	nd	+	+	nd
Production of 2,5-diketo-D-gluconate	v	-	+	-	-	-	-	nd	nd	nd	nd	-	+	nd
Production of a water-soluble brown pigment	v	-	+	-	-	-	-	+	-	-	nd	-	+	-
Production of cellulose	-	-	v	v	-	-	-	nd	-	nd	nd	nd	nd	nd
Flagellation	nm/po	nm/pe	pe	nm	nm/pe	nm/pe	nm	pe	nm	nm	nm	pe	nm	nm
Major ubiquinone	Q10	Q9	Q10	Q10	Q10	Q10	Q10	Q10	Q10	Q10	nd	Q10	Q10	Q10
DNA G+C content (mol%)	54-64	52-64	58-65	56-63	62-63	59-61	56-57	57-60	52-53	63	59	66	66	57

Sugars are completely oxidized via tricarboxylic acid pathway, which is not functional in *Gluconobacter* genus members because of their lack of alpha-ketoglutarate dehydrogenase and succinate dehydrogenase enzymes. On the other hand, *Gluconobacter* species can directly oxidize sugars into glucono-δ-lactone, which is converted into gluconic acid. This reaction is particularly active in *Gluconacetobacter* when it is growing at high sugar concentration. Gluconic acid and its derived lactone, salts, and keto forms are widely applied in pharmaceutical, food, or chemical industries (Kersters et al., 2006).

Moreover, AAB are also able to oxidize several alditols (polyalcohols derived from sugars), such as glycerol, and transform them into other biological compounds (Sievers and Swings, 2005). On the other hand, one of the main characteristics of AAB is their ability to oxidize ethanol to acetic acid. Although the genera *Asaia*, *Neokomagataea*, and *Tanticharoenia* have been described as not producers of acetic acid attending to the phenotypic tests performed under the parameters standardized for their taxonomic classification, they can present this ability in other conditions, even in a low rate (Yamada et al., 2000). Otherwise, *Saccharibacter* and *Granulibacter* present variable phenotype for acetic acid production (Table 3). The ethanol oxidation yields energy via electron transport and oxidative phosphorylation by the respiratory chain. This pathway consists of two sequential steps: in the first step, ethanol is oxidized to acetaldehyde, and the acetaldehyde is oxidized to acetic acid. There are two main enzymes involved in this process (Fig. 2): alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH).

Moreover, other alcohols and acetaldehydes commonly present in the raw materials can serve as substrates for both of these enzymes during vinegar production. The products

obtained from their activity results in acid, ketones, and other products that contribute to the aroma and flavor characteristic of vinegar (Hutkins, 2006).

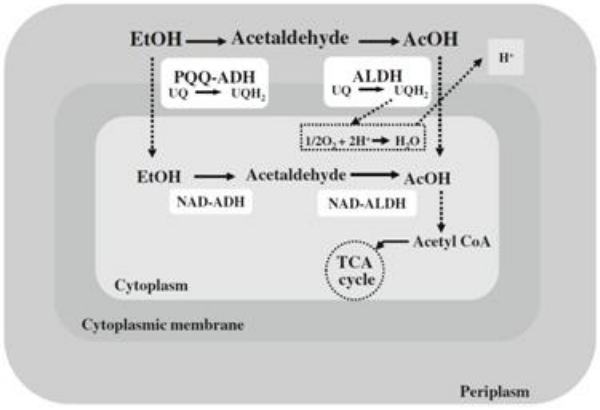

Fig. 2. Ethanol oxidation by PQQ-ADH and ALDH at the outer surface of cytoplasmic membrane and by NAD-ADH and NAD-ALDH in the cytoplasm (from Mamlouk and Gullo, 2013).

Another metabolic aspect to take into account during vinegar production is that strains of *Acetobacter* and *Gluconacetobacter* have been characterized by their ability to oxidize acetic acid via tricarboxylic acid pathway. For that reason, during vinegar elaboration, it is necessary to keep ethanol in enough concentration to inhibit the tricarboxylic acid pathway and avoid the overoxidation of acetic acid into carbon dioxide and water. This overoxidation can be also performed by AAB over other organic acids that could be present in a medium such as lactic acid or malic acid (Matsushita et al., 2004).

1.5. Elaboration of vinegar

Since ancient times, vinegar has been a product appreciated for human consumption because of its use as condiment and preservative and also for its healthy attributes (Solieri and Giudici, 2009). Many different varieties of vinegars are produced all over

the world. It can be made from numerous raw materials, either vegetal (rice, grapes, malt, apple, potatoes) or animal (whey, honey) origin (Bamforth, 2005; Solieri and Giudici, 2009).

The *Codex Alimentarius* (1987) defines *vinegar* as "a liquid, fit for human consumption, produced from a suitable raw material of agricultural origin, containing starch, sugars, or starch and sugars, by the process of double fermentation, first alcoholic and then acetous."

This first biotransformation is alcoholic fermentation; sugars (that can be glucose, fructose, or sucrose) are converted into ethanol and carbon dioxide. Although other microorganisms can be involved in this process, yeasts are mainly responsible to carry out this transformation in food and beverages (Ribéreau-Gayon et al., 2006).

The second transformation is the acetification, the ethanol oxidation highly dependent on the availability of oxygen. This process is carried out by AAB, which are present on the raw material and can survive after alcoholic fermentation despite of the adverse conditions (Du Toit and Pretorius, 2002).

The technological methods used for this elaboration can play an important role in the properties of the final product. Currently, there are mainly two methods in vinegar production: submerged and traditional.

The submerged method is the most common for large-scale vinegar production. It is based on strong aeration of the tanks where acetification is performed. The AAB can grow there under controlled conditions of aeration, temperature, and foam formation (Tesfaye et al., 2002). Although this method allows the fast production of acetic acid reaching in just one or two days the desired acidity for the vinegar, the process favors the loss of volatile compounds, reducing the quality of the final product (Solieri and Giudici, 2009).

The traditional method is one of the most used methods to obtain high-quality vinegar with singular organoleptic properties (Mas et al., 2014). One of the main characteristics of the traditional method for vinegar production is its expensiveness due to the long time it takes to carry on the acetification until the desired acidity is reached. This method is also known as superficial, surface, or Orleans method. It is performed in a static way in barrels or vessels with an open hole that allows air contact with the surface of the liquid. In order to keep on the acetification process and to maintain the AAB metabolically active, it is necessary to draw off some vinegar when the expected acidity is reached. Thereby, new wine is added to the barrel to feed the acetification with more ethanol. This method commonly uses the back-slopping process. It is based on the use of an undefined starter culture obtained from previous vinegar. The advantage of back-slopping is that the microbiota present in the inoculum is adapted to the acetification process and the environmental conditions (Leroy and De Vuyst, 2004). During the acetification step, the selective pressure caused by the environmental extreme conditions in the medium can make that some microorganisms become well adapted and can dominate the process. For that reason, it is considered as the traditional precursor of industrial starters (Solieri and Giudici, 2009).

1.6. Ecological studies of AAB

In recent years, several ecological studies have been performed as screening of the AAB microbiota present in different environments. The recovery of AAB in laboratory culture media is a step that cannot be bypassed in ecological studies at this moment. Among the laboratory media developed for AAB growing, GYC medium is specially indicated for the detection of acetic acid production, which is an important characteristic of AAB and serves as a fast screening for AAB differentiation (Gullo et al., 2006; Hidalgo et al., 2010b). Another AAB selection is the supplementation of the media with

antifungal such as natamycin (also known as pimaricin), commonly used to avoid fungi, especially yeast (Hidalgo et al., 2010b; Vegas et al., 2010). Once the microorganisms are isolated, the next step is the identification of the isolates and the use of fingerprinting techniques to know the genotypic diversity of AAB present in the sample. Different species of AAB have been isolated from grapes or grape must, mainly belonging to the genera *Gluconobacter* and *Acetobacter*. The amount of bacteria recovered from fruits is highly dependent on their soundness (Barbe et al., 2001). It has been demonstrated that healthy grapes allow poor recovery of bacteria and it is usually necessary to use enrichment steps previous to plating isolation (Prieto et al., 2007). Barbe et al. (2001) compared the amount of AAB recovered from sound and botrytized grapes from France, obtaining that in sound grapes, the population of AAB was up to $1.2 \cdot 10^5$ CFU/ml; meanwhile, in the case of botrytized grapes, the concentration of AAB was one order of magnitude higher (up to $5 \cdot 10^6$ CFU/ml), demonstrating that the population of AAB present in infected grapes is increased compared with the sound ones. Moreover, attending to the species identification, which was based on phenotypic features, these authors detected that the majority of the isolates belonged to *Gluconobacter* or *A. pasteurianus* and *A. aceti*. These results were according to those obtained by Joyeux et al. (1984) from French botrytized grapes.

The genera *Acetobacter* and *Gluconobacter* were also isolated and identified from healthy grapes by 16S rRNA gene and 16S–23S rRNA gene ITS region sequences analysis. For instance, Prieto et al. (2007) recovered mainly the species of *G. oxydans* and *A. cerevisiae* from the healthy grapes of Chile, and Navarro et al. (2013) identified five different species of *Gluconobacter* genus as well as three of *Acetobacter* genus from Spanish grape musts (Navarro et al., 2013). These latter authors also reported the presence of strains of *K. baliensis*, and Mateo et al. (2014) detected on healthy and

spoiled grapes from Australian vineyards species belonging to *Asaia* and *Ameyamaea* genera besides *Gluconobacter* and *Acetobacter* members.

In the fermentation process from grape must to wine, González et al. (2004) performed a study by RFLP-PCR of the 16S rRNA in order to identify the population dynamics of AAB. They reported that the species of *G. oxydans* was mainly present in grape must and was significantly reduced in the first day of fermentation until disappearing. One group constituted by *Ga. liquefaciens / Ko. xylinus / Ko. europaeus* was also detected in minor proportions in the beginning of the fermentation as well as *A. aceti*, which gradually increases its population throughout the fermentation. On the other hand, the species *Ko. hansenii* was also recovered by these authors in the beginning to the middle fermentation but not at the end. Similar results were reported by Du Toit and Lambrechts (2002) during white and red wine fermentation, performing the identification using biochemical and physiological tests. The finished wine and the vinegar obtained throughout the acetification process were studied by Vegas et al. (2010). They detected, using RFLP-PCR of the 16S rRNA and sequencing of this gene, the presence of *A. pasteurianus* as the main AAB and also *G. oxydans* and *Ko. europaeus* in wine. Otherwise, in vinegar, *A. pasteurianus* was the predominant species throughout the acetification, and detecting, in some cases, *Ko. europaeus* in minor proportions. Furthermore, also in other fruits and cereal acetification, the predominant species belong to the genera *Acetobacter*, *Gluconobacter*, or *Komagataeibacter* (Hidalgo et al., 2012, 2013a, 2013b; Nanda et al., 2001; Wu et al., 2012).

One of the most interesting reasons for performing ecological studies is the screening of natural environments where AAB are present in order to obtain strains that can be selected for use in starter cultures. The different behaviors and features of the AAB strains even belonging to the same species (Wu et al., 2012) have been reported.

Starter cultures are commonly used in industry in order to maintain the homogeneity and enhance the quality by increasing the control of the processes and allowing the standardization of the final product (Leroy and De Vuyst, 2004; Gullo and Giudici, 2008). Strains with appropriate physiological and metabolic characteristics are selected from the products successfully fermented and are used as starter cultures in the food or beverages industry to make the product more homogeneous and the process faster (Oberman and Libudzisz, 1997). However, few studies are performed in traditional vinegar that use selected strains of AAB in order to obtain a highly controlled production of vinegar (Hidalgo et al., 2010a, 2013a); this is especially important in products like TBV, which is regulated by denomination of origin (Gullo et al., 2009). In acetification, the most common practice to initiate the acetification is the back-slopping process, based on the use of microbiologically undefined cultures (Sokollek and Hammes, 1997). The inoculation studies developed for vinegar production using the previous selected strains from ecological studies (Gullo et al., 2009; Hidalgo et al., 2010b, 2013b) could increase the control of the process and finally result in a higher-quality product (Hidalgo et al., 2013b; Kersters et al., 2006). The results of these studies suggested the use of more than one AAB strain for the inoculation process: one strain belonging to *Acetobacter* to ensure the fast beginning of the acetification and one belonging to *Komagataeibacter*, which is able to grow at higher acetic acid concentration and prevents the process from being stuck (Gullo et al., 2009; Hidalgo et al., 2013b). This idea derives from three studies where a strain of *Acetobacter* was used: a strain of *A. pasteurianus* for the production of wine vinegar (Hidalgo et al., 2010b) and another for TBV production (Gullo et al., 2009), and a strain of *A. malorum* for strawberry vinegar (Hidalgo et al., 2013b). However, in the three studies, species of *Komagataeibacter* were detected at the end of the processes: *Ko. intermedius* in

traditional vinegar and *Ko. europaeus* in submerged method (Hidalgo et al., 2010b); *Ko. europaeus* was also detected in TBV (Gullo et al., 2009) and *Ko. saccharivorans* and *Ko. xylinus* in strawberry vinegar (Hidalgo et al., 2013b). It has been reported that *Komagataeibacter* genus is likely imposed because of its ability to grow in environments with high acidity (Schüller et al., 2000). Species such as *Ga. liquefaciens*, *Ko. europaeus*, *Ko. xylinus*, *Ko. oboediens*, and *Ko. intermedius* have been found in vinegars with high acetic acid concentrations (Du Toit and Lambrechts, 2002; Joyeux et al., 1984; Schüller et al., 2000; Sokollek et al., 1998). Instead, the population of *Acetobacter* remains during the process of vinegar production with low acidity (Nanda et al., 2001).

The selection of strains for starter cultures is based on culture-dependent techniques; however, different culture-independent techniques are applied to get a more accurate vision of the whole microbiota present during the production processes. Several authors carried out their studies comparing the results obtained between culture-dependent and culture-independent techniques. De Vero et al. (2006) identified two species by culture-dependent techniques based on 16S rRNA gene sequence that corresponded to *A. malorum* and *Ko. europaeus.* However, with DGGE, just *A. aceti* and *A. pasteurianus* were detected. The study of Haruta et al. (2006) using DGGE for species detection in rice vinegar production agreed with the results of Nanda et al. (2001), who identified the presence of *A. pasteurianus*. In the study of Vegas et al. (2013), *Ko. europaeus*, *Ko. xylinus*, and *A. pasteurianus* were recovered on plates with different growth media and further 16S rRNA gene sequencing and were also identified by several culture-independent techniques based on the polymorphism of the 16S rRNA gene sequence such as RT-PCR, DGGE-PCR, and RFLP-PCR. These results were similar regardless

the technique used, and furthermore, the integration of all of them resulted in a better understanding of the microbial diversity.

Therefore, culture-dependent and culture-independent techniques should be used in ecological studies because of the complementary information obtained especially when samples come from a hostile environment such as wine or vinegar (De Vero et al., 2006). The development of new molecular techniques has favored the reclassification and also the description of new species and genera, making possible the detection of higher AAB diversity in these environments (Barata et al., 2012; Mateo et al., 2014; Navarro et al., 2013).

1.7. Biofilms

The biofilms are formed by microbial communities that appear associated with a surface or embedded in an extracellular polymer matrix. The microbes appear associated commonly in large number, and this association presents different characteristics in gene expression, protein production or growth compared with their nonattached phenotype (Cloete and Van Der Merwe, 2009). The composition of biofilms is mostly water (80%–90%) and organic matter, generally extracellular polymers (85%–95%) such as polysaccharides, proteins, and extracellular DNA (Pamp et al., 2007). Cellulose is the main EPS, which can be present in a biofilm formed by AAB. In addition, other material can be part of the structure of the biofilm matrix, for example, fimbria, pili, and flagella (Flemming, 1998; Pamp et al., 2007). The biofilm formation is advantageous for bacteria because this kind of growth provides high protection against adverse environment conditions (Allison et al., 2000).

The existence of biofilms related with processes of food fermentation is very common, and there are some studies based on the description of these microbial communities. For instance, there are several studies on the coexistence of yeast and bacteria in biofilm

during the fermentation of fermented foods and beverages such as kombucha, rice vinegar, fermented olives, or kefir (Dobson et al., 2011; Domínguez-Manzano et al., 2012; Teoh et al., 2004; Wang et al., 2012).

The structure of these biofilms has been observed using microscopy, detecting that mixed communities of yeast and bacteria are present, and their distribution is usually described as irregular, being that the cells are mixed through the matrix (Escalante et al., 2008; Wang et al., 2012). In the case of kombucha, the yeasts may be arranged in islets or bands and embedded in EPS matrix, which is secreted primarily by AAB (Anken and Kappel, 1992). In addition, several authors point out the establishment of a symbiotic relationship between the microorganisms present in mixed-species communities (Dobson et al., 2011; Teoh, 2004), which can be supported by this structural disposition.

In the case of vinegar production by the traditional method, the biofilm is formed on the surface of the liquid. The biofilm is organized on a cellulosic pellicle in the air-liquid interface. This solid matrix composed mainly of cellulose is formed by the AAB to be in direct contact with oxygen (Llaguno and Polo, 1991) and probably also in order to survive under stress conditions such as high ethanol or acetic acid concentration (Kanchanarach et al., 2010). The existence of this cellulosic matrix facilitates the oxygen and nutrient supply, since the adsorption properties of the polymers in the biofilm favors the increase of the nutrient concentration in contrast to the aqueous environment (Mamlouk and Gullo, 2013). Biofilm is also used as bed for quick vinegar production (Adams and Twiddy, 1987) in the back-slopping. As far as we know, only in two studies carried out from vinegar obtained by traditional method was the AAB diversity analyzed (Gullo et al., 2009; Nanda et al., 2001). Both studies performed the AAB species identification analysis from the isolates obtained after recovering them from culture media (culture-dependent methods).

1.8. Cellulose production and genes involved

The AAB are able to produce several EPS that are part of the extracellular matrix. This matrix covers the surface of the liquid media. Among the EPS produced by AAB, acetan is a water-soluble heteropolymer that is composed of glucose, mannose, glucuronic acid, and rhamnose in a molar ratio of 4:1:1:1. On the other hand, cellulose is a water-insoluble homopolymer produced in the presence of oxygen and glucose. This polymer has been studied by its structural characteristics, and it is used in the industry because its purity, especially for medical and engineering purposes (Mamlouk and Gullo, 2013).

The production of the homopolysaccharide cellulose has been widely described in the species *Ko. xylinus*, which is considered a model microorganism for the production of bacterial cellulose (BC). The BC presents structural and mechanical characteristics that make it different from plant cellulose (Delmer, 1999). BC is formed by glucose monosaccharide in (1→4) β-glycosidic bonds; several linear chains (up to 250 in some organisms) are arranged in parallel joined by intra- and interchain hydrogen bonds (Ross et al., 1991). This tertiary structure makes the molecule water insoluble and inert to the treatment with either acidic or alkaline solutions (Römling, 2002). In addition, BC can be produced in pure and crystalline form, which makes its recovery relatively easy (Vandamme et al., 1998).

The structure of the BC produced by AAB has been also studied by several authors because of its importance for industrial and medical applications. Purity and crystalline structure are interesting features that are usually analyzed by nuclear magnetic resonance (NMR), X-ray diffraction, and scanning electron microscopy (SEM) (Keshk and Sameshima, 2005; Nguyen et al., 2008; Ramana et al., 2000; Trovatti et al., 2011). Microscope techniques revealed that *Ko. xylinus* secretes a slimy substance within

which, after a short time, cellulose fibers are formed, resulting finally in bundles and bigger ribbons (Chawla et al., 2009).

In cells that actively produce cellulose, the presence of multienzymatic complexes can be observed by an electronic microscope. In *Ko. xylinus*, the protein complex for cellulose synthesis is transmembrane; it is localized over the cytoplasmic and outer membrane because cellulose synthase and the cyclic diguanylic acid (cyclic-di-GMP) binding protein are embedded in the cytoplasmic membrane (Kimura et al., 2001). The protein complexes (approximately 50 per cell) are organized in a single row along the longitudinal axis of the bacterial rod secreting from 12 to 25 glucan chains by each complex. These fibrils will be assembled into larger microfibrils at the site of synthesis (Kimura et al., 2001).

Using SEM, Mikkelsen et al. (2009) described the changes in the structure of the cellulose matrix produced by a strain of *Ko. xylinus* after 48 hours of growth. This strain presented light differences in the microarchitecture created by the cellulose microfibrils depending on the carbon source (glucose, mannitol, glycerol, fructose, sucrose, and galactose). In general, the pellicles were described as densely packed networks of fibers with different spatial orientation and thickness depending on the carbon source used.

The main concern in cellulose production study is focused on how to enhance the yield of this EPS. The improvement has been focused on the modification of growth conditions or in the strains used (Keshk and Sameshima, 2005; Trovatti et al., 2011). A proportional relation between cell growth and cellulose production has been reported, being influenced by the nutrients used in the medium. Abundant carbon source and minimal nitrogen source can enhance the secretion of EPS (Ramana et al., 2000). Different experiments have been performed with variations in the concentration and type of carbon source such as glucose, fructose, maltose, xylose, starch, and glycerol.

More recently, the use of subproducts from other industries such as dry olive mill residue or agroindustrial wastes have been tested in *Ga. sacchari* (Gomes et al., 2013) and *Ko. swingsii* (Castro et al., 2011), respectively. Moreover, the supplementation of the media with ethanol has been analyzed in order to increase the cellulose production and also with a positive effect on the reduction of the spontaneous mutation into cellulose nonproducing phenotype (Son et al., 2001).

The use of glucose as carbon source is very common for cellulose production in AAB, obtaining good results with this carbon source (Mikkelsen et al., 2009; Trovatti et al., 2011). However, some authors have reported that the use of this monosaccharide can mediate the formation of gluconic acid as byproduct in the medium, decreasing the pH of the culture and reducing the cellulose production rate (Keshk and Sameshima, 2006).

Other factors have been studied as possible causes of cellulose biosynthesis modification such as nitrogen source (Ramana et al., 2000), pH (Verschuren et al., 2000), presence of ethanol in the culture medium (Park et al., 2003a), or the effect of long periods of storage at −70°C (Chawla et al., 2009; Zogaj et al., 2003). In all these studies, the results obtained are strongly strain dependent, so the determination of optimal conditions for cellulose production is specific for each strain (Chawla et al., 2009).

Another point to take into account is that the production of other EPS can modify the cellulose production in some species of AAB such as *Ko. xylinus* and *Ko. intermedius*. It has been reported that acetan production could influence the degree of polymerization and the crystalline properties of BC secreted (Ross et al., 1991).

The genes required for the biosynthesis of BC are encoded by the *bcsABZC* operon (also called *acsABZC* or *celABZC*) (Fig. 3). The species *Ko. xylinus* has served for the description of this operon, and most of the information about the cellulose biosynthesis

pathway comes from the study of this species. In AAB and in other bacterial species studied, the presence of at least two genes in this operon has been reported: *bcsA* and *bcsB* (for BC synthesis, also named as *acs* or *cel* genes). The first one, the *bcsA* gene, is the most conserved gene of the operon and codifies for cellulose synthase, which is the catalytic subunit for cellulose biosynthesis. While the other one, the *bcsB* gene, codifies for bis-(3′, 5′) cyclic-di-GMP binding protein, which is less conserved among the bacterial species. In some cases, both genes are fused in one called *bcsAB* (Saxena et al., 1994). The presence of more than one cellulose biosynthesis operon in *Ko. xylinus* has been also reported. Moreover, in the strain AY201 of this species, Saxena and Brown (1995) described the presence of two genes for cellulose synthase although cellulose biosynthesis is mediated only by one of them *in vivo* under laboratory conditions.

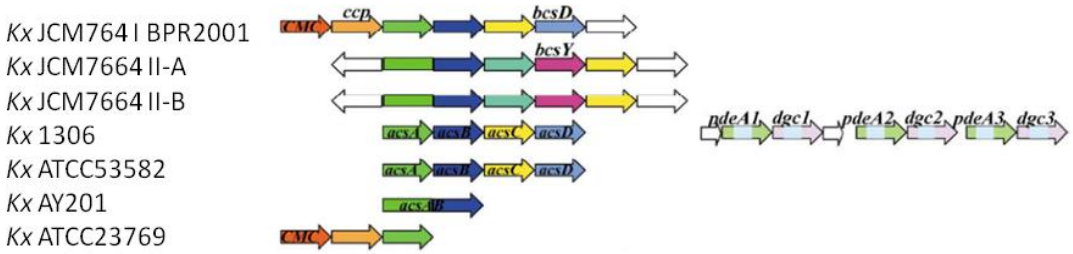

Fig. 3. Confirmed and predicted operon structure for cellulose biosynthesis in different strains of *Ko. xylinus* (*Kx*). The open reading frames (not drawn to scale) corresponding to homologous genes have the same color. The alternative designations for homologous genes are marked as assigned in databases. Gray and white arrows indicate genes not proven to be involved in cellulose biosynthesis. Light blue boxes represent GGDEF domains, putatively involved in cellulose biosynthesis regulation (modified from Römling, 2002).

Other genes could also be involved in the cellulose biosynthesis such as the gene *bcsZ* (synonymous to *celC* in *Ko. xylinus*), which encodes for the enzyme cellulase. Homologous to this gene are present in all the cellulose-producer species and can be inside the operon *bcs* as in the case of enterobacteria or can be outside yet adjacent such

as in *Ko. xylinus* (Standal et al., 1994). Another gene required *in vivo* but not *in vitro* for cellulose production is *bcsC* gene, which has been reported to be present in enterobacterial species, *Pseudomonas* and *Ko. xylinus*. In addition, there are some genes involved in cellulose biosynthesis that demonstrated their presence in concrete species and not in other groups of bacteria such as *bcsD* gene in *Ko. xylinus* (Saxena et al., 1994; Wong et al., 1990) and *celDE* gene in *A. tumefacines* and *Rhizobium leguminosarum* var. *trifolii* (Ausmees et al., 1999; Matthyse et al., 1995).

Regarding the information available from the data of complete genomes sequenced, it is expected that some bacterial strains not described before as cellulose producers are capable of biosynthesizing this polymer under certain conditions because of the presence of the genes for its synthesis (Römling, 2002). Many sequences are available in databases about the genes involved in cellulose production for the species *Ko. xylinus*; however, for other AAB species, it is reduced. Most of the information accessible from these other AAB species comes from whole genome sequencing, but the real functionality of the genes noted as homologous to those for cellulose production has not been proven yet.

The regulation of the cellulose biosynthesis is complex because of the different metabolic routes involved in this process. It has been identified in *Ko. xylinus* that cyclic-di-GMP acts as activator of cellulose biosynthesis (Ross et al., 1987), being responsible for the allosteric activation of BcsA protein (cellulose synthase). The 90% of this cyclic-di-GMP remains bonded to BcsB protein in the cell, and the regulation of the binding state of cyclic-di-GMP to BcsB depends on the action of proteins that share conserved domains with the amino acid sequence GGDEF. This domain is present in different families of bacteria, but the real role of this homology is currently unclear (Römling, 2002).

The cellulose production test (Carr and Passmore, 1979; Navarro et al., 1999) is used as phenotypical trait for the description of novel species of AAB (Table 3). However, the cellulose production is strain dependent and can be variable depending on the growth conditions (Keshk and Sameshima, 2005). Nowadays, the species described as cellulose producers belong mostly to the genera *Gluconacetobacter* and *Komagataeibacter* (Aydın and Aksoy, 2014; Park et al., 2003a; Trovatti et al., 2011), but it has been found that also strains of *Acetobacter* (Gullo et al., 2012; Jung et al., 2010) and *Gluconobacter* (Jia et al., 2004) genera can show this ability.

2. Cell-to-cell communication in bacteria

It is widely known that bacteria are able to detect and act in response to environmental conditions such as temperature changes, nutrient availability, pressure, oxygen concentration, and pH level. Although for a long time, bacterial behavior was considered as individual, in the last three decades, a notable amount of bacterial mechanisms coordinated by all the bacterial population coexisting in the same niche has been described (Waters and Bassler, 2005).

In natural environments, there are different chemical signals synthesized by bacteria that allow monitoring the population density and determining the cooperative behavior of these bacteria. These cell-to-cell signaling processes are named quorum sensing (QS) systems and are controlled by small hormone-like molecules termed autoinducers (Fuqua et al., 1994). The autoinducers are secreted to the extracellular environment by diffusion or active transport to be detected by the surrounding bacteria (Redfield, 2002). When the autoinducer accumulated in the extracellular environment arrives to a threshold concentration, the consequence is the change of the gene expression and a positive feedback that increases the production of autoinducers (Waters and Bassler,

2005). The mechanisms coordinated by QS systems involve an orchestrated multicellular behavior. For a unique cell, the development of the metabolic pathways is costly; however, a cell community supports the investment of resources, obtaining a benefit for the group (Von Bodman et al., 2008).

Although the term QS was first used by Fuqua et al. (1994) for describing the density-dependent system in Gram-negative bacteria by specific autoinducer molecules, it has been also applied for Gram-positive density-dependent response systems. The cell-to-cell communication can be an intra- or interspecific phenomenon. The similarity of the molecules involved in QS communication and the homology of the signalization mechanisms allow the cross talk between species coexisting in the same environment. This fact allows the development of either cooperative QS strategies coordinated by different species or even the blocking of the signalization in a competitive behavior between species (Burmølle et al., 2014). The mechanism of QS inactivation is known as quorum quenching (QQ). The use of QQ strategies has an important interest in microbiology and has even been suggested as a potential strategy for disease control (Dong et al., 2000). Traditionally, the tools for bacterial removal have been based on the use of antibiotics or other substances that can cause the death of the microorganisms. The QQ mechanisms allow interfering in intercellular communication and prevent the metabolic responses triggered under high microbial population (Dong and Zhang, 2005).

The cooperative behavior is found, for example, during biofilm formation, where this behavior benefits the community by improving the colonization and survival (Burmølle et al., 2014). For instance, a dual species biofilm produced by the Gram-negative bacteria *Pseudomonas aeruginosa* and *Burkholderia cepacia* is detected in patients who present cystic fibrosis and are affected by chronic infections of these bacteria

consortium. The production of this biofilm is regulated by QS signalization mediated by specific autoinducers related with Gram-negative bacteria (Riedel et al., 2001). Another association has been reported between *Actinomyces naeslundii* and *Streptococcus oralis*, both Gram-positive bacteria that present a mutualistic relation in oral biofilms mediated by other kinds of autoinducers found in both Gram-negative and Gram-positive bacteria (Rickard et al., 2006). On the other hand, in a recent study, the potential QQ interaction of some *Staphylococcus* species with Gram-negative bacteria has been reported. Some species of these Gram-positive bacteria excrete two molecules (yayurea A and B) that inhibit both the expression of QS-controlled toxins and other compounds in Gram-negative bacteria. These quorum quenchers have in *Staphylococcus* a function in self-protection and competitiveness in natural environments shared with Gram-negatives (Chu et al., 2013).

2.1. Quorum sensing systems

There are a wide variety of processes that have been reported as controlled by QS in bacteria. Since the first system was described, corresponding to *Vibrio fischeri* and its control of bioluminescence (Nealson and Hastings, 1979), the antibiotic production, virulence factors liberation, plasmid transfer by conjugation, motility, cellular division, endospore formation, biofilm formation, EPS synthesis, and others have been reported as QS-dependent mechanisms (Miller and Bassler, 2001; Whitehead et al., 2001).

The genes activated by QS systems can be involved in:

– processes where cell population must be high to achieve a common purpose such as the initiation of virulence response

– processes that require high competition for nutrients, such as the antibiotic production to obstruct the development of other microorganisms

– processes that favor the colonization and the development of complex communities as is the case of biofilm formation.

2.1.1. Autoinducer molecules

The autoinducer molecules are mainly divided into three classes (Fig. 4):

– Acyl homoserine lactone (AHL) molecules, produced by Gram-negative bacteria. These were the first autoinducers described concretely in *V. fischeri* (Eberhard et al., 1981), and currently, they are the best characterized because their structure is highly conserved among all the Gram-negative bacteria (Galloway et al., 2011). They are composed of a homoserine lactone (HSL) ring joined to a fatty acid as lateral chain.

This lateral chain can have from 4 to 18 carbons that can be saturated or present insaturations or even be substituted in the third carbon with an oxo- or hydroxy- group, resulting in a molecule with a high conformational diversity (Whitehead et al., 2001). The synthesis of AHL involves S-adenosyl methionine (SAM) as homoserine lactone ring donor, and for the acyl chain, the precursor molecule is acyl-ACP.

– Autoinducer peptides (AIP) are the molecules related with QS regulation in Gram-positive bacteria. They are small peptides that can be modified after translation. Their size ranges from 5 and 34 amino acids, and their molecular structure is quite complex, containing chemical modifications like the presence of big substituents such as isoprenyl groups (*Bacillus subtilis*) or thiolactone rings (*Staphylococcus* spp.) (Henke and Bassler, 2004). They are actively exported to the medium because of their proteic nature, and there, they will be recognized by membrane detectors in other cells (Fig. 4). The recognition of the AIP is mediated by a two-component signal transduction system, formed by a receptor and a regulator protein. The proteins called *histidine kinases* are the receptors that detect the extracellular AIP. In response to the AIP, these receptors are autophosphorylated, and the phosphate group is transferred from the receptor to the

regulator protein. Then the activation of this protein initiates the signalization, which is meditated by a phosphorylation cascade that finally promotes transcriptional changes.

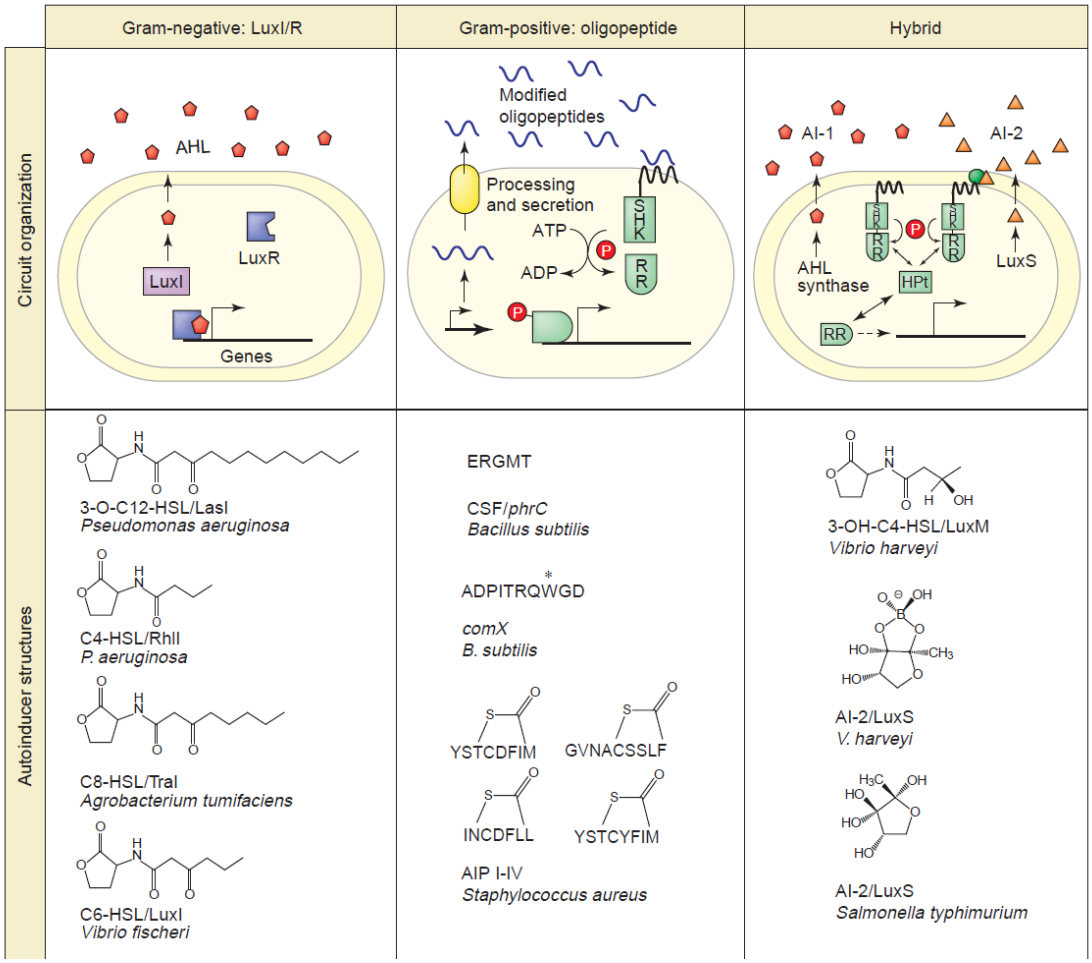

Fig. 4. The three general classes of QS systems attending to the autoinducer molecules used. Red pentagons represent AHLs, wavy blue lines denote AIP, and orange triangles represent AI-2. Representative structures of different autoinducer molecules are detailed attending to the microorganisms that produce them (from Henke and Bassler, 2004).

– The third molecule group is named Autoinducer-2 (AI-2). Its structure is a furanosyl borate diester, and it is present in many bacterial groups both Gram-negative and Gram-positive. The molecule AI-2, often in conjunction with an AHL or AIP, depending on their Gram condition, can control a variety of traits in these bacteria (Bassler and Losick, 2006). The system used for the AI-2 signalization is a mixture of elements from

Gram-negative and Gram-positive QS systems being different among the bacterial species. The common precursor for AI-2 molecules is 4,5-dihydroxy-2,3-pentanedione (DPD), which changes spontaneously, producing several interconverting molecules (Xavier and Bassler, 2005). The recognition system of AI-2 allows bacteria to respond not only to their own produced AI-2 but also to AI-2 produced by other bacterial species. The existence of this mechanism in both Gram-negative and Gram-positive bacteria supports the idea of the existence of interspecies communication via QS (Henke and Bassler, 2004). The existence of other communication systems intermediate that indicate that Gram-positive and Gram-negative bacteria are able to communicate between them has been reported.

Recently, it has been reported that QS molecules could have different functions besides that as autoinducers, such as antibiotic action, ion chelating, or intracellular signalization (Schertzer et al., 2009). For example, the most complex net of QS signalization has been described in *Pseudomonas aeruginosa*. This bacterium has the AHL autoinducer typical from Gram-negative bacteria but it also has other molecules such as cyclic dipeptides (CDPs), diketopiperazines, and the quinolone PQS that present activity in cell communication (Swift et al., 2001; Williams and Cámara, 2009). The molecules of PQS have been described as iron-binding molecules (Bredenbruch et al., 2006) conferring an advantage for this species reducing the growth of other competitor microorganisms that could be in the same environment. These PQS molecules are much more hydrophobic than molecules such as the AI-1 (specific AHL molecule), AI-2, and APS, which are relatively water soluble and can diffuse freely between cells (Mashburn and Whiteley, 2005). The molecules of PQS are carried in outer membrane vesicles, and the formation of these vesicles depends on the presence of PQS, which suggests that PQS molecules can be involved in altering membrane properties besides its QS activity

(Schertzer et al., 2009). Moreover, as quinolones have been related with antibiotic activity, the quinolone PQS could present antibiotic action and also act as QS signal (Fajardo and Martínez, 2008).

2.1.2. LuxI/LuxR system

The LuxI/LuxR system from *V. fischeri* is considered the model of QS system mediated by AHL signalization (Henke and Bassler, 2004). Nealson and Hastings (1979) reported the first study about the process controlled by QS and responsible for bioluminescence production in *V. fischeri*. However, these studies had been started ten years before when the light production was observed with high cell density and not with dilute cell suspensions (Nealson et al., 1970).

The system of *V. fischeri* is composed of two proteins: LuxI and LuxR. The protein LuxI of 193 amino acids is responsible for the synthesis of the AHL signal molecule, an N-oxohexanoyl-L-homoserine lactone (3-oxo-C6-HSL). The protein LuxR of 250 amino acids is the receptor of AHL, and the complex LuxR-AHL activates the genes controlled by QS systems.

The genes involved in the bioluminescence of *V. fischeri* are organized in two operons, *lux*R and *luxICDABEG*, which are shown in Fig. 5. Between these operons is located a regulator region called *lux box* with a binding site for the complex LuxR-AHL (Stevens and Greenberg, 1997). Besides the *luxI* gene, which codifies for the protein LuxI, the other genes of the operon are encoding proteins involved directly in the luminescence production. Thus, the genes *luxA* and *luxB* codify the subunits α and β of the luciferase, the protein involved in the light production. These two genes are disposed close to the *luxI* gene. The genes *luxC*, *luxD*, and *luxE* codify the components of the reductase complex, which is responsible for the synthesis and the recycling of the substrates for the activity of the luciferase enzyme.

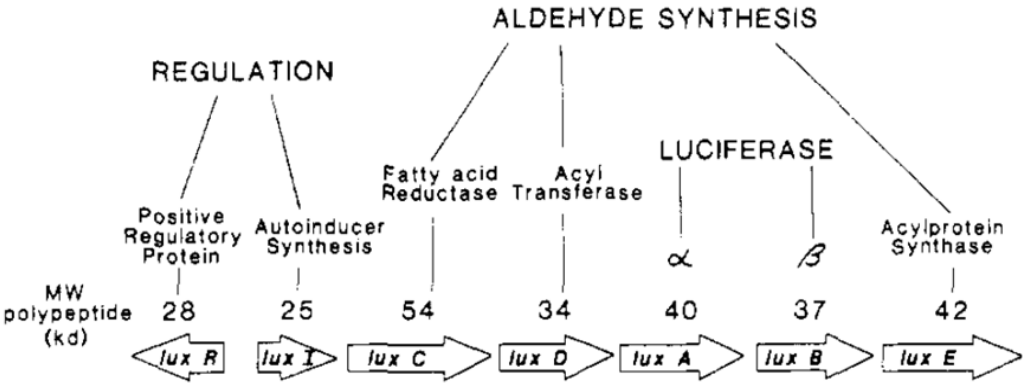

Fig. 5. Organization of the operons *lux* of *V. fischeri* (modified from Devine et al., 1987).

In situations of low cellular population, the transcription level of the *luxICDABEG* operon is reduced. The AHL synthesis is reduced, and consequently, the genes *luxA* and *luxB* are also lowly expressed being that the amount of light produced is inappreciable. When the *V. fischeri* population increases in number considerably, the AHL concentration is accumulated in the extracellular medium until arriving to a threshold concentration. The concentration of AHL is detected by the LuxR receptor, and the complex LuxR-AHL is formed. The interaction between AHL and LuxR produces a conformational change in this receptor protein (LuxR), and this complex (LuxR-AHL) binds to the promoter responsible for the transcription of the operon *luxICDABEG*. The result is not only the production of bioluminescence but also an increase of the signal molecule AHL, creating a positive feedback loop (Miller and Bassler, 2001).

This system can recognize different AHL molecules with different structures. It has been reported that some AHL molecules generate more response than others in each LuxI/LuxR homologous system, so some kind of differentiation should be involved in the AHL recognition by LuxR. Moreover, there are some AHL that could have antagonistic effects on the QS mechanism because of their analogous structure; these molecules bind to the receptor and cause a competitive inhibition of the signalization. In

most cases, the mechanisms of this inhibition remain unknown, but these molecules are probably not able to maintain the stability of the receptor and produce its degradation (Uroz et al., 2009). Therefore, the LuxI/LuxR system has the ability to discriminate between AHL molecules preventing the cross-linked interaction of one group of bacteria with another and also to recognize AHL molecules of other bacteria, which promotes a cooperative behavior between different species (Zhu et al., 1998; Henke and Bassler, 2004).

After the finding of LuxI/LuxR system, several homologous systems have been reported in different Gram-negative bacteria.

2.1.3.Quorum sensing in AAB, GinI/GinR system

Despite that the first QS system was described 30 years ago, the signalization mechanisms of AAB have not been studied until the years 2008 and 2009 when Iida et al. reported the existence of a homologous LuxI/LuxR system in the species of *Ko. intemedius*. The QS system of AAB named GinI/GinR has a degree of homology of 40% with the *V. fischeri* QS system.

Three different AHL molecules with different acyl chains have been described in *Ko.intermedius*: N-decanoyl-L-homoserine lactone (N-C_{10}-HSL), N-dodecanoyl-L-homoserine lactone (N-C_{12}-HSL), and C_{12}-HSL with a single unsaturated carbon bond (Iida et al., 2008a). Via these AHLs, the GinI/GinR QS system regulates the transcription of the gene *ginA* that codifies for a small protein of 89 amino acids named GinA protein. The production of GinA, induced by QS, can modulate the oxidative fermentation (regulating the production of acetic acid and also gluconic acid), the growth of bacteria in medium containing ethanol, and the foam production (Iida et al., 2008b, 2009).

In order to control all these functions, GinA protein induces the production of GmpA protein (Fig. 6), which is known as an outer membrane protein related with the signalization processes. The GmpA protein belongs to the OmpA protein family, which has been related with structural properties of membrane being responsible for its integrity in Gram-negative bacteria (Koebnik et al., 2000).

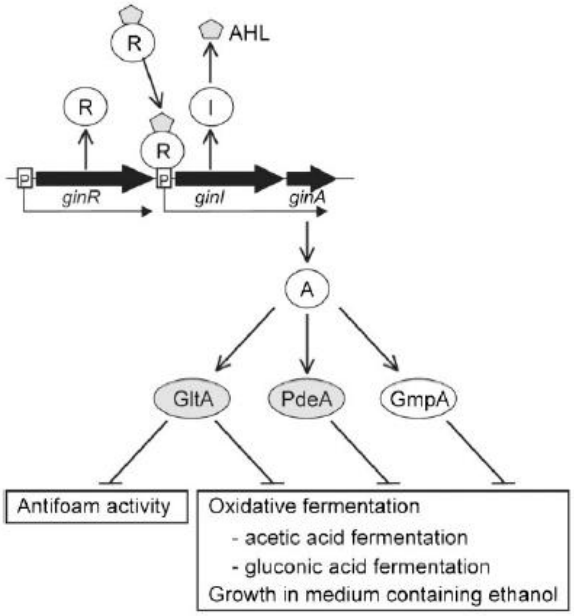

Fig. 6. Proposed regulative mechanism for QS signalization in *Ko. intermedius* (from Iida et al., 2009).

In the genome of AAB, at least four homologous amino acid sequences for OmpA protein has been found. The GinA protein also induces other genes: *gltA* gene, which encodes for a putative glycosyltransferase; *pdeA* gene, for a putative cyclic-di-GMP phosphodiesterase; *pdeB* gene, for a putative phosphodiesterase/diguanylate cyclase; and *nagA*, a putative N-acetylglucosamine-6-phosphate deacetylase (Iida et al., 2009). The activity of *pdeB* gene is not clear and requires further studies to understand its function. However, the genes *gltA* and *pdeA*, together with the GmpA protein, repress the growth in medium with ethanol as well as the production of acetic acid and gluconic acid. The GltA protein from *gltA* gene is also able to repress the antifoam activity, and probably it is involved in the biosynthesis of polysaccharides (Iida et al., 2009).

Iida et al. (2008a), in an attempt to study the QS control for the production of cellulose in AAB, reported that the mutant strains of *Ko. intermedius* for *ginR* and *ginL* genes did not present difference in cellulose production compared with the wild-type strain of *Ko. intermedius* used in the study. However, when *gltA* gene was disrupted, a reduction of the production of polysaccharides was observed modifying the outer membrane of the cell. This change results in a higher diffusion of glucose or ethanol, which has also effect in the oxidative fermentation (Iida et al., 2009).

2.2. Interception of quorum sensing signalization: quorum quenching

Frequently, in nature, there are communities of mixed populations of microorganisms surviving under stress conditions. The lack of nutrients and the difficulties for the obtention of energy in changing environments forces the competition between microorganisms in the same niche (Fuqua et al., 2001). Since bacteria communication by QS systems allows the cooperative behavior in microbial communities, one strategy for blocking the QS signalization of other competitors would be useful to take advantage from them in the same niches, which are competitive environments (Bauer and Robinson, 2002). For that reason, it is not surprising that some of these groups of microorganisms produce molecules that can quench or interfere with the communication of other microorganisms present in the same niche or just to modulate their own growth and gene expression in a changing environment (Zhang and Dong, 2004).

2.2.1. Mechanisms of quorum quenching

The first studies performed about QS inhibition were based on the halogenated furanones, structural analogs of AHL molecules (Manefield et al., 2001). Nowadays, there are several QQ mechanisms described to deactivate the QS signalization (Fig. 7), but those involved in AHL signalization inhibition are the most widely detailed.

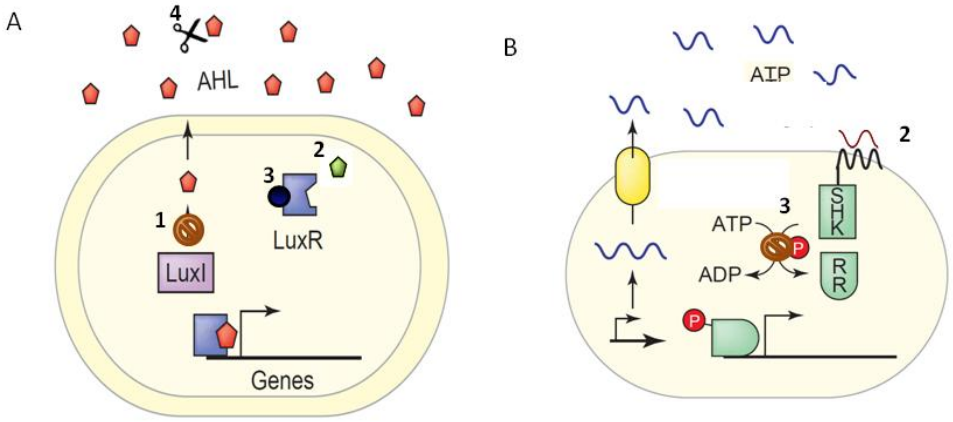

Fig.7. Different QQ mechanisms described at the moment: (1) obstruction of autoinducer synthesis; (2) competitive inhibition of the autoinducer reception; (3) noncompetitive inhibition of the autoinducer reception; (4) enzymatic degradation of the autoinducer in (A) Gram-negative bacteria and (B) Gram-positive bacteria (modified from Henke and Bassler, 2004).

