Identification and partial characterization of acid phosphatases from *Haemophilus parasuis*

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Ph.D. Thesis, 2013

CReSA
Centre de Recerca en Sanitat Animal

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Universitat Autònoma de Barcelona

Identification and partial characterization of acid phosphatases from *Haemophilus parasuis*

Tesi doctoral presentada per Paula Constanza Manrique Ramírez per accedir al grau de Doctor dins del programa de Doctorat en Microbiologia de la Facultat de Biociències de la Universitat Autònoma de Barcelona, sota la direcció de la Dra. Virginia Aragón Fernández.

Bellaterra, 2013

VIRGINIA ARAGÓN FERNÁNDEZ, investigadora del Centre de Recerca en Sanitat Animal (CReSA)

Certifica:

Que la memòria titulada, **"Identification and partial characterization of acid phosphatases from *Haemophilus parasuis"*** presentada per Paula Constanza Manrique Ramírez per a l'obtenció del grau de Doctor, s'ha realitzat sota la seva supervisió en el Centre de Recerca en Sanitat Animal (CReSA) i que la Dra. Susana Campoy Sánchez, professora agregada del Departament de Genètica i de Microbiologia de la Facultat de Biociències de la Universitat Autònoma de Barcelona ha actuat com a tutora.

I per tal que consti a efectes oportuns, firmen el present certificat a Bellaterra, a 20 de Setembre de 2013

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Els estudis de doctorat de Paula Constanza Manrique Ramírez han estat finançats per una beca de COLFUTURO, Fundació per al futur de Colòmbia a Formació, concedida pel· el govern de Colòmbia.

Aquest treball ha estat finançat pels projectes AGL2007-60432 i AGL2010-15232 del *Ministerio de Ciencia e Innovación.*

Alas de seda

Persiguiendo el perfume de risueño retiro,
la fugaz mariposa por el monte revuela,
y en esos aires enciende sutilísima estela
con sus pétalos tenues de cambiante zafiro.

En la ronda versátil de su trémulo giro
esclarece las grutas como azul lentejuela;
y al flotar en la lumbre que en los ámbitos riela,
vibra el sol y en la brisa se difunde un suspiro.

Al rumor de las lianas y al vaivén de las quinas,
resplandece en la fronda de las altas colinas,
polvoreando de plata la florida arboleda;

y la gloriosa en el brillo de sus luces triunfales,
sobre el limpio remanso de sernos cristales
pasa, sin hacer sombra, con sus alas de seda.

José Eustasio Rivera
(Poeta colombiano)

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ACKNOW LEDGEMENTS

This dissertation is a reflection of the work that I have accomplished at the Universidad Autonóma of Barcelona, but I was not without guidance and assistance, throughout the graduate process there were many people that worked tirelessly to help me succeed. Here I acknowledge those who have contributed to my successful completion of this thesis.

First off all would like to extend my great appreciation and admiration to my mentor Dr. Virginia Aragón Fernandez. The art of patience in one person now I believe that it is possible. When I asked for some advice or some general idea of science, you were always willing to listen and give me some advice. Thank you Vicky for taking the direction of this thesis, for your guidance in the conduct and presentation of this research and for allowing me to be part of your research group. Without your help and experience, this work would not have been possible.

Members of the Bacteriology lab who I would like to thank specially is Nuria Galofrè. I would like to thank you greatly for all the techniques and tricks lab that I learned, checking my unending calculations of solutions and dilutions and convey to me all your skills throughout all these years of my stay in the CreSA. As a group I would like to thank the remaining members of the bacter-lab, Ana, Lourdes, Nuria A, Judit, Tere, Marta, Saulo, Karla and Marc; it has been my pleasure to work, learn and share with you guys and for all the time that I spend in the bacteriology/equipment lab. I spent a very pleasant time with all of you in the bacter-parties, calçotada, karaoke, and other several events of the group. I think that we had a great bacteriology lab, which made all of us better scientists.

In the sector of PhD students, I just want to thank each and every one of you guys for share with me some of the millions of times inside and outside of CReSA. The annual calçotadas (I will I lack so much this staff), going out to festivals around Catalunya, trips, tapas, cañas, international dinners, movies, Sitges/costumes!!!, peer session, uffff, etc.. Thank you very much Pamela, Juliana, Tufaria, Mar, Mario, Karla, Joan, Saulo, Gerard, Aida, Max and Fernando, I don´t have words to express how grateful I' am with all the becarios for welcoming me and making me feel at home. For a hug or a word of encouragement one million of thanks. For help me in infections, protocols or just tell me where things were in the lab. And to all the rest of becarios-CreSA, as well thank you very much for your all guys, Kate, Noelia, Feng, Marco, Adriana, Anna, Paula, Alexandra, David, Ariadne, Bernardo, Elisa, Cristina L, Julia and Emma. I learned something from all of you and I catch a nice memory in my heart.

