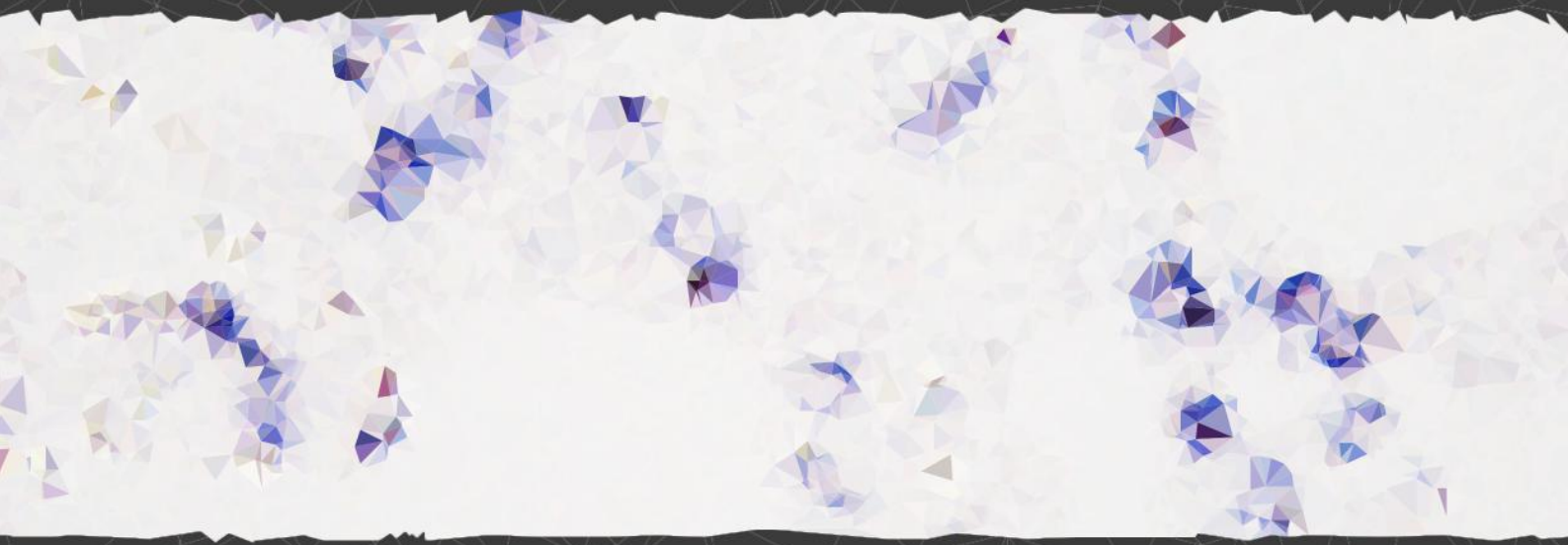
Haemophilus parasuis host-pathogen interactions in the respiratory tract



Bernardo Bello Ortí

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Tesis doctoral presentada por Bernardo Bello Ortí para obtener el grado de Doctor por la Universitat Autònoma de Barcelona (UAB), bajo la dirección de la Dra. Virginia Aragón Fernández. Bernardo Bello Ortí ha cursado los estudios de Doctorado en Microbiología, en el **Departamento de Genética y de Microbiología de la UAB.**

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Bellaterra, 2015

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Certifica:

Que la tesis titulada **"*Haemophilus parasuis* host-pathogen interactions in the respiratory tract",** presentada por Bernardo Bello Ortí para la obtención del título de Doctor, se ha desarrollado bajo mi supervisión en el Centre de Recerca en Sanitat Animal (CReSA) y que el Dr. Francesc Accensi i Alemany, profesor del Departament de Sanitat i d'Anatomia Animals (Facultad de Veterinaria) de la Universitat Autònoma de Barcelona ha actuado como tutor.

Así, autoriza la presentación de la mencionada Tesis Doctoral para su defensa.

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DEDICATION

This thesis is dedicated to the people that contributed to my education, since they had enough patience to teach me and motivated me to find out more.

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SUMMARY

In the veterinary field, Glässer's disease is a common pathogenic process that leads to considerable economic losses. This disease is caused by *Haemophilus parasuis*. Although considerable effort has been focused towards understanding the factors involved in disease outcome, evidences of lack of complete protection of commercial vaccine formulations suggest that more work should be addressed towards understanding this pathogenic process.

To fill this gap in pathogenesis knowledge we developed a series of experiments. It is well known that different *H. parasuis* strains exist, ranging from non-virulent to highly virulent. Particular pathogenic mechanisms are attributed to virulence strains, while non-virulent strains only colonize the upper respiratory tract and are unable to cause disease. It is expected that these different virulence degrees can be appreciated also during the early steps of infection. Using samples from the respiratory tract of piglets infected with two virulent strains (Nagasaki and IT29755) and two non-virulent strains (SW114 and F9), immunohistochemistry and immunofluorescence methods were developed, as well as a double staining targeting *H. parasuis* and macrophage/neutrophil cells. Our results revealed that *H. parasuis* virulent strains were present in nasal, trachea and lung locations. Additional details showed that virulent *H. parasuis* was associated to macrophages and neutrophils in lung, but also to pneumocyte-like cells. Thus, virulent *H. parasuis* was able to attach to respiratory tract epithelia, invade and disseminate into the host. On the contrary, non-virulent strains were barely detected in the respiratory tract.

Biofilm-like patterns were displayed by virulent Nagasaki strain in trachea and this made us question the role of biofilm formation in infection. Since previously published reports indicated that biofilm formation was mainly present in non-virulent strains, we performed additional research in this direction to compare biofilm formation with virulent and non-virulent *H. parasuis* strains. Our results confirmed that the capacity to form biofilm *in vitro* was mainly presented by non-virulent strains. Thus, we sequenced the transcriptome of non-virulent F9 strain under biofilm growth using an *in vitro* model. Results suggested that under biofilm conditions *H. parasuis* showed a low metabolic state, as indicated by the gene expression profile. Some of the genes induced under biofilm conditions were specific of non-

virulent strains, as the filamentous hemagglutinin *fhaB*, which has been associated to biofilm formation in other bacteria.

Additionally, the observation of virulent *H. parasuis* strains in the lung during infection inspired us to sequence the transcriptome of a pathogenic strain in this location. Gene expression was determined after a short *in vivo* infection and after *ex vivo* lung inoculation. Results showed common trends in *H. parasuis* gene expression under *in vivo* and *ex vivo* lung infection, such as reduced metabolism and higher expression of genes involved in nutrient acquisition, which could indicate a survival strategy under these conditions. Genes unique of virulent *H. parasuis* strains coding for outer membrane proteins were also detected during lung infection. These genes would require further characterization as virulent factors and could be also useful to develop new antimicrobials and vaccines. Our results also support the use of lung explants as models for pathogenicity studies of respiratory bacteria.

RESUMEN

En el sector veterinario, la enfermedad de Glässer es un proceso patogénico frecuente que conduce a pérdidas económicas considerables. Esta enfermedad es causada por *Haemophilus parasuis*. Aunque se ha llevado a cabo un esfuerzo importante hacia la comprensión de los factores que intervienen en la evolución de la enfermedad, la falta de completa protección de las vacunas comerciales sugieren que debe dirigirse más trabajo hacia el estudio de este proceso patogénico.

Para aumentar el conocimiento en patogénesis desarrollamos una serie de experimentos. Es bien sabido que existen diferentes cepas de *H. parasuis*, desde no virulentas a altamente virulentas. Ciertos mecanismos patogénicos se atribuyen a la virulencia de algunas cepas, mientras que las cepas no virulentas solamente colonizan el tracto respiratorio superior y no son capaces de causar enfermedad. Estos diferentes grados de virulencia podrían ser apreciados durante los primeros pasos de la infección. De este modo, usando muestras de las vías respiratorias de lechones infectados con dos cepas virulentas (Nagasaki y IT29755) y dos cepas no virulentas (SW114 y F9), se desarrollaron métodos de inmunohistoquímica e inmunofluorescencia, así como una doble tinción de *H. parasuis* y macrófagos/neutrófilos. Nuestros resultados revelaron que las cepas virulentas de *H. parasuis* estaban presentes en cornete nasal, tráquea y pulmón. Detalles adicionales mostraron que las cepas virulentas de *H. parasuis* no solo se asociaron a macrófagos y neutrófilos del pulmón, sino también a células tipo neumocitos. Por lo tanto, las cepas virulentas de *H. parasuis* fueron capaces de adherirse al epitelio de las vías respiratorias, invadir y diseminarse en el huésped. Por el contrario, las cepas no virulentas apenas se detectan en el tracto respiratorio.

La cepa virulenta Nagasaki mostró patrones de biofilm en tráquea, que nos hizo cuestionar el papel de la formación de biofilm en la infección. Dado que la literatura publicada anteriormente indicaba que la formación de biofilm se presentaba principalmente en cepas no virulentas, se realizó una investigación adicional en esta dirección para comparar la formación de biofilm en cepas virulentas y no virulentas de *H. parasuis*. Nuestros resultados confirmaron que la capacidad de formar biofilm *in vitro* se presenta principalmente en cepas no virulentas. Por tanto, se secuenció el transcriptoma de la cepa no virulenta F9 durante su crecimiento en biofilm utilizando un modelo *in vitro*. Los resultados sugieren que bajo

condiciones de biofilm, *H. parasuis* muestra un metabolismo reducido, demostrado por el perfil de expresión génica. Además, algunos de los genes inducidos en condiciones de biofilm eran específicos de las cepas no virulentas, como la hemaglutinina filamentosa *fhaB*, previamente asociada a la formación de biofilm en otras bacterias.

Finalmente, la observación de cepas virulentas de *H. parasuis* en pulmón durante la infección motivó la secuenciación del transcriptoma de una cepa patógena en esta ubicación. Se determinó la expresión génica después de una infección corta *in vivo* y tras la inoculación de pulmón *ex vivo*. Los resultados mostraron tendencias comunes en la expresión génica de *H. parasuis* bajo infección pulmonar *in vivo* y *ex vivo*, como la reducción del metabolismo y la expresión de genes implicados en la adquisición de nutrientes, lo que podría indicar una estrategia de supervivencia en estas condiciones. Durante la infección pulmonar también se detectaron genes únicos de cepas virulentas de *H. parasuis* que codifican para proteínas de membrana externa. Estos genes requerirán una mayor caracterización como factores de virulencia, pudiendo ser también útiles para desarrollar nuevos antibióticos y vacunas. Nuestros resultados también apoyan el uso de explantes de pulmón como modelo para estudios de patogenicidad de otras bacterias respiratorias.

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INTRODUCTION

Relevance of Glässer's disease

In 1910, K. Glässer isolated a bacillus from the lesions of pigs with fibrinous polyserositis. Phenotypic characterization of this bacillus required several years, and it finally was named *Haemophilus parasuis*. The disease caused by this bacterium is characterized by fibrinous polyserositis and arthritis, and it was named after his discoverer. At present, Glässer's disease is common in farms of all countries with pig industry. Since the prevalence of this disease is rising, national and international efforts are directed towards disease control and economic losses prevention [1].

Etiology of Glässer's disease

H. parasuis is a Gram-negative bacterium belonging to the *Pasteurellaceae* family. Molecular methods have been used to further differentiate from related counterparts, such as *Actinobacillus* and *Pasteurella*. Techniques such as 16s rRNA gene sequencing confirmed that *H. parasuis* belongs to a separate species, also revealing that *H. parasuis* species segregate into two distinct clusters [2-4]. Although it is now well established that *H. parasuis* is a different bacterial species than its human homolog *Haemophilus influenzae*, it was not until 1969 when Biberstein and White demonstrated that, as opposed to *H. influenzae, H. parasuis* does not require X factor (hemin) to grow [5].

Diagnosis

Clinical signs are the first indication of disease, followed by the corresponding lesions at necropsy. However, clinical signs and lesions are not pathognomonic of Glässer's disease, and careful diagnosis complemented with laboratory techniques is recommended. *H. parasuis* infection displays similar clinical signs and lesions as other bacterial pathogens such as *Escherichia coli*, *Mycoplasma hyorhinis* and *Streptococus suis*.

To assist Glässer's disease diagnosis, different methods have been developed. Classic methods, such as bacterial isolation using enriched culture media, are common. Alternatively, culture-independent methods can be used. For instance, immunohistochemistry (IHC) and *in situ* hybridization were among the first that were developed [6-8]. While these two have their particular advantages, such as the ability to locate the bacteria in the animal tissues, detection by PCR is faster and more sensitive for routine diagnosis [2, 9-11].

Clinical signs

Clinical outcomes of *H. parasuis* infection range from sudden death to chronic infection. These different outcomes depend on various factors, including pig age, *H. parasuis* strain and acquired maternal immunity. Most frequently, pigs affected are 4- to 8-week-old, but occasionally Glässer's disease can also affect adult naïve pigs, when these are mixed with *H. parasuis* carriers. Peracute disease occurs within the first 48 hours of infection, leading to sudden death without gross typical lesions. On the contrary, acute disease is well characterized by clear clinical signs, which can include high fever, cough, abdominal breathing, swollen joints, lameness and central nervous system signs, such as lateral decubitus, paddling and trembling [8, 12]. Animals that recover from acute disease can be left affected chronically, with rough hair, diminished growth and lameness.

Morbidity and mortality rates are variable and range form 5-10% in conventional farms and up to 75% in naïve animals. Disease prevalence varies in function of stress and concomitant infections, such as the presence of other pathogens commonly associated to impaired immunity. For instance, porcine reproductive and respiratory syndrome (PRRS) virus is commonly found in farms affected by Glässer's disease [13, 14], but this observation has not been replicated in experimental infections. Although it is expected that other bacterial and viral pathogens influence *H. parasuis* colonization outcome, this remains to be studied in detail.

Lesions

Peracute infections lack gross typical lesions, but some cases can present petechial hemorrhages in some tissues [15, 16]. At microscopic level, septicemia-like lesions (such as intravascular coagulation in different tissues including renal glomeruli, liver sinusoids and pulmonary capillaries) and microhemorrhages can be observed [6]. Acute systemic infection is characterized by fibrinous or fibrinopurulent polyserositis, including polyarthritis and

meningitis. The fibrinous exudate can be localized in pleura, pericardium, peritoneum, synovia and meninges [8, 16, 17]. Some animals with acute infection display neurological clinical signs but lack characteristic gross lesions; 80% of these animals present meningitis that can be microscopically detected [18]. Last, chronically affected pigs show fibrous lesions in the implicated serosas.

Laboratory cultivation of H. parasuis

For correct diagnosis of Glässer's disease, confirmation in the laboratory is required. Transportation of the samples to the laboratory needs to be performed under the right conditions; as fast as possible and using Amies medium and refrigeration to maintain bacterial viability since *H. parasuis* is a labile bacterium. *H. parasuis* is a small Gram-negative bacterium that can display different morphologies, ranging from cocobacilli to filamentous forms. Contrary to other *Haemophilus* species, requires NAD but not X factor (hemin) for growth. Cultivation is optimum in enriched chocolate agar, requiring 1-3 days to develop small colonies showing a brown-grey color. Some strains produce capsule after infection [19].

Identification of H. parasuis

An important advantage for diagnostics is the ability to have culture-independent detection techniques. Although these techniques were applied to other bacterial species earlier, it was not until 2001, when Oliveira et al. developed the technique to amplify a fragment of the 16S rRNA of *H. parasuis*, with latter improvements and real-time variants [2, 11]. PCR was a step forward in *H. parasuis* diagnosis, being faster and more specific than traditional microbiological techniques [10, 20]. Other gene-specific PCR to diagnose *H. parasuis* reported the use of genes such as *tbpA* [21], virulence-associated trimeric autotransporters (*vtaA*s), which can be use as an indication of strain virulence [22], putative hemolysin gene operon *hhdBA* [23] and outer membrane protein (OMP) P2 gene [24]. The potential of these PCR methods has been demonstrated by their good community acceptance and the continuous attempts to expand their use for the combined detection with other pathogens [25-27]. Alternatively, another diagnostic method is enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *H. parasuis*. Various *H. parasuis* specific proteins have been tested as antigens for ELISA, such as the oligopeptide permease ABC transporter membrane protein (OppA) [28, 29] and the VtaAs [30]. Last, other diagnostic methods have been reported in the literature, such as immunohistochemistry [6, 7, 31], *in situ* hybridization

[32], nested polymerase chain reaction (PCR) [9] and loop-mediated isothermal amplification (LAMP) of 16S rRNA gene [33], but are not commonly used in routine diagnosis.

Diversity of *H. parasuis* strains

Bacterial genetic diversity is a phenomenon observed for some species, as part of the evolutionary process and fitness adaptation. This process is not always accompanied by enhanced virulence, since the general trend is to evolve towards less virulence, sometimes reaching a symbiotic relationship with the host. In some bacterial species, genotypic diversity can be exploited for diagnostic and population structure analysis purposes. Variability among *H. parasuis* strains is observed both at genotype and phenotype levels. Diverse virulence grades exhibited by this bacterium have to be taken into consideration for diagnostic purposes, since some strains are clearly capable of reproducing disease experimentally while others do not (virulent and non-virulent strains, respectively). Differentiation between virulent and non-virulent strains may be performed at the antigenic and the genetic level. Detection of clinic isolates in a farm allows on-time vaccination for outbreak prevention. To that end, various phenotyping and genotyping methods have been applied to study *H. parasuis* population. The first methods were based on the antigenic properties as defined by agar gel precipitation test (AGPT), using heat-stable antigens and rabbit polyclonal antisera. This serotyping methodology classified the strains in 15 serovars, with a significant number of non-typeable strains [34-37]. A better serotyping technique was developed later, an indirect hemaglutination method, which was able to assign more *H. parasuis* isolates to the 15 serovars previously described [38, 39]. Although these techniques took a step forward *H. parasuis* diversity knowledge, still suffered from serious reproducibility problems, most probably due to the lack of standardization of the antisera and antigenic reagents. Even with these limitations, a consistent result from both methods is that serovars 4 and 5, along with non-typeable isolates, are the most prevalent in different countries [35, 40-42], with the exception of United Kingdom, where serovar 10 is the most abundant [43].

Strain diversity knowledge was further increased thanks to the use of genotyping methods. One of the most important advantages of genotyping *versus* serotyping methods is the ability to type all isolates. Two different types of genotyping methods have been applied to *H. parasuis*: fingerprinting and sequencing methods. Within the fingerprinting methods,

restriction endonuclease pattern (REP) consists on the digestion of the bacterial DNA, whose fragments are then visualized in an electrophoresis agar gel. This technique revealed that more than one strain can be present at the same time in one farm and that the same animal can carry more than one strain [44]. This epidemiological situation constitutes a challenge for diagnosis, since the identification of the specific strain responsible of the clinical outbreak can be hindered by the presence of other non-pathogenic strains. It was also observed that certain strains were only isolated from particular locations, such as systemic and respiratory sites. Another fingerprinting technique, enterobacterial repetitive intergenic consensus (ERIC)-PCR, which consists on the generation of genomic fingerprints using random amplification by PCR, confirmed previous *H. parasuis* REP results, showing a structured and diverse population within this bacterial species [35, 45, 46]. A more advanced genotyping technique based on sequencing of housekeeping genes, multilocus sequence typing (MLST), was developed latter [47]. MLST allows the exchange of genotyping information among distant laboratories, especially through the specific on-line database (http://pubmlst.org/hparasuis) [48]. MLST revealed association of two genotype clusters with the clinical origin of the strains; systemic lesions *versus* nasal isolation [47, 49, 50]. As a result, both ERIC-PCR and MLST are being applied in recent published works, denoting its advantages of speed and cost-efficiency over more modern methods such as whole genome sequencing [22, 51, 52]. Nonetheless, whole genome sequencing has the capability to uncover higher diversity levels. In that way, Howel et al. sequenced a collection of 212 *H. parasuis* isolates revealing various unpredicted diversity characteristics and allowing a better overview of *H. parasuis* genome structure [53]. From that work, it was determined that the pan-genome, i.e. the sum of all different genes present in the isolate collection, comprised 7,431 genes. Of these, 1,049 were found in all isolates and therefore constituted the core genome. Core genome was approximately 0.9 Mb, from the average genome size of 2.2 Mb, and included more than 65,000 single nucleotide polymorphisms (SNPs). Importantly, some SNPs were able to discriminate virulent and non-virulent isolates. On the other side, the accessory genome (those genes found in some but not all strains) comprised 6,300 homology groups, a term that refers to genes expected to encode for proteins with the same function. A considerable number of these homology groups were putative pseudogenes [53]. Similarly, other studies focused on genome comparison of virulent strains, revealing considerable diversity, with a particular gene repertoire for each virulent strain, which could indicate that acquisition of certain genes could be linked to enhanced virulence [54, 55]. All in all, it is expected that

modern and classic techniques will be combined to exploit their particular advantages.

Pathogenesis

When considering infectious diseases one has to take into account that many elements can influence a disease outcome. However, a reductive but nowadays inevitable approach for the study of pathogens is to analyze bacterial virulence assuming constant other variables. This kind of approach is normally carried out using animal infection models with different pathogenic strains, as have been successfully used to study Glässer's disease. Simpler experimental procedure concepts are the *in vitro* models, which have been usefully applied to the study of bacterial virulence factors and pathogenic mechanisms. Both approaches, *in vivo* and *in vitro* models, have been extensively applied for *H. parasuis*, as well as for other bacterial pathogens, yet the classification of *H. parasuis* isolates as virulent or non-virulent is complex, since a large number of isolates have an intermediate virulence grade. Bacterial virulence is flexible, and can be influenced by acquisition of novel virulence factors or even by subtle genetic modifications such as SNPs or gene methylations, commonly known as methylome when taking all methylations of a bacterial cell into account [56-58]. Additionally, the number of different virulence factors as well as variations in the number of some virulence factor in the same strain could enhance virulence, thus denoting to which point bacterial virulence can be variable.

Designing effective antibacterial therapies and vaccines require deep knowledge about bacterial pathogenesis. This approximation has been successfully applied to major bacterial pathogens, including *H. parasuis*. Among the different strategies that bacteria exhibit for host survival, are the offensive virulence factors, such as the ones dedicated for adherence and invasion of host cells structures. Another offensive virulence mechanisms are secretion systems, including toxins, able to modify and damage host membranes. On the other side, defensive virulence factors are dedicated towards immune system evasion, with sophisticated functions that includes phagocytosis resistance, anti-proteolysis activities, host cellular metabolism alterations or phase variation of proteins recognized by the immune system. Other important defensive mechanisms are immunoglobulin (Ig) and complement protease activities, as well as proteins that stabilize bacterial stress during infection. Additional non-classical virulence factors are the ones dedicated to capture important bacterial nutrients, such

as iron or magnesium uptake systems, which are essential for bacterial survival. Besides, transcriptional activation or repression of a virulence factor is a key system for the opportune use of each virulence mechanism at specific infection steps. Previous works have evidenced that pathogenesis knowledge leads to novel therapies [59-61]. For instance, targeting essential virulence offensive mechanisms such as adherence, invasion or secretion systems could lead to efficient prevention of bacterial entry into the host. Moreover, targeting defensive virulence mechanisms such as phagocytosis resistance, or intracellular survival metabolic adaptations could lead to novel effective therapies. A useful tool for the study of potential virulence factors is the well-accepted virulence factors online databases released in the last years, such as the Virulence Factor DataBase (VFDB) or the Pathosystems Resource Integration Center (PATRIC), which allow comparative genomic analysis with curated and updated data [62, 63]. Last, an important advantage that can be extracted from pathogenesis knowledge to therapy approach is that therapies can be directed against virulent strains.

Virulence mechanisms of H. parasuis

It is expected that behind the clinical signs and lesions of Glassër's disease, *H. parasuis* is using various sophisticated pathogenic mechanisms. Pathogenic mechanisms can be divided according to the host barriers that the bacteria have to overcome during the infection process. One of the first steps in infection is bacterial entry into the host, which involves mucosal colonization. To be able to colonize and attach to the respiratory tract mucosa, adhesive capacities are needed. Although the capacity of *H. parasuis* to adhere to eukaryotic cells has been reported using *in vitro* assays [64, 65], these results need further *in vivo* validation. Other mechanism that has been reported to possibly enhance nasal colonization is the ability to form biofilms. Although biofilm formation capabilities are usually associated with virulent isolates in other bacterial species, it seems not to be the case for *H. parasuis*. To get insights into *H. parasuis* biofilm formation as well as its association with virulent or non-virulent isolates, this particular multicellular behavior was investigated by Jin et al, who reported a relation between biofilm formation and virulence, being non-virulent isolates able to form more robust biofilms than virulent ones. Also, non-virulent strains maintained their biofilm phenotype after agar or broth passage, while virulent strains did not show this capacity, even after experimental infection, which is compatible with a phenotype switch in *H. parasuis* from a "colonizer" to an "invasive" state [66] . Although these observations showed a clear pattern for virulent and non-virulent strains, *in vivo* biofilm behavior remains to be clarified.

More recently, Zhang et al. reported that antimicrobial capacity was more frequent in biofilm-formers, in accordance with similar results found in other bacterial species [67, 68]. However, a relationship between biofilm and virulence or serovar was not found [69]. Another essential step to a successful infection is to surpass respiratory tract immune defenses, such as the host IgA. *H. parasuis* is able to degrade purified IgA, yet this protease activity has not been associated to any particular gene since *H. parasuis* lacks homologs of the classic IgA protease genes of *H. influenzae* [70]. After the initial nasal colonization, *H. parasuis* could reach the lung, where bacteria would be required to confront and resist phagocytosis by macrophages. Virulent *H. parasuis* strains are resistant to phagocytosis by alveolar macrophages [71]. Virulent *H. parasuis* strains delay phagocytosis *in vitro* [72], as well as alveolar macrophage activation after pig infection, which is associated to disease progression [73]. Next, when reaching the blood, serum-resistance is a requirement for an effective systemic pathogen. Some *H. parasuis* isolates have this capacity, being normally associated to systemic isolates rather than nasal ones [74]. Systemic invasion by *H. parasuis* is characterized by a significant inflammation, which produces the typical lesions of Glässer's disease. When the systemic infection is established, *H. parasuis* is able to reach other pig essential organs, such as brain [66]. To cause meningitis, *H. parasuis* has to be able to traverse the blood-brain barrier (BBB). *H. parasuis* capacity to interact with and to invade porcine brain microvascular endothelial cells (PBMEC) *in vitro* has been reported, but the protein factors involved in this process remain to be identified [75, 76]. Further characterization of this interaction by Bouchet et al. pointed out that lipooligosaccharide (LOS) plays a partial role in this process, as well as in induction of phenotype changes in PBMECs, such as caspase-3 dependent apoptosis, as well as release of key inflammatory mediators, including IL-8 and IL-6 [77]. Further elucidation of *H. parasuis* ability to interact with endothelial cells and invade adjacent tissues were the objective of a latter study by Frandoloso et al. (2013), who concluded that virulent *H. parasuis* strains exhibit higher adhesion and invasion capacity to porcine aortic endothelial cells (AOC-45 cell line) [65]. Although validation of these pathogenic mechanisms in *H. parasuis* needs to be done in pig infection, they are in agreement with the characteristic clinical signs and lesions of Glässer's disease.

Biofilms as an alternative mode of growth

Before J. Shapiro described 25 years ago the possibility of bacteria to grow as multicellular organisms, bacteria were considered only as isolated and independent cells [78]. Although

there were some exceptions to this rule, such as development stages of *Streptomyces spp*. [79, 80], a wider extension of these multicellular behaviors was not possible until considerable advances in microbiological techniques were achieved. Today, multicellular formations are widely recognized for bacteria, up to the point that it is estimated that one of this manifestation, the biofilms, is the predominant growing style in the majority of bacterial species [78, 81]. Research in this direction has allowed understanding that multicellular patterns are wide, in accordance with the vast genotypic variation that can be found among bacterial species; e.g., the number of multicellular stages, cells of same bacterial species cohabitating at the same time but with particular gene expression patterns, the factors involved in the transition from one phenotype into another, as well as the extracellular components [82].

Some well recognized advantages of growth as clusters or biofilms are protection against predation or stress conditions, as well as improved efficient nutrient usage and acquisition. A good example is the bimodal growth displayed by *Mycobacterium tuberculosis*. This species, when proliferating inside macrophages during infection, changes from single cells to filaments, which is associated to increased survival [83]. This behavior is normally accompanied by arrest of cellular division and a reduced metabolic state, which has also important implications during antibiotic treatment. In fact, an important characteristic associated with biofilm growth is the enhanced ability to tolerate antimicrobial agents. This enhanced antimicrobial tolerance has been linked to the surrounding polymeric matrix that avoids antibiotic entry as well as to the lower metabolic state that biofilm cells display in comparison to planktonic counterparts. The polymeric matrix formed by extracellular material explains the advantages of biofilms growth. This matrix includes 97% water, but can also contain abundant polysaccharides, nucleic acids, proteins, lipids, glycolipids and cations. The composition of the matrix varies among species and growing conditions [84, 85]. For instance, pneumococcal biofilm formation displays different robustness when cultured on plastic surfaces or on a biological layer of epithelial cells [86]. In addition to the above described biofilm characteristics, biofilms have been also associated with different attributes than planktonic cells, such as isolation from nasopharynx, enhanced competence, lower induction of immune response and lower metabolism [87]. To enhance attachment to epithelial cells, biofilms down-regulate capsule, which makes them able to better display adhesins. Although this may allow opsonophagocytosis, it is counteracted by uncovered

virulence factors able to inhibit complement deposition and activation. These phenomena were observed in *S. pneumoniae*, a frequent colonizer of human nasopharynx and one of the best studied biofilms, but may be different in other bacterial species [87]. On the contrary, planktonic bacteria are normally associated with an invasive phenotype, being more similar to bacteria found in blood, lung or brain samples of infected animal [87, 88]. Last, a particular and still controversial feature is the association of biofilms with the virulence of bacterial isolates. Although biofilm formation has been historically associated with virulence, reports of non-virulent biofilm-forming bacteria are available, as it is the case for *H. parasuis* [66]. Biofilm formation has been reported to be an intermediary biological state during host colonization, enhancing a successful infection and disease by *Streptococcus pneumoniae* [88]. Thus, association of biofilm formation with virulence phenotypes varies among bacterial species.

In contrast to the different roles that biofilm plays in each bacterial pathogen, a common theme for all biofilm-forming bacteria is the biofilm 'life cycle'. The dispersed cells, commonly known as planktonic cells, migrate to new surfaces where they start the biofilm formation structure. These first attached cells undergo gene expression changes that result in multiplication and formation of microcolonies, which constitute the primordial phase of mature biofilms. Bacterial replication is coupled with secretion of the extracellular components of the matrix. The mature biofilm includes aggregated cells (that can be alive of dead), exopolysaccharides, extracellular DNA (eDNA) and proteins, and degradation products. In fact, inside the mature biofilm the composition varies, influenced by nutrients and oxygen availability, among others. The transition of a dispersed cell to a biofilm forming bacteria is a highly regulated process that involves major transcriptional changes, being directly seen both at RNA and protein level and evidenced by increased use of exopolysaccharides and fimbria in biofilms, leaving for the dispersal phase other mechanism, such as motility and chemotaxis [89].

Immune responses against H. parasuis

H. parasuis alters host homeostasis at tissue and cellular levels, producing the characteristic clinical signs and lesions. In order to study the molecular mechanisms behind them, various works have focused on immune response after *H. parasuis* experimental infections. Although the role of antibodies is key to prevent infection (see section "Glassër's disease as a

multifactorial process and strategies for disease control"), much of the work has been focused towards understanding the innate immune responses generated against *H. parasuis*.

Modern approximations using microarrays revealed that host genes affected at porcine spleen tissue 7 days after *H. parasuis* infection belonged mainly to an enhanced immune response, including pathways related to inflammasome, adhesion, acute-phase proteins and complement and antigen processing and presentation [90]. A similar study detected 428 genes in host porcine alveolar macrophages, whose expression was altered after 6 days of *H. parasuis* infection. These genes were mainly associated to inflammatory response, immune response, microtubule polymerization, transcript regulation and signal transduction [91]. Other molecular alterations of the immune system by *H. parasuis* have been studied. For instance, Caveolin-1, a protein located in cell membranes, including immune cells, is down-regulated in the brain after *H. parasuis* infection [92]. Since decreased caveolin-1 expression is linked to proinflammatory outcome in murine alveolar and peritoneal macrophages [93], and decreased Caveolin-1 expression is linked to fibrosis and enhanced inflammation [94], its down-regulation could facilitate *H. parasuis* traverse of the blood–brain barrier due to increased permeability, thus facilitating development of meningitis and inflammatory processes. Other participants in the molecular pathways behind the inflammatory response are induced by *H. parasuis*, such as NF-κβ and MAP kinase signaling pathways [95], mediated by toll-like receptors [96]. Finally, the inflammatory reaction mediator S100 calcium-binding protein, variants A4, A6, A8, A9 and A12 were also induced after *H. parasuis* infection [91, 97, 98].

While an adequate and effective immune response is key to counteract infection, exaggerated immune response may lead to excessive inflammation (characteristic of Glässer's disease), as it has been already demonstrated for other bacterial pathogens [99]. In that way, cytokine induction by *H. parasuis* was shown to be different in surviving *versus* severely sick animals. The latter showed augmented IL-1α, a pro-inflammatory cytokine, in spleen, lymph nodes and brain, while animals surviving a *H. parasuis* infection showed higher expression levels of IL-4, IL-10, TNF-α and IFN-γ in these tissues, suggesting the importance of a modulated immune response, as revealed by the anti-inflammatory cytokine profile of IL-4 and IL-10 [100]. In agreement, another work found overexpression of proinflammatory molecules in diseased animals, unable to control disease, such as CCL-2, CXCL-8, IL-1 α, IL-1 β, and IL-6 in tissues including brain, lungs and spleen [101]. Furthermore, high IL-8 and soluble CD163

were observed in sera of sick animals infected with virulent *H. parasuis* strains at 3-4 days after infection [73].

On the other hand, cellular responses give insights at higher level, being complementary to molecular studies. In that regard, Costa-Hurtado et al., studied the relation between the virulence of the infecting *H. parasuis* strain and activation of porcine alveolar macrophages after *in vivo* infection [73]. Interestingly, virulent strains delayed macrophage activation, which could be linked to the previously reported delayed phagocytosis by *H. parasuis* VtaA8 and VtaA9 [72]. Also, induced IL-8 response in sera by the virulent strains matched the enhanced inflammatory response pattern previously found at porcine spleen tissue using microarrays [90]. In a similar fashion, other *in vivo* work focused on the characterization of blood cellular immune response after *H. parasuis* challenge. Martín de la Fuente et al. (2009) found that *H. parasuis* infection induced subtle cell population changes, only increasing the proportion of granulocytes (SWC3+) and B cells (αIgM+), as well as reduction in CD3+ cells, suggesting an increased in inflammatory cell recruitment [102]. In the same line, Frandoloso et al. (2012) reported altered immune cell population changes after *H. parasuis* challenge, with reduction of CD3+γδTCR+ and SLAIIDR+ cells, while increased cellular immune responses mediated by CD172α+CD163+ cells, CD3ε+CD8α+CD8β+ and B cells were observed [103].

H. parasuis virulence factors

A crucial element for successful infection is the presence of virulence factors in the infecting *H. parasuis* strain. The search for bacterial virulence factors has been the scope of multiple works, since they could be useful for antimicrobial and vaccine development. Although the lack of genetic manipulation techniques for *H. parasuis* has limited their identification, genomics and gene expression techniques have been useful as an alternative strategy. Before *H. parasuis* genome sequences were available, putative virulence factor identifications were performed with traditional biochemistry techniques, such as immunoblotting with monoclonal antibodies (mAb) specific to classic virulence factors, such as the P5 outer membrane protein [104].

One of the first *H. parasuis* factors to be characterized was the *tonB* region, which is involved in iron acquisition. Although it can be argued that iron-acquisition is not a virulence mechanism *per se*, it is essential for infection, since it is involved in survival within the host.

Del Rio et al. (2005) identified this genetic region using PCR, with further detection of its up-regulation under iron restriction conditions using immunoblotting [105]. The same strategy was applied later to characterize the siderophore-mediated iron uptake in *H. parasuis*, finding that the genes located in the ferric hydroxamate uptake (*fhu*) region were constitutively expressed in *H. parasuis*, and not regulated by iron concentration [106]. In a similar manner, the transferrin-binding proteins A and B (*tbpA* and *tbpB*), part of the transport system involved in iron acquisition in *H. parasuis*, were also identified and demonstrated to be antigenic, conferring protection when used as vaccine [107-109].

Virulence factors are not restricted to proteins. An important *H. parasuis* virulence factor is the lipooligosaccharide (LOS), involved in adherence to host cells [110]. Additional virulence is given by LOS modification with sialic acid, an acidic sugar that protects against complement-mediated killing in other pathogens [111]. In that regard, Martinez-Moliner et al. (2012) studied the presence of *lsgB* (lipopolysaccharide sialyltransferase) in a set of virulent and non-virulent isolates, revealing the presence of this gene mainly in virulent isolates. The purification of LOS revealed presence of sialic acid in the LOS of virulent Nagasaki strain, thus adding additional evidence of its role in virulence [112]. Another part of bacterial cell surface is the capsule, commonly involved in phagocytosis resistance [113]. In *H. parasuis*, although the majority of bacterial factors that prevent phagocytosis remain to be defined, the role of the capsule in phagocytosis, as well as in serum resistance, has been suggested. There is no direct demonstration between capsule formation and phagocytosis resistance, but capsule is induced after incubation with host porcine alveolar macrophages, thus suggesting a role in phagocytosis resistance [71]. Also, a *capD* deletion mutant (Δ*capD*) showed limited serum resistance, thus denoting the important role of the capsule in this process [114].

Among the characterized *H. parasuis* virulence factors are the outer membrane proteins belonging to the porin family. This protein family has been well studied in other bacterial species, and is involved in numerous pathogenic mechanisms, including central nervous system adhesion and invasion, mucosal respiratory adhesion, evasion of host defenses, as well as innate and adaptive immunity activation, being also protective when included in vaccine formulations [115]. Tadjine et al. (2004) reported the first evidences of OmpA (or P5) presence and its immunogenicity in *H. parasuis*, elegantly demonstrated using a mAb library combined with immune and proteolitic techniques, and terminal sequencing [110]. P5 was later identified and characterized in more detail via immunoblotting with mAbs and N-

terminal sequencing [104]. Another porin protein, the P2, is involved in porcine alveolar cell adhesion as well as in serum complement resistance through inhibition of the activation of the classic pathway [116, 117]. P2 also induces proinflammatory cytokine mRNA expression [118]. Even though *H. parasuis* porin proteins are involved in pathogenesis, their direct association with virulence is not clear since both P2 and P5 are present in all *H. parasuis* isolates, although with considerable heterogeneity in amino acid sequence, possibly adding additional pathogenesis functionality [119].

A common strategy to identify bacterial virulence factors for further characterization is to compare virulent and non-virulent strains, thus obtaining genes only present in virulent *H. parasuis* isolates. Various works in *H. parasuis* field followed this approximation, with different methodologies, such as genome subtraction [120], representational difference analysis [121-123] or suppression subtractive hybridization [124, 125]. With the advent of high throughput nucleotide sequencing, comparative genomics strategies were applied to *H. parasuis* [126-130]. For instance, comparative genomic studies allowed the identification of 13 *vtaAs* in the Nagasaki strain, which were differentially present in virulent and non-virulent strains [131]. Latter research allowed validation of their antigenicity, *in vivo* expression at protein level as well as their protective capacity after *H. parasuis* challenge [30, 132, 133]. In addition, their differential presence in virulent and non-virulent strains allowed the design of a specific PCR [22]. Interestingly, two VtaAs, VtaA8 and 9, are involved in phagocytosis resistance [72]. Other outer membrane autotransporters, the monomeric autotransporters, were also identified by genome sequencing analysis, but their capacity to confer immunological protection needs to be further explored, as well as its role in virulence [134]. More recently, an additional set of virulence factors was identified by genome comparison of five serovar 4 *H. parasuis* isolates with different clinical background [135]. Finally, the recent genomic analysis reported by Howel et al. (2014) using a large number of genome sequences, 212, revealed a set of over 20 virulence factors, including four adhesin genes, three cytolethal distending toxins genes, *fhu*, glycerol uptake facilitator, N-acetylmuramoyl-L-alanine amidase, serine protease and TonB-dependent receptor plug domain protein [53].

Proteomics is another approximation to study the presence of certain virulence factors, and was successfully used for *H. parasuis* combined with immunodetection [136, 137]. Comparative proteomics studies in *H. parasuis* used a low number of isolates, which limits the implication in virulence [138, 139]. Taken together, these studies identified some putative

virulence factors, such as the outer membrane proteins Wza, OmpP2, OmpP5, D15, PalA and the putative adhesin AidA protein, virulence-associated proteins involved in secretion systems (VtaAs), as well as proteins participating in iron-uptake systems such as tonB-dependent siderophore receptor, periplasmic serine protease and putrescine/spermidine ABC transporter substrate-binding protein and iron ABC transporter substrate-binding protein.

Overall, a general observation that can be extracted from the results obtained using these strategies is that although low scale genome/proteome comparisons of virulent and non-virulent isolates identified some genes/proteins present only in the virulent isolates tested, the majority of those molecules were not associated with virulence when tested in a larger set of *H. parasuis* isolates. Thus, the most reliable and statistically significant virulence factors up to day seems to be the ones identified by Howel et al. using large scale genome wide association methods [53].

Gene expression is another powerful technique to identify virulence factors, since bacterial expression of certain gene set at a specific infection step is a clear indication of their role in pathogenesis. The first report on differential expression of genes under growth conditions mimicking those found in the upper respiratory tract identified 7 gene fragments by differential display RT-PCR [140]. Later, the use of selective capture of transcribed sequences (SCOTS) technique allowed the identification of 38 putative virulence factors by their up-regulation in necrotic lung after *H. parasuis* infection [141]. Also using SCOTS, 36 genes involved in survival under iron-restricted conditions were identified [142]. Additional virulence factors were retrieved from similar approximations, such as Representational Difference Analysis (RDA) [143], with some of the up-regulated genes uniquely expressed by virulent isolates [122]. Also mimicking *in vivo* growing conditions, but with microarray technology, 75 up-regulated genes were identified, mainly involved in iron transport, sugar derivatives, DNA metabolism and proteins of unknown function [144].

One of the most widely used techniques to validate virulence factors is production of defined mutants by genetic manipulation. This technique combined with downstream pathogenesis assays, such as adhesion, serum resistance or pathogenicity after experimental infection is a powerful technique to associate a particular pathogenic mechanism to a certain bacterial gene. Even though *H. parasuis* has been shown to be mainly refractory to genetic manipulation, some techniques have been reported, such as electrotransformation with a shuttle vector [145], natural transformation [146, 147] or Tn5-based random mutagenesis method [148].

Product of these studies has been the elucidation of the role of some genes, such as *ompP2,* cytolethal distending toxin *cdt*, gal genes *galU* and *galE*, *capD* (which encodes a polysaccharide biosynthesis protein), and the LOS core biosynthesis *rfaE*, in pathogenesis mechanisms such as adhesion and invasion of eukaryotic cells, serum and complement resistance or biofilm formation. Interestingly, deletion of *ompP2*, enhanced *in vitro* adherence and invasion due to the overexpression of other three proteins: RfaD, ThyA and Mip [114, 149-153]. Last but not least, genome sequencing of SH0165 allowed identification of two copies of cytolethal distending toxin (CDT), being further characterized, showing toxin activity *in vitro*, as revealed by their ability to induce G_2/M cell cycle arrest [154].

Glassër's disease as a multifactorial process and strategies for disease control

Infectious diseases have been traditionally view from a host or pathogen perspectives, having sometimes a reductive approximation to disease causation. More recently, modern works are giving novel insights about new factors involved in the disease process at the host-pathogen interface and the interaction with other microorganisms, including both bacteria and viruses. A good example is *Bordetella bronchiseptica,* which is able to alter *H. parasuis* bacterial-host outcome, increasing nasal colonization and pneumonia [155]. Other example is *A. pleuropneumoniae*, since attenuated *A. pleuropneumoniae* serovar 1 live vaccine protects against lethal *H. parasuis* infection [156]. *Mycoplasma hyorhinis* is another pathogen that possibly influences Glässer's disease outcome, since this *Mycoplasma* species was detected together with *H. parasuis* from polyserositis lesions by PCR [157]. Multiple pathogens can be isolated from the same animal, including *H. parasuis* [158-162], being *H. parasuis* frequently detected in pigs with polyserositis and carrying other pathogens such as *Mycoplasma hyorhinis* or porcine reproductive and respiratory syndrome virus (PRRSV) [14]. The role of porcine reproductive and respiratory syndrome virus (PRRSV) in Glässer's disease outcome has been suggested [163-165] and viceversa [166]. PRRSV is determinant for certain swine infections disease occurrence, since secondary infections with other bacterial pathogens, such as *H. parasuis,* are common [167]. PRRSV also alters alveolar macrophages, augmenting the number of phagosomes and phagolysosomes [168]. In addition, other pathogens can affect *H. parasuis* infections. Using a model of porcine bone marrow dendritic cells (poBMDC), Mussá T. et al. observed that levels of IL-1β, TNF-α, IL-6, IL-12 and IL-10 were different between

cells coinfected with swine influenza virus (SwIV) and *H. parasuis* compared with SwIV infection alone [169]. Last, positive relation between pseudorabies virus (PRV) and *H. parasuis* has also been reported [170].

Besides, influence of host genetic background to control *H. parasuis* infection was the objective of an original work focused on the differentially expressed genes (DEGs) in the lungs of animals reported to be resistant or susceptible to *H. parasuis*, revealing specific pathways to each group of animals, demystifying pathogen-centric perspectives as the only determinant of a Glässer's disease outcome [171]. Another work followed similar idea, reporting that certain polymorphisms in genes involved in the genetic regulation of immune responses in pigs are associated with the risk of infection of PRRSV and *H. parasuis*. [172]. Further, the importance of swine gene polymorphisms in Alpha-(1,2)-fucosyltransferase (FUT1), a gene involved in addition of specific blood group antigens on erythrocytes and epithelial cell surfaces, determines *H. parasuis* colonization [173], as it was already demonstrated for Enterotoxigenic (ETEC) and Verotoxigenic (VTEC) *Escherichia coli* [174].

Additional evidences suggest that presence of *H. parasuis* is not enough for disease causation. Although differences in virulence have been demonstrated for *H. parasuis* strains, other factors influence Glässer's disease development. For instance, concomitant infections, genetic predisposition, antibiotic treatment and maternal immunity, among others, affect infection outcome. In that sense, *H. parasuis* may be present in a pig without showing symptoms, starting the disease when host defenses get impaired. One of the most studied elements that increase Glässer's disease susceptibility, as well as other opportunistic infections, is management. As a starting point for a efficient swine production, an adequate facility is a key point in pig health, including indoor air quality (air temperature, humidity level, ventilation,..), animal density, manure removal and handling, feed and water composition, and the overall farm structural design and construction [1].