Curiously, all the bacteria that have been reported that present QQ enzymes can be divided into these three phyla: *Firmicutes* (*Bacillus*, *Geobacillus*, and *Solibacillus* genera), *Actinobacteria* (*Arthrobacter*, *Microbacterium*, *Rhodococcus*, *Mycobacterium*, and *Streptomyces* genera), and *Proteobacteria* (*Agrobacterium*, *Variovorax*, *Ralstonia*, *Shewanella*, *Pseudomonas*, *Comamonas*, *Burkholderia*, and *Acinetobacter* genera). Besides, all the bacteria that present QQ activities also demonstrated QS signalization routes for their own communication (Chen et al., 2013).

Nowadays, QQ mechanisms could be divided in three groups: (i) obstruction of autoinducer synthesis, (ii) inhibition of autoinducer reception, and (iii) enzymatic degradation of autoinducers. The characteristics of these different mechanisms are as follows:

(i) Obstruction of autoinducer synthesis. There are several enzymes that participate in the synthesis of AHL molecules which pathway involves the activation of fatty acids and SAM. The activities of these enzymes can be blocked by different molecules

stopping the AHL biosynthesis such as molecule analogs of SAM that have the ability to repress the AHL synthase activity in *P. aeruginosa* (Parsek et al., 1999). Other molecules in *P. aeruginosa*, such as triclosan, can inhibit enoyl-ACP reductase, which is an intermediate in the pathway for AHL synthesis (Hoang and Schweizer, 1999). Some antibiotic molecules belonging to the macrolide group could be also responsible to inhibit the AHL synthesis in this bacteria (Pechère, 2001; Tateda et al., 2001).

(ii) Inhibition of autoinducer reception. There are mainly two mechanisms for inhibiting the autoinducer reception: competitive and noncompetitive inhibition.

Competitive inhibition: This first mechanism consists of molecules with a structure that is analogous to AHL. These molecules bind with the AHL receptor, being unable to activate the cascade of signalization.

The first and most studied molecules of this inhibition are halogenated furanones, which were first obtained from *Delisea pulchra*. This marine alga produces up to 30 different types of natural furanones substituted with bromide, chloride, or iodide in different positions (De Nys et al., 1993). These molecules have an effect on bacteria genera that present the LuxI/LuxR system; they compete with AHL for the LuxR autoinducer binding site because of their ability to interact directly with the LuxR receptor. After binding, the proteolytic degradation of the LuxR receptor is accelerated, avoiding their role as transcriptional factor (Manefield and Turner, 2002).

On the other hand, there are different studies that reported the action of molecules derived from natural furanones but chemically synthesized in the laboratory. They have shown an inhibitory effect on QS systems of different microorganisms such as *P. aeruginosa* (Hentzer et al., 2003; Wu et al., 2004), *Chromobacterium violaceum* Cv026 (Martinelli et al., 2004), *Salmonella enterica* (Janssens et al. 2008), and *Streptococcus* spp. (Lönn-Stensrud et al., 2007). Other structural changes of AHL molecules have

been synthetically performed, changing the side of chains or rings from the common AHLs, in order to create structural analogs. Substitution within or at the end of the acyl chain was effective for QS inhibition (Castang et al., 2004; Persson et al., 2005). Moreover, chemical substitution under laboratory conditions of the lactone ring of AHLs by other ring structures like phenyl or benzyl substituents were also able to inhibit the QS systems (Reverchon et al., 2002; Smith et al., 2003). Because of the complexity of the AHL signalization in different organisms, it is not easy to select the adequate antagonistic molecule able to block the LuxI/LuxR system in all the species. In some cases, this AHL analog has other activities in the communication mechanisms, causing other effects that are not always desirable in the cells (Rice et al., 1999).

Noncompetitive inhibition: this second mechanism is based on molecules that are not similar to autoinducers, but they bind to different sites of the receptor, altering the structure of the binding site and avoiding the interaction with the autoinducer.

Malabaricone C is a natural molecule extracted from the nutmeg *Myristica cinnamomea*, which does not present a similar structure to AHL; however, it possesses QQ activity on Gram-negative bacteria inhibiting the receptors for QS signalization in *P. aeruginosa* PA01 (the LasR and RhlR proteins) and also in *C. violaceum* (the CviR protein) (Koh et al., 2013).

Phenolic compounds can act on the histidine kinase proteins from a two-component transduction system of Gram-positive bacteria. These molecules induce the structural alteration of the proteins, causing the protein aggregation and, therefore, the inhibition of their activity (Stephenson et al., 2000). The inhibition of the histidine kinases interrupts the signalization mediated by the autoinducer AIPs in this group of bacteria.

(iii) Enzymatic degradation of autoinducers. The AHL molecules are susceptible of being inactivated spontaneously by changes in the pH of the medium; when AHL

molecules are subjected to alkaline pH, their structure is modified, and they are not active (Yates et al., 2002). However, there are also common enzymatic target sites for AHL cleaving attending to the general structure of AHL.

There are two main possible targets for AHL cleaving enzymes, which affect the HSL ring or the side acyl chain (Fig. 8). The degradation of the HSL ring could be the lactone hydrolysis generated by AHL lactonases or the decarboxylation by decarboxylases. On the other hand, it is possible to cleave the acyl chain off the HSL moiety by hydrolysis of the amide bond mediated by amidases or deaminases. Its modification is also possible, for example, the oxidoreduction that can be produced by oxidases and/or reductases (Dong and Zhang, 2005).

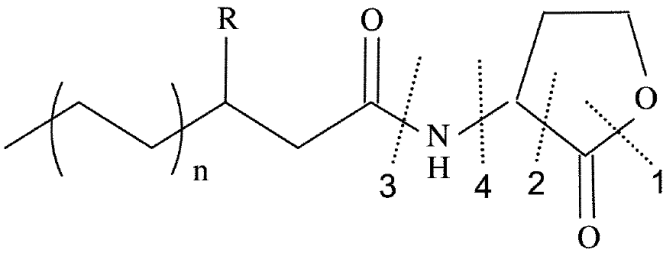

Fig. 8. Potential cleaving sites in the AHL molecule. (1) lactonases cleaving site, (2) cleaving site for decarboxylases, (3) acylase cleaving site, (4) deaminase cleaving site (from Dong and Zhang, 2005).

2.2.2. Enzymatic modification of AHLs signals

As described above, there are different enzymes that have target sites for AHL modification or cleavage. However, only in three of them the ability to modify these AHL molecules has been demonstrated enzymatically and structurally. Two of these activities are AHL-degradation enzymes, corresponding to AHL-lactonases and AHL-

amidases, while the other one belongs to the oxidoreductase group, and they modify the AHL molecule. These three QQ enzymes are detailed below:

(i) *Signal degradation by AHL-lactonases*. These enzymes modify the lactone ring in the homoserine moiety of the AHL without affecting the rest of the molecule. The hydrolysis of the lactone ring, leaving it open, causes the inactivation of the AHL signal (Dong et al., 2001), and this structure cannot fit in the binding site of the receptor. The cleavage reaction is identical to the pH-dependent lactonolysis, and this reaction can be reversed by acidification (Yates et al., 2002). Lactonase activities with QQ ability have been found in different groups of bacteria including Gram-positive and Gram-negative bacteria (Dong and Zhang, 2005; Krysciak et al., 2011). There are two families of lactonases identified in prokaryotes: one is represented by autoinducer inactivator (aiiA) and the other one formed by QsdA lactonase.

The metallo-hydrolase family, represented by aiiA, is formed by enzymes that require Zn^{2+} ions for full functionality (Kim et al., 2005; Thomas et al., 2005). The gene *aiiA* was firstly described in some species of the genus *Bacillus*, codifying for a lactonase able to hydrolyze the lactone ring of AHL molecules independently of the acyl chain length and the substitutions in the C_3 (Dong et al., 2000). The role proposed for this activity is the ecological advantage for colonization of new environments avoiding the competence of other microorganisms by degrading their QS signals (Chen et al., 2013).

In addition, other bacterial species present lactonase activities in their genomes, even in Gram-negative bacteria, although they are also able to produce AHLs for their own QS communication. This is the case of *Agrobacterium tumefaciens*, which produces BlcB (formerly AttM) and AiiB proteins with lactonase activity (Carlier et al., 2003; Zhang et al., 2002), and the protein AhlK, which is also a lactonase produced by *Klebsiella pneumoniae* (Park et al., 2003b). There are other lactonases described from *Sreptomyces*

(Park et al., 2005), *Arthrobacter* (Park et al., 2003b), and *Pseudomonas* (Sio et al., 2006). The existence of lactonase activities codified in the genome of eukaryotes has been also found. The study of mammalian cells revealed their ability of AHL inactivation via paraoxonases (PONs) that can cleave the lactone ring (Yang et al., 2005).

On the other hand, the other lactonase family is QsdA lactonase. The most characterized is the QsdA lactonase from *Rhodococcus erythropolis* strain W2, which is also Zn^{2+} dependent, but it belongs to the phosphotriesterase family (Uroz et al., 2008). This protein has a perfect fitting pocket for the lactone ring and acyl chain; this finding was revealed after the crystallographic study that was performed with this enzyme. The binding site of QsdA protein has particular characteristics that are important for the hydrolysis of the lactone ring and probably for the substrate specificity. This group of activities (phosphotriesterases) can be considered another family of lactonase to which SsoPox activity from *Sulfolobus solfataricus* also belongs (Elias et al., 2008).

(ii) Signal degradation by AHL-amidases. The hydrolysis of the amino bond between the acyl side chain and the HSL, which this enzyme produces, is irreversible. It leads to signal molecule inactivation and suppression of QS communication. They are also called acylases (Uroz et al., 2009) because they present specificity attending to the length of the acyl chain (Lin et al., 2003), but enzymatically, they correspond to amidases enzymes. The first amidase was described from the Gram-negative bacteria *Variovorax paradoxus*, which cleaved the acyl chain of AHL molecules to use them as sole carbon and nitrogen source (Leadbetter et al., 2000).

The AHL amidases can be divided in two groups attending to the chemical mechanism used for hydrolysis reaction: N-terminal nucleophilic hydrolases (Ntn) and carboxylic ester hydrolases. The group of Ntn hydrolases (Dong et al., 2007; Lin et al., 2003) is

synthesized as proenzymes and susceptible of posttranslational processing. The enzymatic precursor undergoes autoproteolysis and becomes a mature acylase with two active subunits. In addition, they exhibit preference for the degradation of long-chain AHLs. On the other hand, carboxylic ester hydrolases do not present this posttranslational modification, as is the case of AiiO protein from *Ochrobactrum* sp. A44 strain. These enzymes are characterized for their ability to break down a wide spectrum of AHLs, including acyl chain of different lengths and oxo-substituted or not oxo-substituted (Jafra et al., 2006).

(iii) Modification of AHL signals by oxydoreductases. These enzymes cause the modification of the chemical structure of AHL molecules oxidizing or reducing the acyl chain. The QS signal is not degraded, but its modification disturbs the QS communication (Chen et al., 2013).

Up to the date, there are few activities reported about oxydoreductases involved in QS interference. The first one was described in *R. erythropolis* W2 strain as reducer of N-oxo-acyl side chains to their 3-hydroxy derivatives; this enzyme modifies AHL molecules ranging from C_8 to C_{14} (Uroz et al., 2005). Two years later, the existence of a monooxygenase enzyme that can oxidize AHL molecules of long acyl chain saturated and unsaturated was reported in *Bacillus megaterium* (Chowdhary et al., 2007). The next one was a short-chain dehydrogenase/reductase derived from a metagenomic library that can modify AHL molecules, and it is NADP dependent (Bijtenhoorn et al., 2011).

In general, there are predictable situations for QQ activities release. For example, in *A. tumefaciens*, AttM molecules are produced during the stationary phase, and they can degrade their own autoinducer with them being able to autoregulate their QS signalization depending on their metabolic state (Zhang et al., 2002). However, in the

case of AAB, currently, there is no information about QQ activities. It has been suggested that the production of QQ activities in bacteria could be related with competition mechanisms for nutrients and ecological niches between different groups of microorganisms, but also with a mechanism of adaptation to environmental changes blocking the own QS response (Zhang and Dong, 2004). AAB are a group of bacteria able to survive in extreme conditions, such as a low pH level, high ethanol, and/or acetic acid concentration where very few microorganisms can be present. This reduced competence could be related with a low production of QQ activities. However, the physiological activity of most of the QQ molecules described is unknown at the moment, and some of these molecules probably do not act in nature as QQ enzymes but present this ability when are tested *in vitro* (Chen et al., 2013).

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Chapter 1

Diversity of acetic acid bacteria present in healthy grapes from the Canary Islands

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Abstract

The identification of acetic acid bacteria (AAB) from sound grapes from the Canary Islands is reported in the present study. No direct recovery of bacteria was possible in the most commonly used medium, so microvinifications were performed on grapes from Tenerife, La Palma and Lanzarote islands. Up to 396 AAB were isolated from those microvinifications and identified by 16S rRNA gene sequencing and phylogenetic analysis. With this method, *Acetobacter pasteurianus*, *Acetobacter tropicalis*, *Gluconobacter japonicus* and *Gluconacetobacter saccharivorans* were identified.

However, no discrimination between the closely related species *Acetobacter malorum* and *Acetobacter cerevisiae* was possible. As previously described, 16S-23S rRNA gene internal transcribed spacer (ITS) region phylogenetic analysis was required to classify isolates as one of those species. These two species were the most frequently occurring, accounting for more than 60% of the isolates. For typing the AAB isolates, both the Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR and (GTG)5-PCR techniques gave similar resolution. A total of 60 profiles were identified. Thirteen of these profiles were found in more than one vineyard, and only one profile was found on two different islands (Tenerife and La Palma).

Keywords: wine, spoilage, vinegar, phylogenetic analysis, genotyping.

1. Introduction

Acetic acid Bacteria (AAB) are well-known microorganisms that proliferate in fruits such as grapes. They are the main spoilage microorganisms in some fermented beverages, such as beer and wine; on the other hand, they are the main microorganisms responsible for the production of vinegar (Guillamon and Mas, 2009). The presence and population dynamics of AAB on grapes and in wines have been analyzed with traditional microbiological methods (Barbe et al., 2001; Drysdale and Fleet, 1989; Joyeux et al., 1984). The species *Gluconobacter oxydans* was detected in grape must, and *Acetobacter aceti* and *Acetobacter pasteurianus* were detected in wine, in which they were responsible for spoilage. The development of new molecular methods has allowed its confirmation along with the discovery of further details, especially new species associated with grapes and/or wine. *Gluconacetobacter liquefaciens* and *Gluconacetobacter hansenii* have been described in South African (DuToit and Lambrechts, 2002) and Spanish wines (González et al., 2004; González 2005); *Acetobacter cerevisiae* has been described on healthy Chilean grapes (Prieto et al., 2007) and *Acetobacter oeni* has been described as the main spoilage microorganism in Portuguese wines (Silva et al., 2006).

The fundamental limitation of ecological studies remains the difficult isolation, cultivation and maintenance of pure cultures. In spite of this, the methods that have been developed to characterize the genetic variation in AAB are based on culture-dependent techniques, as the step of culture cannot be bypassed at this moment. Furthermore, the difficulties in cultivating microorganisms from healthy grapes have been indicated in previous works (Joyeux et al., 1984), and some possible solutions have included an enrichment step (Prieto et al., 2007).

In the present work, we have focused on the diversity of AAB from grapes cultivated in the Canary Islands, where no previous studies of AAB have been performed. In fact, few studies on the microorganisms associated with winemaking in the Canary Islands have been published. González et al. (2007) studied in detail the yeast population dynamics during alcoholic fermentation in wineries located in Tenerife.

The Canary Islands archipelago comprises seven main islands together with a number of smaller islets. They are located in the Atlantic Ocean, near the Tropic of Cancer, off the African coast of Western Sahara. All of the islands are volcanic and oceanic in origin. The isolation between the islands, their age, and their position off the coast of Western Sahara, results in a high degree of endemism that has been studied largely in their flora and fauna.

The Canary Islands have a long tradition of viticulture. Today, there are ten wine-producing regions, each with a "Denominación de Origen" (D.O.). Five of these regions are on Tenerife, and there is one on each of the other islands, except Fuerteventura.

Most of the Canarian wines are produced on the islands of Lanzarote, Tenerife and La Palma. These three islands have very different characteristics. Tenerife is situated near the center of the archipelago; it is large and very populated. La Palma is the most northwestern of the Canary Islands, whereas Lanzarote is the most northeastern and closest to the African coast (100 km). La Palma and Lanzarote are smaller and less populated than Tenerife. La Palma and Tenerife both have an abrupt relief, but Lanzarote does not.

The aim of the present study was to conduct an in-depth analysis, identification and genetic variability characterization of the AAB population isolated from grape bunches obtained aseptically from different vineyards of Tenerife, La Palma and Lanzarote.

However, as direct cultivation of the grape juices did not yield any identifiable AAB colonies, the juices were spontaneously allowed to ferment in microvinifications. After alcoholic fermentation, some AAB colonies were cultured and analyzed. The isolated colonies were typed by analysis of highly conserved repetitive DNA elements, (GTG)5-PCR and Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR. Different typing profiles were grouped by RFLP-16S rRNA gene and identified by 16S rRNA gene sequencing and phylogenetic analysis. When the 16S rRNA was not able to differentiate closely related species, the more variable 16S-23S rRNA gene internal transcribed spacer (ITS) region was used for phylogenetic analyses and putative identification.

2. Materials and methods

2.1 Samples, isolation and culture conditions

Samples of healthy grapes were obtained from three different islands of the Canary Islands, from East to West: Lanzarote, Tenerife and La Palma. Grapes from 34 vineyards were collected during the 2009 harvest season.

In Tenerife, bunches were harvested from 18 vineyards ranging from 60 to 1175 meters above sea level (m.a.s.l.). The vineyards from this island are included in three wine-growing areas with "Denominación de Origen". In La Palma, bunches were harvested from eight vineyards ranging from 365 to 1180 m.a.s.l. Samples from Lanzarote were obtained from eight vineyards ranging from 188 to 380 m.a.s.l. Samples of a red (Listan Negro, LN) and a white grape variety (Listan Blanco, LB, or Malvasía, MV, in Lanzarote) were taken from in each vineyard. All the grape samples used had following parameters: ºBrix, 18-20; pH, from 3.1 to 3.6 and weight of the bunches, >700 g.

The samples were handled by the Applied Microbiology Department of the Instituto Canario de Investigaciones Agrarias (Canary Islands, Spain), where they were processed. Ten grape bunches were randomly and aseptically collected from each grape variety at each vineyard and store in sterile plastic bags. All the samples were transported cooled to the laboratory and directly processed. Each bunch was homogenized in a Stomacher for 2 min and a pool of musts from each variety and vineyard was made. From each pool, aseptic microvinifications were done in 450 ml sterile bottles with 200 ml of must. Trials were carried out in duplicate at 22 ± 1 ºC in a dark room. Fermentations were monitored daily by measuring ºBrix. Prior to sampling, to ensure homogeneity the bottles were stirred and then covered with a cotton cap to allow the gas exchange. A total of 136 microvinifications (34 vineyards, two varieties in each vineyard and each microvinification in duplicate) were analyzed.

Samples were taken at different times of the fermentation process. The samples were plated onto GYC medium (1% yeast extract, 5% glucose, 1.5% $CaCO_3$, 1.5% agar w/v) and incubated at 28 °C under aerobic conditions (Gosselé et. al., 1984). This medium was supplemented with pimaricin (200 mg/L) (Sigma-Aldrich, Madrid) and cycloheximide (20 mg/L) (Fluka, Madrid) to suppress fungal and yeast growth. Each bacterial colony that produced a clear halo on GYC was considered a putative AAB isolate and sent to the Universitat Rovira i Virgili (Tarragona, Spain) for further analysis.

2.2. AAB typing and identification

Total DNA was extracted from each sample by the CTAB method (Cetyltrimethylamonium bromide) described by Ausubel et al. (1992). The concentration and purity of the DNA samples were determined using a NANODROP^{TM}1000 spectrophotometer (Thermo Scientific, Delaware, USA).

2.2.1. ERIC-PCR and (GTG)5-PCR fingerprinting

For AAB genotyping, we used the ERIC-PCR technique described by González et al. (2004) and the $(GTG)_5$-PCR technique (DeVuyst et al., 2008). All amplification reactions were carried out in GeneAmp PCR System 2700 (Applied Biosystems, Foster City, USA), and the products were detected by electrophoresis on 1.5% (w/v) agarose gels. The pattern band lengths were determined by comparison with a DNA XVI 250 bp ladder for the largest bands and a DNA XIV 100 bp ladder for the smallest bands (both from Roche Diagnostics, Mannheim, Germany).

To determine more accurately the number of different profiles obtained, the ERIC-PCR and (GTG)5-PCR profiles were compared by an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany) using the 7500 Labchip and 12000 Labchip, respectively. Sizes of the amplified fragments were determined by comparison with external standards (DNA sizing ladders) and internal standards (DNA markers). This technique has been reported to be an accurate and reproducible method (Panaro et al., 2000) for precise and reliable comparisons between profiles.

Phylogenetic relationships among the obtained profiles were determined using the BioNumerics software program (Version 6.5, Applied Maths, Sint-Martens – Latem, Belgium). Cluster analysis was carried out using the Dice coefficient with 1% of band position tolerance and Unweighted Pair Group Method with Arithmetic average (UPGMA). In addition, all band profiles were carefully determined to be correctly marked by visual inspection.

Diversity was calculated using Simpson's biodiversity index, which shows the probability that two randomly selected isolates are different genotypes. The index was calculated using $1-\Sigma p_i^2$, where p_i is equal to the number of isolates of the same genotype divided by the total number of isolates.

2.2.2. RFLP-PCR 16S rRNA gene, 16S-23S rRNA gene ITS PCR and sequencing

All of the different profiles obtained either by ERIC-PCR or by (GTG)5-PCR were identified after restriction analysis of the 16S rRNA gene amplicons and compared to those of the type strains reported by Ruiz et al. (2000) and González et al. (2006) to group the different profiles for sequence analysis.

The 16S rRNA gene was amplified using the method described by Ruiz et al. (2000). Briefly, 50-100 ng of AAB DNA were amplified with *Taq* DNA polymerase (ECOTAQ; ECOGEN ®, Barcelona, Spain) using a denaturation step at 94 ºC, followed by 30 cycles of denaturation at 94 ºC, annealing at 65ºC, and extension at 72 ºC; and a final extension at 72 ºC, that were performed in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, USA).

For a positive result, a 1450–bp AAB-specific fragment of the 16S rRNA gene must be amplified. These amplified products were digested with two restriction enzymes: *Taq*I, and *Bcc*I (Roche Diagnostics, Mannheim, Germany) (Ruiz et al., 2000; Torija et al., 2010). Amplification and restriction fragments were detected by electrophoresis on 1% and 2.5% (w/v) agarose gels, respectively. In both cases, the DNA XIV 100 bp ladder (Roche Diagnostics, Mannheim, Germany) was used.

When isolates could not be identified by 16S rRNA gene RFLP-PCR, the 16S-23S rRNA gene ITS region was amplified using the method described by Ruiz et al. (2000).

The amplified DNA from the 16S rRNA gene and the 16S-23S rRNA gene ITS was purified and sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. DNA sequences were compared with those in the GenBank databases. They have been deposited in the GenBank database under accession numbers JF346071 to JF346101. Phylogenetic and molecular evolutionary analyses of these

sequences based on the neighbor joining and Kimura 2- parameter methods were conducted using MEGA version 4 (Kimura, 1980; Tamura et al., 2007).

The analysis described in this work was performed in a research lab that meets the 9001 ISO regulation.

3. Results

The initial sugar concentration of the microvinifications was about 180 to 230 g/L in all samples. The microvinifications were considered finished when the density remained stable for several days. Only a few samples completely consumed the available sugar. A total of 513 halo-forming colonies were recovered from 65 microvinifications out of the 136 performed. These colonies were initially considered to be putative AAB.

3.1. AAB identification

Amplification of the 16S rRNA gene showed that of these putative isolates only 396 were AAB. The remaining 117 colonies were identified as belonging to other bacterial groups (data not shown). The RFLP-PCR of the 16S rRNA gene analysis grouped the AAB isolates as *Acetobacter tropicalis*, *A. pasteurianus*, *A. aceti*, *G. oxydans* and *Gluconacetobacter europaeus*. The sequencing of the 16S rRNA amplicon only confirmed the identification of *A. tropicalis* and *A. pasteurianus.* The other three species were identified as *Acetobacter malorum*/*cerevisiae* (tentatively identified as *A. aceti* by RFLP-PCR of 16S rRNA gene analysis*)*, *Gluconobacter japonicus* (tentatively identified as *G. oxydans)* and *Gluconacetobacter saccharivorans* (tentatively identified as *Ga. europaeus*). The 16S rRNA gene sequences of the type strains of these four species (*A. malorum* AJ419844, *A. cerevisiae* AJ419843, *G. japonicus* JF346081 and *Ga. saccharivorans* AB166740) were digested *in silico* with the same two enzymes

used in the RFLP analysis and yielded 16S rRNA restriction profiles identical to those of the isolates.

The 16S rRNA gene sequencing did not allow discrimination between the species *A. malorum* and *A. cerevisiae.* In fact, the 16S rRNA sequences of the type strains of these two species (*A. cerevisiae* LMG 1625 (AJ419843) and *A. malorum* LMG 1746 (AJ419844)) have a difference of only two base pairs between them. The sequences of the isolates were identical to neither of these two type sequences; the base pair at one position corresponded to *A. cerevisiae* and the base pair at the other position corresponded to *A. malorum.* Thus, it was impossible to classify those isolates as one of these species.

To clarify the identification of these isolates, the 16S-23S rRNA gene ITS region was amplified from all the genotypes putatively considered *A. malorum* or *A. cerevisiae*. The phylogenetic tree constructed with the 16S-23S rRNA gene ITS sequences revealed the presence of isolates belonging to the two species, indicated by the presence of two subclusters, one for each species (Fig.1). The grouping of these isolates was supported by bootstrap values higher than 90% (in 1000 trials) in most of the branches. It is interesting to remark that in both species the isolates were grouped separately from the type strain.

Therefore, the 396 AAB isolates could be identified as follows: 175 isolates belonged to the species *A. malorum*, 79 isolates to *A. cerevisiae*, 72 isolates to *A. pasteurianus*, 33 to *A. tropicalis*, 36 to *G. japonicus* and finally, one isolate was identified as *Ga. saccharivorans*.

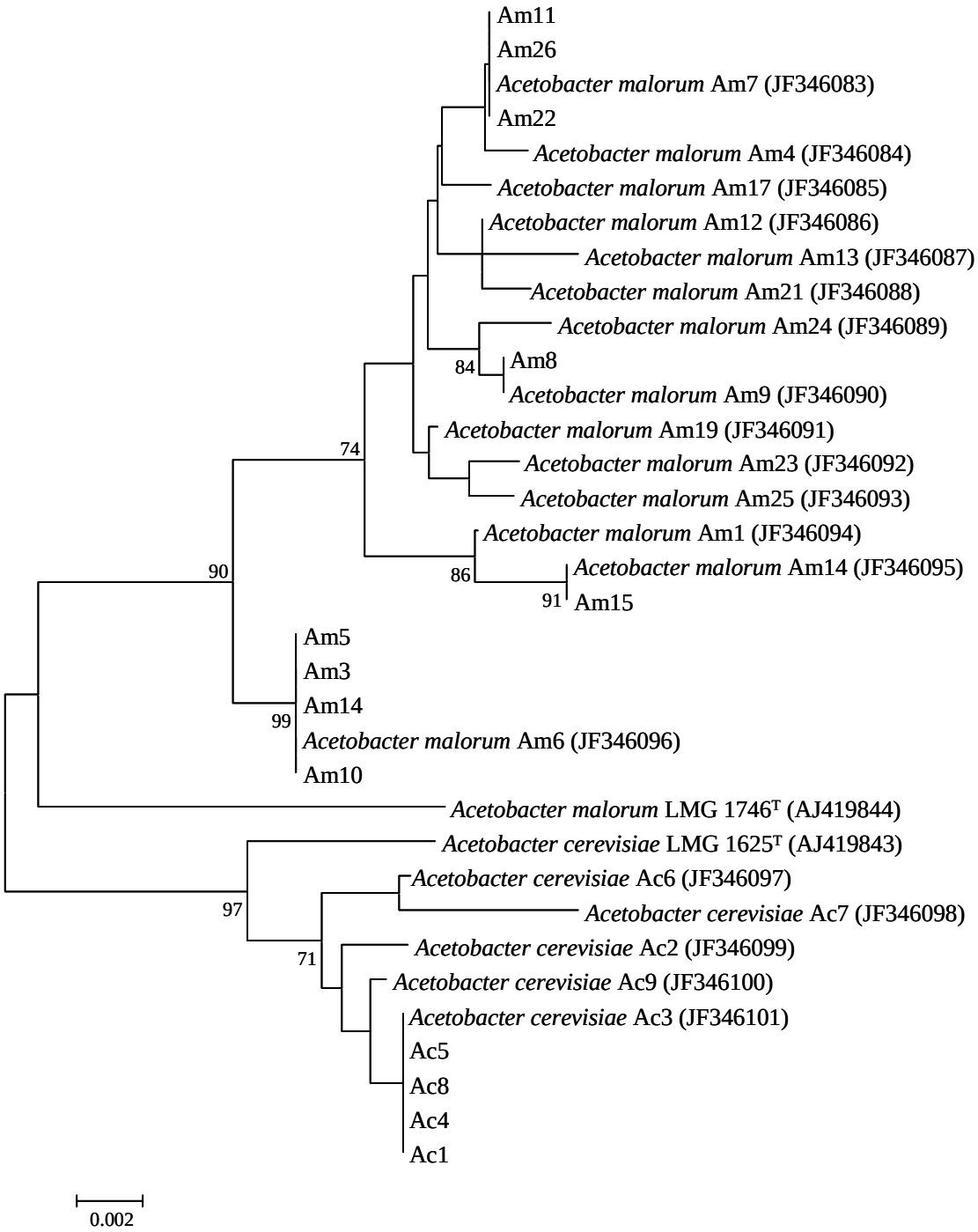

Fig. 1. Phylogenetic relationships of isolates corresponding to *A. malorum* and *A. cerevisiae* species detected on the three islands. The phylogenetic tree based on the 16S-23S rRNA ITS sequences was constructed using the neighbor-joining method. The robustness of the branching is indicated by bootstrap values (%) calculated for 1000 subsets. The entries of the different genotypes include the accession numbers of the GenBank database sequences.

3.2. AAB typing.

All 396 isolates were classified by their ERIC-PCR and $(GTG)_5$-PCR profiles. ERIC-PCR analysis showed profiles that contained up to 12 bands with sizes between 100 and 5000 bp. On the other hand, $(GTG)_5$-PCR products revealed up to 11 bands of sizes ranging from 400 to 8500 bp. Both techniques differentiated a total of 55 profiles, but the isolates classified as equal using each technique were not the same. Fifty profiles yielded the same identities with both techniques; whereas the remaining 10 profiles differed between the techniques (i.e. a single profile with ERIC-PCR had two profiles with $(GTG)_5$-PCR or vice versa)

The profile analysis with the BioNumerics v. 6.5 software package showed similar dendrograms with two different fingerprinting techniques (data not shown). However, the results obtained with both techniques were combined to generate a unique dendrogram using the BioNumerics software (Fig. 2) that differentiated a total of 60 profiles from the 396 AAB isolates. Two profiles were considered to be the same genotype when there was 100% agreement between the results of the two techniques. The percentage of similarity between different pairs of genotypes ranged from 26 to 94%. Of these 60 genotypes, 25 were identified as *A. malorum*, 16 as *A. pasteurianus*, 10 as *A. cerevisiae*, five as *A. tropicalis*, three as *G. japonicus* and one as *Ga. saccharivorans*. In this dendrogram, the genotypes were grouped into three main clusters, corresponding to *A. malorum*, *A. pasteurianus*/*G. japonicus* and *A. tropicalis*, although some genotypes did not group with their species cluster. However, genotypes of *A. cerevisiae* were not grouped into a cluster and were distributed among the three clusters.

3.3. AAB diversity

Table 1 shows the genotypes obtained on the three islands. In Tenerife, the total number of isolates was 225. Among them, *A. malorum* was the main species both in terms of the number of isolates (a total of 122 isolates) and the number of different genotypes (a total of 19 genotypes).

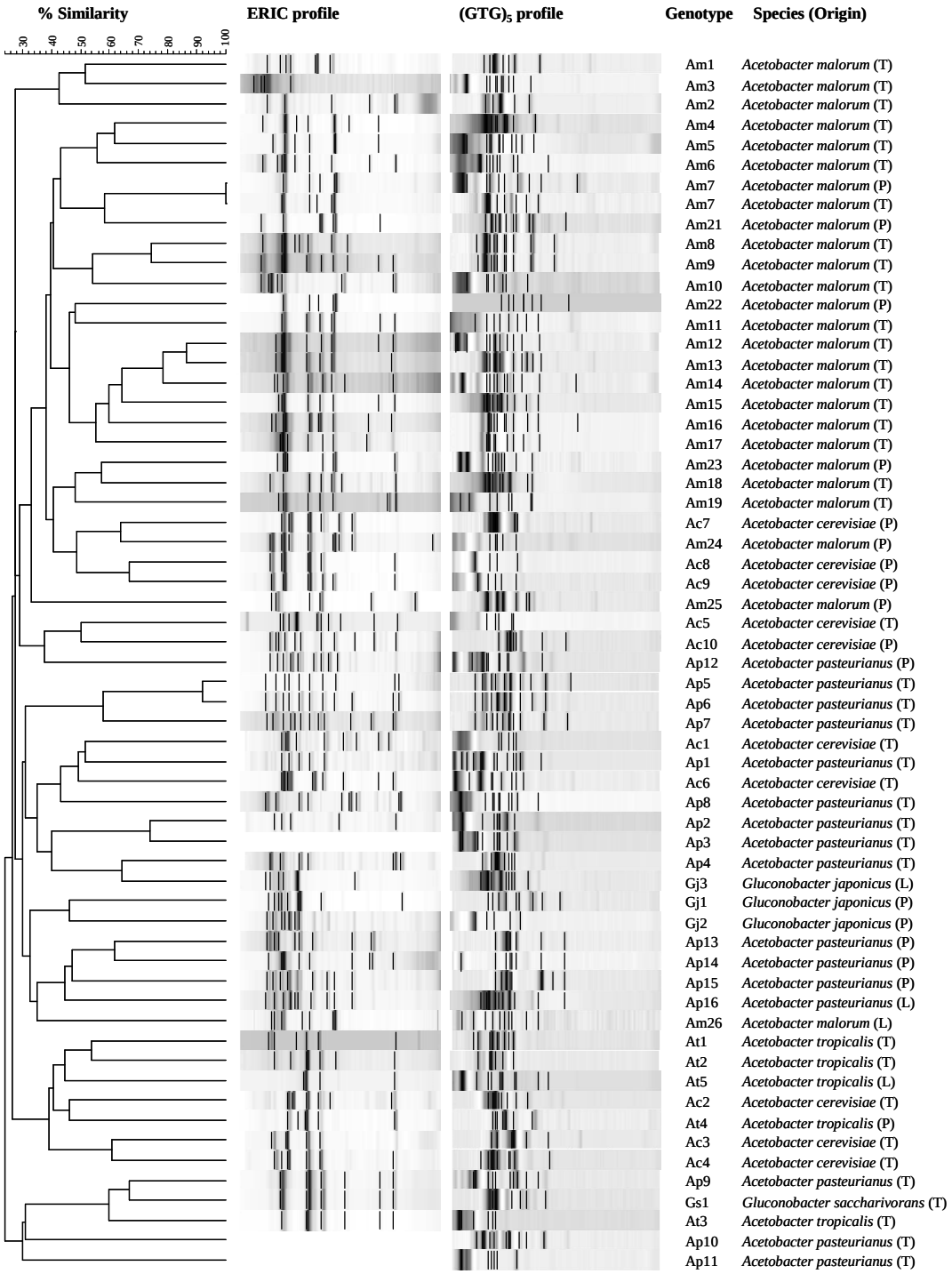

Fig. 2. ERIC-PCR and $(GTG)_5$-PCR profiles from all AAB isolates. The dendrogram was generated after cluster analysis of the digitized fingerprints and was derived from UPGMA linkage of Dice correlation coefficients. The origin of the isolates is indicated in brackets (T: Tenerife; P: La Palma; L: Lanzarote).

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UNIVERSITAT ROVIRA I VIRGILI
DEVELOPMENT OF TECHNIQUES FOR THE ANALYSIS OF ACETIC ACID BACTERIA POPULATIONS AND THEIR INTERACTION
IN DIFFERENT FOOD ENVIRONMENTS.
María José Valera Martínez
Dipòsit Legal: T 1835-2014

Table 1. Identification and typing of AAB isolates from the three islands.

Island of origin	No. of isolates studied	No. of different genotypes	Species (no. of isolates)	GTG_5/ERIC profile (no. of isolates)	Biodiversity Simpson's index
Tenerife	225	40	*Acetobacter malorum*(122)	Am7(25),Am17(25),Am8(15),Am3(10),Am12(10),Am5(8), Am1(7),Am6(4),Am13(4),Am4(2),Am9(2),Am14(1),Am16(2),Am19 (2),Am2(1),Am10(1),Am11(1),Am15(1),Am18(1)	0.95
			Acetobacter cerevisiae (55)	Ac3(22),Ac5(15),Ac2(15),Ac1(1),Ac6(1),Ac4(1)	
			Acetobacter pasteurianus (39)	Ap4(16),Ap2(6),Ap11(6),Ap6(3),Ap3(2),Ap5(1),Ap7(1), Ap1(1),Ap8(1), Ap9(1),Ap10(1)	
			Acetobacter tropicalis (8)	At1(3),At2(3),At3(2)	
			Gluconacetobacter saccharivorans (1)	Gs1(1)	
La Palma	110	17	*Acetobacter malorum* (60)	Am7(19),Am25(16),Am21(12),Am24(6),Am23(4),Am22(3)	0.90
			Acetobacter cerevisiae (8)	Ac7(3),Ac8(3),Ac9(1),Ac10(1)	
			Acetobacter pasteurianus (21)	Ap12(9),Ap15(6), Ap13(5),Ap14(1)	
			Acetobacter tropicalis (1)	At4(1)	
			Gluconobacter japonicus(20)	Gj1(15),Gj2(5)	
Lanzarote	61	4	*Acetobacter malorum* (9)	Am26(9)	0.72
			Acetobacter pasteurianus (12)	Ap16(12)	
			Acetobacter tropicalis (24)	At5(24)	
			Gluconobacter japonicus(16)	Gj3(16)	

A. cerevisiae was the second most frequently isolated species (55 isolates and six genotypes), and *A. pasteurianus* had the second highest number of genotypes (39 isolates and 11 genotypes). *A. tropicalis* and *Ga. saccharivorans* were minor species. The diversity index of Tenerife was 0.95. A total of 40 genotypes were detected, and 10 of them were present in more than one vineyard. The most frequent genotypes were Am7 and Am17, which were recovered in three and four vineyars respectively. The genotype Ac3 was recovered in five vineyards, Ap4 was recovered in three vineyards and the rest of genotype (Am5, Am8, Am1, Ac2, Ap2, and Ap3) were recovered in two vineyards each.

In La Palma, a total of 110 isolates were recovered. *A. malorum* was again the main species isolated (60 isolates and six genotypes) followed by *A. pasteurianus* (21 isolates and four genotypes) and *G. japonicus* (20 isolates and two genotypes). *A. cerevisiae* and *A. tropicalis* were also recovered, albeit in minor amounts. A total of 17 genotypes were identified and the diversity index was 0.90. The Am7 genotype, which was also recovered in Tenerife, was isolated with the highest frequency and detected in three vineyards. Three genotypes (Am22, Am24, and Ac8) appeared in two vineyards each. The other genotypes were each exclusive to one vineyard.

In Lanzarote, the total number of isolates recovered was 61. In this case, *A. tropicalis* was the species with the highest number of isolates recovered. Other species identified, in order of decreasing frequency, were *G. japonicus*, *A. pasteurianus* and *A. malorum*. Each genotype corresponded to a different species and each genotype appeared in one vineyard. As a consequence of this limited variation, the index of diversity was the lowest of the three islands, 0.72.

Table 2. AAB identification and typing from the grape varieties analyzed. The codes for the different typing profiles indicate the abbreviation of the species (Am, Ac, Ap, At, Gj, Gs) and the assigned profile number. The number in brackets indicates the number of isolates detected.

Identified Species	Tenerife		La Palma		Lanzarote[a]	
	LB	LN	LB	LN	MV	LN
Acetobacter malorum	Am7(24),Am17(22), Am8(15),Am3(10), Am12(9),Am5(6), Am13(4),Am1(3), Am4(2),Am9(2), Am19(2),Am11(1), Am14(1),Am15(1), Am18(1)	Am1(4),Am6(4), Am17(3),Am5(2), Am16(2),Am2(1), Am7(1),Am10(1), Am12(1)	Am7(11),Am24(5), Am22(1)	Am25(16),Am21(12), Am7(8)Am23(4), Am22(2),Am24(1)		Am26(9)
Acetobacter cerevisiae	Ac2(14),Ac3(11), Ac5(4),Ac4(1), Ac6(1)	Ac3(11),Ac5(11), Ac1(1),Ac2(1)	Ac8(2)	Ac7(3),Ac8(1), Ac9(1),Ac10(1)		
Acetobacter pasteurianus	Ap4(6),Ap2(2), Ap3(2)	Ap4(10),Ap11(6), Ap2(4),Ap6(3), Ap1(1),Ap5(1), Ap7(1),Ap8(1), Ap9(1),Ap10(1)	Ap12(6)	Ap15(6),Ap13(5), Ap12(3),Ap14(1)		Ap16(12)
Acetobacter tropicalis		At1(3),At2(3), At3(2)		At4(1)		At5(24)
Gluconobacter japonicus			Gj1(15),Gj2(5)		Gj3(16)	
Gluconacetobacter saccharivorans		Gs1(1)				

[a] No isolates from LB variety were obtained

Regarding the grape variety, the results obtained from the isolates from the LB, LN and MV varieties are shown in Table 2. The species recovered from microvinifications carried out with LB grapes were *A. malorum* (18 genotypes, 120 isolates), *A. cerevisiae* (six genotypes, 33 isolates), *A. pasteurianus* (four genotypes, 16 isolates) and *G. japonicus* (two genotypes, 20 isolates). The species recovered from microvinifications of MV grapes was *G. japonicus* (one genotype, 16 isolates). Moreover, the species *G. japonicus* was always the only species found in the microvinifications where it was detected. The isolates from microvinifications of LN grapes belonged to the species *A malorum* (16 genotypes, 71 isolates), *A. cerevisiae* (eight genotypes,

30 isolates), *A. pasteurianus* (15 genotypes, 56 isolates), *A. tropicalis* (five genotypes, 33 isolates) and *Ga. saccharivorans* (one genotype, one isolate). The genotypes Am1, Am5, Am7, Am12, Am17, Am22, Am24, Ac2, Ac3, Ac5, Ac8, Ap2 and Ap12 were recovered in microvinifications of LB and LN.

4. Discussion

The recovery of AAB from grapes is highly dependent on the sanitary status of the grapes (Barbe et al., 2001; González et al., 2005; Joyeux et al., 1984), with poor recoveries from intact, healthy grapes (Joyeux et al., 1984; Prieto et al., 2007). In the case of the Canary Islands, it was not possible to recover any AAB species from healthy grapes, even when the enrichment protocol of Prieto et al. (2007) was used. Thus, only after alcoholic fermentation the status of the AAB was appropriate to grow in plates.

Thus, in this study, microvinifications based on the spontaneous fermentation of grape juices were carried out to analyze in-depth the AAB diversity present in healthy grapes from the Canary Islands, specifically Tenerife, La Palma and Lanzarote islands. The identification of species and the characterization of genetic variability were performed using molecular methods.

Analysis of the 16S rRNA gene sequence is the most common method used to identify AAB. The RFLP-PCR technique based on the amplification of this gene is a useful tool for rapidly processing a large number of samples (González et al., 2006; Ruiz et al., 2000). However, the high degree of homology among AAB species often necessitates the use of more than one or two enzymes (González et al., 2006; Torija et al., 2010), making the technique more time consuming. On the other hand, sequence analysis of the 16S rRNA gene has been reported to be a good method of identification for this group of bacteria (Yamada et al., 2008) because of the high degree of conservation of this region in the genome and the availability of this sequence for practically all the AAB species described. Nevertheless, the high sequence identity between different species (up to 99.9%) makes it difficult to get a correct identification (Cleenwerck and De Vos, 2008).

The species identification of the isolates analyzed in this work was initially carried out by RFLP-PCR and sequencing of the 16S rRNA gene. Although the 16S rRNA RFLP-PCR analysis allowed grouping the related, the sequencing of this gene was necessary to obtain a correct identification. However, phylogenetically close species, like *A. malorum* and *A. cerevisiae*, could not be differentiated by this gene analysis. Difficulties in differentiating these two species by 16S rRNA RFLP-PCR have also been reported by Prieto et al. (2007), but in their study, sequencing the 16S rRNA gene resolved the identification. However, in the present study, 16S rRNA gene analysis did not clarify the identification of very closely related species, and the study of the 16S-23S rRNA gene ITS region was necessary. This non-coding spacer has been described as a sequence with high variability. This characteristic has made it problematic to obtain RFLP profiles specific for each species (González et al., 2006; Ruiz et al., 2000; Trcek and Teuber, 2002). However, in a recent work (González and Mas, 2011), the analysis of this sequence has been described as a useful tool to identify species that are taxonomically very close. In the present work, the analysis of this sequence was also successful in differentiating closely related species.

Some of the species detected in this study have been described in previous studies of grapes and wine. *A. cerevisiae* has been described on healthy grapes (Prieto et al., 2007), although it was first recovered on breweries (Cleenwerck et al., 2002). *A. pasteurianus*, which has been considered to be the typical wine-spoiling microorganism (Bartowsky et al., 2003; Bartowsky and Henschke, 2008; Du Toit and Lambrechts, 2002; Joyeux et al., 1984), has even been proposed as a pioneer starter-culture species that can carry out traditional wine vinegar production (Gullo et al, 2009; Hidalgo et al., 2010). The identification of *A. tropicalis* was not surprising because this species has recently been isolated from spontaneously fermenting Austrian wine followed by acetic fermentation (Silhavy and Mandl, 2006). However, this species was first described from samples of coconut (Lisdiyanti et al., 2000) and later recovered from limes and figs (Cleenwerck et al., 2002). On the other hand, *Ga. saccharivorans* was first

recovered from beet juice (Lisdiyanti et al., 2006) and recently in wine fermentations at the early and middle stages of alcoholic fermentation (Kato et al., 2011).

Nevertheless, we also recovered and identified some species that had not been previously described either in wine or from grapes. In previous works, the species of the genus *Gluconobacter* that was most often identified on grapes and wine was *G. oxydans* (Andorrà et al., 2008; Du Toit and Lambrechts, 2002; González et al., 2005; Joyeux et al., 1984; Renouf et al., 2005). It has to be considered that *G. japonicus*, has been recently described from fruits of Chinese bayberry (Malimas et al., 2009) and, thus, some previous identifications of *G. oxydans* could indeed have been *G. japonicus* due to their high homology. On the other hand, *A. malorum* has been found in traditional balsamic vinegar (De Vero et al., 2006) and pulque, a fermented beverage made from the agave plant (Escalante et al., 2008). This species and *A. cerevisiae* are very closely related to *A. aceti*, and we initially identified them as such by RFLP-PCR of 16S rRNA gene. Thus, it is likely that previous identifications of *A. aceti* in grapes and wines could mask the presence of these recently described species (Cleenwerck et al., 2002).

The typing of strains or different genotypes of AAB is not usually studied in wine; these works have commonly focused only on species identification (Bartowsky and Henschke, 2008). But this knowledge could be useful, as not all strains that belong to the same species have the same abilities (Mas et al., 2007). Typing allows studying the genetic variation between isolates. Vegas et al. (2010) pointed out that the degree of polymorphism detected by $(GTG)_5$-PCR is slightly lower than that detected by ERIC-PCR.

However, in our results, neither ERIC-PCR nor $(GTG)_5$-PCR could discriminate all of the genetically different isolates. The results from these two techniques were not always concordant; some isolates that showed the same ERIC-PCR profile did not show the same profile by $(GTG)_5$-PCR and vice versa. Therefore, we propose that the combination of both techniques makes it possible to uncover a higher degree genetic variability that could be masked by the use of only one typing technique.