Also I would like to thank to all the researchers and technical staff of CReSA for the teachings, knowledge's and advices received. I tried not to bother you with my scientific problems, but rather enjoyed the many conversations that we had about unimportant issues. Especially to Ayub for teaching me and helping me with the monoclonal antibodies, hybridomas. Also for the suggestions and corrections to take into consideration along this thesis and tell me anecdotes or for just ask me existential questions about life. To Tuija, Maria, Francesc and Fernando for their punctual notes and suggestions in the journal meeting of every Monday. I realize that you have higher priorities than me but I greatly appreciate all that you have done in helping me succeed as a scientist. Each of you had a different impact on my my life and research and for that I will be forever grateful.

Finally, to all the friends I meet in Barcelona, thanks for the company, going out to dinner, going out to movies, the mall, the rebajas season!!!!, encouraging me to go to the GYM (Who goes to the SAF???), the beach, the barbecues, for the can paixano, for the unlimited tapas in can eusebio, meals at home, dancing, karaoke, cañas, tapas, soccer games, cocktails night, for several trips, pleasant talks drinking Estrella or wine and all those new sites we discovered together in Barcelona and Europe. Because I conclude that there is no better company than a friend to travel and as Estanislao del Campo says: “I, on this long, and exhausting journey, in my soul will take your beautiful image”.

LIST OF ABBREVIATIONS USED

4-MUP	4-methylumbelliferyl phosphate
ACN	Acetonitrile
ACP	Acid Phosphatase
AlkP	Alkaline Phosphatase
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage – fluid
BHI	Brain hearth infusion
BLAST	Basical Local Alignment Search Tool
BSA	Bovine sera albumin.
CA	Coagglutination technique
CDTs	Cytolethal distending toxins
CV	Column volume
DiFMU	6,8-difluoro-4-methylumbelliferone.
DiFMUP	6,8-difluoro-4-methylumbelliferyl phosphate
DMSO	Dimethyl sulfoxide
EDTA	Etilen-diamine-tetracetic-acid
ELISA	Enzyme-linked immunosorbent assay
ERIC	Enterobacterial Repetitive Sequence Consensus intergenic
FBS	Fetal bovine serum
GPS	Global Protein Server
HCCA	α-cyano-4-hydroxycinnamic acid
Hps	*Haemophilus parasuis*.
IHA	Indirect hemagglutination test
Kb	Kilo base
LB	Luria-Bertani
LOS	Lipooligosaccharide
LPS	Lipopolisacaride

MAbs	Monoclonal antibodies
MALDI-TOF	Matrix Assisted Laser Desorption Time-of0Flight Mass Spectrometry
MAP	Major Acid Phosphatase
MEE	Multilocus Enzyme Electrophoresis.
min	minutes
MS	Mass spectrum
MUP	Methylumbelliferyl phosphate
MW	Molecular weight
NADP+	Nicotinamideadenine dinucleotide phosphate
NADPH	Reduced Nicotinamideadenine dinucleotide phosphate
NCS	Non-crystallographic Symmetry
NH_4 HCO_3	Ammonium bicarbonate
NSAP	Bacterial Non-specific Acid Phosphatase
NSAP	Non-specific acid phosphatases
NT	Non-typeable
OD	Optical Density (nm)
Omp	Outer membrane protein
PAM	Porcine alveolar macrophages
pb	Pare of bases
PBS	Saline phosphate buffer solution
PCR	Polymerase Chain Reaction
PCV2	The porcine circovirus type 2
PEG	Polyethylene Glycol
PEGMME	Polyethylene Glycol Mono-methyl ether
Pi	Inorganic phosphate
PLC	Phospholipase C
pNPP	*para*-nitrophenyl phosphate

PP1	protein phosphatase 1
PP2A	protein phosphatase 2ª
PRRSV	Porcine reproductive and respiratory syndrome virus
PUVEC	Porcine umbilicus vein endothelial cells
r.p.m.	Revolutions per minute
REF	Restriction endonuclease fingerprinting
RFLP	Restriction Fragment Length Polymorphism
RFU	Relative fluorescence units
SDS	Dodium dodecyl sulfate
SDS-PAGE	Dodium dodecyl sulfate polyacrylamide gel electrophoresis
SIV	Swine influenza virus
SPF	Specific-pathogen-free
TAE	Tris Acetate EDTA buffer
TBE	Tris Borate EDTA buffer
TBS	Tris-buffered saline
TCA	Tricloroacétic acid
Tris	Tris (hydroxymethyl) methylamine
URL	Relative light units
UV	Ultraviolet (wave length)

SUMMARY

The Gram-negative bacterium *Haemophilus parasuis* is common in the upper respiratory tract of healthy swine and is the etiological agent of Glässer´s disease, which is characterized by fibrinous polyserositis and polyarthritis. In the last few years the prevalence of respiratory infections, including those by *H. parasuis*, has increased due to management practices, as early weaning, and the emergence of immunosuppressive viruses. Little is known about the pathogenesis and virulence factors of *H. parasuis* and this complicates the control of the disease.