The role of maternal antibodies is crucial to prevent a disease outcome. In that regard, Blanco et al. showed that inoculation of sow-reared piglets with virulent *H. parasuis* were protected by the maternal immunity, contrary to colostrum-deprived piglets [175]. The protective effect of sow vaccination was further demonstrated, since sow vaccination with bacterins increased specific IgG titers, slowed colonization and reduced the number of *H. parasuis* colonizing strains in the piglets [176]. Additionally, piglet vaccination can guarantee the presence of specific antibodies. A direct demonstration of the importance of antibodies in Glässer's

disease prevention was given by an antibody transfer experiment [177] and by the effect of antibody opsonization in phagocytosis susceptibility [68]. Therefore, vaccination can constitute an efficient tool for disease control.

Today, vaccine development is viewed in a refined manner. In that way, once *H. parasuis* genome sequences started to be available, reverse vaccinology strategy was applied to characterize a novel set of outer membrane proteins. Expression and immunogenicity analysis of a set of outer membrane proteins was performed, including three ABC-transporters (OppA, YfeA and PlpA) and 1 curli protein assembly (CsgG), demonstrated to be strong immunogens [178]. Similarly, extracellular serine protease-like protein EspP2 [179], 6-phosphogluconate-dehydrogenase (6PGD), which is involved in adhesion [180], rGAPDH, rOapA, and rHPS-0675 were proposed as vaccine candidates due to their antigenicity and protection capacity [181]. Other subunit vaccines derived from reverse vaccinology were the VtaAs [30, 132, 133] and the outer membrane proteins PalA, P2, D15 and HPS_06257, shown to be protective immunogens [182].

Since development of Glässer's disease is influenced by bacterial virulence and numerous environmental factors that are not easily manageable, epidemiology and vaccines are used as preventive strategies. As opposed to vaccines, antibiotics are used as therapeutic tool against ongoing infections. Although antibiotics are still being used as preventive agents in many countries, the increasing antibiotic resistance leaves vaccination as the best alternative. The main problem associated with antibiotic use is the fast emergence of antibiotic resistance. The probable explanation for this is that antibiotics have only one target while vaccines elicit antibodies against multiple epitopes. Although some vaccine resistance have been reported, pathogens would have to alter all the implicated antigens to become resistant [183]. In veterinary medicine, the number of vaccines has been growing while antibiotic resistance increased. This scenario is also similar in Glässer's disease treatment, where various works reported antimicrobial resistance [184-186], as well as some molecular mechanisms behind them [187-189]. This has been the main reason for vaccine development against Glässer's disease, where a long way has been walked since the first papers were released with evidences of vaccine protection [190]. Subunit vaccine and bacterines have been successfully tested, revealing that both innate and adaptive immune responses are involved in protective responses [103, 133, 191-193].

HYPOTHESIS AND OBJECTIVES

Numerous studies have reported that *H. parasuis* strains display a range of virulence degree; observed in experimental infections as well as under *in vitro* pathogenesis assays. Virulent strains isolated from systemic locations display different pathogenic characteristics, such as phagocytosis and serum resistance. On the contrary, non-virulent strains are normally isolated from the nasopharynx and lack virulence phenotypes associated with virulent strains [194]. These observations lead to the hypothesis that particular colonizing and infectious strategies could be associated with virulent and non-virulent isolates. Therefore, the general objective of this thesis was to analyze differences between virulent and non-virulent strains during infection, especially during the first phases of infection of the respiratory tract. For that reason, the first specific objective of this thesis was to determine different patterns in colonization/infection of the respiratory tract by strains with different virulence potential.

A recurrent theme in the literature is the association of biofilm formation with virulent bacterial isolates and antibiotic resistance. Observations by Jin et al. point to biofilm having an alternative role in *H. parasuis*, since this characteristic is mainly present in non-virulent *H. parasuis* isolates [66]. These observations suggest that non-virulent *H. parasuis* strains could display this phenotype, possibly associated with enhanced colonization capabilities. Since there is still a gap regarding the regulatory networks that participate in the conversion between free growing style and biofilm formation, additional research was needed. Therefore, the second specific objective of this thesis was to compare the gene expression at transcriptomic level to identify the molecular adaptations required to change between these two growing styles.

Regarding Glässer's disease control, vaccination is reported as an effective strategy. However, vaccines are commonly based on bacterines, with lack of protection among different serovars. In that sense, improvement of vaccine formulations is desired. In that way, the third specific objective of this thesis was to identify *H. parasuis* virulence factors expressed during lung infection with the final goal of identifying essential bacterial factors at this early stage of infection.

RESULTS

1. Detection of *Haemophilus parasuis* in the host respiratory tract during infection

Time course *Haemophilus parasuis* infection reveals pathological differences between virulent and non-virulent strains in the respiratory tract

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Abstract

Haemophilus parasuis is a common inhabitant of the upper respiratory tract of pigs and the etiological agent of Glässer's disease. However, the host-pathogen interaction remains to be well understood. In this work, 33 colostrum-deprived pigs were divided in 4 groups and each group was inoculated intranasally with a different *H. parasuis* strain (non-virulent strains SW114 and F9, and virulent strains Nagasaki and IT29755). Animals were necropsied at different times in order to determine the location of the bacteria in the respiratory tract of the host during infection. An immunohistochemistry method was developed to detect *H. parasuis* in nasal turbinates, trachea and lung. Also, the co-localization of *H. parasuis* with macrophages or neutrophils was examined by double immunohistochemistry and double immunofluorescence. Virulent strains showed a biofilm-like growth in nasal turbinates and trachea and were found easily in lung. Some virulent bacteria were detected in association with macrophages and neutrophils, but also inside pneumocyte-like cells. On the other hand, non-virulent strains were seldom detected in nasal turbinates and trachea, where they showed a microcolony pattern. Non-virulent strains were essentially not detected in lung. In conclusion, this work presents data showing differential localization of *H. parasuis* bacteria depending on their virulence. Interestingly, the intracellular location of virulent *H. parasuis* bacteria in non-phagocytic cells in lung could allow the persistence of the bacteria and constitute a virulence mechanism.

Introduction

H. parasuis is an early colonizer of the upper respiratory tract of pigs, but also the causative agent of Glässer's disease, a systemic disease characterized by fibrinous polyserositis and polyarthritis (Aragon et al., 2012). Strains of *H. parasuis* are heterogeneous and include virulent and non-virulent strains. *H. parasuis* is an important swine pathogen but its host-pathogen interactions remain to be well understood.

Since *H. parasuis* is a colonizer of the upper respiratory tract, it is common to find non-virulent strains in this location, but virulent strains can also be found. However, it is not known if the colonization of the respiratory mucosa by strains with different degree of virulence is identical or presents differential characteristics. After experimental inoculation, *H. parasuis* can be easily isolated from the nasal cavity of pigs that remain healthy but it is difficult to isolate from the nose of those animals that develop disease (Amano et al., 1994; Vahle et al., 1995; Aragon et al., 2010). However, in infections with virulent strains, *H. parasuis* can be detected in nasal turbinates with infiltrated intraepithelial and mucosal neutrophils, in lung alveoli within the cytoplasm of macrophage-like cells, and in brain tissue (Amano et al., 1994; Segales et al., 1997; Vahle et al., 1997). In disease outbreaks, colonization of the respiratory tract constitutes an initial step for subsequent spreading to internal organs and systemic disease. From the upper respiratory tract, *H. parasuis* proceeds to the lung (Vahle et al., 1997), where it encounters a line of defence constituted by alveolar macrophages. It is known that virulent strains are resistant to macrophage phagocytosis *in vitro* (Olvera et al., 2009), but the interaction between *H. parasuis* and macrophages or other cells in the lung is not well determined. Thus, the objective of the present study was to explore how *H. parasuis* strains of different virulence interact with the respiratory tract of pigs during infection.

Materials And Methods

Animal infection

Samples were taken from a previous experiment (Costa-Hurtado et al., 2013). Briefly, *H. parasuis* infection was performed by intranasal inoculation with 33 snatch-farrowed,

colostrum-deprived piglets. Animals were obtained from a conventional farm with a standard health status. Sows were seropositive against porcine reproductive and respiratory virus (PRRSV), swine influenza and seronegative against Aujeszky's disease virus. Piglets were seronegative for porcine circovirus 2 (PCV2). Four strains of *H. parasuis* were used in the infection: virulent reference strain Nagasaki (serovar 5), virulent field strain IT25209 (serovar 4), non-virulent reference strain SW114 (serovar 3) and non-virulent field strain F9 (serovar 6). After 1, 2, 4, and 7 days post-inoculation (or at other times if the animals showed signs of suffering) two piglets from each group were euthanized. Lesions were assessed, and samples for bacterial culture were taken.

Histopathology

After post-mortem examination, samples of nasal turbinates, trachea and lung were removed, fixed by immersion in 20% buffered neutral formalin and subsequently embedded in paraffin. Paraffin wax-embedded tissues were sectioned at 4 μm and stained with haematoxylin and eosin (HE). Inflammation was scored from 0 to 3 depending on the severity (normal, mild, moderate or severe) based on the quantity of lymphocytes present in the corresponding tissue. Purulent catarrhal pneumonia and interstitial pneumonia were evaluated as positive or negative.

Detection of H. parasuis, macrophages and neutrophils by immunohistochemistry (IHC)

The IHC to detect *H. parasuis* was optimized using a primary antibody consisting of a mix of two anti-sera against *H. parasuis* strains, SW114 and Nagasaki, which were obtained in rabbit by hyper-immunization with formalin killed bacteria (Cerdà-Cuéllar and Aragon, 2008). Lung, trachea and nasal turbinates of all 33 animals were analyzed by IHC to detect *H. parasuis*. As control, all the samples were also tested with secondary antibody only. Briefly, formalin-fixed, paraffin-embedded tissues were cut into sections of 3-4 μm. After dewaxing and hydration, sections were incubated with 3% H_2O_2-methanol to inhibit endogenous peroxidase. Pre-treatment of tissues was performed with 10 mM citrate buffer (pH 6) at 98ºC for 20 min. After blocking with 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature (RT), *H. parasuis* detection was performed with the antibody mix described above diluted 1:250 in 2% BSA in PBS. After overnight incubation at RT, the sections were washed and incubated with a biotin-labeled goat anti-rabbit IgG (1:200 in PBS) for 1 h at RT. After 3 washes with PBS during 5 min, slides were incubated 60 min at RT with a

peroxidase-conjugated avidin-biotin complex diluted 1:100 in PBS. After 3 washes with PBS, reactions were revealed with a solution of 0.05% diaminobenzidine tetrahydrochloride (DAB) and 100 µl of H_2O_2 in 200 ml of PBS. Tissue sections were counterstained with haematoxylin and mounted with distrene-plasticizer-xylene (DPX). Detection of *H. parasuis* in the samples was semiquantified and results expressed as follows: -, no presence; +, poor and focal detection; ++, multifocal; +++, presence of antigen in the majority of tissue.

Samples from non-infected piglets served as negative control. As an additional negative control, lung samples from an animal infected with *H. parasuis* strain Nagasaki were incubated with pre-immune rabbit serum. In addition, brain tissues with meningitis from a pig infected with *Streptococcus suis* were also included as negative controls.

Simultaneous detection of macrophages and *H. parasuis* by double IHC was also carried out in lungs, nasal turbinates and trachea from pigs euthanized at 1 and 4 days post-inoculation (dpi). Non-inoculated animals (#1 and #2), inoculated with Nagasaki (#3 and #10), IT29205 (#11 and #16), F9 (#19 and #24) or SW114 (#27 and #32) were used. First, *H. parasuis* detection was performed following the protocol described above, but using the Target Retrieval Solution (Dako, Barcelona, Spain) instead of citrate buffer. After pre-treatment and blocking with 2% BSA in PBS for 1 h at room temperature (RT), macrophage detection was performed with α-CD163 monoclonal antibody (mAb), kindly donated by Dr. Javier Dominguez from Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA, Spain). After overnight incubation at 4ºC and washing three times with PBS, sections were incubated for 1 h at RT with alkaline phosphatase (AP) conjugate goat anti-mouse Ig (H+L) (Southern Biotechnology, AL, USA), diluted 1:100 in PBS. The presence of macrophages was visualized in blue using nitro blue tetrazolium chloride 5-bromo-4-chloro-3-indoxyl (NBT-BCIP) (Roche, IN, USA). Samples were not counterstained.

To achieve the detection of neutrophils and macrophages, together with *H. parasuis*, α-myeloid–histiocytic antigen MAC387 mAb (Dako, Barcelona, Spain) was diluted 1:40 with the α-CD163 mAb solution and the protocol described above was followed. Only lung tissue of animal #10 was used in this latter experiment.

Detection of H. parasuis and neutrophils by immunohistofluorescence

At time of necropsy, samples of lung and trachea were embedded in Tissue-Tek O.C.T. (Sakura, The Netherlands) and stored at -80ºC for further processing. Frozen samples were

cut using a cryostat (Leica CM-3050S) at approximately 8-10 μm, air-dried and fixed with 2% paraformaldehyde for 40 min. Sections were then permeabilized with 0.5% Triton X-100 and incubated in blocking solution (3% BSA in PBS). For the detection of neutrophils, undiluted supernatant of mAb 6D10 (Perez et al., 2007), kindly donated by Dr. Javier Dominguez from INIA (Spain), was incubated at 4ºC overnight followed by three washes with PBS for 5 min; bound antibody was detected with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Europe Ltd, UK) for 1h at RT. ***H. parasuis*** was detected in the tissues with a 1:100 dilution of the same mix of hyperimmune rabbit sera utilized in IHC. After overnight incubation at 4ºC, three washes with PBS were performed and tissue samples were incubated with Dylight 549 goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Europe Ltd, UK) for 1 h in 3% BSA-PBS at RT. Finally, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) at 1 μg/mL and samples were mounted with Fluoprep (Biomerieux, France). Fluorescent images were viewed on a Nikon eclipse 90i epifluorescence microscope. Images were processed with ImageJ v1.46d software. Negative controls were performed in each tissue by incubating only with secondary antibodies.

Detection of porcine reproductive and respiratory virus

Piglets were checked for PRRSV infection by ELISA. Also, lung tissues were screened for PRRSV presence by IHC following a previously published protocol (Grau-Roma et al., 2007), using mAb anti-protein N (1AC7, Ingenasa, Madrid, Spain) diluted at 1:200 to detect the virus.

Results

Detection of H. parasuis antigen in the respiratory tract of experimentally infected piglets

Histopathological findings and detection of *H. parasuis* by IHC at different times post-infection in the piglets are summarized in Table 1 and Table 2, respectively. Although some strain-specific locations were found, bacterial detection was similar within the strains of similar virulence (i.e., virulent strains Nagasaki and IT29205 versus non-virulent SW114 and F9).

Table 1. Histological findings in the respiratory tract of pigs after different times post-inoculation with 4 strains of *H. parasuis*: virulent Nagasaki and IT29205, and non-virulent SW114 and F9.

Strain	Pig	DPI[a]	Histological findings		
			Inflammation[b] (Lung/Trachea/ Nasal turbinates)	**CPB**[c]	**IN**[d]
Non-infected	1	0	3/0/0	-	+
	2	0	3/0/1	-	+
Nagasaki	3	1	2/0/1 MF[e]	+	+
	4	1	3/0/0	-	+
	5	2	0/0/0	-	-
	6	2	1/0/1 MF	-	+
	7	3	3/0/1 F[g]	-	+
	8	3	2/0/1 F	-	+
	9	3	1/0/0	-	+
	10	4	3/0/1 MF	+	+
IT29205	11	1	3/0/1 MF	-	+
	12	1	0/0/1 MF	-	-
	13	2	3/0/1 MF	-	+
	14	2	2/0/0	-	+
	15	4	2/0/0	-	+
	16f	4	1/0/0	-	+
	17	7	3/0/1 MF	+	+
	18	7	1/0/1 MF	-	-
F9	19	1	3/0/1 F	-	+
	20	1	3/0/0	-	+
	21	2	3/0/0	-	+
	22	2	3/0/0	-	+
	23	4	2/0/1 F	-	+
	24	4	3/0/1 MF	-	+
	25	7	1/0/1 MF	-	-
	26	7	1/0/0	-	+
SW114	27	1	3/0/1 MF	-	+
	28	1	0/0/1 F	-	-
	29	2	3/0/0	-	+
	30	2	3/0/1 F	-	+
	31	4	2/0/1 F	-	+
	32	4	3/0/1 MF	-	+
	33	7	3/0/0	-	+

[a] DPI, days post-inoculation
[b] Inflammation was evaluated from 1 to 3
[c] CPB, catarrhal purulent bronchopneumonia
[d] IN, interstitial pneumonia
[e] MF: nasal turbinates multifocal inflammation
[f] Pleuritis
[g] F, nasal turbinates focal inflammation

Table 2. Immunohistochemical evaluation of pigs infected with the four *H. parasuis* strains: virulent Nagasaki and IT29205, and non-virulent SW114 and F9, which were detected by immunohistochemistry (IHC) and semiquantified (-: no presence; + to +++, increased presence of antigen). Time indicates days after infection.

Pig	Strain	Time	Nasal turbinates				Trachea			Lung			
			Epi	Submucosa	Vessels	Meatus[d]	Epi	Submucosa	Vessels	Bronch[b]	Epi[c]	Vessels[e]	Alveolar
1	Non-infected	-	-	-	-	-	-	-	-	-	-	-	-
2		-	-	-	-	-	-	-	-	-	-	-	-
3[g]	Nagasaki	1 day	-	-	-	-	+++	-	-	++	+++	-	+
4		1 day	-	-	-	-	++	-	-	-	-	-	-
5		2 days	+	-	-	-	+	-	-	-	-	-	-
6		2 days	-	-	-	-	-	-	-	-	-	-	-
7		3 days	-	-	-	-	++	-	-	-	-	-	-
8		3 days	+	-	-	-	++	-	-	-	-	-	+++
9		3 days	+	++	+++	-	+++	++	++	-	-	+++	+++
10		4 days	+++	+++	+++	-	+++	+	++	++	+++	+++	+++
11	IT29205	1 day	-	-	-	-	++	-	-	-	-	-	-
12		1 day	-	+	-	-	-	-	-	-	-	-	-
13		2 days	++	-	-	+++	++	-	-	-	-	-	-
14		2 days	-	-	-	-	-	-	-	-	-	-	-
15		4 days	-	-	-	-	-	+	-	-	-	-	-
16[a]		4 days	-	-	-	+	++	-	-	++	++	-	+
17		7 days	-	-	-	-	-	-	-	+	+++	-	-
18		7 days	-	-	-	-	+	-	-	-	-	+	+
19	F9	1 day	-	+	-	-	-	-	-	-	-	-	-
20		1 day	-	-	-	-	-	-	-	-	-	-	+
21		2 days	-	-	-	-	+	-	-	-	-	-	-
22		2 days	-	-	-	-	-	-	-	-	-	-	-
23		4 days	-	-	-	-	-	-	-	-	-	-	-
24		4 days	-	++	-	+	-	-	-	-	-	-	-
25		7 days	-	-	-	-	-	-	-	-	-	-	-
26		7 days	-	-	-	-	-	-	-	-	-	-	-
27	SW114	1 day	-	+	-	+++	+	-	-	-	-	-	-
28		1 day	-	-	-	-	-	-	-	-	-	-	-
29		2 days	-	-	-	-	-	-	-	-	-	-	-
30		2 days	-	-	-	-	-	-	-	-	-	-	-
31		4 days	-	-	-	-	-	-	-	-	-	-	-
32		4 days	-	+	-	-	-	-	-	-	-	-	-
33		7 days	-	+	-	-	-	-	-	-	-	-	-

[a] This animal was also positive in pulmonary interstitium and pleura

[b] Bronch, bronchial space

[c] Epi: bronchial, tracheal or nasal turbinate epithelium

[d] Bacteria was found in association with neutrophil-like cells

[e] Bacteria were found inside cells

[f] NC, negative control

[g] Bacteria was also located in bronchial submucosa

Nagasaki strain was detected in the epithelium of nasal turbinates, especially in the cilia, but occasionally within the epithelial cells after 4 dpi (Fig. S1A). Similar results, but at earlier time points, were obtained with strain IT29205, but its presence in epithelium and submucosa was considerably lower than Nagasaki (not shown). In contrast, the non-virulent strains were found in much lesser extent in nasal turbinates, where they were detected inside neutrophil-like cells associated to mucus or located in the submucosa (Fig. S1B).

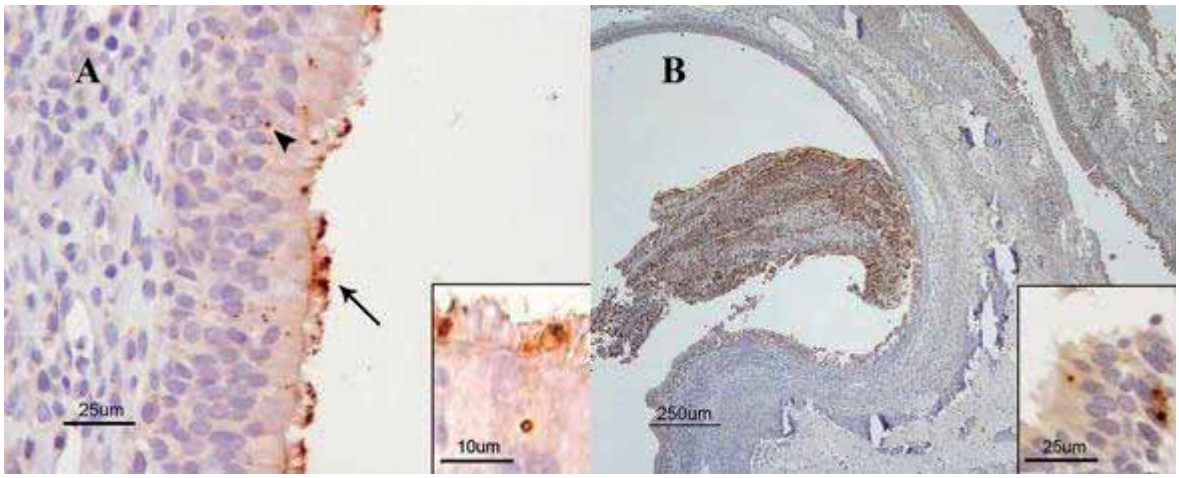


Figure S1. *H. parasuis* immunohistochemistry, nasal turbinates. A. Pig No. 10, Nagasaki strain, 4 days post-inoculation. Bacterial antigen is located multifocally in the cilia (arrow) and within the epithelial cells (arrowhead). Inset: detail at a higher magnification. B. Pig No. 27, SW114 strain, 1 day post-inoculation, nasal turbinates. Bacterial antigen is located within the purulent inflammatory infiltrate (purulent rhinitis) in the nasal meatus. Inset: Pig No. 32, SW114 strain, 4 days post-inoculation, nasal turbinates. Low amount of bacterial antigen is located multifocally within the apical or basal part of epithelial cells.

In trachea, virulent strains were widely detected on the epithelium. Figure 1A shows a representative image of the detection of Nagasaki in tracheal epithelium, but similar pattern was found for IT29205. Occasionally, virulent bacteria were detected in the tracheal submucosa. On the contrary, non-virulent strains were scarcely found in tracheal epithelium (Fig. 1B).

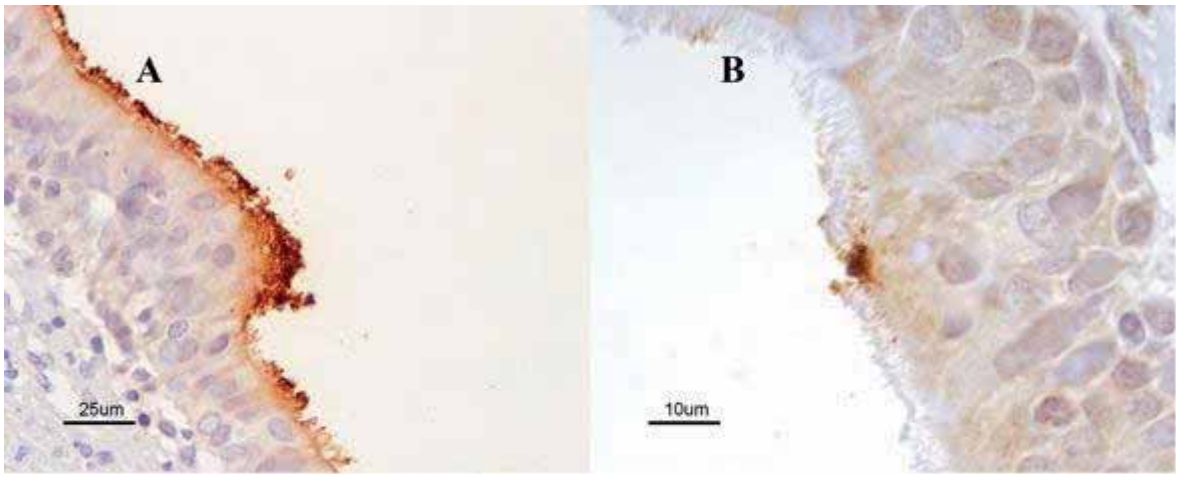


Figure 1. *H. parasuis* immunohistochemistry, trachea. A. Pig No. 10, Nagasaki strain, 4 days post-inoculation. *H. parasuis* antigen is located on the surface of epithelium (cilia) forming a biofilm-like structure. B. Pig No. 21, F9 strain, 2 days post-inoculation. Focal detection of *H. parasuis* antigen on the surface of epithelial cells, associated with loss of cilia.

In lung, differences between virulent and non-virulent strains were even more obvious. Nagasaki was distributed in bronchial lumen and bronchial epithelium (Fig. 2A, 2B and 2C), as well as IT29205 (not shown). Remarkably, at 3 and 4 days, Nagasaki strain was abundant inside alveoli (Fig. 2D). In addition, IT29205 was also detected in pleura and interstitial space at 4 dpi (Fig. 2E). In contrast, non-virulent strains F9 and SW114 were totally absent from lung. Only in one occasion, F9 strain was found in lung at day 1 after infection, but as degraded bacteria inside alveolar macrophage-like cells (Fig. 2F).

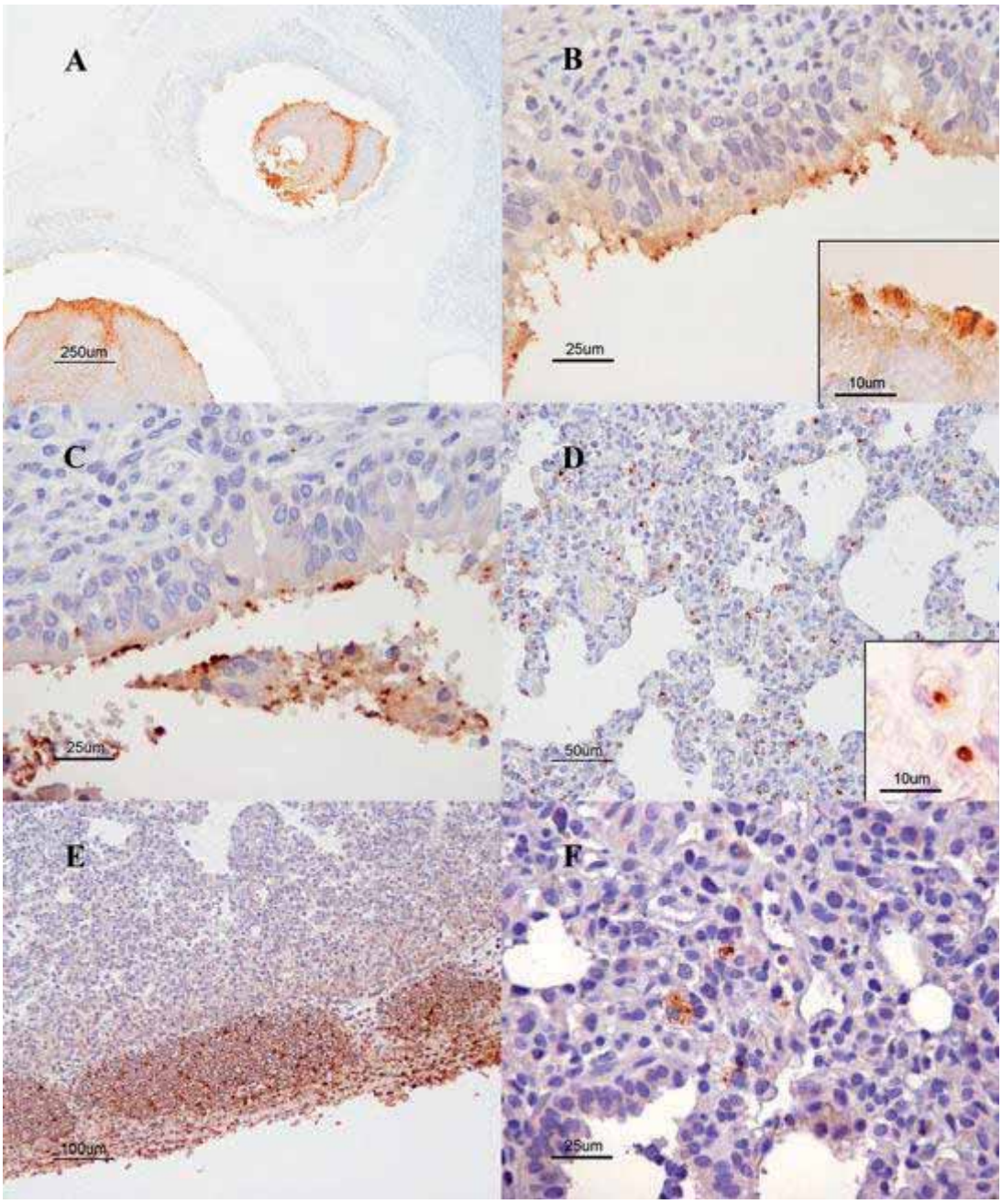


Figure 2. *H. parasuis* immunohistochemistry, lung. A. Pig No. 3, Nagasaki strain, 1 day post-inoculation. High amount of bacterial antigen located within the bronchial purulent inflammatory infiltrate (catarrhal-purulent bronchopneumonia). B. Pig No. 3, Nagasaki strain, 1 day post-inoculation. Bacterial antigen is located multifocally in the cilia and within the apical part of epithelial cells. Inset: detail of antigen located in the cilia of epithelial cells. C. Pig No. 10, Nagasaki strain, 4 days post-inoculation. Higher amount of bacterial antigen located multifocally in the cilia and within the bronchial inflammatory infiltrate. D. Pig No. 10, Nagasaki strain, 4 days post-inoculation. Bacterial antigen located within macrophage-like cells in the alveolar walls of a pig displaying interstitial pneumonia. Inset: detail at a higher magnification. E. Pig No. 16, IT29205 strain, 4 days post-inoculation. Massive amount of *H. parasuis* antigen distributed within the inflammatory infiltrates of the pleura (fibrinous-purulent pleuritis). F. Pig No. 20, F9 strain, 1 day post-inoculation. Bacterial antigen located inside macrophage-like cells in the alveolar wall of a pig with interstitial pneumonia.

Cellular localization of H. parasuis

Double IHC of *H. parasuis* and macrophages or neutrophils showed localization of virulent strains in lung macrophages (Fig. 3A) and neutrophils (Fig. S2). In both cases, some bacteria were found independently of phagocytes (macrophages or neutrophils). To evaluate if bacteria localized also with non-phagocytic cells, simultaneous detection of *H. parasuis*, macrophages and neutrophils was performed. Again, virulent *H. parasuis* was observed inside lung macrophages and/or neutrophils at 4 dpi, but bacteria were also found in cells that did not correspond to macrophages or neutrophils, and whose morphological characteristics make them most probably pneumocytes (Fig. 3B and 3C).

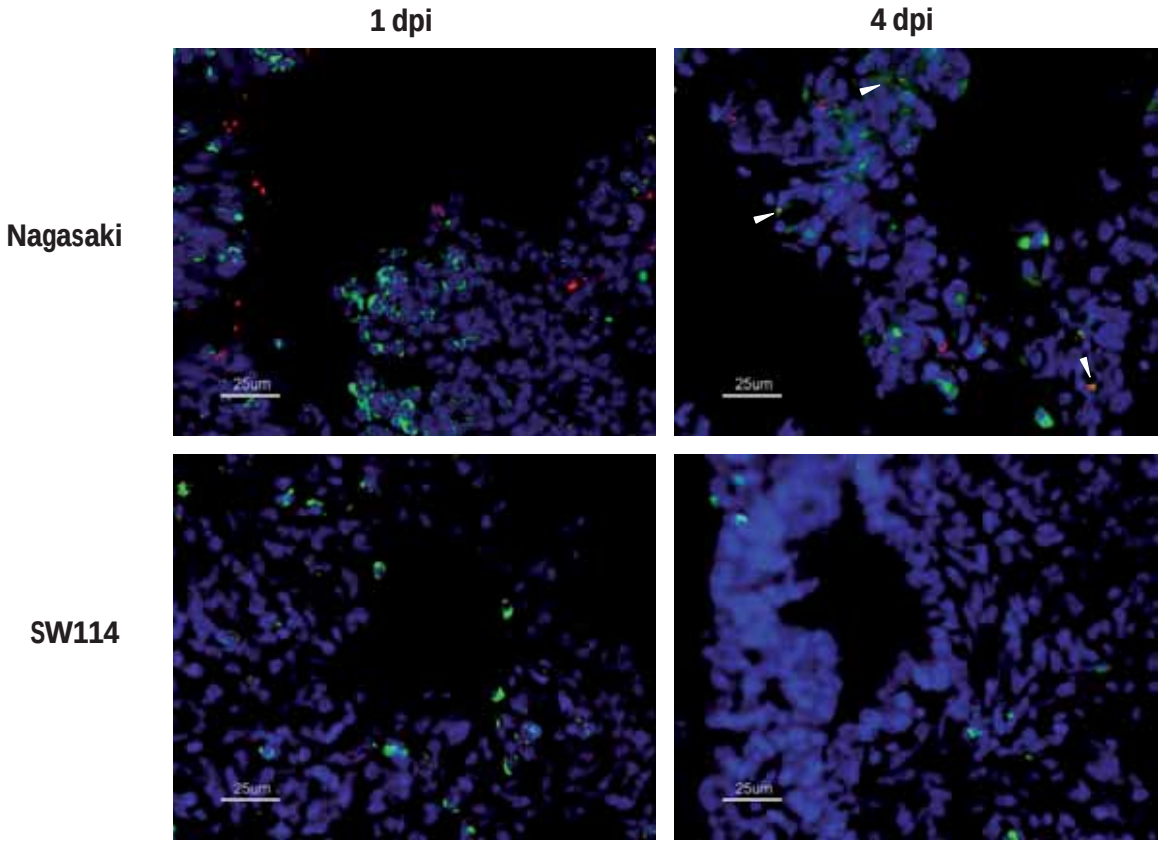


Figure S2. Simultaneous detection of *H. parasuis* and neutrophils by double immunofluorescence in lung tissue sections from piglets inoculated with the virulent strain Nagasaki and the non-virulent strain SW114 at 1 day post-inoculation (dpi) and 4 dpi. Neutrophils were labeled with mAb 6D10 followed by anti-mouse-FITC (green signal). *H parasuis* was labeled with a rabbit anti-*H. parasuis* hyperimmune serum followed by a Dylight 549 goat anti-rabbit (red signal) and nuclei were counterstained with DAPI (blue signal). Upper panels show the presence of neutrophils in the lung infected with Nagasaki strain at 1dpi (left panel) or 4 dpi (right panel). White arrow heads point to bacteria co-localizing with neutrophils. Lower panels show the presence of neutrophils in lung infected with SW114 strain at 1dpi (left panel) or 4 dpi (right panel). Scale bars, 25 μm. The images showing the individual fluorescences are presented in supplemental Figure S3 (ANNEX II).

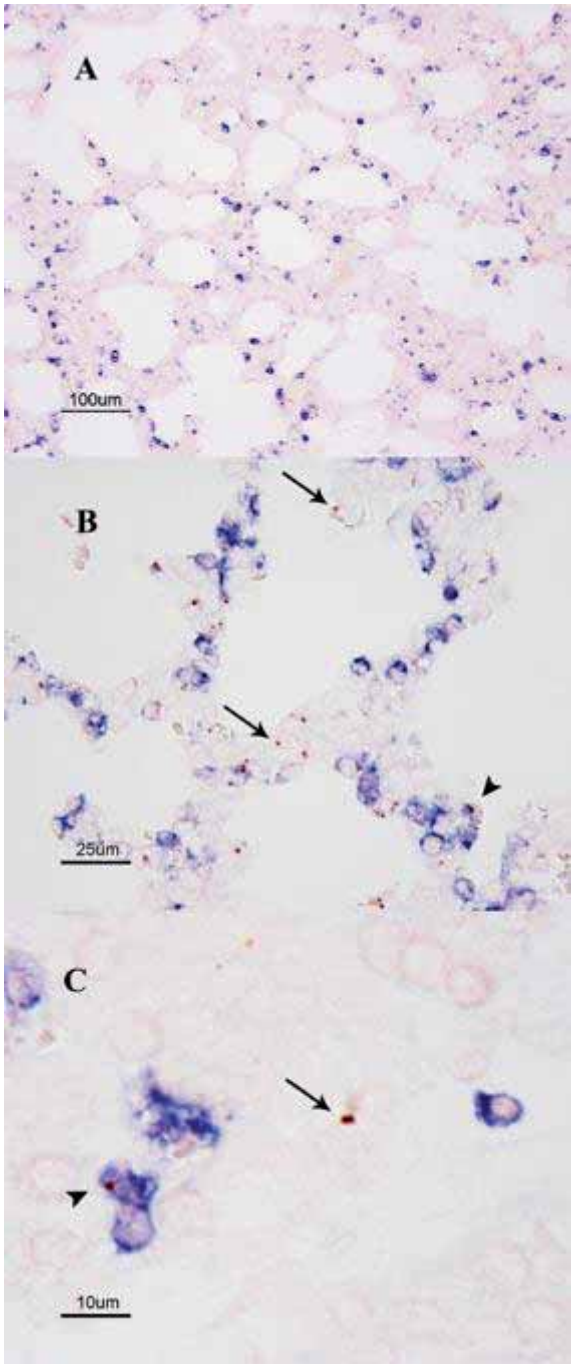


Fig. 3. Simultaneous detection of *H. parasuis*, macrophages and neutrophils by double immunohistochemistry in lung. (A) Pig No. 10, Nagasaki strain, 4 days post-inoculation. Representative view of the labeling of lung macrophages with anti-CD163 (blue). (B) and (C) Pig No. 10. Nagasaki strain, 4 days post-inoculation. Combination of anti-CD163 and MAC387 antibodies for detection of macrophages and neutrophils (blue). *H. parasuis* (in brown) was detected inside pneumocyte-like cells (arrows) and in association with macrophage or neutrophil

Concomitant PRRSV infection

During histopathological examination of the lung sections, interstitial pneumonia, suggestive of a viral infection, was observed in the majority of the animals, including the negative, non-infected controls. PRRSV is a common viral infection of swine and so the animals were tested by ELISA and IHC. PRRSV antibodies were detected in all but 3 piglets, and the presence of PRRSV antigen in lung could be confirmed by IHC in 18 out of 33 pigs (data not shown).

Discussion

In the present study, the progression of infection of the respiratory tract of snatch-farrowed, colostrum-deprived piglets by four different strains of *H. parasuis* demonstrate differences in the interaction between the host and the *H. parasuis* strains depending on the bacterial virulence.

The clinical outcome was directly associated with the virulence of the *H. parasuis* strains, as described previously (Costa-Hurtado et al., 2013). However, the presence of a previous infection by the PRRSV in the animals used may have affected the speed of infection, as recently reported (Yu et al., 2012).

To study the presence and location of the bacteria in the respiratory tract at different times after infection, an IHC was developed with a mix of two hyper-immune sera produced in rabbit against whole bacteria and a citrate pre-treatment of the tissue samples, which was suitable for *H. parasuis* detection by immunochemistry in pig lung, nasal turbinates, trachea and meninges. Virulent strains Nagasaki and IT29205 were easily detected in the tracheal and nasal turbinate epithelium, while strains F9 and SW114 were scarce and showed a microcolony-like pattern in these locations. Biofilm formation in the upper respiratory tract has been described for numerous bacterial respiratory pathogens (Trivedi et al., 2011; Hanke et al., 2012). Although the present work cannot confirm that *H. parasuis* forms a real biofilm *in vivo,* these results suggest that virulent *H. parasuis* strains could use this type of growth for colonization. The possibility that virulent strains form a biofilm in the trachea deserves further investigations to examine the role of this type of growth in the pathogenesis of Glässer's disease.

Contrary to the abundance of *H. parasuis* in the nose of the animals inoculated with non-virulent strains observed by isolation (not shown), presence of non-virulent bacteria F9 and SW114 in the epithelium of trachea and nasal turbinates was very low and could be due to the predominant presence of these bacteria in the mucus without a tight interaction with the epithelium. This mucus layer, and the bacteria within, may be lost after processing of the tissue samples for IHC.

In lung samples only virulent strains were frequently detected, while non-virulent strains were almost absent. These results confirm the finding of virulent *H. parasuis* strains by IHC in previous works (Amano et al., 1994; Segales et al., 1997; Vahle et al., 1997) and

are in agreement with the different susceptibility of the strains to phagocytosis by alveolar macrophages (Olvera et al., 2009). In the current study, neutrophils were found in the lungs of infected animals at early time-points, especially inside bronchia, corresponding to the recruitment of neutrophils into the infected tissue. At later time points, animals infected with virulent strains showed higher levels of neutrophils in lung tissue than animals infected with non-virulent strains, which were not longer detected in the lung.

Molecular mechanisms enhancing adhesion to epithelial surfaces are usually very specific, involving selected molecules (Trivedi et al., 2011; Edwards et al., 2012). The detection of virulent *H. parasuis* in the epithelium and submucosa probably requires specific interactions between *H. parasuis* and nasal/tracheal epithelium, as previously described for other bacteria species and also suggested for *H. parasuis* (Bouchet et al., 2008; Bencurova et al., 2011; Frandoloso et al., 2012). In addition, the capacity of strains Nagasaki and IT29205 to form a stable biofilm-like structure could be a key component in *H. parasuis* infection, possibly altering the immune response in mucosal surfaces, as it has been observed in other bacteria (Hanke et al., 2012). On the other hand, it has been reported that biofilm formation is more prevalent in non-pathogenic *H. parasuis* strains than in pathogenic strains *in vitro* (Jin et al., 2006). However, *in vitro* studies may not represent the *in vivo* conditions, as genes possibly involved in biofilm formation have been detected to be transcribed in the lung of animals infected by a virulent strain (Jin et al., 2008).

Virulence mechanisms, including phagocytosis resistance, serum resistance and capsule formation, have been reported in *H. parasuis* (Cerdà-Cuéllar et al., 2008; Olvera et al., 2009) and other bacteria (Celli et al., 2002), to be essential to reach the lung, survive the host defenses and disseminate to the rest of the body. The present findings confirm that virulent strains can reach the lung and multiply there, while non-virulent strains are barely detected in this location.

Initial observations of tissue sections indicated that *H. parasuis* was found in macrophage- or neutrophil-like cells, although not always. The interaction of *H. parasuis* with lung macrophages and neutrophils has been previously observed in *in vitro* assays (Olvera et al., 2009), *in vivo* experiments (Amano et al., 1994) and in co-infection with PRRSV (Segales et al., 1998; Solano et al., 1998). However, a detailed study identifying the cellular types involved in the interaction was missing. Here, we have showed that *H. parasuis* can be found in macrophages and neutrophils, but more importantly in pneumocyte-like cells. The

invasion of pneumocytes may constitute an virulence mechanism of *H. parasuis*, as it has been already described for the human pathogen *Haemophilus influenzae* (King, 2012).

In summary, IHC results showed that the colonization and infection of the respiratory tract by *H. parasuis* was different depending on the virulence of the infecting strain. Virulent strains were observed in trachea and lung, while non-virulent strains were barely seen at these locations. In addition, the presence of virulent *H. parasuis* in non-immune cells in the lung may constitute a virulence mechanism of these strains.

References

Amano, H., Shibata, M., Kajio, N., Morozumi, T., 1994. Pathologic observations of pigs intranasally inoculated with serovar 1, 4 and 5 of *Haemophilus parasuis* using immunoperoxidase method. J. Vet. Med. Sci. 56, 639-644.

Aragon, V., Cerda-Cuellar, M., Fraile, L., Mombarg, M., Nofrarias, M., Olvera, A., Sibila, M., Solanes, D., Segales, J., 2010. Correlation between clinico-pathological outcome and typing of *Haemophilus parasuis* field strains. Vet. Microbiol. 142, 387-393.

Aragon, V., Segales, J., Oliveira, S., 2012. Glässer's disease. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), Diseases of Swine, 10th Ed. Wiley-Blackwell, Iowa, USA, pp. 760–769.

Bencurova, E., Mlynarcik, P., Bhide, M., 2011. An insight into the ligand-receptor interactions involved in the translocation of pathogens across blood-brain barrier. FEMS Immunol. Med. Microbiol. 63, 297-318.

Bouchet, B., Vanier, G., Jacques, M., Gottschalk, M., 2008. Interactions of *Haemophilus parasuis* and its LOS with Porcine Brain Microvascular Endothelial Cells. Vet. Res. 39, 42.

Celli, J., Finlay, B.B., 2002. Bacterial avoidance of phagocytosis. Trends Microbiol. 10, 232-237.

Cerdà-Cuéllar, M., Aragon, V., 2008. Serum-resistance in *Haemophilus parasuis* is associated with systemic disease in swine. Vet. J. 175, 384-389.

Costa-Hurtado, M., Olvera, A., Martinez-Moliner, V., Galofre-Mila, N., Martinez, P., Dominguez, J., Aragon, V., 2013. Changes in macrophage phenotype after infection of pigs with *Haemophilus parasuis* strains with different levels of virulence. Infect. Immun. 81, 2327-2333.

Edwards, A.M., Massey, R.C., Clarke, S.R., 2012. Molecular mechanisms of *Staphylococcus aureus* nasopharyngeal colonization. Mol. Oral Microbiol. 27, 1-10.

Frandoloso, R., Martinez-Martinez, S., Gutierrez-Martin, C.B., Rodriguez-Ferri, E.F., 2012. *Haemophilus parasuis* serovar 5 Nagasaki strain adheres and invades PK-15 cells. Vet. Microbiol. 154, 347-352.

Grau-Roma, L., Segales, J., 2007. Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine influenza virus and Aujeszky's disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain. Vet. Microbiol. 119, 144-151.

Hanke, M.L., Kielian, T., 2012. Deciphering mechanisms of staphylococcal biofilm evasion of host immunity. Front Cell Infect Microbiol 2, 62.