In the fingerprinting studies, the main difficulty is finding a rapid and reliable technique for establishing categories that allow the appropriate characterization of microorganisms. Currently, different techniques have been tested for AAB typing (Cleenwerck et al., 2009; Cleenwerck et al., 2010; Fernández-Pérez et al., 2010; González et al., 2004; Nanda et al., 2001; Papalexandratou et al., 2009; Trcek et al., 1997), among them ERIC-PCR and $(GTG)_5$-PCR. The former was described as an appropriate method for AAB typing (González et al., 2004; Gullo et al., 2009; Nanda et al., 2001) but unable for grouping in species cluster (Fernández-Pérez et al., 2010). The $(GTG)_5$-PCR was reported to be a good technique to differentiate between AAB species (De Vuyst et al., 2008) and at the strain level (Papalexandratou et al., 2009). However, these authors reported that not all strains of the same species clustered together. Our typing results from isolates recovered in natural environments confirmed the impossibility of grouping all the profiles into species clusters by either $(GTG)_5$-PCR or by ERIC-PCR. Only some species were grouped clearly; for example, *A. malorum* presented a big cluster. Our inability to assign isolates to different species according to the typing profile clearly shows the limitation of these techniques, which should be considered appropriate for typing but not appropriate for identification.

The results obtained were also analyzed to detect if there was any island-specific trend with respect to isolated AAB. In the phylogenetic analysis, the typing profiles were not grouped according to the island of origin. *A. malorum* was the main species in Tenerife and La Palma. All of the genotypes of *A. malorum* isolated from these two islands were grouped in one big cluster. The only genotype of *A. malorum* that fell outside of this cluster was the one detected in Lanzarote (Am26). In fact, most of the isolates recovered from this island belonged to *A. tropicalis*. Furthermore, a high degree of biodiversity is found on the three islands. It is remarkable that most of the genotypes found were recovered from one vineyard isolates and that only one genotype (Am7) was detected in vineyard isolates from two islands (Tenerife and La Palma), showing a high frequency of isolation.

This is the first in-depth study of AAB from healthy grapes from the Canary Islands. The environmental peculiarities of these islands resulted in a high degree of biodiversity. Species that had not previously been detected either on grapes or in wines were recovered. In addition, the combination of more than one typing technique is recommended to analyze AAB genetic diversity.

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Chapter 2

Acetobacter malorum and *Acetobacter cerevisiae* identification and quantification by Real-Time PCR with TaqMan-MGB probes

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Abstract

The identification and quantification of *Acetobacter malorum* and *A. cerevisiae* in wine and vinegar was performed using the Real-Time PCR (RT-PCR) with two TaqMan-MGB probes designed to amplify the internal transcribed spacer (ITS) region between the 16S–23S rRNA genes. The primers and probes were highly specific, with a detection limit of 10^2 cells/ml for both species, and the efficiency of the technique was >80%. The RT-PCR technique with these two new TaqMan-MGB probes, together with the five (*A. aceti*, *A. pasteurianus*, *G. oxydans*, *Ga. hansenii* and *Ga. europaeus*) that are already available (Torija et al., 2010), were validated on known concentrations of Acetic Acid Bacteria (AAB) grown in glucose medium (GY) and in inoculated matrices of wine and vinegar. Furthermore, this technique was applied to evaluate the AAB population in real wine samples collected in the Canary Islands. PCR enrichment performed prior to RT-PCR increased the accuracy of quantification and produced results similar to those detected with SYBR-Green. In real wine samples, the total AAB enumeration ranged from 9×10^2 to 10^6 cells/ml, and the seven AAB species tested were detected in more than one sample. However, AAB recovery on plates was poor; the isolates obtained on plates were *A. malorum*, *G. oxydans*, *A. cerevisiae* and *A. pasteurianus* species. RT-PCR with TaqMan-MGB probes is an accurate, specific and fast method for the identification and quantification of AAB species commonly found in wine and vinegar.

Keywords: acetic acid bacteria, Real Time-PCR, MGB probes

1.- Introduction

Acetic Acid Bacteria (AAB) are taxonomically classified as α-Proteobacteria, within the *Acetobacteraceae* family. In recent years, the classification of these microorganisms has been substantially changed several times, including additions of new genera and species descriptions (Yukphan et al., 2010; Malimas et al. 2009a; 2009b; Kommanee et al., 2011) and reclassifications of strains from culture collections into new species (Lisdiyanti et al., 2006; Malimas et al., 2008). Currently, 13 genera have been described in the AAB group (Yuphan et al, 2011). However, all of the species detected on grapes and in wine or vinegar belong to the genera *Acetobacter (A.)*, *Gluconobacter (G.)* or *Gluconacetobacter (Ga.)* (De Vero et al., 2006; Bartowsky and Henschke, 2008; Vegas et al., 2010, Valera et al., 2011).

Knowledge of the AAB populations present during the production of wine and vinegar is required for controlling the production process and maintaining the quality of the products. Both culture-dependent and culture-independent methods have been used to study AAB population dynamics during wine and vinegar production (De Vero et al., 2006; Andorrà et al., 2008; Hidalgo et al., 2010; Vegas et al., 2010; Jara et al., 2012). To obtain strains that can be selected for use in starter cultures, it is necessary to perform ecological studies in which the ability to recover and effectively isolate AAB in culture media is essential. However, it is well known that recovery of these microorganisms by growth in laboratory media is low, especially for samples derived from extreme environments, including conditions involving high ethanol and/or acetic acid concentrations (Sievers et al., 1992; Sokollek et al., 1998; Millet and Lonvaud-Funel, 2000; Trcek, 2005). This low recovery often results in the incomplete representation of the true microbial diversity of these niches.

In recent years, a wide variety of culture-independent techniques have been developed, providing a broader view of the AAB populations present in both wine and vinegar. Several of these techniques, such as Denaturing Gradient Gel Electrophoresis (DGGE) and Temporal Temperature Gradient Gel Electrophoresis (TTGE) (Lopez et al., 2003; De Vero et al., 2006; Andorrà et al., 2008; Ilabaca et al., 2008), focus on the identification of AAB at the species level, whereas other techniques, such as Fluorescence in Situ Hybridization (FISH) (Blasco et al., 2003; Franke-Whittle et al., 2005) or Real-Time PCR (RT-PCR) (Gammon et al., 2006; Gonzalez et al., 2006; Andorrà et al., 2008), aim to detect and quantify AAB. The RT-PCR technique involving TaqMan-MGB probes combines high specificity with exceptional sensitivity, and using this technique allowed us to successfully identify and quantify five of the most commonly species found in wine and vinegar: *A. aceti*, *A. pasteurianus*, *G. oxydans*, *Ga. hansenii* and *Ga. europaeus* (Torija et al., 2010).

The recent description of new species in the AAB group made it necessary to revise the species-specificity of the previously designed TaqMan-MGB probes to ensure the detection of the recently described species, many of which are commonly identified in wine and vinegar (Valera et al., 2011; Xu et al., 2011; Hidalgo et al., 2012a, 2012b). *Acetobacter* species are commonly detected on grapes, in wine and in vinegar, with *A. aceti* and *A. pasteurianus* being the most abundant species. However, the detection of these two species has recently decreased, while the reported detection of other *Acetobacter* species has increased, including *A. oeni* in wine (Silva et al., 2006), *A. cerevisiae* on grapes (Prieto et al., 2007; Valera et al; 2011) and in persimmon vinegar (Hidalgo et al., 2012a), and *A. malorum* on grapes (Valera et al., 2011) and in different varieties of vinegars (De Vero et al., 2006; Hidalgo et al., 2012a; 2012b). The close

phylogenetic relationship between some of the AAB species could have confounded the proper identification of the recently described species.

The most common target sequence used to design primers and probes in AAB is the 16S rRNA gene. The 16S gene is highly conserved in all bacterial genomes, and sequence information for this gene is publically available for practically all known species of AAB, making this gene a very convenient target. Nevertheless, the high sequence similarity (up to 99.9%) in the 16S rRNA region between closely related AAB species (Cleenwerck and De Vos, 2008) makes difficult to discern differences between these species using this gene (Valera et al., 2011). A number of studies based on the 16S-23S rRNA gene Internal Transcribed Spacer (ITS) sequence have enabled the resolution of closely related AAB species (González and Mas, 2011; Valera et al., 2011) by adding new sequences corresponding to this region to the public databases. Therefore, the ITS between the 16S-23S rRNA genes is a promising new target for the identification and detection of species belonging to this group of bacteria.

In the present work, two new species-specific TaqMan-MGB probes were designed and validated for *A. malorum* and *A. cerevisiae*. These species have been detected frequently both on grapes and in wine or vinegar within recent years. The TaqMan-MGB probes were designed to amplify the 16S-23S rRNA gene ITS region because the 16S rRNA gene sequences were too similar and did not allow for species differentiation using only this gene. These two new TaqMan-MGB probes along with the five probes already available for AAB species identification were tested in wine and vinegar matrices inoculated with known concentrations of these AAB species. Finally, the probes were also used to evaluate the AAB population in real samples, specifically, wine samples collected in the Canary Islands.

2.- Materials and Methods

2.1.- Bacterial strains and growth conditions

For this study, a total of 121 strains belonging to 30 AAB species were analyzed *in silico* and/or using the genomic DNA by RT-PCR (Table 1). AAB strains grown and tested in the laboratory were obtained from the following sources: 46 strains belonging to 22 AAB species from culture collections, and 54 strains isolated from microfermentations of healthy grapes from the Canary Islands (Spain) (Table 1). Furthermore, 6 strains of different lactic acid bacteria (LAB) species and 13 strains of diverse yeast species obtained from different culture collections were also tested. AAB strains were routinely grown at 28 °C for 2-3 days on glucose medium (GY; 1% yeast extract, 1% glucose [w/v]), whereas LAB and yeast were grown at 28 °C on MRS (Oxoid, Hampshire, United Kingdom) and on YPD (2% glucose, 2% peptone, 1% yeast extract [w/v]), respectively.

2.2.- Design of primers and Taqman-MGB probes

Primers and TaqMan-MGB probes for *A. malorum* and *A. cerevisiae* were designed from the consensus sequence obtained using the 16S-23S rRNA gene ITS sequences available in the GenBank databases for these species. The alignments of these sequences were performed with the ClustalW multiple sequence alignment program (Thompson et al., 1994), and the primer-probe design was conducted with the Primer Express software package (v. 3.0, Applied Biosystems, Foster City, CA, USA).

Primers were the same for both species: forward primer MACER-F (5’ CTGGTTTGAGGCTTGAGTGATATG 3’) and reverse primer MACER-R (5’ TGCTCTCCCAGCTGAGCTATG 5’). The specific probe designed to detect *A. malorum* was MAL-S (5’ 6FAM-AGCAGCGCTCTGGT-MGB 3’), whereas that used to detect *A. cerevisiae* was CER-S (5’ VIC-AGCAGCATTCTGGT-MGB 3’).

Table 1. Strains used to test by RT-PCR and *in silico* aligment the probes and primers designed in this study

Species	Collection Designation or Strain[a]	Origin or isolation source	Region	GeneBank database accession number
A. cerevisiae	LMG 1625^T	Beer (ale) in storage	16S rRNA gene	AJ419843
			16S-23S rRNA gene ITS	FR716475
	Ac3;(Ac1;Ac4;Ac5;Ac8)	Microfermentation of healthy grapes[c]	16S rRNA gene	JF346071; (-)
			16S-23S rRNA gene ITS	JF346101; (-)
	Ac2; Ac6; Ac7; Ac9	Microfermentation of healthy grapes[c]	16S-23S rRNA gene ITS	JF346097-JF346100
A. malorum	LMG 1746^T	Rotting apple	16S rRNA gene	AJ419844
			16S-23S rRNA gene ITS	FR716476
	Am7; (Am1;Am22;Am26)	Microfermentation of healthy grapes[c]	16S rRNA gene	JF346074; (-)
			16S-23S rRNA gene ITS	JF346083; (-)
	Am1;Am4; Am6; Am9; Am12; Am13; Am14; Am17; Am19; Am21; Am23; Am24; Am25; (Am3;Am5;Am10; Am8; Am15)	Microfermentation of healthy grapes[c]	16S-23S rRNA gene ITS	JF346084- JF346096; (-)
A. pasteurianus	LMG 1262^T	Beer	16S rRNA gene	JF793964
	DSM 46617	-	-	-
	LMG 1605	Vinegar brews	-	-
	LMG 1553	Spoiled beer	-	-
	DSM 2006	-	-	-
	Ap8; Ap15; (Ap 1-Ap7; Ap9-Ap14; Ap 16)	Microfermentation of healthy grapes[c]	16S rRNA gene	JF346078; JF346079; (-)
	LMG 1590 [b]	-	16S-23S rRNA gene ITS	GU205102
	LMG 1591 [b]	-	16S-23S rRNA gene ITS	GU205103
	NBRC 13754 [b]	-	16S rRNA gene	AB680508

	LMG 1925; NBRC 16470[T]	Coconut juice	16S rRNA gene	AB681066
A. tropicalis	LMG 1663 [b]	Fermenting putrified meat sample	16S-23S rRNA gene ITS	FR716480
	At3; At4; At5; At1	Microfermentation of healthy grapes[c]	16S rRNA gene	JF346075; JF346076; JF346077; -
A. aceti	DSM 3508[T]; LMG 1261[T]	Alcohol turned to vinegar	16S rRNA gene	JF793947
			16S-23S rRNA gene ITS	AJ007831
	LMG 1512 [b]; IAM 1802 [b]	Film in fermentor of rice vinegar	16S-23S rRNA gene ITS	AB161358
	LMG 1531 [b]	-	16S rRNA gene	AJ419840
	LMG 1496	-	-	-
	LMG 1505	Quick vinegar	-	-
	CCM 3620 [b]	-	16S-23S rRNA gene ITS	AJ621845
A. oeni	LMG 21952[T]; NBRC 105207[T]	Spoiled red wine	16S rRNA gene	AB682244
A. lovaniensis	LMG 1579[T]	Sewage on soil	16S rRNA gene	AJ419837
A. cibinongensis	LMG 21418[T]; NBRC 16605[T]	*Annona montanae*	16S rRNA gene	AB681085
			16S-23S rRNA gene ITS	FR716481
A. pomorum	LMG 18848[T]	Cider vinegar fermentation	16S rRNA gene	AJ419835
			16S-23S rRNA gene ITS	EU449498
A. indonesiensis	LMG 19824[T]; NRIC 0313[T]	Fruit of zirzak	16S rRNA gene	AB032356
			16S-23S rRNA gene ITS	FR716479
A. peroxidans	LMG 1635[T]	Ditch water	16S rRNA gene	JF793969
			16S-23S rRNA gene ITS	FR716485
A. syzygii	LMG 21419[T]; NBRC 16604	*Syzygium malaccense*	16S rRNA gene	AB681084
			16S-23S rRNA gene ITS	FR716487

A. orleanensis	LMG 1583^{T}	Beer	16S rRNA gene	AJ419845
			16S-23S rRNA gene ITS	FR716478
A. estunensis	LMG 1626^{T}	Cider	16S rRNA gene	AJ419838
			16S-23S rRNA gene ITS	FR716484
A. orientalis	LMG 21417^{T}; NBRC 16606^{T}	*Canna hybrida*	16S rRNA gene	AB681086
			16S-23S rRNA gene ITS	FR716482
A. nitrogenifigens	LMG 23498^{T}; NBRC 105050^{T}	Kombucha tea	16S rRNA gene	AB682235
			16S-23S rRNA gene ITS	FR716483
Ga. europaeus	DSM 6160^{T}; LMG 18890^{T}	Submerged culture vinegar generator	16S rRNA gene	AB205220
			16S-23S rRNA gene ITS	X85406
	DSM 2004	-	-	-
Ga. hansenii	DSM 5602^{T}; LMG 1527^{T}	Vinegar	16S rRNA gene	JF793987
			16S-23S rRNA gene ITS	AJ007832
	LMG 1524	Vinegar	-	-
	LMG 1529	Malt vinegar brewery acetifiers	-	-
Ga. xylinus	LMG 1515$^{T\,b}$; NBRC 15237$^{T\,b}$	Mountains ash berries	16S rRNA gene	AB205216
			16S-23S rRNA gene ITS	X85405
	DSM 2325	-	-	-
Ga. liquefaciens	DSM 5603^{T}; IFO 12388^{T};	Dried fruit	16S rRNA gene	X75617
	LMG 1382^{T}		16S-23S rRNA gene ITS	AJ007833
Ga. intermedius	LMG 18909$^{T\,b}$	Commercially available tea fungus beverage (Kombucha)	16S rRNA gene	JF793990
Ga. oboediens	LMG 18849$^{T\,b}$	Red wine vinegar fermentation	16S rRNA gene	JF794004
			16S-23S rRNA gene ITS	FR716490

Ga. johannae	DSM 13595^{T}	Rhizosphere of coffee plants	16S rRNA gene	JF793992
			16S-23S rRNA gene ITS	FR716502
G. oxydans	DSM 7145^{T}; NBRC 14819^{T}; DSM 3503^{T}	Beer	16S rRNA gene	X73820
			16S-23S rRNA gene ITS	AB163869
	DSM 2003	-	-	-
	LMG 1674$^{T\,b}$	Beer	16S-23S rRNA gene ITS	GU205104
G. thailandicus	NBRC 100600$^{T\,b}$	Flower, *Millingtonia hortensis*	16S rRNA gene	AB681206
	F142-1^{b}; F149-1^{b}	-	16S-23S rRNA gene ITS	AB127941; AB127942
G. japonicus	NBRC 3271$^{T\,b}$	Fruit, *Myrica rubra*	16S rRNA gene	AB253435
	Gj1;Gj3; Gj2	Microfermentation of healthy grapes^{c}	16S rRNA gene	JF346080; JF346081;-
G. albidus	NBRC 103509$^{T\,b}$	Fruit of apple guava, *Psidium guajava*	16S rRNA gene	AB682096
G. roseus	NBRC 3990$^{T\,b}$	-	16S rRNA gene	AB178429
G. sphaericus	NBRC 12467$^{T\,b}$	Grape	16S rRNA gene	AB178431
G. cerinus	NBRC 3267$^{T\,b}$	Cherry, *Prunus sp.*	16S rRNA gene	AB178406
			16S-23S rRNA gene ITS	AB111899

[a] Some strains were obtained from different culture collections (^{T}, Type strain): DSM-DSMZ, German Collection of Microorganisms and Cell Cultures; BCCM-LMG, Belgian coordinated collections of microorganisms; NBRC, Biological Resource Center. National Institute of Technology and Evaluation, Japan; IFO, Institute for fermentation. Osaka, Japan; NRIC Nodai Research Institute Culture Collection,, Tokyo, Japan; CCM, Czech Collection of Microorganisms, Masaryk University, Brno, Czeck Republic; IAM, Institute of Applied Microbiology, University of Tokyo, Institute of molecular and cellular bioscience, Tokyo, Japan.

[b] These strains were tested only *in silico*

[c] These strains were recovered from healthy grapes collected in the Canary Islands (Spain).

All primers and TaqMan-MGB probes were synthesized by Applied Biosystems (Foster City, CA, USA).

2.3.-DNA extraction and conditions of RT-PCR with Taqman-MGB probes

Genomic DNA from AAB was extracted according to the CTAB method (Cetyltrimethylammonium bromide) described by Ausubel et al. (1992). In the case of LAB and yeast, the DNA extraction methods were described by Reguant and Bordons (2003) and Querol et al. (1992), respectively. DNA concentration and purity were determined using a NanoDrop^{TM} 1000 Spectrophotometer (Thermo Scientific, Whaltham, MA, USA).

Different combinations of primer concentrations (100 nM, 200 nM) and TaqMan-MGB probe concentrations (100 nM, 200 nM, 300 nM) were tested to optimize the RT-PCR.

The amplification reactions were performed in a total volume of 25 µl. The PCR mixture was prepared with 1X TaqMan Universal Master Kit (Applied Biosystems, Foster City, CA, USA), 5 µl of extracted DNA (ranging from 50 to 100 ng/µl) and the empirically determined concentrations of primers and probes for each species. A protocol that included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 15 s, and annealing at 62 °C for 1 min was performed for all analyses in triplicate on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The cycle threshold (C_T) was determined automatically. Three samples without a template were always included as a no template control (NTC).

2.4.- Standard curves

Standard curves were created by plotting the C_T values of the RT-PCR performed on a dilution series of AAB DNA and cells (10^7 to 10^2 cells/ml) against the log of input cells/ml. The standard curves were constructed with AAB grown on GY medium. The

confidence intervals were calculated using Student's t-test, with a significance level of 5%.

2.5.- PCR enrichment for target DNA

Enrichment PCR amplification was performed with the forward primer MACER-F (described above) and specific reverse primers designed in the 16S-23S rRNA gene ITS region using the sequences available in the GenBank databases. The reverse primer for *A. malorum* was MAL-R (5' CCATGCGACTAAGTGACACGTCT 3'), whereas that for *A. cerevisiae* was CER-R (5' CCATGCCACTAAGTGACACTTCC 3'). All the primers were synthesized by Applied Biosystems (Foster City, CA, USA). The PCR conditions were the same as those described by Torija et al. (2010). Briefly, a 0.3 µM concentration of the specific primers was used to amplify 50-100 ng of AAB DNA with 0.5 U EcoTaq DNA Polymerase (Ecogen, Spain) using a Gene AmpPCR System 2700 (Applied Biosystems, Foster City, CA, USA) with the following program: 1 cycle at 94 °C for 5 min; 15 cycles at 94 °C for 15 s and at 62 °C for 30 s.

2.6.- Samples inoculated with AAB

Samples of red wine and wine vinegar were sterilized with disposable cellulose acetate filter units of 0.2 µm pore diameter (Whatman GmbH. Dassel, Germany). These two matrices, in addition to GY medium, were inoculated with a mixture of seven AAB species: *A. aceti*, *A. pasteurianus*, *A. malorum*, *A. cerevisiae*, *Ga. hansenii*, *Ga. europaeus* and *G. oxydans*. The concentration of each inoculum was 10^6 cells/ml. The samples were incubated for 24 h at 4°C to prevent cell growth, and the population of each was determined by enumeration under a microscope. The matrix effect was assayed by detecting different AAB species using the TaqMan-MGB probes designed in this study and the five previously described by Torija et al., (2010).

These wine and vinegar samples were treated with PVP-EDTA (0.15 M NaCl, 0.1 M EDTA and 2% w/v PVP (polyvinyl pyrrolidone; Sigma-Aldrich, Madrid)) to eliminate PCR inhibitors (Jara et al., 2008). The samples were centrifuged, and the pellets were washed twice with PVP-EDTA and once again with sterile water. The DNA isolation of the samples was performed in triplicate by the CTAB method (Ausubel et al., 1992).

The detection and enumeration for *A. malorum* and *A. cerevisiae* were performed as described above and the procedure used for the species *A. aceti*, *A. pasteurianus*, *Ga. hansenii*, *Ga. europaeus* and *G. oxydans* was conducted using the RT-PCR TaqMan-MGB probes proposed by Torija et al. (2010), PCR mixture (25 µl) contained 100 nM of each primer, 200 nM of each probe, 1X TaqMan Universal Master Kit (Applied Biosystems, Foster City, CA, USA) and 5 µl of extracted DNA (ranging from 50 to 100 ng/µl). The thermal cycling program was the same as in section 2.3, and was performed on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

The enumeration of the total AAB populations was determined by RT-PCR with SYBR-Green fluorescence detection following the method described by González et al. (2006). The calibration curves for each type strain of AAB species were constructed using ten-fold dilution series of DNA extracted from pure cultures of 10^8 cells/ml. As reported by González et al. (2006) and confirmed in this study, all AAB strains yielded equivalent calibration curves using this method. The primers used for the amplification were AQ1F and AQ2R (González et al., 2006), and the reactions were carried out in a total volume of 25 µl, containing 5 µl of DNA solution, 12.5 µl SYBR-Green universal PCR master mix (Applied Biosystems, Foster City, CA, USA), 600 nM of each primer and 4.5 µl of sterile H_2O. The amplification was performed on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions took place after 2 min at 50°C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

The C_T value was determined automatically by the instrument, and NTC reactions were used as negative PCR controls. The linearity of the reactions was conserved in a range from 10^7 to 10^2 cells/ml.

2.7.- Environmental samples from wines produced in the Canary Islands

The study of a total of 19 different wine samples used samples recovered from cellars at different stages of vinification: samples 1 and 2 from the middle of fermentation, and the others from finished wines. These samples were collected from 14 different wineries located in Tenerife (Canary Islands, Spain) by the Applied Microbiology Department of the Instituto Canario de Investigaciones Agrarias (Canary Islands, Spain) and were sent to the Universitat Rovira i Virgili (Tarragona, Spain) for their analysis.

One and a half milliliters of every sample was centrifuged, and the pellets were treated with PVP-EDTA, as described in section 2.6. After DNA extraction, which was performed by the CTAB method (Ausubel et al., 1992), the samples were analyzed by RT-PCR with the seven TaqMan-MGB probes and with SYBR-Green as described above.

Every wine sample was also plated onto GY medium (1% yeast extract, 5% glucose, 1.5% Agar) supplemented with natamycin (100 mg/L) (Delvocid, DSM; Delft, The Netherlands) to suppress fungal growth. After 2 days of incubation at 28 ^oC, the colonies were observed under the microscope and were grown on GYC medium (10% glucose, 1% yeast extract, 2% $CaCO_3$, 1.5% Agar). The production of acid was determined by the dissolution of $CaCO_3$ precipitates on the plates. The colonies with a clear halo around them were subjected to the catalase test; positive results supported their putative identity as AAB. These isolates were picked from plates, used to inoculate liquid GY medium, and grown for 48 h at 28^oC for DNA extraction (Ausubel et al., 1992). For AAB identification, the 16S rRNA gene and 16S-23S rRNA gene ITS were

amplified using the method described by Ruiz et al. (2000). The amplified DNA from both regions was purified and sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. The DNA sequences were compared with those in the GenBank databases.

The analyses described in this work were performed in a research laboratory that meets the 9001 ISO regulation.

3. - Results

3.1. - Design and specificity of RT-PCR with TaqMan-MGB probes for A. malorum and A. cerevisiae

Primers and probes for *A. malorum* and *A. cerevisiae* species were designed to detect 16S-23S gene ITS region using the sequences available in the GenBank database. The sequences of different strains from the same species were aligned with ClustalW software to determine the consensus sequence and design the primers and probes for *A. malorum* and *A. cerevisiae*. The percentage of similarity in the 16S-23S gene ITS sequences between the type strains of both species, LMG 1746^T (*A. malorum*) and LMG 1625^T (*A. cerevisiae*), is very high (98%); there are only 16 different base pairs between them, which made the design of the primers and probes difficult. Due to this high level of similarity, the primers for both species have the same sequence, and species-specificity is determined by the probes.

Although several concentrations of the primers and the probes were assayed, the best results with the target species were obtained with 100 nM for each primer and 200 nM for the probes.

The specificity of the primers and probes was tested *in silico* and using the genomic DNA of AAB species from different culture collections (Table 1). Most of these strains

belonged to the genus *Acetobacter* because they were closely related to the target species *A. malorum* and *A. cerevisiae*.

In silico, there was at least a two-base-pair difference between the TaqMan-MGB probes and the available sequences of the species tested; therefore, only AAB-specific fluorescent signals were expected. However, in the case of the *A. orleanensis* type strain, a positive fluorescent signal with the *A. cerevisiae* probe was observed, despite the presence of a two-base-pair difference with the probe. This difference was located at the 5'- end but was not sufficient to make the probe specific only for *A. cerevisiae*.

Because there are few strains of *A. malorum* and *A. cerevisiae* deposited in culture collections, we also tested the specificity of the probes with 54 AAB strains isolated from microfermentations of healthy grapes from the Canary Islands (Spain) (Table 1). All of the strains were previously identified by 16S rRNA gene and 16S-23S gene ITS sequencing. In the RT-PCR assay, they yielded positive results only with their specific probes (data not shown).

AAB commonly coexist with other groups of microorganisms in different environments. In the case of grapes, wine and vinegar, these microorganisms are often LAB and yeast. For this reason, the specificity of these probes was determined with six strains of different LAB species (*Oenococcus oeni* (CECT217^T); *Pediococcus (P.) parvulus* (CECT813), *P. pentosaceus* (CECT4695^T), *Leuconostoc mesenteroides* (CECT219^T), *Lactobacillus (L.) plantarum* (CECT220), *L. brevis* (CECT4121^T)) and 13 strains belonging to 11 different yeast species (*Candida (C.) boidini, C. stellata, C. mesenterica, C. sake, Dekkera anomala, Hanseniaspora (H.) uvarum, H. guillermondii, Issatchenkia terricola, Saccharomyces cerevisiae, Torulaspora delbrueckii, Zygosaccharomyces rouxii*). None of the probes detected any organisms except for the

A. cerevisiae probe, which showed a faint signal for a highly concentrated DNA from *C. sake.*

3.2. - Accuracy, sensitivity and quantification limit of RT-PCR with TaqMan-MGB probes for A. malorum and A. cerevisiae

Fig. 1 shows the standard curves for *A. malorum* and *A. cerevisiae* species obtained using 10-fold serially diluted genomic DNA extracted from 10^8 cells/ml of each species. For both of these species, the detection limit was 10^2 cells/ml (C_T = 30.8 – 32.0), and the efficiency, estimated by the formula ($E = 10^{-1/slope} - 1$), was >80%. Therefore, this technique was able to detect a DNA concentration as low as 5×10^{-5} ng/ml in the most dilute solution.

Additionally, we tested the accuracy of quantification for these two new probes in the presence of DNA extracted from 10^7 cells/ml of non-target AAB species. The presence of other AAB species did not produce any interference in the quantification, which yielded the same values that we expected to detect for the target species. However, we observed a strong interference between the species *A. malorum* and *A. cerevisiae* when 100-fold excess of non-target DNA was present in the sample, producing unexpected C_T values.

This issue was solved by increasing the amount of target DNA compared to non-target DNA by PCR enrichment of the target DNA in an earlier step. This enrichment was a more species-specific PCR consisting of 15 cycles of amplification in a regular thermocycler (end-point PCR); the amplicons obtained were then used for the RT-PCR.

To optimize and test this new step in the protocol, a standard curve for each species was generated with and without PCR enrichment. In all cases, the standard curves with the specific RT-PCR maintained linearity in the range of 10^7 to 10^2 cells/ml and showed good correlation with regression values (r^2) > 0.98 (Fig. 1). Therefore, the detection

limit and the efficiency remained at the same levels as those obtained without PCR enrichment (10^2 cells/ml and > 80%).*3.3. - Specificity of RT-PCR with previously designed TaqMan-MGB probes*

We also evaluated the species-specificity of the previously reported TaqMan-MGB probes for *A. aceti*, *A. pasteurianus*, *Ga. hansenii*, *Ga. europaeus* and *G. oxydans* (Torija et al., 2010) and addressed the changes in the taxonomy of AAB and the appearance of new species in this group of bacteria.

The *A. pasteurianus* probe, as reported in Torija et al. (2010), is 100% identical to the closely related species of *A. pomorum* and *A. aceti*, as well as *A. oeni* and *A. nitrogenificens*. No other similarities were detected in this study. However, because the primer used for RT-PCR and/or for the PCR enrichment does not anneal to these closely related sequences, the primers had specificity for the target species. The probe designed for *Ga. hansenii* was reported to hybridize exclusively with sequences found in this species; however, in this study, it was observed *in silico* that the same sequence region in *Ga. kombuchae* is 100% identical to this probe. The probe designed for *Ga. europaeus* anneals *in silico* to *Ga. saccharivorans* sequences, as well as to other, previously reported species (*Ga. swingsii*, *Ga. rhaeticus*, *Ga. oboediens* and *Ga. nataicola*). Nevertheless, the primers for PCR enrichment make this probe specific, and no amplification was detected by RT_PCR. Finally, for *G. oxydans*, the probe and primers were reported as identical for all the species of *Gluconobacter* genus; however, it was observed *in silico* that the ITS region for the PCR enrichment is identical to the sequence of *G. albidus* but not to those of *G. cerinus*, *G. japonicus*, *G. nephelii*, *G. roseus* and *G. thailandicus*. The DNA of *G. japonicus* strains was tested, and no signal was detected.

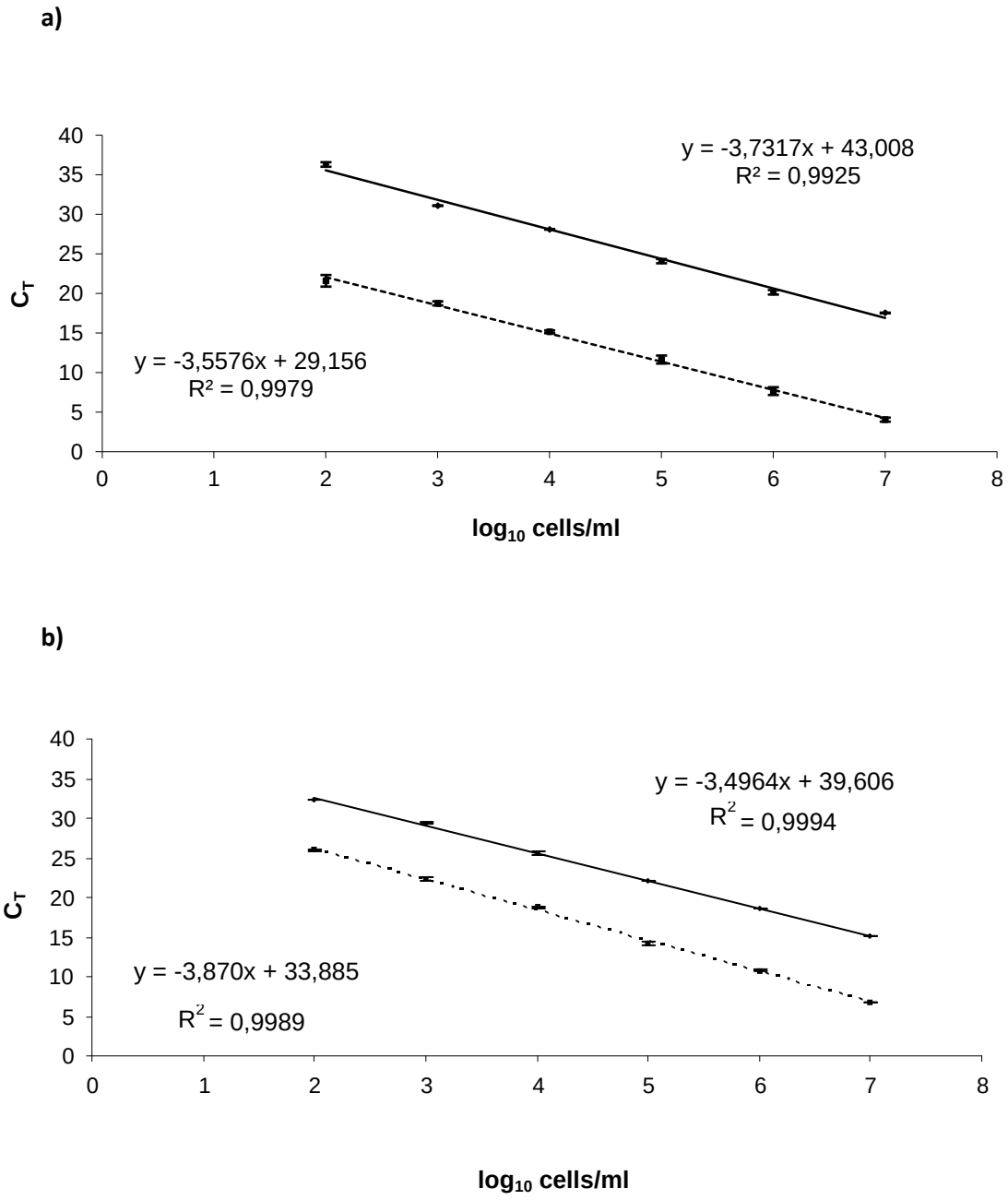

Fig. 1. Standard curves obtained from 10-fold serial DNA dilutions of *A. malorum* (a) and *A. cerevisiae* (b). The RT-PCR was performed with (dotted line) and without (solid line) PCR enrichment. Standard errors are represented by vertical bars.

3.4. - Effect of the matrix on the quantification of specific DNA in the presence of exogenous DNA

The aim of this assay was to test the effect of the complex matrices, such as wine or vinegar, in the enumeration of *A. malorum* and *A. cerevisiae*. In addition, to evaluate the interference resulting from the presence of other AAB species in these samples, three different matrices (wine, vinegar and GY medium) were inoculated with a mixture of *A. aceti*, *A. pasteurianus*, *A. malorum*, *A. cerevisiae*, *Ga. hansenii*, *Ga. europaeus* and *G. oxydans* at a concentration of 10^6 cells/ml each. The seven available TaqMan-MGB probes were used to study the matrix effect.

The RT-PCRs were performed with and without PCR enrichment. When the assays were performed without PCR enrichment, the quantity of detected organisms in the GY medium was close to the known quantity of organisms, but this was not true in wine or vinegar. However, when the PCR enrichment was applied, the enumeration of every species was closer to the expected values in all media (Table 2). The standard curves in wine and vinegar presented efficiencies higher than 80%, and the detection limits were similar to the limit observed in GY medium for all of the TaqMan-MGB probes.

3.5. - AAB analysis from wine samples produced in the Canary Islands

The 19 wine samples from the Canary Islands were analyzed by RT-PCR, using SYBR-Green fluorescence to quantify the total number of AAB and the seven TaqMan-MGB probes for species enumeration. The latter technique was always used with PCR enrichment, due to the higher accuracy observed with this step.

Table 3 shows the quantification results obtained in the wine samples. The total AAB observed was between 9×10^2 and 10^6 cells/ml, and the seven species tested were detected in more than one sample.

Table 2. AAB enumeration by RT-PCR in different matrices (wine, vinegar and GY medium) inoculated with 10^6 cells/ml of each species. Effect of the PCR enrichment on the AAB species quantification with RT-PCR TaqMan-MGB probes.

Species detected by TaqMan-MGB probes	Wine (cell/ml)		Vinegar (cell/ml)		GY medium (cell/ml)	
	Without PCR enrichment	With PCR enrichment	Without PCR enrichment	With PCR enrichment	Without PCR enrichment	With PCR enrichment
A. cerevisiae	6,62E+02	2,37E+05	1,51E+03	1,01E+05	4,42+04	4,28E+05
A. malorum	3,22E+04	7,43E+05	1,32E+02	1,15E+05	4,03E+05	1,33E+06
A. pasteurianus	2,31E+04	3,91E+05	8,11E+04	2,74E+05	1,25E+06	7,22E+05
A. aceti	7,52E+04	2,87E+05	8,92E+02	6,98E+04	9,72E+05	5,97E+05
G. oxydans	7,96E+04	2,33E+06	5,25E+04	7,90E+05	2,43E+06	3,40E+06
Ga. hansenii	1,23E+04	1,08E+06	1,24E+05	1,87E+05	1,25E+06	2,01E+06
Ga. europaeus	9,21E+02	1,31E+06	3,55E+02	1,49E+05	8,11E+03	3,55E+05
Sum of TaqMan-MGB probes quantification	2,24E+05	6,38E+06	2,60E+05	1,69E+06	6,31E+06	8,83E+06
AAB quantification by SYBR-Green[a]	3,83E+06		1,33E+06		9,26E+06	

[a]This value is the mean of the SYBR-Green quantifications performed using each species for constructing the standard curve.

In most samples, the sum of the quantities determined by the seven TaqMan-MGB probes in each sample was very similar to the total AAB determined by SYBR-Green. In only four samples (13-15, 17), the sum was far from the total abundance. Species of *G. oxydans*, *Ga. hansenii* and *Ga. europaeus* were detected in almost all the samples, with enumeration values between 10^2 and 10^6 cells/ml. However, the *Acetobacter* species were detected in fewer samples, especially *A. aceti* (only detected in two samples); these species also exhibited lower enumeration values (between 10^2-10^4 cells/ml). All of these samples were also plated onto GY medium, and 48 colonies with different morphologies were observed under a microscope. These isolates were plated onto GYC medium, where 14 of them produced a clear halo and were classified as putative AAB by a positive catalase test.

These 14 isolates were recovered from only four wine samples (2, 10, 17, and 18) (Table 3). After DNA isolation, these isolates were identified by PCR amplification and sequencing of the 16S rRNA and 16S-23S rRNA gene ITS. The isolates belonged to four different species which can be ordered in terms of isolation frequency: *A. malorum*, *G. oxydans*, *A. cerevisiae* and *A. pasteurianus*. The *A. malorum* and *A. cerevisiae* isolates had identical 16S rRNA gene sequences that differed from their type strain sequences (*A. cerevisiae* LMG 1625 (AJ419843) and *A. malorum* LMG 1746 (AJ419844)). These type strains have only two base pairs of difference in their 16S rRNA gene sequences, and the isolates have a base pair at one position corresponding to *A. cerevisiae* and a base pair at the other position corresponding to *A. malorum*. The identification of these isolates was made possible by analyzing the 16S-23S rRNA gene ITS sequence.

In addition, these 14 isolates were tested by the TaqMan-MGB probes, and in all cases, the results matched with those obtained previously.

Table 3. Results of AAB analysis from wine samples produced in the Canary Islands.

	TaqMan-MGB probes RT-PCR							Summa TaqMan-MGB quantification	SYBR-Green quantification Total AAB	Species identified of isolates (num of colonies)
	A. cerevisiae cells/ml	*A. malorum* cells/ml	*A. pasteurianus* cells/ml	*A. aceti* cells/ml	*G.oxydans* cells/ml	*Ga. hansenii* cells/ml	*Ga. europaeus* cells/ml			
B1	<100	<100	4,92E+02	<100	1,16E+02	<100	<100	6,08E+02	8,76E+02	nr
B2	<100	1,40E+02	<100	<100	3,94E+03	6,02E+05	6,56E+04	6,72E+05	1,69E+05	*G. oxydans* (1)
B3	1,03E+02	9,32E+02	<100	<100	5,69E+04	1,22E+05	1,86E+04	1,99E+05	3,25E+05	nr
B4	<100	1,06E+02	<100	<100	1,72E+03	8,81E+03	1,23E+03	1,19E+04	2,35E+04	nr
B5	<100	<100	<100	<100	6,31E+02	7,45E+03	7,74E+02	8,85E+03	1,72E+04	nr
B6	<100	2,47E+02	<100	2,47E+02	8,25E+02	8,10E+03	6,55E+02	1,23E+04	3,42E+04	nr
B7	<100	3,29E+02	<100	<100	5,18E+02	1,67E+03	<100	2,51E+03	5,83E+03	nr
B8	<100	7,20E+02	<100	<100	1,56E+03	1,93E+04	<100	2,15E+04	5,64E+03	nr
B9	<100	1,99E+02	<100	<100	1,51E+03	5,27E+03	5,36E+02	7,51E+03	2,92E+04	nr
B10	1,52E+03	9,26E+03	<100	<100	3,09E+04	7,48E+04	1,16E+05	2,32E+05	3,92E+04	*A. malorum* (6) *G. oxydans* (4)
B11	<100	<100	<100	<100	7,32E+02	3,34E+03	1,63E+03	5,71E+03	1,15E+04	nr
B12	1,27E+03	1,12E+03	4,98E+02	<100	3,68E+02	2,63E+05	5,50E+03	2,75E+05	1,16E+05	nr
B13	5,47E+02	4,37E+02	6,51E+02	<100	2,24E+03	2,72E+02	3,44E+02	4,49E+03	9,73E+04	nr
B14	<100	<100	<100	<100	3,43E+02	1,45E+03	1,74E+03	3,53E+03	1,05E+05	nr
B15	<100	<100	<100	<100	1,14E+03	1,75E+03	8,90E+02	3,78E+03	2,40E+04	nr
B16	9,28E+02	4,73E+03	7,92E+02	4,73E+04	1,10E+04	9,59E+05	2,20E+05	1,24E+06	1,17E+06	nr
B17	7,84E+02	<100	<100	<100	2,08E+03	3,55E+04	2,07E+03	4,04E+04	1,38E+05	*A. pasteurianus* (1)
B18	1,72E+02	<100	6,99E+02	<100	1,19E+03	3,62E+04	1,27E+03	3,95E+04	5,08E+04	*A. cerevisiae* (2)
B19	<100	<100	2,45E+05	<100	<100	3,49E+03	2,44E+02	2,48E+05	7,11E+05	nr

nr: No recovery from plates were obtained.

4.- Discussion

The detection, quantification and identification of AAB during wine or vinegar production are important to better understand AAB population dynamics, as well as to maintain the quality of the final product. The difficulties in cultivating AAB using common laboratory media, especially when the samples come from extreme environments, such as wine and vinegar, are commonly reported (Sievers et al., 1992; Sokollek et al., 1998; Millet and Lonvaud-Funel, 2000; Trcek, 2005). Culture-independent techniques bypass this culture step and enable the identification of microorganisms that are undetectable by traditional methods.

However, the isolation of strains and their maintenance in pure cultures is useful for industrial purposes. Both approaches are complementary and should be taken into account.

In our previous work, we described five TaqMan-MGB probes designed to detect, enumerate and identify different species of AAB (Torija et al., 2010). The main advantage of using TaqMan-MGB probes is their high specificity and their ability to discriminate between two sequences that differ by only one base pair (Kutyavin et al., 2000; van Hoeyveld et al., 2004; Yao et al., 2006; Alonso et al., 2007). For AAB, this characteristic is especially useful to distinguish between closely related species in which the commonly analyzed genes are very similar. Recent taxonomical changes in the AAB group and the description of new species complicate the identification of species by analysis of the commonly used 16S rRNA gene. The Multilocus Sequence Analysis (MLSA), which classifies strains of AAB into their taxonomical groups by housekeeping genes, involves the analysis of more than one gene (Cleenwerck et al., 2009). It is a powerful tool for discriminating between species, but the lack of availability of gene sequences makes species comparisons difficult. Furthermore,

organism isolation is required for the species analysis of each single strain. An alternative technique proposed to identify AAB species when the 16S rRNA gene is not sufficient to resolve differences is the sequencing of 16S-23S rRNA gene ITS (González and Mas, 2011; Valera et al., 2011). This technique has the advantage of abundant sequence information available for this region of AAB genomes in public databases. In the case of very closely related species, such as *A. malorum* and *A. cerevisiae*, analyses of the 16S rRNA gene were not sufficient to identify the isolates belonging to these species. However, comparisons of the 16S-23S rRNA gene ITS were able to resolve isolate classifications, as has been observed previously (Valera et al., 2011).

The detection of *A. malorum* and *A. cerevisiae* species was first reported in isolates from rotting apples and breweries, respectively, (Cleenwerck et al., 2002). Their detection has increased notably in the intervening years in different fruit vinegars or fermented beverages (Escalante et al., 2008; Hidalgo et al., 2012a), and they are commonly found on healthy grapes from Chile (Prieto et al., 2007) or the Canary Islands (Valera et al., 2011). These two species are phylogenetically close to *A. aceti*, and it is likely that some earlier reports of *A. aceti* detection could refer to either of these other two species (Valera et al., 2011). In the present study, TaqMan-MGB probes were designed in the ITS region to detect *A. malorum* and *A. cerevisiae* by an RT-PCR culture-independent method and to discriminate between them. The high sequence similarity (98%) in this ITS sequence demonstrates the phylogenetical closeness of these species, whereas the variability between strains belonging to the same species in this intergenic sequence made the design of species-specific probes difficult. Despite this high intraspecific variability there are regions that correspond to transfer RNA ($tRNA_{Ile}$ and $tRNA_{Ala}$) which are highly conserved within strains of the same species

(Sievers et al., 1996) and which are adequate for primers and probes design (González and Mas, 2011). The primers and probes for *A. malorum* and *A. cerevisiae* designed in this study are inside these regions or close of them (data not shown).

The specificity of the probes was a notable success, with the ability to differentiate between any strains of these species without interference from the presence of other non-target species, except for *A. orleanensis* with the *A. cerevisiae* probe. The 16S rRNA gene sequence of the *A. orleanensis* type strain LMG 1583^T (AJ419844) has only two base pair differences, compared with the same gene in *A. malorum* LMG 1746^T (AJ419844) and *A. cerevisiae* LMG 1625^T (AJ419843); however, the sequences of the 16S-23S rRNA gene ITS are 91% similar. Despite the fact that there was a two-base-pair difference between the sequences, the *A. cerevisiae* probe yielded a positive result for the *A. orleanensis* type strain. The *A. orleanensis* species had been initially considered an *A. aceti* subsp. *orleanensis* (De Ley and Frateur, 1974), and it was isolated from beer and rice vinegar (Cleenwerck et al., 2002) but never on grapes or in wine.

Due to the identification of new species in the AAB group, the specificity of previously designed probes (Torija et al., 2010) was tested again in this study *in silico*. All of the probes maintained the specificity, except for the species *Ga. kombuchae* and *Ga. saccharivorans*, which hybridized with *Ga. hansenii* and *Ga. europaeus* probes, respectively. The species *Ga. saccharivorans* was recently described both in wine fermentation (Kato et al., 2011) and on healthy grapes (Valera et al., 2011); however, *Ga. kombuchae* has been recovered only in kombucha tea thus far (Dutta and Gachhui 2007).