Acid phosphatases are ubiquitous in eukaryotic and prokaryotic organisms and much attention has been given to these enzymes in order to understand their structures, functions, and catalytic mechanisms. In many Gram-negative bacteria these enzymes have been determined to play a critical role in numerous processes, including pathogenesis. Previous results by our group showed the presence of a secreted acid phosphatase in the culture supernatant of some strains of *H. parasuis*. Interestingly, the secretion of the phosphatase activity was lost after passages of the bacteria in the laboratory, indicating a role of this enzyme in the infection. Therefore the main objective of this thesis was to identify and characterize the genes responsible for this secreted phosphatase activity. Screening of a genomic library from the virulent strain ER-6P of *H. parasuis* identified 2 clones with phosphatase activity. These clones contained genes *aphA* and *pgpB*, respectively. The subsequent cloning and analysis of the genes demonstrated that their products were in fact phosphatases. Specifically, AphA presented characteristics of bacterial class B acid phosphatases, including the typical catalytic domain (motif F-D-I-D-D-TV-L-F-S-S-P, located in the N-terminal moiety, and Y-G-D-[AS]-D-X-D-[IV] located near the C-terminus), a predicted molecular weight of 24.33 KDa and inhibition by EDTA. Bacterial class B acid phosphatases are a group of homotetrameric enzymes, which are present in a minority of bacteria, most of which are important pathogens. PgpB is a phosphatidylglycerophosphate phosphatase, with six transmembrane domains in the predicted protein, which would explain its location in the bacterial cell. Both

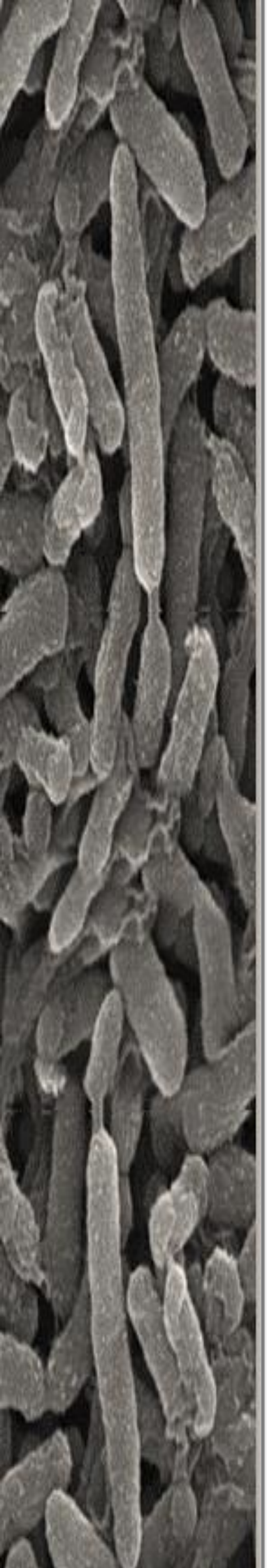
proteins, AphA and PgpB, showed a different optimal pH than the activity secreted by *H. parasuis* into the supernatant. In an attempt to purify the phosphatase from the *H. parasuis* supernatant, a size exclusion chromatography was performed and fractions with phosphatase activity were analyzed by SDS-PAGE and MALDI-TOF-TOF. A hypothetical protein HPS_05483 was identified in the fractions with phosphatase activity, but cloning and native expression of the gene was not achieved. However, the protein HPS_05483 was purified as a His-tagged protein and antibodies in infected pigs were detected against it, indicating that this protein is expressed during infection. Monoclonal antibodies (mAb) were produced against a phosphatase-positive supernatant from *H. parasuis* and an initial screening of the mAb identified several hybridomas with reaction against AphA. Further screenings are needed to identify mAb against the secreted phosphatase found in the supernatant of *H. parasuis*. Finally, the effect of the phosphatase-positive supernatant on porcine alveolar macrophages was studied. The level of macrophage surface markers CD163, SLAI, SLAII, sialoadhesin and SWC3 was not affected by incubation with supernatants from *H. parasuis* or supernatant from a clone secreting AphA. In conclusion, we have identified and partially characterize 2 phosphatases of *H. parasuis*, AphA and PgpB. More studies are needed to determine if the hypothetical protein HPS_05483 is in fact a phosphatase. The role of these enzymatic activities in the biology of *H. parasuis* needs to be further studied.