Jin, H., Wan, Y., Zhou, R., Li, L., Luo, R., Zhang, S., Hu, J., Langford, P.R., Chen, H., 2008. Identification of gene transcribed by *Haemophilus parasuis* in necrotic porcine lung through the selective capture of transcribed sequences (SCOTS). Environ. Microbiol. 10, 3326-3336.

Jin, H., Zhou, R., Kang, M., Luo, R., Cai, X., Chen, H., 2006. Biofilm formation by field isolates and reference strains of *Haemophilus parasuis*. Vet. Microbiol. 118, 117-123.

King P., 2012. *Haemophilus influenzae* and the lung (*Haemophilus* and the lung). Clin. Transl. Med. 1, 10.

Olvera, A., Ballester, M., Nofrarias, M., Sibila, M., Aragon, V., 2009. Differences in phagocytosis susceptibility in *Haemophilus parasuis* strains. Vet. Res. 40, 24.

Perez, C., Revilla, C., Alvarez, B., Chamorro, S., Correa, C., Domenech, N., Alonso, F., Ezquerra, A., Dominguez, J., 2007. Phenotypic and functional characterization of porcine granulocyte developmental stages using two new markers. Dev. Comp. Immunol. 31, 296-306.

Segales, J., Domingo, M., Balasch, M., Solano, G.I., Pijoan, C., 1998. Ultrastructural study of porcine alveolar macrophages infected *in vitro* with porcine reproductive and respiratory syndrome (PRRS) virus, with and without *Haemophilus parasuis*. J. Comp. Pathol. 118, 231-243.

Segales, J., Domingo, M., Solano, G.I., Pijoan, C., 1997. Immunohistochemical detection of *Haemophilus parasuis* serovar 5 in formalin-fixed, paraffin-embedded tissues of experimentally infected swine. J. Vet. Diagn. Invest. 9, 237-243.

Solano, G.I., Bautista, E., Molitor, T.W., Segales, J., Pijoan, C., 1998. Effect of porcine reproductive and respiratory syndrome virus infection on the clearance of *Haemophilus parasuis* by porcine alveolar macrophages. Can. J. Vet. Res. 62, 251-256.

Trivedi, K., Tang, C.M., Exley, R.M., 2011. Mechanisms of meningococcal colonisation. Trends Microbiol. 19, 456-463.

Vahle, J.L., Haynes, J.S., Andrews, J.J., 1995. Experimental reproduction of *Haemophilus parasuis* infection in swine: clinical, bacteriological, and morphologic findings. J. Vet. Diagn. Invest. 7, 476-480.

Vahle, J.L., Haynes, J.S., Andrews, J.J., 1997. Interaction of *Haemophilus parasuis* with nasal and tracheal mucosa following intranasal inoculation of cesarean derived colostrum deprived (CDCD) swine. Can. J. Vet. Res. 61, 200-206.

Yu, J., Wu, J., Zhang, Y., Guo, L., Cong, X., Du, Y., Li, J., Sun, W., Shi, J., Peng, J., Yin, F., Wang, D., Zhao, P., Wang, J., 2012. Concurrent highly pathogenic porcine reproductive and respiratory syndrome virus infection accelerates *Haemophilus parasuis* infection in conventional pigs. Vet. Microbiol. 158, 316-321.

2. RNA expression of *Haemophilus parasuis* in biofilm

Biofilm formation by virulent and non-virulent strains of *Haemophilus parasuis*

Bello-Ortí and Deslandes et al., 2014. Vet Res 45:104

Abstract

Haemophilus parasuis is a commensal bacterium of the upper respiratory tract of healthy pigs. It is also the etiological agent of Glässer's disease, a systemic disease characterized by polyarthritis, fibrinous polyserositis and meningitis, which causes high morbidity and mortality in piglets. The aim of this study was to evaluate biofilm formation by well-characterized virulent and non-virulent strains of *H. parasuis*. We observed that non-virulent strains isolated from the nasal cavities of healthy pigs formed significantly ($p < 0.05$) more biofilms than virulent strains isolated from lesions of pigs with Glässer's disease. These differences were observed when biofilms were formed in microtiter plates under static conditions or formed in the presence of shear force in a drip-flow apparatus or a microfluidic system. Confocal laser scanning microscopy using different fluorescent probes on a representative subset of strains indicated that the biofilm matrix contains poly-N-acetylglucosamine, proteins and eDNA. The biofilm matrix was highly sensitive to degradation by proteinase K. Comparison of transcriptional profiles of biofilm and planktonic cells of the non-virulent *H. parasuis* F9 strain revealed a significant number of up-regulated membrane-related genes in biofilms, and genes previously identified in *Actinobacillus pleuropneumoniae* biofilms. Our data indicate that non-virulent strains of *H. parasuis* have the ability to form robust biofilms in contrast to virulent, systemic strains. Biofilm formation might therefore allow the non-virulent strains to colonize and persist in the upper respiratory tract of pigs. Conversely, the planktonic state of the virulent strains might allow them to disseminate within the host.

Introduction

Haemophilus parasuis is a Gram-negative bacterium and a commensal organism of the upper respiratory tract of healthy pigs. It is also the etiological agent of Glässer's disease, a systemic disease characterized by polyarthritis, fibrinous polyserositis and meningitis, which causes high morbidity and mortality in piglets [1]. Glasser's disease is recognized as one of the main causes of economic loss in the pig industry.

The heterogeneity among *H. parasuis* strains has been demonstrated by multiple methods, including multilocus sequence typing (MLST) which has shown a genetic lineage associated with polyserositis (cluster A) and another lineage associated with nasal colonization (cluster C) [2]. Serum resistance [3], phagocytosis resistance [4], and invasion of endothelial cells [5, 6] have been associated with virulence of *H. parasuis*. Some putative virulence factors have been reported [7, 8]. Those include the capsule [4], lipooligosaccharide (LOS) [9-11] and other genes involved in polysaccharide production, such as *galE* and *galU*, which have been associated with serum resistance and biofilm production [12]. Genes involved in sialic acid utilization were identified as potential virulence factors [13]. The sialytransferase gene *lsgB* was predominantly present in systemic isolates and not in nasal isolates, and sialylation of the LOS was observed in the virulent strain *H. parasuis* Nagasaki. In addition, a family of trimeric autotransporters, designated virulence associated trimeric autotransporters (VtaA) has been described [14-15] and these can be divided into three groups based on their translocation domains [14]. Group 3 *vtaA* gene is highly conserved among invasive and non-invasive strains, whilst groups 1 and 2 *vtaA* genes were detected only in virulent strains.

Bacterial biofilms are structured clusters of bacterial cells enclosed in a self-produced polymer matrix that are attached to a surface [16-18]. Bacteria can adhere to biotic surfaces (e.g. cells at the mucous layer) as well as abiotic surfaces (e.g. floor or equipment found at a farm). The polymer matrix is often composed of exopolysaccharides, proteins and nucleic acids. The biofilm protects bacteria from hostile environmental conditions. Bacteria within a biofilm can resist attack from the host immune response, and are less sensitive than planktonic cells to desiccation and to the action of biocides. Biofilm formation by nasal strains of *H. parasuis* has been previously reported [19]. Although the role of biofilm in *H. parasuis* pathogenesis is not clear, expression of genes with putative

function in biofilm formation was detected during pulmonary infection [20]. The aims of this study were to compare biofilm formation by well-characterized virulent and non-virulent strains of *H. parasuis* and to analyse the gene expression during biofilm growth. Such analyses would help explore the possible role of biofilms in the pathogenesis of *H. parasuis*.

Materials And Methods

Bacterial strains

The *H. parasuis* strains used in this study and their main phenotypic and genotypic characteristics are listed in Table 1. Bacteria were grown on Brain Heart Infusion (BHI; Oxoid Ltd, Basingstoke, Hampshire, UK) agar containing 10 μg/mL NAD or on chocolate agar (Biomerieux, Madrid, Spain). Plates were incubated overnight at 37ºC with 5% CO_2.

Table 1. *Haemophilus parasuis* strains used in the present study.

Strains	Susceptibility[1] to		*lsgB*[2]	group1 *vtaA*[2]	MLST[3]	Serovar
	Serum	Phagocytosis				
Nasal strains						
F9	S	S	-	-	C	6
ND14-1	S	S	-	-	C	7
SC14-1	S	S	-	-	C	15
MU21-2	S	S	-	-	C	7
SL3-2	S	S	-	+	B	10
Strains from lesions						
Nagasaki	R	R	+	+	A	5
P015/96	R	R	+	+	A	5
ER-6P	R	R	+	+	A	15
264/99	R	R	+	+	A	10
IT29205	R	R	+	+	A	4
2725	S	R	+	+	A	10
373/03A	I	R	-	+	A	7
PV1-12	S	R	-	+	B	15
9904108	S	R	-	-	C	4

[1] (S) sensitive; (I) intermediate; (R) resistant
[2] (+) gene present; (-) gene absent
[3] Cluster associated with isolates from systemic lesions (A); with nasal isolation (C); or cluster with no statistical association with clinical origin (B) [2].

Biofilm assays

Static conditions

Biofilms were cultured in 96-well microtiter plates as described by Wu et al. [21], with some modifications. Briefly, colonies from overnight agar cultures were resuspended in BHI-NAD containing 5 μg/mL NAD, and the suspension was aliquoted (100 μL) in triplicate in a flat-bottom 96-well polystyrene plate (Costar® 3599, Corning, NY, USA) and incubated for 40h at 37ºC. Wells containing sterile broth were used as negative control. Following incubation, biofilms were treated as described before [22] with some modifications. Briefly, the liquid medium was removed using a vacuum and unattached cells were removed by immersing the plate once in MilliQ water. The water was removed with a vacuum and excess water was removed by inverting plates onto a paper towel. Biofilms were then stained with 0.1% (w/v) crystal violet for 2 min. Biofilms were washed once with distilled water and then dried at 37°C for 15 min. The stain was then released with 100 μL of 70% (v/v) ethanol and the amount of released stain was quantified by

measuring the absorbance at 590 nm with a microplate reader (Powerwave; BioTek Instruments, Winooski, VT, USA).

Shear force conditions

Biofilms were cultured in a drip-flow apparatus (DFR 110 Biofilm Reactor, BioSurface Technologies Corp. Bozeman, MT, USA) as described by Goeres et al. [23] with some modifications [24]. Briefly, colonies of *H. parasuis* strains F9, MU21-2, ER-6P or Nagasaki on BHI-NAD agar were resuspended into 13 mL of fresh BHI-NAD to an OD_{600} of 0.1 and 12.5 mL of this inoculum was transferred into a channel containing a glass slide (Catalogue #48300-025, VWR, Ville Mont-Royal, QC, Canada). The apparatus was incubated for 24h at 37°C with 5% CO_2 to allow the biofilm to form under static conditions. The apparatus legs were then attached to create a 10° downward slope. The apparatus was then connected to the nutrient system containing pre-warmed (37°C) BHI-NAD. The flow (~25 mL per hour per channel) of the medium was then initiated and maintained for 24h at 37°C. After 24h, the glass slide was removed and gently washed once with sterile MiliQ water. The biofilms were resuspended in 1.5 mL of MiliQ water, centrifuged and dried with a DNA 120 Speed Vac® (Thermo Scientific, Ottawa, ON, Canada). The weight of dry biofilms was then measured.

Biofilms were also cultured in a microfluidic system. Growth of biofilms in the BioFlux 200 device (Fluxion Biosciences, South San Francisco, CA, USA) was adapted from Benoit et al. [25] and the manufacturer's recommendations. Briefly, colonies of *H. parasuis* strains F9, MU21-2, ER-6P or Nagasaki on BHI-NAD agar were resuspended into 2.5 mL of fresh BHI-NAD to an OD_{600} of 1.0 and this suspension was serially diluted to OD_{600} of 0.5 and 0.25. The microfluidic channels were wetted with BHI-NAD and were then inoculated with different bacterial suspensions (OD_{600} 1.0, 0.5 or 0.25). The microfluidic plate was incubated for 2h at 37°C to allow bacteria to bind to the surface. The flow of fresh medium was then initiated and was set from 0.5 dyne/cm^2 to 1.0 dyne/cm^2. Growth of the biofilms was monitored for up to 24h and, in some cases, fresh medium was added and the "waste" outlet was emptied to ensure that wells would not dry or spill. Images of BioFlux biofilms were obtained using an inverted fluorescence microscope (Olympus CKX41, Markham, ON, Canada), a digital camera (Retiga EX; Q Imaging, Surrey, BC, Canada), and the software provided with the BioFlux 200 device.

Confocal laser scanning microscopy (CLSM)

Biofilms were prepared under static conditions as described above and were stained with FilmTracer™ FM®1-43 Green biofilm cell stain (Molecular Probes, Eugene, OR, USA) according to manufacturer's instructions. To determine the composition of the biofilm matrix, biofilms were stained with Wheat Germ Agglutinin (WGA-Oregon Green 488, Molecular Probes; binds to N-acetyl-D-glucosamine and N-acetylneuraminic acid residues), FilmTracer™ SYPRO® Ruby biofilm matrix stain (Molecular Probes; labels most classes of proteins) or BOBO™-3 iodide (Molecular Probes; stains extracellular DNA) according to manufacturer's instructions. After a 30 min incubation at room temperature, the fluorescent marker solution was removed, biofilms were washed with water and the wells were then filled with 100 µL of water or PBS for WGA-stained biofilms. Stained biofilms were visualized by CLSM (Olympus FV1000 IX81, Markham, ON, Canada).

Dispersion of biofilm by enzymatic treatments

A biofilm dispersion assay was performed as described previously [26]. Briefly, biofilms were grown under static conditions as described above, and after the 40h incubation, 50 µl of DNase I (500 µg/mL in 150 mM NaCl, 1 mM $CaCl_2$), 50 µl of dispersin B (100 ug/mL in PBS; Kane Biotech Inc., Winnipeg, MB, Canada), or 50 µl of proteinase K (500 ug/mL in 50 mM Tris-HCl pH 7.5, 1mM $CaCl_2$) were added directly to the biofilms. Control wells were treated with 50 µl of the buffer without the enzyme. Wells treated with dispersin B were incubated for 5 min at 37ºC, and those treated with proteinase K or DNase I were incubated for 1 h at 37ºC. After the treatments, the biofilms were stained with crystal violet as described above.

Effect of fibrinogen and fibronectin on biofilm formation

Biofilms were prepared under static conditions as described above with some modifications. Prior to inoculation, various concentrations of fibrinogen (bovine, porcine or human) (0.5, 1, 2 and 5 mg/ml) or fibronectin (human) (0.5, 1 and 2 mg/ml) were added to the biofilm medium. Plates were prepared in duplicate for each experiment: one plate was used to measure biofilm formation and the other plate was used to measure growth of the bacteria in the presence of proteins. Both plates were incubated and one plate was stained as described before. The unstained replicate plate was used to evaluate growth by measuring the absorbance at 600 nm.

Genome sequencing and assembly

Genomic DNA of the high biofilm producer strain F9 was prepared using the Blood and Tissue DNeasy kit (Qiagen) according to the manufacturer's instructions. Illumina libraries were prepared using 500 ng of genomic DNA and modified Illumina protocols [27, 28]. Paired-end sequencing was performed on an Illumina HiSeq 2000 analyzer for 75 cycles at the Wellcome Trust Sanger Institute (Hinxton, Cambridge, UK). The fastq reads were mapped to the complete SH0165 genome using Stampy to check for quality and purity of the sequence before any analyses [29, 30].

A custom-made bioinformatics pipeline was used to assemble the draft genome sequence. Cutadapt was used to remove the adaptor sequences from the sequence reads that were previously introduced during the library preparation [31]. Any undetermined nucleotides (N's) were removed from reads and the program sickle [32] was used to trim low-quality sequences found at the ends of paired-end sequence reads resulting in a minimum length of 31bp, using the program's default quality thresholds for the reads. Next, a custom Perl script was used to screen out low quality fastq reads and produce a single fastq file containing the good paired reads, and a separate file containing good quality single reads. Finally, we used Velvet [33] and VelvetOptimiser 2.2.0 [34] to assemble the fastq files into the de-novo genome assembly, made up of contiguous sequences (contigs). Assembly parameters were optimised to produce the highest quality assembly (i.e. highest n50 value) using VelvetOptimiser, which runs through all possible k-mer values from 19 to 71 in increments of 2. The draft genome sequence was annotated using the automatic annotation software Prokka [35], including the rfam option. This Whole Genome Shogun project has been deposited [DDBJ/EMBL/GenBank: JHQI00000000]. The version described in this paper is version JHQI01000000.

Transcriptomics analysis

The high biofilm producer strain F9 was chosen for transcriptomic analysis. Planktonic and biofilm samples were obtained under static conditions as described above with some modifications. Bacteria were cultured in 6-well microtiter plates and after 36h, planktonic cultures were transferred to sterile tubes, whereas biofilm bacteria were collected by scraping the surface of the wells with lysis buffer (2% SDS in PBS). Bacteria from both samples, biofilm or planktonic, were recovered by centrifugation and pellets were used for RNA extraction. For comparison, F9 was grown with shaking (220 rpm) until the culture

reached stationary phase and this culture was then processed for RNA extraction. This is considered to be the "stationary culture" sample.

To extract RNA, bacterial pellets were resuspended in 2% SDS, 16 mM EDTA, 10 mM Tris (pH 8.0) and incubated for 5 min at 100 ºC. Afterwards, samples were processed by two hot acid phenol-chloroform extractions, followed by two chloroform/ isoamyl alcohol extractions. RNA was then precipitated with 0.6 volumes of isopropanol, 0.1 volumes of 5M ammonium acetate and 1µl of glycogen. After centrifugation, the pellet was washed with 70% ethanol, dried and resuspended in warmed RNase-free water. To ensure that contaminating bacterial DNA was eliminated from the samples, treatment with RNase-free DNase (Qiagen) was performed. In addition, ribosomal RNA was eliminated with the Ribo-Zero rRNA removal kit (Epicentre Biotechnologies, Madison, WI, USA) following manufacturer instructions. PCR reactions using primers specific for *H. parasuis* 16S rRNA gene [36] were carried out to ensure that no bacterial DNA was left in the sample. Final RNA quality was verified with a Nanodrop spectrophotometer and the integrity was analyzed using Agilent Bioanalyzer 2100 (Agilent technologies). Libraries were generated using an Ion Torrent RNA-Seq v2 kit (Life Technologies) and sequenced using an Ion Torrent PGM (Life Technologies) with an Ion 318 chip (Life technologies) at the Centre for Research in Agricultural Genomics (CRAG, Campus de Bellaterra-UAB, Spain).

Bioinformatic analysis was performed following the count-based differential expression method. Briefly, reads quality control was performed using FastQC [37] and FASTX-Toolkit [38], and reads were then mapped to *H. parasuis* F9 genome using the recommended Torrent Mapping Alignment Program (TMAP) v3.4.1 with map2 setting [39]. Alignments were inspected using SAMtools [40] and Integrative Genomics Viewer (IGV) [41]. HTSeq v0.5.4p3 [42] was used for feature counting with intersection-nonempty setting and discarding non-protein coding CDS. Differentially expressed genes (DEGs) were identified with edgeR R package [43] with a 5% P-value cut-off, using an assigned dispersion value of 0.04.

To perform gene set enrichments, a custom Gene Ontology (GO) database was built. Protein coding genes were BLASTed using BLASTp (version 2.2.28) against the non-redundant NCBI database (April 2014), e value of 10^{-3} and keeping first 20 hits. GO terms were mapped to Blast hits using Blast2GO [44]. The most significantly up-regulated genes ($P<0.05$) were identified as candidates for classic Fisher's exact testing through Blast2GO.

Tests were performed for biological process (BP), cellular component (CC) and molecular function (MF) with $P < 0.05$. Conservation of membrane-related genes among all 14 *H. parasuis* sequences available in GenBank [GenBank: APCA00000000.1, APBW00000000.1, ABKM00000000.2, APBX00000000.1, AZQU00000000.1, APBZ00000000.1, AOSU00000000.1, APBY00000000.1, APBV00000000.1, CP001321.1, APBT00000000.1, APBU00000000.1, APCB00000000.1, CP005384.1] was achieved as follows: whole F9 proteome was analyzed using Phobius [45] via Blast2GO, positively predicted membrane-related genes were blasted against the 14 *H. parasuis* genomic sequences using tBLASTn with the following settings: e value of 10^{-5}, alignment length > 70% and match identity > 60%. Whole-genome BLASTp comparisons were performed between *H. parasuis* and *Actinobacillus pleuroneumoniae* serovar 5b strain L20 [GenBank: NC_009053.1], using the following settings: e value of 10^{-5}, alignment length > 90% and match identity > 40%. All transcriptomic data were deposited in the Gene Expression Omnibus database [GEO: GSE56428].

Results

Biofilm formation under static conditions

The phenotypic and genotypic characteristics of the *H. parasuis* strains used in the present study are given in Table 1. Five non-virulent strains were recovered from the nasal cavities of healthy pigs while nine virulent strains were recovered from lesions of pigs with Glässer's disease. The ability of *H. parasuis* to form biofilms at the solid-liquid interface in polystyrene microtiter plates was determined for each of the virulent and non-virulent strains (Figure 1A). Interestingly, the nasal, non-virulent strains formed significantly ($p < 0.05$) more biofilms than the virulent strains isolated from lesions of pigs with Glässer's disease (Figure 1B). We also noticed that biofilm production was stronger in strains sensitive to serum compared to resistant strains (Additional file 1A, $p = 0.059$); in strains negative for *vtaA* group 1 genes compared to strains positive for these genes (Additional file 1B, $p = 0.189$); in strains belonging to MLST cluster C compared to strains belonging to MLST cluster A (Additional file 1C, $p = 0.202$), and in strains negative for the sialyltransferase gene *lsgB* compared to strains positive for *lsgB* (Additional file 1D, $p = 0.228$). These differences however were not statistically significant probably due to the small number of strains in some of the groups that were compared.

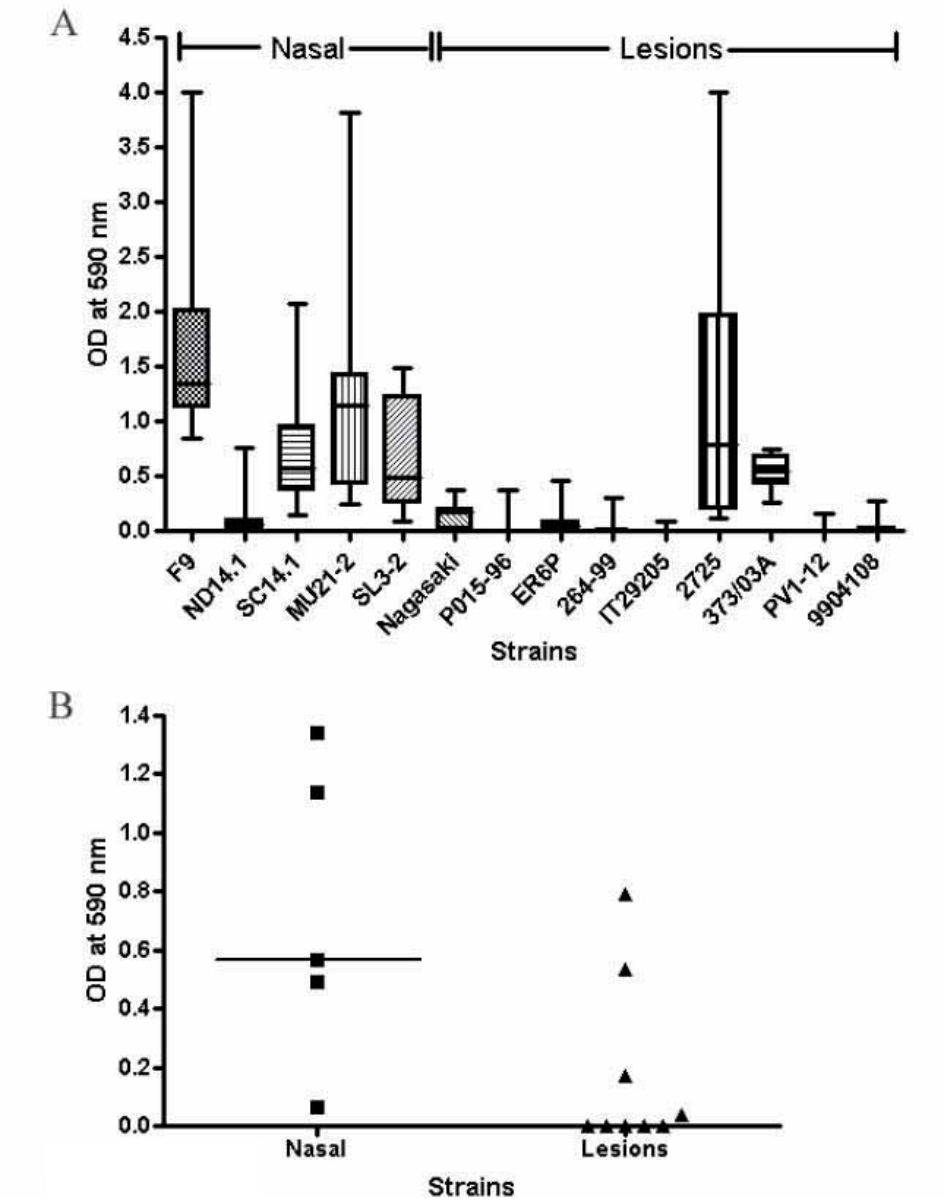


Figure 1. Biofilm formation by *H. parasuis* isolates. (A) Biofilm formation under static conditions in microtiter plates for *Haemophilus parasuis* nasal strains (n = 5) and strains isolated from lesions of pigs with Glässer's disease (n = 9). (B) Medians of biofilm formation for *H. parasuis* strains isolated from the nasal cavities of healthy pigs (n = 5) or for strains isolated from the lesions of pigs with Glasser's disease (n = 9). Difference between the median of the two groups of strains was statistically significant ($p<0.05$).

Confocal laser scanning microscopy using different fluorescent probes was performed with the nasal, high-biofilm producer strains F9 and MU21-2 and the weak-biofilm producer virulent strains Nagasaki and ER-6P. The biofilm cells were stained with FilmTracer^{TM}FM$^{®}$1-43, which becomes fluorescent once it is inserted in the cell membrane. The biofilms were also stained with fluorescent probes to label poly-N-acetylglucosamine (PGA), proteins, or extracellular DNA that could be present in the biofilm matrix. The data indicated that the biofilm matrix of these strains contains PGA, proteins, and eDNA; strain MU21-2 seemed however to produce less PGA than the other three strains (Figure 2). To further characterize the biofilms, 15 images of biofilm layers were recorded and stacked, and 3D-images of the biofilms were generated (Figure 3). Based on these reconstructions, the thickness as well as the biomass of the biofilm produced by each strain was evaluated. The thickness of the weak-biofilm producer strains Nagasaki and ER-6P was approximately 40 μm while the thickness of the high-biofilm producer strains MU21-2 and F9 was 50 μm and 70 μm, respectively. This was in agreement with the biomass ($\mu m^3/ \mu m^2$) which was 17 for the weak-biofilm producer strain Nagasaki while the biomass of the high-biofilm producer strain F9 was 34.

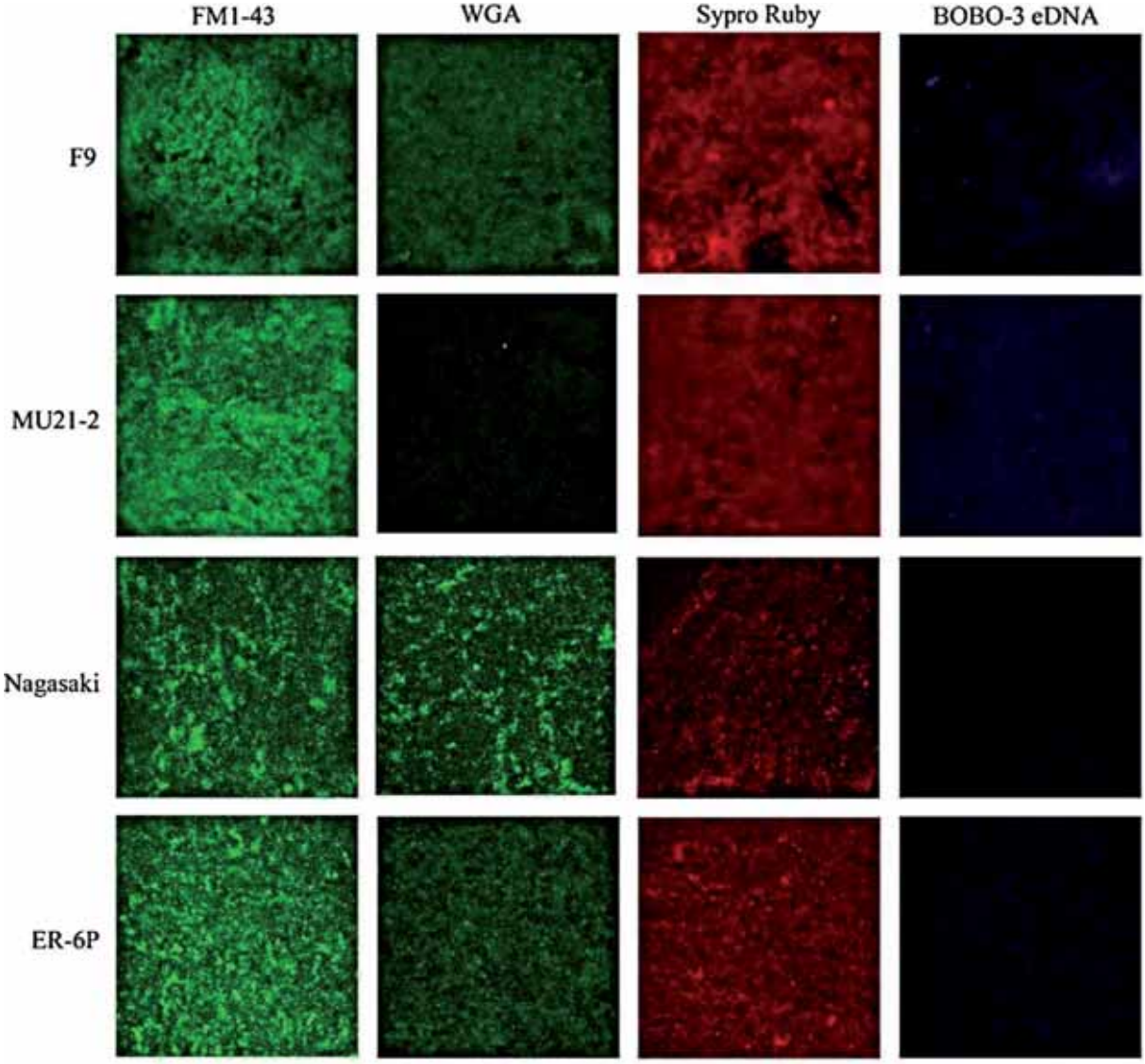


Figure 2. Images of *H. parasuis* biofilms obtained by CLSM. Confocal laser scanning microscopy of *Haemophilus parasuis* strains F9, MU21-2, Nagasaki and ER-6P biofilms formed under static conditions in wells of microtiter plates. Biofilms were stained with FilmTracer™ FM 1-43, wheat-germ agglutinin (WGA)-Oregon green 488 (for poly-*N*-acetyl glucosamine), SYPRO Ruby (for proteins) and BOBO-3 (for eDNA).

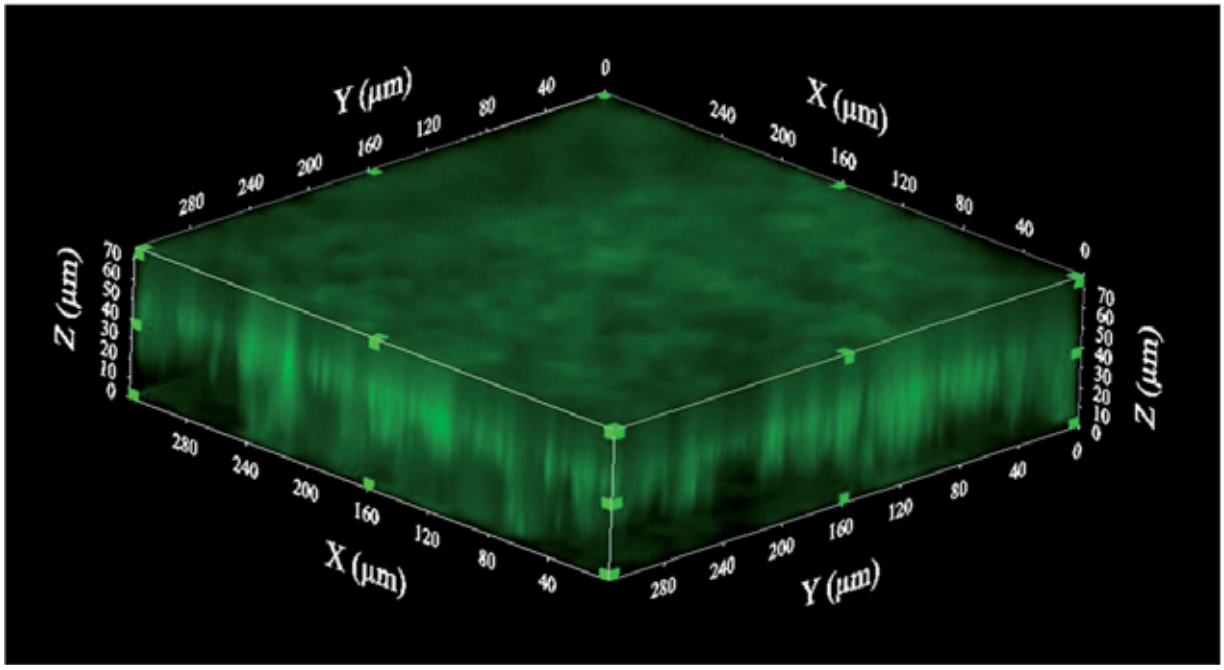


Figure 3. 3D images of *H. parasuis* strain F9 obtained by CLSM. Confocal laser scanning microscopy three-dimensional images of *Haemophilus parasuis* strain F9 biofilm formed under static conditions in wells of a microtiter plate. Biofilm was stained with wheat-germ agglutinin (WGA)-Oregon green 488. Stack of sections of the X-Z plane of the biofilm.

Biofilms of strains F9, MU21-2, Nagasaki and ER-6P were digested with enzymes to further characterize the composition of the matrix. All strains were resistant to dispersin B (more than 70% of the biofilm remained after treatment) (Figure 4A). All strains were however sensitive to the proteinase K treatment (Figure 4B), with strain Nagasaki showing the lowest sensitivity (~80% of the biofilm remaining after treatment) and strain ER-6P showing the highest sensitivity (less than 20% of the biofilm remaining after treatment). Most strains were not affected by DNase I treatment (more than 70% of the biofilm remained after treatment) except strain F9 which was highly sensitive (with less than 20% of the biofilm remaining after treatment) (Figure 4C). This suggested that proteins, and, in one strain, extracellular DNA play a larger role than PGA in *H. parasuis* biofilm formation.

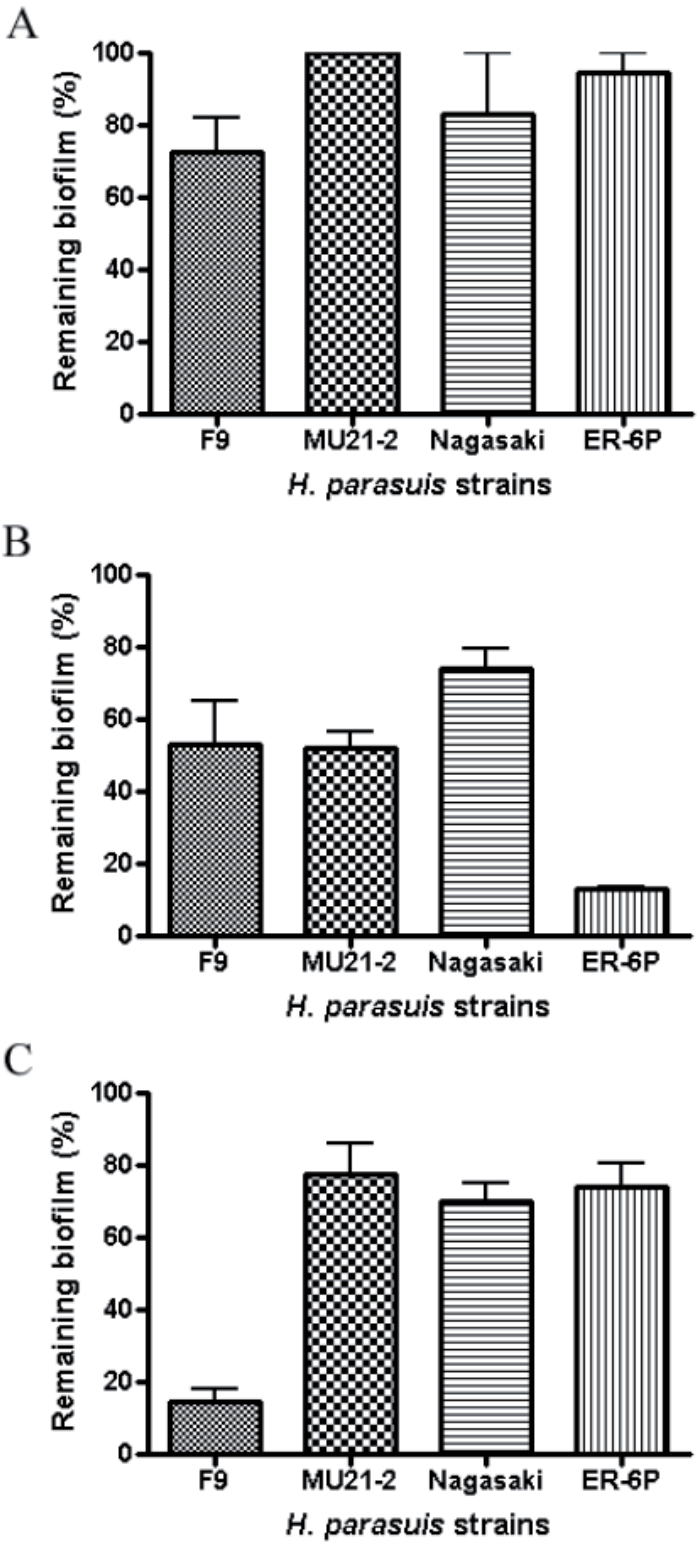


Figure 4. Effect of enzymatic treatments on *H. parasuis* biofilms. Dispersion of *Haemophilus parasuis* biofilms formed under static conditions in microtiter plates by (A) dispersin B, (B) proteinase K, and (C) DNase I.

Biofilm formation under shear force conditions

Previous experiments with *A. pleuropneumoniae* and the drip-flow apparatus indicated that this biofilm reactor could be used to grow biofilm of fastidious *Pasteurellaceae* isolated from the upper respiratory tract [24]. The conditions (50mL/channel/hour; 50% BHI-NAD) used to grow *A. pleuropneumoniae* biofilms in the drip-flow apparatus were used for the first test. However, these did not support the growth of *H. parasuis* biofilms. Given that *H. parasuis* is more fastidious than *A. pleuropneumoniae*, a full strength BHI-NAD was used and the speed of the flow was reduced by half. These conditions allowed the two strains with a strong-biofilm phenotype, MU21-2 and F9, to produce visible biofilms (Figure 5). Although a thin film was observable for Nagasaki and ER-6P, which are strains with a weak-biofilm phenotype under static conditions, the conditions of the drip-flow apparatus did not enhance the biofilm formation by these strains (Figure 5). As indicated by the biofilm dry weight, strain MU21-2 produced a larger biomass than strain F9 under these flow conditions (Table 2).

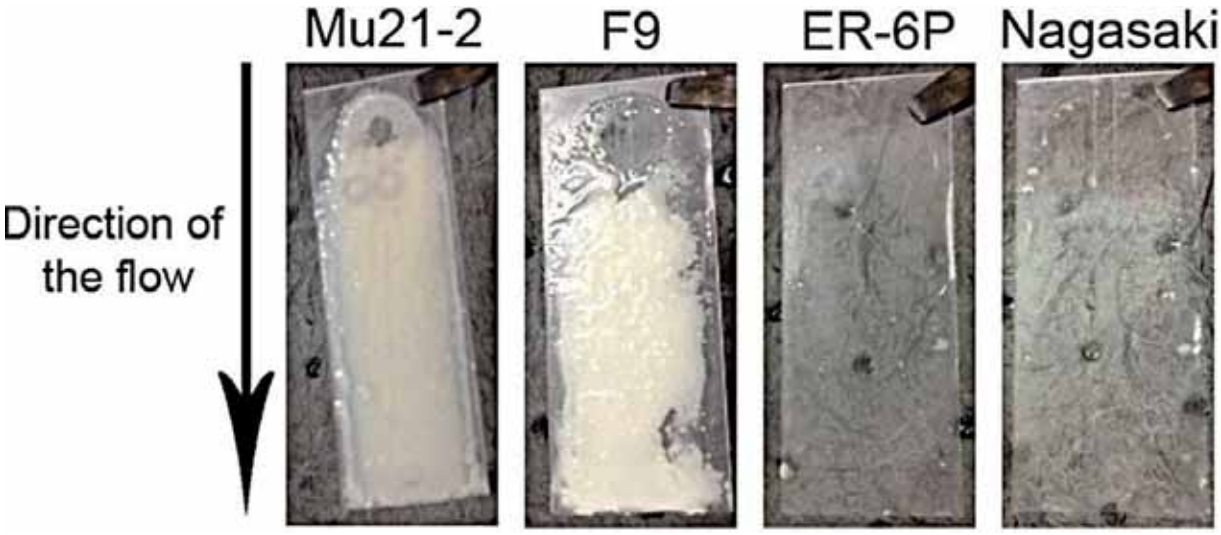


Figure 5. Biofilm formation by *H. parasuis* in a drip-flow apparatus. Biofilm formation under low shear force in a drip-flow apparatus. Images of typical biofilms for *Haemophilus parasuis* strains MU21-2, F9, ER-6P, and Nagasaki visible after 24h of incubation with continuous flow (25 mL/h).

Table 2. Average dry weight (in mg) for drip-flow biofilms formed after 24h of continuous flow by 4 different strains of *Haemophilus parasuis*.

Strain	**Biofilm Dry Weight (±Standard Error of the Mean)**
MU21-2	8.33 (±3.23)
F9	3.37 (±0.38)
Nagasaki	ND[1]
ER-6P	ND

[1]The amount of biofilm was below the detection limit.

The BioFlux 200 flowthrough device is a high throughput microfluidic system that has been recently tested for the growth of bacterial biofilms [25]. This system has yet to be tested with *Pasteurellaceae* and could provide certain advantages over the drip-flow apparatus. For example, the system requires smaller volumes, which range in μL to mL, and can be used for high throughput screens. Initial density of the inoculum (OD_{600} of 1.0, 0.5 and 0.25) and the incubation time (2h and 4h) for the initial attachment were the first parameters tested with a relatively low shear force (0.5 dyne/cm^2). An OD_{600} of 0.25 and an incubation time of 2h for the initial attachment were sufficient for strains MU21-2 and F9 to form biofilms that block the microfluidic channel after 6 hours (Figure 6); however, strains Nagasaki and ER-6P did not form biofilms (Figure 6). To prevent blocking of the channel, a range of shear force was tested with an inoculum at OD_{600} of 0.25 and an incubation time of 2h for the initial attachment. Both strains MU21-2 and F9 were not able to form a biofilm when the shear force was equal or above 0.7 dyne/cm^2. Between 0.5 and 0.7 dyne/cm^2, both strains always blocked the channel within 12 hours but it took longer as the shear force was increased. Therefore, in our hands, this microfluidic system can only be used to study biofilm formation of *H. parasuis* during short incubation periods.

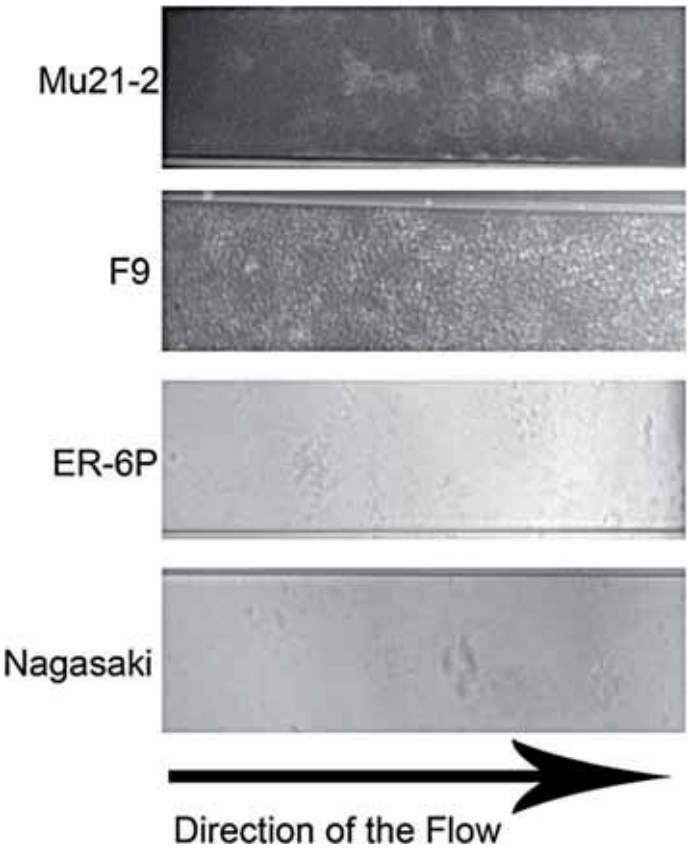


Figure 6. Biofilm formation by *H. parasuis* in a microfluidic system. Biofilm formation under controlled shear force in a BioFlux 200 microfluidic system. Phase-contrast images of typical biofilms of *Haemophilus parasuis* non-virulent strains MU21-2 and F9, and virulent strains ER-6P and Nagasaki obtained after 24h of incubation with an inoculum of OD_{600} of 0.25 and a shear force of 0.5 dyne/cm^2.

Effect of fibrinogen and fibronectin on biofilm formation

It has been shown that supplementing the culture medium with fibrinogen induces biofilm formation of *Streptococcus suis*, another important swine pathogen [46]. Thus, we evaluated the effect of supplemental fibrinogen on *H. parasuis* biofilm formation. As shown in Figure 7, fibrinogen (at a concentration of 1 mg/mL) inhibited biofilm formation by all four strains. Fibronectin had no effect on bioflm formation (data not shown). Inhibition of biofilm formation by fibrinogen was not related to an inhibition of growth since fibrinogen did not affect growth of *H. parasuis* (data not shown).