The sensitivity of this RT-PCR technique reached values of 10^2 cells/ml, without prior concentration of the sample. A previous study, which used culture-independent

techniques, reported that the total amount of AAB in vinegar ranges from 10^6 to 10^8 cells/ml, and that in wine ranges from 10^4 to 10^5 cells/ml (Andorrà et al., 2008). In the presence of other non-target AAB species, the accuracy of RT-PCR with these probes is not reduced, except when *A. cerevisiae* and *A. malorum* species are together. The specificity between these two species is determined only by the probes because the primers are the same for the two species and, thus, both are amplified. This fact reduced the sensitivity of the probe for the target DNA when more than a 100-fold concentration of non-target DNA was present in comparison to the target DNA. The use of a PCR enrichment step solved this issue. This pre-amplification step was applied by Torija et al. (2010) to increase the specificity of TaqMan-MGB probes whenever other non-target DNA was overly abundant in the samples, and it was also used by González et al. (2005b) to detect the minor microorganisms present in environmental samples.

The use of this PCR enrichment technique is also adequate to overcome the difficulties of the AAB detection by RT-PCR in wine or vinegar. Although the extraction method was optimized for the direct detection of AAB in wine and vinegar (Jara et al., 2008), the presence of PCR inhibitors (polyphenols, tannins and polysaccharides) affects the DNA quality and, therefore, negatively affects the PCR (De Vero et al., 2006; Hierro et al., 2006; Ilabaca et al., 2008; Jara et al., 2008; Mamlouk et al., 2011). The effect of these matrices on DNA extraction, together with the high amount of non-target species, was reflected in the low accuracy of the AAB species quantification, which was also observed by Torija et al. (2010). In this study, the application of a PCR enrichment step before the RT-PCR increased the number of copies of target DNA and decreased the concentration of inhibitors, which resulted in more accurate quantification values for wine and vinegar samples. After solving these issues, the environmental samples from wines produced in the Canary Islands were successfully enumerated by the RT-PCR

technique with TaqMan-MGB probes specific to AAB species after PCR enrichment. The total numbers of AAB cells resulting from the addition of the seven species that were analyzed individually were similar to those obtained by the RT-PCR with SYBR-Green.

This latter method allowed for a good estimation of the total AAB population, detecting between 10^2-10^5 cell/ml in mid-fermentation samples and between 10^2-10^3 cell/ml at the end of the fermentation (Joyeux et al., 1984; Du Toit and Lambrechts, 2002; Bartowsky et al., 2003; González et al., 2004). However, in the latter samples, higher populations of AAB (up to 10^4-10^5 cell/ml) have been detected when culture-independent techniques were used (Andorrà et al., 2008). Among the analyzed samples, two reached concentrations of over 7×10^5 cells/ml, indicating that they could be spoiled wines or with high risk of spoilage.

The highest AAB species enumeration by RT-PCR was observed for *Ga. hansenii* and *Ga. europaeus*. These species were present in most of the samples, as well as *G. oxydans*. This latter species has been reported as one of the most commonly recovered AAB species in grape must, and its presence decreased during the fermentation, practically disappearing at the end of the fermentation (Joyeux et al., 1984). In this study, this species was also among the most frequently isolated; however, it was observed that the population was always approximately 10^3 cells/ml by RT-PCR. It is important to note that the TaqMan-MGB probe targeted for *G. oxydans* also hybridizes with all the species belonging to the genus *Gluconobacter* that have been described to date. Therefore, it is possible that other species were present in the samples, although the recovery of such species was impossible.

Ga. hansenii was another species that has been previously reported in wines in mid-fermentation (González et al., 2005a) and also at the end of fermentation (Du Toit and

Lambrechts, 2002). Curiously, *Ga. europaeus* was commonly found in vinegar (Sievers et al., 1992; Trcek et al., 2000; Vegas et al., 2010) but not in wines. Another species frequently identified in wines at the end of fermentation was *A. pasteurianus* (Du Toit and Lambrechts, 2002; Bartowsky et al., 2003), which was also detected in some of the wine samples; the recovery of this species on plate was possible from a single sample, despite its low abundance. *A. aceti*, which was often detected in wines from must to the end of fermentation, was poorly quantified in these samples. In microfermentations of grapes collected from the Canary Islands (Valera et al., 2011), no isolates were identified as *A. aceti* species. Furthermore, *A. malorum* and *A. cerevisiae*, which were the main isolates obtained on plates both in this study and in the previous study of Valera et al. (2011), were quantified at low levels by RT-PCR (always below 10^4 cells/ml), although they were detected in a large number of samples. When these two species were present in the same sample, we could detect both, although there were 10-fold higher populations of one of the species. The closest related environments in which *A. malorum* had also been detected was in Traditional Balsamic Vinegar (Gullo et al., 2006) and in Shanxi aged vinegar from China (Wu et al., 2012), whereas *A. cerevisiae* had been detected in a previous study of Chilean grapes (Prieto et al., 2007). In addition, some isolates that were classified as *A. aceti* in previous studies may be considered *A. malorum* or *A. cerevisiae* because these other species were not described at the time of those studies.

The results obtained by total enumeration of AAB with SYBR-Green were similar to the sum of the partial results for each species obtained with TaqMan-MGB probes. This fact demonstrates the accuracy of the quantification using this method. However, in three samples, the value of the sum of the results obtained using TaqMan-MGB probes was 10 times lower than the total amount determined by SYBR-Green. It is possible that

other major species were present in the samples but none of the probes could detect them.

Growth of organisms on plates was poor compared with the results obtained from the RT-PCR. Only some colonies were identified as AAB from four wine samples. In agreement with our findings, culture-dependent approaches allow for poor recoveries on plates relative to the total enumeration by techniques such as microscopy (Millet and Lonvaud-Funel, 2000; Bartowsky et al., 2003).

Using TaqMan-MGB probes, we can specifically detect two very closely related species, *A. malorum* and *A. cerevisiae*. The quantification of these microorganisms is reliable over a range wide enough to be suitable for wine and vinegar samples. Performing PCR enrichment prior to RT-PCR improves the accuracy of the microorganism quantification, especially in wine and vinegar samples.

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Chapter 3

Acetic Acid Bacteria from Biofilm of Strawberry Vinegar Visualized by Microscopy and Detected by Complementing Culture-Dependent and Culture-Independent Techniques

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Abstract

Acetic acid bacteria (AAB) usually develop biofilm on the air-liquid interface of the vinegar elaborated by traditional method. This is the first study in which the AAB microbiota present in a biofilm of vinegar obtained by traditional method was detected by pyrosequencing. Direct genomic DNA extraction from biofilm was set up to obtain suitable quality of DNA to apply in culture-independent molecular techniques. The set of primers and TaqMan – MGB probe designed in this study to enumerate the total AAB population by Real Time – PCR detected between 8×10^5 and 1.2×10^6 cells/g in the biofilm. Pyrosequencing approach reached up to 10 AAB genera identification. The combination of culture-dependent and culture-independent molecular techniques provided a broader view of AAB microbiota from the strawberry biofilm, which was dominated by *Ameyamaea*, *Gluconacetobacter*, and *Komagataeibacter* genera. Culture-dependent techniques allowed isolating only one genotype, which was assigned into the *Ameyamaea* genus and which required more analysis for a correct species identification. Furthermore, biofilm visualization by laser confocal microscope and scanning electronic microscope showed different dispositions and cell morphologies in the strawberry vinegar biofilm compared with a grape vinegar biofilm.

Keywords: wine, vinegar, next-generation sequencing, PCR-DGGE, 16S rRNA gene.

1. Introduction

Acetic acid bacteria (AAB) are obligate aerobic microorganisms able to efficiently convert ethanol to acetic acid and, the main bacteria responsible for vinegar elaboration.

There are two main vinegar production methods: traditional and submerged. Traditional method is characterized by slow acetification carried out in wood barrels resulting in high-quality vinegar (Solieri and Giudici, 2009). It is a static method, the so-called surface culture method or Orleans method, where AAB are placed on the air-liquid interface, developing a biofilm in direct contact with oxygen. Part of this biofilm, also called "vinegar mother", is used as starter culture to inoculate a new batch for vinegar production through a back-slopping procedure (Gullo et al., 2009).

Biofilm formation is commonly observed in several traditional fermentation processes (Domínguez-Manzano et al., 2012; Wang et al., 2012). This structure has been related with cell-cell communication via quorum-sensing signalization in some bacterial species (Davies et al., 1998), and it provides a protective growth environment that allows to tolerate the extreme conditions and to survive in a hostile medium as vinegar (Solieri and Giudici, 2009). Biofilm produced by AAB is constituted by different exopolysaccharides (EPS), with cellulose as the main EPS (Chawla et al., 2009). The production of cellulose by *Komagataeibacter xylinus* (formerly *Gluconacetobacter xylinus*; Yamada et al., 2012) has been widely studied due to its industrial biosynthesis applicability. These studies were mainly focused on the composition and structure of these EPS pellicles (Chawla et al., 2009; Krishnamachari et al., 2011). However, other AAB genera have also been reported as cellulose producers, like *Gluconacetobacter* (Aydın and Aksoy, 2013) or *Acetobacter* (Gullo et al., 2012).

Molecular techniques have demonstrated their usefulness to determine the AAB diversity in ecological vinegar studies. Culture-dependent techniques are efficient for typing and monitoring AAB strains throughout vinegar elaboration process (Hidalgo et al., 2013; Wu et al., 2012), whereas culture-independent techniques are a good approach to analyze total AAB diversity present in vinegar production (Mamlouk et al., 2011; Xu et al., 2011). Moreover, some studies reported a good correlation between the results obtained by both approaches (Jara et al., 2013; Vegas et al., 2013). As far as we know, there are no studies which detect the AAB population in biofilm of traditional vinegar using culture-independent techniques. The studies from these vinegar biofilms are limited and had always been carried out after a step on culture media (Gullo et al., 2009; Nanda et al., 2001).

The culture-independent techniques based on next-generation sequencing systems, such as pyrosequencing, have been described as a more complete alternative to capture the whole complexity of the communities present in different fermented products (Illeghems et al., 2012; Jung et al., 2011; Roh et al., 2010) and very recently in one of famous vinegars in China obtained by solid-state fermentation technology (Nie et al., 2013). However, it has never been used to study the microbiota present in any other vinegar either in the liquid or in the biofilm matrix.

Therefore, the aim of this study was to analyze the presence of AAB in a biofilm from strawberry vinegar obtained by traditional method, using different molecular methods, including next-generation techniques. The disposition of microbial cells in the strawberry biofilm was also analyzed by laser confocal microscope (LCM) and scanning electronic microscope (SEM) and was compared with a grape vinegar biofilm also obtained by traditional method. The technological aspects of the elaboration of this

strawberry vinegar and the analysis of the yeast and AAB diversity responsible for its production were previously reported (Hidalgo et al., 2010, 2013).

2. Materials and Methods

The strawberry vinegar biofilm analyzed in this study and also the grape vinegar biofilm used for microscopy study were obtained from Mas dels Frares Experimental Cellar (Constantí, Tarragona, Spain). Both pieces of biofilms were collected from the surface of vinegar elaborated in wood barrels using the traditional method. They were samples of approximately 300 mm long × 200 mm wide and 50 grams, each one. The samples were aseptically deposited in a sterile tube, transported to the laboratory, and stored at 4°C before being analyzed. The laboratory where this research was performed meets the ISO 9001 regulations.

2.1. Microscopic study

2.1.1. Laser confocal microscope

Three samples of 25 mm^2 from different parts of each vinegar biofilms (from both ends and from the middle of biofilm) were carefully and aseptically cut and disposed on a glass slide. One microliter of Syto9 dye from LIVE/DEAD BacLight kit (Molecular Probes, Eugene, OR, USA) was directly added to each piece of biofilm and incubated in the dark for 20 minutes. After that, each sample was washed with 2 μL of washing buffer (20 mM Tris-HCl, 0.9; 0.9 M NaCl and 0.1% sodium dodecyl sulfate) to eliminate the excess of dye and visualized in a LCM (Nikon, model TE2000-E).

2.1.2. Scanning electronic microscope

Other three samples of 25 mm^2 from each biofilms were fixed in glutaraldehyde solution (6% glutaraldehyde in 0.1 M phosphate-buffered saline, pH 7.3). They were treated with 1% of osmium tetroxide for 2 hours. After that, the samples were dehydrated using a graded ethanol series. Ethanol was replaced for amyl-acetate before drying to the critical point. The samples were then gold coated for their visualization in a SEM (JEOL model JSM-6400).

2.2. Plate growing

The strawberry vinegar biofilm was plated by triplicate streaking the loop with three samples of approximately 100 mm^2 onto the GY medium (5% glucose, 1% yeast extract, and 1.5% agar) supplemented with natamycin (100 mg/L) (Delvocid, DSM; Delft, The Netherlands) to suppress fungal growth. After 2 days of incubation at 28°C, the colonies were grown on the GYC medium (10% glucose, 1% yeast extract, 2% $CaCO_3$, and 1.5% agar) to determine their acid production by the dissolution of $CaCO_3$ precipitates on plates. The colonies with a clear halo around them were subjected to the catalase test; positive results supported their putative identity as AAB and were identified by molecular methods.

2.3. Genomic DNA extraction

Colonies grown on plates were processed for DNA extraction using the protocol of cetyltrimethylammonium bromide (CTAB) method (Ausubel et al., 1992).

On the other hand, the remaining biofilm was analyzed by culture-independent techniques for which, DNA from biofilm was directly extracted using a protocol that consisted of a pre-treatment of the sample and the method of Ausubel et al., (1992)

modified. The biofilm was aseptically cut in a total of nine portions, each one of five grams. Each biofilm portion was processed to release cells from the matrix as follows: it was stirred at 200 rpm overnight in 100 mL of buffer (100 mM Tris-HCl [pH 8.0], 100 mM Na-EDTA [pH 8.0], 1.5 M NaCl, and 0.1% Tween 80), and then 1 g of glass beads was added to the samples and vortexed for 10 minutes. The resultant solution was vacuum filtered through a paper membrane filter grade 1 (Whatman, Maidstone, UK). Then the membrane was washed twice with 0.1 M NaCl, and the filtrated solution was centrifuged at 4500 rpm for 15 minutes at 4°C. Pellets were washed twice with polyvinylpyrrolidone-EDTA (0.15 M NaCl, 0.1 M EDTA, and 2% w/v polyvinylpyrrolidone; Sigma-Aldrich, Munich, Germany) and once with sterile water for polyphenols elimination. Genomic DNA extraction from these pellets was performed by CTAB method described by Ausubel et al. (1992), with some modifications. Briefly, the pellet was resuspended with 520 μL of TE buffer (10 mM Tris-HCl, 100 mM EDTA, and 0.80 M NaCl, pH 8), 40 μL of lysozyme (20 mg/mL), and 10 μL of RNAse (1 mg/mL) and incubated at 37°C during 1 hour. A quantity of 30 μL sodium dodecyl sulfate 20% and 6 μL of proteinase K (20 mg/mL) was added and incubated at 50°C for 30 minutes. The lysis concluded with CTAB, and the steps of purification with fenol-chloroform-isoamilic alcohol (25:24:1) repeating at least twice as well as DNA precipitation were carried out, as described by Ausubel et al. (1992). DNA was finally resuspended with 50 μL of TE buffer, treated with 2.5 μL of RNAse (1 mg/mL) and stored at −20°C until processed.

DNA concentration and purity were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Whaltham, MA, USA).

2.4. ERIC-PCR fingerprinting

The AAB colonies recovered on plates were genotyped by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR technique as described González et al. (2004). All amplification reactions were carried out using GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA), and the amplification products were detected by electrophoresis on 1.5% w/v agarose gels. The pattern band lengths were determined by comparison with a DNA XIV 100 bp ladder (Roche Diagnostics, Mannheim, Germany).

2.5. 16S rRNA gene and 16S-23S rRNA gene Internal Transcribed Spacer (ITS) region analysis

The DNA samples of colonies that presented different ERIC-PCR profiles were identified at species level by the 16S rRNA gene and 16S-23S rRNA gene ITS region sequencing. The amplifications of these genes were performed in the GeneAmp PCR System 2700 (Applied Biosystems) as described by Ruiz et al. (2000). These samples were then purified and sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. The sequences obtained were compared with those in the GenBank databases of Basic Local Alignment Search Tool (BLAST).

On the other hand, the 16S rRNA gene analysis was also applied to DNA obtained directly from biofilm. The samples were analyzed by PCR-RFLP of the 16S rRNA gene and sequencing. The 16S rRNA gene amplifications were carried out in the GeneAmp PCR System 2700 (Applied Biosystems) using the method described by Ruiz et al. (2000). Amplified DNA was digested with two restriction enzymes: *Bcc*I and *Alu*I (Roche Diagnostics) by incubation during 3 hours at 37°C. The generated fragments were detected by 3% w/v agarose electrophoresis gel using the DNA XIV 100 bp ladder (Roche). The patterns obtained were then compared with those previously reported by

Ruiz et al. (2000) and Torija et al. (2010). Furthermore, the 16S rRNA gene amplicons were also purified and sequenced by Macrogen Inc., and the sequences were compared by BLAST with those in the GenBank databases.

2.6. PCR-DGGE

The strawberry biofilm DNA samples were amplified using the primer pair WBAC1GC/WBAC2 (Lopez et al., 2003). Polymerase chain reaction (PCR) amplifications were carried out in a Gene Amp PCR System 2700 (Applied Biosystems), and electrophoretic conditions were 170 V for 4 hours at a constant temperature of 60°C in TAE buffer 0.5× and in a denaturing gradient from 30% to 60% of urea and formamide. The DNA fragments from the PCR-DGGE gels were excised, purified and transferred into 50 μL of sterile water. After overnight incubation at 4°C to allow diffusion of the DNA, one microliter of this eluted DNA was used for reamplification with the previously mentioned primers but without the GC clamp. The PCR products were purified and sequenced by Macrogen Inc. using an ABI3730 XL automatic DNA sequencer. The sequences were compared by BLAST with those in the GenBank databases.

2.7. Enumeration of AAB by Real-Time PCR with TaqMan-MGB probe

The detection and the quantification of total AAB population present in the strawberry biofilm samples were performed in triplicate by Real Time - PCR using primers and a specific TaqMan-MGB probe designed in this study. The sequences of the AAB 16S rRNA gene were obtained from the GenBank database and aligned with ClustalW multiple-sequence alignment program (Thompson et al., 1994). The primers and probe were designed from the consensus sequence using the Primer Express software package version 3.0 (Applied Biosystems). The forward primer AAB-F (5′

TGAGAGGATGATCAGCCACACT 3′), the reverse primer AAB-R (5′ TCACACACGCGGCATTG 5′), and the TaqMan-MGB probe AAB-S (5′ NED-ACTGAGACACGGCCCA-MGB 3′) were synthesized by Applied Biosystems. The PCR mixture was prepared in a total volume of 25 mL with 100 nM of each primers, 100 nM of TaqMan-MGB probe, 1× TaqMan Universal Master Kit (Applied Biosystems), and 5 mL of extracted DNA (ranging from 50 to 100 ng/mL). The amplification was performed in triplicate on an ABI Prism 7300 real-time PCR system (Applied Biosystems) using the conditions of 95°C for 10 minutes, 40 cycles of denaturing at 95°C for 15 seconds, and 62°C for 1 minute. The cycle threshold (C_T) was determined automatically, and no template control by triplicate was included for each amplification.

Furthermore, AAB species quantification was carried out for *Acetobacter pasteurianus*, *Acetobacter aceti*, *Acetobacter malorum*, *Acetobacter cerevisiae*, *Komagataeibacter europaeus*, *Komagataeibacter hansenii*, and *Gluconobacter oxydans* by Real Time - PCR using TaqMan-MGB probes (Torija et al., 2010; Valera et al., 2013). The presence of these seven AAB species in the strawberry biofilm was evaluated by triplicate on an ABI Prism 7300 real-time PCR system (Applied Biosystems), and for all the cases, a PCR enrichment for the target DNA of 15 cycles of amplification was previously carried out in the GeneAmp PCR System 2700 (Applied Biosystems). PCR reagents and amplification conditions for each species were performed as described Torija et al. (2010) and Valera et al. (2013).

2.8. Pyrosequencing of the V3-V5 hypervariable regions

The pyrosequencing analysis for the strawberry biofilm was carried out with DNA samples directly extracted from biofilm. To obtain the required quantity and high

quality of DNA, the nine DNA samples were pooled and divided in two aliquots (F1 and F2). These two samples were purified using the Plant DNA easy mini kit (Qiagen) columns and sent to LifeSequencing S.L. (Valencia, Spain) for their analysis. The technique briefly consisted in the amplification of the V3-V5 hypervariable regions in the 16S rRNA gene of bacteria using the pyrosequencing system Roche 454 FLS GS Titanium sequencer at LifeSequencing S.L. and the construction of two libraries, each marked with a different molecular identifier tag (Roche) to differentiate the sequences from each sample. After that, the amplified DNA was quantified using Quant-IT PicoGreen kit (Invitrogen) to generate and equimolecular pool to proceed with the sequencing.

The analysis of the sequences was also developed by LifeSequencing S.L. The resulting sequences were trimmed to remove the primers sequences, and among these clean sequences, just the ones with more than 300 nucleotides were used for further analysis. The different sequences obtained were compared with those in the Ribosomal Database Project. Taxonomic assignments of each operational taxonomic unit (OTU) were performed using the Ribosomal Database Project and contrasted with the taxonomical levels described for those organisms by the National Center for Biotechnology Information databases. The confidence cutoff was 70% for each OTU.

From the results provided by LifeSequencing S.L. (Valencia, Spain), microbial biodiversity at the genus level was calculated using Simpson's biodiversity index, which shows the probability that two randomly selected sequences correspond to different genera. The index was calculated using $1 - \Sigma p_i^2$, where p_i is equal to the number of sequences grouped into the same genus divided by the total number of sequence.

2.9. Phylogenetic analysis

The different sequences obtained from the 16S rRNA gene amplicons and DGGE-excised bands sequencing were used to perform phylogenetic analysis. All these sequences were aligned with those of the related AAB species type strains available in the GenBank database using the ClustalW multiple sequence alignment program (Thompson et al., 1994).

On the other hand, some sequences of the V3-V5 hypervariable regions from PCR-pyrosequencing analysis were used to carry out a phylogenetic analysis for AAB group. All the AAB trimmed sequences were aligned with the type strains of the AAB species, which presented a higher similarity in the BLAST analysis. Finally, only two sequences of those grouped as the same AAB species were used to construct the phylogenetic dendrogram.

Phylogenetic and molecular evolutionary analyses based on the neighbor joining and Kimura two-parameter methods were conducted using MEGA version 4 (Tamura et al., 2007).

3. Results

3.1. Cellular distribution in strawberry vinegar biofilm

Strawberry and grape vinegar biofilms were analyzed by LCM and SEM microscopes, and the images obtained are showed in Fig. 1. Different cellular morphologies were distinguished in strawberry biofilm (Fig. 1a) comparing with the homogeneous matrix of bacterial cells observed in grape biofilm (Fig. 1b). Bacterial cells from grape biofilm were elongated and rod shaped, separately arranged and mainly embedded in the cellulose fibers.

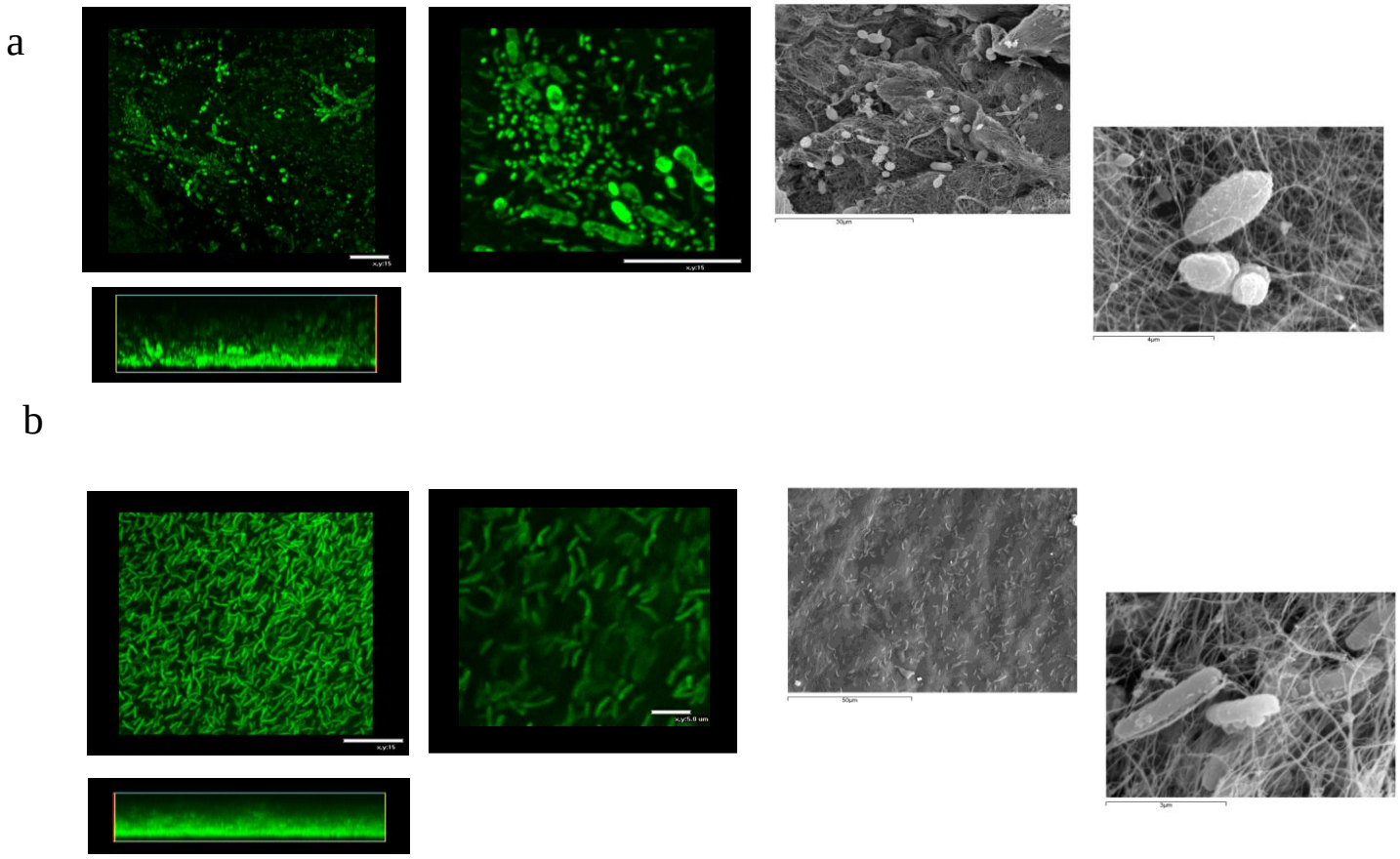

Fig. 1. Images of strawberry vinegar biofilm (a) and grape vinegar biofilm (b) by Laser Confocal Microcope (left) and Scanning Electronic Microscope SEM (right). Reconstruction in 3-D by LCM for transversal section and general vision with zoom. Cellulose fibers with cells and zoom of a group of cells visualized by SEM.

However, in strawberry biofilm the diversity and distribution of the cells were very different. From the images obtained by LCM of this biofilm, a bacterial cells layer could be distinguished in the background and bigger cells on the top, some of them could be identified like yeast by SEM, even fragments of fungal hyphae were also detected (data not shown). Aggregates of cells in this strawberry biofilm were frequently observed by both techniques.

3.2. Culture-dependent identification of AAB

From the strawberry vinegar biofilm, only 24 isolates were recovered on the GY medium. All of them formed a clear halo onto the GYC medium, were catalase positive, and presented the same band pattern after ERIC-PCR. The species identification of this unique genotype by 16S rRNA gene sequencing resulted in a homology (or sequence similarity) of 98% with the species *Amayemaea chiangmaiensis* and *Asaia krungthepensis*. Nevertheless, when a dendogram was constructed with the 16S rRNA gene sequences (Fig. 2), this sequence was grouped in the cluster of *Am. chiangmaiensis* and *Tanticharoenia sakaeratensis* type strains, but outside of it. On the other hand, the sequencing of the 16S-23S rRNA gene ITS region resulted in a homology of 87% with *Neoasaia chiangmaiensis* AC28^{T} strain followed by 85% with several strains classified as *Gluconobacter cerinus*

3.3. Culture-independent identification of AAB

The microbiota of strawberry vinegar biofilm was also analyzed without a previous culture growth. Although different DNA extraction methods previously reported for vinegar samples (Jara et al., 2008; Mamlouk et al., 2011) were initially performed in strawberry vinegar biofilm samples, they did not yield suitable amplification results by PCR; therefore, a new DNA extraction protocol was designed in this study. The biofilm

was divided into nine pieces, and the extracted DNA from each one was analyzed by PCR-RFLP and 16S rRNA gene sequencing, PCR-DGGE, Real Time - PCR with TaqMan-MGB probes, and PCR pyrosequencing of the V3-V5 hypervariable regions. In the latter technique, the DNA from the nine pieces of biofilm was necessary pooled and collected in two DNA samples to perform the analysis.

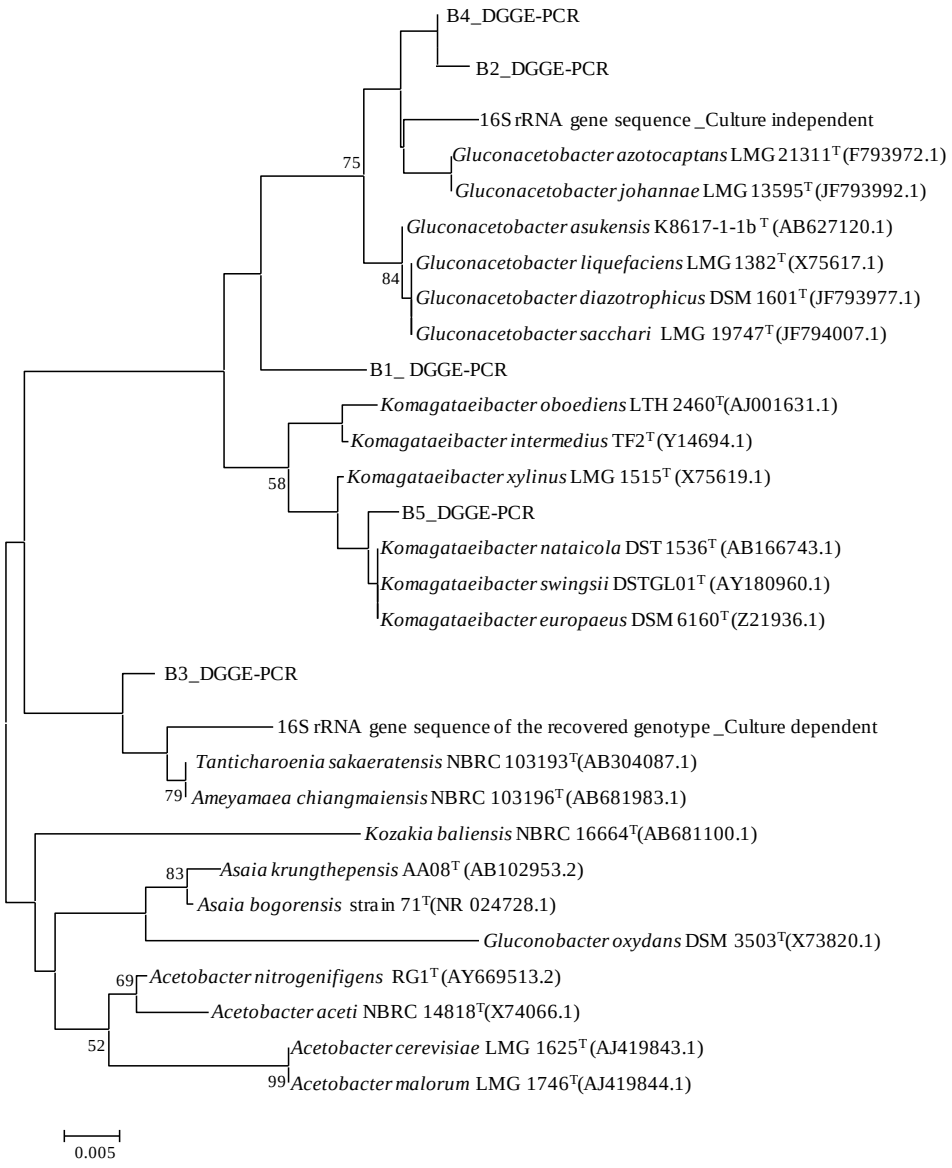

Fig. 2. Phylogenetic relationships of Acetic Acid Bacteria generated from sequences of PCR-DGGE bands and 16S rRNA gene sequences in both culture-dependent and culture-independent analysis. Dendrogram constructed using the neighbor-joining method; the robustness of the branching is indicated by bootstrap values (%) calculated for 1000 subsets.

3.3.1. PCR-RFLP and 16s rRNA gene sequencing

The 16S rRNA gene amplification and enzyme digestion of the DNA obtained from the nine biofilm samples resulted in a single profile for each restriction enzyme used. In the case of *Bcc*I, two bands of approximately 1000 and 500 bp were visualized. According to the *in silico* analysis, this profile could correspond to *Gluconacetobacter diazotrophicus*, *Gluconacetobacter liquefaciens*, *Gluconacetobacter sacchari*, *Gluconacetobacter asukensis*, *Am. chiangmaiensis*, *T. sakaeratensis*, *Ko. xylinus*, *Gluconobacter oxydans*, and *Acetobacter cerevisiae*. For *Alu*I, the restriction profile presented seven bands of 800, 500, 250, 220, 180, 120, and 70 bp, which could correspond to *Gluconacetobacter azotocaptans*, *Gluconacetobacter johannae*, *Gluconacetobacter sacchari*, *Ga. liquefaciens*, *Ga. asukensis*, *Komagataeibacter nataicola*, *Am. chiangmaiensis*, *A. cerevisiae*, *Acetobacter malorum*, *As. bogorensis* and *Asaia krungthepensis*. Therefore, crossing the data from both restriction profiles, the matching species were *Ga. liquefaciens*, *Ga. sacchari*, *Ga. asukensis*, and *Am. chiangmaiensis*.

On the other hand, the 16S rRNA gene sequencing of these samples (DNA directly extracted from the biofilm) resulted in a unique sequence, which was clear enough to be identified by comparing it with the GenBank database. This sequence presented a 97% of homology with *Ga. liquefaciens* and *Ga. azotocaptans* and was grouped close to *Ga. azotocaptans* and *Ga. johannae* type strain sequences in the 16S rRNA gene dendogram constructed (Fig. 2).

3.3.2. PCR-DGGE

From the nine DNA samples analyzed, only four of them amplified the fragment of the approximately 340 bp expected. The DGGE of these amplicons revealed two similar

profiles. One profile comprised four bands, and the other profile was formed by five bands, the same four bands and another new one. The sequencing of the different fragments obtained was compared with those available in the GenBank database, and the results are detailed in Table 1. Three bands were identified belonging to the *Gluconacetobacter* genus, and the other two corresponded to the *T. sakaeratensis*/*Am. chiangmaiensis* and *Komagataeibacter* genus.

Table 1. Sequencing results of bands obtained from the two PCR-DGGE profiles of strawberry vinegar biofilm. The bands are ordered from lower to higher electrophoretic mobility.

Band	Species (% homology)
B 1[a]	*Ga. johannae; Ga.azotocaptans* (99%)
B 2	*Ga. johannae; Ga. azotocaptans; Ga. liquefaciens; Ga. diazotrophicus; Ga. sacharii* (99%)
B 3	*T. sakaeratensis; Am. chiangmaiensis* (99%)
B 4	*Ga. johannae; Ga. azotocaptans; Ga. liquefaciens; Ga. diazotrophicus* (99%)
B 5	*Ko. europaeus; Ko. xylinus; Ko. swingsii; Ko. nataicola* (99%)

[a] This band was only detected in one of the DGGE profiles

This identification was confirmed by the 16S rRNA gene dendogram because these sequences were clustered in the corresponding branches (groups) of these genera (Fig. 2).

3.3.3. Real Time - PCR quantification and identification of AAB

The same four samples used in PCR-DGGE were the ones quantified by Real Time - PCR. No amplification was obtained with the other five samples.

In this study, a set of primers and TaqMan-MGB probe for the enumeration of total AAB population were designed. Their specificity was tested both *in silico* and in laboratory conditions with different species of yeast, lactic acid bacteria, and other bacteria such as *Escherichia coli*, none of them gave signal. The standard curves

obtained using 10-fold serially diluted DNA extracted from 10^8 cells/mL of any AAB species maintained linearity in the range of 10^7–10^2 cells/mL, and the regression values (r^2) were >0.98 (data not shown). The AAB total quantification for the four biofilm samples ranged between 8×10^5 and 1.2×10^6 cells/g.

On the other hand, in the quantification of AAB species, only positive results were obtained with two TaqMan-MGB probes (*Ko. europaeus* and *Ko. hansenii*) in the four samples. The population size of *Ko. europaeus* and *Ko. hansenii* in these samples was on average 1.71×10^4 cells/g and 5.20×10^3 cells/g, respectively. The *A. pasteurianus*, *A. aceti*, *A. malorum*, *A. cerevisiae*, and *G. oxydans* species were not detected in any samples.

3.3.4. Pyrosequencing

The two DNA samples (F1 and F2) obtained from the pool of the initial nine samples were amplified in the V3-V5 hypervariable regions of 16S rRNA gene and massive sequenced.

Table 2. Data sets of 454- pyrosequencing technique from strawberry vinegar biofilm samples.

Sample	Raw reads		Clean reads		No. genera affiliated with OTUs	Simpson's Diversity Index by genera
	No. Sequences	Average length (ntds)	No. Sequences	Average length (ntds)		
F1	14,698	426.53	11,233	501.33	42	0.68
F2	14,918	421.47	11,034	504.80	51	0.71

The rarefaction curves at the family level indicated enough number of sequences to detect all the variability present in both samples (data not shown). The general result of the analysis was detailed in Table 2. From the raw reads, the 76.43% of sequences for sample F1 and the 73.96% for sample F2 were retained for analysis after sequence

cleaning. These trimmed sequences with an average length of 500 bp presented similar results for both samples. The Simpson's diversity index at the genus level was 0.68 for F1 and slightly higher for F2 (0.71), differentiating 42 and 51 genera, respectively. All the genera affiliated with OTUs were grouped in 32 families of bacteria; in both samples, the majority of them (77%) belonged to the *Proteobateria* phylum followed by *Firmicutes* (19%), and around a 4% corresponded to the *Acidobacteria* phylum.

In both samples, the most abundant genus was *Gluconacetobacter*, which comprised 51.80% of the sequences in F1 and 49.23% in F2. The second genus was *Bacillus* (19% of the sequences in both samples), and the third one was *Ameyamaea* (9.32% in F1 and 10.49% in F2) (Fig. 3).

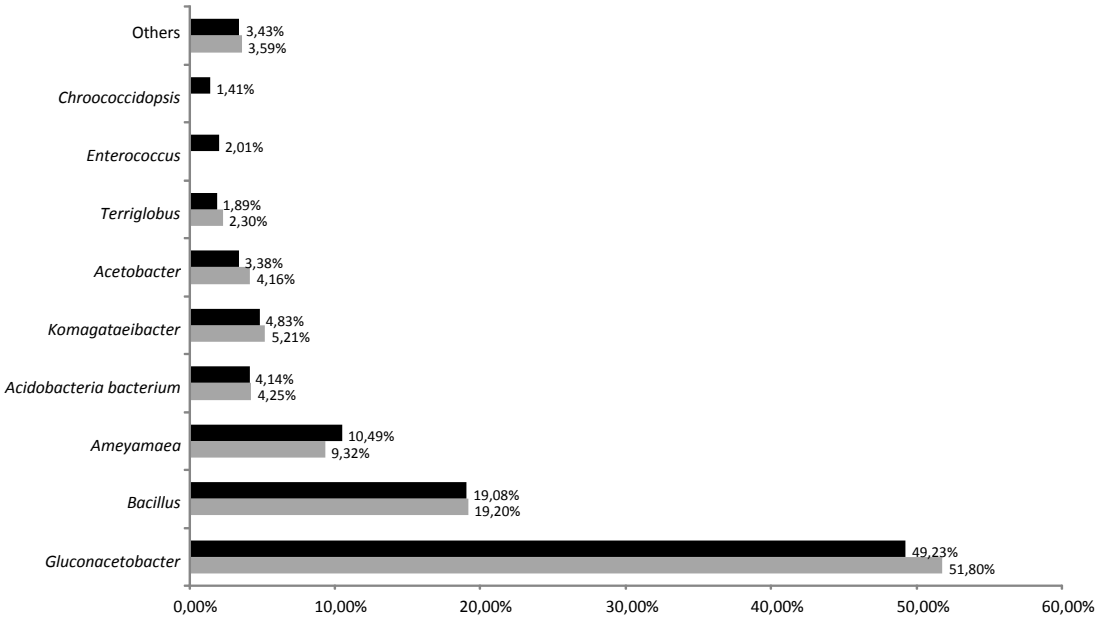

Fig. 3. Relative abundance of genera obtained by pyrosequencing identification attending to the V3-V5 hypervariable regions of 16S rRNA gene amplification. Sample F1 is presented in gray bars and sample F2 in black bars.

Among all the genera detected, 10 corresponded to the *Acetobacteraceae* family, belonging to AAB group. These AAB genera from highest to lowest percentage were *Gluconacetobacter*, *Ameyamaea*, *Komagatabacter*, and *Acetobacter*, and the AAB

genera grouped into "others" were *Acidomonas*, *Tanticharoenia*, *Gluconobacter*, *Kozakia*, *Asaia*, and *Neoasaia*.

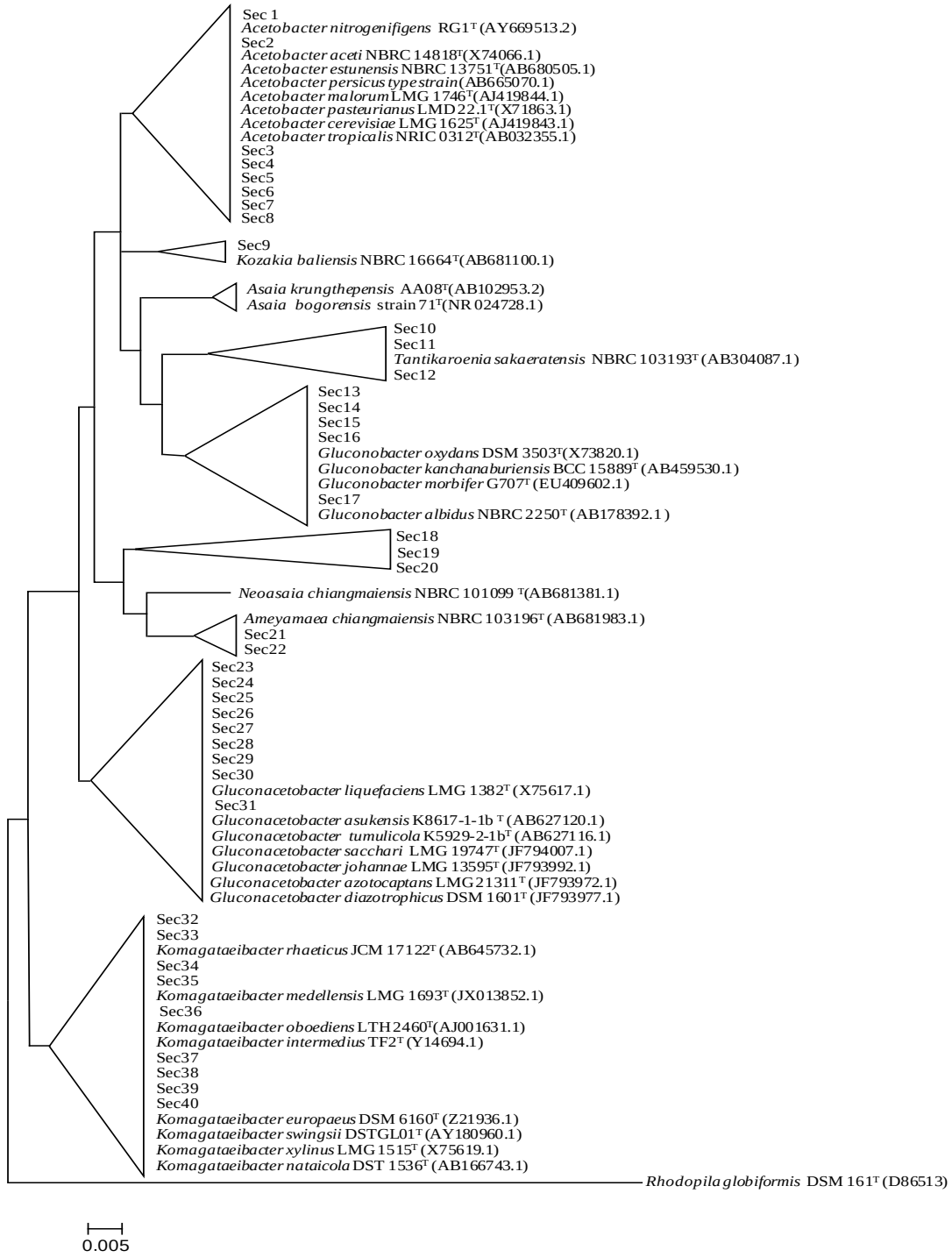

Fig. 4. Phylogenetic relationships of Acetic Acid Bacteria generated from trimmed sequences from the V3-V5 hypervariable regions of 16S rRNA gene pyrosequencing. Dendrogram constructed using the neighbor-joining method; the robustness of the branching is indicated by bootstrap values (%) calculated for 1000 subsets.

A phylogenetic dendrogram was constructed with AAB trimmed sequences belonging to the *Acetobacteraceae* family (Fig. 4). These sequences were aligned with the 16S rRNA sequences from the type strains of some AAB species. The dendrogram grouped the sequences derived from the metagenomic analysis in seven clusters closely related with the type strains of *Acetobacter*, *Kozakia*, *Tanticharoenia*, *Gluconobacter*, *Ameyamaea*, *Gluconacetobacter*, and *Komagataeibacter* genera. Furthermore, three sequences were grouped together but not associated with any AAB type strain. These sequences, Sec 18, Sec 19, and Sec 20, showed the highest similarity by BLAST to *Kozakia baliensis* (96% of homology), *As. krungthepensis* (97% of homology), and *Ga. diazotrophicus* (94% of homology), respectively. Although they were more homologous to different species and even different genera in the database, they appeared grouped together in a separate cluster when the dendrogram with all the sequences was constructed.

3.4. Comparative analysis of the bacterial community

Comparing the results obtained with the different techniques used, only four of the 10 AAB genera identified by pyrosequencing were also detected by the other techniques (Table 3). Three of these four genera had the highest percentage of detection by pyrosequencing within the AAB group (Fig. 3). The genus *Ameyamaea* was identified on plates and by PCR-DGGE. The *Gluconacetobacter* genus was detected by PCR-RFLP and 16S rRNA gene sequencing and PCR-DGGE. The presence of *Komagataeibacter* was revealed by PCR-DGGE and Real Time - PCR. Finally, *Tanticharoenia* was only detected by PCR-DGGE.

Table 3. Acetic acid bacteria genera detected in strawberry vinegar biofilm by culture-dependent and culture-independent molecular techniques.

Genera	Culture-dependent	Culture-independent			
	16S rRNA sequencing	RFLP-PCR and sequencing of 16S rRNA gene	PCR-DGGE	RT-PCR with TaqMan-MGB	PCR-454 pyrosequencing
Acetobacter					**x**
Acidomonas					**x**
Ameyamaea	**x**		**x**		**x**
Asaia					**x**
Gluconacetobacter		**x**	**x**		**x**
Gluconobacter					**x**
Komagataeibacter			**x**	**x**	**x**
Kozakia					**x**
Neoasaia					**x**
Tanticharoenia			**x**		**x**

4. Discussion

Vinegar biofilm is developed by AAB (Solieri and Giudici, 2009) and mainly composed by cellulose and other EPS (Chawla et al., 2009). Some studies have been performed about the composition and structure of the AAB biofilm (Aydın and Aksoy, 2013; Gullo et al., 2012; Krishnamachari et al., 2011), but few studies have been focused on its microbiota analysis from vinegar samples (Gullo et al., 2009; Nanda et al., 2001) and none of them using culture-independent molecular techniques. Therefore, in this study, an in-depth analysis was performed to find out the microbial distribution and

complexity, mainly of AAB, present in a biofilm from strawberry vinegar elaborated by traditional method.

The strawberry vinegar biofilm was observed under microscope and compared with grape vinegar biofilm. The images obtained by SEM and LSM showed heterogeneous cell distribution and the presence of yeast, bacteria, and fungi morphology in strawberry vinegar biofilm in contrast with the uniformity observed in grape vinegar biofilm, which was like a homogeneous bacterial layer. In natural environments, biofilms are expected to be formed by more than one microorganism. In fact, the presence of mixed communities of yeast, bacteria, and even hypha irregularly distributed and mixed through the EPS matrix has been reported in several studies of biofilms (Dominguez-Manzano et al., 2012; Wang et al., 2012). The symbiotic relationship between yeast and AAB is well established in kombucha production, where yeast may be arranged in islets or bands and embedded in the laminae of EPS matrix primarily secreted by AAB to survive throughout the adverse conditions generated from the alcoholic fermentation and acetification bioprocesses that happen almost simultaneously (Teoh et al., 2004). However, traditional wine vinegar production is usually performed with filtered wines, which are inoculated with "vinegar mother" taken from a previously acetified batch. Therefore, only AAB can proliferate in these conditions, transforming ethanol in acetic acid and forming a homogeneous biofilm of this group of bacteria. In the case of strawberry vinegar production, the acetification was carried out with unfiltered strawberry wine (Hidalgo et al., 2010); therefore, some yeasts from alcoholic fermentation or other organisms from environment could survive to the acetification of wine embedded in the biofilm, which act as a protective environment for these stress condition but likely without a defined function during the process.

AAB identification in this strawberry vinegar biofilm was carried out by culture-dependent and culture-independent molecular methods. One of the main hurdles for a good coverage of the microbial diversity is the recovery of representative and high-quality DNA from the matrix. In a biofilm, the cells are mainly trapped in the EPS matrix, which hinders the DNA isolation.

Moreover, in a vinegar biofilm, the removal of polyphenols from the raw material is also important (Mamlouk et al., 2011). The DNA extraction protocol applied in this study to the biofilm of strawberry vinegar seemed to be appropriated to obtain enough amount of DNA with adequate quality to successfully perform all the culture-independent molecular techniques assayed.

The fingerprinting analysis carried out by ERIC-PCR detected a unique genotype from the only 24 isolates recovered from plates. It is largely known that the results obtained in culture-dependent studies are likely biased because of the low AAB recovery on plates when the samples come from extreme media such as vinegar (Torija et al., 2010; Valera et al., 2013). Therefore, these results should be complemented with the application of culture-independent techniques to have a better approach for the identification of the bacteria community (Jara et al., 2013; Vegas et al., 2013).

In this study, the 16S rRNA gene analysis was carried out as a common tool used to perform phylogenetic studies and species identification of AAB (Yamada et al., 2012). The 16S rRNA identification of the unique ERIC-PCR genotype detected was not accurate because its highest homology (98%) was with *Am. chiangmaiensis* and *As. krungthepensis*, but in the dendrogram of 16S rRNA gene sequences, this genotype was placed close to the *Am. chiangmaiensis* and *T. sakaeratensis* cluster but outside of it. On the other hand, a mixed restriction profile and an overlapped sequence were expected

after the analysis of the 16S rRNA gene from DNA directly obtained from biofilm samples. However, the nine biofilm samples resulted in a single and well-defined restriction profile for each enzyme in PCR-RFLP and a clean enough sequence of 16S rRNA gene. Even so, it was not possible to provide an accurate species identification because the highest homology was 97%. This could be explained because although the sequence obtained was good due to the high similarity in this gene between AAB, the presence of a mixed AAB population indicated that some positions in the sequence were undetermined. However, these techniques confirmed the presence of *Gluconacetobacter* species in the biofilm. The 16S rRNA gene amplicons were also analyzed by cloning in *E. coli* (Ilabaca et al., 2008), resulting in a library of clones where the more representative species corresponded to the *Enterobacteriaceae* family (data not shown). Therefore, this technique was not efficient for AAB analysis because the cloning of other species present in this biofilm seemed most favored. As a summary of results obtained from the 16S rRNA gene analysis, mainly from those of culture-dependent methods, a new AAB species could be present in this strawberry biofilm. The study of the 16S-23S rRNA gene ITS region for this genotype did not clarify its species identification presenting low homology with the described AAB species; therefore, more analyses are required to identify if it might be a novel AAB species.

PCR-DGGE has been reported as a good tool to detect major species present in vinegar (Gullo et al., 2009; Xu et al., 2011). In the present study, similar DGGE profiles were obtained from all analyzed biofilm samples. The detection of five DGGE bands indicated the presence of at least five different AAB species belonging to the *Gluconacetobacter*, *Komagataeibacter*, and *Ameyamaea*/*Tanticharoenia* genera after sequencing the excised bands. This technique provided more information than that obtained using the previous technique, but the high homology between AAB-related

species (up to 99.9%) together with the small size of the fragment analyze (340 bp) only allowed to identify the biofilm samples at the genus level.

An accurate detection and quantification of seven AAB species, usually present in wine and vinegar, is possible using Real Time - PCR with TaqMan-MGB probes (Torija et al., 2010; Valera et al., 2013). In biofilm samples, among the seven species tested, only *Ko. europaeus* and *Ko. hansenii* were detected and quantified, both species with concentrations higher than 5×10^3 cells/g. Therefore, the presence of *A. aceti*, *A. cerevisiae*, *A. malorum*, *A. pasteurianus*, and *G. oxydans* was discarded. Moreover, a new TaqMan-MGB probe for total AAB quantification was successfully designed. The use of this new set of primers and probe provided a more accurate quantification than Real Time - PCR with SYBR-Green designed by González et al. (2006), because their primers also annealed with *E. coli* which is a ubiquitous bacterium. When the total AAB population detected in strawberry vinegar biofilm (10^6 cells/g) was compared with the sum of the individual species quantification, the former was two magnitude orders higher. Therefore, the main drawback of this technique is the impossibility to detect and quantify other AAB species that could be present in the biofilm different from these seven ones of which specific primers and probes were designed. However, this technique confirmed the presence of the *Komagataeibacter* species in the strawberry biofilm, previously detected by PCR-DGGE.

Recently, next-generation sequencing techniques have been reported adequate to find out the microbial complexity present in a community (Nie et al., 2013; Roh et al., 2010). Among them, the platform 454 pyrosequencing allows the large-scale metagenomic sequencing of DNA (Illeghems et al., 2012; Jung et al., 2011). In this study, the regions V3-V5 from the 16S rRNA gene of bacteria were analyzed. The length of the sequences obtained was in average 500 nucleotides, longer than the used in

other metagenomic bacteria studies (200 nucleotides) (Nie et al., 2013; Roh et al., 2010). Although this longer sequence provides an assignation of taxa more accurate (Bohorquez et al., 2012), the identification was only possible up to the genera level because the AAB species detected presented low homology with their respective type strain (90%–98%). The dendrogram construction with the AAB trimmed sequences could provide slightly higher accuracy to these identifications. Few studies have applied metagenomic techniques to analyze the microbiota present in fermented products (Illeghems et al., 2012; Jung et al., 2011; Roh et al., 2010), and only one of them was carried out in vinegar (Nie et al., 2013), with its biodiversity index lower than that obtained in the samples of strawberry vinegar biofilm. In the present study, 10 AAB genera were identified and also genera of bacteria corresponding to other families. All the AAB genera detected by the other techniques applied in this study were also identified by pyrosequencing being them as majority.

The genera *Acetobacter*, *Komagataeibacter*, and *Gluconacetobacter* have been usually associated with traditional vinegar (Gullo et al., 2009; Hidalgo et al., 2013; Nie et al., 2013, Wu et al., 2012). Moreover, the presence of *Komagataeibacter* genus was expected because Hidalgo et al. (2013) detected *Ko. xylinus* and *Ko. saccharivorans* in the final stage of strawberry vinegar production. However, the *Ameyamaea* and the *Tanticharoenia* genera were not previously detected in vinegar, just in flowers (Yukphan et al., 2009) and soil (Yukphan et al., 2008), respectively. The *Asaia* genus, not previously described in vinegar, has been isolated from flowers (Yamada et al., 2000), grapes (Mateo et al., 2014) wines (Bae et al., 2006), or spoilage microorganisms in strawberry-flavored bottled water (Kregiel et al., 2012).

The presence of *Kozakia*, *Acidomonas*, and *Neoasaia* genera were identified in a minor amount and only by pyrosequencing technique. None of them were previously reported

as vinegar associated microbiota. *Kozakia* was first recovered from brown palm sugar (Lisdiyanti et al., 2002) and then from grape must (Navarro et al., 2013) harvested in the same cellar where the strawberry vinegar biofilm was produced. *Neoasaia* was recovered from red ginger flowers (Yukphan et al., 2005), and *Acidomonas* (formerly classified as *Acetobacter*) was first isolated in sludges of a yeast fermentation (Uhlig et al., 1986).

Among the bacteria corresponding to other families, the genus *Bacillus* was highly represented with 19% of the reads followed by *Terriglobus* with lower percentage. The *Bacillus* genus was reported as part of the endophytic microbiota of strawberry fruit (De Melo et al., 2012) and *Terriglobus* from tundra soil (Männistö et al., 2011); therefore, their presence may be expected as environment contaminant in this strawberry vinegar biofilm.

Although the drawbacks of PCR-based methods, such as the preferential annealing of the primers or the representativity and quality of DNA, can partly bias the information obtained about the real composition of a mixed bacterial population (Bohorquez et al., 2012), in our study, all the molecular techniques, both culture dependent and culture independent, presented concordant results. Moreover, the use of several techniques allowed a better understanding of the AAB microbiota complexity present in strawberry vinegar biofilm. The metagenomic sequencing technique provided a more global vision of bacteria genera diversity that inhabits in this community, whereas the remaining techniques gave complementary information to one another about the main AAB genera and species. Among the latter, the culture-dependent method enabled the isolation of an AAB strain that could be further studied to recognize as novel species while the culture-independent methods, which are usually applied for AAB identification, have proven to be efficient in the detection of the main AAB that harbor the biofilm of strawberry

vinegar. It was the first study where the microbiota from a biofilm of vinegar elaborated by traditional method, using next-generation sequencing and others molecular techniques, has been determined, being the main AAB genera: *Ameyamaea*, *Gluconacetobacter*, and *Komagataeibacter*.

Acknowledgments

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Chapter 4

Cellulose production and cellulose synthase gene detection in acetic acid bacteria

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Abstract

The ability of acetic acid bacteria (AAB) to produce cellulose has gained much industrial interest due to the physical and chemical characteristics of bacterial cellulose. The production of cellulose occurs in the presence of oxygen and in a glucose-containing medium, but it can also occur during vinegar elaboration by the traditional method. The vinegar biofilm produced by AAB on the air-liquid interface is primarily composed of cellulose and maintains the cells in close contact with oxygen. In this study, we screened for the ability of AAB to produce cellulose using different carbon sources in the presence or absence of ethanol. The presence of cellulose in biofilms was confirmed using the fluorochrome Calcofluor by microscopy. Moreover, the process of biofilm formation was monitored under epifluorescence microscopy using the Live/Dead BacLight Kit. A total of 77 AAB strains belonging to 35 species of *Acetobacter*, *Komagataeibacter*, *Gluconacetobacter*, and *Gluconobacter* were analysed, and 30 strains were able to produce a cellulose biofilm in at least one condition. This cellulose production was correlated with the PCR amplification of the *bcsA* gene that encodes cellulose synthase. A total of eight degenerated primers were designed, resulting in one primer pair that was able to detect the presence of this gene in 27 AAB strains, 26 of which formed cellulose.

Keywords: vinegar, biofilm, epifluorescence microscopy, *bcsA* gene

1. Introduction

Acetic acid bacteria (AAB) belong to the *Acetobacteraceae* family and are the main bacteria involved in vinegar production due to their ability to synthesise acetic acid from ethanol in the presence of oxygen. This group of bacteria is also involved in the production of compounds for the food, textile, medical, and construction industries (Mamlouk and Gullo, 2013). The ability of AAB to produce cellulose is one of their most interesting features. The structural and mechanical characteristics of bacterial cellulose (BC) make it different from plant cellulose, and BC is considered especially valuable in industry (Chawla et al., 2009).

In the traditional method of vinegar elaboration, AAB are located at the air-liquid interface where they develop a biofilm in direct contact with oxygen (Laguno and Polo, 1991), which may help them survive under stressful growth conditions such as high ethanol or acetic acid concentrations (Kanchanarach et al., 2010). In bacteria, biofilm production has been related to cell-to-cell communication via quorum-sensing signalling that controls the formation of the biofilm and its detachment in response to different factors such as lack of nutrients (Davies et al., 1998). The vinegar biofilm produced by AAB is composed of various exopolysaccharides (EPS), including cellulose (Moonmangmee et al., 2002). Several AAB species belonging to the genera *Gluconacetobacter* (*Ga.*), *Komagataeibacter* (*Ko.*) (Aydın and Aksoy, 2014; Castro et al., 2011, 2013; Park et al., 2003a, 2003b), *Acetobacter* (*A.*) (Dayal et al., 2013; Gullo et al., 2012; Ibnaof et al., 2011; Jung et al., 2010), or *Gluconobacter* (*G.*) (Jia et al., 2004) have been reported to be cellulose producers. The species *Ko. xylinus* (formerly *Ga. xylinus*) (Yamada et al., 2012) is considered a model microorganism for BC production (Chawla et al., 2009; Römling, 2002).

Therefore, the studies of cellulose production in AAB have focused on the composition and structure of the EPS biofilms produced by *Ko. xylinus* strains (Mikkelsen et al., 2009). Microscopy techniques have shown that *Ko. xylinus* secretes a slimy substance within which the cellulose fibres are formed. These initial fibres then form bundles and larger ribbons (Chawla et al., 2009; Zhang, 2013). Several images of this structure were obtained by scanning electron microscopy (Gomes et al., 2013; Mikkelsen et al., 2009; Trovatti et al., 2011). However, while the development of several biofilms has been studied using fluorescent dyes like SYTO 9 or live/dead stains (Cooper, 2014; Neu and Lawrence, 1997), to our knowledge, these methods have not been applied specifically to study AAB biofilms. The molecular structure of BC allows the use of specific dyes like Congo red or Calcofluor to screen for cellulose-producing strains (Römling, 2002; Zogaj et al., 2003).

Cellulose production studies have primarily focused on how to increase its formation by changing the growth conditions or the strains used (Keshk and Sameshima, 2005; Trovatti et al., 2011). The growth conditions can induce changes in the cellulose production rate (Chawla et al., 2009). The effects of nitrogen and phosphorus sources (Gomes et al., 2013; Ramana et al., 2000), pH (Verschuren et al., 2000), and ethanol concentrations (Naritoni et al., 1998; Park, 2003a) on cellulose production by AAB have been evaluated. However, one of the most studied factors has been the influence of the carbon source, and different monosaccharides, disaccharides, and even alcohols have been tested (Keshk and Sameshima, 2005; Mikkelsen et al., 2009). More recently, the use of by-products from other industries, such as dry olive mill residue or agro-industrial wastes, has been tested with *Ga. sacchari* (Gomes et al., 2013) and *Ko. swingsii* (formerly *Ga. swingsii*) (Castro et al., 2011), respectively. The results obtained

in these studies are strongly strain dependent, so the determination of optimal conditions for cellulose production should be performed for single strains (Chawla et al., 2009).

The genes required for the biosynthesis of BC are encoded by the *bcsABZC* operon (also called *acsABZC* or *celABZC*). In all of the species where some form of this operon has been found, the *bcsA* and *bcsB* genes (bacterial cellulose synthesis, also named as *acs* or *cel* genes) are present. The *bcsA* gene is the most well-conserved gene of the operon. It encodes cellulose synthase, the catalytic subunit required for cellulose biosynthesis. The *bcsB* gene encodes a bis-(3', 5') cyclic diguanylic acid (c-di-GMP)-binding protein and is less conserved among bacterial species. In some cases, the genes are fused into one gene referred to as *bcsAB* (Saxena et al., 1994). The availability of *bcs* operon sequences from AAB species in databases is reduced, and most of the information is specific for *Ko. xylinus*. Although it has been reported that several strains of this species contain more than one operon for cellulose biosynthesis (Ogino et al., 2011; Römling, 2002), under laboratory conditions, only one of the cellulose synthase genes present in any of these operons is expressed (Saxena and Brown, 1995). As previously mentioned, the *bcsA* gene is the most conserved gene of the operon; therefore, it could be used for the molecular identification of putative cellulose-producing bacteria. Although both the N- and C-terminal portions of the protein encoded by this gene are poorly conserved, there is a 350-amino acid region with high homology (Römling, 2002); it may be possible to design primers against the DNA sequence encoding this region.

In the present study, we evaluated the effects of different carbon sources (fructose, glucose, and sucrose) and the presence or absence of ethanol in controlled media for the production of cellulose biofilms by various AAB strains. The presence of cellulose in the biofilm and the viability of the AAB cells were visualised by microscopy using

specific fluorochromes. The tested AAB strains belonged to the *Acetobacter*, *Komagataeibacter*, *Gluconacetobacter*, and *Gluconobacter* genera. Moreover, the correlation of this phenotypical screening with the amplification of *bcsA* gene homologues was studied.

2. Materials and methods

2.1. Bacterial strains and growth conditions

In this study, 77 strains from 35 species of AAB were tested for the ability to produce cellulose; 52 strains belonging to 34 AAB species were obtained from different culture collections, and 25 strains of six AAB species were isolated from microfermentations of healthy grapes, blueberry vinegar, or grape vinegar produced by the traditional method (Table 1). The work was performed in a research laboratory that meets the ISO 9001 regulations.

Strains stored at −80ºC were recovered on GY medium (1% yeast extract, 5% glucose) at 28°C for two days. For cellulose production analysis, all of the strains were grown in triplicate in 20 ml tubes with five ml of six different broths containing one of the following carbon sources: glucose medium (GY; 1% yeast extract, 1% glucose [w/v]), fructose medium (FY; 1% yeast extract, 1% fructose [w/v]), sucrose medium (SY; 1% yeast extract, 1% sucrose [w/v]), and these three media supplemented with 1% ethanol, (GYE, FYE, and SYE, respectively). The tubes were incubated at 28°C for 72 hours under shaking conditions.

2.2. Cellulose production and visualisation by epifluorescence microscopy

The tubes containing the 77 AAB strains inoculated in the six different types of media were analysed for cellulose production by visual inspection every 24 hours.

After 72 hours of incubation, the cellulose production in the tubes was checked by staining with the fluorochrome Calcofluor White Stain (Fluka, Switzerland), which binds with the cellulose in the cell walls of fungi and other organisms such as AAB. One millilitre of each AAB strain culture was recovered by centrifugation at 5,000 rpm for five minutes, and one microlitre of the dye was added to the pellet. After incubation (one minute in dark), the cellulose presence was analysed using epifluorescence microscopy.

Epifluorescence microscopy was also applied to monitor the viability of the *Ko. europaeus* strain *Gae02* during the production of the cellulose biofilm. This strain, which was previously recovered from vinegar elaborated by the traditional method, was grown in a static culture in 60 mm plates with 10 ml of GY liquid medium at 28ºC. The sample was inoculated, and biofilm samples of approximately 25 mm^2 were aseptically recovered on a cover after 24, 48 and 72 hours. They were stained with one microlitre of SYTO 9 dye and one microlitre of propidium iodide (PI) dye from the Live/Dead BacLight Kit (Molecular Probes, Eugene, OR, USA). After incubation in the dark for 20 minutes, each sample was washed with two microlitres of water to eliminate the excess dye, and the biofilm structure was observed under epifluorescence microscopy.

2.3. DNA extraction

Genomic DNA was extracted from all of the AAB strains using the cetyltrimethylammonium bromide (CTAB) method described by Ausubel et al. (1992). The DNA concentration and purity were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Table 1. Results of biofilm formation and homologous *bcsA* gene region amplification in all the strains used in this study.

Species	Collection Designation or Strain	Origin or isolation source	Biofilm production in different culture media[a]																		PCR result
			GY			GYE			FY			FYE			SY			SYE			
			24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h	
A. cerevisiae	LMG 1625$^{T\,b}$	Beer (ale) in storage	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ac2	Microfermentation of healthy grapes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Ac3		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ac5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ac7		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. malorum	LMG 1746$^{T\,b}$	Rotting apple	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Am1	Microfermentation of healthy grapes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Am3		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Am5		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Am7		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Am8		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Am12		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Am17		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	LMG 1262$^{T\,b}$	Beer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	DSM 46617^{b}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	LMG 1582^{b}	Beet juice	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	LMG 1605^{b}	Vinegar brews	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	LMG 1553^{b}	Spoiled beer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. pasteurianus	DSM 3509$^{T\,b}$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ap2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
	Ap3		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ap4	Microfermentation of healthy grapes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Ap7		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ap8		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Ap11		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ap0	Blueberries vinegar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. tropicalis	LMG 1663$^{T\,b}$	Fermenting putrified meat sample	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	At1	Microfermentation of healthy grapes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	At3		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. aceti	DSM 3508$^{T\,b}$	Alcohol turned to vinegar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	LMG 1504^{b}	Beech-wood shavings of vinegar plant	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	LMG 1512^{b}	Film in fermentor of rice vinegar	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

	LMG 1496[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	LMG 1372[b]	-	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	-	+	+	+	
	LMG 1531[b]	Non-cellulose-producing mutant derived from strain Carr Ac 142 (LMG 1530)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. oeni	LMG 21952[T b]	Spoiled red wine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. lovaniensis	LMG 1579[T b]	Sewage on soil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. cibinongensis	LMG 21418[T b]	Annona montanae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. pomorum	LMG 18848[T b]	Cider vinegar fermentation	-	+	+	-	+	+	-	+	+	-	-	+	-	+	+	-	+	+	-	
A. indonesiensis	LMG 19824[T b]	Fruit of zirzak	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. peroxydans	LMG 1635[T b]	Ditch water	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A. syzygii	LMG 21419[T b]	Syzygium malaccense	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A. orleanensis	LMG 1583[T b]	Beer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. estunensis	LMG 1626[T b]	Cider	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. orientalis	LMG 21417[T b]	Canna hybrid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
A. nitrogenifigens	LMG 23498[T b]	Kombucha tea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	DSM 6160[T b]	Submerged culture vinegar	-	+	+	-	+	+	-	-	+	-	-	+	-	-	+	-	-	-	+	
Ko. europaeus	LMG 18890[T b]	Submerged culture vinegar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	DSM 2004[b]	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Gae02	Traditional wine vinegar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Ko. hansenii	DSM 5602[T b]	Vinegar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	LMG 1524[b]	Vinegar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	LMG 1529[b]	Malt vinegar brewery acetifiers	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ko. xylinus	LMG 1515[T b]	Mountains ash berries	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DSM 2325[b]	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	LMG 1518[b]	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ko.nataicola	LMG 1536[T b]	Nata de coco	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ko.rhaeticus	LMG 22126[T b]	Organic apple juice	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ko.swingsii	LMG 1674[T b]	Beer	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Ko.saccharivorans	LMG 1582[T b]	Beet juice	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
	Gs1[c]	Microfermentation of healthy grapes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ko. oboediens	LMG 18849[T b]	Red wine vinegar fermentation	-	+	+	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+
Ga.sacchari	LMG 19747[T b]	Sugar cane, leaf sheath	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ga.azotocaptans	LMG 21311[T b]	Coffea arabica L., rhizosphere	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ga.diazotrophicus	LMG 7603[T b]	Saccharum officinarum, root	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ga. liquefaciens	DSM 5603[T b]	Dried fruit	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ga. johannae	DSM 13595[T b]	Rhizosphere of coffee plants	+	+	+	+	+	+	-	+	+	-	-	+	-	+	+	-	-	-	+

G. oxydans	DSM 7145[T b]	Beer	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	LMG 1408[T b]	Beer	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DSM 2003[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G.frateurii	LMG 1365[T b]	Fragaria ananassa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G. thailandicus	LMG 23137[T b]	Flower, *Millingtonia hortensis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G. japonicus	Gj1	Microfermentation of healthy grapes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Gj2		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Gj3		+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+
G. albidus	LMG 1356[T b]	Fruit of apple guava, *Psidium guajava*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G. cerinus	NBRC 3267[T b]	Cherry, *Prunus sp.*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] The culture media used to evaluate biofilm production: GY, glucose medium; GYE, glucose medium supplemented with 1% of ethanol; FY, fructose medium; FYE, fructose medium supplemented with 1% of ethanol; SY, sucrose medium; SYE sucrose medium supplemented with 1% of ethanol.

[b] The strains were obtained from the following culture collections: DSMZ, German Collection of Microorganisms and Cell Cultures; BCCM-LMG, Belgian Coordinated Collections of Microorganisms; NBRC, Japan Society for Culture Collections; [T], type strain.

2.4. Primer design

Degenerate primers were designed using the AAB sequences available in the GenBank database for the cellulose synthase gene (named *bscA*, *acsA*, or *celA*). These sequences were aligned with the ClustalW multiple sequence alignment programme (Thompson et al., 1994), and the primers were designed with the assistance of the Primer Express software package (v. 3.0, Applied Biosystems, Foster City, CA, USA). A total of eight degenerate primers were designed based on either the DNA sequences (primer pairs CELS1 for a 793-bp fragment), the amino acid sequences (three primers, CELS2 for a fragment of 492 bp or 369 bp), or both the DNA and amino acid sequences (three primers, CELS3 for a fragment of 492 bp or 369 bp) (Table 2).

Table 2.- Primers used for homologous *bcsA* gene region amplification and accession numbers of the sequences used for primers design.

Oligonucleotide	Name	Sequence (5'→ 3')	PCR product	GeneBank database Accession Number
Forward Primer	CELS1_Fw	GCMAAGGCSGGTYAAYCTBAAC		FJ263059.1; M37202.1; AB010645.1; AB091060.1; X54676.1
Reverse Primer	CELS1_Rv	GCACCAGGAACAGCGCCAT	793 bp	
Forward Primer	CELS2_Fw	CAYGCNAARGCNGGNAAY		BAA31463.1; ZP_08901181.1; EFG85216.1; Q9WX75.1; ZP_08899535.1; BAK85161.1; AAA85264.1; BAK83199.1; ZP_08318442.1; ZP_08897031.1
Reverse Primer	CELS2a_Rv	CATNCCNCKNGCCCANCK	492 bp	
Reverse Primer	CELS2b_Rv	NGTRTGNGCRTCYTCNGT	369 bp	
Forward Primer	CELS3_Fw	CAYGCMAAGGCSGGTAAY		BAA31463.1; ZP_08901181.1; EFG85216.1; Q9WX75.1; ZP_08899535.1; BAK85161.1; AAA85264.1; BAK83199.1; ZP_08318442.1; ZP_08897031.1; FJ263059.1; M37202.1; AB010645.1; AB091060.1; X54676.1
Reverse Primer	CELS3a_Rv	CATSCCRCGBGCCCAGCG	492 bp	
Reverse Primer	CELS3b_Rv	GGTATGSGCRTCYTCBGT	369 bp	

2.5. PCR conditions

Different conditions were tested to optimise the PCR amplification. Three concentrations of $MgCl_2$ (1.5 mM, 2.5 mM, and 3 mM) and different annealing temperatures (55ºC, 57ºC, 60ºC, and 62ºC) were assayed.

The final amplification reactions were performed in a total volume of 50 µl. The PCR mixture was tested with a primer concentration of 400 mM, 200 mM of each of the four dNTPs (Roche Diagnostics GmBh, Manheim, Germany), 1 × PCR reaction buffer (Ecogen, Spain), 1.5 mM $MgCl_2$, 0.4 U EcoTaq DNA Polymerase (Ecogen, Spain), and 2 µl of DNA template (approximately 50 ng/ml). An initial denaturation step at 95ºC for 5 minutes, followed by 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 62ºC for 25 seconds and extension at 72ºC for 30 seconds, and a final extension cycle at 72ºC for 5 minutes were performed in a GeneAmp PCR System 2700 (Applied Biosystems). The PCR products were visualised by electrophoresis in 1% (w/v) agarose gels using a DNA XIV 100 bp ladder (Roche Diagnostics, Mannheim, Germany).

2.6. Sequencing and sequence alignment analysis

All of the positive PCR products were purified and sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. Phylogenetic and molecular evolutionary analyses of these sequences based on the neighbor-joining and Kimura 2-parameter methods were conducted using MEGA version 4 (Kimura, 1980; Tamura et al., 2007).

Moreover, the predicted amino acid sequences were analysed. The DNA sequences were translated into amino acid sequences using ExPASy, the SIB bioinformatics resource portal (Artimo et al., 2012), and these sequences were compared using the BLAST program (Altschul, 1990) and the GenBank databases. The amino acid sequences were assembled using ClustalW and visualised using ESPript software (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi).

3. Results

3.1. Cellulose production by AAB in different carbon sources

Cellulose production was assessed for 77 strains of 35 different AAB species belonging to the *Acetobacter*, *Komagataeibacter*, *Gluconacetobacter*, and *Gluconobacter* genera. These strains were inoculated into six different types of media containing different carbon sources. All of the strains grew in all of the media tested, and 30 strains were able to produce a biofilm in at least one condition after 72 hours of growth at 28ºC (Table 1). Of these 30 strains, 26 formed a biofilm under all six conditions; 21 strains produced a biofilm after 24 hours of growth, while five strains needed between 48 and 72 hours to produce the biofilm. The additional growth time required to produce a biofilm was most common in media that contained fructose or sucrose instead of glucose. Of the 26 strains that produced a biofilm in all of the different types of media, there were strains belonging to all *Komagataeibacter* species tested, two of the five *Gluconacetobacter* species (*Ga. sacchari* and *Ga. azotocaptans*), two of the six *Gluconobacter* species (*G. oxydans* and *G. japonicus*) and six of the 16 *Acetobacter* species tested (*A. pasteurianus*, *A. aceti*, *A. pomorum*, *A. peroxydans*, *A. syzygii*, and *A. orientalis*). None of the strains from the remaining ten species of *Acetobacter* produced biofilms in any media.

On the other hand, two strains, one of *Ko. europaeus* and the other of *Ga. johannae*, produced biofilm in all media except the SYE medium. Two other strains produced biofilm after 72 hours and only under specific conditions. One strain of *A. pasteurianus* formed a biofilm when sucrose was the carbon source, and a strain of *Ko. saccharivorans* produced a biofilm in the presence of glucose.

The genus with the most strains that formed biofilms was *Komagataeibacter*; 15 strains out of the 16 strains tested (93.7%) were able to produce a biofilm. The other genera tested presented a lower number of strains with this phenotype. Three out of five *Gluconacetobacter* strains (60%) produced biofilms, three out of 10 *Gluconobacter* strains (30%) produced biofilms, and only nine out of 46 *Acetobacter* strains produced biofilms (19.5%).

The biofilm formation presented different forms depending on the strain (Fig. 1a). In some cases, it was a matrix located on the surface of the liquid, even when the culture was stirred, and in other cases, this matrix was a single aggregate or multiple small aggregates in the liquid. This characteristic was not strain dependent because the same strains could develop both phenotypes randomly.

To determine if the matrix secreted by the bacteria corresponded to the EPS cellulose, samples from all tubes were taken at 72 hours, stained with Calcofluor dye and visualised under an epifluorescence microscope. All of the strains that were able to produce biofilm produced cellulose-containing biofilms. This staining was not observed when there was a lack of visible biofilm.

The strain Gae02 of *Ko. europaeus* presented an intense cellulose biofilm formation in both shaken and static cultures. In shaken cultures and in all of the carbon sources tested, the cells grew inside the cellulose matrix while the medium remained clear. Biofilm sampling from the static culture was conducted over 72 hours and photographed under epifluorescence microscopy (Fig. 1b). The amount of AAB cells in the matrix progressively increased with time; the cells formed aggregates, which were larger and closely arranged throughout the time course. After 72 hours, most of cells were green-coloured, representing live cells, and appeared aggregated inside a matrix.

The use of Calcofluor dye allowed us to observe that the cellulose fibrils were primarily found in regions with a high density of cells.

a

b

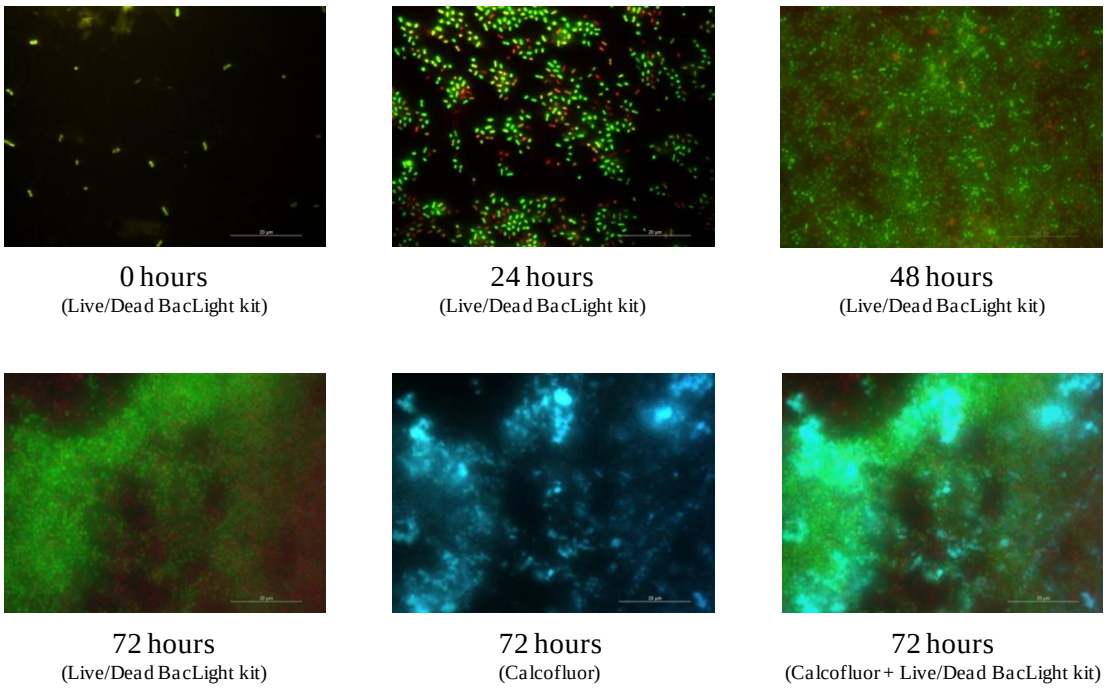

Fig. 1. Phenotypes of AAB strains: (a) biofilm formation in FY, GY, and SY culture media for *Ko. rhaeticus* strain LMG 22126 (the first three tubes) and *A. pasteurianus* strain Ap4 (the last three tubes); (b) progressive biofilm formation of *Ko. europaeus* strain Gae02 in GY broth. The changes in cell viability were visualised by epifluorescence microscopy with the Live/Dead BacLight Kit, cell clumping and the arrangement of Calcofluor-stained cellulose fibrils.

3.2. PCR optimisation and detection of the cellulose synthase gene in AAB

A PCR assay was designed to detect the presence of the cellulose synthase gene in the 77 AAB strains that were analysed for cellulose production. Eight different primers were designed using the sequences available in GenBank databases. All of these primers targeted the same region of the gene, although the fragment size was variable. Optimisation of PCR with these different primer pairs was initially performed using five strains from three different genera that were positive for cellulose production in any of the media tested (*Ko. oboediens* LMG 18849^T; *Ko. nataicola* LMG 1536^T; *Ko. saccharivorans* LMG 1582^T; *Ga. sacchari* LMG 19747^T; *A. pomorum* LMG 18848^T) prior to testing the remaining strains. This optimisation was necessary due to difficulties with the specificity and sensitivity of the assay.

The primer pair CELS1_Fw/CELS1_Rv was designed using the DNA sequences of the cellulose synthase genes available in the GenBank database. PCR amplification generated the expected fragment of 793 bp at annealing temperatures of 55ºC, 57ºC, and 60ºC, but additional unspecific bands with longer and shorter sizes were also present. Attempts to make the conditions more restrictive by using an annealing temperature of 62ºC and a lower $MgCl_2$ concentration (1.5 mM instead of the initial 3 mM) was tested without any improvement in the specificity.

The combinations of primers CELS2_Fw/CELS2a_Rv and CELS2_Fw/CELS2b_Rv were designed using the amino acid sequences designated in the GenBank database as cellulose synthase, and the degeneracy of the primers was higher than the previous primer pair. Multiple PCR conditions were assayed; however, no amplification fragments were obtained with any of the primer pairs.

Finally, the combinations of primers CELS3_Fw/CELS3a_Rv and CELS3_Fw/CELS3b_Rv were designed by comparing both the amino acid sequences and DNA sequences. In this case, the primers' degeneracy was lower than that obtained with the CELS2 primers. Positive amplification products were detected for the two primer pairs in all PCR conditions tested. However, reliable results were obtained using the first primer combination, CELS3_Fw/CELS3a_Rv, with a 62ºC annealing temperature and a concentration of 1.5 mM $MgCl_2$. Under these conditions, the expected size of 492 bp was obtained, and the reproducibility and the intensity of the band were adequate for the first five AAB strains assayed. Therefore, the remaining strains were analysed using these primers and PCR conditions.

Of the 77 AAB strains used in this study, we were able to amplify the cellulose synthase gene for 27 strains, and 26 of these strains had formed cellulose-containing biofilms in the previous experiment. The remaining strain belonged to *A. cerevisiae*; although we were able to amplify the gene, no biofilm production was observed in any media. The PCR assay failed to amplify the cellulose synthase gene from four strains in which a visible biofilm was observed, including two strains of *Acetobacter* species (*A. pasteurianus* and *A. orientalis*) and two strains of *Komagataeibacter* (*Ko. europaeus* and *Ko. swingsii*) (Table 1).

3.3. Analysis of the cellulose synthase gene sequences from AAB

All of the amplicons of the cellulose synthase gene were sequenced and compared with the sequences available in the GenBank database. Only 21 of the 27 amplicons were correctly sequenced; the sequences of the other six were not clear due to overlapping nucleotides.

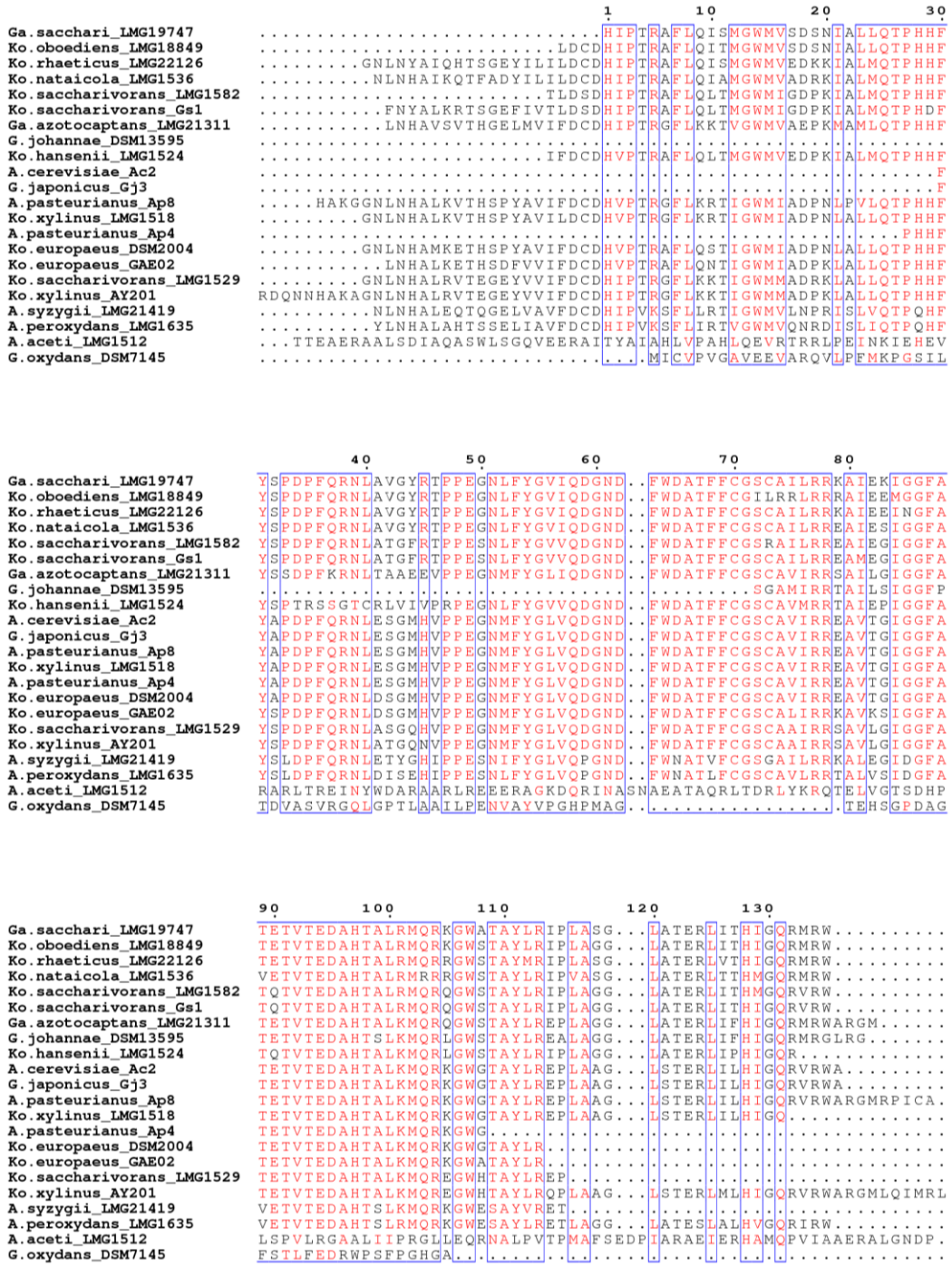

Fig. 2. Amino acid sequence alignment of cellulose synthase from 21 AAB strains and the cellulose synthase protein of *Ko. xylinus* AY201 strain (GenBank accession number U15957) characterised enzymatically. The red characters signify similarity in a group, and the blue frames signify similarity across groups.

The 21 sequences presented a percentage of homology ranging from 67% to 99% with those sequences available in the GenBank database compared to the *bscA* gene or their homologous *acsA* from different species of AAB.

A dendrogram was constructed with the 21 DNA sequences and with several sequences available in the GenBank database to identify the similarities between these sequences. The phylogenetic analysis showed several clusters that could not be clearly associated with a specific species or genus (data not shown). All of the amino acid sequences analysed, except for those of *A. aceti* strain LMG 1512 and *G. oxydans* strain DSM 7145, contained domains homologous to the glycosyltransferase family A proteins (GT-A). The alignment of sequences presented several similarity motifs that ranged between one and 21 amino acids (Fig. 2).

4. Discussion

AAB develop a polymer matrix at the liquid-air interface of vinegars elaborated by the traditional method. Cellulose is the primary EPS in these matrices. In fact, AAB is one of the bacterial groups that is able to produce a cellulose form designated BC, and *Ko. xylinus* is considered a model microorganism for the production of this cellulose (Chawla et al., 2009; Römling, 2002). Moreover, an improvement of the media has been reported to increase the yield of cellulose production in some AAB species; alterations of the carbon source is the most well-studied factor (Keshk and Sameshima, 2005; Mikkelsen et al., 2009; Trovatti et al., 2011). In the present study, a cellulose biofilm production screening was performed with different carbon sources for 77 strains belonging to 35 AAB species; in most of these strains, cellulose production had never previously been analysed by any method other than the cellulose production test (Carr and Passmore, 1979) that carried out for the description of novel species of AAB. The

presence of cellulose in the biofilm was assessed using the Calcofluor fluorochrome. This test is generally performed prior to the use of molecular techniques (Römling, 2002). It can be applied directly to the plates or by microscope preparation (Zogaj et al., 2003). In present study, all of the biofilms produced by the AAB strains were positive for cellulose under microscopy after staining with Calcofluor. This method allowed us to visualise the fibrils generated in the matrix.

When glucose was the primary carbon source, more AAB strains were able to produce cellulose, and the supplementation of the media with ethanol did not change this preference. It has been reported that glucose is one of the best sources for cellulose production by *Ko. xylinus* (Keshk and Sameshima, 2005; Mikkelsen et al., 2009) and by *Ko. sacchari* (Trovatti et al., 2011). However, some studies have identified sucrose as a better carbon source for cellulose production (Ramana et al., 2000), although growth in sucrose delays the cellulose production. In accordance with these results, some of the AAB strains used in this study produced cellulose biofilm later when they grew on sucrose media. Some authors suggested that this delay in cellulose production was because sucrose must be transported to the periplasm and hydrolysed to glucose and fructose prior to being used for cellulose production (Mikkelsen et al., 2009). In contrast, the results obtained with fructose were similar to those with glucose for all the strains, as was previously observed for *Ko. xylinus* strains (Keshk and Semeshima, 2005; Mikkelsen et al., 2009; Nguyen et al., 2008). In the present study, the effect of ethanol was not favourable. Ethanol supplementation has been reported to improve the quantity of cellulose in fructose (Naritomi et al., 1998) or glucose media (Park et al., 2003a). However, the presence of ethanol has also been related to a reduction in the growth of the cells that caused an initial delay in cellulose production that was corrected

or even increased after the fifth day when compared with the media without ethanol (Park et al., 2003a).

Cellulose biofilm production was tested in 35 AAB species from the *Komagataeibacter*, *Gluconacetobacter*, *Acetobacter*, and *Gluconobacter* genera, and 19 of these species yielded positive results after three days. Species of the *Komagataeibacter* genus are considered to be the main BC producers, particularly strains belonging to the species *Ko. xylinus*, *Ko. hansenii*, *Ko. swingsii*, *Ko. rhaeticus*, and *Ko. nataicola* (Aydın and Aksoy, 2014; Castro et al., 2013; Park et al., 2003b). In this study, all of the strains tested from these species produced cellulose biofilms. Furthermore, strains of *Ko. oboediens*, *Ko. saccharivorans*, and three out of four strains of *Ko. europaeus* also produced cellulose. The species of *Gluconacetobacter* are more related to nitrogen fixation, and *Ga. diazotrophicus*, *Ga. azotocaptans*, and *Ga. johannae* have been reported to fix nitrogen (Pedraza, 2008). However, cellulose production has been also reported for *Ga. sacchari* and *Ga. intermedius* (Castro et al., 2013). In our hands, cellulose production was observed for the tested *Ga. sacchari*, *Ga. azotocaptans*, and *Ga. johannae* strains but not for the *Ga. diazotrophicus* or *Ga. liquefaciens* strains. Within the *Acetobacter* genus, some strains from *A. pasteurianus* (Gullo et al., 2012), *A. aceti* (Dayal et al., 2013), and *A. tropicalis* (Ibnaof et al., 2011) have been described as polysaccharide biofilm producers. In this study, only the 23% and 33% of the strains for the first two species, respectively, were cellulose biofilm producers. However, the strains of *A. pomorum*, *A. peroxydans*, *A. syzygii*, and *A. orientalis* that we tested were also able to produce a visible cellulose matrix. Within the *Gluconobacter* genera, ten strains belonging to six different species were tested, and only two of *G. oxydans* strains and one *G. japonicus* strains produced visible cellulose biofilms. Of the *Gluconobacter*

species, cellulose production has been previously reported only for *G. oxydans* (Jia et al., 2004).

Although a large amount of information about cellulose production by AAB has been reported over the last years, especially for *Ko. xylinus*, cellulose production has not been accurately associated with the presence of any specific gene. The *bscA* gene, which encodes a cellulose synthase enzyme, is the most well-conserved cellulose enzyme among the bacterial species. This gene encodes a protein with an amino acid sequence that is highly conserved in some regions (Römling, 2002; Saxena et al., 1994). Therefore, this gene is one of the most appropriate targets to try to correlate the production of cellulose with the presence of the *bcs* operon by PCR amplification. In fact, it was previously used to study the cellulose production by enterobacteria (Zogaj et al., 2003). The lack of sequences available from the AAB and the reduced homology in the available gene sequences were the initial challenges for the design of primers.

Of the eight primers tested in this study, only the CELS3_Fw/CELS3a_Rv degenerate primer pair amplified the *bcsA* gene with reliable results. This set of primers was designed using a combined approach between the DNA and protein sequences. The use of degenerate primers presents some hurdles such as reduced specificity. However, with this primer pair, the expected size of 492 bp fragments was obtained for most of the AAB strains that presented the cellulose production phenotype. The *bcsA* gene was not amplified from four strains (two strains of *Acetobacter* and two strains of *Komagataeibacter*) that produced cellulose. The genome of *Ko. xylinus* encodes more than one gene for cellulose synthase (Römling, 2002). These genes, *acsAB* and *acsAII*, have a high degree of homology, but their DNA sequences are not similar enough to hybridise using the same probe (Saxena and Brown, 1995). Thus, it is likely that some

of the strains that did not amplify with our set of primers could use alternative cellulose synthase genes.

On the other hand, in one strain of *A. cerevisiae* species that has never been studied in terms of its cellulose production, the presence of the *bcs*A gene was detected, but a visible biofilm was not produced. This may be because the cellulose production is a multistep process that is specifically regulated and involves many enzymes and catalytic complexes (Chawla et al., 2009). The presence of the *bcsA* gene may be necessary, but other genes are also involved. Moreover, in addition to the growth medium, there are environmental conditions and other metabolic pathways that could affect the production of cellulose (Chawla et al., 2009; Zogaj et al., 2003). Furthermore, the conditions used for the preservation of the strains have been reported to determine the loss or delay of the cellulose-forming phenotype (Wiegand and Klemm, 2006). Therefore, a longer time of study would have been necessary to determine if this strain required more than three days for cellulose production.

As expected by the low homology between the cellulose synthase gene sequences available for AAB in the GenBank database, the analysis of the sequences from the *bcsA* gene amplicons did not show high homologies between strains of the same species, and therefore, it was not possible to obtain clusters by species or genera. However, the amino acid sequences for most of the strains were similar. They shared homologous motifs and grouped into the GT-A family. The cellulose synthase enzyme contains motifs that are conserved with other β-glycosyltransferases (Römling, 2002; Saxena et al., 1994).

The analysis of *bcsA* gene expression by Real Time PCR would complement the function of this gene and the correlation of its expression with the production of

cellulose. However, the low homology between *bcsA* gene sequences at the species and even strain levels makes it difficult to design primers for AAB and to assess changes in gene expression. Thus, no appropriate sequence has been found for the design of primers or TaqMan probes for Real Time PCR for AAB.