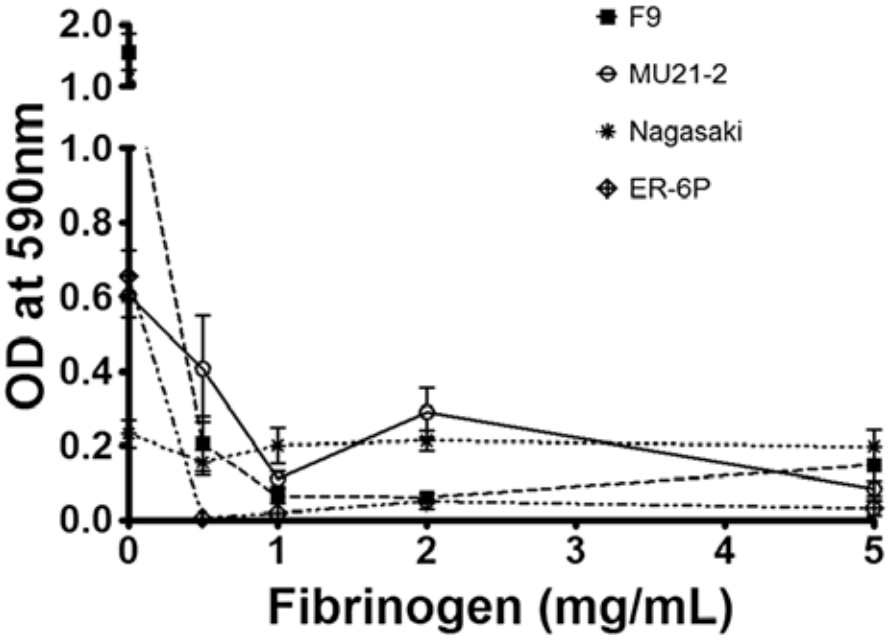


Figure 7. Effects of fibrinogen on biofilm formation by *H. parasuis*. Effects of various concentrations of fibrinogen added to the culture medium on biofilm formation by *Haemophilus parasuis* strains F9, MU21-2, Nagasaki and ER-6P under static conditions in microtiter plates. Assays were performed in triplicate, and the means ± standard deviations are indicated.

Transcriptional profile of H. parasuis F9 grown in biofilm

Given that the genome of strain F9 was not previously sequenced, the genome of this high-biofilm producer was sequenced to facilitate transcriptional analysis. The assembly size of the F9 genome was 2.49 Mb, with an n50 of 44,023 and 644 contigs, with a G+C content of 39%, which is comparable to that of the draft and complete genomes of *H. parasuis* [30, 47]. Transcriptomic analysis was performed with an average of 2M sequence reads per mRNA sample. More than 75% of the reads for each sample were mapped. The majority of reads mapped with a mapping quality (MAPQ) ≥ 20, and only reads mapping with a MAPQ ≥ 10 were further processed for differential gene expression analysis (Additional file 2A). Some reads mapped in non-protein coding sequences, mainly in tRNA gene sequences, and were particularly high in the stationary culture sample (Additional file 2B). Differential expression analysis found 425 DEGs in biofilm (B) when compared to planktonic growth (P) (Table 3). When B or P condition was compared to the stationary-phase culture (S), a notable increase in the number of up-regulated genes was observed (Fig. 8 and Table 3). Filtered lists of DEGs for B *vs* P, P *vs* S and B *vs* S are shown in Additional files 3, 4, 5, respectively. A large number of up- and down-regulated genes were shared between B *vs* S and P *vs* S comparisons (Fig. 9A and 9B), although a

considerable amount was unique to each condition. Fifty-five genes were up-regulated in all three comparisons, which included 8 ribosomal proteins. On the other hand, 56 up-regulated genes were unique to the biofilm and included, among others, six transcriptional regulators, possibly involved in biofilm formation (Additional file 6).

Table 3. Summary of the differential expression analysis performed with edgeR tool (P-value<0.05). Percentages of differentially expressed genes are also shown for each comparison.

Comparison	**Up**	**Down**	**Total**	**[4]Genome (%)**
[1]B *vs* [2]P	212	213	425	19
B *vs* [3]S	538	571	1109	49
P *vs* S	376	417	793	35

[1]Biofilm
[2]Planktonic
[3]Stationary phase
[4]2259 of the 2317 annotated protein-coding genes were taken as total (at least one count per million (cpm) in at least two samples).

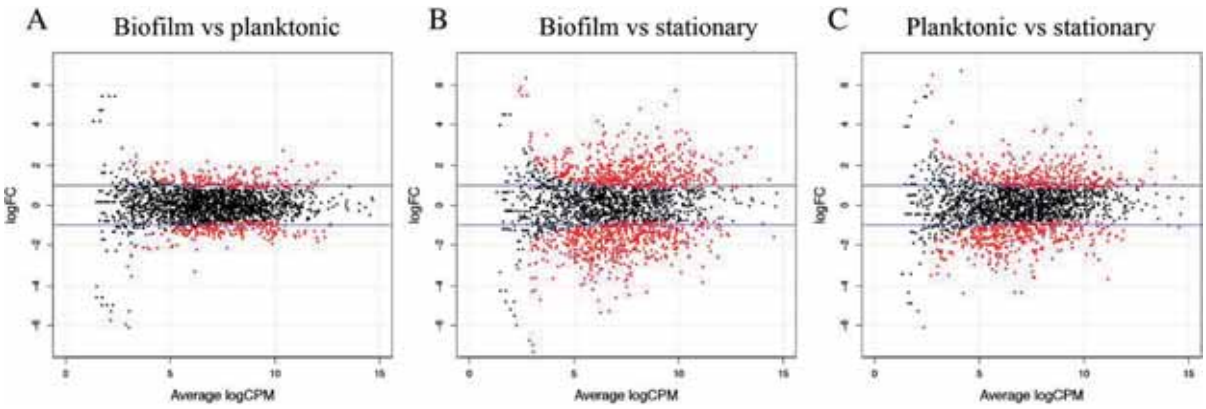


Figure 8. Identification of *H. parasuis* genes differentially expressed. MA plots generated by EdgeR showing transcript expression profiles in the three comparisons performed: biofilm vs planktonic (A), biofilm vs stationary phase (B) and planktonic vs stationary phase (C). For each gene, $\log_2$(fold change) between the two conditions is plotted (M, y axis) against the gene's $\log_2$(average expression) in the two samples (A, x axis). The blue lines indicate 2-fold changes. Red dots highlight the genes at 5% P-value.

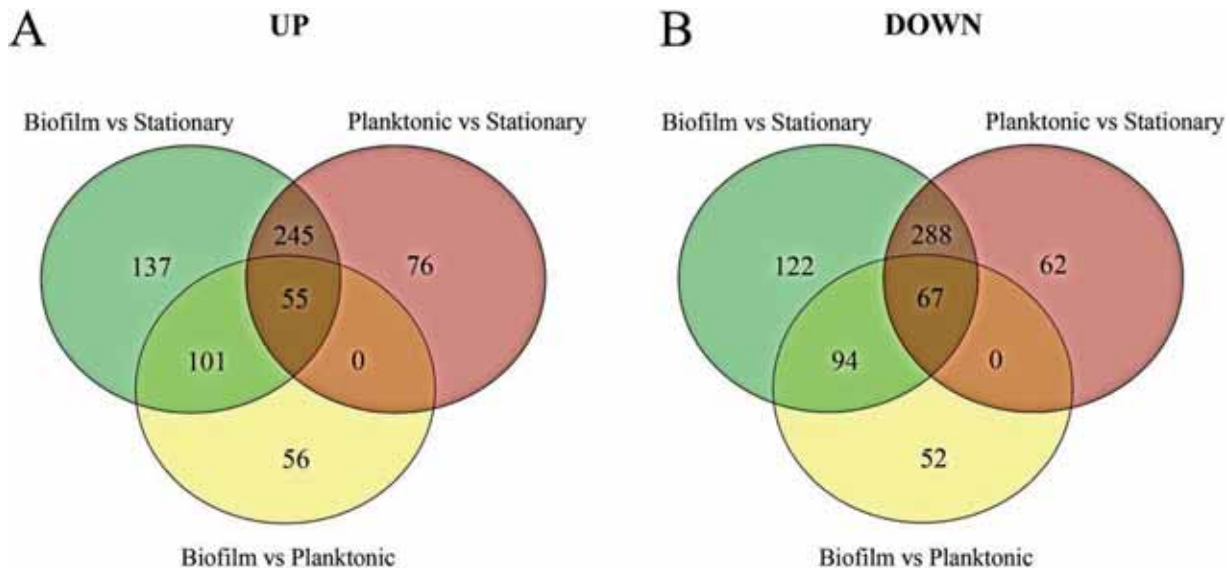


Figure 9. Comparison of *H. parasuis* genes that were up- or down-regulated. Venn diagrams of *Haemophilus parasuis* genes identified as up- (A) and down-regulated (B) under different growth states.

Blast2GO allowed 76% of GO term assignment to *H. parasuis* proteome, with a good GO level distribution (mean level = 6.8; SD = 2.7) and more than 8K annotations. Enrichment analysis of the DEGs ($P<0.05$) identified a large number of up- or down-regulated pathways (Additional file 7). Analysis was visualized performing Venn diagrams (Additional files 8 and 9). All three comparisons shared the enriched up-regulated GO term 'structural constituent of ribosome'. Enrichment of B *vs* S and P *vs* S shared the following GO terms: activation of response to oxidative stress, iron ion binding, ribosome biogenesis, unfolded protein binding and peroxidase activity pathways, indicating the importance of these functions for both biofilm and planktonic growth. Interestingly, protein folding, cell outer membrane, protein secretion, and sequence-specific DNA binding (transcriptional regulators) GO terms were up-regulated specifically in B *vs* S and B *vs* P comparisons, which could indicate specific biofilm pathways. Other GO terms were specific to each comparison. A total of 20 up-regulated GO terms were specific to B *vs* P comparison, such as lipopolysaccharide transport, response to stress or DNA-mediated regulation of transcription (transcriptional regulators) (Additional file 9). The same analysis was performed for down-regulated genes and showed that membrane-related genes were over-represented among these genes in all three comparisons. These membrane-related genes were related to transport, more especially to sodium ion transport or phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS). B *vs* S and P *vs* S detected down-regulated genes related to nutrient transport, such as ions, amino acids or monosaccharides, which indicate common pathways in biofilm and planktonic conditions. On the other hand, some enriched GO terms were specific to biofilm, as evidenced by the B *vs* P comparisons. For instance, GOs related to translation (ribosomal proteins) were among the most significantly down-regulated, which suggest a possible low metabolic state, but these enriched GO terms were caused by different ribosomal proteins than the ones responsible for the shared enriched up-regulated GO term (Additional file 9).

The F9 surface-associated proteins were predicted using Phobius and the conservation of the up-regulated ones was examined by tBLASTn in the 14 *H. parasuis* genomic sequences available in GenBank. The number of predicted membrane proteins up-regulated in F9 grown under biofilm conditions (B *vs* S) was 148 from a total of 538. The majority of the predicted membrane proteins were well conserved among the majority of strains, including a 28 kDa outer membrane protein (HS372_00711), Omp P5 precursor (HS372_01222), PilQ (HS372_02002) and Omp85 (HS372_00165) (Additional file 10).

Others were found exclusively in non-virulent isolates, such as OmpW (HS372_00498), prophage CP4-57 integrase (HS372_00596), filamentous hemagglutinin FhaB (HS372_01074) or five hypothetical proteins (HS372_00147, HS372_01332, HS372_02390, HS372_00611 and HS372_02391) (Additional file 10). Additionally, FhaC (annotated as TpsB) was also found only in the non-virulent strains (Additional file 10).

H. parasuis gene expression during biofilm and planktonic growth was compared to transcriptomic data of *A. pleuropneumoniae*, another swine pathogen member of the *Pasteurellaceae* family, from a previous study [24]. Although preliminary analysis showed that *A. pleuropneumoniae* and *H. parasuis* F9 shared only 60% of the genome, some common up-regulated genes were found under biofilm condition (Table 4), but no predominant pathways were detected, suggesting different regulatory networks for these two species. Notably, proteins related to anaerobic metabolism such as cytochrome c-type protein NapC (HS372_02085) or putative electron transport protein yccM (HS372_02091) and some lipoproteins (HS372_01222 and HS372_00366) were found in both bacteria.

Table 4. Common up-regulated genes between *Haemophilus parasuis* F9 and *Actinobacillus pleuropneumoniae* [22] when grown in biofilm condition.

***H. parasuis* F9**	**App**	**Product**
HS372_02083	APL_1425	Cytochrome c-type protein NapC
HS372_02085	APL_1427	Putative electron transport protein yccM
HS372_02091	APL_1821	50S ribosomal protein L31 type B
HS372_00945	APL_1440	High-affinity zinc uptake system protein znuA precursor
HS372_00147	APL_1894	hypothetical protein
HS372_02009	APL_1423	Putative esterase
HS372_02012	APL_0433	Peptide methionine sulfoxide reductase MsrB
HS372_01222	APL_0460	Outer membrane protein P5 precursor
HS372_00060	APL_1206	putative ribonuclease FitB
HS372_01900	APL_1173	Nicotinamide riboside transporter pnuC
HS372_00666	APL_0442	vancomycin high temperature exclusion protein
HS372_01587	APL_0484	Alpha-aminoadipate--lysW ligase lysX
HS372_02062	APL_0038	hypothetical protein
HS372_00364	APL_0222	Putative lipoprotein/NMB1162 precursor
HS372_01892	APL_0133	Cys regulon transcriptional activator
HS372_00988	APL_1295	Arginine repressor
HS372_02252	APL_1873	Succinyl-diaminopimelate desuccinylase
HS372_02064	APL_0036	hypothetical protein
HS372_01893	APL_0134	hypothetical protein
HS372_01521	APL_1320	Thiamine import ATP-binding protein ThiQ
HS372_02387	APL_1059	Integrase core domain protein
HS372_00061	APL_1207	prevent-host-death family protein
HS372_00916	APL_0423	Ribonuclease HI
HS372_01200	APL_0593	Inosine-5'-monophosphate dehydrogenase
HS372_01950	APL_1574	Putative hydrolase ydeN
HS372_02244	APL_0254	Cytosol non-specific dipeptidase
HS372_01281	APL_1230	Phosphoserine phosphatase
HS372_01385	APL_0967	Glutamate permease
HS372_01220	APL_0463	Putative phosphinothricin acetyltransferase YwnH
HS372_01490	APL_0928	hypothetical protein
HS372_01179	APL_1499	Threonine synthase
HS372_02366	APL_0395	Sigma-E factor negative regulatory protein
HS372_01208	APL_0895	Formate dehydrogenase-N subunit gamma
HS372_01221	APL_0461	putative phosphatase YwpJ
HS372_02265	APL_0687	D-lactate dehydrogenase
HS372_01342	APL_1448	Spermidine/putrescine import ATP-binding protein PotA
HS372_00366	APL_0220	Putative lipoprotein/NMB1164 precursor
HS372_00099	APL_1853	Ketol-acid reductoisomerase

Discussion

In the present study, we used well-characterized virulent and non-virulent strains of *H. parasuis* from our collection [13]. We found that most nasal strains (4/5) were strong-biofilm producers whereas most systemic strains (8/9) formed no biofilm or were very weak-biofilm producers. Similarly, a strong association was found between MLST clusters [2] and biofilm formation. Strains of MLST cluster C (which are associated with nasal isolation) formed more biofilms than strains of MLST cluster A (which are isolated from systemic lesions). Similarly, the presence of autotransporter *vtaA* group 1 genes and of sialyltransferase *lsgB* gene associated with virulent strains [13, 48], and was not associated with strong biofilm formation. Overall, our results are in agreement with Jin et al. [19] who observed that, generally, serovars considered non-virulent showed a higher degree of biofilm formation than virulent serovars. Although it is important to note that a strict relationship between serovars and virulence in *H. parasuis* has not been demonstrated [1].

The use of CLSM and fluorescent probes showed the presence of PGA, proteins, and eDNA in the biofilm matrix of *H. parasuis* nasal, high-biofilm producer strains F9 and MU21-2 and of the weak-biofilm producer strains Nagasaki and ER-6P recovered from lesions of pigs with Glässer's disease. This is, to the best of our knowledge, the first report of the presence of PGA and proteins in the biofilm matrix of *H. parasuis*. PGA is the major component of the biofilm matrix of several bacteria including other *Pasteurellaceae* members such as *A. pleuropneumoniae* and *Aggregatibacter actinomycetemcomitans* [18, 49]. Nevertheless all 4 strains tested were resistant to dispersin B. All strains were however sensitive to a proteinase K treatment suggesting that proteins play a larger role than PGA in *H. parasuis* biofilm formation. Tang et al. [50] have shown that treatment with the staphylococcal nuclease NUC1 decreased slightly the biofilm formation of *H. parasuis* strain 0322. This is in agreement with results of this study showing the presence of eDNA in the biofilm matrix of *H. parasuis* and the high sensitivity of strain F9 biofilm to DNase I digestion.

This is also the first description of *H. parasuis* biofilm formation under shear force in a drip-flow reactor and a microfluidic system. Interestingly, similar biofilm phenotypes (i.e. high-producer vs weak-producer) were observed when static or controlled flow conditions

were used. The drip-flow apparatus is a system that it is thought to create an environment with an air-liquid interface which closely resembles the lung environment [23]. Under the conditions we selected, the two strains with a strong-biofilm phenotype, MU21-2 and F9, produced visible biofilms while a thin film was observable for Nagasaki and ER-6P, which are strains with a weak-biofilm phenotype under static conditions. We also used the BioFlux flowthrough device, a high throughput microfluidic system that has been recently tested for the growth of dental plaque bacteria biofilms [51] and *Pseudomonas aeruginosas* biofilms [25]. Again, strains Nagasaki and ER-6P did not form biofilms under the conditions tested but strains MU21-2 and F9 formed biofilms that rapidly blocked the microfluidic channel. This microfluidic system can therefore be used to study biofilm formation of *H. parasuis* but for short incubation periods. This potentially can be used to investigate genes involved in the early steps of biofilm formation and, if the appropriate tools are combined, to study real-time gene expression during the early steps as demonstrated in *Staphylococcus aureus* [52].

The genes involved in *H. parasuis* biofilm formation are currently not known. Recently, it was reported that both *galU* and *galE* genes seemed to play a role in biofilm formation of *H. parasuis* [12]. A *galU* mutant was unable to form biofilm in a glass tube while a *galE* mutant produced more biofilm than the parent wild-type strain. Surprisingly however, the *galU* mutant also showed an increased tendency to autoagglutinate which is usually associated with a greater ability to form biofilm. Here, the transcriptome of *H. parasuis* F9 strain showed that static biofilm and planktonic cultures are in similar biological states but not identical, whereas greater differences in gene expression were evident when compared to stationary-phase culture. Subtle differences in gene expression between biofilm and planktonic cells have been also reported for other bacterial species [53, 54]. In addition to identifying DEGs related to metabolism, gene enrichment allowed the identification of a large proportion of membrane-related genes among the up-regulated genes in biofilm, including some that have been reported for other bacterial species [53, 54]. The conservation of some highly expressed membrane-protein genes in biofilms among the 14 *H. parasuis* genomes available indicates that those may not specifically associate with biofilm formation. A sub-set of these up-regulated membrane-protein genes, such as the type IV pilus biogenesis protein *pilQ*, may be involved in adhesion to different abiotic and biotic surfaces. Interestingly, some up-regulated genes, such as *fhaB* and *fhaC* (*tpsB*) or *ompW*, were only found in the genome of non-virulent strains. This finding, together with

the fact that non-virulent strains formed stronger biofilms, suggests a possible role of these genes in biofilm formation. In fact, Fha and OmpW, together with type IV pilus, have been shown to play a role in biofilm formation in other bacteria [55, 56]. Additionally, some lipoproteins, such as lipoprotein Plp4, as well as signs of anaerobic metabolism were evidenced in the biofilms of *H. parasuis* and *A. pleuropneumoniae*, which may indicate a potential role in biofilm formation of both bacterial species. The function of specific genes in biofilm formation will need further confirmation.

Interestingly, experimental infections in snatch-farrowed, colostrum-deprived piglets showed that the *H. parasuis* strains that proceeded to invade the host were not maintained well in the nasal cavities of the piglets [57]. This suggests changes in the bacteria from a "colonizing state" to an "invasive state", which could be modulated by the ability of each animal to control the infection. In the light of the results of the present study, these changes from a "colonizing state" to an "invasive state" could also be due to the ability of a given bacterial strain to form or not a robust biofilm. We propose that biofilm formation might allow the non-virulent strains to colonize and persist in the upper respiratory tract of pigs. Conversely, the predominant planktonic state of the virulent strains might allow them to disseminate within the host. This latter statement is supported by the inhibition of biofilm formation by fibrinogen. It is worth noting that a recent study on the human pathogen *Streptococcus pneumoniae* showed that biofilm formation *in vivo* is associated with reduced invasiveness and a dampened cytokine response [58]. High-biofilm production phenotype might therefore not always be linked to virulence.

References

1. Aragon V, Segalés J, Oliveira S: **Glässer's disease.** In *Diseases of Swine*, 10th edition. Edited by Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW. Chichester, UK: Wiley-Blackwell; 2012:760-769.
2. Olvera A, Cerdà-Cuéllar M, Aragon V: **Study of the population structure of *Haemophilus parasuis* by multilocus sequence typing.** *Microbiology* 2006, **152**:3683-3690.
3. Cerdà-Cuéllar M, Aragon V: **Serum-resistance in *Haemophilus parasuis* is associated with systemic disease in swine.** *Vet J* 2008, **175**:384–389.
4. Olvera A, Ballester M, Nofrarias M, Sibila M, Aragon V: **Differences in phagocytosis susceptibility in *Haemophilus parasuis* strains.** *Vet Res* 2009, **40**:24.
5. Aragon V, Bouchet B, Gottschalk M: **Invasion of endothelial cells by systemic and nasal strains of *Haemophilus parasuis*.** *Vet J* 2010, **186**:264–267.
6. Vanier G, Szczotka A, Friedl P, Lacouture S, Jacques M, Gottschalk M: ***Haemophilus parasuis* invades porcine brain microvascular endothelial cells.** *Microbiology* 2006, **152**:135-142.
7. Zhang B, Tang C, Liao M, Yue H: **Update on the pathogenesis of *Haemophilus parasuis* infection and virulence factors.** *Vet Microbiol* 2014, **168:**1-7.
8. Costa-Hurtado M, Aragon V: **Advances in the quest for virulence factors of *Haemophilus parasuis*.** *Vet J* 2013, **198:**571-576.
9. Bouchet B, Vanier G, Jacques M, Gottschalk M: **Interactions of *Haemophilus parasuis* and its LOS with porcine brain microvascular endothelial cells.** *Vet Res* 2008, **39:**42.
10. Bouchet B, Vanicer G, Jacques M, Auger E, Gottschalk M: **Studies on the interactions of *Haemophilus parasuis* with porcine epithelial tracheal cells: limited role of LOS in apoptosis and pro-inflammatory cytokine release.** *Microb Pathog* 2009, **46:**108-113.
11. Xu C, Zhang L, Zhang B, Feng S, Zhou S, Li J, Zou Y, Liao M: **Involvement of lipooligosaccharide heptose residues of *Haemophilus parasuis* SC096 strain in serum resistance, adhesion and invasion.** *Vet J* 2013, **195:**200-204.
12. Zou Y, Feng S, Xu C, Zhang B, Zhou S, Zhang L, He X, Li J, Yang Z, Liao M: **The role of *galU* and *galE* of *Haemophilus parasuis* SC096 in serum resistance and biofilm formation.** *Vet Microbiol* 2013, **162**:278-284.
13. Martínez-Moliner V, Soler-Llorens P, Moleres J, Garmendia J, Aragon V: **Distribution of genes involved in sialic acid utilization in strains of *Haemophilus parasuis*.** *Microbiology* 2012, **158**:2117-2124.
14. Pina S, Olvera A, Barcelo A, Bensaid A: **Trimeric autotransporters of *Haemophilus parasuis*: generation of an extensive passenger domain repertoire specific for pathogenic strains.** *J Bacteriol* 2009, **191**:576-587.
15. Costa-Hurtado M, Ballester M, Galofré-Milà N, Darji A, Aragon V: **VtaA8 and VtaA9 from *Haemophilus parasuis* delay phagocytosis by alveolar macrophages.** *Vet Res* 2012, **43**:57.
16. Costerton JW, Stewart PS, Greenberg EP: **Bacterial biofilms: a common cause of persistent infections.** *Science* 1999, **284**:1318-1322.

17. Hall-Stoodley L, Costerton JW, Stoodley P: **Bacterial biofilms: from the natural environment to infectious diseases.** *Nat Rev Microbiol* 2004, **2**:95-108.
18. Jacques M, Aragon V, Tremblay YDN: **Biofilm formation in bacterial pathogens of veterinary importance.** *Anim Health Res Rev* 2010, **11**:97-121.
19. Jin H, Zhou R, Kang M, Luo R, Cai X, Chen H: **Biofilm formation by field isolates and reference strains of *Haemophilus parasuis*.** *Vet Microbiol* 2006, **118**:117-123.
20. Jin H, Wan Y, Zhou R, Li L, Luo R, Zhang S, Hu J, Langford PR, Chen H: **Identification of gene transcribed by *Haemophilus parasuis* in necrotic porcine lung through the selective capture of transcribed sequences (SCOTS).** *Environ Microbiol* 2008,**10:**3326-3336.
21. Wu C, Labrie J, Tremblay YDN, Haine D, Mourez M, Jacques M: **Zinc as an agent for the prevention of biofilm formation by pathogenic bacteria.** *J Appl Microbiol* 2013, **115:**30-40.
22. Labrie J, Pelletier-Jacques G, Deslandes V, Ramjeet M, Auger E, Nash JH, Jacques M: **Effects of growth conditions on biofilm formation by *Actinobacillus pleuropneumoniae*.** *Vet Res* 2010, **41**:03.
23. Goeres DM, Hamilton MA, Beck NA, Buckingham-Meyer K, Hilyard JD, Loetterle LR, Lorenz LA, Walker DK, Stewart PS: **A method for growing a biofilm under low shear at the air-liquid interface using the drip flow biofilm reactor.** *Nat Protoc* 2009, **4**:783-788.
24. Tremblay YDN, Deslandes V, Jacques M: ***Actinobacillus pleuropneumoniae* genes expression in biofilms cultured under static conditions and in a drip-flow apparatus.** *BMC Genomics* 2013, **14**:364.
25. Benoit MR, Conant CG, Ionescu-Zanetti C, Schwartz M, Matin A: **New device for high-throughput viability screening of flow biofilms.** *Appl Environ Microbiol* 2010, **76**:4136-4142.
26. Tremblay YDN, Lamarche D, Chever P, Haine D, Messier S, Jacques M: **Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms.** *J Dairy Sci* 2013, **96**:234-246.
27. Quail MA, Kozarewa I, Smith F, Scally A, Stephens PJ, Durbin R, Swerdlow H, Turner DJ: **A large genome centre's improvements to the Illumina sequencing system.** *Nat Methods* 2008, **5:**1005–1010.
28. Quail M, Swerdlow H, Turner DJ: **Improved protocols for the Illumina genome analyzer sequencing system.** *Curr Protoc Hum Genet* 2009, **18:**1–27.
29. Lunter G, Goodson M: **Stampy : A statistical algorithm for sensitive and fast mapping of Illumina sequence reads**. *Genome Res* 2011, **21:**936–939.
30. Xu Z, Yue M, Zhou R, Jin Q, Fan Y, Bei W, Chen H: **Genomic characterization of *Haemophilus parasuis* SH0165, a highly virulent strain of serovar 5 prevalent in China.** *PloS One* 2011, **6:**e19631.
31. Martin M: **Cutadapt removes adapter sequences from high-throughput sequencing reads.** *EMBnet.journal* 2011, **17(1):**10–12.
32. Joshi N: UC Davis Bioinformatics Core [https://github.com/ucdavis-bioinformatics/sickle]

33. Zerbino DR, Birney E: **Velvet: algorithms for de novo short read assembly using de Bruijn graphs.** *Genome Res* 2008, **18(5):**821–829.

34. Gladman S: [http://www.vicbioinformatics.com/velvetoptimiser.manual.txt]

35. Seemann T: [http://bioinformatics.net.au/ prokka-manual.html]
36. Oliveira S, Galina L, Pijoan C: **Development of a PCR test to diagnose *Haemophilus parasuis* infections.** *J Vet Diagn Invest* 2001, **13:**495-501.
37. FastQC [http://www.bioinformatics.babraham.ac.uk/projects/fastqc/]

38. FASTX-Toolkit [http://hannonlab.cshl.edu/fastx_toolkit/]

39. Torrent Mapping Alignment Program: [https://github.com/iontorrent/TMAP]

40. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R and 1000 Genome Project Data Processing Subgroup: **The sequence alignment/map (SAM) format and SAMtools.** *Bioinformatics* 2009, **25:**2078-2079.

41. Thorvaldsdóttir H, Robinson JT, Mesirov JP: **Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration.** *Brief Bioinform* 2013, **14(2):**178-192.
42. HTSeq v0.5.4p3: [https://pypi.python.org/pypi/HTSeq]

43. Robinson MD, McCarthy DJ, Smyth GK: **edgeR: a Bioconductor package for differential expression analysis of digital gene expression data.** *Bioinformatics* 2010, **26:**139-140.

44. Conesa A, Götz S, Garcia-Gomez JM, Terol J, Talon M, Robles M: **Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research.** *Biofinformatics* 2005, **21:**3674-3676.

45. Käll L, Krogh A, Sonnhammer EL: **A combined transmembrane topology and signal peptide prediction method.** *J Mol Biol* 2004, **338:**1027-36.

46. Bonifait L, Grignon L, Grenier D: **Fibrinogen induces biofilm formation by *Streptococcus suis* and enhances its antibiotic resistance.** *Appl Environ Microbiol* 2008, **74**:4969-4972.
47. Mullins MA, Register KB, Bayles DO, Dyer DW, Kuehn JS, Phillips GJ: **Genome sequence of Haemophilus parasuis strain 29755.** *Stand Genomic Sci* 2011, **5:**61-68.
48. Olvera A, Pina S, Macedo N, Oliveira S, Aragon V, Bensaid A: **Identification of potentially virulent strains of *Haemophilus parasuis* using a multiplex PCR for virulence-associated autotransporters (*vtaA*).** *Vet J* 2012, 191:213-218.
49. Kaplan JB, Velliyagounder K, Ragunath C, Rohde H, Mack D, Knobloch JK, Ramasubbu N: **Genes involved in the synthesis and degradation of matrix polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* biofilms.** *J Bacteriol* 2004, 186:8213–8220.
50. Tang JN, Kang MS, Chen HC, Shi XM, Zhou R, Chen J, Du YW: **The staphylococcal nuclease prevents biofilm formation in *Staphylococcus aureus* and other biofilm-forming bacteria.** *Sci China Life Sci* 2011, 54:863-869.
51. Nance WC, Dowd SE, Samarian D, Chludzinski J, Delli J, Battista J, Rickard AH: **A high-throughput microfluidic dental plaque biofilm system to visualize and**

quantify the effect of antimicrobials. *J Antimicrob Chemother* 2013, **68**:2550-2560.

52. Moormeier DE, Endres JL, Mann EE, Sadykov MR, Horswill AR, Rice KC, Fey PD, Bayles KW: **Use of microfluidic technology to analyze gene expression during *Staphylococcus aureus* biofilm formation reveals distinct physiological niches.** *Appl Environ Microbiol* 2013, **79**:3413-3424.
53. Yadav MK, Kwon SK, Cho CG, Park S-W, Chae S-W, Song J-J: **Gene expression profile of early in vitro biofilms of *Streptococcus pneumoniae.*** *Microbiol Immunol* 2012, **56:**621-629.
54. Dötsch A, Eckweiler D, Schniederjans M, Zimmerman A, Jensen V, Scharfe M, Geffers R, Häussler S: **The *Pseudomonas aeruginosa* transcriptome in planktonic cultures and static biofilms using RNA sequencing.** *PLoS One* 2012, **7(2):**e31092.
55. Ritter A, Com E, Bazire A, Goncalves Mdos S, Delage L, Le Pennec G, Pineau C, Dreanno C, Compère C, Durour A: **Proteomic studies highlight outer-membrane proteins related to biofilm development in the marine bacterium *Pseudoalteromonas sp.* D41.** *Proteomics* 2012, **12:**3180-92.
56. Irie Y, Mattoo S, Yuk MH: **The Bvg virulence control system regulates biofilm formation in *Bordetella bronchiseptica.*** *J Bacteriol* 2004, **186**:5692-5698.
57. Aragon V, Cerdà-Cuéllar M, Fraile L, Monbarg M, Nofrarias M, Olvera A, Sibila M, Solanes D, Segalés J: **Correlation between clinico-pathological outcome and typing of *Haemophilus parasuis* field strains.** *Vet Microbiol* **142**:387-393.
58. Blanchette-Cain K, Hinojosa CA, Suresh Babu RA, Lizcano A, Gonzalez-Juarbe N, Munoz-Almagro C, Sanchez CJ, Bergman MA, Orihuela CJ: ***Streptococcus pneumoniae* biofilm formation is strain dependent, multifactorial, and associated with reduced invasiveness and immunoreactivity during colonization.** *mBio* 2013, doi:10.1128/mBio.00745-13.

Additional Files

Additional files can be found in the electronic version of this thesis.

Additional file 1 (.tif)

***Haemophilus parasuis* biofilm formation under static conditions in microtiter plates.** **(A)** Medians of biofilm formation for strains that are sensitive or show intermediate resistance to serum (n = 9) or for strains that are resistant to serum (n = 5). **(B)** Medians of biofilm formation for strains negative (n = 5) or positive (n = 9) for *vtaA* group 1 genes. **(C)** Medians of biofilm formation for strains belonging to MLST cluster C (n = 5) or strains belonging to MLST cluster A (n = 7). **(D)** Medians of biofilm formation for strains negative (n = 8) or positive (n = 6) for the sialyltransferase gene *lsgB*. Differences between the median of the two groups of strains were not statistically significant (A: p = 0.059; B: p = 0.189; C: p = 0.202; D: p = 0.228).

Additional file 2 (.tif)

Mapping of RNA sequencing reads to the *H. parasuis* strain F9 genome.

Overview of the mapping (A) and read counts (B) results. Mapping quality (MAPQ) shows that most of the reads were aligned with MAPQ ≥ 20 but a considerable percentage of reads were not taken into account for differential expression because mapping to non-protein coding regions, particularly for stationary culture sample.

Additional file 3 (.xls)

List of *H. parasuis* strain F9 genes differentially expressed in biofilm vs planktonic cells.

Differentially expressed genes in biofilm versus planktonic *H. parasuis* cells (FDR<0.05). Sorted by logFC. *Actinobacillus pleuropneumoniae* (App) first BLASTp hits are also shown.

Additional file 4 (.xls)

List of *H. parasuis* strain F9 genes differentially expressed in planktonic vs stationary phase cells.

Differentially expressed genes in planktonic versus stationary *H. parasuis* cells (FDR<0.05). Sorted by logFC. *Actinobacillus pleuropneumoniae* (App) first BLASTp hits are also shown.

Additional file 5 (.xls)

List of *H. parasuis* strain F9 genes differentially expressed in biofilm vs stationary phase cells.

Differentially expressed genes in biofilm versus stationary *H. parasuis* cells (FDR<0.05). Sorted by logFC. *Actinobacillus pleuropneumoniae* (App) first BLASTp hits are also shown.

Additional file 6. (.xls)

List of *H. parasuis* strain F9 up-regulated genes or unique to biofilm.

Haemophilus parasuis F9 genes up-regulated in all three comparisons (biofilm vs planktonic; biofilm vs stationary phase; planktonic vs stationary phase) or unique to biofilm.

Additional file 7 (.xls)

List of gene ontology (GO) terms following enrichment analysis of *H. parasuis* strain F9 differentially expressed genes.

Enriched Gene Ontology biological process and cellular component nodes among up-regulated genes (FDR<0.001). No enriched CC GO terms were found for biofilm vs planktonic comparison.

Additional file 8 (.tif)

Visualization of gene ontology (GO) terms following enrichment analysis of *H. parasuis* strain F9 differentially expressed genes.

Venn diagrams of *Haemophilus parasuis* enriched Gene Ontology (GO) terms among the subsets of differentially expressed genes identified as up- (A) and down-regulated (B) under different growth states. Only most specific GO terms are shown.

Additional file 9 (.xls)

List of *H. parasuis* strain F9 GO terms shown in Additional file 8 diagrams.

H. parasuis F9 GO terms results from Additional file 8 Venn diagrams.

Additional file 10 (.xls)

List of *H. parasuis* strain F9 membrane-related genes differentially expressed in biofilm compared to planktonic or stationary phase cells.

Conservation of *Haemophilus parasuis* F9 membrane-related genes differentially expressed (P<0.05) in biofilms vs stationary culture or biofilms vs planktonic culture among 14 *H. parasuis* isolates. Genes are sorted by logFC. *Truncated.

3. RNA expression of *Haemophilus parasuis* in the lung

Metatranscriptomics reveals metabolic adaptation and induction of virulence factors by *Haemophilus parasuis* during lung infection

Bello-Ortí et al., 2015. Vet Res (in press)

Abstract

Haemophilus parasuis is a common inhabitant of the upper respiratory tract of pigs, and the causative agent of Glässer's disease. This disease is characterized by polyserositis and arthritis, produced by the severe inflammation caused by the systemic spread of the bacterium. After an initial colonization of the upper respiratory tract, *H. parasuis* enters the lung during the early stages of pig infection. In order to study gene expression at this location, we sequenced the *ex vivo* and *in vivo H. parasuis* Nagasaki transcriptome in the lung using a metatranscriptomic approach. Comparison of gene expression under these conditions with that found in conventional plate culture showed generally reduced expression of genes associated with anabolic and catabolic pathways, coupled with up-regulation of membrane-related genes involved in carbon acquisition, iron binding and pathogenesis. Some of the up-regulated membrane genes, including ABC transporters, virulence-associated autotransporters (*vtaA*s) and several hypothetical proteins, were only present in virulent *H. parasuis* strains, highlighting their significance as markers of disease potential. Finally, the analysis also revealed the presence of numerous antisense transcripts with possible roles in gene regulation. In summary, this data sheds some light on the scarcely studied *in vivo* transcriptome of *H. parasuis*, revealing nutritional virulence as an adaptive strategy for host survival, besides induction of classical virulence factors.

Introduction

Haemophilus parasuis is the causative agent of Glässer's disease, an infectious disease of pigs characterised by fibrinous polyserositis. The current strategies for disease control are based on rapid diagnostics, the use of antibiotics and to a lesser extend vaccines [1]. Antibiotics have been extensively used for this purpose, but current recommendations focus on reduction of their use to avoid the emergence of drug resistance [2-4]. Antibodies can control disease [5] in a mechanism that, at least in part, relies on opsonisation, which renders the virulent phagocytosis resistant strains susceptible to killing by alveolar macrophages [6]. Vaccines, as well as probiotics, are candidates to replace antimicrobials as preventive agents [7,8]. Virulence factors, especially those important for the initial stages of infection, are ideal targets for vaccine design in order to block the pathogenesis potential of bacteria. In that regard, some *H. parasuis* virulence factors have been reported in the literature, and were reviewed recently [9,10].

Numerous works have indirectly linked specific *H. parasuis* genes to its pathogenicity, but direct demonstration of their role during infection is still lacking. In addition, these studies have been typically driven by the homology to previously reported virulence factors in other bacterial species from the *Pasteurellaceae* family. Moreover, pathogenic mechanisms, such as immunomodulation or mechanisms for nutrient acquisition during host infection, could be linked to unsuspected virulence factors [11,12]. After intranasal inoculation, virulent *H. parasuis* can be detected in the lung, from where it can spread causing systemic infection, with the consequent severe inflammation [13,14]. In the lung, *H. parasuis* is detected inside macrophages and neutrophils, but also within epithelial cells [14]. Survival of *H. parasuis* in the lung environment seems to be linked to the phagocytosis resistance capacity of the strain, but other unknown virulence mechanisms cannot be ruled out [14,15]. To address this issue, *in vivo* approaches coupled with hypothesis generating strategies, such as high-throughput RNA sequencing (RNA-seq), could add additional insights into *H. parasuis* pathogenic mechanisms. To our knowledge, no studies have been reported regarding transcriptomic analysis of *H. parasuis* during infection. Few papers have been published in the *Pasteurellaceae* family, but only Jorth et al. [17] applied high-resolution transcriptomics [16-19]. To fill this gap in *H. parasuis* infection control, we have used a metatranscriptomic approach to study *H. parasuis*

pathogenesis in the pig lung. Gene expression profiling, and more recently RNA-seq, has been established as the *de facto* gold standard technique to tackle the survival strategies of numerous bacterial pathogens [20-22]. The specific objective of this work was to study *H. parasuis* gene expression during lung infection, with a special focus on previously reported virulence factors [10]. We found that *H. parasuis* changes its global gene expression during lung infection. A down-regulation of *H. parasuis* metabolism in the lung was accompanied by the induction of the expression of known virulence-factors together with genes of unknown function.

Materials And Methods

RNA samples and sequencing

The virulent *H. parasuis* Nagasaki strain was chosen for transcriptomic analysis [GenBank: ANKT01000000]. This strain was originally isolated from the meninges of a pig with a systemic infection by *H. parasuis* in Japan. Gene annotations are based on previous analysis [23]. Further pathway inspection was performed with Integrated Microbial Genomes (IMG) [24] and BioCyc [25].

Animal experiments were performed in accordance with the regulations required by the Ethics Commission in Animal Experimentation of the Generalitat de Catalunya (Approved Protocol number 5796).

To examine gene expression during lung infection, *ex vivo* incubation of the bacteria in porcine lungs was carried out. Nagasaki grown overnight on chocolate agar plates was resuspended in a final volume of 20 ml sterile PBS (aprox. 10^7 CFU/mL). A healthy 6 week old pig was euthanized and lungs were extracted under sterile conditions and transported to the laboratory, where they were inoculated with the bacterial suspension. After 2 h of incubation at 37 °C with 5% CO_2, bacteria were recovered from the lung by lavage with 100 mL of cold sterile PBS. Colony counts were performed before and after incubation. Next, the brochoalveolar lavage fluid (BALF) was subjected to differential centrifugation to eliminate pulmonary cells (460 × *g*, 10 min) and recover bacteria in a second centrifugation (3220 × *g*, 20 min). Bacterial pellet was processed for RNA extraction following two hot phenol extractions, previously described [26]. The protocol included depletion of host and bacterial rRNA with a Ribo-Zero kit (Epicentre). This

bacterial RNA sample is referred as the *ex vivo* sample for simplicity through the rest of the text. A sequencing library was generated using an Ion Torrent RNA-Seq v2 kit (Life Technologies) and RNA was sequenced using an Ion Torrent PGM instrument (Life Technologies) with an Ion 316 chip (Life technologies) at the Centre for Research in Agricultural Genomics (CRAG, Campus de Bellaterra-UAB, Spain). As control, RNA from the Nagasaki strain grown overnight on chocolate agar plates was purified and sequenced in the same manner. A replicate of the complete experiment was processed in the same manner.

To assess the validity of the *ex vivo* model, RNA was obtained from Nagasaki recovered from the lungs of a pig after a short *in vivo* infection. Nagasaki grown overnight at 37 °C and 5% CO_2 on chocolate agar plates was resuspended in 20 mL of PBS (aprox. 10^7 CFU/mL). This bacterial suspension was intratracheally inoculated in a 6 week old pig. A second pig was mock inoculated with the same quantity of PBS. After two hours, the two pigs were euthanized and lungs were processed as above to obtain a bacterial pellet. This bacterial sample is referred as the *in vivo* sample for simplicity through the rest of the text. The pellets from the low speed centrifugation containing alveolar macrophages were processed to study the expression of surface markers CD163, sialoadhesin (or CD169), SLAI and SLAII by flow cytometry following previous described protocol [27]. Bacterial RNA was purified as above and libraries were generated using NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina®. Sequencing in one paired-end Illumina MiSeq run was performed at Institut de Biotecnologia i Biomedicina (IBB, Campus de Bellaterra-UAB, Spain). As control, RNA from the Nagasaki strain grown overnight on chocolate agar plates was purified and sequenced in the same manner. The NEBNext Ultra Directional kit uses the dUTP method to preserve "directionality of the library".

Transcriptomic analysis

RNA-seq analysis was based on the *H. parasuis* Nagasaki draft genome. The genome is 2.3 Mb in length and was assembled in 47 scaffolds [23]. The Nagasaki genome encodes 2260 protein-coding genes (with 40 additional putative pseudogenes): 329 annotated as transporters, 754 connected to KEGG pathways and 1418 to KEGG orthology (KO), 1,571 are COG annotated and 1918 associated to Pfam. This information is available in IMG.

For both *in vivo* and *ex vivo* experiments, bioinformatic analysis was performed following the count-based differential expression method [28], with some modifications [26]. The procedure was the same used previously; however, BWA aligner v0.7.9a-r786 was used [29] and minimum mapping quality (MAPQ) was increased to 20. SAMStat was used to get additional mapping statistics [30]. Only protein coding genes with one or more counts per million (cpm) in at least one of the samples compared were used. EdgeR tool was chosen for differential gene expression, defining differentially expressed genes at $FDR < 0.05$. EdgeR biological coefficient of variation (BCV) was calculated using the data from the *ex vivo* experiment, and was also used to analyse the *in vivo* data. Enrichments were performed using BLAST2GO built-in tool [31], using $P < 0.05$ as threshold. Enrichment results were further processed using REVIGO online tool [32].

In an attempt to detect putative virulence factors, up-regulated genes in the *in vivo* or *ex vivo* experiments compared to agar plate growth were further analysed. Transmembrane domains and presence of signal peptide motifs were predicted using Phobius [33]. In addition, presence of these membrane-related genes in a database containing 10 virulent and 14 non-virulent isolates of *H. parasuis* was also studied. This database contains some of the *H. parasuis* genomes that were recently published [34] [EMBL:ERS132054-EMBL:ERS132066, EMBL:ERS132069, EMBL:ERS132073, EMBL:ERS132075, EMBL:ERS132076, EMBL:ERS132078- EMBL:ERS132084]. Automated genome annotation was performed by Howell et al. [34]. The predicted proteome of Nagasaki was compared against the database of *H. parasuis* proteins using BLASTP with a cut-off value of $E = 1 \times 10\text{-}5$. Proteomes were parsed from GenBank files using a Biopython script. Settings to define homology were: identities ≥ 90% and query coverage ≥ 80% per high-scoring segment pair (HSP). All transcriptomic data were deposited in the Gene Expression Omnibus database [GEO: GSE63851].

Non-coding RNAs (ncRNAs) were also examined. Read mapping to the complement strand were analysed with the same pipeline as the sense reads, including differential expression and enrichment. Antisense RNAs (asRNAs) have been reported in the literature overlapping more than one gene, but also partially overlapping the target gene, commonly known as *cis*-encoded sRNAs. For simplicity, in this study we took one gene as the unit of analysis, and therefore we did not take into account the possible overlap of an asRNA with more than one gene. Reverse transcription (RT)-PCR was performed to validate four

asRNAs using SuperScriptTM III Reverse Transcriptase (Life Technologies). Only a forward primer was used in the RT step. Each putative asRNA was validated in plate and *in vivo* RNA samples, using Nagasaki DNA as positive control. To ensure DNA absence, samples without prior reverse transcription were also amplified.