The biofilm formation of the 30 AAB strains presented different characteristics depending on the strain and growth conditions. Nguyen et al. (2008) described the different forms of biofilm produced by a strain of *Ko. xylinus* in static or shaken cultures, even under the same conditions. Park et al. (2003a) reported that the biofilm aggregates presented different morphologies ranging from small pellets or clumps to a thick layer depending on the growth. The monitoring of biofilm formation by the strain Gae02 was successfully visualised by epifluorescence microscopy using the Live/Dead BacLight Kit. These dyes have been applied to investigate the development of several biofilms (Cooper, 2014; Neu and Lawrence, 1997), but, to the best of our knowledge, have never been used to monitor AAB biofilms. The strain Gae02 produced cellulose very quickly; large amounts of cellulose floating in the medium were detected after a few hours of growth. Observations using epifluorescence microscopy showed that the cellulose matrix wrapped around the AAB cells. The biofilm formation changed from isolated single cells to cell aggregates in a few hours, as was previously described for *Ko. hansenii* (Park et al., 2003a).

In summary, the formation of cellulose biofilms by AAB and its relationship to cell viability were successfully monitored by epifluorescence microscopy and the use of fluorochromes such as Calcofluor and the Live/Dead BacLight Kit. The use of six different types of culture media was adequate to evaluate the production of cellulose biofilm in strains of AAB species that have never been studied before. Furthermore, the

design of degenerate primers allowed for the detection of the homologous *bcs*A gene in the majority of the AAB strains that produced cellulose. This gene encodes cellulose synthase, the catalytic subunit for cellulose biosynthesis. These results can be used for the selection of strains in biotechnological, biomedical, or high-technology applications where the production of cellulose is sought. Likewise, it is of interest in the production of vinegar, where lower cellulose-producing strains are desirable.

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Chapter 5

First evidence that a prephenate dehydratase is involved in quorum quenching of AHL molecules and cellulose-biofilm formation in the strain Gae02

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Abstract

The genome of the cellulose overproducer strain Gae02 of *Komagataeibacter europaeus* was screened to detect quorum sensing (QS) and quorum quenching (QQ) activity. In addition to two fosmid clones with putative QS activity detected, an open reading frame designed *gqqA* that interfere with bacterial QS was detected and subsequently analysed.

This *gqqA* gene presented ability to modify pyocyanin production and swarming motility, QS regulated features in the *P. aeruginosa* strain PAO1.Aas well as the purified GqqA protein, obtained from the *E. coli* strain BL21 (DE3) with the vector pET-21a::*gqq*A, was able to interfere the QS of the reporter strains of *A. tumefaciens* NTL4 and *C. violaceum* Cv026. The presence of this protein altered the cellulose production phenotype of the Gae02 strain and other AAB strains. The predicted amino acid sequence of this GqqA protein, as well as DNA sequence of the original ORF, presented the highest homology with Prephenate dehydratase. However, the complementation assays performed with two defective mutants of *E. coli* did not corroborate the metabolic function of this enzyme. Therefore, further analyses are required to characterize this protein of 30 KDa isolate from an AAB strain involved in QQ of AHL molecules.

Keywords: Homoserine lactone, Acetic acid bacteria, Vinegar, Quorum quenching, genomic fosmid library.

1. Introduction

Quorum-sensing (QS) is a cell density-dependent system, which allows coordinated expression of genes to regulate diverse physiological functions in microorganisms such as motility, production of extracellular proteins, biofilm formation, pathogenicity, and others (Shank and Kolter, 2009). This cell-cell communication is mediated by autoinducers such as N-acylhomoserine lactones (N-AHLs), which are the best-characterized quorum sensors used by many gram-negative bacteria, and their general mechanism of synthesis is well understood (Fuqua and Greenberg, 2002). In recent years, more than 20 molecules from QS and non-QS microbes have been reported as quorum quenchers for its capacity to interfere with these autoinducers (Bijtenhoorn et al., 2011a, 2011b; Dong and Zhang, 2005, Tannières et al., 2013). They could act as antagonists of the native autoinducer or as enzymes that produce the degradation of the autoinducer molecule, and thereby, inhibit the QS signalization (Dong and Zhang, 2005). However, the physiologically activity of most of these quorum-quenching (QQ) molecules is not clear (Chen et al., 2013).

Acetic Acid Bacteria (AAB) are a group of gram-negative aerobic bacteria within the *Acetobacteraceae* family. They are involved in the partial oxidation of carbohydrates and alcohols and the release of the organic acids as end products into the media (Guillamon and Mas, 2009). They are largely known for its ability to produce acetic acid on ethanol containing substrates, resulting vinegar. In the elaboration of vinegar by traditional method, AAB tend to be placed on the air-liquid interface, developing a cellulose pellicle, to be in direct contact with oxygen (Llaguno and Polo, 1991; Allison et al., 2000) and probably also in order to survive under stress conditions such as high ethanol or acetic acid concentration (Kanchanarach et al., 2010). Recently, the presence

of a QS N-AHL-dependent system termed GinI/GinR which is homologous to LuxI/LuxR described in *Vibrio fischeri* has been reported for *Komagataeibacter intermedius* (formerly *Gluconacetobacter intermedius*) (Iida et al., 2008a). Three different AHLs molecules with different acyl chains have been described: N-decanoyl-L-homoserine lactone (N-C_{10}-HSL), N-dodecanoyl-L-homoserine lactone (N-C_{12}-HSL) and C_{12}-HSL with a single unsaturated carbon bond (Iida et al., 2008a). Via these AHLs, the GinI/GinR QS system induce an *Omp*A family protein and other two more proteins throughout GinA protein, causing the repression of acetic acid and gluconic acid production as well as antifoam activity (Iida et al., 2008b; Iida et al., 2009). Moreover, the GinI/GinR QS system by induction of *nagA* gene accelerates the growth rate in the exponential growth phase (Iida et al., 2009). These are the only three studies about QS that has been reported in AAB; although biofilm production has been related with QS signalization in some bacterial species (Davies et al., 1998; Parsek and Greenberg, 2005) the identification and characterization of target genes involved is yet unknown in this group of bacteria, as well as there is not any information about their QQ ability.

In order to contribute in the knowledge of cell-cell communication of AAB, and concretely in the proteins present in this group of bacteria which interfere the bacterial QS signals, the aim of this study was to screen for QS and QQ activities in the genome of the *Komagataeibacter europaeus* strain Gae02 (formerly *Gluconacetobacter europaeus*), cellulose overproducer and biofilm-former strain isolated from vinegar produced by traditional method.

2. Materials and methods

2.1. - Bacterial strains, plasmids and culture conditions

During the present study different bacterial strains and constructs were used (Table 1). The *Escherichia coli* strains DH5α, EPI300™ and BL21(DE3) strains as well as the *Pseudomonas aeruginosa* strain PAO1 strain were grown in LB medium (1% Tryptone; 0.5% NaCl; 0.5% Yeast Extract) at 37ºC. Antibiotics were added in the medium depending on the vector used: chloramphenicol (final concentration of 12.5 mg/ml) for clones containing the fosmid vector CopyControl pCC1FOS™ Vector, ampicillin (final concentration 100 mg/ml) for clones containing the vector pBlueScript II SK (+) and the vector pET-21a, and gentamycin (final concentration 50 mg/ml) for clones containing the broad host range vector pBBR1MCS-5.

Plasmid transformation in *E. coli* was carried out following standard heat shock protocol (Sambrook and Russell, 2001) and by electroporation (Smith and Iglewski, 1989) in the case of *P. aeruginosa*.

The strain NTL4 of *Agrobacterium tumefaciens* (Luo et al., 2001), carrying a *traI-lacZ* promoter fusion on vector pCF372 (Fuqua and Winans, 1996) and extra copies of traR on vector pCF218 (Fuqua and Winans, 1994) was maintained at 30ºC in LB or AT medium (Tempe et al., 1977) containing 0.5% glucose per liter. Spectinomycin (final concentration 50 mg/ml) and tetracycline (final concentration 4.5 mg/ml) were added to maintain the vectors. The strain Cv026 of *Chromobacterium violaceum* was grown at 30ºC in LB medium. All AAB strains used in this study were grown in GY medium (1% yeast extract, 5% glucose) at 28ºC. When all these media were used as solid media, they were supplemented with 1.5% agar.

Table 1.- Bacterial strains, constructs and vectors used in this study

Bacterial Strain	Description	Reference or source
Escherichia coli EPI300™	F⁻ *mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ⁻ rpsL (StrR) nupG trfA dhfr*	Epicentre Biotechnologies, Madison, WI
Escherichia coli DH5α	F⁻ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK⁻, mK⁺) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen, Karlsruhe, Germany (Hanahan 1983)
Escherichia coli BL21(DE3)	F⁻ ompT hsdS_B (r_B^- m_B^-) gal dcm (DE3)	Novagen, Darmstadt, Germany
Pseudomonas aeruginosa PAO1	Wild type strain; Amp^r	Holloway et al., 1979
Agrobacterium tumefaciens NTL4 (pCF218)(pCF372)	Reporter strain for AHL detection; traI::lacZ Tet^r Sp^r	Fuqua et al., 1994; Fuqua et al., 1996; Luo et al., 2001
Chromobacterium violaceum CV026	Reporter strain for autoinducer I; mini-Tn5 in *cvi*I	McClean, 1997
Escherichia coli KA197	Phenylalanine auxotroph λ⁻, e14- , pheA97, relA1, spoT1, thiE1	Hoekstra et al., 1974
Escherichia coli JW2580-1	Phenylalanine auxotroph *F⁻ Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), *λ⁻ , ΔpheA762::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Baba et al., 2006
Acetobacter nitrogenifigens LMG 23498	Type strain. Cellulose producer	Dutta et al., 2006
Acetobacter pasteurianus Ap4	Strain recovered from healthy grapes microfermentation. Celullose producer	Valera et al., 2011
Acetobacter syzygii LMG 21419	Type strain. Cellulose producer	Lisdiyanti et al., 2001
Acetobacter orientalis LMG 21417	Type strain. Cellulose producer	Lisdiyanti et al., 2001
Komagataeibacter europaeus DSM 2004	Cellulose producer	Leibniz-Institut DSMZ, Germany
Komagataeibacter hansenii LMG 1524	Strain recovered from vinegar. Cellulose producer	BCCM LMG Collection, Belgium
Komagataeibacter rhaeticus LMG 22126	Type strain. Cellulose producer	Dellaglio et al., 2005
Komagataeibacter europaeus Gae02	Strain recovered from vinegar. Cellulose producer	This study
Vector/construct		
CopyControl pCC1FOS™	F-factor single-copy origin of replication and the inducible high-copy *ori*V	Epicentre Biotechnologies, Madison, WI
pBlueScript II SK (+)	Standard cloning vector (phagemid excised from lambda ZAP). The f1 (+) orientation allows rescue of sense strand ssDNA	Stratagene, La Jolla, CA, USA
pDrive	TA-cloning vector, *oriEc*, P_{lac}*lacZ*, Amp^R, Kan^R, T7-promotor	QIAGEN (Hilden, Germany)
pET21a	Expression vector, *lacI*, Amp^R, T7-promotor, C-terminal His_6-tag coding sequence	Novagen, Darmstadt, Germany
pET21a::*gqqA*	pET21a containing *gqqA* gene cloned into *Nde*I and *Xho*I restriction sites	This study
pBBR1MCS-5	Broad host range expression vector, *rep*, *mob*, *lacZ*, Gm^R	Kovach *et al.* 1995
pBBR1MCS-5::*gqqA*	pBBR1MCS containing *gqqA* cloned into *BamH*I and *Xho*I restriction sites	This study

2.2. - Komagataeibacter europaeus strain Gae02 strain fosmid library construction

The strain Gae02 of *Ko. europaeus* stored in our collection was recovered in GY medium (1% yeast extract, 5% glucose) at 28ºC, for 48h in shaken conditions (150 rpm). The genomic DNA of this strain was extracted with the DNA Isolation Kit for Cells and Tissues (Roche), and for its genomic fosmid library construction, the Copy Control™ HTP Fosmid Library Production kit with pCC1FOS™ Vector (Epicentre Biotechnologies, Madison, WI) was performed according to the manufacturer's instruction. The cells of the *E.coli* strain EPI300™ strain were spread on LB agar medium with chloramphenicol and incubated overnight at 37ºC. The transformed colonies were transferred into 96-well microtiter plates containing 150 µl of LB medium with chloramphenicol and were incubated overnight at 37ºC. After this, 50 µl of 86% glycerol were added to each well, and microtiter plates were stored at -70°C.

2.3. - Screening for N-AHL-degrading clones using Agrobacterium tumefaciens NTL4 reporter strain

The fosmid clones from genomic library of the strain Gae02 strain were screened, at least three times, for its capacity to inactivate AHLs or to block AHL receptors/promoters. This AT soft agar screening was performed with the *A. tumefaciens* NTL4 strain which carries a *traI-lacZ* reporter and does not synthesize autoinducers being incapable of reporting the presence of traI-inducing metabolites (Bijtenhoorn et al., 2011a; Schipper, et al., 2009). The concentration of 3-oxo-C8-HSL (Sigma-Aldrich, Heildelberg, Germany) used to supplement the soft AT agar medium was determined with a previous titling experiment without the presence of the fosmid clones. Concentrations from 10^{6} to 10^{-4} nM were tested determining 10 nM as threshold concentration.

2.4. - Genetic analysis of positive clones and subcloning analysis

The genetic analysis of positive clones was carried out after the AT soft agar screening to know the correct transformation of fosmid clones with DNA of the strain Gae02. Ends of inserts, of approximately 42 kb, were sequenced for positive tested fosmid clones using PCC1-Fos Rev and T7 promoter primers (Table 2) and automated sequencing technology of ABI3730XL DNA Analyzer (Applied Biosystems, Foster City, CA).

For detection of the concrete Open Reading Frames (ORFs) involved in QS inhibition, subcloning of fosmid clones was performed. The enzyme *Eco*RV (Fermentas, St-Leon-Rot, Germany) was used to obtain fragments which were ligated with T4 DNA ligase (Promega, Mannheim, Germany) in the plasmid pBlueScript II SK (+) and transformed into the *E. co*li strain DH5α strain.

Table 2.- Primers used in this study

Primer name	Sequence (5'→3')	Reference
PCC1-Fos Rev	CTC GTA TGT TGT GTG GAA TTG TGA GC	Epicentre Biotechnologies, Madison, WI
T7 Promotor	TAA TAC GAC TCA CTA TAG GG	Eurofins MWG Operon (Ebersberg, Germany)
M13-20 for	GTA AAA CGA CGG CCA GT	Eurofins MWG Operon (Ebersberg, Germany)
M13 rev	CAG GAA ACA GCT ATG ACC	Eurofins MWG Operon (Ebersberg, Germany)
gqqA Fw	CAT ATG AAC GGG GAA CGC ATC ATC	This study
gqqA Rv	CTC GAG GGG TTT GCG CCG GAA	This study

All the clones produced by subcloning were assayed for AT soft agar screening with *A. tumefaciens* strain NTL4 strain as above described. In addition, all of them were tested for pyocyanin production assays with the *P. aeruginosa* strain PAO1 strain as

previously described by Gallager et al. (2002), and for motility test with the transformed strain DH5α of *E.coli* using swarming agar as described Harshey and Matsuyama (1994). Cultures were grown for 16 h at 37ºC prior to the pyocyanin and motility assays; both analyses were carried out at least three times.

The positive clones in these screenings were completely sequenced using primers M13-20 for and M13 rev (Table 2). Gaps were closed by primer walking. Nucleotide and amino acid sequence comparisons were carried out using the BLAST program (Altschul, 1990) and the GenBank databases.

2.5. - Purification of His-tagged proteins

Considering the results obtained from the screening with reporter strains, an ORF named *gqq*A was selected. It was amplified using the primer pairs gqqA Fw and gqqA Rv (Table 2), the fragment was initially cloned into pDrive vector and then was excised and cloned into the expression vector pET-21a. Both amplicon and vector were digested with *Nde*I and *Xho*I and ligated directionally, yielding pET21a::*gqqa*. This construct was transformed into the *E. coli* BL21 strain (DE3), which was growth at 37ºC in LB medium with ampicillin to an OD600 of 0.5 to 0.8 values. Expression was induced by addition of 0.8 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside) (Sigma-Aldrich, Heildelberg, Germany) and cultures were incubated overnight at 28°C and 150 rpm. Cells were harvested by centrifugation at 10,000 rpm for 15 min and 4°C and resuspended in LEW buffer (50 mM NaH_2PO_4; 300 mM NaCl). Cells disruption through French press was performed three times at 1,100 Bar and the lysate was centrifuged at 15,000 rpm for 15 min and 4°C. The supernatant obtained was purified using Protino Ni-TED 2000 packed columns (Macherey-Nagel, Dueren, Germany) following the manufacturer's protocol. The protein purity and molecular mass were

determined by SDS-gel electrophoresis and its concentration using the Bradford protein assay (Bradford, 1976).

2.6. – GqqA protein effect in β-galactosidase activity using the Agrobacterium tumefaciens reporter strain NTL4

The ortho-Nitrophenyl-β-D-galactopyranoside (ONPG) test was also carried out in *A. tumefaciens* strain NTL4 with purified GqqA protein extracts. Both 3-oxo-C8-HSL and 3-oxo-C12-HSL (Sigma-Aldrich, Heildelberg, Germany) were tested by triplicate as previously described (Schipper et al., 2009) with minor modifications. A volume of 5 µl of AHLs with concentration of 10 nM or 100 nM was added to 100 µl of purified GqqA protein extracts (1 mg/ml) in 100 mM potassium phosphate buffer at pH 8.0, and was incubated at 30 °C for 2 h. This solution was added to 5 ml of AT medium with the *A. tumefaciens* strain NTL4, previously adjusted to 10^7 cells/ml, and incubated at 30ºC for 17h. The culture was measured at OD_{600}, and then, 1 ml of this culture was mixed with 20 µl of toluene and vortexed for 3 min. To 800 µl of this solution, the ONPG previously dissolved in Z-buffer was added as a substrate to a final concentration of 1mg/ml and it was incubated for 20 min at roon temperature. Finally, the reaction was stopped by addition of 400 µl of 1M Na_2CO_3 solution and after the centrifugation during 2 min at 13000 rpm, the absorbance of the uppter layer was measured at 420 nm with a SmartSpec Plus Spectrometer (Bio-Rad Laboratories, Munich, Germnay). The measured values for OD_{420} were divided by the values for OD_{600}.

Negative controls were performed using crude cell extract of *E.coli* BL21(DE3) strain instead GqqA at the same final concentration.

2.7. – GqqA protein effect in swarming motility and pyocyanin production of Pseudomonas aeruginosa PAO1 *reporter strain*

In order to analyze the effect of the *gqq*A gene in the motility and pyocyanin production of the *P. aeruginosa* strain PAO1, it was cloned into the broad host range vector pBBR1MCS-5. The ORF was amplified using the primer combinations gqqFw and gqqRv (Table 2) and the pBBR1MCS-5::*gqqA* construct was transferred to the *P. aeruginosa* strain PAO1 by electroporation.

The swarming motility test was performed in agar with M9 medium (Sambrook and Russell, 2001) and 0.05% glutamic acid but without NH_4Cl and solidified with 0.5% Eiken Agar (Eiken Chemical, Tokyo). One microliter with 1×10^7 cells of an overnight *P. aeruginosa* PAO1 culture was applied to the middle of the agar plate. Swarming phenotype was documented by photography after incubation at 37ºC during 16h. Triplicates of all the analysis were compared with controls using the strain PAO1 carrying the vector pBBR1MCS-5 recircularized.

2.8. – GqqA protein effect in violacein production by Chromobacterium violaceum Cv026 reporter strain

The strain Cv026 of *C. violaceum* was also used to analyze the purified GqqA protein effect. Both five and 15 µl of purified GqqA protein at 2 mg/ml were incubated with four different concentrations of C6-HSL (Sigma-Aldrich, Heildelberg, Germany) 10^4, 10^3, 10^2 and 10 µM separately. After an incubation of 3 h at 30ºC, this mixture was added to a tube containing 2 ml of LB medium and 10 µl of culture of the strain Cv026, and was incubated for 20 h at 30 ºC and 150 rpm.

Two different controls were used: in one of them, the molecule of C6-HSL was not added in order to see the residuary production of violacein by the strain Cv026, and in the other one, the protein was substituted by crude cell extract of the *E.coli* strain BL21 (DE3) strain (no transformed). Triplicates of violacein production were performed and photographed; absence or impairment of purple coloration indicated lack of QS activity.

2.9. - GqqA protein effect in growing and cellulose production phenotype of AAB

The effect of GqqA protein was also tested both in *Ko.europaeus* Gae02 and in other seven cellulose-producer strains of AAB, four belonging to *Acetobacter* and three to *Komagataeibacter* genera (Table 1). A culture of each strain was obtained in GY medium during 48h at 28ºC.

For the analysis of the growth of *Ko.europaeus* strain Gae02 growing, 50 µl of its culture were inoculated in tubes with 5 ml of GY medium and with three different concentration of purified GqqA protein extract (25µg, 50 µg and 100 µg). Controls were performed with three concentrations (25 µg, 50 µg and 100µg) of crude cell extract obtained from the not transformed *E.coli* strain BL21 (DE3) at the three concentrations (25µg, 50 µg and 100 µg). All the conditions were performed by triplicate. The growth of the strain Gae02 was monitored at 16h, 24h, 48h and 72h by measuring the OD_{600} of the medium, microscope counting, plating onto GY solid medium and visual inspection.

For the analysis of the growth of AAB strains, 50 µl of each strain culture were inoculated in 24-well microplates with 2 ml of GY medium and were supplemented with 100 µg of purified GqqA protein extract. Controls were carried out with 100 µg of Albumin from Bovine Serum (BSA) (Sigma-Aldrich, Heildelberg, Germany) instead GqqA protein. All the conditions were performed by triplicate. The results were photographed after 16h of growing.

2.10. - Complementation assays in auxothrophic Escherichia coli strains

This test was carried out with *E. coli* auxotrophic strains for phenylalanine, which were obtained from the Coli Genetic Stock Center (www.cgsc.biology.yale.edu). The strain JW2580-1, in-frame, single-gene knockout from the Keio Collection (Baba et al., 2006)

and the strain KA197 (Hoekstra et al., 1974) are described in Table 1. These strains were recovered in LB medium overnight at 37ºC. Their phenylalanine auxotrophy was checked by growing on minimal medium M9 supplemented with and without D-phenylalanine (10 μM) (Sigma-Aldrich, Heildelberg, Germany).

Both strains were transformed with the constructed vector pET21a::*gqqa* following the protocol described by Dagert and Erlich (1979). These transformed strains and auxothophics ones were grown in LB medium overnight at 37°C and 150 rpm, five milliliters of the cultures were centrifuged, cells were washed with sterile water and recovered after centrifugation in 1 ml of sterile water. Then, 75 μl of a 1:100 dilution were plated onto M9 medium with and without phenylalanine.

2.11. - Screening for N-AHL-producer clones using the Agrobacterium tumefaciens reporter strain NTL4 (Quorum Sensing analysis)

All the fosmid clones from the genomic library of the strain Gae02 were screened again, at least three times, in this case for their ability to produce AHLs. The AT soft agar screening was performed using plates of 96 wells with 200μl of AT soft agar (Schipper et al., 2009; Bijterhoorn et al., 2011a). The strain NTL4 of *A. tumefaciens*, previously grown in 5 mL of AT medium (Tempe et al., 1977) supplemented with spectinomycin (final concentration, 60 g/ml) and tetracycline (final concentration, 10 g/ ml) during 16h at 30 ºC, was added to the ATsoft agar with a final cell density of 10^7 cells/ml. The plates were inoculated with the fosmid clones from the genomic library, which were previously grown overnight at 37ºC in LB media supplemented with chloramphenicol, and incubated for 20h at 37ºC. The development of a blue colour medium signifies AHL production, therefore, QS activity.

The ONPG test was also carried out with clones derived from the genome of the strain Gae02, positive for the streening in AT soft agar. These clones were grown until a concentration of OD600 of 1 and volunes of 100µl and 400µl of these cultures were tested by triplicate (Schipper et al., 2009). The test tubes with a final volume of 5 ml of AT medium were incubated for 16 h at 30°C and after that, the cell density was calculated in accordance to their OD600. Moreover, 1 mL of this culture was mixed with 20 µL toluene and vortexed for 3 min. The ONPG solution previously dissolved in Z-buffer (Schipper et al., 2009) was added to a final concentration of 1 mg/mL, incubated for 20 min at room temperature and finally, the reaction was stopped by addition of 400 µL of 1 M Na_2CO_3 solution. After the centrifugation during 2 min at 13,000 rpm, the absorbance of the upper layer was measured at 420 nm with a SmartSpec™ Plus Spectrometer (Bio-Rad Laboratories, Munich, Germany).

In both assays, negative controls were performed using the *E.coli* strain EPI300™ without any fosmid vector instead of the fosmid clones at the same OD and quantity. Positive controls were constructed with the AT media supplemented with 3-oxo-C8-HSL (Sigma-Aldrich, Heildelberg, Germany) in a final concentration of 10^9nM whithout any other supplementation.

3. Results

3.1. - Detection of QS interfering clones and genetic analysis

A fosmid library constructed from *Ko. europaeus* Gae02 strain generated a total of 1,824 fosmid clones (Fig. 1). These clones were tested using *A. tumefaciens* NTL4 reporter strain carrying a *traI-lacZ* reporter gene (ATsoft agar screening) for QQ activities.

A total of 13 fosmid clones consistently gave a positive result for QS inhibition in soft AT agar medium, seven of them were digested and subcloned, and the obtained subclones were tested again with *A. tumefaciens* NTL4 strain in soft AT agar medium.

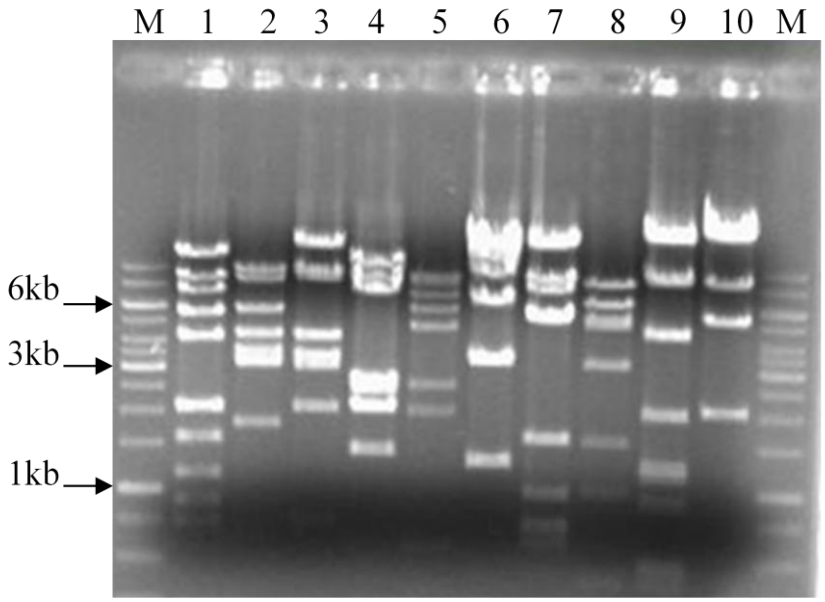

Fig. 1..- Restriction patterns of ten fosmid clones from genomic library of *Ko. europaeus* Gae02 strain obtained with *Bam*HI enzyme (lanes 1-10); GeneRuler 1Kb DNA ladder (Thermo Scientific) (lane M). The probability to contain all the genome in the whole library was 99.99 % calculated according to the manufacturer's instructions.

From this initial screening, 16 positive clones were then analyzed also using *P. aeruginosa* PAO1 reporter strain for pyocyanin production and *E.coli* DH5α transformed strain for motility test. All of them were sequenced and compared with the NCBI database (data not shown). Two clones were selected because they presented the same sequence and strong QS inhibiting phenotype in the assays performed. The sequence insert of these two clones with a size of 1.8 kb was analyzed. Thereby three ORFs were detected: ORF1 encoded for 3-deoxy-D-manno-octulosonate cytidylyltransferase, ORF2 encoded for prephenate dehydratase and ORF3 encoded for

dihydropicolinate synthase. However, only ORF2 encoded a complete protein that was labeled as GqqA (Fig. 2A).

The sequence of ORF2 with 846 bp corresponded to 281 amino acids and was similar to the gene *phe*A annotated in the genomes of several *Gluconacetobacter* spp. The highest similarity was found with *Ko. europaeus* strain LMG 18494 (WP_010507907.1) on the amino acid level with a predicted prephenate dehydratase (PDT) protein. No conserved domains known to be involved in lactonases or any other described AHL-degrading molecule were identified in the *gqqA* gene sequence. A phylogenetic analysis suggested that GqqA formed a distinct cluster of proteins within the known QQ proteins (Fig. 2B). The alignment of the sequence of GqqA with PDT amino acid sequences of other microorganisms showed that the GqqA protein conserves some residues, which are also present in the homologous region of PDT family proteins. These residues corresponded from 6 to 183 amino acid position and an ACT domain between residues 195 and 273 present in all the PDT sequences (Fig. 3).

PDTs are involved in the metabolic pathway of the aromatic amino acids, convert prephenate to phenylpyruvate in L-phenylalanine biosynthesis. In order to confirm this identity and corroborate the proposed activity for this enzyme, two defective *E. coli* strains (JW2580-1 and KA197) for the gene *phe*A obtained from the *E. coli* Genetic Stock Center were used for the complementation assay. Both *E. coli* mutants were transformed with the plasmid pET21a::*gqqa*, however, they were not able to grow in the M9 minimal medium. Supplementing this medium with phenylalanine allowed their growth. This indicated that the activity of the *gqqA* gene product was not coincident with the predicted PDT enzyme activity.

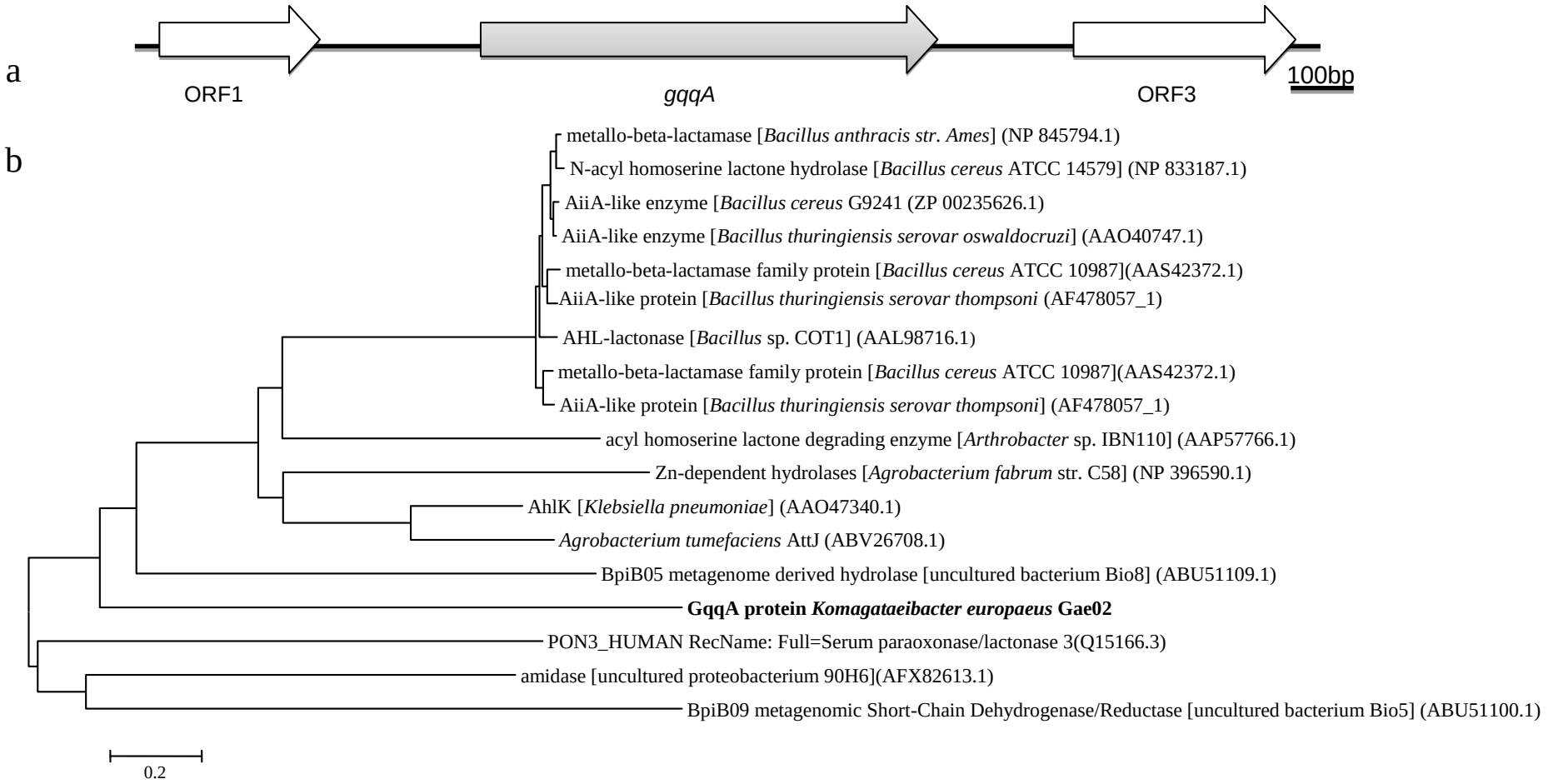

Figure 2. – A) Physiological map of the predicted ORFs in the insert (1.8kb) obtained after subcloning which contained the selected positive QQ clone.The position and the direction of transcription are indicated for ORFs. B) Phylogenetic analysis of GqqA protein and 17 proteins with QQ activity described. Amino acid sequences were obtained from NCBI GenBank and accession numbers are in brackets. The dendrogram was constructed after clustalW alignment using the neighbor-joining and Kimura two-parameter methods subjected to 1,000 bootstrap trials and conducted with MEGA software version 4 (Kimura, 1980; Tamura et al., 2007).

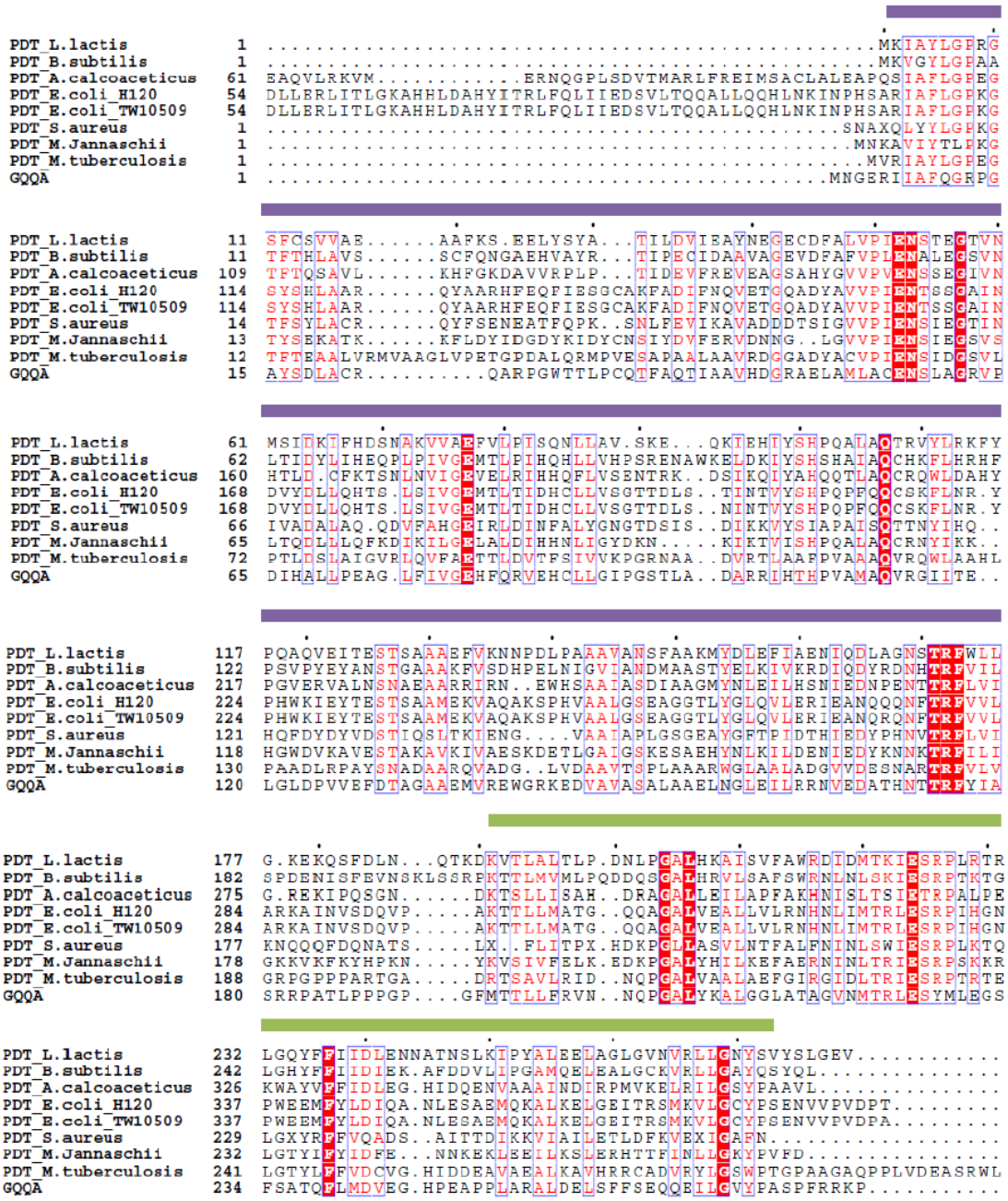

Fig. 3.- Amino acid sequence alignment of GqqA protein and eight PDT proteins characterized enzymatically (GenBank accession number in brackets) from *Lactococcus lactis* (CAA55182.1); *Bacillus subtilis* (AAA22507.1); *Acinetobacter calcoaceticus* (AAA22507.1); *Escherichia coli* H120 (EGB42307.1); *E.coli* TW10509 (EGB73575.1); *Staphylococcus aureus* Mu50 (2QMW_A); *Methanocaldococcus jannaschii* DSM2661 (Q58054.1); *Mycobacterium tuberculosis* H37Ra (ABQ75667.1). Strictly conserved residues are highlighted with red boxes; red characters mean similarity in a group and blue frames similarity across groups. Region homologous to PDT family is marked with a purple box and the green one covers the ACT domain. The sequence alignments were assembled using ClustalW and visualized using ESPript software (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi).

The genome of *Ko. xylinus* NBRC 3288 strain available in GenBank was carefully analyzed, and the presence of three sequences noted as PDT were found: one labeled as GLX_05460 (position from 637225 to 638070), other as GLX_17400 (from 1952934 to 1953836) and the third one as GLX_23070 (from 2541951 to 1542931). Among them, attending to the flaking sequences of the ORFs in the fosmid clone, this *gqqA* gene corresponds with the PDT sequence labeled as GLX_05460.

3.2. - Heterologous expression of GqqA protein

To further characterize the potential QQ gene *gqq*A was amplified from the corresponding clone obtained after the subcloning step using specific primers pair (gqqFw and gqqRv). This fragment of 846 bp was inserted into the expression vector pET-21a and the correctness of the construct was verified by DNA sequencing.

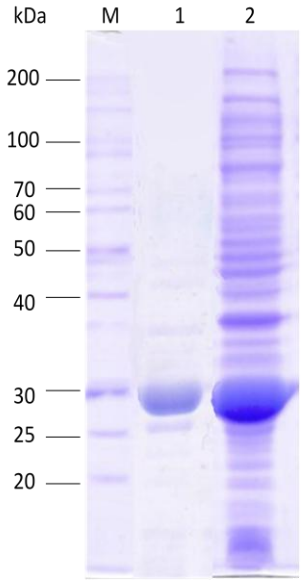

Fig. 4.- SDS-PAGE image of the recombinant GqqA protein expression. Lane 1: Molecular marker (Unstained protein ladder (Fermentas)); Lane 2: purified GqqA his-tagged protein (5µg). Lane 3: protein extract from transformed *E. coli* BL21 (DE3) strain induced for GqqA overexpression.

The protein was induced for recombinant expression with IPTG in the *E. coli* BL21 (DE3) strain, purified from the soluble fraction, and finally, analyzed by SDS-PAGE indicating that the protein was homogenous, with only minor contamination by other proteins (Fig. 4). The purified protein presented a molecular weight of about 30kDa, which was in accordance with the calculated molecular weight of 30.52 kDa.

3.3. – β-galactosidase assays in Agrobacterium tumefaciens NTL4 reporter strain

In presence of the GqqA protein, a decrease of the added N-AHLs was detected with the ONPG test performed with *A. tumefaciens* NTL4 strain. Two different concentrations of 3-oxo-C8-HSL and 3-oxo-C12-HSL molecules were incubated for 2h with purified and recombinant GqqA, and in both cases the levels of detected N-AHLs were reduced in comparison to the controls (Fig. 5A).

The highest β-galactosidase activity reduction was detected with the molecule of 3-oxo-C8-HSL at 100 nM. When the concentration of 10 nM was tested, the reduction was not so strong compared with the control. The analysis tested with the molecule of 3-oxo-C12-HSL presented a higher activity reduction with the smallest concentration. In general, the reduction of the β-galactosidase activity ranged between 20% and 48%.

3.4. – Swarming motility and pyocyanin production tests in Pseudomonas aeruginosa PAO1 reporter strain

The effect of *gqq*A gene was tested for motility and pyocyanin production of *P. aeruginosa* PAO1 strain which was transformed with construct pBBR1MCS-5::*gqq*A, and as control the broad host vector pBBR1MCS-5 re-circularized was used. DNA sequencing verified the correctness of the *gqq*A insert in the vector.

The presence of *gqq*A gene had an effect upon the motility inhibition tested on swarming agar in comparison to the control (Fig. 5B). The pyocyanin pigment production was also reduced in *P. aeruginosa* PAO1 transformed with pBBR1MCS-

5::*gqq*A. A reduction of 88% (relative absorbance measured at 520nm) was detected in pyocyanin production with respect to the control (Fig. 5C).

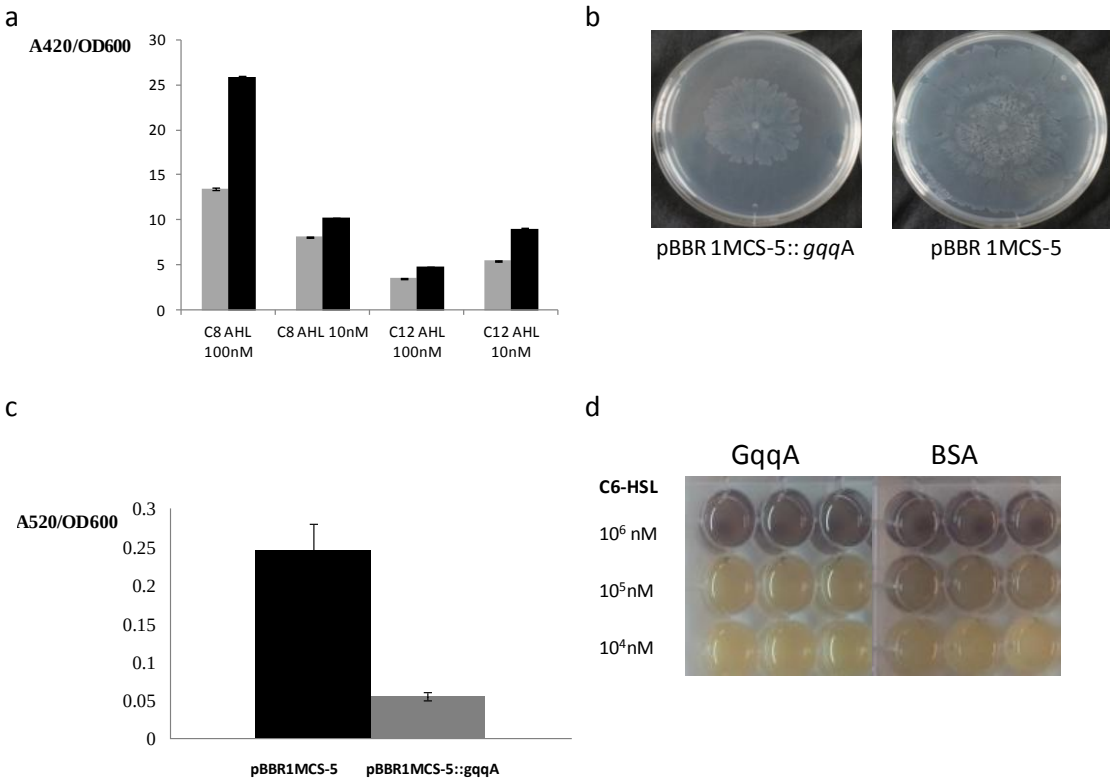

Fig. 5.- a) β-galactosidase activity of *A. tumefaciens* NTL4 strain incubated with different N-AHLs and measured by absorbance at 420nm using ONPG substrate and expressed in function of the cellular growing measured at 600nm. Black bars represent samples previously incubated with GqqA purified protein and grey bars with crude cell extract of *E. coli* BL21(DE3) strain with the same protein concentration. Error bars represent standard deviation. b) Motility tests performed on swarming agar with *P. aeruginosa* PAO1 strain transformed with pBBR1MCS-5::*gqqA* and vector pBBR1MCS-5 re-circularized. c) Pyocyanin production of PAO1 strain transformed with pBBR1MCS-5::*gqqA* and pBBR1MCS-5 empty vector measured as relative absorbance at 520nm corrected by the cell growing as optical density at 600nm. Error bars represent standard deviation. d) Difference in violacein production by *C. violaceum* Cv026 strain using different concentrations of C6-HSL by triplicate in presence of GqqA purified protein and BSA.

3.5. – Violacein production by Chromobacterium violaceum Cv026 reporter strain

The tests carried out in *C. violaceum* Cv026 strain reveled that purified GqqA protein was highly active reducing the production of violacein compared with the control (Fig. 3D). The QQ activity of this protein was tested with four different concentrations of C6-HSL, and with the highest one (10mM) the amount of purified GqqA protein used (20 μg/ml and 6.7 μg/ml) was not enough to reduce the production of the violacein pigment. However, when lower concentration of C6-HSL was assayed (1mM) the QQ activity of purified GqqA protein was clearly appreciable with both concentrations tested. The smaller concentrations of C6-HSL were not able to induce the violacein pigment production of *C. violaceum* strain Cv026 in the control; therefore, we could not evaluate the effect of purified GqqA protein.

3.6. – Growth and cellulose production phenotype of AAB in presence of GqqA protein

Ko. europaeus Gae02 strain grows in GY medium forming strong cellulose aggregates maintaining the broth without turbidity. Its growth was tested in presence of the purified GqqA protein extract and compared with the presence of crude cell extract of *E. coli* BL21 (DE3) strain as control. The growth was clearly different. The turbidity in the culture with purified GqqA protein increased through the time with compared to the control, which medium remained without turbidity but with cellulose aggregation; this aggregate was not visible in the tubes with GqqA protein (Fig. 6A). Three different concentrations of purified GqqA protein extract were tested observing the same result after 72h of incubation. However, with the highest concentration (100μg/5mL), the effect of the protein was detected at initial stages (16h of incubation). The turbidity of the media was also measured at 600nm detecting this increase (Fig. 6B). In addition, microscopy and plate counting were performed obtaining similar results. The cell population counted by microscopy ranged from 10^8 cells/ml (24h) to $5 \cdot 10^9$ cells/ml

(72h) for the culture with purified GqqA protein while in the controls was more difficult to determine due to the presence of aggregates, counting approximately from $2 \cdot 10^5$ cells/ml to 10^6 cells/ml at the end of the experiment. The population recovered on plates was between $8 \cdot 10^7$ cfu/ml (24h) and $3.5 \cdot 10^9$ cfu/ml (72h) for the culture with purified GqqA protein, whereas the control presented lower values, from $2.3 \cdot 10^5$ cfu/ml to $7.9 \cdot 10^5$ cfu/ml at the end of the experiment.

The effect of the purified GqqA protein was also tested by the addition of the supernatant of a previous pure culture of *Ko. europaeus* Gae02 strain but, this addition did not produce any distinguishable effect compared with the control (data not shown).

On the other hand, seven strains of AAB species, selected as cellulose producers, were also tested with this purified GqqA protein. The effect of GqqA protein (100 µg /2ml) in the cellulose production and growth phenotype observed after 16h of incubation was different for each strain (Fig. 6C). In the case of *Acetobacter nitrogenifigens* LMG 23498, *Acetobacter orientalis* LMG 21417, *Komagataeibacter europaeus* DSM2004 and *Komagataeibacter hansenii* LMG 1524 strains no visible change in the growth phenotype was detected with respect to the BSA control. The *Acetobacter pasteurianus* Ap4 strain presented the same turbidity but the cellulose aggregate seems to be smaller in the culture supplemented with GqqA compared with the BSA control.

Nevertheless, *Acetobacter syzigii* LMG 21419 and *Komagataeibacter rhaeticus* LMG 22126 strains presented more turbidity in presence of GqqA protein compared with the BSA control. This behavior is similar to *Ko. europaeus* Gae02 which was included as positive control in this test.

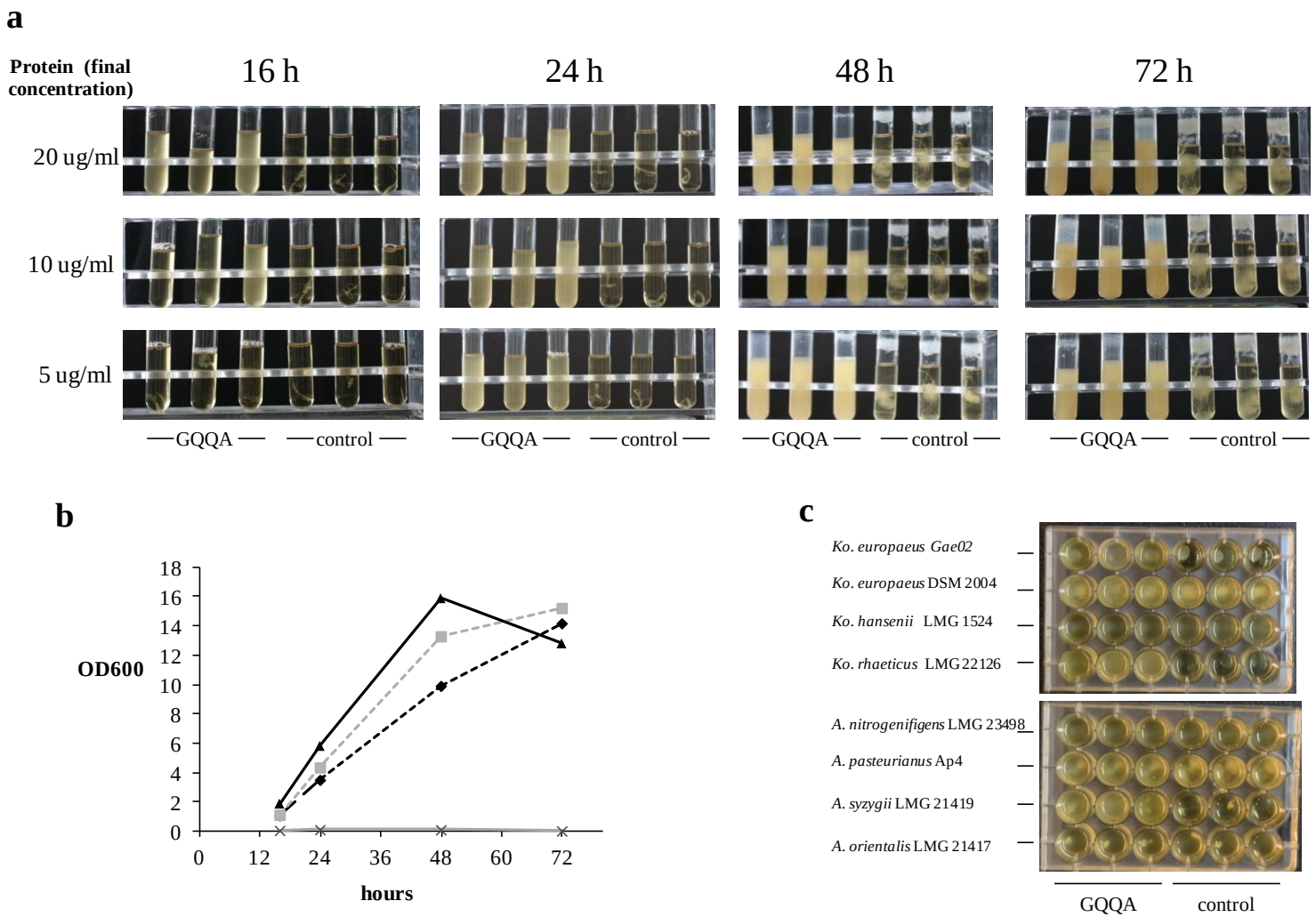

Fig. 6. - a) Phenotypes growth and cellulose production of *Ko. europaeus* Gae02 strain with three different concentrations of purified GqqA protein after 16h, 24h, 48h and 72h. b) Turbidity measures at 600nm during the growing of *Ko. europaeus* Gae02 strain. Black solid bar represents Gae02 strain growth with 20µg/ml of purified GqqA protein; grey dotted line corresponds to 10µg/ml of purified GqqA protein; black dotted line to 5µg/ml of purified GqqA protein and grey solid line corresponds to the control with 20µg/ml of crude cell extract of *E.coli* BL21 (DE3) strain. c) Phenotypes growth and cellulose production of eight AAB strain with purified GqqA protein and BSA as control (final concentration of 50 µg/ml, in both cases)

3.7. Detection of QS-active clones

The 1,824 fosmid clones of the genomic library were screened for the detection of potential QS activities. The AT soft agar screening detected two fosmid clones (clone 7 and clone 12) with potential QS activities (Fig 7A). The ONPG test of these two clones revealed that lower concentration of the fosmid clone 12 yielded more QS activity compared with the control and also with clone 7. On the other hand, clone 7 presented, with lower concentration, higher QS activity compared with the control and the clone 12 (Fig 7B).

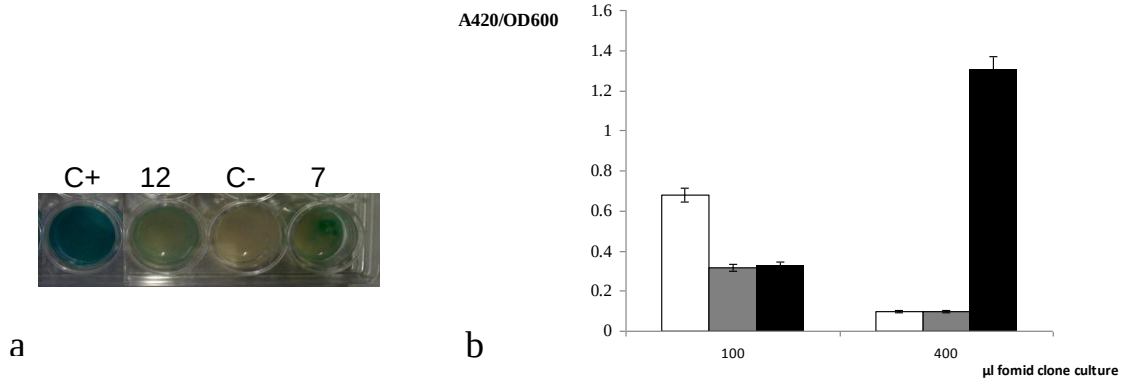

Fig. 7. – Effect of putative QS activity of the fosmid clones 7 and 12 on the strain NTL4 of *A. tumefaciens*. a) Blue color represents AHL production on AT soft agar. Possitive control included 3-oxo-C8-HSL and without any *E.coli* strain and negative control was the *E.coli* strain EPI300^{TM} untransformed in the same concentration as the fosmid clones b) β-galactosidase activity of the *A. tumefaciens* strain NTL4 incubated with the positive fosmid clones in two different concentrations and measured by absorbance at 420nm using ONPG substrate and expressed in function of the cellular growing measured at 600nm. White bars represent clone 12 activity, black bars represent clone 7 and grey bars correspond to the negative control.

4. Discussion

QS system is regulated by autoinducer molecules and in the last years enzymatic degradation of these molecules has been widely reported as a good method to interfere in QS signalization (Rasmussen and Givskov, 2006; Dong and Zhang, 2005). The

genome of different microorganisms such as those belonging to *Pseudomonas, Streptomyces, Bacillus, Staphylococcus, Agrobacterium, Rhizobium* genera (Sio et al., 2006; Park et al., 2005; Dong et al., 2002; Chu et al., 2013; Carlier, et al. 2003; Krysciak et al., 2011) and also metagenomic libraries (Bijtenhoorn et al., 2011; Tannieres et al., 2013) have been screened in order to find new enzymatic activities with anti-QS potential.

AAB are ubiquitous microorganism due to the variety of substrates that can use as energy source as well as their high resistance to acid media. They are mainly known to be involved in vinegar production where develop a pellicle on the air-liquid interface when the vinegar elaboration was carried out by traditional method (Guillamon and Mas, 2009). In the last years, a N-AHL-dependent QS system named GinI/GinR system has been reported as responsible of acetic acid and gluconic acid production repression, antifoam activity, and growth rate acceleration in the exponential growth phase (Iida et al., 2008a, Iida et al., 2008b; Iida et al., 2009). In this study two fosmid clones with putative QS activity were detected from the genome of Gae02 strain. The behavior of the fosmid clones was different which could be related with the type of AHLs produced by these fosmid clones. Although the QS receptors can recognize several AHL molecules, they commonly present different affinity for specific side chain length among the AHLs (Bassler and Losick, 2006). In fact as described by Iida et al, (2008) the specie *Ko. intermedius* produces long side chain AHLs (from 10 to 12 carbons) but the natural receptor of *A.tumefaciens* NTL4 recognizes better 3-oxo-C8-HSL. Further analysis are required for understand the QS activity of these two clones and the whole genome sequencing of this AAB strain could improve the study of QS activity in AAB.

On the other hand, to the date there was not any study about molecules of QQ activities performed either from the any AAB genome or from habitats where this group of

bacteria are frequently present. Therefore, in this work, a screening of QQ activity was carried out from the genome of *Ko. europaeus* Gae02, an cellulose overproducer AAB strain and a protein termed GqqA was purified from the fosmid library constructed and its potential QQ role was characterized.

The first screening, performed with the reporter strain NTL4 of *A. tumefaciens* to the fosmid library of *Ko. europaeus* Gae02 genome, showed QS interference in the 1% of the total fosmid clones obtained. This low percentage of QQ activities could be associated with the high ability to partially oxidise a variety of sugars and alcohols and the capacity of AAB to survive in extreme conditions, such as low pH, high ethanol and/or acetic acid concentration where very few microorganisms can be present. Although the physiological activity of most of the QQ molecules described is unknown by the moment (Chen et al, 2013), it is suggested that the production of molecules with QQ activities could be related with competition mechanisms for nutrients and ecological niches between different groups of microorganisms, but also with a mechanism of adaptation to environmental changes blocking its own QS response (Zhang and Dong, 2004).

The AT soft agar test of the *A. tumefaciens* strain NTL4 (Zhang et al., 1993), pyocyanin production of *P. aeruginosa* PAO1 (Schaber et al., 2004) or motility of *E. co*li DH5α (Harshey and Matsuyama, 1994) which are QS dependent physiological functions, enabled to select two clones with a strong potential QQ activity, even subcloned from different fosmid clone. This fact supported the consistent effect of the protein encoded by the gene termed *gqq*A.

The presence of this *gqq*A gene in the transformed *P. aeruginosa* strain PAO1, presented a clear phenotype change compare with the control, both in swarming motility

and in pyocyanin production. It is generally accepted that QS systems influences motility and pyocyanin production in *P. aeruginosa* (Shrout et al., 2006; Wagner et al., 2007; Dietrich et al., 2006) using 3-oxo-C12-HSL and C4-HSL as autoinducer molecules (Wagner et al., 2007). Therefore, these results ensured the ability of the *gqq*A gene to modify the QS regulated features in the *P. aeruginosa* strain PAO1.

The QQ activity of this *gqq*A gene was also detected in the analysis of the purified GqqA protein codified by this gene. The vector pET-21a::*gqq*A was introduced in the *E. coli* strain BL21 (DE3) to obtain GqqA protein, and the QQ effect of this protein was tested with two different reporter strains, *A. tumefaciens* NTL4 and *C. violaceum* Cv026.

With the first one, two different molecules 3-oxo-C8-HSL and 3-oxo-C12-HSL were tested in two different concentrations, and the GqqA protein presented higher effect on β-galactosidase activity with 3-oxo-C8-HSL. It was expected because the wild-type *A. tumefaciens* recognizes 3-oxo-C8-HSL specifically although other AHL molecules also affect its QS system (Zhang et al., 1998). When less concentration of 3-oxo-C8-HSL was tested, the effect of the GqqA protein was smaller with respect to the control which could be explained because the threshold concentration of this autoinducer was not achieve for the *A. tumefaciens* strain NTL4, and the effect of GqqA was not clearly detected. The GqqA protein effect observed was the opposite with 3-oxo-C12-HSL. In this case, with lower concentration of autoinducer the protein affected more to β-galactosidase activity compared with the control, suggesting that with a higher amount of 3-oxo-C12-HSL, a similar quantity of autoinducter could be degraded by GqqA protein in the same time but the effect detected will be lower.

On the other hand, the GqqA protein also presented an evident QQ effect with the reporter strain *C. violaceum* Cv026. This is a mutant strain that presents violacein pigment production in response to several AHL from with N-acyl side chains of four to eight carbons (Winson et al., 1995). In this case, the autoinducer used to test the QQ effect was C6-HSL and it was observed that the GqqA protein presented the ability to interfere with this molecule reducing the violacein production compared with the control.

Additional assays were performed in order to see a possible effect of purified GqqA protein on the behavior of the *Ko. europaeus* strain Gae02 strain as well of other AAB cellulose producers strains. A relevant change was detected in the growth phenotype of the *Ko. europaeus* strain Gae02. The cells of this strain tend to aggregate in a cellulose pellicle in habitual culture conditions but when GqqA protein was introduced in the medium no distinguishable aggregates were formed and the turbidity of the medium increased with time. Moreover, a higher concentration of protein introduced in the medium generated a faster presence of turbidity in the medium. However, when a supernatant of a previous culture of this strain was introduced, in order the purified GqqA protein, no difference respect to the control was detected. This result suggests that this GqqA protein also has effect in the cellulose production of this strain; however, it is not liberated to the media during normal growth conditions. On the other hand, only in three of the seven strains of *Komagataeibacter* and *Acetobacter* genera changes in the growth phenotype respect to cellulose production were detected in the conditions tested. Even so, further studies are necessary to understand the effect of GqqA protein on AAB metabolism.

It has been reported the presence of a QS system GinI/GinR in *Ko. intermedius* (Iida et al., 2008a) regulated by long chain N-AHL molecules such as N-C10-L-HSL and N-

C12-L-HSL. This system is able to control oxidative fermentation and antifoam activity in these bacteria (Iida et al., 2009). Nevertheless, at the moment there is no evidence of the relation between cellulose pellicle formation and QS system in AAB. However, it is commonly accepted that the production of biofilm is QS dependent in several microorganisms (Davies et al., 1998; Parsek and Greenberg, 2005).

The predicted amino acid sequence of this GqqA protein as well as DNA sequence of the ORF selected from the fosmid clone presented the highest homology with Prephenate dehydratase, an enzyme involved in the metabolic pathway of the aromatic aminoacids. PDTs belong to a group of enzymes that catalyze the breakage of a carbon-oxygen bond leading to unsaturated products via the removal of water (EC 4.2.1.-), the molecular mechanism is mediated by a descarboxilation reaction (Zhang, 1998).

Although PDT activities from several microorganisms have been faithfully characterized (Zhang et al., 1998; Prakash et al., 2005; VanVleet et al., 2010), their biochemical mechanism is not completely understood (Kleeb et al., 2007). Some residues appear highly conserved throughout the species. Especially, Phe-174 is considered the most highly conserved hydrophobic residue in the PDT family (Zhang et al., 2000). The protein GqqA showed a homologous region with PDT family and an ACT domain.

The purified GqqA protein presented a molecular weight of approximately 30 kDa, according to the predicted by the amino acid sequence, and the PDT protein described in other bacteria ranged values of 22kDa and 32kDa in the case of *E. coli* (Zhang et al., 1998), 33KDa in *M. tuberculosis* (Vivan et al., 2006) or 30kDa and 31kDa in *Staphylococcus aureus* and *Chlorobium tepidum*, respectively (Tan et al., 2008). The dendrogram constructed from amino acid PDT sequences reveled low homology with

those sequences that correspond to proteins with PDT activity enzymatically characterized. Moreover, the complementation assays performed with two defective mutants of *E. coli* reveled that the *gqq*A gene presence could not restore the production of phenylalanine in these auxotrophic strains, indicating the absence of this metabolic function. The presence of three different genes homologous to PDT protein noted in the genome of the *Ko. xylinus* strain NBRC 3288 could mean that just one of them has this activity *in vivo*. These results conducted to assume that even the GqqA protein is homologous to PDT family GqqA has not prephenate as substrate and might well be another protein with carboxilase activity.

There are three main types of microbial enzymes whose activity has been proven on the *N*-AHLs signals interference: oxidoreductases, acylases, and lactonases. The most characterized group of enzymes able to cleavage the N-AHL molecules correspond to lactonases, which can hydrolyze the lactone ring in a reversible way (Krysciak et al., 2011). Although in several works it was predicted the possibility of hydrolyze the lactone ring of N-AHL by decarboxilation (Dong and Zhang, 2005), it has been never demonstrated this reaction enzymatically before. Therefore the GqqA protein could be the first descarboxilase enzyme identified as QQ activity.

In summary, a fosmid library from genome of the *Ko. europaeus* strain Gae02 was screened to detect QS and QQ activity. The fosmid clones detected for their putative QS activity require more analysis however; in the QQ study a ORF which codified for the protein named GqqA was selected. The assays with different reporter strains widely studied reveled that this GqqA protein interferes with several N-AHL molecules both long and short lateral acyl-chain in the QS mechanisms of communication. Further analyses are required in order to know the identity of this novel QQ enzyme and its

interaction with the autoinductors, as well as a deeper understanding of its effect on the QS mechanism of the *Ko. europaeus* strain Gae02 and of other AAB strains.

Acknowledgments

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GENERAL DISCUSSION

The AAB are a group of bacteria characterized by its wide metabolic capacities. Their ability to oxidize high variety of sugars and even alcohols in order to produce different metabolites is highly appreciated in the food and biotechnological industry.

The continuous development of ecological studies applying new methodologies has allowed the association of new AAB species or genera with niches where they have not been previously detected. These new molecular methods have been also useful for microbial control of several processes. The AAB strain selection obtained by previous ecological studies could be used for the design of starter cultures in order to implement the homogeneity and reliability of the final product (Leroy and De Vuyst., 2004; Gullo and Giudici, 2008).

However, AAB are considered *fastidious microorganisms*. The difficult recovery in laboratory media, especially when the samples come from extreme environments such as wine or vinegar (Entani et al., 1985), is a hurdle for the performance of ecological studies.

Several AAB biodiversity studies have been made at the moment, especially on fruits such as grapes or in wine from worldwide wine regions and in vinegars from different fruits; however, there are niches that have not been studied yet. This is the case of two studies carried out in this thesis, in which an in-depth study of the AAB population was performed both on grapes from the Canary Islands where a high degree of endemism in plants and animals due to the special geographic characteristics has been reported, and in a biofilm from strawberry vinegar elaborated by traditional method that is the matrix that AAB performs to place in the air-liquid interface providing a close contact with oxygen and protective growth environment (Solieri and Giudici, 2009).

A previous enrichment step was necessary for the detection of AAB species on healthy grapes from the Canary Islands; microvinifications of grapes were performed to

increase the plate recovery. This strategy of enrichment was also performed in other studies, such as Prieto et al. (2007) and Bae et al. (2006), which used specific enrichment cultures for the analysis of Chilean grapes and Australian wine grapes, respectively. In some cases, the physicochemical characteristics of samples could exhibit an autoenrichment such as in the study of Navarro et al. (2013), who performed the analysis of grape musts from Spain that probably initiated alcoholic fermentation, or in the study of Mateo et al. (2014), who analyzed Australian grapes with different infection status, which probably presented spontaneous fermentation effect.

The selection of the media used for the AAB recovery is critical in ecological studies. Not all the species and not all the strains are able to grow in the same conditions, and sometimes the use of a culture media favors the growth of a concrete strain or species (Gullo et al., 2006). In fact, adapted media for AAB from vinegar were designed such as AE-medium and RAE-medium, which incorporate acetic acid and ethanol to their composition, reproducing the conditions of wine or vinegar (Sievers and Teuber, 1995; Sokollek and Hammes, 1997). However, these media were not always satisfactory, as reported by Vegas et al. (2013), who compared four different culture media and only the, GY and YPM (MM) media allowed the growth of AAB. Often, the enumeration of AAB by plate disagrees with the microscopy recounts, indicating that these media do not allow the isolation of all the AAB population present in the sample. Among the possible explanations, the cells proceeding from an extreme medium would be adapted to these conditions, and although the cells were alive, they could not grow under laboratory conditions (Torija et al., 2010). It could be because an adequate and specific medium for AAB growth has not been already designed. Few studies have been performed to describe media with this aim (Entani et al., 1985; Sokollek and Hammes, 1997; Sokollek et al., 1998). Therefore, different nutrient sources such as carbon,

nitrogen, or phosphates; the requirement of oxygen, or the pH conditions in the medium should be analyzed in an attempt to know the nutritional requirements of these bacteria and to design appropriate media for this bacterial group. New technologies developed during the last years could approach and simplify this research. The use of monitored microtiter plates by automated growth curve analysis or even by robotic platforms offers advanced, proven, and reliable systems that could be very useful.

The plate recovery is essential for future studies on the isolated strains that would not otherwise be possible. Before the use of these potential strains for biotechnological applications, their characterization by genotyping is necessary. The main difficulty in the fingerprinting is to find a rapid and reliable technique; among all the molecular techniques tested for AAB typing (Cleenwerck et al., 2009, 2010; Fernández-Pérez et al., 2010; González et al., 2004; Nanda et al., 2001; Papalexandratou et al., 2009; Trček et al., 1997), ERIC-PCR and $(GTG)_5$-PCR are the most commonly used. Some authors reported that the $(GTG)_5$-rep-PCR was able to discriminate less profiles compared with ERIC-PCR (Mateo et al., 2014; Vegas et al., 2013). In the study of healthy grapes from the Canary Islands, the results were complementary; moreover, it was suggested that the use of more than one technique for strain classification is recommendable in order to distinguish very similar strain profiles, specially isolated from the same niche. Although the genotyping of AAB allowed in some cases the clustering of strains attending to their species identification (De Vuyst et al., 2008) or the origin where they have been isolated (Bartowsky and Henske, 2008), the grouping of the AAB genotypes from the Canarian grapes did not match with any of these characteristics as it was also observed by other authors after (Mateo et al., 2014).

The culture-independent techniques are developed with the aim to reveal the real population of AAB that cannot be detected by the culture-dependent approaches.

Therefore, a good strategy could be the initial use of culture-independent techniques to obtain a broader view of the microbiota rather than analyses from plate culture. The information obtained from the culture-independent techniques on the presence of specific species can be used later for the application of culture-dependent techniques, which can be species specific. We could then analyze the isolates and recover the strains that could be used with biotechnological purposes.

In this thesis, with the works carried out in wine samples produced in the Canary Islands and also in biofilm samples from strawberry vinegar, both types of techniques were performed. Using culture-independent techniques, one of the main drawbacks is the necessity of a high-quality DNA. Thus, specific protocols for DNA isolation of AAB are adapted to the composition of the matrix and the technique that will be used for AAB identification (Jara et al., 2008; Mamlouk et al., 2011). The DNA extraction protocol of Ausubel et al. (1992) was applied as reported by Jara et al. (2008) because it was adequate for the analysis of the Canarian wine samples; however, in strawberry vinegar biofilm samples, a new DNA extraction protocol was adapted. The cellulose of this type of matrix interferes in the DNA extraction (Navarro et al., 1999) as well as other compounds present in strawberry vinegar that negatively affect the quality of the DNA. A pretreatment of samples was necessary in the biofilm because the cells are entrapped in a solid matrix, and some modifications were carried out from the protocol of Ausubel et al. (1992) to result in a pure DNA with enough quality and quantity to perform the culture-independent approaches.

Among the culture-independent techniques, RT-PCR allows not only the detection but even the total and species-specific quantification of AAB (González et al., 2006; Torija et al., 2010). The design of RT-PCR is based also on the most common identification techniques available. The identification techniques for AAB are commonly based on the

analysis of the ribosomal genes. Among them, the most frequently used is the 16S rRNA gene because of the high availability of sequences belonging to this region in public databases. However, the discrimination between closely related species could not be possible regarding the 16S rRNA gene as it was observed in the isolates from grapes of the Canary Islands belonging to *A. malorum* and *A. cerevisiae* species. These isolates identification was finally achieved using the 16S–23S rRNA gene ITS region, which allowed ascribing the isolates to a single species, either *A. malorum* or *A. cerevisiae*. This DNA region has been previously described for AAB identification; it presents higher variability than the 16S rRNA gene due to its nature as a noncodifying sequence (González and Mas, 2011). However, there are regions that correspond to tRNA in this ITS region (tRNAIle and tRNAAla) that are highly conserved within strains of the same species (Sievers et al., 1996) and that are adequate for primers and probes design (González and Mas, 2011). The primers and TaqMan-MGB probes for *A. malorum* and *A. cerevisiae* designed in this thesis for their application by RT-PCR were inside these regions or near them.

These two species seem to be present in the natural microbiota of different fruits, and they can survive during the wine fermentation or acetification steps as it was detected on grapes and also in strawberry and persimmon vinegars (Hidalgo et al., 2012, 2013b; Prieto et al., 2007) and they were also used for inoculation of a traditional vinegar production process (Hidalgo et al., 2013b). For this reason, the design of primers and TaqMan-MGB probes to follow the dynamics of these AAB species by RT-PCR could be useful through the process. Furthermore, these two probes together with the other five species-specific probes previously described by our research group (Torija et al., 2010) allowed the detection of the microbiota commonly present in wine samples from

the Canary Islands. However, in the biofilm of strawberry vinegar, only two of these seven species were detected, and moreover, they were not the main ones.

The capacity of the TaqMan-MGB probes to detect mismatches of one base results in a methodology with high specificity, which could be interesting to follow up the AAB strains used as starter cultures. The main hurdle for this application is to find a polymorphism, which is conserved in just one AAB strain. One possible approach to detect a strain-specific sequence could be the analysis of the fragments amplified by fingerprinting techniques.

A handicap of the RT-PCR applied for AAB detection is the interference between the target and the nontarget AAB species in the primer annealing, which is stronger when the species are phylogenetically closer, and also the presence of inhibitors from the samples of wine or vinegar. To solve these drawbacks, an enrichment PCR has been proposed prior to RT-PCR. In this study, this previous amplification improved the specific AAB species detection, decreasing the effect of these interferences.

The work of this thesis has been focused on the application of culture-independent methodologies, especially in the development of RT-PCR. Most of these techniques have been performed analyzing the 16S rRNA gene sequence such as DGGE-PCR, RFLP-PCR, and pyrosequencing. Regarding DGGE-PCR, it has been reported as a good method for the detection of major species but not for the minor ones (Andorrà et al., 2008). The length of the fragments of the 16S rRNA sequences that were analyzed by this technique were too short for an accurate identification at the level of species, making the identification possible just at the genus level. This low accuracy in the AAB species identification was also observed with the other techniques.

Next-generation sequencing is a very new tool to obtain an overview of the whole microbiota present in a concrete environment and the relative abundance of each group

of bacteria (Mardis, 2008). However, the primers commonly used are targeted for all the bacteria in general, and it could introduce an overestimation of some bacterial groups because of preferably annealing among some bacterial species. This drawback has been previously reported for other culture-independent techniques based on PCR (Ercolini, 2004). The design of primers for the AAB group detection could enable a more focused analysis in samples where these bacteria are expected. However, this technique is widely used for the study of poorly characterized environments (Bohorquez et al., 2011), and as a consequence, the methodologies applied have been developed with this finality. The obtained sequences undergo a comparison with those from databases, and the resulting homology is frequently low even when compared with the trimmed sequences. In the pyrosequencing study carried out in the strawberry vinegar biofilm, the differences between the OTUs analyzed just allowed the accurate identification at genus level of AAB. It is frequently observed that the sequence identification that provides the highest homology could not be the most appropriate, and the species detected may not be expected in these niches. Consequently, the appropriate analyses must be a balance between the data homology sets and the previous knowledge that comes from the studied niche.

The molecular approaches allowed the identification at the level of genera or species. In the studies performed with healthy grapes, wine, and strawberry vinegar biofilm, species or even genera of AAB previously described were detected, as well as others never reported in these niches. The genera of *Acetobacter*, *Gluconobacter*, and *Komagataeibacter* that are commonly associated with these three environments were also detected in these studies. Among *Komagataeibacter* species, *Ko. europaeus* and *Ko. hansenii* were found in both strawberry vinegar biofilm and in Canarian wines; they have been commonly associated with the microbiota present in vinegars, especially at

the end of the acetification (Gullo et al., 2009; Hidalgo et al., 2010b, 2012) because of their ability to grow under high acidity conditions (Schüller et al., 2000). Moreover, the detection of *Ko. saccharivorans* species identified on healthy grapes from the Canary Islands was not surprising because it was previously reported in wine (Kato et al., 2011).

In the genus *Gluconobacter*, the species *G. japonicus* has been recently described. The detection of this species on healthy grapes from the Canary Islands and later in grape musts from Spain (Navarro et al., 2013) led us to consider that because of the high homology with *G. oxydans*, some of the previous identifications assigned to *G. oxydans* could be indeed *G. japonicus*. In fact, *G. oxydans* has been one of the species commonly associated with grape and wine as well as *A. aceti* and *A. pasteurianus* (Barbe et al., 2001; Drysdale and Fleet, 1988; Joyeux et al., 1984). The presence of these *Acetobacter* species was also detected in the Canary Islands studies together with *A. cerevisiae* and *A. tropicalis*, which were previously identified on Chilean grapes (Prieto et al., 2007) and Austrian wines (Silhavy and Mandl, 2006), respectively. The species *A. malorum* was not described before on healthy grapes or in grape wine until the performance of the Canary Islands studies. It was just reported on rotten grapes (Barata et al., 2012; Mateo et al., 2014) and in other fruit wines or vinegars (De Vero et al., 2006; Hidalgo et al., 2012, 2013a).

Besides these three common genera, others such as *Gluconacetobacter*, *Asaia*, *Ameyamaea*, or *Tanticharoenia* were present in the studied vinegar biofilm. This high AAB diversity detection in these three niches indicates the high colonization capacity of this bacterial group. On the other hand, the finding of species belonging to these three genera can be related with the lack of accuracy in the identifications because of the

approaches performed, such as next-generation sequencing, which can induce an improper interpretation of the results.

The bacterial community present in the strawberry vinegar biofilm is complex, and several species share the same environment, allowing the establishment of relations between them. The definition of biofilm includes the idea that microorganisms involved in these environments display emergent properties derived from the community and the idea of an altered phenotype due to changes in their gene expression. One characteristic of the biofilms is their heterogeneity; the microorganisms at different positions carry out different metabolic tasks creating specialized domains across the matrix, resulting in the presence of different substrate, oxygen, or pH gradients (Aldeek et al., 2013; Jefferson, 2004). The altruistic behavior of the microorganisms is common in this matrix, presenting a low growth rate to save nutrients available for the group, resulting in an advantage for the community (Kreft, 2004). Evident differences in biofilm structure, in properties, and in the time necessary for its synthesis can derive from the strains of a single or multiple species that formed it or even from small changes in environmental conditions (Branda et al., 2005). The biofilm formation in traditional food or beverage fermentation processes is widely described (Dobson et al., 2011; Gullo et al., 2009; Kawarai et al., 2007; Wang et al., 2012). The hostile environments favor the production of the biofilm in order to create protective environments where the microbial community can survive. This is the case of wine used to produce vinegar, where the pH conditions, the presence of high ethanol, and, consequently, high acetic acid concentration or the low oxygen availability result in the development of a biofilm formed by AAB disposed in the air-liquid interphase to protect the cells from these extreme conditions.

In this thesis, the biofilms produced by AAB were successfully observed by different microscopy techniques such as SEM, LCM and epifluorescence microscopy. These techniques allowed the study of the organization of the cells and their morphology and position in the cellulose matrix. The differences in the cells' organization could be an effect of the cellulose matrix composition; the grape vinegar biofilm was more fluidic, whereas the strawberry vinegar biofilm was more compact. This feature probably could influence the distribution of cells inside the cellulose matrix. Regarding the differences in the cell morphologies observed, it has been reported that the AAB can vary their shape attending to the ethanol and acetic acid amount present in the medium (Trček et al., 2007). Finally, attending to the cell distribution in the cellulosic matrix, the differences found between grape and strawberry vinegars could be related to different states of maturity in the biofilms.

Nowadays, appropriate technologies are available to monitor the biofilm formation that could be applied for AAB, such as continuous-flow-cell systems to take image or even video (Bijtenhoorn et al., 2011). Concretely, among the methodologies for biofilm study, phase contrast and dark field microscopy have been used for *Ko. xylinus* (Brown, 2013).

The biofilm frame formed in traditional vinegar is constituted mainly by cellulose. Several AAB species have been reported as cellulose producers, mainly belonging to the *Komagataeibacter* genus (Aydın and Aksoy, 2013; Castro et al., 2013; Park et al., 2003). In this thesis, species from this genus were also the main producers. However, species from *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter* were able to produce a visible cellulose biofilm. Most of the strains analyzed were type strains, and although one of the tests often performed for the AAB species description is the phenotypic characterization of cellulose production (Carr and Passmore, 1979), it is not very

accurate because of the instability of this trait, which could be affected by the culture media or the growth conditions used. The growth conditions can vary the cellulose-producer phenotype of each strain; thus, the study of different nutritional sources could be useful to archive strains not described as cellulose producers but, attending to their requirements, will be able to form this EPS. Furthermore, in order to corroborate the cellulose production, different methodologies have been proposed such as calcofluor white staining (Römling, 2002) or the dissolution of the EPS after boiling in NaOH (Navarro et al., 1999). The use of the calcofluor was successfully used in the present work.

The cellulose production phenotype could be lost because of the storing and the growth conditions (Chawla et al., 2009; Coucheron, 1991) in a similar way as it has been observed with the ability to produce acetic acid (Takemura et al., 1991). These changes have been associated with production of spontaneous mutations (Azuma et al., 2009; Okumura et al., 1985) and, in particular, with the presence of mobile genetic elements or transposons that are inserted into the genes encoding the key enzymes of these pathways (Gullo et al., 2012).

The study of the best system to store and maintain AAB strains is important for industrial applications mostly because of the preservation of the traits for which they were selected. Several proposals have been reported since the first study carried by Sokollek et al., (1998) who used liquid nitrogen, malt extract, and liofilization as storage strategies. For instance, recent works performed by Ndoye et al. (2007) used liofilization with mannitol as cryoprotector. For many decades, the low survival rate and high mutation frequency of AAB strains stored at 4°C or −20°C have been reported (López et al., 1961; Sourek and Kulhanek, 1969). It is really necessary to develop a project focused on the description of different storage and maintenance methodologies.

Both commercial and traditional protocols could be assayed: liofilization, cellular culture freezing, or concentrated and refrigerated liquid cultures. Moreover, the selection of appropriate cryoprotectors is critical for the maintenance of cell viability. Besides the aforementioned (malt extract and mannitol), other cryoprotectors such as skimmed milk, saccharose, glycerol, and so on, could be interesting to test.

The loss of the cellulose production ability could be one of the possible explanations for the result obtained with the strain Ac2 of *A. cerevisiae*, which was not able to produce cellulose in any condition tested, but the PCR amplified the *bcsA* gene for cellulose synthase. Despite this result, the concordance obtained between the cellulose production and the presence of this gene was highly coincident. This gene is the most conserved among the bacterial species, so it was proposed as a good target for the primer design (Römling, 2002); in fact, it has been used in previous works for cellulose production analysis in enterobacteria (Zogaj et al., 2003). Most of the information about the genes involved in BC production is mainly supported by the deep knowledge of *Ko. xylinus* (Chawla et al., 2009; Römling, 2002) and the lack of data for other AAB. For this reason, the primers' design for *bcsA* gene of AAB must be conducted using DNA and protein sequences resulting in degenerated primers; from the total eight degenerated primers assayed, only one archived reliable amplification products. The good results obtained from this study suggested that this gene could be used for rapid screening of the capacity to produce cellulose.

However, the environmental conditions and other metabolic pathways can interfere, inactivate or even delay the cellulose production (Chawla et al., 2009; Zogaj et al., 2003) indicating the complexity of the synthesis of this polysaccharide. Actually, the cellulose biofilm production has been reported as a mechanism controlled by cell-to-cell communication (QS systems); the paradigm of this control is the biofilm formation by

P. aeruginosa (Davies et al., 1998). In the case of AAB, there is no evidence of the relation between QS and cellulose production (Iida et al., 2008a); there is also no information about QQ activity in this bacterial group. Therefore, in this thesis, in an attempt to identify potential QS and QQ activities, functional screening was performed using the genome of *Ko. europaeus* strain Gae02 isolated from wine vinegar.

The preliminary tests of QS revealed two candidates with the ability of AHL production. These results are very promising; however, they are subjected to further analysis to know the sequences involved in them. In the quorum study carried out in this thesis, the results obtained for QQ activity detected in AAB are worth noting. The functional QQ screening revealed the presence of several genes that could present this kind of activity. Among them, one called *gqqA* was selected because of its stronger QQ phenotype as demonstrated by the tests performed with the reporter strains. The GqqA protein derived from this gene presented interference in the cell-cell communication of the *P. aeruginosa* strain PAO1 (motility and pyocyanin production), the *A. tumefaciens* strain NTL4 (β-galactosidase activity), the *C. violaceum* strain Cv026 (violacein production), and in several AAB strains of different species even on strain Gae02 (cellulose production).

This protein presented high sequence homology with prephenate dehydratase (PDT), an enzyme related with the biosynthesis of phenylalanine that decarboxylates the prephenate into phenylpyruvate. The amino acid sequence of this protein was analyzed, and no conserved domain known to be involved in QQ activity protein was detected. Furthermore, the enzymatic assays described for the characterization of PDT activity did not yield the expected results (data not shown). These assays are not reliable because of the lack of stability of the barium prephenate used as substrate and can be spontaneously decarboxylated, making it difficult to accurately measure enzymatic

activity. However, the results obtained after the complementation assays hinted that this enzyme could not be a real PDT enzyme. In the genome of *Ko. xylinus* NBRC 3288 strain, three different genes are annotated as PDT. This suggests that the GqqA protein could not be correctly assigned. Although this protein presents homology with PDT family, it could present decarboxylase activity. Decarboxylation is one of the predicted mechanisms for AHL cleavage involved in potential QQ that has not been demonstrated experimentally (Dong and Zhang, 2005).

It is not known if the real *in vivo* function of the QQ enzymes detected by screening *in vitro* would be the interference of the cell-to-cell communication (Chen et al., 2013). Therefore, it is possible that the GqqA protein has no real QQ function inside the environments inhabited by AAB. In fact, in preliminary assays, it was observed that this protein was not released to the medium. Consequently, a better knowledge of the QS mechanisms present in AAB would clarify the QQ activity over themselves. For example, the construction of mutants without the ability of AHL production could be a good tool in order to test the mechanisms related with the interaction among cells.

The presence of QQ activities has been associated with competitive strategies between bacteria that share the same environments (Zhang and Dong, 2004). The AAB harbor niches with extreme conditions, and then the competitiveness with other bacteria is low because these habitats are not tolerated by most of the microorganisms. Otherwise, QQ has also been associated with mechanisms of cell adaptation in response to changes in the environment, autoregulating their own metabolism at the community level (Zhang and Dong, 2004). In the case of AAB, it could be related with vinegar; the population size does not exceed 10^9 cell/mL, and it is not related with the nutrients' availability because although more nutrients are supplied, the population does not increase.

Therefore, this growing limitation could be determined probably by quorum signalization.

The knowledge of QS and QQ mechanisms allows better understanding of the interaction between microorganisms. The studies in AAB about QS signalization carried out by Iida et al., (2008a, 2008b; 2009), together with the QQ studies performed in this thesis, open the field for the research about the interaction between cells for this group of bacteria.

The knowledge on AAB is limited; although many studies have focused on the characterization of their metabolism and its regulation, some aspects related with this bacterial group and their potential activities remain unclear. This thesis has tried to open new perspectives in studying the relations established between cells in complex environments. The future applications of AAB will depend on the advancement of the knowledge that this thesis has tried to develop: the improvement of the molecular techniques and the comprehension of the mechanisms that regulate the relations among the individuals of a given population. At this point, it is worth to remember that the industrial applications of this group of microorganisms are very wide, not limited to the production of vinegars, but they also have a broad variety of biotechnological applications.

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GENERAL CONCLUSIONS

- The molecular approaches allowed the identification of species or even genera of AAB which had not been previously described in the niches analyzed: healthy grapes, wine and strawberry vinegar biofilm. The species of *Gluconobacter japonicus* and *Acetobacter malorum* were firstly detected on healthy grapes and in grape wine of the Canary Islands, and the genera of *Asaia*, *Ameyamaea* or *Tanticharoenia*, not previously identified in vinegar, were present in the studied vinegar biofilm.

- The 16S-23S rRNA gene internal transcribed region sequence enables the identification of closely related species such as *Acetobacter cerevisiae* and *Acetobacter malorum* and their quantification by the design of specific primers and TaqMan-MGB probes for Real-Time PCR technique.

- The results of AAB diversity obtained from culture dependent and independent techniques yielded complementary results and provided accurate information on the real population present in Canarian wine and in strawberry biofilm.

- The carbon sources of glucose, fructose and sucrose, even when supplemented with ethanol, were appropriate to analyze the cellulose production by AAB strains. This production of cellulose was successfully correlated with the presence of sequences homologous to *bcsA* gene, which codifies for cellulose synthase, in most of the AAB strains screened.

- Quorum quenching activity was determined for first time in AAB. The GqqA protein derived from the genome of *Komagataeibacter europaeus* Gae02 and homologous to prephenate dehydratase presented quorum quenching activity on *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa* and *Chromobacterium violaceum* and interfered the phenotype of cellulose production on several AAB strains of different species including the Gae02 strain.

APPENDIXES

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Appendix 1

Culture media

GY medium (Glucose Yeast)
GY is a general medium to grow AAB.

Liquid medium:
Glucose 100 g/L in distilled water
Yeast Extract 10 g/L

Solid medium:
Glucose 100 g/L in distilled water
Yeast Extract 10 g/L
Agar 15 g/L

The medium is autoclaved at 121ºC for 15 min. Once the medium is warm, natamycin (100 mg/L) can be added to avoid yeast growth.

FY medium (Fructose Yeast)
FY is used in the chapter 4 to grow AAB.

Liquid medium:
Fructose 100 g/L in distilled water
Yeast Extract 10 g/L

SY medium (Sucrose Yeast)
SY is used in the chapter 4 to grow AAB.

Liquid medium:
Sucrose 100 g/L in distilled water
Yeast Extract 10 g/L

GYC medium (Glucose Yeast Calcium carbonate)
GYC is a general medium to grow AAB.

Liquid medium:
Glucose 100 g/L in distilled water
Yeast Extract 10 g/L

Solid medium:
Glucose 100 g/L in distilled water
Yeast Extract 10 g/L
$CaCO_3$ 20 g/L
Agar 15 g/L

Calcium carbonate is used to detect acid production. When acid is produced, a halo is formed around the colony.

The medium is autoclaved at 121ºC for 15 min. Once the medium is warm, natamycin (100 mg/L) can be added to avoid yeast growth.

LB medium (Sambrook, 2001)
LB medium is a common medium for *Escherichia coli*, *Chromobacterium violaceum*, *Pseudomonas aeruginosa*
Tryptone 10 g/L
Yeast extract 5 g/L
NaCl 10 g/L
Agarose 1.5% (w/v) (Solid medium)
For Blue/White screening the medium autoclaved is supplemented with 500µl of ampiciline (100mg/ml), 500µl of IPTG (50mg/mL) and 1mL of X-gal (40mg/mL) sterile filtered.

AT medium
AT medium is a minimal medium for *Agrobacterium tumefaciens* NTL4

AT salt solution (1L 20x)
$(NH_4)_2SO_4$ 40 g
$MgSO_4$ x 7 H_2O 3.2 g
$CaCl_2$ x 2 H_2O 0.2 g
$FeSO_4$ x 7 H_2O 0.1 g
$MnSO_4$ x 7 H_2O 0.024 g

AT buffer (1L 20x)
KH_2PO_4 214 g
Adjust pH to 7

Glucose solution (100 mL 50%,sterile filtered)
Glucose 50 g

Following components are autoclaved or filtered sterile and afterwards combined according to the details below. For 1 L of AT medium, 50 mL AT salt solution (20x), 50 mL AT buffer (20x), 10 mL glucose solution (50%) and 890 mL H_2O are combined.

AT soft agar
AT soft agar is used for the screening of QS and QQ activities in genomic and metagenomic libraries using *A. tumefaciens* NTL4

To prepare 100mL of AT soft agar

Eiken Agar+H_2O (autoclaved separately)	1g +89ml H_2O_{bidest}
ATbuffer(20x)	5ml
AT salt solution(20x)	5ml
50% Glucose	1ml
Spectinomycine(50 mg/mL)	120 µl
X-Gal(50 mg/mL)	120 µl
A.tumefaciens NTL4(10^9cells /mL)	to 10^7cells/mL

Swarming agar for *E.coli*

Swarming agar for *E. coli* is prepared from LB agar solidified with 0.4% Eiken agar. Glucose is prepared as a stock solution filtered sterile.

For 1L of swarming medium:

NaCl	10 g
Tryptone	10 g
Yeast extract	5 g
Eiken agar	15 g
Nutrient broth	18 g

After autoclaving the medium is supplemented with glucose to a final concentration of 4 g/L.

Swarming agar for *P. aeruginosa* PAO1

For the determination of swarming motility of PAO1, the following solutions are prepared and combined after filtration.

Solution 1(100mL)

Glucose	4 g

Solution 2(100mL)

$MgSO_4$ x 7 H_2O	2 g

Solution 3(100mL)

$CaCl_2$ x 2 H_2O	0.2 g

Solution 4 (100mL)

Na_2HPO_4	7 g
KH_2PO_4	3 g
NaCl	0.5g

Solution 5 (100mL)

$C_5H_8NNaO_4$ x H_2O	5.5% (w/v)

The solutions sterile filtered are stored at room temperature until used. For 100 mL of swarming or swimming medium, the following volumes have to be added to freshly autoclaved Eiken agar.

For 100mL of swarming agar

Solution 1	10 mL
Solution 2	1 mL
Solution 3	1 mL
Solution 4	10 mL
Solution 5	1 mL
Eiken agar	0.5% in 77 mL

Miminal medium (Miller, 1972)

This medium is used for the complementation assay with modified auxotrophs of *E.coli*.

K_2HPO_4 10.5 g/L
KH_2PO_4 4.5 g/L
$(NH4)_2SO_4$ 1g/L
$MgSO_4$ 0.12 g/L

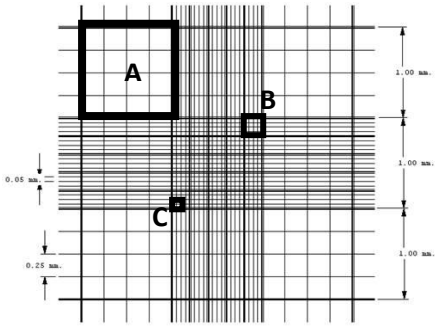
Na citrate 0.5 g/L
Glucose 2g/L
The medium for control with Phe was supplemented Thiamine-HCl 5mg/mL (stock 50mg/mL) and 50µg/mL of phenylalanine (both sterile filtered).

Stock and working concentration of supplements
The following additives are used to supplement the different media in this concentrations and they are previously prepared in a stock concentration, diluted in the adient solvent sterile filtered and stored frozen.

	Abbreviation	End concentration in the medium µg/mL			Solvent	Stock solution mg/mL
		E.coli	*P.aeruginosa*	*A.tumefaciens*		
Ampicillin	**Amp**	**50-100**			**H_2O**	**100**
Chloranphenicol	**Cm**	**25-50**			**EtOH**	**25**
Gentamycin	**Gm**	**10**	**50**		**H_2O**	**50**
Kanamycin	**Km**	**20-25**		**100**	**H_2O**	**25**
Spectinomycin	**Sp**			**50**	**H_2O**	**50**
Tetracyclin	**Tc**	**5**		**4.5**	**EtOH70%**	**5**
X-Gal		**50**	**50**	**60**	**DMF**	**50**
IPTG		**40-100**	**100**		**H_2O**	**100**

Cell recount

Microscope counting
The number of total microorganisms can be determined counting the cells with a microscope and a Neubauer chamber. Following there is a representation of a Neubauer chamber with the squares marked as A, B, C attending to theirsize. For AAB, 20 C-squares are used to count the cells.



Relation among different squares,
A = 16 B
B = 25 C
Profundity = 0.1 mm
Volume A = 1 x 1 x 0.1 = 0.1 mm^3 = 10^{-4} ml
Volume B = 0.25 x 0.25 x 0.1 x 10^{-3} = 6.25 10^{-6} ml
Volume C = 0.05 x 0.05 x 0.1 x 10^{-3} = 2.5 10^{-7} ml

cells/ml=(Num cells) / (Num squares x Square volume)

Plate counting
Different microorganisms are plated in a medium and incubated during specific time and conditions, after this incubation time each cell will form a colony and counting the colonies will be obtained the number of colony form units (UFC/ml). Serial dilutions (1:10) are performed and plated in order to obtain between 10-100 colonies in every plate to be counted.

Determination of cell density by absorbance
Growth of liquid cell cultures was determined by optical density (OD) measurement with an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany). The OD is measured at a wavelength of 600 nm (OD600) using one-way cuvettes (10x4x45 mm,

Sarstedt, Nuembrecht, Germany) with a path length of 1 cm. Pure medium is used as reference. Cell cultures have to be diluted when required so a measured optical density of 0.8 is not exceeded. An OD600 of 0.1 corresponds to a cell density of approximately $1x10^8$ cells/mL for *E. coli*.

Maintenance and strain storage

Strain storage of pure cultures
For conservation purposes, glycerol stocks are prepared to preserve strains for long-term storage. For these stocks, cultures are grown in liquid medium overnight at appropriate temperatures, 900µl of culture are mixed with 500 µl of 87% sterile glycerol in screw-cap tubes and stored at -70°C or -80°C.