RT-qPCR

To validate *in vivo* and *ex vivo* RNA-seq runs, primers were designed for selected genes (listed in Additional file 1). Also, some putative virulence factors were validated in these two samples, as well as in additional *in vivo* samples from a previous experiment [27]. Briefly, snatch-farrowed colostrum-deprived piglets were intranasally inoculated with 5×10^6 CFU of Nagasaki. After 1, 2 and 3 days post infection (dpi), pigs were euthanized and lungs were recovered. Bronchoalveolar lavage was performed and bacteria were obtained for RNA purification as described above. Samples were retrotranscribed using SuperScript® VILO™ Master Mix (Life Technologies). qPCRs were performed in triplicates in the 7500 Fast Real-Time PCR System with SYBR® Green Real-Time PCR Master Mix (Life Technologies). Each sample belonged to one animal at the specific time post infection. Expression of the selected genes was compared to plate culture. Results were analysed using the $\Delta\Delta C_t$ method.

Results

As a facultative anaerobe, *H. parasuis* Nagasaki is expected to have the ability to generate energy via fermentation or respiration. In fact, when metabolic pathways were inspected using IMG and BioCyc databases, genes involved in both aerobic and anaerobic respiration were detected (oxygen and nitrate as terminal electron acceptors), as well as formate and acetate fermentation genes. Sugar transport systems such as ATP-binding cassette (ABC) transport complexes and phosphotransferase systems (PTS) were encoded in its genome, as well as the genes associated to glycolysis, gluconeogenesis, tricarboxylic acid (TCA) cycle and the pentose phosphate pathway. From the anabolic side, pathways for the biosynthesis of amino acids, nucleotides, cofactors, fatty acids as well as heme were present. Natural competence, adherence and secretion capabilities, lipooligosaccharide (LOS) biosynthesis, proteases and iron acquisition completed the repertoire of genes that should give Nagasaki strain the potential to colonize and survive during host infection.

In order to mine RNA-seq results, a custom GO term database was built to include more functionally annotated genes and therefore be potentially more suitable for pathway mining than the KEGG annotations assigned by IMG. Using this custom database, at least one GO term was assigned to 74% of the 2260 protein coding genes. A good GO level distribution (mean level = 6.8; SD = 2.7) and more than 8000 annotations were used for pathway mining. Since 25% of the genes did not have any associated GO term, they were not taken into account when performing enrichments, and a separate analysis had to be performed to mine possible up-regulated membrane-related genes.

RNA-seq run statistics

A range of 2.8-3.2M and 2.6-3.7M reads were obtained with Ion Torrent from the *ex vivo* samples and the corresponding plate samples, respectively. Alignment to the Nagasaki genome was successful for 53-87% of the reads from the *ex vivo* samples and 61-90% of the reads from the pure plate cultures. To perform differential expression, BCV was calculated and a value of 0.3 was obtained. Differential expression analysis of the *ex vivo* samples revealed 765 differentially expressed genes (DEGs) with statistical significance (*FDR*<0.05). From these, 393 were up-regulated and 372 down-regulated after 2 h incubation in lung explants (Figure 1A and Additional file 2A). RNA-seq results were

validated by RT-qPCR using selected genes belonging to key altered pathways, such as cofactor biosynthesis (6-pyruvoyl tetrahydrobiopterin synthase), TCA cycle (type II citrate synthase), phenylalanine, tyrosine and tryptophan biosynthesis (anthranilate synthase component II), heme export (heme exporter protein D), mannose transport (PTS system mannose-specific transporter subunit IID) and nutrient transport (ABC transporter inner membrane subunit), being the latter also a putative virulence factor (Table 1). Differential gene expression was confirmed with a significant correlation between RT-qPCR and RNA-seq (r=0.95, $P < 0.05$).

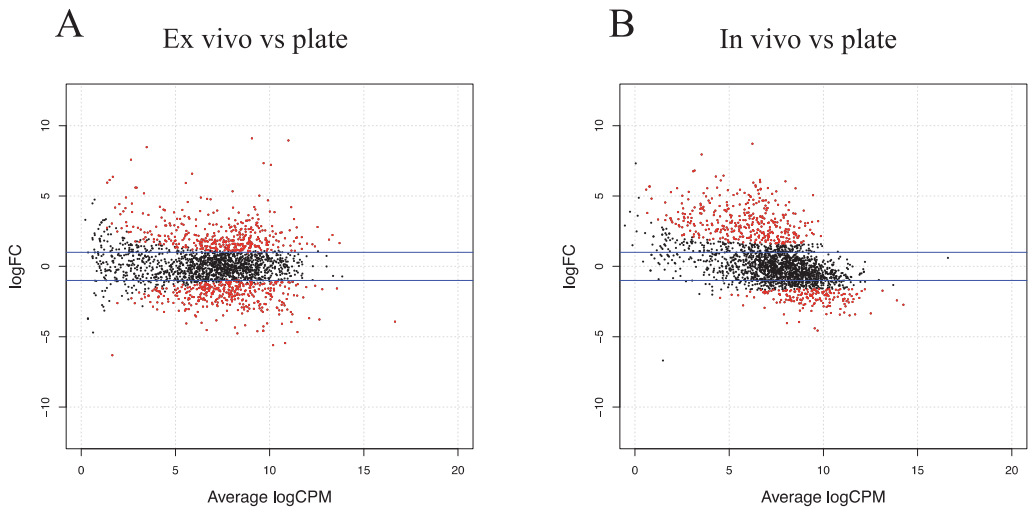


Figure 1. MA plots generated by EdgeR. Transcript expression profiles in the two comparisons performed: *ex vivo* vs plate culture (A) and *in vivo* vs plate culture (B). For each gene, $\log_2$(fold change) between the two conditions is plotted (M, y axis) against the gene's $\log_2$(average expression) in the two samples (A, x axis). Horizontal lines indicate 2-fold changes. Grey dots highlight the genes at 5% *FDR*.

For the *in vivo* experiment, high-resolution Illumina sequencing was chosen. A total of 18M and 15M reads were obtained for the *in vivo* sample and the corresponding plate sample, respectively. From the total, 15% and 83% mapped to the Nagasaki genome, for *in vivo* and plate culture, respectively. This 15% accounted for 2.7M reads that mapped to the Nagasaki genome from the total *in vivo* reads. To perform differential expression, a BCV of 0.3 was used, as obtained with the *ex vivo* data. Differential expression analysis between both samples yielded 542 DEGs: 369 were up-regulated and 173 down-regulated after 2h pig infection (Figure 1B and Additional file 2B). The differential expression was validated by RT-qPCR with the same genes used for the *ex vivo* experiment (Table 1). The correlation between the results by RT-qPCR and RNAseq was lower in this case but still statistically significant ($r=0.8$, $P < 0.05$).

Table 1. Genes whose expression was validated using RT-qPCR with purified RNA from *H. parasuis* Nagasaki recovered after 2h incubation in lung explants (*ex vivo*), 2h after intratracheal infection (*in vivo*) and lung samples from a time-course intranasal infection (1dpi, 2dpi and 3 dpi).

Locus tag	Gene type	RT-qPCR[1]					Product	RNAseq	
		ex vivo	*in vivo*	1dpi	2dpi	3dpi		*ex vivo* logFC	*in vivo* logFC
HPNK_10441	virulence factor	ND[2]	4.8	4.2	3.7	4.6	ABC transporter, permease protein	4.2	4.0
HPNK_06205	virulence factor	ND	4.9	4.6	2.9	4.9	hypothetical protein	2.1	6.0
HPNK_09829	virulence factor	ND	3.6	4.1	1.4	3.3	hypothetical protein	5.2	2.8
HPNK_10446	virulence factor	ND	3.3	3.5	2.4	3.1	hypothetical protein	3.1	5.5
HPNK_11461	virulence factor	ND	5.2	2.1	2.2	2.9	hypothetical protein	1.8	3.8
HPNK_06565	virulence factor	ND	5.4	HC_T[3]	HC_T	3.7	Na+/H+ antiporter NhaC	2.3	3.3
HPNK_01698	virulence factor	ND	4.1	3.8	3.2	3.0	VtaA2	1.6	3.1
HPNK_03728	key pathway	2.3	5.0	ND	ND	ND	6-pyruvoyl tetrahydrobiopterin synthase	2.6	1.7
HPNK_08953	key pathway	4.3	2.4	ND	ND	ND	ABC transporter inner membrane subunit	7.3	3.4
HPNK_06845	key pathway	3.9	5.3	ND	ND	ND	anthranilate synthase component II	9.1	1.8
HPNK_01064	key pathway	1.3	5.9	ND	ND	ND	heme exporter protein D	1.2	2.0
HPNK_10256	key pathway	-2.8	0.6	ND	ND	ND	PTS mannose-specific transporter subunit	-3.2	-1.9
HPNK_05784	key pathway	-4.2	-0.6	ND	ND	ND	type II citrate synthase	-4.7	-3.4
HPNK_02274	reference	ND	-	-	-	-	BirA repressor	-0.1	-0.1
HPNK_07913	reference	ND	-	-	-	-	MurE ligase	-0.1	0.1
HPNK_00732	reference	-	ND	ND	ND	ND	50S ribosomal protein L5	-0.5	-1.7
HPNK_07703	reference	-	ND	ND	ND	ND	translocation protein TolB	-0.2	-0.4
HPNK_09689	reference	-	ND	ND	ND	ND	50S ribosomal protein L2	0.7	-0.6
HPNK_01728	reference	-	ND	ND	ND	ND	pyruvate dehydrogenase subunit E1	0.0	-1.7

[1] log2 fold changes; [2]Not determined; [3] the gene was amplified but the high CT values obtained were not sufficient to calculate differential expression.

Differential gene expression and enrichment

Gene set enrichments for housekeeping, up-regulated and down-regulated genes were performed in order to have an overview of *H. parasuis* transcriptomic response in the lung. With regard to the *ex vivo* incubation, analysis revealed 1430 genes whose expression remained constant with respect to their expression on agar plate. These stable genes were mainly related to intracellular processes, as evidenced by the enriched GO term "metabolic process", encompassing the majority of the genes associated to biosynthetic processes. Among them, the most representative categories were amino sugar metabolism and other metabolic processes involved in amino acid, nucleic acid or ncRNA. Interestingly, some processes related to cell wall biogenesis were also constant, such as phospholipid, peptidoglycan and lipooligosaccharide biosynthetic processes, including lipid A metabolic process (Additional file 3A). Among the up-regulated genes in *ex vivo* conditions, GO enrichment revealed that genes related to "transporter activity", within the "membrane" category, were enriched, as it was also found in the *in vivo* experiment (see below). Some of these membrane-related genes were associated to pathogenesis functions (Additional file 4). Other *ex vivo* GO terms within the up-regulated genes were related to RNA processing, nucleic acid metabolic process, RNA metabolic process, including 21 genes related to tRNA processing (Additional file 3B). Among the most induced genes ($FDR < 0.001$) were: anthranilate synthase component I and II, glycerol-3-phosphate transporter, 2 hypothetical proteins, ABC transporter inner membrane subunit, anthranilate phosphoribosyltransferase, phage capsid scaffolding protein, and phage phi-c31 gp35-like protein. Notably, a high induction (>64 fold) of the operon related to anthranilate metabolism was observed after *ex vivo* incubation. Anthranilate is a key intermediate in aromatic amino acid biosynthesis and pyruvate production, and induction of its operon was also observed after *in vivo* infection (see below). On the other side, the GO terms overrepresented among the set of down-regulated genes were related to intracellular pathways, with metabolism as the most represented, evidenced by the presence of GO terms related to stress response, sugar and ion transport, carbohydrate catabolism, tricarboxylic acid (TCA) cycle, nucleoside biosynthetic process, glyoxylate metabolic process and dicarboxylic acid metabolic process (Additional file 3C). Among the most repressed genes ($FDR < 0.001$) were: long-chain fatty acid transport protein P1/47 kDa outer membrane protein, methyl-galactoside ABC transporter galactose-binding periplasmic protein MglB, cold shock-like protein CspD, type II citrate synthase, universal

stress protein A, maltose ABC transporter periplasmic protein, sigma 54 modulation protein and a hypothetical protein.

In an attempt to validate the *ex vivo* model, a short *in vivo* incubation was performed. Comparison of the DEGs detected in both conditions showed considerable overlap; 120 genes were up-regulated in both conditions, belonging mainly to membrane–associated genes, mobile genetic elements and hypothetical proteins (Table 2 and Additional file 3D). In contrast to the stable genes, which were mainly related to intracellular metabolism, up-regulated genes after the *in vivo* infection consisted predominantly of the category "membrane"; with 57 genes that encoded membrane proteins, and 38 of those directly tagged as transporters. In addition, 14 of the up-regulated membrane genes were specific of the "outer membrane", including several virulence associated trimeric autotransporters *vtaA*. Most of these genes were also up-regulated *ex vivo* (Additional file 4) and included functions usually related to virulence such as nutrient transport, drug export, adhesion and mobile genetic elements including transposases and phages. Some of the genes induced in both conditions were ABC transporter inner membrane subunit, ABC transporter permease protein, Na+/H+ antiporter, biopolymer transport ExbD protein, sn-glycerol-3-phosphate dehydrogenase subunit A, a drug/metabolite transporter (DMT) superfamily permease and several VtaA. In addition, the analysis also found genes that were induced only under one of the two conditions studied. Thus, genes uniquely induced *ex vivo*, although with subtle changes *in vivo*, included an iron-uptake permease inner membrane protein, an iron(III) ABC transporter ATP-binding protein, an oligopeptide ABC transporter permease and the peptide transport periplasmic protein SapA. On the other hand some genes were only induced *in vivo*, but again with subtle changes *ex vivo*, such as the recombination protein 2, Mu-like phage gp25, heme/hemopexin utilization protein C/outer membrane receptor protein, Tfp pilus assembly pathway component PilC, competence protein E and phosphatidylglycerophosphatase B. Among the *in vivo* down-regulated genes, "metabolic process" was the most represented category with 125 genes, comprising both anabolic and catabolic processes. However, these genes were different from the ones found with stable expression. The most abundant metabolic categories were those related to translation, cellular carbohydrate metabolic process, TCA cycle, electron transport chain, nucleotide metabolic process, glycosyl compound biosynthetic process and monocarboxylic acid metabolic process (Additional file 3E). Forty two genes in the "membrane" category were also found down-regulated, including carbohydrate and ion transporters. Key indicators of

low growth rate during lung infection compared to plate culture were also revealed by the down-regulation of the ATP synthase subunits, responsible for generating ATP via the electrochemical gradient, as well as glucose and manose phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS). Also, 3 subunits of the Na(+)–NQR complex were down-regulated, suggesting anaerobic growth, as previously reported for other bacterial species [35]. The Na(+)–NQR complex plays an important role in bacterial energy metabolism [36].

Table 2. Results of the comparison between the sets of differentially expressed genes (DEGs) and enriched Gene Ontology (GO) terms between Nagasaki recovered after 2 h incubation in lung explants (*ex vivo*) or recovered 2 h after intratracheal infection (*in vivo*).

Gene group	Unique *ex vivo*	Shared	Unique *in vivo*
DEGs UP	273	120	249
DEGs DOWN	273	99	74
GOs UP	74	5	46
GOs DOWN	68	115	102

To support this model of short *in vivo* infection, alveolar macrophages from the BALF sample were analysed. Alveolar macrophages recovered after the 2 h infection, showed increased expression level of CD163, SLAI and SLAII when compared to the macrophages recovered from the non-infected pig. Sialoadhesin did not show different expression in the alveolar macrophages of the infected and the non-infected piglet (Figure 2). These results indicate that this short infection was enough for the animal to detect the presence of bacteria and respond to it.

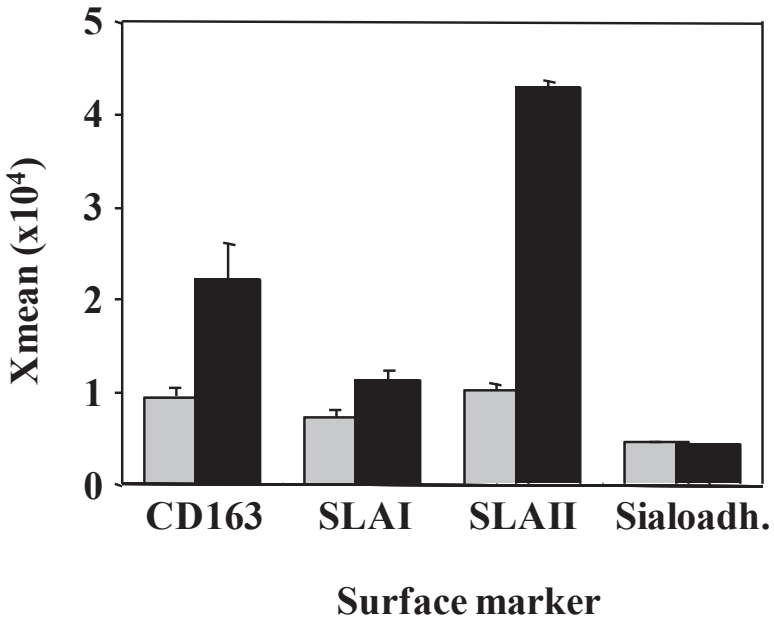


Figure 2 Expression of surface CD163, SLAI, SLAII and sialoadhesin on alveolar macrophages. The pig was intratracheally inoculated with *H. parasuis* strain Nagasaki (black bars). Infection was allowed to proceed during 2 h, when the animal was euthanized and alveolar macrophages were obtained from the lung by lavage. A control non-infected pig was included as control (gray bars). Level of expression of the surface markers was measured with specific monoclonal antibodies by flow cytometry. Results (mean of the x-mean ± standard deviation) are representative of two independent analyses run with duplicate samples.

Up-regulated membrane genes and their conservation in virulent H. parasuis strains

The observation of numerous membrane-related genes that were up-regulated in both *ex vivo* and *in vivo* conditions was studied in more detail. Transmembrane topology and signal peptides were predicted in the putative proteome of Nagasaki using Phobius. A total of 225 putative proteins were predicted to have signal peptide, 360 to have transmembrane domains and 42 to have both signal peptide and transmembrane domains. These 627 genes are referred as "membrane-related genes" for simplicity through the rest of the text. All these membrane-related genes in Nagasaki genome were compared to the up-regulated genes *in vivo* or *ex vivo*, confirming the high proportion observed using GO terms. A total of 115 and 108 of those 627 were up-regulated ex-vivo and in-vivo, respectively. Comparison of these up-regulated membrane-related genes between *ex vivo* and *in vivo* confirmed the tendency observed with GO terms, with 36 shared genes. Next, in order to explore if these genes were unique to virulent strains, the list of up-regulated membrane-related genes was compared to a database of virulent and non-virulent genomic sequences of *H. parasuis*. We found 41 up-regulated membrane genes that were only present in

virulent strains, or at most in one non-virulent strain. Twelve of these 41 genes were up-regulated *in vivo* and *ex vivo*, while 22 and 7 genes were uniquely up-regulated *in vivo* and *ex vivo*, respectively (Table 3). Shared up-regulated genes between the *in vivo* and *ex vivo* conditions included an ABC transporter permease, four genes of unknown function, Na+/H+ antiporter *nhaC*, a transposase mutator family protein and five virulence-associated autotransporters VtaAs (*vtaA*1, *vtaA*2, *vtaA*4, *vtaA*7 and *vtaA*11). The fact that *vtaAs* are assembled with no gaps in the Nagasaki genome used in the analysis strengthens the value of these results. The 22 genes uniquely up-regulated under *in vivo* conditions, included bacteriophage tail protein GPT, nine hypothetical proteins, three ISAs1 family transposases, putative iron compound ABC transporter permease, putative phage DNA replication protein O, two pyruvate kinases, two Sel1 domain repeat-containing proteins, and three *vtaAs* (*vtaA*3, *vtaA*8 and *vtaA*9). On the other hand, the 7 genes uniquely up-regulated in the *ex vivo* experiment included those encoding a CDP-diglyceride pyrophosphorylase, copper-transporting P-type ATPase, exonuclease III, two hypothetical proteins, phage-like minor tail protein and polysaccharide biosynthesis protein CapD (Table 3). Due to the essential role of pyruvate kinase enzyme in glycolysis, lack of this gene in non-virulent strains would be unlikely. The two genes annotated as pyruvate kinases and found only in virulent strains were HPNK_07393 and HPNK_09849. Further InterPro inspection results revealed that none of these two genes really belongs to the pyruvate kinase family (IPR001697), thus being their protein annotation incorrect. Although these two genes have positive prediction for signal peptide, InterPro functional annotation was scarce. Their predicted domains are not specific to a particular pathway: a tetratricopeptide-like helical domain (IPR011990) for HPNK_07393 and a polyketide cyclase SnoaL-like domain (IPR009959) for HPNK_09849. In fact, there are at least three more genes annotated as “pyruvate kinase” in *H. parasuis* Nagasaki: HPNK_06225, HPNK_05314 and HPNK_07388. Only HPNK_06225 is specific to virulent strains, having a polyketide cyclase SnoaL-like domain (IPR009959). The other two, HPNK_05314, the largest one, seems to be the true pyruvate kinase (IPR001697), while HPNK_07388, again with a wrong annotation, has no predicted domains.

Table 3 List of up-regulated membrane genes (*FDR* < 0.05) unique of virulent *H. parasuis* strains (V) or present at most in one non-virulent strain (one NV).

Locus	*in vivo*/*ex vivo*[1]	Product	Unique V/one NV
HPNK_10441	*in vivo*/*ex vivo*	ABC transporter, permease protein	unique V
HPNK_03268	*in vivo*	Bacteriophage tail protein GPT	unique V
HPNK_04602	*ex vivo*	CDP-diglyceride pyrophosphorylase	unique V
HPNK_01144	*ex vivo*	Copper-transporting P-type ATPase	unique V
HPNK_07008	*ex vivo*	Exonuclease III	unique V
HPNK_00812	*in vivo*/*ex vivo*	Hypothetical protein	one NV
HPNK_06205	*in vivo*/*ex vivo*	Hypothetical protein	unique V
HPNK_09829	*in vivo*/*ex vivo*	Hypothetical protein	unique V
HPNK_10446	*in vivo*/*ex vivo*	Hypothetical protein	unique V
HPNK_09189	*ex vivo*	Hypothetical protein	one NV
HPNK_00632	*ex vivo*	Hypothetical protein	unique V
HPNK_00572	*in vivo*	Hypothetical protein	unique V
HPNK_05179	*in vivo*	Hypothetical protein	unique V
HPNK_00277	*in vivo*	Hypothetical protein	unique V
HPNK_08083	*in vivo*	Hypothetical protein	one NV
HPNK_10351	*in vivo*	Hypothetical protein	unique V
HPNK_00312	*in vivo*	Hypothetical protein	one NV
HPNK_11566	*in vivo*	Hypothetical protein	one NV
HPNK_11461	*in vivo*	Hypothetical protein	one NV
HPNK_04137	*in vivo*	Hypothetical protein	one NV
HPNK_04889	*in vivo*	ISAs1 family transposase	one NV
HPNK_00080	*in vivo*	ISAs1 family transposase	one NV
HPNK_04984	*in vivo*	ISAs1 family transposase	one NV
HPNK_06565	*in vivo*/*ex vivo*	Na+/H+ antiporter NhaC	unique V
HPNK_11451	*ex vivo*	Phage-like minor tail protein	one NV
HPNK_07568	*ex vivo*	Polysaccharide biosynthesis protein CapD	unique V
HPNK_05239	*in vivo*	putative iron compound ABC transporter permease	unique V
HPNK_00090	*in vivo*	Putative phage DNA replication protein O	one NV
HPNK_09849	*in vivo*	Polyketide cyclase SnoaL-like domain protein	unique V
HPNK_07393	*in vivo*	Tetratricopeptide-like helical domain protein	unique V
HPNK_07408	*in vivo*	Sel1 domain protein, repeat-containing protein	unique V
HPNK_07403	*in vivo*	Sel1 domain protein, repeat-containing protein	unique V
HPNK_00210	*in vivo*/*ex vivo*	Transposase, Mutator family protein	one NV
HPNK_09439	*in vivo*/*ex vivo*	VtaA1	unique V
HPNK_01479	*in vivo*/*ex vivo*	VtaA11	unique V
HPNK_01698	*in vivo*/*ex vivo*	VtaA2	unique V
HPNK_07258	*in vivo*	VtaA3	unique V
HPNK_10146	*in vivo*/*ex vivo*	VtaA4	unique V
HPNK_02582	*in vivo*/*ex vivo*	VtaA7	unique V
HPNK_01967	*in vivo*	VtaA8	unique V
HPNK_10812	*in vivo*	VtaA9	unique V

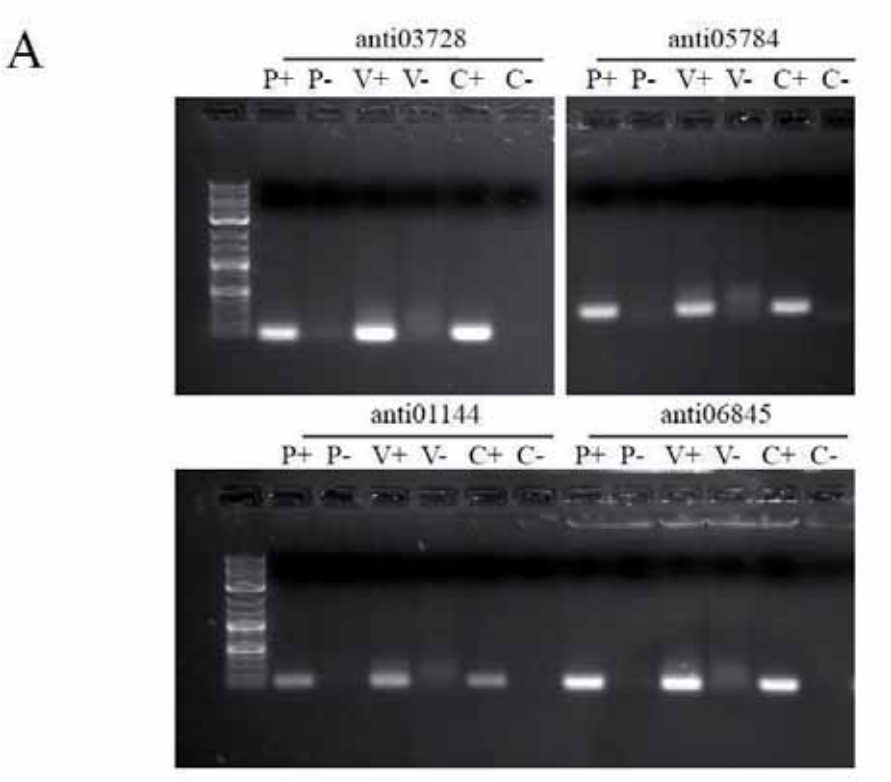
[1] up-regulated *in vivo*, *ex vivo* or in both samples (*in vivo*/*ex vivo*).

Expression of four genes of unknown function, the *vtaA*2, the Na+/H+ antiporter *nhaC* and an ABC transporter permease protein was validated via RT-qPCR in the *in vivo* sample (Table 1). These genes were selected because they were found up-regulated in both *in vivo* and *ex vivo* conditions and were only detected in virulent strains. The up-regulation was confirmed for all seven genes, and positive correlation was found between RT-qPCR and RNA-seq (r=0.8, $P < 0.05$). Increase in RT-qPCR fold change correlated with increase in RNA-seq fold change. In addition, the transcription of these genes was examined by RT-qPCR in BALF samples from a previous experimental infection with the Nagasaki strain. These genes were found to be up-regulated in bacteria recovered at 1, 2 and 3 dpi from the lung of piglets intranasally inoculated with Nagasaki, with exception of the Na+/H+ antiporter *nhaC*, whose expression was too low for evaluation by this method.

ncRNAs in H. parasuis and their differential expression

Antisense expression was observed for all six sequenced samples, and was processed for further investigation. Counts files from counting step were used [GEO: GSE63851]. Determination of sense/antisense expression ratio was performed. Due to presence of some extreme values for antisense transcripts, median values were used. For *ex vivo* samples, sense to antisense ratios varied between 13 and 19, while a ratio of 43-68 was observed for their corresponding plate samples; thus evidencing a clear change in the sense to antisense ratio during *ex vivo* incubation. This tendency was also observed for the *in vivo* sample, where sense to antisense ratios of 4 and 15 were obtained for *in vivo* and its corresponding plate sample, respectively. These observations suggest that proportions were more equilibrated in the *in vivo* and *ex vivo* samples, while plate samples were biased towards sense expression. Normality of sense and antisense counts was assessed using the Shapiro-Wilk test. In all 6 cases *P* values were below 0.05 indicating non-normality. Therefore, correlations were assessed using the non-parametric Spearman rank-correlation test. The results indicated that *in vivo* sense and antisense expression was positively correlated (r=0.43, $P < 0.05$). Similarly, plate sense and antisense expression was also positively correlated (r=0.66, $P < 0.05$). However, this tendency was not observed for the *ex vivo* and their corresponding plate samples (r=0.07-0.15, $P < 0.05$). All calculations were carried out using R statistical software [37]. Since a negative correlation would have been expected by reducing sense expression via sense/asRNA pairing, these unexpected results suggest that no sense/antisense regulation is occurring and that there are other factors

influencing an increase of antisense expression. Pervasive transcription has been reported both for prokaryotes and mammals [38,39], but it has not yet been described for *H. parasuis*. To validate some of the observed asRNAs, RT-PCRs were performed. Four asRNAs were validated both in the *in vivo* and plate culture samples. These asRNAs overlapped with genes annotated as 6-pyruvoyl tetrahydrobiopterin synthase, type II citrate synthase, copper-transporting P-type ATPase and anthranilate synthase component II (Figure 3A and Additional file 1). The positive correlation sense-asRNA observed for the *in vivo* sample was studied in more detail. Antisense differential expression was calculated, finding a considerable number of differentially expressed asRNAs ($FDR < 0.05$), 213 and 268 up- and down-regulated, respectively (Additional file 2C). GO enrichments were also performed for the set of differentially expressed asRNAs (Additional files 3F and 3G) and were compared to sense enrichment results. We found an important overlap between the enriched GO terms of the repressed asRNAs and sense RNAs, with 135 matches (Figure 3B). These pathways were mainly related to intracellular genes, mostly metabolism. Among them, carbohydrate catabolic process, phosphoenolpyruvate-dependent sugar phosphotransferase system, translation, tricarboxylic acid cycle, glycosyl compound biosynthetic process, purine ribonucleoside triphosphate biosynthetic process and monocarboxylic acid metabolic process were the main representatives. These results confirm that the positive correlation of sense and antisense expression was also linked to some common pathways. Some overlap was also observed between the pathways of the induced asRNAs and sense RNAs, with 6 shared GO terms including pathogenesis and pilus organization.



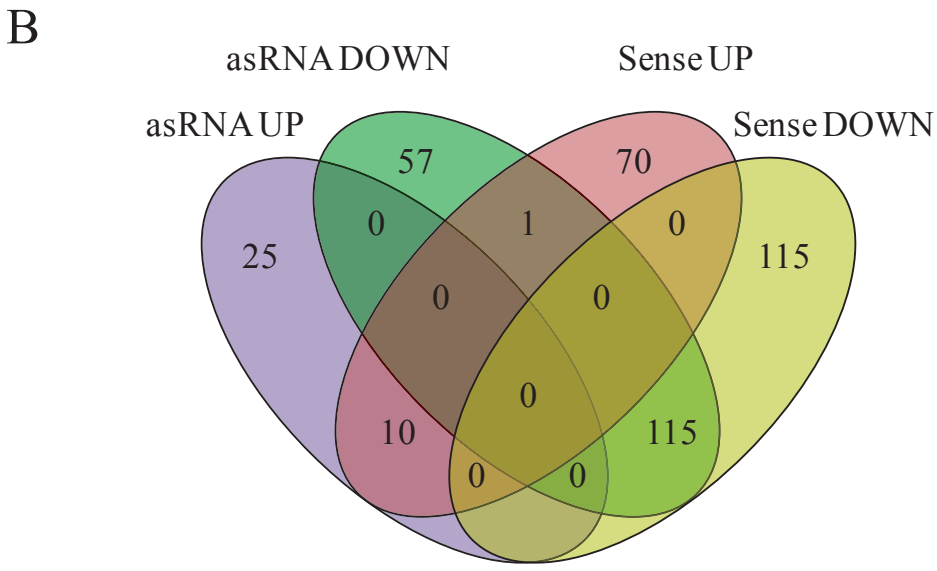


Figure 3 Validation of antisense RNAs (asRNAs) expressed *in vivo* and in plate culture. (A) Electrophoresis gel of four asRNAs amplified by RT-PCR showing amplification results for RNA purified from plate culture with RT step (P+), plate culture without RT step (P-), 2 h *in vivo* sample with RT (V+) or without RT step (V-). Genomic DNA was included as positive control (C+) and water as negative control (C-). (B) Venn diagram showing overlapping Gene Ontology (GO) enriched terms for up- and down-regulated asRNAs or sense RNAs at 5% *FDR*.

Discussion

After an initial colonization of the upper respiratory tract, lung infection is one of the early steps in *H. parasuis* pathogenesis. Therefore, a deep understanding of the factors involved in survival of this bacterium in the lung will be useful for disease control. In this study, we have shown that the Nagasaki strain of *H. parasuis* modulates its gene expression during *in vivo* lung infection, but also in an *ex vivo* lung infection model. Nutrient acquisition, expression of genes related to pathogenesis and a reduced metabolism are the main signatures of *H. parasuis* adaptation to the lung environment. Some of the up-regulated genes during lung infection were only found in the genomes of virulent strains, indicating their potential as virulence factors. In addition, previously reported *H. parasuis* virulence factors were also up-regulated in the lung.

One of the most important limitations to elucidate bacterial gene expression during host colonization is the low RNA quality that is usually obtained from field or experimental infections. Moreover, the proportionally low quantity of mRNA relative to rRNA makes transcriptomics a challenging strategy. In our case, we were not able to obtain enough RNA of good quality for high-throughput sequencing from samples taken at 1 to 4 days post-inoculation from an infection previously performed by us [14,27]. To overcome these limitations, we decided to use a short *in vivo* infection and an *ex vivo* lung infection model. Processing of BALF samples for RNA purification coupled with additional enrichment steps such as differential centrifugation to remove host cells and removal of host and bacterial rRNA resulted in a modest but sufficient RNA quantity to study bacterial gene expression by metatranscriptomics. In our study, 3M reads from the *ex vivo* sample and 18M reads from the *in vivo* sample were enough to study the gene expression of 95% of the genes. These sequencing depths resulted sufficient to reach the coverage recommended to quantify bacterial gene expression [40].

In vivo incubation revealed numerous genes that were differentially expressed. Even prior to pathway analysis, visualization of MA differential expression plot showed a particular "wave" shape, thus pointing to up-regulation of genes normally not expressed during laboratory agar plate growth, and, conversely, down-regulation of the most expressed ones. Increased nutrient capture, together with metabolism repression were the main adaptive responses of *H. parasuis* in the lung. Interestingly, a similar picture has been observed in

other *in vivo* transcriptome studies of other bacterial species from the *Pasteurellaceae* family, such as *Mannheimia hemolytica*, responsible of bovine pneumonic pasteurellosis [16] or for *Actinobacillus pleuropneumoniae*, the etiological agent of porcine pleuropneumonia [18], thus suggesting slower metabolism and replication rate *in vivo* compared to growth in a rich culture media. Deslandes et al. [18] also found that the "Transport and Binding Protein" functional class was overrepresented among the up-regulated genes in *A. pleuropneumoniae* under *in vivo* conditions. Here, we found that the most represented cellular component among the up-regulated genes in the lung was the one related to membrane proteins. Some of these genes were distributed in different categories of nutrient capture, such as sugar and amino acid transport. These findings indicate that "nutritional virulence", previously reported for other bacterial pathogens [41], could be an important survival strategy for *H. parasuis* during lung colonization. Moreover, some of these genes were involved in iron, nitrate and sulfonate acquisition, which are vital for energy generation and have been previously reported to be up-regulated in *H. parasuis* grown under iron-restriction *in vitro* [42]. Furthermore, up-regulation of cytochrome biogenesis, heme transport and some components of the electron transport chain add additional value to the hypothesis that maintenance of anabolic/catabolic balance could be important for survival in the host. Additionally, ABC transporters, which have been found to be up-regulated in the current study, have been reported to be essential elements for bacterial survival in the host [43,44]. Interestingly, some ABC transporters could have been acquired via horizontal gene transfer in *H. parasuis* [23]. While ABC transporters were found up-regulated, the PTS sugar transports systems were down-regulated, indicating that different sugar transporters could be used depending on the environmental conditions, by inverse regulation of these two ATP-dependent transporters [45].

One of the main questions we tried to answer in this work was if any of the previously reported virulence factors were up-regulated *in vivo*. Genes involved in surface polysaccharides, lipid A or proteases did not show an overall increased expression. However, iron, hemin, hemopexin or transferring binding proteins showed a tendency of up-regulation. Also, mining for up-regulated membrane-related genes that were unique to virulent strains revealed 42 putative virulence factors. Several of these gene categories have been previously linked to virulence in bacteria, such as iron acquisition or ABC transporters [46], frequently subjected to horizontal genetic transfer via mobile genetic elements [47]. On the other hand, some proteins previously reported as putative virulence

factors in *H. parasuis*, such as outer membrane protein P2 (OmpP2), cytolethal distending toxins, heptosyltransferases, which have been involved in adhesion, invasion or serum resistance [48-50], showed subtle down-regulation. This down-regulation could indicate that these molecules do not play a specific role in lung survival. Finally, *vtas* were up-regulated *in vivo*, including *vtaA8* and *vtaA9*, which have been demonstrated to play a role in phagocytosis resistance in *H. parasuis* [15]. Due to the complex structure of these genes, with repetitions, common domains and variability of passenger domain [51], careful analysis should be performed to determine their presence in different strains. Previous knowledge about these genes indicates that *vtaA*1 to *vtaA*11 are associated with virulence [52]. We should highlight that a total of 128 up-regulated genes are currently annotated as genes of unknown function. Some of these genes of unknown function were detected by Phobius as membrane-related and were only found in virulent strains. These genes of unknown function require further research, since they could play a role in novel pathogenic strategies [53]. Additionally, the validation of up-regulation of 4 of these genes of unknown function in other *in vivo* samples supports the role of these membrane proteins during infection as well as their use as vaccine candidates.

Development of *ex vivo* models has been reported as useful to mimic *in vivo* conditions [54]. Currently, an *ex vivo* infection model is absent for *H. parasuis*. In this work we tried to bypass the current limitations of *in vivo* experiments by developing an *ex vivo* lung infection model. A 2 h *ex vivo* incubation resulted in high yield of bacterial RNA recovery. Importantly, DEGs were detected in similar pathways as in the *in vivo* sample, validating this strategy as a good alternative for *H. parasuis* host-pathogen interaction studies at the respiratory tract. However, the comparison of the results from the *ex vivo* and *in vivo* infections also found differences between these samples, indicating that the true expression of some genes during infection can only be revealed *in vivo*. The advent of ultra-high throughput sequencing would allow previously unimagined sequencing depths, which will be useful in the analysis of samples from *in vivo* experiments. For instance, analysis of other sample types containing lower number of bacterial cells, such as samples from latter stages of infection, will open new possibilities to study the progression of disease in the lung.

One of the key factors to develop an effective vaccine to control a bacterial infection is to know its pathogenic lifestyle. We found that *H. parasuis* changes its global gene

expression during lung infection, with an overall tendency to up-regulate membrane-related genes involved in carbon acquisition, iron binding and pathogenesis. On the other hand, a strong down-regulation of metabolism was observed, suggesting an importance of a lower replicative rate as an adaptation for host survival. These metabolic adaptations were accompanied by induction of well-known virulence factors as well as other genes that, although less explored, could be behind novel virulence mechanisms. In summary, this work serves as a useful infection model for *H. parasuis,* adding value to some up-regulated virulence factors for their use as vaccine candidates.

References

1. Aragon V, Segalés J, Oliveira S: **Glässer's disease: In Diseases of swine.** 10th edition. Edited by Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW. Iowa: Wiley-Blackwell publishing, 2012, 760–769.

2. San Millan A, Escudero JA, Catalan A, Nieto S, Farelo F, Gibert M, Moreno MA, Dominguez L, Gonzalez-Zorn B: **Beta-lactam resistance in *Haemophilus parasuis* is mediated by plasmid pB1000 bearing blaROB-1**. *Antimicrob Agents Chemother* 2007, **51**:2260-2264.

3. Zhang Q, Liu J, Yan S, Yang Y, Zhang A, Jin M: **Fluoroquinolone-resistant *Haemophilus parasuis* isolates exhibit more putative virulence factors than their susceptible counterparts**. *J Clin Microbiol* 2013, **51**:3130-3131.

4. Chen LP, Cai XW, Wang XR, Zhou XL, Wu DF, Xu XJ, Chen HC: **Characterization of plasmid-mediated lincosamide resistance in a field isolate of *Haemophilus parasuis***. *J Antimicrob Chemother* 2010, **65**:2256-2258.

5. Nedbalcova K, Kucerova Z, Krejci J, Tesarik R, Gopfert E, Kummer V, Leva L, Kudlackova H, Ondriasova R, Faldyna M: **Passive immunisation of post-weaned piglets using hyperimmune serum against experimental *Haemophilus parasuis* infection**. *Res Vet Sci* 2011, **91**:225-229.

6. Olvera A, Ballester M, Nofrarias M, Sibila M, Aragon V: **Differences in phagocytosis susceptibility in *Haemophilus parasuis* strains**. *Vet Res* 2009, **40**:24.

7. Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L, Yuan Z: **Antibiotic alternatives: the substitution of antibiotics in animal husbandry?** *Frontiers in microbiology* 2014, **5**:217.

8. Vouloumanou EK, Makris GC, Karageorgopoulos DE, Falagas ME: **Probiotics for the prevention of respiratory tract infections: a systematic review**. *Int J Antimicrob Agents* 2009, **34**:197 e191-110.

9. Costa-Hurtado M, Aragon V: **Advances in the quest for virulence factors of *Haemophilus parasuis***. *Vet J* 2013, **198**:571-576.

10. Zhang B, Tang C, Liao M, Yue H: **Update on the pathogenesis of *Haemophilus parasuis* infection and virulence factors**. *Vet Microbiol* 2014, **168**:1-7.

11. Ruter C, Hardwidge PR: **'Drugs from bugs': bacterial effector proteins as promising biological (immune-) therapeutics**. *FEMS Microbiol Lett* 2014, **351**:126-132.

12. Eisenreich W, Dandekar T, Heesemann J, Goebel W: **Carbon metabolism of intracellular bacterial pathogens and possible links to virulence**. *Nature reviews Microbiology* 2010, **8**:401-412.

13. Vahle JL, Haynes JS, Andrews JJ: **Interaction of *Haemophilus parasuis* with nasal and tracheal mucosa following intranasal inoculation of cesarean derived colostrum deprived (CDCD) swine**. *Can J Vet Res* 1997, **61**:200-206.

14. Bello-Orti B, Costa-Hurtado M, Martinez-Moliner V, Segales J, Aragon V: **Time course *Haemophilus parasuis* infection reveals pathological differences between virulent and non-virulent strains in the respiratory tract**. *Vet Microbiol* 2014, **170**:430-437.

15. Costa-Hurtado M, Ballester M, Galofre-Mila N, Darji A, Aragon V: **VtaA8 and VtaA9 from *Haemophilus parasuis* delay phagocytosis by alveolar macrophages**. *Vet Res* 2012, **43**:57.

16. Sathiamoorthy S, Hodgins DC, Shewen PE, Highlander SK, Lo RY: **A snap-shot of *Mannheimia hemolytica* A1 gene expression during infection in the bovine host**. *FEMS Microbiol Lett* 2011, **325**:148-154.

17. Jorth P, Trivedi U, Rumbaugh K, Whiteley M: **Probing bacterial metabolism during infection using high-resolution transcriptomics**. *J Bacteriol* 2013, **195**:4991-4998.

18. Deslandes V, Denicourt M, Girard C, Harel J, Nash JH, Jacques M: **Transcriptional profiling of *Actinobacillus pleuropneumoniae* during the acute phase of a natural infection in pigs**. *BMC genomics* 2010, **11**:98.

19. Boyce JD, Wilkie I, Harper M, Paustian ML, Kapur V, Adler B: **Genomic scale analysis of *Pasteurella multocida* gene expression during growth within the natural chicken host**. *Infect Immun* 2002, **70**:6871-6879.

20. Kim S, Park J, Lee J, Shin D, Park DS, Lim JS, Choi IY, Seo YS: **Understanding pathogenic *Burkholderia glumae* metabolic and signaling pathways within rice tissues through *in vivo* transcriptome analyses**. *Gene* 2014, **547**:77-85.

21. Son MS, Matthews WJ, Jr., Kang Y, Nguyen DT, Hoang TT: ***In vivo* evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients**. *Infect Immun* 2007, **75**:5313-5324.

22. Camejo A, Buchrieser C, Couve E, Carvalho F, Reis O, Ferreira P, Sousa S, Cossart P, Cabanes D: ***In vivo* transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection**. *PLoS pathogens* 2009, **5**:e1000449.

23. Bello-Orti B, Aragon V, Pina-Pedrero S, Bensaid A: **Genome comparison of three serovar 5 pathogenic strains of *Haemophilus parasuis*: insights into an evolving swine pathogen**. *Microbiology* 2014, **160**:1974-1984.

24. Markowitz VM, Chen IM, Palaniappan K, Chu K, Szeto E, Grechkin Y, Ratner A, Jacob B, Huang J, Williams P, Huntemann M, Anderson I, Mavromatis K, Ivanova NN, Kyrpides NC: **IMG: the integrated microbial genomes database and comparative analysis system**. *Nucleic Acids Res* 2012, **40**:D115-122.

25. Caspi R, Altman T, Billington R, Dreher K, Foerster H, Fulcher CA, Holland TA, Keseler IM, Kothari A, Kubo A, Krummenacker M, Latendresse M, Mueller LA, Ong Q, Paley S, Subhraveti P, Weaver DS, Weerasinghe D, Zhang P, Karp PD: **The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases**. *Nucleic Acids Res* 2014, **42**:D459-471.

26. Bello-Orti B, Deslandes V, Tremblay YD, Labrie J, Howell KJ, Tucker AW, Maskell DJ, Aragon V, Jacques M: **Biofilm formation by virulent and non-virulent strains of *Haemophilus parasuis***. *Vet Res* 2014, **45**:104.