Storage of fosmid clones
Colonies obtained by construction of the fosmid clone library are picked from agar plates into sterile microtiter plates (96-wells) containing 150 µL LB broth supplemented with chloranphenicol (25 µg/mL). The microtiter plates are incubated at 37°C overnight without shaking. For preservation 50 µL sterile glycerol (87%) are added to each well, mixed well and stored at -70°C.

DNA extraction

DNA extraction of AAB from culture media, wine and vinegar (Ausubel et al., 1992; Jara et al., 2008)
Cells are harvested from culture medium, wine or vinegar and centrifugued for 5 min in at 14000 rpm.
When the samples are wine or vinegar, specially when they show high pigmentation, they are washed twice with a solution of EDTA-PVP (0.15M NaCl, 0.1 M EDTA and 2% (v/w) polyvinyl pyrrolidone (PVP)).
When the samples present cellulose, 100 µl of cellulase 25 mg/mL were added and incubated for 1 h at 37ºC and then centrifuged.
-The pellet is resuspended in 520 µl of TE Buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8).
-Add 30 µl of SDS 20% and 6 µl of proteinase K (20 mg/mL). Mix using the vortex
- Incubate the mixture during 1h at 37ºC
- Add 150 µl of NaCl 5M and 140 µl of 10% CTAB in 0,7M NaCl
- Incubate the suspension during 10 min at 65ºC
- Incubate on ice for 10-15 min
- Add one volume of chloroform:isoamyl alcohol (24:1) and mix manually until homogenize
- Incubate the mixture on ice for 5 min
- Centrifuge at 4ºC and 10000rpm for 10 min (wash again with chloroform:isoamyl alcohol until not observe the inter-phase)
- Transfer the aqueous phase to an eppedorf tube with 380 µl of isopropanol
- Mix until observe the precipitation of the DNA
- Incubate at -20ºC for 5 min
- Centrifuge at 4ºC at 10000 rpm for 10 min
- Eliminate the supernatant

- Add 150 µl of cold ethanol 70% (v/v)
- Centrifuge at 4ºC at 10000 rpm for 5 min
- Pipette off the ethanol and air dry the pellet or using the speed-vac for 10-15 min
- Resuspend the pellet in 50 µl of TE buffer and keep overnight for a better resuspension

DNA extraction of AAB from biofilm

- Cut 5g of biofilm and leave it overnight in 10 mL of extraction buffer (100mM Tris-HCl pH 8; 100mM Na-EDTA pH 8; 1,5 M NaCl; 0,1% Tween 80) at 37ºC-stirred at 200 rpm.
- Vortex for 15 min with glass pearls
- Filter the solution through a paper membrane Whatman grade 1 and wash with NaCl 1M three times and centrifuge for 15 min at 4500 rpm
-Wash the pellet twice with PVP-EDTA and once again with modified TE (10mM Tris-Hcl; 100mM EDTA, 0,80M NaCl, pH 8)
-Resuspend the pellet in 520 µL of modified TE and add 40 µL of lysozyme (20 mg/mL) and 10 µL of RNAsa (1 mg/mL) incubate at 37°C for 1 h.
-Add 30 µL of SDS at 20% and 6 µL of Proteinase K (20 mg/ml). Vortex it.
- Incubate the mixture for 30 min at 50ºC
- Add 150 µL of 5M NaCl and 140 µL of CTAB in 0,7M NaCl.
- Incubate the suspenssion for 10 min at 65ºC and briefly incubate on ice for 10-15 min
-Add one volumen of phenol:chloroform:isoamyl alcohol (25:24:1) and mix until homogenize
-Add one volumen phenol:chloroform:isoamyl alcohol (24:1) homogenize and incubate the mixture on ice for 5 min
-Centrifuge at 8ºC and 10000rpm for 10 min
-Repeat the step twice or until not observe an interphase
-Transfer the aquous phase (top) into a new tube with 0.6 volumes of isopropanol
-Inverse the tuve until precipítate the DNA and incúbate at -20ºC for 3 h
-Centrifuge at 4ºC 10000 rpm for 10 min and pipette off the supernatant
-Add 150µL of cold ethanol 70% (v/v) and centrifuge again in the same conditions
-Eliminate the supernatant and air dry the samples to evaporate the remaining ethanol at room temperature
-Resuspend in 50 µL of TE
-Add 2.5 µL of RNAsa (1 mg/mL)

Identification and typing techniques for AAB

Identification

Amplification of the 16S rRNA gene (Ruiz et al., 2000)

Amplification mix to a final volume of 50 µl:

Primer Aceti I (10 ρM)	1.5 µl
Primer Aceti IV (10 ρM)	1.5 µl
dNTPs (each dNTP 10 mM) (Boehringer Mannheim)	1 µl
$MgCl_2$ (100 mM) (Ecotaq)	3 µl
DMSO (Dimethyl sulfoxide)	5 µl

BSA (Bovine serum albumin) (20 mg/mL)	0.4 µl
Buffer Taq 10x, without Mg. (Ecotaq)	5 µl
Taq DNA polymerasa (Ecotaq)	0.4 µl
H_2O milli-Q	29.2 µl
DNA	3 µl

PCR conditions:
The samples are incubated for 1 min at 94ºC and then cycled 35 times at 94ºC for 1 min, 60ºC for 45 seconds, and 72ºC for 2 min. The samples are incubated for 10 min at 72ºC for final extension and kept at 4ºC until tested.
Five microliters of the amplified DNA are mixed with 2 µL of bromophenol blue and detected by electrophoresis on a 1% (w/v) agarose gel (Boehringer Mannheim).

PCR-RFLP 16S rRNA (Ruiz et al., 2000)
Once the amplified fragment is obtained, the digestion is performed. The following restriction endonucleases used are: *Taq*I, *Alu*I (Roche diagnostics), and *Bcc*I (Biolabs).

Enzyme	1 µl
H_2O milli-Q (or 6.8 µl for *Bcc*I)	2 µl
Specific buffer for each enzyme	7 µl
BSA (Bovine serum albumin) (only for *Bcc*I)	0.2 µl
DNA amplified	10 µl

Samples are incubated for 3 hours at 37ºC (for *Alu*I and *Bcc*I) or 65ºC (for *Taq*I). The digested DNA is mixed with 4 µL of bromophenol blue and detected by electrophoresis on a 3% (w/v) agarose gel. The length of the restriction fragment is determined by comparison with a 100 bp DNA ladder (Marker XIV, Roche).

Amplification of the 16S-23S rRNA gene ITS region (Ruiz et al., 2000).
Primers used to amplify the ITS 16S-23S rDNA are:
ITS1, 5'-ACCTGCGGCTGGATCACCTCC-3'
ITS2, 5'-CCGAATGCCCTTATCGCGCTC-3'.

Amplification mix to a final volume of 50 µl:

Primer ITS1 (10 ρM)	1.5 µL
Primer ITS2 (10 ρM)	1.5 µL
dNTPs (each dNTP 10 mM) (Boehringer Mannheim)	1 µL
$MgCl_2$ (100 mM) (Ecotaq)	3 µL
Buffer Taq 10x, without Mg. (Ecotaq)	1 µL
Taq DNA polymerasa (Ecotaq)	0.5 µL
H_2O milli-Q	40.5 µL
DNA	1 µL

PCR conditions:
The samples are incubated for 5 min at 94°C and then cycled 35 times at 94°C for 30 s, 65°C for 30 s and 72°C for 1 min. The samples are then incubated for 7 min at 72°C for a final extension and kept at 4°C until tested.
Five microliters of the amplified DNA are mixed with 2 µL of bromophenol blue and detected by electrophoresis gel on a 1% (w/v) agarose gel (Boehringer Mannheim). The length of the amplification product is determined by comparison with a 100 bp DNA ladder (Gibco-BRL, Eggenstein, Germany)

SYBR-Green Real-time PCR(González et al., 2006)

Master mix	12.5 µL
Primer forward(Stock 10 µM)	1.5 µL
Primer reverse(Stock 10 µM)	1.5 µL
H_2O	4.5 µL
DNA	5 µL

PCR conditions:
The PCR program consists in incubate the mixture at 95ºC during 10 min, and then, 40 cycles of 15 s at 95ºC and 1 min at 62ºC

TaqMan-MGB probes Real-time PCR(Torija et al., 2010)
The primers for the detection and quantification of AAB by Real-time PCR are:

Species	Oligonucleotide	Name	Sequence (5'→3')
A.pasteurianus	Forward primer	PASTEU-F	TCAAGTCCTCATGGCCCTTATG
	RT-PCR reverse primer	PASTEU-R	TCGAGTTGCAGAGTGCAATCC
	Probe	PASTEU-S	6FAM-TGGTGACACCATGCTGA-MGB
	Pre-PCR reverse primer	Apa-R	CCCATAGGAACCGGCAGTCT
A.aceti	Forward primer	ACETI-F	TGGAGCATGTGGTTTAATTCGA
	RT-PCR reverse primer	ACETI-R	GCGGGAAATATCCATCTCTGAA
	Probe	ACETI-S	VIC-CAGCCTCTCCATACAA-MGB
	Pre-PCR reverse primer	AAc-R	GCCGATTGCTCAGCTCATG
G.oxydans	Forward primer	OXYD-F	CCCAGTGTAGAGGTGAAATTCGT
	RT-PCR reverse primer	OXYD-R	CCAGGGTATCTAATCCTGTTTGCT
	Probe	OXYD-S	6FAM-TGGCTCGATACTGAC-MGB
	Pre-PCR reverse primer	GO-R	GTCATCCAACTTCGTGGAGACA
Ko.hansenii	Forward primer	HANSEN-F	GTCCACGCTGTAAACGATGTGT
	RT-PCR reverse primer	HANSEN-R	TGTGCTTATCGCGTTAACTACGA
	Probe	HANSEN-S	VIC-TGGCCAAGCCATCCA-MGB
	Pre-PCR reverse primer	ITS-GAH	CCACGAGAAGACAGTTCCTTGGCT
Ko.europaeus	Forward primer	EUR-F	GGGTTAAGTCCCGCAACGA
	RT-PCR reverse primer	EUR-R	ACCTTCCTCCGGCTTGTCA
	Probe	EUR-S	VIC-TAGTTGCCATCACGTCTG-MGB
	Pre-PCR reverse primer	ITS-GAE	CAGTCCAGTTACCCAGACCATCCTC
A.malorum	Forward primer	MACER-F	CTGGTTTGAGGCTTGAGTGATATG
	RT-PCR reverse primer	MACER-R	TGCTCTCCCAGCTGAGCTATG
	Probe	MAL-S	6FAM-AGCAGCGCTCTGGT-MGB
	Pre-PCR reverse primer	MAL-R	CCATGCGACTAAGTGACACGTCT
A.cerevisiae	Forward primer	MACER-F	CTGGTTTGAGGCTTGAGTGATATG
	RT-PCR reverse primer	MACER-R	TGCTCTCCCAGCTGAGCTATG
	Probe	CER-S	VIC-AGCAGCATTCTGGT-MGB
	Pre-PCR reverse primer	CER-R	CCATGCCACTAAGTGACACTTCC
Total AAB	Forward primer	AAB-F	TGAGAGGATGATCAGCCACACT
	RT-PCR reverse primer	AAB-R	TCACACACGCGGCATTG
	Probe	AAB-S	NED- ACTGAGACACGGCCCA -MGB
Total AAB	Forward primer	AQ1F	TCAAGTCCTCATGGCCCTTATG
	Reverse primer	AQ2R	CGCCATTGTAGCACGTGTGTA

Enrichment PCR

Amplification mix to a final volume of 50 µl:

Primer forward (10 ρM)	1.5 µL
Primer reverse (10 ρM)	1.5 µL
dNTPs (each dNTP 10 mM) (Boehringer Mannheim)	1 µL
$MgCl_2$ (100 mM) (Ecotaq)	3 µl

Buffer Taq 10x, without Mg. (Ecotaq)	1 µl
Taq DNA polymerasa (Ecotaq)	0.5 µl
H_2O milli-Q	40.5 µl
DNA	5 µl

PCR conditions:
The samples are incubated for 5 min at 94°C and then cycled 15 times at 94°C for 15 s, 62°C for 30 s. The samples are then kept at 4°C until used.

Real-time-PCR
To a mixture volume of 50 µL:

Master mix	12.5 µL
Primer forward(Stock 10 µM)	0.25 µL
Primer reverse(Stock 10 µM)	0.25 µL
Probe(Stock 10 µLM)	0.75 µL or 0.5 µL (*A.cerevisiae* and A.*malorum*)
H_2O	6.25 µL or 6.5 µL (*A.cerevisiae* and *A.malorum*)
DNA	5 µL

PCR conditions:
The PCR program consists in incubate the mixture at 95ºC during 10 min, and then, 40 cycles of 15 s at 95ºC and 1 min at 62ºC(*Ko. europaeus* at 60ºC)

DGGE-PCR(Denaturing Gradient Gel Electrophoresis) (Lopez et al., 2003; De Vero et al., 2006)
The PCR amplification of the region V7 to V8 of the 16S rRNA gene is performed using the following primers: WBAC1 (5'-GTCGTCAGCTCGTGTCGTGAGA-3'; nt 1069–1090) with GC-clamp and WBAC2 (5'-CCCGGGAACGTATTCACCGCG-3'; nt 1374–1394).

PCR conditions:
The samples are incubated for 5 min at 95°C and then cycled 30 times at 95°C for 1min, 67°C for 30 s and 72°C for 1 min. The samples are then incubated for 5 min at 72°C for a final extension and kept at 4°C until tested.
Once the amplicons are obtained, the next step is to prepare the denaturing gradient gel.

Solutions for DGGE:

TAE 50 X

Trizma base	242 g
Acetic acid glacial	57.1 g
EDTA 0.5 M (pH8)	100 mL
dH_20 up to 1000 mL	

Autoclave 121ºC during 15 min

EDTA 0.5 M pH 8

EDTA	186.12 g
Adjust the pH at 8 with NaOH	
dH_20 up to 1000 mL	
Autoclave 121ºC during 15 min	

0% denaturing solution 40%

bisAcrilamida	10 mL
50X TAE	1 mL
dH_20 to 50 ml	

100% denaturing solution

urea	21 g
formamide	20 mL
40% bisAcrilamide	10 mL
50X TAE	1 mL
dH_20 to 50mL	

10% Ammonium persulphate (APS)
0.1 g ammonium persulphate in 1 ml dH_20

TEMED (N,N,N,N'-tetra-methyl-ethylenediamine)

DGGE of PCR products is performed on an 8% (w/v) polyacrylamide gel with urea and formamide as denaturants. Once the gel assembly is ready, the acrylamide gel can be prepared. It is prepared with a mixer gradient pump. Both 0% and 100% denaturing solutions are needed, and approximately 20 ml of each solution is kept on ice while the gel is built. Into each 20 ml solution, 20 µl TEMED and 200 µl APS are added. Fifty microliters of colorant can be added to the 100% denaturing solution to see the denaturing gradient when it is created. The denaturing gradient is prepared with an interval between 40% and 60% (v/v). The gel is left for 1 hour to polymerize.
Electrophoresis is performed in 1X Tris-acetate EDTA (TAE) buffer at 60°C at a constant voltage of 200 V for 4 h. Subsequently, the gel is stained with ethidium bromide in 250 ml of 1X TAE buffer for 15 minutes. The gel is then destained with 250 ml of 1X TAE buffer for 20 minutes and photographed.
Individual PCR-DGGE bands are cut out from gel and incubated overnight at 4°C in 30µl H_2O. An aliquot (1 µl) is used in a PCR reaction with the same primer set used for DGGE but without the GC-clamp attached to the WBAC1 primer.

Typing

ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus-PCR) for AAB (González et al., 2004)
Amplification mix to a final volume of 25 µl:

Primer Eric I (10 ρM)	1 µl
Primer Eric II (10 ρM)	1 µl
dNTPs (each dNTP 10 mM) (Boehringer Mannheim)	1.25 µl
5XGB (1M $(NH_4)_2SO_4$; 1M Tris-HCl; 1M $MgCl_20.5$M EDTA (pH 8.8); β-mercaptoethanol 14.4M)	5 µl
BSA (Bovine serum albumin) (20 mg/mL)	0.2 µl
DMSO (Dimethyl sulfoxide)	2.5 µl
H_2O milli-Q	10.65 µl
Taq DNA polymerasa (Ecotaq)	0.4 µl
DNA	3 µl

PCR conditions:
The samples are incubated for 5 min at 94°C and then cycled 30 times at 94°C for 30 s, 57°C for 30 s and 65°C for 4 min. The samples are then incubated for 8 min at 65°C for a final extension and kept at 4°C until tested.
Eight microliters of the amplified DNA is mixed with 2 μL of bromophenol blue and detected by electrophoresis on a 1.5% (w/v) agarose gel (Boehringer Mannheim).
DNA Molecular Weight Marker XIV and DNA Molecular Marker XVI (Roche, Germany) were used to determine the weight of the bands amplified.

$(GTG)_5$-PCR for AAB (De Vuyst et al., 2008)
The amplification mix contains a final volume of 25 μl:
PCR conditions:
The samples are incubated for 5 min at 94°C and then cycled 30 times at 94°C for 1min, 40°C for 1 min and 65°C for 8 min. The samples are then incubated for 16 min at 65°C for a final extension and kept at 4°C until tested.
Eight microliters of the amplified DNA is mixed with 2 μL of bromophenol blue and detected by electrophoresis on a 1.5% (w/v) agarose gel (Boehringer Mannheim).To determine the weight of the largest fragments DNA Molecular Marker XIV and XVI (Roche, Germany) were used.

Primer $(GTG)_5$ (10 ρM)	1 μl
5XGB (1M $(NH_4)_2SO_4$; 1M Tris-HCl; 1M MgCl2; 0.5M EDTA (pH 8.8); β-mercaptoethanol 14.4M)	5 μl
BSA (Bovine serum albumin) (20 mg/mL)	0.4 μl
dNTPs (each dNTP 10 mM) (Boehringer Mannheim)	1.25 μl
DMSO (Dimethyl sulfoxide)	2.5 μl
H2O milli-Q	13.45 μl
Taq DNA polymerasa (Ecotaq)	0.4 μl
DNA	1 μl

Molecular methods for strain transformation

Alkaline lysis for plasmid extraction (Sambrook, 2001)
- Centrifuge 1 to 5 mL of the cellular culture for 30 s at 12000 rpm and discard the supernatant
- Resuspend the cells in 100μl of P1 buffer
- Add 200μl of P2 buffer, mix by invertion, and incubate for 1 min at room temperature
- Add 200μl of chlorophorm and mix well
- Add 150 μl of P3 buffer
- Incubate at room temperature for 1 min and centrifuge during 15 min at 12000 rpm
- Transfer the supernatant to a sterile tube
- Precipitate the DNA with 1 mL of cold ethanol 96% (v/v)
- Let stay at least for 30 min at -20°C
- Centrifugar a 13.000 rpm a 4°C durante 20 minutos
- Eliminar el sobrenadante. El pellet se lava 2 veces con 200μl de etanol (70%) centrifugando a 13.000 rpm a 4°C durante 2 minutos
- Discard the supernatant, dry the pellet and resuspend in sterile water (50-100 μl)

P1buffer:EDTA 10mM; Tris-HCl 50mM; RNAsa 100mg/100ml; H_2O 25 ml pH 8

P2 buffer: NaOH 200mM; SDS 1% (w/v); H_2O 50ml

P3 buffer: KAc 3M; H_2O aprox. 50 ml pH5.5

Ligation of PCR products in pDrive Cloning Vector

For subcloning purposes and for insertion of additional restriction sites, purified PCR products obtained are ligated with the QIAGEN PCR Cloning Kit into pDrive Cloning Vector (linearized and equipped with U overhangs) according to manufacturer's instructions.

The mix for pDrive ligation (10 µL) contains:
DNA PCR product 0.5 - 2 µL
pDrive vector 0.5 µL
Ligation master mix 2.5 µL
H_2Obidest ad 5 µL
The ligation reaction was incubated at 16°C for 2 h and continued with transformation into competent cells.

Insertion of DNA fragments in a vector

Vector and insert restriction

Vectors and plasmids used to transform bacterial strains are cut with two restriction enzymes. In order to perform the cut with both activities at the same time it is used an special buffer if they do not work with the same buffer.

Plasmidic DNA	15µL
Enzyme 1	2 µL
Enzyme 2	2 µL
Tango buffer	9 µL (it must be used either 2x or 1x, see instructions)

Vector desphosphorylation

In order to prevent the superrolling and increase the effectiveness of the subsequent ligation it is necessary the dephosphorilation of the vector after cutting.
Fast AP Thermosensitive Alcaline Phosphatase is used in a final volume of 40 µL.
The reaction is prepared as follows:

DNA	15 µl
Buffer Fast AP	4 µl
Fast AP	2 µl
H_2O	19 µl

Incubate for 10 min at 37ºC although it can be incubated 20 min if there is a high amount of vector DNA

Inactivation of plasmids and vectors

Plamids and vectors are inactivated in order to stop the activity of the restriction enzymes and the alcaline phosphatase incubating the samples at the temperature indicated for each enzyme. They are purified from an agarose gel (0.8% in TAE buffer).

Ligation
Vector and insert are ligated by T4 DNA ligase (Thermo Scientific) using this reaction mixture:

H_2O	9 µL
DNA (90 µg/ml)	6 µL
Vector (100 µg/ml)	2 µL
Buffer T4 DNA ligase	2 µL
T4 DNA ligase	1 µL

Incubate 4 h at 16°C or overnight at 4ºC

Competent cells preparation

E.coli chemocompetent cells (Dagert and Ehrlich, 1979)
- Grow the *E.coli* strain in 50mL of LB medium and incubate until an OD 0.2
- Cool the culture at 0ºC for 10min and centrifuge
- Resuspend the pellet in 20mL of CaCl2 0.1M at 0ºC and incubate at this temperature
- Centrifuge and resuspend the pellet in 0.5 mL of CaCl2 of 0.1M
- Store at 24h at 0-4ºC

P.aeruginosa electrocompetent cells
- Grow the culture to exponential phase OD600 0.3-0.5
- Centrifuge 10min at 4ºC
- Wash with 1 volume of 300mM sucrose (20mL)
- Centrifuge 10 min at 4ºC 10min
- Wash 0.5 volumes of 300mM sucrose (10mL)
- Resuspend in 0.01 volumes of 100mM sucrose (100 µL)
- Cool the suspension for 30 min at 0ºCs
- Transfer 40 µL of cells and 5 µL of DNA of the plamid to a BioRad cuvette and make the electric shock

Heat shock transformation
Competent *E. coli* cells are thawed on ice for 5 min. Five µL of plasmid DNA are added to the competent cells and incubated on ice for 30 min. The heat shock is performed for exactly 90 sec at 42°C. The cells are transferred back on ice for 5 min, resuspended with 1 mL LB broth and incubated for 1 h at 37°C. Volumes between 50–100 µL of the cell suspension are plated on selective LB agar plates and incubated overnight at 37°C.

Protein purification

Crude cell extract preparation
To obtain crude cell extracts from *E. coli* for purification of His-tagged proteins, *E. coli* BL21 (DE3) cells harboring target DNA were cultivated.The induction of the production is produce by the addition of IPTG 800mM when the OD of the culture is 0.6. After overnight growing at 30ºC, cells are then harvested by centrifugation at 10000 rpm, 4°C for 20 min, washed and resuspended in 1x LEW buffer, where 2 mL of buffer were used per 1 g cell pellet.

Cell disruption
The crude cell extract is produced in 3 intervals of 1100 Bar each, with a French Pressure Cell Press (American Instrument Company, Silver Spring, MD, USA). After

cell disruption, the lysate is transferred into sterile centrifugation tubes and centrifuged at 4500 rpm, 4°C for 30 min.

Purification of histidine-tagged proteins by Protino Ni-TED columns
The obtained supernatants are purified under native conditions using Protino Ni-TED 2000 packed columns (Macherey-Nagel, Dueren, Germany) following the manufacturers protocol. First, the column was equilibrated with 1x LEW buffer, the clarified lysate was loaded on the column and allow draining by gravity, the bound protein is eluted with 1x elution buffer containing imidazole. If necessary, a dialysis was carried out to remove excess imidazole from the eluted proteins. The levels of protein purity as well as the molecular mass are determined by SDS-PAGE.
1x LEW buffer(50 mM NaH_2PO_4; 300 mM NaCl ; pH 8.0)
1x Elution buffer(50 mM NaH_2PO_4; 300 mM NaCl; 250 mM imidazole; pH 8.0)

Concentration by Vivaspin concentrator
Therefore, the Vivaspin 6 concentrator (10000 MWCO PES, Satorius Stedim Biotech GmbH, Goettingen, Germany) is filled with max. 6 mL of the protein extract and centrifuged at 4500 rpm, 4°C until the desired concentration is reached. The supernatant is removed from the concentrator and could be used for further assays.

Biochemical methods for working with proteins

Quantitative determination of protein content (Bradford 1976)
The measurement of concentration of protein solutions was carried out by using the Bradford protein assay. The method is based on the shift of the absorbance in the dye Coomassie Brilliant Blue G-250 (absorbs at 595 nm), when the previously red form Coomassie reagent (absorbs at 465 nm) changes and stabilizes into Coomassie blue. 10 µl of purified protein are added to 1 mL of Bradford solution (see below), mixed and incubated for 15 min at RT in the dark. The extinction is measured at a wavelength of 595 nm in a one-way cuvette with an Eppendorf BioPhotometer or a SmartSpec Plus Spectrometer. The buffer used for dialysis serves as a reference. A direct correlation between extinction and protein concentration is only given in the range of linearity of the calibration curve. Samples above this range have to be diluted either with buffer or H_2Obidest. Prior to the measurements, a calibration curve is generated with BSA.

Bradford solution (sterile filtered) Coomassie Brilliant Blue G250 100 mg Ethanol (95%, v/v) 50 mL H3PO4 (85%, w/v) 100 mL H_2Obidest ad 1000 mL

SDS-Polyacrylamidgelelectrophoresis (SDS-PAGE)
After dialysis, the eluted proteins were separated due to their molecular weight by SDS-PAGE. The proteins were treated with SDS (sodium dodecyl sulfate), which neutralizes and covers them with negative charges. The SDS-PAGE was carried out in gel electrophoresis chambers (Bio-Rad Laboratories, Munich, Germany).

For preparation of the SDS-PAGE gels, the following solutions have to be prepared:

Acrylamide stock solution Acrylamide 30 g Bisacrylamide 0.8 g H_2Obidest ad 100 mL

Ammonium Persulphate (APS) 10% (w/v) in H_2Obidest

N,N,N',N'-Tetramethylene ethylene diamine (TEMED) supplied by Bio-Rad Laboratories, Munich, Germany

Resolving gel stock solution Tris (1.5 M) 45.4 g SDS (0.4%, w/v) 0.4 g H2Obidest ad 50 mL pH 8.8 (with HClconc.)

Stacking gel stock solution Tris (500 mM) 6.1 g 1 g SDS (0.4%, w/v) H2Obidest ad 100 mL pH 6.8 (with HClconc.)

10x electrophoresis buffer 4x SDS loading buffer Tris 30.3 g Glycerol 7.5 mL Glycine 144.1 g β-Mercaptoethanol 2.5 mL SDS 10 g SDS 1.2 g H_2Obidest ad 1000 mL

Bromophenol blue (0.2%,w/v) 0.5 mL pH 8.4 (with Glycine) Tris 0.4 g H_2Obidest ad 50 mL pH 6.8 (with HClconc.)

The resolving and stacking gel are prepared in a percentage of 12 and 7% respectively attending to the specific application of the SDS-PAGE. After mixing all components in a falcon tube, the resolving gel is poured between two glass plates, which are previously cleaned with 70% ethanol and inserted into a stand. Water is poured on the top of the resolving gel to level the gel edge. After the resolving gel polymerized, water is decanted and all components for the stacking gel are mixed.

Composition of SDS-PAGE gels used in this study

	12% resolving gel	7% stacking gel
Resolving gel stock solution	1.25 mL	-
Stacking gel stock solution	-	0.48 mL
Acrylamide stock solution	1.5 mL	0.35 mL
APS	23 µL	10 µL
TEMED	5 µL	3 µL
H_2O	2.23 mL	1.17 mL

Preparation of protein samples and gel electrophoresis

Aliquots of 20 µL of obtained protein extracts were mixed with 5 µL of SDS loading buffer and incubated at 95°C for 5 min. For detection of molecular masses of proteins, protein markers (Fermentas, St.Leon-Rot, Germany) it had to be incubated at 95°C for 5 min. Prior to loading of samples, the gel was inserted into the electrophoresis chamber (Bio-Rad Laboratories, Munich, Germany) and the chamber was filled with 1x electrophoresis buffer. each sample was then loaded carefully into the gel pocket and electrophoresis was carried out at 150 mV for the stacking gel and was increased to 200 mV for the resolving gel.

Loading buffer

Stacking buffer	4mL
Glycerol	10mL
β-mercaptoethanol	5mL
SDS	2g
Bromophenol Blue 1%	1mL

Coomassie staining of SDS-PAGE gels

After the electrophoresis, the gel was carefully removed from the glass plates and stained with Coomassie stain overnight under gentle shaking. Destaining of the gel was accomplished with 20% acetic acid until bands were visible.

Coomassie stain (1 L):
Coomassie brilliant blue powder 1 g
Acetic acid 100 mL
Ethanol 400 mL
H_2O bidest 500 mL

Screening of QQ activity

ATsoft-Agar-Screening

- Inoculate *A. tumefaciens* NTL4 on LB supplemented with spectinomycin (50µg/mL), and incubate at 28 or 30°C overnight with shaking at 250 rpm.
- Inoculate 5 mL of AT medium supplemented with spectinomycin and tetracycline were inoculated with 1-5% of the overnight culture of NTL4, and incubate at 28 or 30ºC overnight with shaking at 250 rpm.
-In a sterile microtiter plate of 1mL wells (deep well plates) add 1 mL of LB and chloranphenicol (12.5 µg/mL), and then transfer the metagenomics clones to the plate, and incubate at 37 ºC overnight with shaking at 250 rpm.
-To determine the OD_{600} of the NTL4 culture, and calculate the volum for AT_{soft}-Agar in order to get 10^7 cells/ml in each well.
- Fill the wells of a microtiter plate with 240 µl AT_{soft}-Agar, and then transfer the precultures of the metagenomics clones from the growed cultures to the new microtiter plate and incubate at 30ºC overnight.
- Set the positive control with AHL solution.

ONPG Test for AHLs in Culture Supernatant

- Grow NTL4 in 5 ml LB with Sp (50 µg/ml) and Tc (4.5 µg/ml) overnight at 28°C and positive clone in suitable LB.
- Add different volume culture of positive clones into 5 ml of a freshly grown NTL4 culture in AT media with Sp and Tc (NTL4: $1x10^7$cell/ml), and incubate at 28ºC for 17 or 24 h.
- Measure OD600 for each sample.
- 1 ml of the cell suspension was mixed with 20 µl toluene and vortexed for 3 min.
- Mix 800 µl cell suspension (avoiding the top layer containing undissolved toluene) with 200 µl freshly prepared ONPG-solution (4 mg/ml in Z-buffer), incubate 20 min at room temperature (at 28°C up to 30 min).
- Add 400 µl 1M Na_2CO_3-solution.
- Centrifuge 2 min 13,000 rpm
- Measure OD of supernatant at 420 nm.
- Calculate OD420/OD600.

Z buffer for 100 ml:

$Na_2HPO_4 \times 7H_2O$	1.61 g
$NaH_2PO_4 \times H_2O$	0.55 g

KCl	0.075 g
$MgSO_4 \times 7H_2O$	0.0246 g
ß – mercaptoethanol	0.27 ml

Adjust pH to 7.0. Do not autoclave, store in refrigerator

Inhibition of swarming motility in *E. coli*

To narrow the number of clones tested positively in the ATsoft screening, the influence of these candidate clones on QS-dependent swarming motility directly in their *E. coli* EPI300 and *E. coli* DH5α hosts is analyzed.

Selected clones are cultivated in 5 mL LB medium with ampicillin overnight at 37°C. OD600 is measured and aliquots with $1x10^9$ cells/mL are centrifuged shortly. The supernatant is pipetted off completely and the pellet is resuspended in 10 µL fresh LB medium. The agar plates are prepared and allowed to solidify under sterile conditions. Then, 1 µL of the cell suspension was pipetted on the center of each plate. Incubation is carried out at 30°C and 37°C for 20 h. Swarming behavior is evaluated visually.

Swarming motility inhibition in *P. aeruginosa* PAO1

For motility assays PAO1 is used as its motility like swarming and swimming or biofilm formation is QS-dependent.

Selected clones are cultivated in 5 mL LB medium with ampicillin overnight at 37°C. A preculture of PAO1 was necessary to assay its motility, therefore it is cultivated in LB at 37ºC overnight. Either crude cell or protein extracts are added to the agar. Therefore, the swarming agar is prepared as described above, autoclaved and cooled to 40°C. Extracts are then added to the agar, gently vortexed and poured into Petri dishes. After 3 h at room temperature, the plates could be used for inoculation with PAO1. Alternatively, extracts are spread over the surface of already solidified swarming agar. Plates were dried under sterile condition until the extracts were completely absorbed by the agar surface. The prepared swarming agar supplemented with either crude cell extract or protein extract is inoculated with PAO1. Therefore, OD600 is measured and an aliquot of OD600 1 is configured from the preculture. The cells are harvested, the supernatant is pipetted off and cells are resuspended in 10 µL LB medium. One µL of the PAO1 cell suspension is pipetted exactly in the center of each swarming or swimming agar and plates are incubated at 37°C for 16 h. Swarming behavior is evaluated visually.

Pigment inhibition in liquid medium with CV026

A preculture of Cv026 was cultivated in LB medium at 30ºC. Different volumes of crude cell extracts and purified protein are mixed with 5 µL of 3-oxo-C6-HSL (0.1 mM) and incubated for 3 h. The 48-well plates with fresh LB are inoculated with Cv026 and the suspensions previously incubated with AHL added. They are incubated overnight at 30°C. When AHLs are absent in the medium or are degraded by putative QQ, the medium became white, otherwise it turned to purple.

Pyocyanin assay (Gallagher et al., 2002)

The blue compound pyocyanin produced by PAO1 in liquid and solid cultures is also regulated by QS-dependent processes (subordinated to biofilm formation). In the absence of QQ active compounds in PAO1 cultures, the blue compound is produced that is is why cultures appear to be green. By extraction with chloroform, these compound gives a strong blue color, while inhibited production of pyocyanin results in

colorless or pale blue extracts. The strain PAO1 is cultivated, test tubes are filled with 5 mL liquid LB medium and supplemented with either crude cell extracts or protein extracts in varying concentrations. The supplemented test tubes were inoculated with a 5% aliquot of the preculture and incubated at 30°C, 140 rpm for 16 h. Then, 1mL is used to measure the OD600 and 4 ml from the cultures grown are extracted with 3 ml of chloroform; 2.5 ml of the chloroform phase is then further extracted with 0.5 ml of 0.2 N HCl, and the OD520 of the aqueous phase is measured.

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Appendix 2

CAPÍTULO DE LIBRO: María José Valera; Federico Laich; Sara S. González; María Jesús Torija; Estibaliz Mateo; Albert Mas.Identificación y tipificación de bacterias acéticas presentes en uvas de las Islas Canarias. Actualizaciones enInvestigación Vitivinícola: XI Congreso Nacional de Investigación Enológica. pp. 185 - 188. (España): El Boletín, 2011. ISBN 978-84-938945-6-6

Identificación y tipificación de bacterias acéticas presentes en uvas de las Islas Canarias

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Resumen

Las Islas Canarias presentan una serie de características ecológicas y peculiaridades climáticas relacionadas con su orografía que les confieren un alto grado de endemismo. El objetivo del estudio fue la identificación y tipificación de bacterias acéticas procedentes de uvas sanas de diferentes viñedos de Lanzarote, Tenerife y La Palma. En los medios de cultivo habitualmente empleados en el laboratorio no fue posible recuperar bacterias directamente de las uvas sanas, por lo que se realizaron microvinificaciones de estas uvas en condiciones estériles y se recuperaron 396 aislamientos de BA. Se identificaron por secuenciación del gen 16S rRNA: *Acetobacter pasteurianus*, *Acetobacter tropicalis*, *Gluconobacter japonicus*, *Gluconacetobacter saccharivorans y Acetobacter malorum/cerevisiae*. Las especies muy relacionadas, como es el caso de las dos últimas, se diferenciaron tras el análisis del espaciador intergénico (ITS) entre los genes 16S-23S rRNA.. Por otro lado, la tipificación de los aislamientos por técnicas moleculares reveló 60 perfiles distintos.

Palabras clave:

Vino, alteración, análisis filogenético, genotipado

1. Introducción

Las bacterias acéticas (BA), además de ser responsables de la producción de vinagre (Gillamon y Mas 2009), son los principales microorganismos que participan en la alteración de algunas bebidas fermentadas, como cerveza y vino. La especie *Gluconobacter oxydans* ha sido considerada tradicionalmente como la principal BA presente en uvas sanas y *Acetobacter aceti* ha sido aislada en uvas dañadas y durante gran parte de la fermentación alcohólica (Joyeux et al., 1984). También *Acetobacter pasteurianus* ha sido relacionada con la alteración de vino en botella y la producción de vinagre (Bartowsky et al., 2003). Estos resultados se han ido modificando sustancialmente con el uso de métodos moleculares describiéndose nuevas especies como por ejemplo *Acetobacter oeni* responsable de la alteración de vinos de Portugal (Silva et al., 2006). El análisis molecular ha permitido un notable incremento del número de géneros y especies, siendo en la actualidad 12 géneros y 58 especies dentro de la Familia *Acetobacteraceae*

Las islas Canarias tienen una larga tradición en viticultura reconociéndose actualmente 10 regiones productoras de vino con Denominación de Origen (D.O.). La mayor parte del vino que se produce en las Islas Canarias procede de Lanzarote, Tenerife y La Palma. Estas tres islas tienen características muy diferentes, por su tamaño, localización geográfica y relieve, afectando éste último a la climatología creando diferentes zonas. El archipiélago canario por el aislamiento entre islas, su edad, y la posición que ocupan hace que tenga un alto grado de endemismo que ha sido estudiado ampliamente en cuanto a su flora y fauna (Kelly et al., 2001). Sin embargo, son pocos los estudios publicados en relación a los microorganismos asociados al vino (González 2007) y ninguno sobre BA.

El objetivo de este estudio fue realizar un análisis en profundidad para identificar y caracterizar la variabilidad genética de la población de BA presente en diferentes variedades de uvas procedentes de viñedos de tres islas de las Islas Canarias: Tenerife, La Palma y Lanzarote.

2. Material y Métodos

2.1 Muestras, aislamiento y condiciones de cultivo.

Durante la vendimia de 2009 se recogieron de manera aséptica uvas sanas de 34 viñedos distintos provenientes de diferentes regiones de Tenerife (18 viñedos), La Palma (ocho viñedos) y Lanzarote (ocho viñedos). Se analizaron diferentes variedades de uva. En Tenerife y La Palma se recogieron Listán Blanco (LB) y Listán Negro (LN), mientras que en Lanzarote, además de éstas dos se recogieron también uvas de la variedad Malvasía (MV).

Debido a que no se obtuvo crecimiento de bacterias a partir de las uvas recién recolectadas, se llevaron a cabo microvinificaciones de los mostos de dichas uvas. Para ello, se tomaron diez racimos de uvas de cada variedad y viñedo y cada uno de ellos se homogeneizó en el Stomacher, para realizar después una mezcla de mostos. De esta mezcla, 200 ml se fermentaron en contenedores separados por duplicado. En total se realizaron 136

microvinificaciones de las que se tomaron muestras al comienzo, mitad y final de fermentación. Estas muestras se sembraron en medio GYC (1% extracto de levadura, 5% glucosa, 1,5% $CaCO_3$, 1,5% agar m/v) y se incubaron a 28ºC en condiciones aerobias. Los aislamientos que produjeron halo en GYC se consideraron posibles BA.

2.2 Tipificación e identificación de BA.

Para realizar el análisis molecular de los aislamientos se extrajo el DNA total por el método CTAB descrito por Ausubel et al. (1992). La tipificación o determinación del perfil genómico de todos los aislamientos se realizó utilizando las técnicas ERIC-PCR (González et al., 2004) y $(GTG)_5$-PCR (De Vuyst et al., 2007). La visualización de los perfiles se llevó a cabo tanto por electroforesis en gel de agarosa como por el bioanalizador Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Alemania). Se realizó un análisis informático de las relaciones filogenéticas con el programa BioNumerics software (Version 6.5, Applied Maths, Sint-Martens – Latem, Bélgica). La diversidad fue calculada por el indice de biodiversidad de Simpson ($1-\Sigma p_i^2$), donde pi es el número de aislamientos del mismo genotipo dividido entre el número total de aislamientos).

La identificación a nivel de especie de los diferentes perfiles obtenidos se llevó a cabo por la técnica RFLP-PCR del gen 16S rRNA (Ruiz et al., 2000) utilizando los enzimas de restricción *Taq*I, y *Bcc*I (Ruiz et al., 2000, Torija et al., 2010) y por la secuenciación de dicho gen. En los casos en que el análisis del gen 16S rRNA no permitió la identificación de los aislamientos, se amplificó y se secuenció la región intergénica (ITS) entre los genes 16S-23S rRNA. Los análisis filogenéticos de las secuencias de los diferentes genotipos se llevaron a cabo utilizando el programa MEGA version 4 (Tamura et al., 2007).

3. Resultados y Conclusiones

La recuperación de BA en uva depende del estado sanitario de éstas (González et al., 2005; Joyeux et al., 1984), describiendose una baja recuperación en uvas sanas (Joyeux et al., 1984; Prieto et al., 2007). En este estudio, no fué posible recuperar BA de las uvas y fue necesario realizar un proceso de microvinificación igual al descrito por Prieto et al. (2007). Tras estas microvinificaciones el total de aislamientos recuperados en placa que formaron halo fue de 513. Estos aislamientos que se consideraron como posibles BA procedían tan solo de 65 microvinificaciones de las 136 que se realizaron.

3.1 Identificación de BA

El análisis del gen 16S rRNA reveló que sólo 396 aislamientos eran BA. El RFLP-PCR 16S rRNA permitió agrupar los aislamientos en *A. pasteurianus* (72 aislamientos/ 396 aislados totales), *Acetobacter tropicalis* (33/396), *A. aceti* (254/396), *G. oxydans* (36/396) y *Gluconacetobacter europaeus* (1/396). La secuenciación del gen 16S rRNA sólo confirmó la identificación de las dos primeras especies e identificó las otras tres como *Acetobacter malorum/cerevisiae, Gluconobacter japonicus* y *Gluconacetobacter saccharivorans*, respectivamente. A pesar de que el análisis del gen 16S rRNA ha sido descrito como una buen método para identificación de BA (Ruiz et al., 2000; Gonzalez et al., 2004), existe un alto grado de homología en esta región (Cleenwerck y De Vos, 2008) que hace imposible la discriminación entre especies muy cercanas filogenéticamente, este es el de caso *A. malorum/cerevisiae*. Para clarificar la identificación de los aislamientos pertenecientes a estas dos especies , se analizó el

ITS 16S-23S rRNA descrito recientemente como una buena herramienta para identificar especies muy cercanas (Gonzalez y Mas, 2011). El árbol filogenético de estas secuencias (Fig. 1) reveló que 175 aislamientos pertenecían *A. malorum* y 79 a *A. cerevisiae*.

Varias de las especies como *A. pasteurianus* (Bartowsky et al., 2003), *A. tropicalis* (Silhavy y Mandl, 2006), *A. cerevisiae* (Prieto et al., 2007) y *Ga. Saccharivorans* (Kato et al., 2011) han sido previamente descritas en uvas y vino. Sin embargo, la especie *G .japonicus* muy cercana a *G. oxydans* y *A. malorum* junto con *A. cerevisiae* cercanas a *A. aceti* al no haber sido descritas previamente en vino hace pensar que identificaciones previas podrían haber correspondido a estas nuevas y recién descritas especies.

3.2 Tipificación y Diversidad de AB

La tipificación de los 396 aislamientos se realizó por ERIC-PCR y por $(GTG)_5$-PCR. El análisis de los perfiles mediante el programa BioNumerics generó dendrogramas parecidos con las dos técnicas sin conseguir, al igual que otros autores (De Vuyst et al., 2007, Fernández-Pérez et al., 2010) agrupar claramente perfiles correspondientes a la misma especie. Combinando ambas técnicas se obtuvieron 60 perfiles diferentes: 25 perfiles de *A. malorum*, 16 de *A. pasteurianus*, 10 de *A. cerevisiae*, cinco de *A. tropicalis*, tres de *G. japonicus* y uno de *Ga. saccharivorans*.

Los dendrogramas no agruparon claramente los perfiles por islas excepto el grupo de aislamientos de *A. malorum* provenientes de Tenerife y La Palma. En estas dos islas esta especie fue la mayoritaria y además, se detecto el genotipo (Am7) de *A. malorum* aislado con mayor frecuencia y el único que apareció en más de una isla. La proximidad de estas dos islas y su abrupta orografía pueden explicar las diferencias encontradas con respecto a Lanzarote que al ser más plana da lugar a una menor variación climática, lo cual podría haber influido en la diversidad de las BA.

La biodiversidad encontrada en las tres islas es alta, siendo en Tenerife la mayor. (Tabla 1). En esta isla se detectó un total de 40 genotipos, y 10 de ellos estaban presentes en más de un viñedo: Am7 se encontró en tres viñedos, Am17 en cuatro, Ac3 en cinco, Ap4 en tres y el resto de los genotipos (Am5, Am8, Am1, Ac2, Ap2, y Ap3) en dos viñedos cada uno. En La Palma, de los 17 genotipos encontrados tres de ellos (Am22, Am24, y Ac8) aparecieron en dos viñedos cada uno. En Lanzarote cada genotipo correspondió a una especie diferente y apareció en un viñedo.

Tabla 1. *Identificación y tipificación de BA de las tres islas*

Isla de origen	Nº aislamientos	Nº genotipos	Especies (nº aislamientos)	Perfil GTG_5/ERIC (nº de aislamientos)	Índice de Biodiversidad de Simpson
Tenerife	225	40	*A.malorum* (122) *A.cerevisiae* (55) *A.pasteurianus* (39) *A.tropicalis* (8) *Ga.saccharivorans* (1)	Am7(25),Am17(25),Am8(15),Am3(10),Am12(10), Am5(8), Am1(7),Am6(4),Am13(4),Am4(2),Am9(2),Am14(1),Am16(2), Am19(2),Am2(1),Am10(1),Am11(1),Am15(1),Am18(1) Ac3(22),Ac5(15),Ac2(15),Ac1(1),Ac6(1),Ac4(1) Ap4(16),Ap2(6),Ap11(6),Ap6(3),Ap3(2),Ap5(1),Ap7(1), Ap1(1),Ap8(1), Ap9(1),Ap10(1) At1(3),At2(3),At3(2) Gs1(1)	0.95
La Palma	110	17	*A.malorum* (60) *A.cerevisiae* (8) *A.pasteurianus* (21) *A.tropicalis* (1) *G.japonicus*(20)	Am7(19),Am25(16),Am21(12),Am24(6),Am23(4), Am22(3) Ac7(3),Ac8(3),Ac9(1),Ac10(1) Ap12(9),Ap15(6), Ap13(5),Ap14(1) At4(1) Gj1(15),Gj2(5)	0.90
Lanzarote	61	4	*A.malorum* (9) *A.pasteurianus* (12) *A.tropicalis* (24) *G.japonicus*(16)	Am26(9) Ap16(12) At5(24) Gj3(16)	0.72

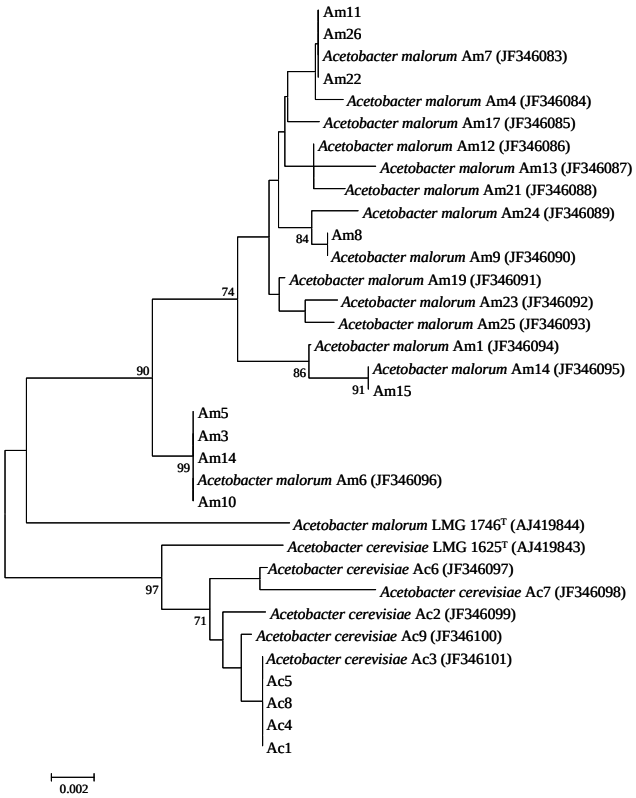

Figura 1. Relaciones filogenéticas de los aislamientos correspondientes a las especies A. malorum y A. cerevisiae detectados en las tres islas.

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