27. Costa-Hurtado M, Olvera A, Martinez-Moliner V, Galofre-Mila N, Martinez P, Dominguez J, Aragon V: **Changes in macrophage phenotype after infection of pigs with *Haemophilus parasuis* strains with different levels of virulence**. *Infect Immun* 2013, **81**:2327-2333.

28. Anders S, McCarthy DJ, Chen Y, Okoniewski M, Smyth GK, Huber W, Robinson MD: **Count-based differential expression analysis of RNA sequencing data using R and Bioconductor**. *Nature protocols* 2013, **8**:1765-1786.

29. Li H, Durbin R: **Fast and accurate long-read alignment with Burrows-Wheeler transform**. *Bioinformatics* 2010, **26**:589-595.

30. Lassmann T, Hayashizaki Y, Daub CO: **SAMStat: monitoring biases in next generation sequencing data**. *Bioinformatics* 2011, **27**:130-131.

31. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M: **Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research**. *Bioinformatics* 2005, **21**:3674-3676.

32. Supek F, Bosnjak M, Skunca N, Smuc T: **REVIGO summarizes and visualizes long lists of gene ontology terms**. *PloS one* 2011, **6**:e21800.

33. Kall L, Krogh A, Sonnhammer EL: **A combined transmembrane topology and signal peptide prediction method**. *J Mol Biol* 2004, **338**:1027-1036.

34. Howell KJ, Weinert LA, Chaudhuri RR, Luan S, Peters SE, Corander J, Harris D, Angen O, Aragon V, Bensaid A, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW, Holden MT, Tucker AW, Maskell DJ: **The use of genome wide association methods to investigate pathogenicity, population structure and serovar in *Haemophilus parasuis***. *BMC genomics* 2014, **15**:1179.

35. Fadeeva MS, Yakovtseva EA, Belevich GA, Bertsova YV, Bogachev AV: **Regulation of expression of Na+ -translocating NADH:quinone oxidoreductase genes in *Vibrio harveyi* and *Klebsiella pneumoniae***. *Arch Microbiol* 2007, **188**:341-348.

36. Juarez O, Morgan JE, Nilges MJ, Barquera B: **Energy transducing redox steps of the Na+-pumping NADH:quinone oxidoreductase from *Vibrio cholerae***. *Proc Natl Acad Sci U S A* 2010, **107**:12505-12510.

37. Team RDC: **R: A language and environment for statistical computing**. 2012.

38. Wade JT, Grainger DC: **Pervasive transcription: illuminating the dark matter of bacterial transcriptomes**. *Nature reviews Microbiology* 2014, **12**:647-653.

39. Clark MB, Amaral PP, Schlesinger FJ, Dinger ME, Taft RJ, Rinn JL, Ponting CP, Stadler PF, Morris KV, Morillon A, Rozowsky JS, Gerstein MB, Wahlestedt C, Hayashizaki Y, Carninci P, Gingeras TR, Mattick JS: **The reality of pervasive transcription**. *PLoS biology* 2011, **9**:e1000625; discussion e1001102.

40. Haas BJ, Chin M, Nusbaum C, Birren BW, Livny J: **How deep is deep enough for RNA-Seq profiling of bacterial transcriptomes?** *BMC genomics* 2012, **13**:734.

41. Abu Kwaik Y, Bumann D: **Microbial quest for food *in vivo*: 'nutritional virulence' as an emerging paradigm**. *Cellular microbiology* 2013, **15**:882-890.

42. Xie Q, Jin H, Luo R, Wan Y, Chu J, Zhou H, Shi B, Chen H, Zhou R: **Transcriptional responses of *Haemophilus parasuis* to iron-restriction stress *in vitro***. *Biometals* 2009, **22**:907-916.

43. Mazzariol A, Tokue Y, Kanegawa TM, Cornaglia G, Nikaido H: **High-level fluoroquinolone-resistant clinical isolates of *Escherichia coli* overproduce multidrug efflux protein AcrA**. *Antimicrob Agents Chemother* 2000, **44**:3441-3443.

44. Martinez JL, Sanchez MB, Martinez-Solano L, Hernandez A, Garmendia L, Fajardo A, Alvarez-Ortega C: **Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems**. *FEMS Microbiol Rev* 2009, **33**:430-449.

45. Untiet V, Karunakaran R, Kramer M, Poole P, Priefer U, Prell J: **ABC transport is inactivated by the PTS(Ntr) under potassium limitation in *Rhizobium leguminosarum* 3841**. *PloS one* 2013, **8**:e64682.

46. Rodriguez GM, Smith I: **Identification of an ABC transporter required for iron acquisition and virulence in *Mycobacterium tuberculosis***. *J Bacteriol* 2006, **188**:424-430.

47. Frost LS, Leplae R, Summers AO, Toussaint A: **Mobile genetic elements: the agents of open source evolution**. *Nature reviews Microbiology* 2005, **3**:722-732.

48. Xu C, Zhang L, Zhang B, Feng S, Zhou S, Li J, Zou Y, Liao M: **Involvement of lipooligosaccharide heptose residues of *Haemophilus parasuis* SC096 strain in serum resistance, adhesion and invasion**. *Vet J* 2013, **195**:200-204.

49. Zou Y, Feng S, Xu C, Zhang B, Zhou S, Zhang L, He X, Li J, Yang Z, Liao M: **The role of galU and galE of *Haemophilus parasuis* SC096 in serum resistance and biofilm formation**. *Vet Microbiol* 2013, **162**:278-284.

50. Zhang B, He Y, Xu C, Xu L, Feng S, Liao M, Ren T: **Cytolethal distending toxin (CDT) of the *Haemophilus parasuis* SC096 strain contributes to serum resistance and adherence to and invasion of PK-15 and PUVEC cells**. *Vet Microbiol* 2012, **157**:237-242.

51. Pina S, Olvera A, Barcelo A, Bensaid A: **Trimeric autotransporters of *Haemophilus parasuis*: generation of an extensive passenger domain repertoire specific for pathogenic strains**. *J Bacteriol* 2009, **191**:576-587.

52. Olvera A, Pina S, Macedo N, Oliveira S, Aragon V, Bensaid A: **Identification of potentially virulent strains of *Haemophilus parasuis* using a multiplex PCR for virulence-associated autotransporters (vtaA)**. *Vet J* 2012, **191**:213-218.

53. Shahbaaz M, Hassan MI, Ahmad F: **Functional annotation of conserved hypothetical proteins from *Haemophilus influenzae* Rd KW20**. *PloS one* 2013, **8**:e84263.

54. Harrison F, Muruli A, Higgins S, Diggle SP: **Development of an *ex vivo* porcine lung model for studying growth, virulence, and signaling of *Pseudomonas aeruginosa***. *Infect Immun* 2014, **82**:3312-3323.

Additional Files

Additional files can be found in the electronic version of this thesis.

Additional file 1

Primers. Primers used to validate expression pattern of RNA-seq data.

Additional file 2

Differential expression of Nagasaki genes. Differential expression of genes *ex vivo* versus plate culture (A), *in vivo* versus plate culture (B) and antisense RNAs *in vivo* versus plate culture (C).

Additional file 3

GO enrichments. Gene set enrichments for housekeeping, up-regulated and down-regulated genes. Enriched GO categories among the up- and down-regulated antisense RNAs *in vivo* are also shown.

Additional file 4

Membrane genes. Membrane genes up-regulated *in vivo*, *ex vivo* or in both conditions. Differential expression is also shown.

Table 1 Genes whose expression was validated using RT-qPCR with purified RNA from *H. parasuis* Nagasaki recovered after 2 h incubation in lung explants (*ex vivo*), 2 h after intratracheal infection (*in vivo*) and lung samples from a time-course intranasal infection (1 dpi, 2 dpi and 3 dpi).

Locus tag	Gene type	RT-qPCR[1]					Product	RNAseq	
		ex vivo	*in vivo*	1dpi	2dpi	3dpi		*ex vivo* logFC	*in vivo* logFC
HPNK_10441	virulence factor	ND[2]	4.8	4.2	3.7	4.6	ABC transporter, permease protein	4.2	4.0
HPNK_06205	virulence factor	ND	4.9	4.6	2.9	4.9	hypothetical protein	2.1	6.0
HPNK_09829	virulence factor	ND	3.6	4.1	1.4	3.3	hypothetical protein	5.2	2.8
HPNK_10446	virulence factor	ND	3.3	3.5	2.4	3.1	hypothetical protein	3.1	5.5
HPNK_11461	virulence factor	ND	5.2	2.1	2.2	2.9	hypothetical protein	1.8	3.8
HPNK_06565	virulence factor	ND	5.4	HC_T[3]	HC_T	3.7	Na+/H+ antiporter NhaC	2.3	3.3
HPNK_01698	virulence factor	ND	4.1	3.8	3.2	3.0	VtaA2	1.6	3.1
HPNK_03728	key pathway	2.3	5.0	ND	ND	ND	6-pyruvoyl tetrahydrobiopterin synthase	2.6	1.7
HPNK_08953	key pathway	4.3	2.4	ND	ND	ND	ABC transporter inner membrane subunit	7.3	3.4
HPNK_06845	key pathway	3.9	5.3	ND	ND	ND	anthranilate synthase component II	9.1	1.8
HPNK_01064	key pathway	1.3	5.9	ND	ND	ND	heme exporter protein D	1.2	2.0
HPNK_10256	key pathway	-2.8	0.6	ND	ND	ND	PTS mannose-specific transporter subunit	-3.2	-1.9
HPNK_05784	key pathway	-4.2	-0.6	ND	ND	ND	type II citrate synthase	-4.7	-3.4
HPNK_02274	reference	ND	-	-	-	-	BirA repressor	-0.1	-0.1
HPNK_07913	reference	ND	-	-	-	-	MurE ligase	-0.1	0.1
HPNK_00732	reference	-	ND	ND	ND	ND	50S ribosomal protein L5	-0.5	-1.7
HPNK_07703	reference	-	ND	ND	ND	ND	translocation protein TolB	-0.2	-0.4
HPNK_09689	reference	-	ND	ND	ND	ND	50S ribosomal protein L2	0.7	-0.6
HPNK_01728	reference	-	ND	ND	ND	ND	pyruvate dehydrogenase subunit E1	0.0	-1.7

[1] log2 fold changes; [2] Not determined; [3] the gene was amplified but the high CT values obtained were not sufficient to calculate differential expression.

DISCUSSION

It is well known that strains with different virulence grades exist in *H. parasuis*, as revealed by the clinical data. Also, some of the associated pathogenic mechanisms behind these contrasting clinical manifestations have been discovered, such as serum resistance and phagocytosis resistance by the virulent strains. In addition, the exaggerated immune response in the pig is part of the pathogenic response as a response to the inability to clear the virulent bacteria, enhancing damage to host tissues. Other pathogenic mechanisms have also been reported and attributed to virulent strains, such as invasion and adhesion of eukaryotic cells. However, all these virulence mechanisms have been reported using *in vitro* assays and need further support with *in vivo* data. In particular, biofilm formation has been reported to be more frequent in non-virulent strains [66], but these findings need further *in vivo* validation, being also necessary to confirm if virulent strains form biofilms *in vivo*. Moreover, *H. parasuis* may be using novel strategies during infection and may not have been reported yet due to the limitations of *in vitro* techniques such as the ability to mimic host cell structures. In order to confirm the pathogenesis mechanism reported for *H. parasuis*, give additional insights about colonization of both virulent and non-virulent strains, as well as discover novel pathogenic mechanisms, we designed a 7-day time course infection, performed with strains of different virulence grades. Our results showed that clinical outcome was strain-dependent. While animals inoculated with non-virulent strains remained healthy, some of the ones inoculated with virulent strains presented clinical signs of infection and sepsis, such as fever and prostration. Those clinical signs were accompanied by inflammation patterns in nasal turbinates, trachea and lung as well as fibrinous polyserositis, in agreement with previous *H. parasuis* clinico-pathological data [49]. Using immunohistochemistry, we were also able to associate strain virulence with particular infection sites. While virulent strains were found in nasal turbinates, trachea and lung, non-virulent strains were almost absent in these locations, although they were isolated in the laboratory from the nasal cavities [73].

Virulent and non-virulent isolates show a different colonization pattern of the respiratory tract

In the respiratory tract, it is well known the myriad of anatomical, cellular and molecular barriers that mammalian tissues have developed to prevent bacterial colonization or subsequent invasion of the blood [195]. *H. parasuis*, as an early colonizer, is commonly found. If specific host conditions appear, virulent strains can use their pathogenic mechanisms to subvert host barriers and produce disease. One of these barriers is IgA, present in all respiratory mucosa. IgA prevents bacterial adhesion to the host mucosa. To counteract this barrier, bacterial pathogens own specific tools, such as IgA proteases. No classic IgA protease genes have been identified in *H. parasuis*, but since IgG protease activity has been detected, dedicated proteases may exist [70]. Although we have not tested if this activity is used *in vivo*, the fact that *H. parasuis* virulent strains were found attached and inside nasal mucosa indicates that these barriers can be broken. Also, since both virulent and non-virulent strains were found by isolation culture [73], subversion of host IgA barrier can be assumed to both types of strains, independent of their virulence. It is also worth to note that cross-reacting antibodies to other bacterial pathogens could be also useful to stop *H. parasuis* entry. In our case the use of colostrum-deprived piglets involves absence of specific IgA response since no previous contact with *H. parasuis* happened. Next, to establish infection, *H. parasuis* needs to adhere and invade host upper airway cells and tissue structures, such as nasal and tracheal epithelial and mucosal cells. To reach this goal, pathogenic mechanisms such as adhesion and invasion are required. Previous reports indicate that *H. parasuis* is able to adhere and invade eukaryotic cells *in vitro* [64, 65]. Also, some evidences exist *in vivo* [7, 31]. We found that only virulent strains had this capacity, contrary to non-virulent strains. These capacities were observed in nasal, tracheal and lung locations, since bacteria were found attached to respiratory epithelium, inner mucosal tissues, as well as in the blood vessels associated to nasal, tracheal and bronchia. Some virulence factors have been directly linked to this pathogenic mechanism via deletion mutants [150, 151, 153]. Interestingly, the genes encoding for one of those factors, the CDT, have been shown to be associated with clinical isolates [53].

Next step in *H. parasuis* infection may involve invasion of other types of cells, such as pneumocytes, which were in some cases populated with *H. parasuis*. Also, finding

bacterial cells inside macrophages suggest that phagocytosis resistance capacity is used *in vivo*, previously reported using *in vitro* assays [71, 72]. Bacteria were also found inside host neutrophilic cells at tracheal space, thus denoting that *H. parasuis* may be able to resist different types of phagocyte cells. However, to which extent bacteria were dead or alive is more arguable. In our case, we found better evidences depending on tissue and cell type were *H. parasuis* was found. Our IHC results show that rod-shape bacterial cells were clearly observed attached to respiratory epithelium. This shape was more difficult to observe in submucosa and vessels of nasal turbinates, trachea and lung. Similarly, this shape was less conserved inside alveolar macrophages, thus indicating that bacteria were under degradation. However, rod-shapes were displayed inside lung pneumocytes, thus indicating a possible novel pathogenic mechanism that could facilitate survival in the harsh conditions of the lower respiratory tract, with enhanced immune responses, such as alveolar macrophages action. Apart form being a survival mechanism, live bacteria in pneumocytes could be modifying pneumonyctes behaviors at molecular and cellular levels, thus avoiding pneumocytes apoptosis as a survival mechanism. This hypothesis, as well as the effect on neighbor adjacent cells, would need further investigation. For instance, validation using electronic microscope or studying host-pathogen interactions at omics levels such as RNA or metabolome would be interesting to validate *H. parasuis* adaptations, as well as the response induced in host cells.

From the host side, modulation of immune response is an important pathogenic mechanism in other bacterial pathogens, and can be carried out in both directions, exaggerating immune response or suppressing inflammation [196, 197]. In our case, bacterial multiplication as results of survival to macrophage phagocytosis and serum resistance could be key to the increased immune response in the animals with clinical signs of Glassër's disease. This response was evidenced by the increased number of inflammatory cells in the lower respiratory tract as well as in bronchial space. Also, some *H. parasuis* structural proteins virulence factors, such as the outer membrane protein P2 have been directly involved in the induction of proinflammatory cytokine expression in porcine alveolar macrophages [118]. An overall observation was that animals inoculated with non-virulent strains were able to clear the inoculated bacteria, preventing clinical signs. On the other hand, catarrhal bronchopneumonia, a characteristic lesion in acute Glassër's disease, was observed in animals inoculated with virulent strains. In that case, *H. parasuis* infection was revealed via strong IHC staining at different lower respiratory tract locations, which

was accompanied by a strong immune response, as revealed by the increased number of immune cells, with inflammation patterns characteristic of catarrhal bronchopneumonia. Inflammation patterns due to uncontrolled infection have been reported during *H. parasuis* infection, *in vitro* with porcine epithelial PK-15 cells [96] and *in vivo* at spleen [90] and lung locations [73]. Although previous reports point to an increased immune cellular response in *H. parasuis* [73, 103], immune response during chronic infection may be different, with inhibited inflammatory responses, as reported for other bacterial pathogens, which could be part of future work [197]. From our results can be understood that efficient blockage of bacterial lung colonization is key to avoid further bacterial invasion and immune response exacerbation. Last, observation of bacterial cells inside blood vessels at different locations in the respiratory tract indicated that serum resistance capabilities were used, as reported earlier using *in vitro* assays [74] and also demonstrated via deletion mutants of key virulence factors [114, 150-153].

Biofilm formation by virulent and non-virulent strains of *H. parasuis*

Biofilm formation enhances virulence in some bacterial pathogens, but in *H. parasuis* this multicellular behavior is more frequent in non-virulent strains, as revealed using *in vitro* assays [66]. In contrast, we observed the ability of *H. parasuis* virulent strains to adhere to tracheal epithelial surfaces, revealing microcolony patterns as well as some thicker biofilm-like structures. It is well known that biofilm formation is a key strategy to colonize host surfaces and plays a role in the infection process in the *Pasteurellaceae* family, including *Haemophilus influenzae* [198] and *Histophilus somni* [199], as well as in other bacterial pathogens such as *Pseudomonas aeruginosa* [200] or *Streptococcus pneumoniae* [88]. In that regard, we designed a series of experiments to get additional insights about the different biofilm-forming abilities of virulent and non-virulent strains. Contrary to our *in vivo* observations, *in vitro* results showed that non-virulent strains formed more robust biofilms, in agreement with previous reports [66]. *H. parasuis in vitro* biofilms contained compounds such as poly-N-acetylglucosamine, proteins and extracellular DNA (eDNA), as it has been described for other bacterial species [85].

In vitro models are ideal since allow simplification of the more complex conditions bacteria find during natural infection, but some methodological aspects need to be taken into account. First, the methodology used to obtain multicellular aggregates is critical and possibly determinant for aerobes and anaerobes, thus being the oxygen concentration adapted according to the conditions bacteria finds *in vivo*. Since *H. parasuis* is a facultative anaerobe, is expected that in our microtiter plate model biofilm phenotypes would be exposed to anaerobic growth at the bottom of the well. On the other side, planktonic bacteria may be growing aerobically closer to the surface, where oxygen concentration is higher. Second, another factor that may also influence biofilm formation patterns is the starting nutrient concentration. A third limitation of this *in vitro* model is that cross-contamination of both samples, planktonic and biofilm bacteria, may occur during pipetting, constituting a confounding factor for the subsequent transcriptome analysis. Nevertheless, this contamination is expected and assumed to be low, making these phenotypes distinguishable. Although *in vitro* models may differ from the conditions that bacteria face *in vivo* [201], the strong biofilms formed by non-virulent strains made us further study the molecular mechanism governing conversion between biofilm and planktonic growth.

Overall, transcriptomic results agreed with those previously reported for other biofilm-forming bacteria growing in similar conditions [89]. Although it is important to mention that when comparing results from different studies one has to be cautious, since the bacteria to be compared and the methodology used can influence substantially the RNA-seq profiles. The general trend we observed when analyzing RNA-seq data and performing pathway enrichments was that *H. parasuis* biofilm and planktonic bacteria constitute similar biological states, characterized by a low metabolic rate, which could be directly related to low nutrient and oxygen availability after 36h of incubation. These data suggest that bacterial replication may be low at these growing conditions, as reported earlier, being key to survive in nature under adverse conditions, such as poor nutrient environments [89]. Nevertheless, the subtle enhanced activity of planktonic *versus* biofilm bacteria could also be linked to the change to colonizing phenotypes, where enhance ability to capture nutrients and rapid replication would be crucial. These subtle changes observed between biofilm and planktonic samples were substantially pronounced when comparing to stationary culture, which showed more active metabolism. Although stationary phase has been described as similar to biofilm in *P. aeruginosa* and *E. coli* [202, 203], we were not

able to replicate these observations for *H. parasuis*. This disagreement may be due to the use of *H. parasuis* samples from an earlier stationary phase, with better oxygen and nutrient access than those used in the *P. aeruginosa* and *E. coli* studies. In our case, we observed that *H. parasuis* in stationary phase changed to a different growing style balancing the cost of producing more nutrient transporters with the enhanced replicative rate pay off. Thus, our results suggest that bacteria growing under conventional laboratory culture conditions may be far from the biofilm-planktonic model presented, and assuming that this model is closer to the *in vivo* growing conditions, would be suitable for further host-pathogen interaction studies. Apart from the low metabolic state observed for both biofilm and planktonic samples, particular profiles were also detected. Biofilm was associated with lipid transport, protein secretion and folding, as well as response to stress. On the other side, planktonic sample revealed a tendency to up-regulate pathways with a signature of enhance metabolism such as carbohydrate transporters, electron transport chain, translation, glycerol and nitrogen transporters. All in all, these trends generally agree with the ones reported earlier for biofilm-forming bacteria and, more importantly, with the ones observed for the *in vitro* biofilms of *Actinobacillus pleuropneumoniae* [204]. Our results showed that *H. parasuis* has the ability to grow at different rates, thus allowing flexible metabolism and dynamic environmental adaptation, which could be crucial for survival during infection. In addition, the expression of outer membrane genes was detected in biofilm, which could be associated with the adhesion required to establish and maintain the biofilm. Some of these up-regulated genes coding for outer membrane proteins were present only in non-virulent *H. parasuis* strains, indicating its possible role in biofilm formation. A good example is the filamentous hemagglutinin FhaB, which has been associated to biofilm formation in other bacteria [205] and it was only detected in *H. parasuis* non-virulent strains. It is also important to note that the list of genes involved in biofilm formation in *H. parasuis* may be longer since some genes could be present in virulent and non-virulent strains but repressed in virulent strains due to regulatory mechanisms, and thus not able to participate in biofilm formation *in vitro*.

From the perspective of colonization or infection, if multicellular behaviors play an important role in *H. parasuis* during these steps is a controversial issue. Since we observed that *in vitro* biofilms were mostly formed by non-virulent isolates, results go in line with the alternative view of biofilms as not always associated to virulence. The fact that other bacterial species use biofilm as a strategy to persist in the host until adequate conditions

are present to start colonizing, invading and starting disease, gives alternative roles for biofilm formation in each bacterial species [88]. Virulent strains were not able to form biofilms *in vitro*, thus biofilm formation may not be a key virulence strategy for *H. parasuis*. On the other hand, non-virulent strains, which were able to form biofilm *in vitro*, were barely detected in the respiratory tract attached to respiratory epithelium. The lack of detection of non-virulent strains in the lung can be explained by their susceptibility to phagocytosis by alveolar macrophages. However, non-virulent strains were not visualized by IHC in the nasal cavities, from where they are successfully recovered by bacterial culture. This could be associated to the presence of non-virulent strains in the nasal mucus, which could be lost during IHC sample preparation. On the contrary, during the experimental infection, virulent strains were detected adhered to respiratory tract epithelial cells as well as in mucosal traps associated to epithelial cells. The surface of the trachea showed aggregates of virulent *H. parasuis*, resembling biofilms, suggesting that virulent strains are able to form biofilms *in vivo*, which could be a virulence strategy to survive in the host and one of the first stages of infection. These mucus traps with bacteria were detected located within the bronchial purulent inflammatory infiltrate (catarrhal-purulent bronchopneumonia) and within the bronchial inflammatory infiltrate. Although these biofilms differ from the classic biofilms, this could be due to particular *in vivo* conditions, being more complex than classic biofilm studies, since are biological surfaces. The observation that virulent strains were within mucus embedded with inflammatory cells suggests that inflammatory cells, detached epithelial cells might be key part of these structures, as described for nontypeable *Haemophilus influenzae* (NTHi) [206].

In conclusion, if biofilm-like structures formation capabilities *in vivo* play a role in *H. parasuis* infection is arguable. Our *in vitro* studies revealed that biofilm capacity might not be linked to virulence, yet the experimental infection suggests that *H. parasuis* caused Glässer's disease through biofilm-like structures, or at least by bacterial microcolonies attached to respiratory epithelium. Although biofilm formation may not be as disease-determinant as other factors, such as the presence of certain virulence factors or presence of maternal antibodies, could be directed towards increased virulence capacity. These observations may be also important when designing antibiotics targeting *H. parasuis*. Biofilms have been reported as interesting targets for antibiotic developments, but since non-virulent *H. parasuis* strains may confer benefit to the host, focusing on virulent strains may be a better alternative.

Virulent Nagasaki strain exhibits dynamic gene expression within the host

Lung infection seems to be an important step in Glässer's disease development. The majority of previous observations of the interaction of *H. parasuis* with its host when leading to Glässer's disease have been focused on describing their clinical signs and lesions. However, an important gap exists regarding bacterial adaptations, especially at molecular level. To better understand the molecular underpinnings behind *H. parasuis* abilities to survive in the host, we selected a transcriptomics strategy. *H. parasuis* gene expression was studied during a 2h *ex vivo* infection and was compared to plate culture. To validate this *ex vivo* model we also designed a 2h *in vivo* infection in pigs, its natural host. Our results indicated that *H. parasuis* was able to adapt its gene expression, both *in vivo* and *ex vivo*. Interestingly, similar gene expression patterns were observed during these two conditions, thus adding value to our *ex vivo* model. Up-regulated genes were mainly related to nutrient capture, as some iron and ABC transporters were found induced. Thus, our results indicate that a novel pathogenic mechanism may be used by *H. parasuis*; the ability to adapt its metabolic rate during infection. This phenomenon, known as "nutritional virulence", has been reported for other well-adapted bacterial pathogens, able to survive harsh growing conditions, with limited nutrient availability [207, 208]. Also, some induced genes had unknown function, possible being novel virulence factors. Moreover, some of the induced genes were unique of virulent strains, indicating its role in virulence. Among them, the *vtaAs*, known to be antigenic and involved in phagocytosis delay were notable [72, 132]. On the other side, previously reported *H. parasuis* virulence factors that were characterized via mutants in processes such as adhesion, invasion or serum resistance were found down-regulated or with stable expression, indicating that these may not play a role in the lung or alternatively they may be regulated by sRNAs [209].

Perusal of the literature showed a lack of works regarding *H. parasuis* gene expression in the lung. One study, using capture of transcribed sequences (SCOTS), reported genes transcribed by *H. parasuis* in necrotic porcine lungs [141], but methodological aspects are quite distant, such as time of infection, and limit the possible comparison with our results. Although the number of differentially expressed genes was smaller, an overall observation is that similar patterns were detected, such as alteration of genes belonging either to

intracellular or membrane functions, including cell surface iron regulated transporters, and genes involved in intracellular metabolic adaptation. Similar patterns were reported under *in vitro* growth mimicking the *in vivo* environment [144]. Lack of standardization when reporting these results makes difficult to validate if the altered genes are the same ones, since homology would need to be validated before making conclusions. Similar picture is observed when trying to compare the *in vivo* transcriptomic profiles of other bacterial pathogens. Further works will be needed to validate these gene expression trends during *H. parasuis* infection.

Future work

Novel questions arise when comparing the current progress in Glässer's disease research with other respiratory pathogens, where farfetched molecular mechanisms have been revealed. Among them, bacterial adaptations for an efficient carbon metabolism as well as the alteration of host metabolites [210], manipulation of innate immunity to enhance infection [211] or the case of *S. pneumoniae,* able to release hydrogen peroxide and other cytotoxic reagents causing DNA damage in alveolar epithelial cells, leading to apoptosis [212]. Our results suggest that similar scenario could be happening in *H. parasuis*. From the host side, alteration of host immune cell system was observed at microscopic level, with enhanced inflammatory reaction. From the pathogen, *H. parasuis* Nagasaki showed different gene expression in the lung. However, bacterial cells could be distributed at different sites, which could be an additional cause of gene expression variation in order to adapt to particular host environment, such as inside alveolar macrophages or pneumocytes. Although may not necessary to go up to single-cell approximations [213], sampling bacteria at specific locations with sophisticated techniques such as laser sampling could be part of future work. Also, time could be an additional cause of gene expression variation, even at the same host location. Thus, the reader should note that combination of sampling sites and different time points would be more accurate, thus generating valuable information, especially if combined with host response data, since pathogens can alter host responses at cellular and molecular levels. Although it is true that these studies will have their own caveats, such as sequencing depth and complexity of data analysis, its potential is undeniable. We also think that our IHC findings will be useful to guide these studies. It is also important to note that these findings at RNA level would need further validation at

protein level, as well as using mutants lacking identified virulence factors. All in all, knowing more about how chameleonic bacteria use protein repertoire would also have important implications for the development of antibiotics and vaccines.

CONCLUSIONS

1- *H. parasuis* isolates present particular colonization patterns depending on the virulence of the strain; virulent isolates were found in multiple locations in the respiratory tract, while non-virulent *H. parasuis* were barely detected.

2- The capacity of virulent *H. parasuis* strains to adhere and invade eukaryotic cells *in vitro* was confirmed *in vivo*, where they could be detected within cells, such as pneumocytes.

3- Non-virulent *H. parasuis* strains display stronger biofilm formation capacities than virulent isolates *in vitro*. In the biofilm, *H. parasuis* cells presented a low metabolic state, which may be associated to persistence.

4- The use of lung explants constitute a good model to study *H. parasuis* pathogenicity in the lung, since it revealed similar bacterial RNA profiles than those observed in *in vivo* lung infection.

5- *H. parasuis* adapts its metabolism during lung infection, possibly as a survival strategy, involved in "nutritional virulence".

6- *H. parasuis* outer membrane is modified during lung infection, including overexpression of virulence factors, such as the virulence associated trimeric autotransporters (VtaA).

7- *H. parasuis* displays non-coding RNAs that are differentially expressed in the lung, which may play a role during infection.

BIBLOGRAPHY

1. Aragon V, Segales J, Oliveira S. **Glässer's disease**. 10 ed. Diseases of Swine. Ames, Iowa: Wiley-Blackwell; 2012.
2. Angen Ø, Oliveira S, Ahrens P, Svensmark B, Leser TD: **Development of an improved species specific PCR test for detection of *Haemophilus parasuis***. *Vet Microbiol* 2007, **119**:266-276.
3. Olvera A, Calsamiglia M, Aragon V: **Genotypic Diversity of *Haemophilus parasuis* Field Strains**. *Appl Environ Microbiol* 2006, **72**:3984-3992.
4. Olvera A, Cerda-Cuellar M, Mentaberre G, Casas-Diaz E, Lavin S, Marco I, Aragon V: **First isolation of *Haemophilus parasuis* and other NAD-dependent Pasteurellaceae of swine from European wild boars**. *Vet Microbiol* 2007, **125**:182-186.
5. Biberstein EL, Mini PD, Gills MG: **Action of *Haemophilus* Cultures on Delta-Aminolevulinic Acid**. *J Bacteriol* 1963, **86**:814-819.
6. Amano H, Shibata M, Kajio N, Morozumi T: **Pathologic observations of pigs intranasally inoculated with serovar 1, 4 and 5 of *Haemophilus parasuis* using immunoperoxidase method**. *J Vet Med Sci* 1994, **56**:639-644.
7. Segales J, Domingo M, Solano GI, Pijoan C: **Immunohistochemical detection of *Haemophilus parasuis* serovar 5 in formalin-fixed, paraffin-embedded tissues of experimentally infected swine**. *J Vet Diagn Invest* 1997, **9**:237-243.
8. Vahle JL, Haynes JS, Andrews JJ: **Experimental reproduction of *Haemophilus parasuis* infection in swine: clinical, bacteriological, and morphologic findings**. *J Vet Diagn Invest* 1995, **7**:476-480.
9. Jung K, Ha Y, Kim SH, Chae C: **Development of polymerase chain reaction and comparison with *in situ* hybridization for the detection of *Haemophilus parasuis* in formalin-fixed, paraffin-embedded tissues**. *J Vet Med Sci* 2004, **66**:841-845.
10. Oliveira S, Galina L, Pijoan C: **Development of a PCR test to diagnose *Haemophilus parasuis* infections**. *J Vet Diagn Invest* 2001, **13**:495-501.
11. Turni C, Pyke M, Blackall PJ: **Validation of a real-time PCR for *Haemophilus parasuis***. *J Appl Microbiol* 2010, **108**:1323-1331.
12. Neil DH, McKay KA, L'Ecuyer C, Corner AH: **Glasser's disease of swine produced by the intracheal inoculation of *Haemophilus suis***. *Can J Comp Med* 1969, **33**:187-193.
13. Li JX, Jiang P, Wang Y, Li YF, Chen W, Wang XW, Li P: **Genotyping of *Haemophilus parasuis* from diseased pigs in China and prevalence of two coexisting virus pathogens**. *Prev Vet Med* 2009, **91**:274-279.
14. Palzer A, Ritzmann M, Wolf G, Heinritzi K: **Associations between pathogens in healthy pigs and pigs with pneumonia**. *Vet Rec* 2008, **162**:267-271.
15. Peet RL, Fry J, Lloyd J, Henderson J, Curran J, Moir D: ***Haemophilus parasuis* septicaemia in pigs**. *Aust Vet J* 1983, **60**:187.
16. Riley MG, Russell EG, Callinan RB: ***Haemophilus parasuis* infection in swine**. *J Am Vet Med Assoc* 1977, **171**:649-651.
17. Oliveira S, Galina L, Blanco I, Canals A, Pijoan C: **Naturally-farrowed, artificially-reared pigs as an alternative model for experimental infection by *Haemophilus parasuis***. *Can J Vet Res* 2003, **67**:146-150.
18. Thomson. Bones and joints. In: Maxie M, editor. Pathology of Domestic Animals. Saunders; 2007. p. 2-184.
19. Morozumi T, Nicolet J: **Morphological variations of *Haemophilus parasuis* strains**. *J Clin Microbiol* 1986, **23**:138-142.

20. Oliveira S, Pijoan C: ***Haemophilus parasuis*: new trends on diagnosis, epidemiology and control**. *Vet Microbiol* 2004, **99**:1-12.
21. de la Puente Redondo VA, Navas Mendez J, Garcia del Blanco N, Ladron Boronat N, Gutierrez Martin CB, Rodriguez Ferri EF: **Typing of *Haemophilus parasuis* strains by PCR-RFLP analysis of the *tbpA* gene**. *Vet Microbiol* 2003, **92**:253-262.
22. Olvera A, Pina S, Macedo N, Oliveira S, Aragon V, Bensaid A: **Identification of potentially virulent strains of *Haemophilus parasuis* using a multiplex PCR for virulence-associated autotransporters (vtaA)**. *Vet J* 2012, **191**:213-218.
23. Assavacheep P, Assavacheep A, Turni C: **Detection of a putative hemolysin operon, hhdBA, of *Haemophilus parasuis* from pigs with Glasser disease**. *J Vet Diagn Invest* 2012, **24**:339-343.
24. McDowall R, Slavic D, MacInnes JI, Cai HY: **Evaluation of a real-time polymerase chain reaction assay of the outer membrane protein P2 gene for the detection of *Haemophilus parasuis* in clinical samples**. *Can J Vet Res* 2014, **78**:150-152.
25. Hricinova M, Holoda E, Mudronova D, Ondrasovicova S: **Multiplex PCR assay for detection of *Actinobacillus pleuropneumoniae*, *Pasteurella multocida* and *Haemophilus parasuis* in lungs of pigs from a slaughterhouse**. *Folia Microbiol (Praha)* 2010, **55**:635-640.
26. Frandoloso R, Martinez-Martinez S, Rodriguez-Ferri EF, Gutierrez-Martin CB: **Comparison of real-time PCR and culture isolation in colostrum-deprived pigs immunized and challenged with *Haemophilus parasuis***. *Lett Appl Microbiol* 2012, **54**:149-152.
27. Kang I, Kim D, Han K, Seo HW, Oh Y, Park C, Lee J, Gottschalk M, Chae C: **Optimized protocol for multiplex nested polymerase chain reaction to detect and differentiate *Haemophilus parasuis*, *Streptococcus suis*, and *Mycoplasma hyorhinis* in formalin-fixed, paraffin-embedded tissues from pigs with polyserositis**. *Can J Vet Res* 2012, **76**:195-200.
28. Chen S, Chu Y, Zhao P, He Y, Jian Y, Liu Y, Lu Z: **Development of a recombinant OppA-based indirect hemagglutination test for the detection of antibodies against *Haemophilus parasuis***. *Acta Trop* 2015, **148**:8-12.
29. Galina Pantoja L, Stammen B, Minton B, Amodie D: **Serologic profiling of *Haemophilus parasuis*-vaccinated sows and their litters using a novel oligopeptide permease A enzyme-linked immunosorbent assay reveals unexpected patterns of serological response and maternal antibody transfer**. *J Vet Diagn Invest* 2014, **26**:125-130.
30. Olvera A, Martinez-Moliner V, Pina-Pedrero S, Perez-Simo M, Galofre-Mila N, Costa-Hurtado M, Aragon V, Bensaid A: **Serum cross-reaction among virulence-associated trimeric autotransporters (VtaA) of *Haemophilus parasuis***. *Vet Microbiol* 2013, **164**:387-391.
31. Vahle JL, Haynes JS, Andrews JJ: **Interaction of *Haemophilus parasuis* with nasal and tracheal mucosa following intranasal inoculation of cesarean derived colostrum deprived (CDCD) swine**. *Can J Vet Res* 1997, **61**:200-206.
32. Jung K, Chae C: **In-situ hybridization for the detection of *Haemophilus parasuis* in naturally infected pigs**. *J Comp Pathol* 2004, **130**:294-298.
33. Zhang JM, Shen HY, Liao M, Ren T, Guo LL, Xu CG, Feng SX, Fan HY, Li JY, Chen JD, Zhang B: **Detection of *Haemophilus parasuis* isolates from South China by loop-mediated isothermal amplification and isolate characterisation**. *Onderstepoort J Vet Res* 2012, **79**:E1-6.
34. Morozumi T, Nicolet J: **Some antigenic properties of *Haemophilus parasuis* and a proposal for serological classification**. *J Clin Microbiol* 1986, **23**:1022-1025.

35. Oliveira S, Blackall PJ, Pijoan C: **Characterization of the diversity of *Haemophilus parasuis* field isolates by use of serotyping and genotyping**. *Am J Vet Res* 2003, **64**:435-442.
36. Kielstein P, Rosner H, Muller W: **Typing of heat-stable soluble *Haemophilus parasuis* antigen by means of agargel precipitation and the dot-blot procedure**. *Zentralbl Veterinarmed B* 1991, **38**:315-320.
37. Kielstein P, Rapp-Gabrielson VJ: **Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts**. *J Clin Microbiol* 1992, **30**:862-865.
38. Del Rio ML, Gutierrez CB, Rodriguez Ferri EF: **Value of indirect hemagglutination and coagglutination tests for serotyping *Haemophilus parasuis***. *J Clin Microbiol* 2003, **41**:880-882.
39. Tadjine M, Mittal KR, Bourdon S, Gottschalk M: **Development of a new serological test for serotyping *Haemophilus parasuis* isolates and determination of their prevalence in North America**. *J Clin Microbiol* 2004, **42**:839-840.
40. Angen Ø, Svensmark B, Mittal KR: **Serological characterization of Danish *Haemophilus parasuis* isolates**. *Vet Microbiol* 2004, **103**:255-258.
41. Cai X, Chen H, Blackall PJ, Yin Z, Wang L, Liu Z, Jin M: **Serological characterization of *Haemophilus parasuis* isolates from China**. *Vet Microbiol* 2005, **111**:231-236.
42. Turni C, Blackall PJ: **Comparison of the indirect haemagglutination and gel diffusion test for serotyping *Haemophilus parasuis***. *Vet Microbiol* 2005, **106**:145-151.
43. Morris S, Carrington L, Guttierez-Martin C, Jackson G, Tucker A, editors. Characteristion of field isolates of *Haemophilus parasuis* from the UK. 19th IPVS Congress; 2006; Copenhagen.
44. Smart NL, Miniats OP, MacInnes JI: **Analysis of *Haemophilus parasuis* isolates from southern Ontario swine by restriction endonuclease fingerprinting**. *Can J Vet Res* 1988, **52**:319-324.
45. Rafiee M, Bara M, Stephens CP, Blackall PJ: **Application of ERIC-PCR for the comparison of isolates of *Haemophilus parasuis***. *Aust Vet J* 2000, **78**:846-849.
46. Ruiz A, Oliveira S, Torremorell M, Pijoan C: **Outer membrane proteins and DNA profiles in strains of *Haemophilus parasuis* recovered from systemic and respiratory sites**. *J Clin Microbiol* 2001, **39**:1757-1762.
47. Olvera A, Cerda-Cuellar M, Aragon V: **Study of the population structure of *Haemophilus parasuis* by multilocus sequence typing**. *Microbiology* 2006, **152**:3683-3690.
48. Mullins MA, Register KB, Brunelle BW, Aragon V, Galofre-Mila N, Bayles DO, Jolley KA: **A curated public database for multilocus sequence typing (MLST) and analysis of *Haemophilus parasuis* based on an optimized typing scheme**. *Vet Microbiol* 2013, **162**:899-906.
49. Aragon V, Cerda-Cuellar M, Fraile L, Mombarg M, Nofrarias M, Olvera A, Sibila M, Solanes D, Segales J: **Correlation between clinico-pathological outcome and typing of *Haemophilus parasuis* field strains**. *Vet Microbiol* 2010, **142**:387-393.
50. Olvera A, Cerda-Cuellar M, Nofrarias M, Revilla E, Segales J, Aragon V: **Dynamics of *Haemophilus parasuis* genotypes in a farm recovered from an outbreak of Glasser's disease**. *Vet Microbiol* 2007, **123**:230-237.
51. Dijkman R, Wellenberg GJ, van der Heijden HM, Peerboom R, Olvera A, Rothkamp A, Peperkamp K, van Esch EJ: **Analyses of Dutch *Haemophilus parasuis* isolates by serotyping, genotyping by ERIC-PCR and *Hsp60* sequences and the presence of the virulence associated trimeric autotransporters marker**. *Res Vet Sci* 2012, **93**:589-595.

52. Macedo N, Rovira A, Oliveira S, Holtcamp A, Torremorell M: **Effect of enrofloxacin in the carrier stage of *Haemophilus parasuis* in naturally colonized pigs**. *Can J Vet Res* 2014, **78**:17-22.
53. Howell KJ, Weinert LA, Chaudhuri RR, Luan S, Peters SE, Corander J, Harris D, Angen O, Aragon V, Bensaid A, Williamson SM, Parkhill J, Langford PR, Rycroft AN, Wren BW, Holden MT, Tucker AW, Maskell DJ: **The use of genome wide association methods to investigate pathogenicity, population structure and serovar in *Haemophilus parasuis***. *BMC genomics* 2014, **15**:1179.
54. Bello-Orti B, Aragon V, Pina-Pedrero S, Bensaid A: **Genome comparison of three serovar 5 pathogenic strains of *Haemophilus parasuis*: insights into an evolving swine pathogen**. *Microbiology* 2014, **160**:1974-1984.
55. Lawrence P, Bey R: **Map-based comparative genomic analysis of virulent *Haemophilus parasuis* serovars 4 and 5**. *Journal of genomics* 2015, **3**:59-71.
56. Sanchez-Romero MA, Cota I, Casadesus J: **DNA methylation in bacteria: from the methyl group to the methylome**. *Curr Opin Microbiol* 2015, **25**:9-16.
57. Young BC, Golubchik T, Batty EM, Fung R, Larner-Svensson H, Votintseva AA, Miller RR, Godwin H, Knox K, Everitt RG, Iqbal Z, Rimmer AJ, Cule M, Ip CL, Didelot X, Harding RM, Donnelly P, Peto TE, Crook DW, Bowden R, Wilson DJ: **Evolutionary dynamics of *Staphylococcus aureus* during progression from carriage to disease**. *Proc Natl Acad Sci U S A* 2012, **109**:4550-4555.
58. Olsen RJ, Sitkiewicz I, Ayeras AA, Gonulal VE, Cantu C, Beres SB, Green NM, Lei B, Humbird T, Greaver J, Chang E, Ragasa WP, Montgomery CA, Cartwright J, Jr., McGeer A, Low DE, Whitney AR, Cagle PT, Blasdel TL, DeLeo FR, Musser JM: **Decreased necrotizing fasciitis capacity caused by a single nucleotide mutation that alters a multiple gene virulence axis**. *Proc Natl Acad Sci U S A* 2010, **107**:888-893.
59. Durmus S, Cakir T, Ozgur A, Guthke R: **A review on computational systems biology of pathogen-host interactions**. *Frontiers in microbiology* 2015, **6**:235.
60. Heroven AK, Dersch P: **Coregulation of host-adapted metabolism and virulence by pathogenic *yersiniae***. *Frontiers in cellular and infection microbiology* 2014, **4**:146.
61. Holt JF, Kiedrowski MR, Frank KL, Du J, Guan C, Broderick NA, Dunny GM, Handelsman J: ***Enterococcus faecalis* 6-phosphogluconolactonase is required for both commensal and pathogenic interactions with Manduca sexta**. *Infect Immun* 2015, **83**:396-404.
62. Yang J, Chen L, Sun L, Yu J, Jin Q: **VFDB 2008 release: an enhanced web-based resource for comparative pathogenomics**. *Nucleic Acids Res* 2008, **36**:D539-542.
63. Gillespie JJ, Wattam AR, Cammer SA, Gabbard JL, Shukla MP, Dalay O, Driscoll T, Hix D, Mane SP, Mao C, Nordberg EK, Scott M, Schulman JR, Snyder EE, Sullivan DE, Wang C, Warren A, Williams KP, Xue T, Yoo HS, Zhang C, Zhang Y, Will R, Kenyon RW, Sobral BW: **PATRIC: the comprehensive bacterial bioinformatics resource with a focus on human pathogenic species**. *Infect Immun* 2011, **79**:4286-4298.
64. Frandoloso R, Martinez-Martinez S, Gutierrez-Martin CB, Rodriguez-Ferri EF: ***Haemophilus parasuis* serovar 5 Nagasaki strain adheres and invades PK-15 cells**. *Vet Microbiol* 2012, **154**:347-352.
65. Frandoloso R, Pivato M, Martinez-Martinez S, Rodriguez-Ferri EF, Kreutz LC, Martin CB: **Differences in *Haemophilus parasuis* adherence to and invasion of AOC-45 porcine aorta endothelial cells**. *BMC veterinary research* 2013, **9**:207.
66. Jin H, Zhou R, Kang M, Luo R, Cai X, Chen H: **Biofilm formation by field isolates and reference strains of *Haemophilus parasuis***. *Vet Microbiol* 2006.
67. Stewart PS, Costerton JW: **Antibiotic resistance of bacteria in biofilms**. *Lancet* 2001, **358**:135-138.

68. Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O: **Antibiotic resistance of bacterial biofilms**. *Int J Antimicrob Agents* 2010, **35**:322-332.
69. Zhang J, Xu C, Shen H, Li J, Guo L, Cao G, Feng S, Liao M: **Biofilm formation in *Haemophilus parasuis*: relationship with antibiotic resistance, serotype and genetic typing**. *Res Vet Sci* 2014, **97**:171-175.
70. Mullins MA, Register KB, Bayles DO, Butler JE: ***Haemophilus parasuis* exhibits IgA protease activity but lacks homologs of the IgA protease genes of *Haemophilus influenzae***. *Vet Microbiol* 2011, **153**:407-412.
71. Olvera A, Ballester M, Nofrarias M, Sibila M, Aragon V: **Differences in phagocytosis susceptibility in *Haemophilus parasuis* strains**. *Vet Res* 2009, **40**:24.
72. Costa-Hurtado M, Ballester M, Galofre-Mila N, Darji A, Aragon V: **VtaA8 and VtaA9 from *Haemophilus parasuis* delay phagocytosis by alveolar macrophages**. *Vet Res* 2012, **43**:57.
73. Costa-Hurtado M, Olvera A, Martinez-Moliner V, Galofre-Mila N, Martinez P, Dominguez J, Aragon V: **Changes in macrophage phenotype after infection of pigs with *Haemophilus parasuis* strains with different levels of virulence**. *Infect Immun* 2013, **81**:2327-2333.
74. Cerdà-Cuéllar M, Aragon V: **Serum-resistance in *Haemophilus parasuis* is associated with systemic disease in swine**. *Vet J* 2008, **175**:384-389.
75. Vanier G, Szczotka A, Friedl P, Lacouture S, Jacques M, Gottschalk M: ***Haemophilus parasuis* invades porcine brain microvascular endothelial cells**. *Microbiology* 2006, **152**:135-142.
76. Aragon V, Bouchet B, Gottschalk M: **Invasion of endothelial cells by systemic and nasal strains of *Haemophilus parasuis***. *Vet J* 2010, **186**:264-267.
77. Bouchet B, Vanier G, Jacques M, Gottschalk M: **Interactions of *Haemophilus parasuis* and its LOS with Porcine Brain Microvascular Endothelial Cells**. *Vet Res* 2008, **39**:42.
78. Shapiro JA: **Bacteria as multicellular organisms**. *Sci Am* 1988, **256**:82–89.
79. Verstraeten N, Braeken K, Debkumari B, Fauvart M, Fransaer J, Vermant J, Michiels J: **Living on a surface: swarming and biofilm formation**. *Trends Microbiol* 2008, **16**:496-506.
80. Kearns DB: **A field guide to bacterial swarming motility**. *Nature reviews Microbiology* 2010, **8**:634-644.
81. Shapiro JA: **Thinking about bacterial populations as multicellular organisms**. *Annu Rev Microbiol* 1998, **52**:81-104.
82. Claessen D, Rozen DE, Kuipers OP, Sogaard-Andersen L, van Wezel GP: **Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies**. *Nature reviews Microbiology* 2014, **12**:115-124.
83. Chauhan A, Madiraju MV, Fol M, Lofton H, Maloney E, Reynolds R, Rajagopalan M: ***Mycobacterium tuberculosis* cells growing in macrophages are filamentous and deficient in FtsZ rings**. *J Bacteriol* 2006, **188**:1856-1865.
84. Karatan E, Watnick P: **Signals, regulatory networks, and materials that build and break bacterial biofilms**. *Microbiol Mol Biol Rev* 2009, **73**:310-347.
85. Flemming HC, Wingender J: **The biofilm matrix**. *Nature reviews Microbiology* 2010, **8**:623-633.
86. Marks LR, Parameswaran GI, Hakansson AP: **Pneumococcal interactions with epithelial cells are crucial for optimal biofilm formation and colonization *in vitro* and *in vivo***. *Infect Immun* 2012, **80**:2744-2760.

87. Gilley RP, Orihuela CJ: **Pneumococci in biofilms are non-invasive: implications on nasopharyngeal colonization**. *Frontiers in cellular and infection microbiology* 2014, **4**:163.
88. Chao Y, Marks LR, Pettigrew MM, Hakansson AP: ***Streptococcus pneumoniae* biofilm formation and dispersion during colonization and disease**. *Frontiers in cellular and infection microbiology* 2014, **4**:194.
89. McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S: **Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal**. *Nature reviews Microbiology* 2012, **10**:39-50.
90. Chen H, Li C, Fang M, Zhu M, Li X, Zhou R, Li K, Zhao S: **Understanding *Haemophilus parasuis* infection in porcine spleen through a transcriptomics approach**. *BMC genomics* 2009, **10**:64.
91. Wang Y, Liu C, Fang Y, Liu X, Li W, Liu S, Liu Y, Liu Y, Charreyre C, Audonnet JC, Chen P, He Q: **Transcription analysis on response of porcine alveolar macrophages to *Haemophilus parasuis***. *BMC genomics* 2012, **13**:68.
92. Liu XD, Chen HB, Tong Q, Li XY, Zhu MJ, Wu ZF, Zhou R, Zhao SH: **Molecular characterization of caveolin-1 in pigs infected with *Haemophilus parasuis***. *J Immunol* 2011, **186**:3031-3046.
93. Wang XM, Kim HP, Song R, Choi AM: **Caveolin-1 confers antiinflammatory effects in murine macrophages via the MKK3/p38 MAPK pathway**. *Am J Respir Cell Mol Biol* 2006, **34**:434-442.
94. Del Galdo F, Sotgia F, de Almeida CJ, Jasmin JF, Musick M, Lisanti MP, Jimenez SA: **Decreased expression of caveolin 1 in patients with systemic sclerosis: crucial role in the pathogenesis of tissue fibrosis**. *Arthritis Rheum* 2008, **58**:2854-2865.
95. Chen Y, Jin H, Chen P, Li Z, Meng X, Liu M, Li S, Shi D, Xiao Y, Wang X, Zhou Z, Bi D, Zhou R: ***Haemophilus parasuis* infection activates the NF-κB pathway in PK-15 cells through IκB degradation**. *Vet Microbiol* 2012.
96. Chen Y, Liu T, Langford P, Hua K, Zhou S, Zhai Y, Xiao H, Luo R, Bi D, Jin H, Zhou R: ***Haemophilus parasuis* induces activation of NF-kappaB and MAP kinase signaling pathways mediated by toll-like receptors**. *Mol Immunol* 2015, **65**:360-366.
97. Chen H, Lunney JK, Cheng L, Li X, Cao J, Zhu M, Zhao S: **Porcine S100A8 and S100A9: molecular characterizations and crucial functions in response to *Haemophilus parasuis* infection**. *Dev Comp Immunol* 2011, **35**:490-500.
98. Li X, Tang J, Xu J, Zhu M, Cao J, Liu Y, Yu M, Zhao S: **The inflammation-related gene S100A12 is positively regulated by C/EBPbeta and AP-1 in pigs**. *International journal of molecular sciences* 2014, **15**:13802-13816.
99. Pirofski LA, Casadevall A: **The damage-response framework of microbial pathogenesis and infectious diseases**. *Adv Exp Med Biol* 2008, **635**:135-146.
100. Martín de la Fuente AJ, Ferri EF, Tejerina F, Frandoloso R, Martinez SM, Martin CB: **Cytokine expression in colostrum-deprived pigs immunized and challenged with *Haemophilus parasuis***. *Res Vet Sci* 2009, **87**:47-52.
101. Frandoloso R, Martinez-Martinez S, Rodriguez-Ferri EF, Yubero S, Rodriguez-Lazaro D, Hernandez M, Gutierrez-Martin CB: ***Haemophilus parasuis* subunit vaccines based on native proteins with affinity to porcine transferrin prevent the expression of proinflammatory chemokines and cytokines in pigs**. *Clinical & developmental immunology* 2013, **2013**:132432.
102. Martín de la Fuente AJ, Gutierrez-Martin CB, Rodriguez-Barbosa JI, Martinez-Martinez S, Frandoloso R, Tejerina F, Rodriguez-Ferri EF: **Blood cellular immune response in pigs immunized and challenged with *Haemophilus parasuis***. *Res Vet Sci* 2009, **86**:230-234.

103. Frandoloso R, Martinez-Martinez S, Yubero S, Rodriguez-Ferri EF, Gutierrez-Martin CB: **New insights in cellular immune response in colostrum-deprived pigs after immunization with subunit and commercial vaccines against Glasser's disease**. *Cell Immunol* 2012.
104. McVicker JK, Tabatabai LB: **Isolation and characterization of the P5 adhesin protein of *Haemophilus parasuis* serotype 5**. *Prep Biochem Biotechnol* 2006, **36**:363-374.
105. del Rio ML, Gutierrez-Martin CB, Rodriguez-Barbosa JI, Navas J, Rodriguez-Ferri EF: **Identification and characterization of the TonB region and its role in transferrin-mediated iron acquisition in *Haemophilus parasuis***. *FEMS Immunol Med Microbiol* 2005, **45**:75-86.
106. del Rio ML, Navas J, Martin AJ, Gutierrez CB, Rodriguez-Barbosa JI, Rodriguez Ferri EF: **Molecular characterization of *Haemophilus parasuis* ferric hydroxamate uptake (*fhu*) genes and constitutive expression of the FhuA receptor**. *Vet Res* 2006, **37**:49-59.
107. Huang X, Li Y, Fu Y, Ji Y, Lian K, Zheng H, Wei J, Cai X, Zhu Q: **Cross-protective efficacy of recombinant transferrin-binding protein A of *Haemophilus parasuis* in guinea pigs**. *Clinical and vaccine immunology : CVI* 2013, **20**:912-919.
108. Martinez S, Frandoloso R, Rodriguez-Ferri EF, Gonzalez-Zorn B, Gutierrez-Martin CB: **Characterization of a recombinant transferrin-binding protein A (TbpA) fragment from *Haemophilus parasuis* serovar 5**. *FEMS Microbiol Lett* 2010.
109. Frandoloso R, Martinez-Martinez S, Calmettes C, Fegan J, Costa E, Curran D, Yu RH, Gutierrez-Martin CB, Rodriguez-Ferri EF, Moraes TF, Schryvers AB: **Nonbinding site-directed mutants of transferrin binding protein B exhibit enhanced immunogenicity and protective capabilities**. *Infect Immun* 2015, **83**:1030-1038.
110. Tadjine M, Mittal KR, Bourdon S, Gottschalk M: **Production and characterization of murine monoclonal antibodies against *Haemophilus parasuis* and study of their protective role in mice**. *Microbiology* 2004, **150**:3935-3945.
111. Hood DW, Makepeace K, Deadman ME, Rest RF, Thibault P, Martin A, Richards JC, Moxon ER: **Sialic acid in the lipopolysaccharide of *Haemophilus influenzae*: strain distribution, influence on serum resistance and structural characterization**. *Mol Microbiol* 1999, **33**:679-692.
112. Martinez-Moliner V, Soler-Llorens P, Moleres J, Garmendia J, Aragon V: **Distribution of genes involved in sialic acid utilization in strains of *Haemophilus parasuis***. *Microbiology* 2012, **158**:2117-2124.
113. Domenico P, Salo RJ, Cross AS, Cunha BA: **Polysaccharide capsule-mediated resistance to opsonophagocytosis in *Klebsiella pneumoniae***. *Infect Immun* 1994, **62**:4495-4499.
114. Wang X, Xu X, Wu Y, Li L, Cao R, Cai X, Chen H: **Polysaccharide biosynthesis protein CapD is a novel pathogenicity-associated determinant of *Haemophilus parasuis* involved in serum-resistance ability**. *Vet Microbiol* 2013, **164**:184-189.
115. Confer AW, Ayalew S: **The OmpA family of proteins: roles in bacterial pathogenesis and immunity**. *Vet Microbiol* 2013, **163**:207-222.
116. Zhang B, Feng S, Xu C, Zhou S, He Y, Zhang L, Zhang J, Guo L, Liao M: **Serum resistance in *Haemophilus parasuis* SC096 strain requires outer membrane protein P2 expression**. *FEMS Microbiol Lett* 2012, **326**:109-115.
117. Zhang B, Xu C, Liao M: **Outer membrane protein P2 of the *Haemophilus parasuis* SC096 strain contributes to adherence to porcine alveolar macrophages cells**. *Vet Microbiol* 2012, **158**:226-227.

118. Zhou S, He X, Xu C, Zhang B, Feng S, Zou Y, Li J, Liao M: **The outer membrane protein P2 (OmpP2) of *Haemophilus parasuis* induces proinflammatory cytokine mRNA expression in porcine alveolar macrophages**. *Vet J* 2014, **199**:461-464.
119. Mullins MA, Register KB, Bayles DO, Loving CL, Nicholson TL, Brockmeier SL, Dyer DW, Phillips GJ: **Characterization and comparative analysis of the genes encoding *Haemophilus parasuis* outer membrane proteins P2 and P5**. *J Bacteriol* 2009, **191**:5988-6002.
120. Wang X, Xu X, Zhang S, Guo F, Cai X, Chen H: **Identification and analysis of potential virulence-associated genes in *Haemophilus parasuis* based on genomic subtraction**. *Microb Pathog* 2011, **51**:291-296.
121. Del Rio ML, Navas-Mendez J, Gutierrez-Martin CB, Rodriguez-Barbosa JI, Rodriguez-Ferri EF: **Identification of sulI allele of dihydropteroate synthase by representational difference analysis in *Haemophilus parasuis* serovar 2**. *Lett Appl Microbiol* 2005, **40**:436-442.
122. Metcalf DS, MacInnes JI: **Differential expression of *Haemophilus parasuis* genes in response to iron restriction and cerebrospinal fluid**. *Can J Vet Res* 2007, **71**:181-188.
123. Sack M, Baltes N: **Identification of novel potential virulence-associated factors in *Haemophilus parasuis***. *Vet Microbiol* 2009, **136**:382-386.
124. Zhou H, Yang B, Xu F, Chen X, Wang J, Blackall PJ, Zhang P, Xia Y, Zhang J, Ma R: **Identification of putative virulence-associated genes of *Haemophilus parasuis* through suppression subtractive hybridization**. *Vet Microbiol* 2010.
125. Yu J, Wu J, Zhang Y, Du Y, Peng J, Chen L, Sun W, Cong X, Xu S, Shi J, Li J, Huang B, Zhu X, Wang J: **Identification of putative virulence-associated genes among *Haemophilus parasuis* strains and the virulence difference of different serovars**. *Microb Pathog* 2014, **77**:17-23.
126. Yue M, Yang F, Yang J, Bei W, Cai X, Chen L, Dong J, Zhou R, Jin M, Jin Q, Chen H: **Complete genome sequence of *Haemophilus parasuis* SH0165**. *J Bacteriol* 2009, **191**:1359-1360.
127. Li Y, Kwok AH, Jiang J, Zou Y, Zheng F, Chen P, Hou C, Leung FC, Jiang P: **Complete Genome Analysis of a *Haemophilus parasuis* Serovar 12 Strain from China**. *PloS one* 2013, **8**:e68350.
128. Brockmeier SL, Register KB, Kuehn JS, Nicholson TL, Loving CL, Bayles DO, Shore SM, Phillips GJ: **Virulence and draft genome sequence overview of multiple strains of the swine pathogen *Haemophilus parasuis***. *PloS one* 2014, **9**:e103787.
129. Mullins MA, Register KB, Bayles DO, Dyer DW, Kuehn JS, Phillips GJ: **Genome sequence of *Haemophilus parasuis* strain 29755**. *Stand Genomic Sci* 2011, **5**:61-68.
130. Kuehn JS, Register KB, Phillips GJ: **Draft Genome Sequences for 10 Isolates of the Swine Pathogen *Haemophilus parasuis***. *Genome announcements* 2013, **1**.
131. Pina S, Olvera A, Barcelo A, Bensaid A: **Trimeric autotransporters of *Haemophilus parasuis*: generation of an extensive passenger domain repertoire specific for pathogenic strains**. *J Bacteriol* 2009, **191**:576-587.
132. Olvera A, Pina S, Perez-Simo M, Oliveira S, Bensaid A: **Virulence-associated trimeric autotransporters of *Haemophilus parasuis* are antigenic proteins expressed *in vivo***. *Vet Res* 2010, **41**:26.
133. Olvera A, Pina S, Perez-Simo M, Aragon V, Segales J, Bensaid A: **Immunogenicity and protection against *Haemophilus parasuis* infection after vaccination with recombinant virulence associated trimeric autotransporters (VtaA)**. *Vaccine* 2011, **29**:2797-2802.

134. Pina-Pedrero S, Olvera A, Perez-Simo M, Bensaid A: **Genomic and antigenic characterization of monomeric autotransporters of *Haemophilus parasuis*: an ongoing process of reductive evolution**. *Microbiology* 2012, **158**:436-447.
135. Lawrence PK, Wiener BL, Kolander-Bremer T, Bey RF, Stine DL, Kittichotirat W, Bumgarner RE: **Genome-Wide Association Studies of Virulent and Avirulent *Haemophilus parasuis* Serotype 4 Strains**. *Genome announcements* 2014, **2**.
136. Zhou M, Zhang A, Guo Y, Liao Y, Chen H, Jin M: **A comprehensive proteome map of the *Haemophilus parasuis* serovar 5**. *Proteomics* 2009, **9**:2722-2739.
137. Yu Y, Wu G, Zhai Z, Yao H, Lu C, Zhang W: **Fifteen novel immunoreactive proteins of Chinese virulent *Haemophilus parasuis* serotype 5 verified by an immunoproteomic assay**. *Folia Microbiol (Praha)* 2015, **60**:81-87.
138. Zhang L, Wen Y, Li Y, Wei X, Yan X, Wen X, Wu R, Huang X, Huang Y, Yan Q, Liu M, Cao S: **Comparative proteomic analysis of the membrane proteins of two *Haemophilus parasuis* strains to identify proteins that may help in habitat adaptation and pathogenesis**. *Proteome science* 2014, **12**:38.
139. Wei X, Cao S, Zhang L, Wen X, Wen Y, Wu R, Huang X, Huang Y, Yan Q: **Comparative proteome analysis of the extracellular proteins of two *Haemophilus parasuis* strains Nagasaki and SW114**. *Biochem Biophys Res Commun* 2014, **446**:997-1001.
140. Hill CE, Metcalf DS, MacInnes JI: **A search for virulence genes of *Haemophilus parasuis* using differential display RT-PCR**. *Vet Microbiol* 2003, **96**:189-202.
141. Jin H, Wan Y, Zhou R, Li L, Luo R, Zhang S, Hu J, Langford PR, Chen H: **Identification of gene transcribed by *Haemophilus parasuis* in necrotic porcine lung through the selective capture of transcribed sequences (SCOTS)**. *Environmental microbiology* 2008, **10**:3326-3336.
142. Xie Q, Jin H, Luo R, Wan Y, Chu J, Zhou H, Shi B, Chen H, Zhou R: **Transcriptional responses of *Haemophilus parasuis* to iron-restriction stress *in vitro***. *Biometals* 2009, **22**:907-916.
143. Lancashire JF, Turni C, Blackall PJ, Jennings MP: **Rapid and efficient screening of a Representational Difference Analysis library using reverse Southern hybridisation: identification of genetic differences between *Haemophilus parasuis* isolates**. *J Microbiol Methods* 2007, **68**:326-330.
144. Melnikow E, Dornan S, Sargent C, Duszenko M, Evans G, Gunkel N, Selzer PM, Ullrich HJ: **Microarray analysis of *Haemophilus parasuis* gene expression under *in vitro* growth conditions mimicking the *in vivo* environment**. *Vet Microbiol* 2005, **110**:255-263.
145. Chen L, Wu D, Cai X, Guo F, Blackall PJ, Xu X, Chen H: **Electrotransformation of *Haemophilus parasuis* with *in vitro* modified DNA based on a novel shuttle vector**. *Vet Microbiol* 2012, **155**:310-316.
146. Bigas A, Garrido ME, de Rozas AM, Badiola I, Barbe J, Llagostera M: **Development of a genetic manipulation system for *Haemophilus parasuis***. *Vet Microbiol* 2005, **105**:223-228.
147. Zhang L, Li Y, Dai K, Wen Y, Wen X, Wu R, Huang X, Cao S: **The confirmation of the DNA uptake signal sequence needed for genetic manipulation in *Haemophilus parasuis***. *Vet Microbiol* 2014, **173**:395-396.
148. Luan SL, Chaudhuri RR, Peters SE, Mayho M, Weinert LA, Crowther SA, Wang J, Langford PR, Rycroft A, Wren BW, Tucker AW, Maskell DJ, Consortium BRT: **Generation of a Tn5 transposon library in *Haemophilus parasuis* and analysis by transposon-directed insertion-site sequencing (TraDIS)**. *Vet Microbiol* 2013, **166**:558-566.

149. Zhang B, Xu C, Zhang L, Zhou S, Feng S, He Y, Liao M: **Enhanced adherence to and invasion of PUVEC and PK-15 cells due to the overexpression of RfaD, ThyA and Mip in the DeltaompP2 mutant of *Haemophilus parasuis* SC096 strain**. *Vet Microbiol* 2013, **162**:713-723.
150. Zhang B, He Y, Xu C, Xu L, Feng S, Liao M, Ren T: **Cytolethal distending toxin (CDT) of the *Haemophilus parasuis* SC096 strain contributes to serum resistance and adherence to and invasion of PK-15 and PUVEC cells**. *Vet Microbiol* 2012.
151. Xu C, Zhang L, Zhang B, Feng S, Zhou S, Li J, Zou Y, Liao M: **Involvement of lipooligosaccharide heptose residues of *Haemophilus parasuis* SC096 strain in serum resistance, adhesion and invasion**. *Vet J* 2013, **195**:200-204.
152. Zou Y, Feng S, Xu C, Zhang B, Zhou S, Zhang L, He X, Li J, Yang Z, Liao M: **The role of *galU* and *galE* of *Haemophilus parasuis* SC096 in serum resistance and biofilm formation**. *Vet Microbiol* 2013, **162**:278-284.
153. Zhang B, Yu Y, Zeng Z, Ren Y, Yue H: **Deletion of the *rfaE* gene in *Haemophilus parasuis* SC096 strain attenuates serum resistance, adhesion and invasion**. *Microb Pathog* 2014, **74**:33-37.
154. Zhou M, Zhang Q, Zhao J, Jin M: ***Haemophilus parasuis* encodes two functional cytolethal distending toxins: CdtC contains an atypical cholesterol recognition/interaction region**. *PloS one* 2012, **7**:e32580.
155. Brockmeier SL: **Prior infection with *Bordetella bronchiseptica* increases nasal colonization by *Haemophilus parasuis* in swine**. *Vet Microbiol* 2004, **99**:75-78.
156. Fu S, Ou J, Zhang M, Xu J, Liu H, Liu J, Yuan F, Chen H, Bei W: **The live attenuated *Actinobacillus pleuropneumoniae* triple-deletion mutant DeltaapxIC DeltaapxIIC DeltaapxIV-ORF1 strain, SLW05, Immunizes pigs against lethal challenge with *Haemophilus parasuis***. *Clinical and vaccine immunology : CVI* 2013, **20**:134-139.
157. Palzer A, Ritzmann M, Hafner-Marx A, Wolf G, Heinritzi K: **[Detection of *Haemophilus parasuis* and *Mycoplasma hyorhinis* in swine and association of those pathogens with clinical and pathological-anatomic findings]**. *Dtsch Tierarztl Wochenschr* 2006, **113**:227-230.
158. Zhao Z, Wang C, Xue Y, Tang X, Wu B, Cheng X, He Q, Chen H: **The occurrence of *Bordetella bronchiseptica* in pigs with clinical respiratory disease**. *Vet J* 2011, **188**:337-340.
159. Fablet C, Marois C, Dorenlor V, Eono F, Eveno E, Jolly JP, Le Devendec L, Kobisch M, Madec F, Rose N: **Bacterial pathogens associated with lung lesions in slaughter pigs from 125 herds**. *Res Vet Sci* 2012, **93**:627-630.
160. Hillen S, von Berg S, Kohler K, Reinacher M, Willems H, Reiner G: **Occurrence and severity of lung lesions in slaughter pigs vaccinated against *Mycoplasma hyopneumoniae* with different strategies**. *Prev Vet Med* 2014, **113**:580-588.
161. Risco D, Serrano E, Fernandez-Llario P, Cuesta JM, Goncalves P, Garcia-Jimenez WL, Martinez R, Cerrato R, Velarde R, Gomez L, Segales J, Hermoso de Mendoza J: **Severity of bovine tuberculosis is associated with co-infection with common pathogens in wild boar**. *PloS one* 2014, **9**:e110123.
162. Fablet C, Marois C, Kuntz-Simon G, Rose N, Dorenlor V, Eono F, Eveno E, Jolly JP, Le Devendec L, Tocqueville V, Queguiner S, Gorin S, Kobisch M, Madec F: **Longitudinal study of respiratory infection patterns of breeding sows in five farrow-to-finish herds**. *Vet Microbiol* 2011, **147**:329-339.
163. Yu J, Wu J, Zhang Y, Guo L, Cong X, Du Y, Li J, Sun W, Shi J, Peng J, Yin F, Wang D, Zhao P, Wang J: **Concurrent highly pathogenic porcine reproductive and**

respiratory syndrome virus infection accelerates *Haemophilus parasuis* infection in conventional pigs. *Vet Microbiol* 2012, **158**:316-321.
164. Solano GI, Segales J, Collins JE, Molitor TW, Pijoan C: **Porcine reproductive and respiratory syndrome virus (PRRSv) interaction with *Haemophilus parasuis***. *Vet Microbiol* 1997, **55**:247-257.
165. Segales J, Domingo M, Solano GI, Pijoan C: **Porcine reproductive and respiratory syndrome virus and *Haemophilus parasuis* antigen distribution in dually infected pigs**. *Vet Microbiol* 1999, **64**:287-297.
166. Done SH, Paton DJ: **Porcine reproductive and respiratory syndrome: clinical disease, pathology and immunosuppression**. *Vet Rec* 1995, **136**:32-35.
167. Zimmerman JJ, Yoon KJ, Wills RW, Swenson SL: **General overview of PRRSV: a perspective from the United States**. *Vet Microbiol* 1997, **55**:187-196.
168. Segales J, Domingo M, Balasch M, Solano GI, Pijoan C: **Ultrastructural study of porcine alveolar macrophages infected in vitro with porcine reproductive and respiratory syndrome (PRRS) virus, with and without *Haemophilus parasuis***. *J Comp Pathol* 1998, **118**:231-243.
169. Mussa T, Rodriguez-Carino C, Sanchez-Chardi A, Baratelli M, Costa-Hurtado M, Fraile L, Dominguez J, Aragon V, Montoya M: **Differential interactions of virulent and non-virulent *H. parasuis* strains with naive or swine influenza virus pre-infected dendritic cells**. *Vet Res* 2012, **43**:80.
170. Narita M, Kawashima K, Matsuura S, Uchimura A, Miura Y: **Pneumonia in pigs infected with pseudorabies virus and *Haemophilus parasuis* serovar 4**. *J Comp Pathol* 1994, **110**:329-339.
171. Wilkinson JM, Sargent CA, Galina-Pantoja L, Tucker AW: **Gene expression profiling in the lungs of pigs with different susceptibilities to Glasser's disease**. *BMC genomics* 2010, **11**:455.
172. Wang SJ, Liu WJ, Yang LG, Liu HB, Sargent CA, Affara NA, Zhang SJ: **Association of multi-pathogenic infections with BAT2, CXCL12, Mx1 and EHMT2 variations in pigs**. *Mol Biol Rep* 2012, **39**:8169-8176.
173. Wang SJ, Liu WJ, Yang LG, Sargent CA, Liu HB, Wang C, Liu XD, Zhao SH, Affara NA, Liang AX, Zhang SJ: **Effects of FUT1 gene mutation on resistance to infectious disease**. *Mol Biol Rep* 2012, **39**:2805-2810.
174. Boren T, Falk P, Roth KA, Larson G, Normark S: **Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens**. *Science* 1993, **262**:1892-1895.
175. Blanco I, Galina-Pantoja L, Oliveira S, Pijoan C, Sanchez C, Canals A: **Comparison between *Haemophilus parasuis* infection in colostrums-deprived and sow-reared piglets**. *Vet Microbiol* 2004, **103**:21-27.
176. Cerdá-Cuéllar M, Naranjo JF, Verge A, Nofrarias M, Cortey M, Olvera A, Segales J, Aragon V: **Sow vaccination modulates the colonization of piglets by *Haemophilus parasuis***. *Vet Microbiol* 2010.
177. Nedbalcova K, Kucerova Z, Krejci J, Tesarik R, Gopfert E, Kummer V, Leva L, Kudlackova H, Ondriasova R, Faldyna M: **Passive immunisation of post-weaned piglets using hyperimmune serum against experimental *Haemophilus parasuis* infection**. *Res Vet Sci* 2011, **91**:225-229.
178. Hong M, Ahn J, Yoo S, Hong J, Lee E, Yoon I, Jung JK, Lee H: **Identification of novel immunogenic proteins in pathogenic *Haemophilus parasuis* based on genome sequence analysis**. *Vet Microbiol* 2011, **148**:89-92.

179. Zhang NZ, Chu YF, Gao PC, Zhao P, He Y, Lu ZX: **Immunological Identification and Characterization of Extracellular Serine Protease-Like Protein Encoded in a Putative espP2 Gene of *Haemophilus parasuis***. *J Vet Med Sci* 2012.
180. Fu S, Yuan F, Zhang M, Tan C, Chen H, Bei W: **Cloning, expression and characterization of a cell wall surface protein, 6-phosphogluconate dehydrogenase, of *Haemophilus parasuis***. *Res Vet Sci* 2012, **in press**.
181. Fu S, Zhang M, Xu J, Ou J, Wang Y, Liu H, Liu J, Chen H, Bei W: **Immunogenicity and protective efficacy of recombinant *Haemophilus parasuis* SH0165 putative outer membrane proteins**. *Vaccine* 2013, **31**:347-353.
182. Zhou M, Guo Y, Zhao J, Hu Q, Hu Y, Zhang A, Chen H, Jin M: **Identification and characterization of novel immunogenic outer membrane proteins of *Haemophilus parasuis* serovar 5**. *Vaccine* 2009, **27**:5271-5277.
183. McLean AR: **Vaccines and their impact on the control of disease**. *Br Med Bull* 1998, **54**:545-556.
184. Xu C, Zhang J, Zhao Z, Guo L, Zhang B, Feng S, Zhang L, Liao M: **Antimicrobial susceptibility and PFGE genotyping of *Haemophilus parasuis* isolates from pigs in South China (2008-2010)**. *J Vet Med Sci* 2011, **73**:1061-1065.
185. Zhou X, Xu X, Zhao Y, Chen P, Zhang X, Chen H, Cai X: **Distribution of antimicrobial resistance among different serovars of *Haemophilus parasuis* isolates**. *Vet Microbiol* 2010, **141**:168-173.
186. Aarestrup FM, Seyfarth AM, Angen O: **Antimicrobial susceptibility of *Haemophilus parasuis* and *Histophilus somni* from pigs and cattle in Denmark**. *Vet Microbiol* 2004, **101**:143-146.
187. Guo L, Zhang J, Xu C, Zhao Y, Ren T, Zhang B, Fan H, Liao M: **Molecular characterization of fluoroquinolone resistance in *Haemophilus parasuis* isolated from pigs in South China**. *J Antimicrob Chemother* 2011, **66**:539-542.
188. Zhang Q, Zhou M, Song D, Zhao J, Zhang A, Jin M: **Molecular characterisation of resistance to fluoroquinolones in *Haemophilus parasuis* isolated from China**. *Int J Antimicrob Agents* 2013, **42**:87-89.
189. Liu Y, Chen P, Wang Y, Li W, Cheng S, Wang C, Zhang A, He Q: **Transcriptional profiling of *Haemophilus parasuis* SH0165 response to tilmicosin**. *Microbial drug resistance* 2012, **18**:604-615.
190. Smart NL, Miniats OP: **Preliminary assessment of a *Haemophilus parasuis* bacterin for use in specific pathogen free swine**. *Can J Vet Res* 1989, **53**:390-393.
191. Frandoloso R, Martinez S, Rodriguez-Ferri EF, Garcia-Iglesias MJ, Perez-Martinez C, Martinez-Fernandez B, Gutierrez-Martin CB: **Development and characterization of protective *Haemophilus parasuis* subunit vaccines based on native proteins with affinity to porcine transferrin and comparison with other subunit and commercial vaccines**. *Clin Vaccine Immunol* 2011, **18**:50-58.
192. Fu S, Zhang M, Ou J, Liu H, Tan C, Liu J, Chen H, Bei W: **Construction and immune effect of *Haemophilus parasuis* DNA vaccine encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mice**. *Vaccine* 2012, **30**:6839-6844.
193. Hu M, Zhang Y, Xie F, Li G, Li J, Si W, Liu S, Hu S, Zhang Z, Shen N, Wang C: **Protection of piglets by a *Haemophilus parasuis* ghost vaccine against homologous challenge**. *Clinical and vaccine immunology : CVI* 2013, **20**:795-802.
194. Zhang B, Tang C, Liao M, Yue H: **Update on the pathogenesis of *Haemophilus parasuis* infection and virulence factors**. *Vet Microbiol* 2014, **168**:1-7.
195. Whitsett JA, Alenghat T: **Respiratory epithelial cells orchestrate pulmonary innate immunity**. *Nature immunology* 2015, **16**:27-35.

196. Bauler TJ, Chase JC, Wehrly TD, Bosio CM: **Virulent *Francisella tularensis* destabilize host mRNA to rapidly suppress inflammation**. *Journal of innate immunity* 2014, **6**:793-805.
197. Kravchenko VV, Kaufmann GF: **Bacterial inhibition of inflammatory responses via TLR-independent mechanisms**. *Cellular microbiology* 2013, **15**:527-536.
198. Langereis JD, Hermans PW: **Novel concepts in nontypeable *Haemophilus influenzae* biofilm formation**. *FEMS Microbiol Lett* 2013, **346**:81-89.
199. Sandal I, Shao JQ, Annadata S, Apicella MA, Boye M, Jensen TK, Saunders GK, Inzana TJ: ***Histophilus somni* biofilm formation in cardiopulmonary tissue of the bovine host following respiratory challenge**. *Microbes and infection / Institut Pasteur* 2009, **11**:254-263.
200. van Gennip M, Christensen LD, Alhede M, Qvortrup K, Jensen PO, Hoiby N, Givskov M, Bjarnsholt T: **Interactions between polymorphonuclear leukocytes and *Pseudomonas aeruginosa* biofilms on silicone implants *in vivo***. *Infect Immun* 2012, **80**:2601-2607.
201. Leuck AM, Johnson JR, Dunny GM: **A widely used in vitro biofilm assay has questionable clinical significance for enterococcal endocarditis**. *PloS one* 2014, **9**:e107282.
202. Hentzer M, Eberl L, Givskov M: **Transcriptome analysis of *Pseudomonas aeruginosa* biofilm development: anaerobic respiration and iron limitation**. *Biofilms* 2005, **2**:37-61.
203. Ito A, May T, Kawata K, Okabe S: **Significance of *rpoS* during maturation of *Escherichia coli* biofilms**. *Biotechnol Bioeng* 2008, **99**:1462-1471.
204. Tremblay YD, Deslandes V, Jacques M: ***Actinobacillus pleuropneumoniae* genes expression in biofilms cultured under static conditions and in a drip-flow apparatus**. *BMC genomics* 2013, **14**:364.
205. Serra DO, Conover MS, Arnal L, Sloan GP, Rodriguez ME, Yantorno OM, Deora R: **FHA-mediated cell-substrate and cell-cell adhesions are critical for *Bordetella pertussis* biofilm formation on abiotic surfaces and in the mouse nose and the trachea**. *PloS one* 2011, **6**:e28811.
206. Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sorensen SR, Moser C, Kuhl M, Jensen PO, Hoiby N: **The *in vivo* biofilm**. *Trends Microbiol* 2013, **21**:466-474.
207. Abu Kwaik Y, Bumann D: **Microbial quest for food *in vivo*: 'nutritional virulence' as an emerging paradigm**. *Cellular microbiology* 2013, **15**:882-890.
208. Abu Kwaik Y, Bumann D: **Host Delivery of Favorite Meals for Intracellular Pathogens**. *PLoS pathogens* 2015, **11**:e1004866.
209. Ortega AD, Quereda JJ, Pucciarelli MG, Garcia-del Portillo F: **Non-coding RNA regulation in pathogenic bacteria located inside eukaryotic cells**. *Frontiers in cellular and infection microbiology* 2014, **4**:162.
210. Eisenreich W, Dandekar T, Heesemann J, Goebel W: **Carbon metabolism of intracellular bacterial pathogens and possible links to virulence**. *Nature reviews Microbiology* 2010, **8**:401-412.
211. Diacovich L, Gorvel JP. Bacterial manipulation of innate immunity to promote infection. Nat Rev Microbiol. vol 2. England2010. p. 117-128.
212. Rai P, Parrish M, Tay IJ, Li N, Ackerman S, He F, Kwang J, Chow VT, Engelward BP: ***Streptococcus pneumoniae* secretes hydrogen peroxide leading to DNA damage and apoptosis in lung cells**. *Proc Natl Acad Sci U S A* 2015, **112**:E3421-3430.
213. Saliba AE, Westermann AJ, Gorski SA, Vogel J: **Single-cell RNA-seq: advances and future challenges**. *Nucleic Acids Res* 2014, **42**:8845-8860.

ANNEX I - Genome comparison of three serovar 5 pathogenic strains of *Haemophilus parasuis*: insights into an evolving swine pathogen

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Genome comparison of three serovar 5 pathogenic strains of *Haemophilus parasuis*: insights into an evolving swine pathogen

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Haemophilus parasuis is the causative agent of Glässer's disease, a systemic disorder characterized by polyarthritis, polyserositis and meningitis in pigs. Although it is well known that *H. parasuis* serovar 5 is the most prevalent serovar associated with the disease, the genetic differences among strains are only now being discovered. Genomes from two serovar 5 strains, SH0165 and 29755, are already available. Here, we present the draft genome of a third *H. parasuis* serovar 5 strain, the formal serovar 5 reference strain Nagasaki. An *in silico* genome subtractive analysis with full-length predicted genes of the three *H. parasuis* serovar 5 strains detected 95, 127 and 95 strain-specific genes (SSGs) for Nagasaki, SH0165 and 29755, respectively. We found that the genomic diversity within these three strains was high, in part because of a high number of mobile elements. Furthermore, a detailed analysis of large sequence polymorphisms (LSPs), encompassing regions ranging from 2 to 16 kb, revealed LSPs in virulence-related elements, such as a Toll-IL receptor, the AcrA multidrug efflux protein, an ATP-binding cassette (ABC) transporter, lipopolysaccharide-synthetizing enzymes and a tripartite ATP-independent periplasmic (TRAP) transporter. The whole-genome codon adaptation index (CAI) was also calculated and revealed values similar to other well-known bacterial pathogens. In addition, whole-genome SNP analysis indicated that nucleotide changes tended to be increased in membrane-related genes. This analysis provides further evidence that the genome of *H. parasuis* has been subjected to multiple lateral gene transfers (LGTs) and to fine-tuning of virulence factors, and has the potential for accelerated genome evolution.

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INTRODUCTION

Haemophilus parasuis is a Gram-negative bacterium from the *Pasteurellaceae* family and is the causative agent of Glässer's disease, characterized by fibrinous polyserositis, arthritis and meningitis in pigs. Serovar 5 is frequently associated with virulent *H. parasuis* strains, but no direct correlation has been found between each serovar and virulence (Aragon *et al.*, 2012).

Abbreviations: ABC, ATP-binding cassette; ACLAME, A CLAssification of Mobile genetic Elements; CAI, codon adaptation index; GO, Gene Ontology; LGT, lateral gene transfer; LSP, large sequence polymorphism; nsSNP, non-synonymous SNP; SSG, strain-specific gene; TRAP, tripartite ATP-independent periplasmic; VFDB, Virulence Factors Database.

The GenBank/EMBL/DDBJ accession number for the genome sequence of *Haemophilus parasuis* strain Nagasaki is ANKT01000000.

One supplementary figure and three supplementary tables are available with the online version of this paper.

Although the knowledge of the evolutionary history of *H. parasuis* genome is still in its infancy, interesting results have been reported with genotyping techniques such as MLST (Mullins *et al.*, 2013; Olvera *et al.*, 2006), 16S rRNA gene sequencing (Dewhirst *et al.*, 1992) and analysis of selected outer-membrane proteins (Mullins *et al.*, 2009). These techniques are rapid and useful for establishing *H. parasuis* phylogenies and for strain identification, but they do not provide an overview of the evolutionary state of the genome. A more in-depth study was possible when the *H. parasuis* SH0165 and 29755 genomes became available. Whole orthologous comparisons between *H. parasuis* and other micro-organisms within the *Pasteurellaceae* family showed that the swine pathogens *H. parasuis* and *Actinobacillus pleuropneumoniae* occupy close positions in this phylogenetic analysis, suggesting that the two species are probably derived from a recent common ancestor (Xu *et al.*, 2011). In addition, a comparative analysis of monomeric autotransporters from three serovar 5 invasive strains (SH0165,

29755 and Nagasaki) suggested a reductive evolutionary state for these genes (Pina-Pedrero *et al.*, 2012). More recently, a genome comparison of a *H. parasuis* serovar 12 strain (ZJ0906) with the serovar 5 strains SH0165 and 29755 showed that gene rearrangements are frequent in this species (Li *et al.*, 2013).

Studies of genetic differences are crucial for understanding how bacterial genomes evolve (Joseph *et al.*, 2011) and can provide insights into the bacterial adaptation to the host and its immune system (Wilson, 2012). The specific objectives of this work were to present the draft genome of a third serovar 5 strain, the formal serovar 5 reference strain Nagasaki, and to study the genetic differences among three serovar 5 strains.

METHODS

Genome assembly and annotation. Sequencing and genome assembly were performed as described previously, using a shotgun strategy combining plasmid and fosmid libraries (Costa-Hurtado *et al.*, 2012; Pina *et al.*, 2009) and the Phred–Phrap and Consed software tools (Gordon *et al.*, 1998). The genome of *H. parasuis* strain SH0165 (GenBank accession no. NC_011852.1) was used for Nagasaki contig fusion. In addition, Nagasaki contigs were mapped with the Mauve contig mover module, using default settings (Rissman *et al.*, 2009), to the SH0165 genome to check for gene synteny and detection of possible mis-assemblies. Contigs were submitted to the Prokaryotic Genome Annotation Pipeline at the National Center for Biotechnology Information (Angiuoli *et al.*, 2008). This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. ANKT00000000. The version described in this paper is version ANKT01000000. The Nagasaki strain was isolated from the meninges of a pig with a systemic infection by *H. parasuis* in Japan.

***In silico* genome-subtraction analysis with predicted proteins: determination of large sequence polymorphisms (LSPs).** Genome sequences for strains SH0165 and 29755 were obtained from GenBank (accession no. ABKM00000000.1). Strain SH0165 was isolated from a pig with Glässer's disease in China, and strain 29755 was isolated from a pig with Glässer's disease in the USA. *In silico* genome-subtraction analysis was carried out with mGenomeSubtractor (http://202.120.12.132/mGS/), a web-based tool for parallel *in silico* subtractive analysis of multiple bacterial genomes (Shao *et al.*, 2010). Using the BLASTP option with a cut-off value of 1×10^{-5}, each predicted proteome was BLAST-searched against the other two strains' predicted proteomes. Settings to define strain-specific genes (SSGs) were the following: identities $\leqslant 60\,\%$ and matching length/query length $\leqslant 70\,\%$, and for core genome identities $\geqslant 80\,\%$ and matching length/query length $\geqslant 80\,\%$. SSGs were confirmed by TBLASTN using the same settings as above (Altschul *et al.*, 1990). Next, LSPs were manually selected when four or more SSGs were found to be contiguous. Adjacent genes in the 5′ and 3′ regions of selected LSPs from the Nagasaki strain were verified by mapping against the other two genomes, using BLASTP (Altschul *et al.*, 1990), TBLASTN and WebACT (http://www.webact.org/WebACT/home). Gene Ontology (GO) terms were retrieved using the InterProScan built-in tool in Blast2GO (Conesa *et al.*, 2005).

Mobile genetic elements and polymorphic sites analysis. Both core genomes and SSGs were BLAST-searched against the A CLAssification of Mobile genetic Elements (ACLAME) database (Leplae *et al.*, 2004), the Antibiotic Resistance Genes Database (Liu & Pop, 2009), the Database of Essential Genes (Luo *et al.*, 2014), and the Virulence Factors Database (VFDB) (Yang *et al.*, 2008) using a value of 1×10^{-5} as the cut-off. Further mining of lateral gene transfer (LGT) was performed using the CodonW tool (v1.4.4 at http://codonw.sourceforge.net/) to calculate the codon adaptation index (CAI) and G+C content for each gene. The CAI was calculated following the method proposed by Wright (1990). Briefly, putative optimal codons were separately calculated for each strain from all ribosomal-protein-encoding genes, thus assuming that the major codon usage trend is due to selection for optimal translation. Predicted proteins containing incomplete 5′ or 3′ ends were discarded. DNA-uptake sequences were detected using the Perl program repeat finder developed by Redfield *et al.* (2006).

In addition, whole-genome SNPs were analysed with the kSNP tool (Gardner & Hall, 2013), and membrane localization and signal peptides were predicted for each putative protein using Phobius with default settings (Käll *et al.*, 2004).

RESULTS

General features of the Nagasaki genome

The Nagasaki strain draft genome was assembled with 34 619 reads (34 Mb), resulting in 47 contigs. The N50 and N90 contig sizes were 122 and 30 kb, respectively. The average and largest contig lengths were 49 and 366 kb, respectively, and the sum of all contigs was 2.3 Mb. Sequencing coverage was 14×, assuming a 2.3 Mb genome length. The ends of contigs contained mainly repetitive elements such as transposases, bacteriophages or repetitive sequences within coding regions. G+C content averaged 40 mol%. Automatic annotation of the Nagasaki genome detected 2260 genes, with 40 additional putative pseudogenes, annotated as 'potential frameshifts'.

Genome organization comparison

Whole-genome alignments were performed using Mauve and showed more rearrangements for the Nagasaki strain (Fig. 1a) than for the 29755 strain (Fig. 1b) when both genomes were compared to the SH0165 genome. Although overall synteny conservation was observed among the three strains, numerous gene rearrangements were detected, especially at two highly variable regions located at 600 kb and 1900 kb (Fig. 1).

A complete comparison of orthologous genes was performed with 2260, 2021 and 2244 predicted genes for the Nagasaki, SH0165 and 29755 strains, respectively. The core genome was composed of 1637 genes (Table S1, available in the online Supplementary Material), and 95, 127 and 95 genes were confirmed as truly specific for Nagasaki, SH0165 and 29755, respectively (Table S2). Thus, 4–6 % of the genome content consisted of strain-specific accessory genes. SSGs encoded mainly hypothetical proteins and mobile genetic elements, such as phages, phage-associated proteins, restriction-modification enzymes and transposases. Transposases were especially abundant in Nagasaki, with two annotated transposases among its SSGs. Further manual annotation with the ACLAME database revealed one copy of an IS4 family transposase and 16 copies of an IS256 family transposase, commonly annotated as TnpA. Notably, 12 copies of TnpA were potentially functional, i.e. not truncated.

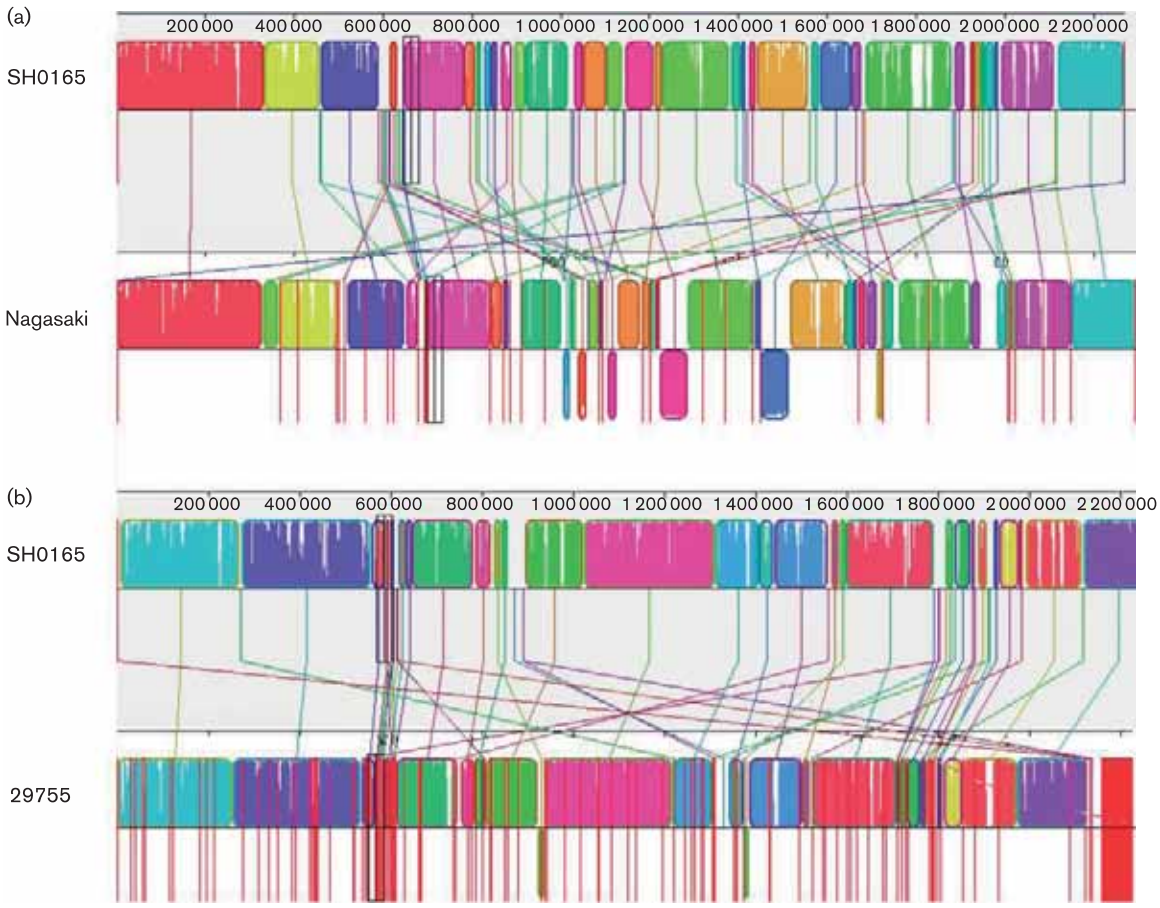

Fig. 1. Whole-genome alignments performed using the Mauve contig mover module. The genome of strain SH0165 was aligned with the Nagasaki (a) and with the 29755 (b) draft genomes. White areas indicate low-identity regions between strains. Regions with the same colour indicate high-similarity syntenic blocks and are connected by the same colour bars. Inverted blocks are shown below the centreline of the Nagasaki or 29755 genomes. Red bars indicate boundaries of the original contigs. The numbers above the alignments indicate the nucleotide positions in the SH0165 genome.

This TnpA was further investigated and a full-length copy was found with ACLAME in *Yersinia pestis* plasmid pG8786 (GenBank accession no. NC_006323.1). Genes directly involved in virulence were also detected (see below).

Competence-related genes, previously reported for the SH10165 strain, *comA*, *comE*, *comL*, *comM*, *pilA*, *pilB*, *pilC*, *pilD* and *pilF*, *rec2*, *tfoX* (encoding a Sxy-type regulator), *dprA* and *comEA* (Xu *et al.*, 2011) were present in all three strains. In addition, a DNA-uptake signal sequence, ACAAGCGGT, already found in strain SH0165 (Xu *et al.*, 2011) was also detected in the other two strains.

Gene functionality overview

The distribution of gene functional categories in the three strains was analysed using GO terms. Whole-genome comparison of GO 'biological process' term annotations showed highly similar profiles for the three strains studied (Fig. S1). Although 40 % of the genes did not have an assigned GO term, the main represented categories observed in the core genome belonged to the terms 'biosynthetic process', 'DNA metabolic process', 'transport', 'catabolic process' and 'translation and response to stress'.

For the core genome, a total of 80 hits to antibiotic resistance genes were found with searches in the Antibiotic Resistance Genes Database (Table S1). In addition, among Nagasaki and 29755 SSGs were also detected one (HPNK_00457) and two (HPS_08627 and HPS_10020) additional hits, respectively (Table S2). Manual inspection revealed genes associated with resistance to macrolides, tetracycline, penicillin and vancomycin, among others. In addition, the VFDB screening showed that the principal virulence factors present within the core genome were ATP ABC/iron transporters, polysaccharide-biosynthesis-related genes and transposases (Table S1).

Host adaptation overview: CAI and G+C content

In order to find evidence for host adaptation, the CAI and the G+C content were calculated for all of the predicted

coding sequences from the three genomes. All three strains had similar G+C content but differed slightly in average CAI, ranging from 0.43 to 0.52. For SSGs, the CAI was lower, 0.4, and the G+C content in SSGs tended to deviate from the average G+C content in the three genomes, suggesting that LGT has taken place in these subgroups of genes. This was further confirmed by BLASTP of unique genes in ACLAME (Fig. 2 and Table S2). Remarkably, a G+C content bias (Fig. 3) was observed for genes in LSP_NK4, such as an ABC transporter substrate-binding protein (HPNK_11292) and in LSP_NK5, such as lipooligosaccharide biosynthesis protein lex-1 (HPNK_11756). Unusual G+C contents of 26 and 50 % were also observed for a putative Toll-IL receptor (HAPS_1951 in LSP_SH8) and an AcrA protein (HPS_08627 in LSP_AM2), respectively.

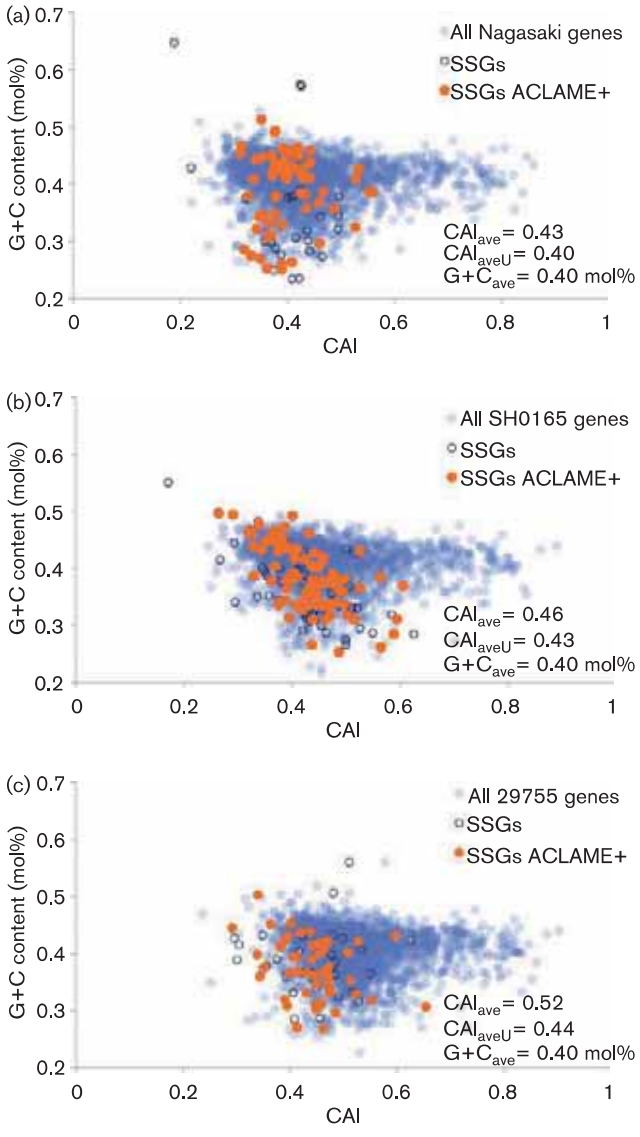

Fig. 2. Whole-genome CAI versus G+C content for the three *H. parasuis* strains studied, Nagasaki (a), SH0165 (b) and 29755 (c). SSGs: strain-specific genes; CAI$_{ave}$: CAI average for each genome; CAI$_{aveU}$: CAI average for SSG; G+C$_{ave}$: average G+C content for each genome. SSGs ACLAME+: SSGs found in the ACLAME database.

Unique genes and LSPs

Some SSGs were clearly located in blocks of strain-specific LSPs, with a range from 2 to 6 kb, 2 to 16 kb and 2 to 5 kb for the Nagasaki, SH0165 and 29755 strains, respectively (Table 1). Mapping of LSPs boundaries was performed using WebACT. Mobile genetic elements were always present in LSPs as predicted by ACLAME (Table 1), suggesting that these genome regions are hot spots of genetic rearrangements. Virulence factors were also found in LSPs (detailed below).

The largest LSP (LSP_NK5) in Nagasaki had a length of 5219 bp (Table 1 and Fig. 3a) and contained various genes involved in LPS synthesis, such as *lgtA*, *rfaJ* and *lex1* (*lex1* was duplicated, with one copy inverted). These genes belong to the glycosyltransferase families 2 (*lgtA*), 8 (*rfaJ*) and 25 (*lex1*). Interestingly, in the other two strains, different genes for LPS biosynthesis were found at the homologous genomic region (Fig. 3a), as previously reported by Xu *et al.* (2011). Both SH0165 and 29755 strains had *lpsA* (or *wzyE*), *wabH*, *rfaG*, *lbgA*, *rfaF2* and *lbgB*, which belong to glycosyltransferase families 1 (*rfaG* and *wabH*), 9 (*lbgB* and *rfaF2*) and 25 (*lbgA* and *lpsA*). The G+C content in these LPS-synthesis genes averaged 25 mol% in the Nagasaki strain and 28 mol% in both SH0165 and 29755, percentages that were lower than the mean G+C content in the whole *H.* parasuis genomes (40 mol%). Syntenic boundaries were found in LSP_NK5, but two genes, *prfB* and *cmoB*, were located at a different genomic position in SH0165. This rearrangement could be explained by the activity of *ISAma3* transposable elements next to *rpmE* and *cmoB* in SH0165. In addition, a full-length recombinase gene (*recG*) was located next to *rpmE* in Nagasaki and was truncated in the SH0165 and 29755 strains. This pseudogene is not annotated in SH0165, whereas it is annotated as a hypothetical protein (HPS_07468) in strain 29755.

LSP_NK4 had a length of 3.8 kb (Table 1 and Fig. 3b) and was located at the end of a contig. This region contained two ABC transporter subunits: the nucleotide-binding domain *abcA* and the substrate-binding domain *abcS*. Three hypothetical proteins (HPNK_11272, HPNK_11277 and HPNK_11282), a thiamine-biosynthesis-related gene (*thiF*) and a tRNA-Leu were also found in LSP_NK4. The G+C content in the LSP_NK4 region was lower than in the whole genome (26 vs 40 mol%). The upstream syntenic boundaries were defined by an ATPase involved in chromosome partitioning (*parA*), a hypothetical protein (HPNK_11262) and a truncated IS5 family transposase. The *parA* gene was disrupted by a frameshift in the SH0165 and 29755 strains, and HPNK_11262 is not annotated in these two strains. Downstream of LSP_NK4, the homologue of HPS_01627 and HAPS_0775, HPNK_02891, was found at the end of a different Nagasaki contig and flanked

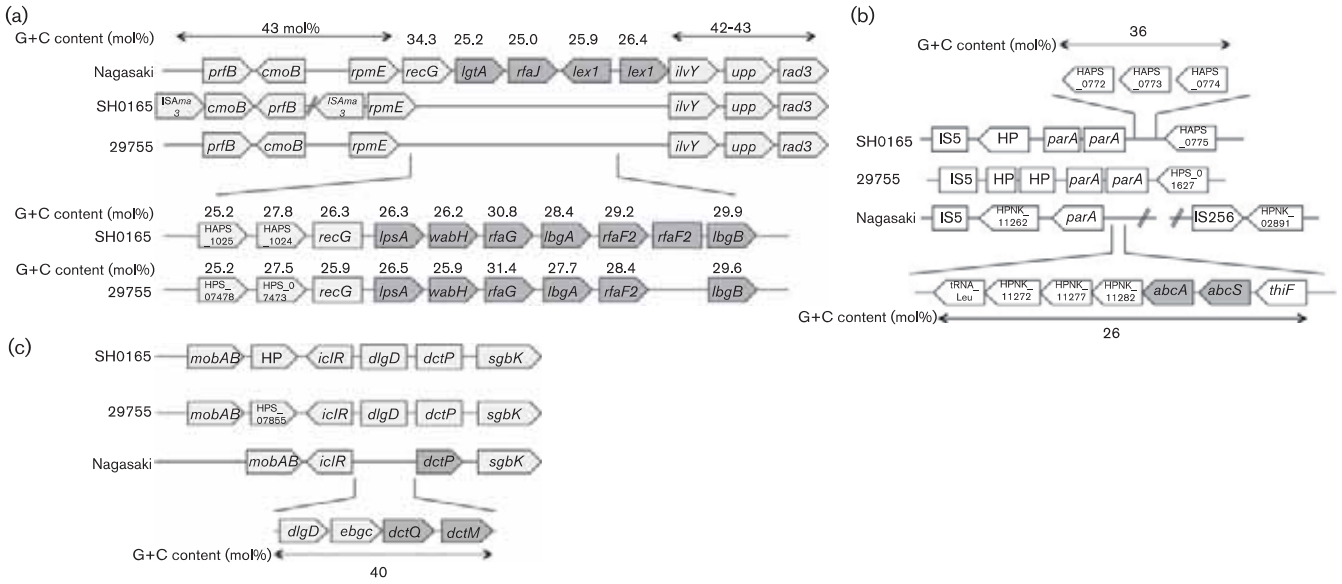

Fig. 3. Schematic representation of three LSPs found in the *H. parasuis* Nagasaki strain and comparisons with the same genomic regions in the SH0165 and 29755 strains. (a) LSP_NK5 comprises several genes involved in LPS synthesis (shown in grey). Lower bracket shows the genes found in the corresponding SH0165 and 29755 genomic regions. G+C content is shown for all genes and for adjacent syntenic genes averaged for all three strains. ISA*ma3*, insertion sequence transposase; *prfB*, peptide chain release factor RF-2; *cmoB*, *S*-adenosyl-L-methionine-dependent methyltransferase; *rpmE*, 50S ribosomal protein L31; *recG*, ATP-dependent DNA helicase, *lgtA*, lacto-*N*-neotetraose biosynthesis glycosyltransferase; *rfaJ*, lipopolysaccharide 1,2-glucosyltransferase; *lex1*, UDP-Gal-lipooligosaccharide (LOS) galactosyltransferase; *ilvY*, DNA-binding transcriptional regulator; *upp*, uracil phosphoribosyltransferase; *rad3*, ATP-dependent helicase; *lpsA*, LOS glycosyltransferase; *wabH*, glycosyltransferase; *rfaG*, glycosyltransferase, LPS biosynthesis glycosyltransferase; *rfaF2*, ADP-heptose:LPS heptosyltransferase II; *lbgB*, LOS D-glycero-D-manno-heptosyltransferase. HAPS_1025, HAPS_1024, HPS_07478 and HPS_07473 are hypothetical proteins. (b) LSP_NK4 contains two ABC transporter subunits: the nucleotide-binding domains *abcA* and the substrate-binding domain *abcS* (both shown in grey). The double slash indicates unassembled ends of contigs. G+C mol% content is shown for the LSP_NK4 region and for additional SH0165 genes. IS5, IS256, transposases; *parA*, ATPase involved in chromosome partitioning; tRNA-Leu, tRNA; *abcA,* ABC-type transport system ATP-binding protein; *abcS,* ABC transporter substrate-binding protein; *thiF*, thiamine biosynthesis protein; HP, hypothetical protein (HAPS_0772, HAPS_0773, HAPS_0774, HAPS_0775, HPS_01627, HPNK_02891, HPNK_11272, HPNK_11277 and HPNK_11282). (c) LSP_NK3 (shown in grey) encodes a TRAP transporter. Lower bracket shows the genes found in this genomic region of the Nagasaki strain. G+C mol% content is shown for the LSP_NK3 region. *mobAB,* putative bifunctional molybdopterin-guanine dinucleotide; *iclR*, transcriptional regulator; *dlgD*, 2,3-diketo-L-gulonate reductase; *ebgC*, beta-D-galactosidase subunit beta; *dctQ*, TRAP C4-dicarboxylate transport system subunit DctQ; *dctM*, TRAP dicarboxylate transporter subunit DctM; *dctP*, TRAP dicarboxylate transporter subunit DctP; *sgbK*, L-xylulose kinase; HP, hypothetical protein (HPS_07855). Length of genes is not adjusted to scale. Pseudogenes are represented by rectangles.

by a mobile genetic element. Three additional hypothetical genes were also found in the SH1065 strain (HAPS_0772, HAPS_0773 and HAPS _0774).

Nagasaki LSP_NK3 had a length of 4.2 kb and contained subunits of a putative tripartite ATP-independent periplasmic (TRAP) transporter: *dctQ*, *dctM* and *dctP* (Table 1 and Fig. 3c). Other genes, *dlgD* (encoding 2,3-diketo-L-gulonate reductase) and *ebgC* (cryptic beta-D-galactosidase, beta subunit) were also found in LSP_NK3. The TRAP subunits *dctQ* and *dctM* were not found in SH0165 and 29755, and *dctP*, the third essential TRAP subunit, is truncated in these two strains. The G+C content for the LSP_NK3 region was similar to that in the whole genome (40 mol%). Syntenic boundaries were found for all three strains, with *mobAB* and *icIR* at the 5′ and *sgbK* at the 3′ end of LSP_NK3. A hypothetical protein (HPS_07855) was found in strains 29755 and SH0165.

Other SSGs not included in the LSPs but related to virulence, as predicted according to the VFDB and ACLAME databases (Table S2), were the following: a transcriptional regulator/antitoxin MazE (HPS_02559), a putative maltose *O*-acetyltransferase (HPS_10020) and a hypothetical protein HPS_01342 in strain 29755; an ABC transporter ATP-binding protein (HPNK_00457) in Nagasaki; and a drug/metabolite exporter family transporter (HAPS_0404), a putative inner-membrane protein (HAPS_1150), a putative

Table 1. LSPs in Nagasaki, SH0165 and 29755 strains

Results of PFAM, ACLAME and VFDB databases searches are also shown. +, a hit was found in the database; −, no hit was found in the database.

LSP strain no.	LSP length (nt)	Locus tag	Product	PFAM	ACLAME	VFDB
Nagasaki (LSP_NK1)	2334	HPNK_00587	Hypothetical protein	PF05565.6	+	−
		HPNK_00592	Hypothetical protein	−	+	−
		HPNK_00597	Hypothetical protein	−	+	−
		HPNK_00602	Hypothetical protein	−	−	−
Nagasaki (LSP_NK2)	1859	HPNK_03338	Phage protein	PF14470.1	+	−
		HPNK_03343	Hypothetical protein	PF05901.6	+	−
		HPNK_03348	Hypothetical protein	−	−	−
		HPNK_03353	Hypothetical protein	−	−	−
Nagasaki (LSP_NK3)	4280	HPNK_06125	2,3-Diketo-L-gulonate reductase DlgD	PF02615.9	+	−
		HPNK_06130	EbgC	PF04074.7	+	−
		HPNK_06135	TRAP transporter subunit DctQ	PF04290.7	+	−
		HPNK_06140	TRAP transporter subunit DctM	PF06808.7	+	+
		HPNK_06145	TRAP transporter subunit DctP	PF03480.8	+	+
Nagasaki (LSP_NK4)	6031	HPNK_11267	ATPase involved in chromosome partitioning	PF01656.18	+	+
		HPNK_11272	Hypothetical protein	−	+	−
		HPNK_11277	Hypothetical protein	−	−	−
		HPNK_11282	Hypothetical protein	−	−	−
		HPNK_11287	ABC transporter nucleotide-binding domain	PF13207.1	+	−
		HPNK_11292	ABC transporter substrate-binding	−	−	−
		HPNK_11297	Possible molybdopterin/thiamine biosynthesis protein (ThiF)	PF00899.16	+	−
Nagasaki (LSP_NK5)	5219	HPNK_11741	ATP-dependent DNA helicase RecG	PF04326.9; PF13749.1	+	−
		HPNK_11746	Lipopolysaccharide synthesis (LgtA)	PF00535.21	+	+
		HPNK_11751	Lipopolysaccharide synthesis (RfaJ)	PF01501.15	+	−
		HPNK_11756	Lipopolysaccharide synthesis (Lex1)	PF01755.12	+	+
		HPNK_11761	Lipopolysaccharide synthesis (Lex1)	PF01755.12	+	+
SH0165 (LSP_SH1)	3176	HAPS_0243	Hypothetical protein	−	−	−
		HAPS_0245	Hypothetical protein	−	−	−
		HAPS_0246	Hypothetical protein	−	+	+
		HAPS_0247	Bacteriophage Mu GP27-like protein	PF11985.3	+	−
SH0165 (LSP_SH2)	2240	HAPS_0562	Hypothetical protein	−	−	−
		HAPS_0563	Hypothetical protein	−	−	−
		HAPS_0564	Hypothetical protein	PF07927.7	+	−
		HAPS_0565	Putative bacteriophage protein transcription regulator, cro/C1-type DNA-binding domain	PF03681.10	+	−
		HAPS_0566	Hypothetical protein	−	−	−
SH0165 (LSP_SH3)	7735	HAPS_0585	Hypothetical protein	−	−	−
		HAPS_0586	Hypothetical protein	−	−	−
		HAPS_0587	Phage tail protein/putative Fels-1 prophage host specificity protein	PF13550.1; PF09327.6	+	−

Table 1. cont.

LSP strain no.	LSP length (nt)	Locus tag	Product	PFAM	ACLAME	VFDB
		HAPS_0589	Hypothetical protein	–	–	–
SH0165 (LSP_SH4)	3890	HAPS_0817	Hypothetical protein	PF12728.2	–	–
		HAPS_0818	Hypothetical protein	–	–	–
		HAPS_0819	Hypothetical protein	–	–	–
		HAPS_0820	Hypothetical protein	PF01541.19	+	–
		HAPS_0821	Hypothetical protein	–	–	–
		HAPS_0822	Transcriptional regulator/helix–turn–helix domain-containing protein	PF12844.2	+	–
		HAPS_0823	CP4-57 prophage, integrase	PF13356.1; PF14659.1; PF00589.17	+	+
SH0165 (LSP_SH5)	10242	HAPS_0859	Bacteriophage transcriptional regulator	PF08765.6	+	–
		HAPS_0860	Mu-like phage E16 protein	PF06252.7	+	–
		HAPS_0862	Hypothetical protein	–	+	–
		HAPS_0863	Hypothetical protein	–	+	–
		HAPS_0864	Host-nuclease inhibitor protein	PF07352.7	+	–
		HAPS_0865	Hypothetical protein	–	–	–
		HAPS_0866	ExeA protein, phage-like protein	PF13401.1	+	+
		HAPS_0867	Putative phage-associated protein	PF00665.21	+	+
		HAPS_0868	Putative phage-associated protein	–	+	+
		HAPS_0869	Putative phage-like DNA-binding protein	PF09339.5	+	–
		HAPS_0870	Hypothetical protein	–	–	–
		HAPS_0871	Hypothetical protein	–	–	–
		HAPS_0872	Putative phage-associated protein	–	+	–
		HAPS_0873	Hypothetical protein	–	–	–
		HAPS_0874	Hypothetical protein	–	–	–
		HAPS_0875	Phage protein	PF04245.8	+	–
		HAPS_0876	Putative phage-like membrane protein	–	+	–
SH0165 (LSP_SH6)	4097	HAPS_0894	Putative phage anti-repressor protein	–	+	–
		HAPS_0895	Hypothetical protein	–	–	–
		HAPS_0896	Hypothetical protein	PF10109.4	+	–
		HAPS_0897	Hypothetical protein	PF06528.7	+	–
		HAPS_0898	Hypothetical protein	–	–	–
		HAPS_0899	Putative bacteriophage tail protein/phage tail tape measure protein, TP901 family	PF10145.4	+	–
SH0165 (LSP_SH7)	16706	HAPS_1825	LysR family transcriptional regulator	PF00126.22; PF03466.15	+	+
		HAPS_1826	Type III restriction-modification system *Eco*PI enzyme subunit res	PF04851.10	+	–
		HAPS_1827	Type III restriction-modification system *Eco*PI enzyme mod (M.*Eco*PI) (*Eco*PI methyltransferase)	PF01555.13	+	–
		HAPS_1828	Putative ATPase (AAA+ superfamily)	PF13173.1; PF13635.1	–	–
		HAPS_1829	Hypothetical protein	–	–	–
		HAPS_1830	MerR family transcriptional regulator	PF13411.1	+	–

Table 1. cont.

LSP strain no.	LSP length (nt)	Locus tag	Product	PFAM	ACLAME	VFDB
		HAPS_1832	MerR family transcriptional regulator	PF13411.1	+	+
		HAPS_1833	Hypothetical protein	–	–	–
		HAPS_1834	Hypothetical protein	PF03713.8	+	–
		HAPS_1835	2-Nitropropane dioxygenase	PF03060.10	+	–
		HAPS_1836	Putative methionine sulfoxide reductase A	PF01541.19	–	–
		HAPS_1837	Putative cytoplasmic protein	PF06250.6	+	–
		HAPS_1839	Thiamine-phosphate pyrophosphorylase	PF02581.12	+	–
		HAPS_1840	Phosphomethylpyrimidine kinase	PF08543.7	+	–
		HAPS_1841	Hydroxyethylthiazole kinase	PF02110.10	+	–
SH0165 (LSP_SH8)	11147	HAPS_1950	Type I site-specific DNase S subunit, restriction-modification system	PF01420.14; PF01420.14	+	–
		HAPS_1951	Toll-IL receptor (TIR)	PF13676.1	+	–
		HAPS_1953	Putative metal-dependent hydrolase (Mdh)	PF01863.12	+	–
		HAPS_1954	Adenine-specific methyltransferase (ecoRIM)	PF13651.1	–	–
		HAPS_1955	HNH endonuclease (Hnh)	PF03235.9; PF01844.18	+	–
		HAPS_1956	Type IIS restriction enzyme protein (BcgIB)	PF01420.14; PF01420.14	+	–
29755 (LSP_AM1)	2411	HPS_11365	Hypothetical protein	PF13490.1	–	–
		HPS_11370	RNA polymerase sigma factor, ECF subfamily protein	PF04542.9; PF08281.7	+	+
		HPS_11375	RNA polymerase sigma factor	PF09836.4	–	–
		HPS_11380	Hypothetical protein	PF05114.8	+	–
		HPS_11385	Hypothetical protein	–	–	–
		HPS_11390	Hypothetical protein	PF07681.7	–	–
29755 (LSP_AM2)	3615	HPS_08627	AcrA protein	PF12700.2	+	–
		HPS_08632	Aldo/keto reductase(Akr)	–	–	–
		HPS_08637	Predicted ATP-dependent endonuclease (AtpE)	PF13304.1	+	–
		HPS_08642	Truncated predicted ATP-dependent endonuclease (AtpE)	–	–	–
29755 (LSP_AM3)	2402	HPS_05348	Hypothetical protein	–	–	–
		HPS_05353	Hypothetical protein	–	–	–
		HPS_05358	Hypothetical protein	PF14281.1	–	–
29755 (LSP_AM4)	1648	HPS_10020	Putative maltose *O*-acetyltransferase	PF12464.3; PF00132.19	+	+
		HPS_10030	Hypothetical protein	PF13411.1	+	–
		HPS_10035	Transmembrane pair domain protein	PF05232.7; PF05232.7	+	–
		HPS_10040	Transcriptional regulator, MarR family protein	PF01047.17	+	–
		HPS_10045	Hypothetical protein	PF00583.19	–	–
		HPS_10050	Hypothetical protein	–	–	–
29755 (LSP_AM5)	5777	HPS_09855	Hypothetical protein	–	–	–
		HPS_09860	Hypothetical protein	–	–	–
		HPS_09870	Hypothetical protein	–	–	–
		HPS_09875	Hypothetical protein	PF04352.8	+	–
		HPS_09890	Predicted EndoDNase RusA	PF05866.6	+	–
		HPS_09895	Hypothetical protein	PF07102.7	+	–

Table 1. cont.

LSP strain no.	LSP length (nt)	Locus tag	Product	PFAM	ACLAME	VFDB
		HPS_09900	YcfA family protein	PF07927.7	+	−
		HPS_09905	Hypothetical protein	PF03681.10	+	−
		HPS_09915	Hypothetical protein	–	−	−
		HPS_09920	Hypothetical protein	PF05869.6; PF05869.6	+	−
29755 (LSP_AM6)	2258	HPS_00350	Hypothetical protein	–	−	−
		HPS_00355	Hypothetical protein	–	−	−
		HPS_00360	Hypothetical protein	–	+	−
		HPS_00365	Hypothetical protein	–	+	−
		HPS_00370	Hypothetical protein	PF13328.1	+	+

outer-membrane colicin Js receptor (HAPS_1568), a putative iron-regulated lipoprotein (HAPS_1569), an AraC family transcriptional regulator (HAPS_1638) and a Toll-IL receptor (HAPS_1951) in SH0165.

Polymorphic sites

The genomes of all three *H. parasuis* strains were scanned for SNPs and 16 235 SNPs were detected, with 12 588 being located in coding regions (Table S3). Of these, 3010 were non-synonymous SNPs (nsSNP) that were located in 1232 genes coding for proteins, but with significant differences in abundance among these genes. Because of the poorly conserved sequence of prophages in bacterial genomes, genes annotated as prophages were excluded from further investigation, resulting in a total of 868 genes with non-synonymous SNPs (nsSNPs). Of those, 120 were annotated as hypothetical proteins. Genes with five or more nsSNP were mainly annotated as membrane-related genes, which were confirmed by Phobius. In descending order by number of SNPs, we found a putative tubulin-binding protein (HAPS_0221, with 18 nsSNPs), the Nagasaki monomeric autotransporter encoded by *bmaA2* (Pina-Pedrero *et al.*, 2012) and annotated in SH0165 as 'putative pertactin family virulence factor outer-membrane autotransporter/Type V secretory pathway adhesin AidA' (HAPS_0753, 12 nsSNPs), an outer-membrane lipoprotein A (HAPS_1356, 10 nsSNPs), a monomeric autotransporter *bmaA1*, annotated as 'putative serine protease' (HAPS_0648, 10 nsSNPs), a neuraminidase *nanH* (HAPS_1616, 9 nsSNPs) and a cysteine/glutathione ABC transporter membrane/ATP-binding protein (HAPS_0224, 7 nsSNPs), among others. We also identified several regulators that contained five or more nsSNPs, such as a tyrosine kinase, chain length regulator in capsular polysaccharide biosynthesis (HAPS_0051, 6 nsSNPs), a transcriptional regulator IclR (HAPS_1810, 6 nsSNPs), a HipA-like protein (HAPS_1822, 6 nsSNPs) and an ATP-dependent transcriptional regulator (HAPS_1001, 5 nsSNPs).

DISCUSSION

H. parasuis is a pathogen whose known host is exclusively the pig. Since *H. parasuis* comprises clinical and non-clinical isolates, it is expected that different *H. parasuis* strains are undergoing host adaptations. The low CAI values for the three genomes studied are similar to previously reported CAI values for other pathogenic bacteria including another pig-associated pathogen, *A. pleuropneumoniae* (Botzman & Margalit, 2011). CAI values were slightly different among the three *H. parasuis* strains, suggesting a particular evolutionary state for each strain. In addition, a low CAI suggested that multiple LGT events have occurred recently. It is well known that LGT events can improve bacterial fitness (Gogarten & Townsend, 2005). Consistent with LGT, the CAI observed for some SSGs was even lower, and their G+C content showed an overall deviation from that of the genome in which they resided, providing

additional evidence of multiple recent recombination events.

H. parasuis serovar 5 strains are frequently associated with Glässer's disease (Cai *et al.*, 2005; Castilla *et al.*, 2012). However, detailed genetic heterogeneity within a given serovar has not been studied yet at the genomic scale. *In silico* genome-subtraction analysis is a valuable tool for identifying specific differences in bacterial genomes. The three *H. parasuis* strains in the present study all belonged to serovar 5, but differences in their predicted proteins and mobile genetic elements were detected. Interestingly, we found that strains from serovar 5 differed in LPS-synthesis genes, suggesting that these genes are not involved in defining serovar traits. The fact that the G+C content in these putative LPS-synthesis genes was lower than the mean G+C content in the whole *H. parasuis* genomes is indicative of LGT. The synteny of genes surrounding LSP_NK5 indicated that there are preferred regions for integration of foreign DNA, and that different LGT events can affect *H. parasuis* strains of the same serovar. LGT of LPS- and capsule-encoding genes have been observed in other bacterial species (Coffey *et al.*, 1991; Patil & Sonti, 2004), and it has been suggested that the virulence-associated trimeric autotransporter (*vtaA*) genes in *H. parasuis* are also subjected to LGT (Pina *et al.*, 2009). Mobile genetic elements have been associated with LGT of glycosyltransferases, adhesion-like proteins and ABC transporters among different bacterial species (Lurie-Weinberger *et al.*, 2012). Transposases or other insertion elements normally flank genes subjected to LGT (Schwendener & Perreten, 2011). In LSP_NK4, we identified two ORFs related to ABC transporters, flanked by a complete (IS256) and a truncated (IS5) insertion sequence. This suggests that these ABC transporters may be subjected to LGT among *H. parasuis* strains or even among more distantly related bacteria. Mobile genetic elements, such as transposases and bacteriophages, seem to be playing an important role in modelling the *H. parasuis* genome. Accordingly, genes *prfB* and *cmoB* appear to be subjected to translocation in SH0165, as suggested by the nearby presence of the transposable element *ISAma3*. The fact that transposases such as TnpA are apparently functional in the Nagasaki strain and truncated in the other strains, and that transposons varied in copy number among the strains indicates that each strain is in a different evolutionary state (Pina-Pedrero *et al.*, 2012). Moreover, expansion of mobile genetic elements and presence of multiple pseudogenes is often associated with bacterial adaptation to the host (Mira & Pushker, 2005; Mira *et al.*, 2006).

Whereas LGT increases gene diversity, genome reduction acts in the opposite direction. Both phenomena seem to be concomitantly shaping the genome of *H. parasuis*. It has been reported by Pina-Pedrero *et al.* (2012) that monomeric autotransporters genes (*bma*) are undergoing genomic reduction in *H. parasuis*. Here, by showing that the TRAP transporter operon in LSP_NK3 from the Nagasaki strain was partially deleted in strains SH0165 and 29755, we provide additional evidence suggesting that genome reduction affects more regions of the *H. parasuis* genome.

As stated above, *H. parasuis* is found exclusively in swine, and the interaction with this host is expected to have a large impact on *H. parasuis* evolution. It has been demonstrated that secreted or surface-exposed bacterial proteins evolve at higher rates than the rest of the genome (Nogueira *et al.*, 2012). Here, we have provided evidence for this trend in *H. parasuis* genomes by showing that nsSNPs accumulate in the genes for membrane-associated proteins. Thus, these subtle changes could improve adaptation of *H. parasuis* to its host.

During the preparation of this paper, ten additional *H. parasuis* genome drafts, including one of the Nagasaki strain genome, have been deposited in GenBank (Kuehn *et al.*, 2013). In the study by Kuehn *et al.* (2013), sequencing of the Nagasaki strain resulted in assembly of 78 contigs, making the draft genome of the present study more suitable for genome comparison. Nonetheless, it is expected that the analysis of a higher number of genomes will shed more light on the genetics of this bacterium.

Conclusion

In summary, this work confirms the hypothesis that *H. parasuis* is still adapting to its host, as can be observed by numerous genomic rearrangements, gene translocations, presence of mobile genetic elements and LGT events.

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REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Angiuoli, S. V., Gussman, A., Klimke, W., Cochrane, G., Field, D., Garrity, G., Kodira, C. D., Kyrpides, N., Madupu, R. & other authors (2008). Toward an online repository of Standard Operating Procedures (SOPs) for (meta)genomic annotation. *OMICS* **12**, 137–141.

Aragon, V., Segales, J. & Oliveira, S. (2012). Glässer's disease. In *Diseases of Swine*. 10 edn. pp. 760–769. Edited by J. J. Zimmerman, L. A. Karriker, A. Ramirez, K. J. Schwartz & G. W. Stevenson. Ames, IA: Wiley-Blackwell.

Botzman, M. & Margalit, H. (2011). Variation in global codon usage bias among prokaryotic organisms is associated with their lifestyles. *Genome Biol* **12**, R109.

Cai, X., Chen, H., Blackall, P. J., Yin, Z., Wang, L., Liu, Z. & Jin, M. (2005). Serological characterization of *Haemophilus parasuis* isolates from China. *Vet Microbiol* **111**, 231–236.

Castilla, K. S., de Gobbi, D. D., Moreno, L. Z., Paixão, R., Coutinho, T. A., dos Santos, J. L. & Moreno, A. M. (2012). Characterization of *Haemophilus parasuis* isolated from Brazilian swine through serotyping, AFLP and PFGE. *Res Vet Sci* **92**, 366–371.

Coffey, T. J., Dowson, C. G., Daniels, M., Zhou, J., Martin, C., Spratt, B. G. & Musser, J. M. (1991). Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. *Mol Microbiol* **5**, 2255–2260.

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M. & Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**, 3674–3676.

Costa-Hurtado, M., Ballester, M., Galofré-Milà, N., Darji, A. & Aragon, V. (2012). VtaA8 and VtaA9 from *Haemophilus parasuis* delay phagocytosis by alveolar macrophages. *Vet Res* **43**, 57.

Dewhirst, F. E., Paster, B. J., Olsen, I. & Fraser, G. J. (1992). Phylogeny of 54 representative strains of species in the family *Pasteurellaceae* as determined by comparison of 16S rRNA sequences. *J Bacteriol* **174**, 2002–2013.

Gardner, S. N. & Hall, B. G. (2013). When whole-genome alignments just won't work: kSNP v2 software for alignment-free SNP discovery and phylogenetics of hundreds of microbial genomes. *PLoS ONE* **8**, e81760.

Gogarten, J. P. & Townsend, J. P. (2005). Horizontal gene transfer, genome innovation and evolution. *Nat Rev Microbiol* **3**, 679–687.

Gordon, D., Abajian, C. & Green, P. (1998). Consed: a graphical tool for sequence finishing. *Genome Res* **8**, 195–202.

Joseph, B., Schwarz, R. F., Linke, B., Blom, J., Becker, A., Claus, H., Goesmann, A., Frosch, M., Müller, T. & other authors (2011). Virulence evolution of the human pathogen *Neisseria meningitidis* by recombination in the core and accessory genome. *PLoS ONE* **6**, e18441.

Käll, L., Krogh, A. & Sonnhammer, E. L. (2004). A combined transmembrane topology and signal peptide prediction method. *J Mol Biol* **338**, 1027–1036.

Kuehn, J. S., Register, K. B. & Phillips, G. J. (2013). Draft genome sequences for 10 isolates of the swine pathogen *Haemophilus parasuis*. *Genome Announc* **1**, e00739-13

Leplae, R., Hebrant, A., Wodak, S. J. & Toussaint, A. (2004). ACLAME: a CLAssification of Mobile genetic Elements. *Nucleic Acids Res* **32** (Database issue), D45–D49.

Li, Y., Kwok, A. H., Jiang, J., Zou, Y., Zheng, F., Chen, P., Hou, C., Leung, F. C. & Jiang, P. (2013). Complete genome analysis of a *Haemophilus parasuis* serovar 12 strain from China. *PLoS ONE* **8**, e68350.

Liu, B. & Pop, M. (2009). ARDB—Antibiotic Resistance Genes Database.

Luo, H., Lin, Y., Gao, F., Zhang, C. T. & Zhang, R. (2014). DEG 10, an update of the database of essential genes that includes both protein-coding genes and noncoding genomic elements. *Nucleic Acids Res* **42** (Database issue), D574–D580.

Lurie-Weinberger, M. N., Peeri, M. & Gophna, U. (2012). Contribution of lateral gene transfer to the gene repertoire of a gut-adapted methanogen. *Genomics* **99**, 52–58.

Mira, A. & Pushker, R. (2005). The silencing of pseudogenes. *Mol Biol Evol* **22**, 2135–2138.

Mira, A., Pushker, R. & Rodríguez-Valera, F. (2006). The Neolithic revolution of bacterial genomes. *Trends Microbiol* **14**, 200–206.

Mullins, M. A., Register, K. B., Bayles, D. O., Loving, C. L., Nicholson, T. L., Brockmeier, S. L., Dyer, D. W. & Phillips, G. J. (2009). Characterization and comparative analysis of the genes encoding *Haemophilus parasuis* outer membrane proteins P2 and P5. *J Bacteriol* **191**, 5988–6002.

Mullins, M. A., Register, K. B., Brunelle, B. W., Aragon, V., Galofré-Mila, N., Bayles, D. O. & Jolley, K. A. (2013). A curated public database for multilocus sequence typing (MLST) and analysis of *Haemophilus parasuis* based on an optimized typing scheme. *Vet Microbiol* **162**, 899–906.

Nogueira, T., Touchon, M. & Rocha, E. P. (2012). Rapid evolution of the sequences and gene repertoires of secreted proteins in bacteria. *PLoS ONE* **7**, e49403.

Olvera, A., Cerdà-Cuéllar, M. & Aragon, V. (2006). Study of the population structure of *Haemophilus parasuis* by multilocus sequence typing. *Microbiology* **152**, 3683–3690.

Patil, P. B. & Sonti, R. V. (2004). Variation suggestive of horizontal gene transfer at a lipopolysaccharide (lps) biosynthetic locus in *Xanthomonas oryzae* pv. *oryzae*, the bacterial leaf blight pathogen of rice. *BMC Microbiol* **4**, 40.

Pina, S., Olvera, A., Barceló, A. & Bensaid, A. (2009). Trimeric autotransporters of *Haemophilus parasuis*: generation of an extensive passenger domain repertoire specific for pathogenic strains. *J Bacteriol* **191**, 576–587.

Pina-Pedrero, S., Olvera, A., Pérez-Simó, M. & Bensaid, A. (2012). Genomic and antigenic characterization of monomeric autotransporters of *Haemophilus parasuis*: an ongoing process of reductive evolution. *Microbiology* **158**, 436–447.

Redfield, R. J., Findlay, W. A., Bossé, J., Kroll, J. S., Cameron, A. D. & Nash, J. H. (2006). Evolution of competence and DNA uptake specificity in the *Pasteurellaceae*. *BMC Evol Biol* **6**, 82.

Rissman, A. I., Mau, B., Biehl, B. S., Darling, A. E., Glasner, J. D. & Perna, N. T. (2009). Reordering contigs of draft genomes using the Mauve aligner. *Bioinformatics* **25**, 2071–2073.

Schwendener, S. & Perreten, V. (2011). New transposon Tn6133 in methicillin-resistant *Staphylococcus aureus* ST398 contains *vga*(E), a novel streptogramin A, pleuromutilin, and lincosamide resistance gene. *Antimicrob Agents Chemother* **55**, 4900–4904.

Shao, Y., He, X., Harrison, E. M., Tai, C., Ou, H. Y., Rajakumar, K. & Deng, Z. (2010). mGenomeSubtractor: a web-based tool for parallel *in silico* subtractive hybridization analysis of multiple bacterial genomes. *Nucleic Acids Res* **38** (Web Server issue), W194–W200.

Wilson, D. J. (2012). Insights from genomics into bacterial pathogen populations. *PLoS Pathog* **8**, e1002874.

Wright, F. (1990). The 'effective number of codons' used in a gene. *Gene* **87**, 23–29.

Xu, Z., Yue, M., Zhou, R., Jin, Q., Fan, Y., Bei, W. & Chen, H. (2011). Genomic characterization of *Haemophilus parasuis* SH0165, a highly virulent strain of serovar 5 prevalent in China. *PLoS ONE* **6**, e19631.

Yang, J., Chen, L., Sun, L., Yu, J. & Jin, Q. (2008). VFDB 2008 release: an enhanced web-based resource for comparative pathogenomics. *Nucleic Acids Res* **36** (Database issue), D539–D542.

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ANNEX II - Supplemental Figure S3

Figure S3. Simultaneous detection of *H. parasuis* and neutrophils by double immunofluorescence in lung tissue sections from piglets inoculated with the virulent strain Nagasaki and the non-virulent strain SW114, at 1 day post inoculation (dpi) and 4 dpi. Detection of neutrophils with the specific monoclonal antibody 6D10 is shown in green (first panels); nuclei counterstained with DAPI are shown in blue (second panels); bacteria (Nagasaki or SW114) detected with a rabbit antiserum anti-*H. parasuis* are visualized in red (third panels); merged images are shown in the last panels. Scale bars, 25 µm.