RESUMEN

Haemophilus parasuis es una bacteria Gram negativa común en el tracto respiratorio superior de cerdos sanos y es el agente etiológico de la enfermedad de Glässer, que se caracteriza por poliserositis fibrinosa y poliartritis. En los últimos años, la prevalencia de las infecciones respiratorias, incluyendo la producida por *H. parasuis,* ha aumentado debido a prácticas de manejo de los animales, como el destete precoz, y a la aparición de virus inmunosupresores. Poco se sabe acerca de la patogénesis y los factores de virulencia de *H. parasuis* y esto complica el control de la enfermedad.

Las fosfatasas ácidas son ubicuas en organismos eucariotas y procariotas y han recibido mucha atención con la finalidad de entender sus estructuras, funciones y mecanismos catalíticos. En bacterias Gram negativas estos enzimas tienen un papel esencial en numerosos procesos, incluyendo la patogénesis bacteriana. Resultados previos de nuestro grupo demostraron la presencia de fosfatasa ácida secretada en sobrenadantes de ciertas cepas de *H. parasuis.* De especial interés fue la observación de que la secreción de la fosfatasa se perdía tras pases de la bacteria en el laboratorio, indicando un papel de este enzima en la infección. Por lo tanto el objetivo principal de esta tesis fue identificar y caracterizar los genes responsables de esta actividad fosfatasa. El análisis de una genoteca de la cepa virulenta *H. parasuis* ER-6P permitió la identificación de 2 clones con actividad fosfatasa. Estos clones contenían los genes *aphA* y *pgpB,* respectivamente. El clonaje y análisis posterior de estos genes confirmó que codificaban dos fosfatasas. Específicamente, AphA muestra características típicas de fosfatasas ácidas bacterianas de clase B, incluyendo el dominio catalítico típico (motivo F-D-I-D-D-TV-L-F-S-S-P, localizado en el N-terminal y el motivo Y-G-D-[AS]-D-X-D-[IV] localizado cerca del C-terminal), un peso molecular teórico de 24,33 KDa e inhibición por EDTA. Las fosfatasas ácidas bacterianas de clase B son un grupo de enzimas que forman homotetrámeros y están presentes en una minoría de bacterias, que incluye patógenos importantes. Por otro lado, PgpB es una fosfatasa de fosfatidilglicerofosfato, con seis dominios

transmembrana, que pueden explicar su localización celular en la bacteria. Ambas proteínas, AphA y PgpB, mostraron un pH óptimo distinto que la actividad fosfatasa secretada por *H. parasuis* al sobrenadante. En un intento de purificar la fosfatasa secretada, sobrenadantes de *H. parasuis* fueron fraccionado por cromatografía de flujo por tamaño y las fracciones con actividad fosfatasa fueron analizadas por SDS-PAGE y MALDI-TOF-TOF. La proteína hipotética HPS_05483 se identificó en las fracciones con actividad fosfatasa, pero su expresión nativa recombinante no fue posible. Sin embargo, sí pudo ser purificada con una cola de His y se detectaron anticuerpos frente a ella en cerdos infectados, indicando que ésta proteína se expresa durante la infección. Se produjeron anticuerpos monoclonales (Acm) frente a un sobrenadante de *H. parasuis* con actividad fosfatasa y una selección inicial detectó Acm frente a Apha. Sin embargo, se debe realizar una selección más exhaustiva de los hibridomas para identificar Acm específicos frente a la fosfatasa del sobrenadante de *H. parasuis*. Finalmente, se estudió el efecto de sobrenadantes con actividad fosfatasa sobre los macrófagos. La expresión de marcadores de superficie de macrófagos alveolares CD163, SLAI, SLAII, sialoadhesina y SWC3 no se vio afectada por incubación con sobrenadantes de *H. parasuis* o sobrenadante de un clon que secretaba AphA. En conclusión, hemos identificado y caracterizado parcialmente dos fosfatasas de *H. parasuis*, AphA y PgpB. Sin embargo, más estudios son necesarios para determinar si la proteína hipotética HPS_05483 es realmente una fosfatasa. El papel de estos enzimas en la biología de *H. parasuis* debería ser estudiado en más profundidad.



1. INTRODUCTION

1. INTRODUCTION

1.1. Glässer's disease

1.1.1. Importance

Swine production has changed dramatically in the last century. New trends in production, including early weaning of piglets and the management of specific pathogen free herds, have contributed to an increase in the prevalence and severity of bacterial diseases, including those caused by *Haemophilus parasuis* (Aragon et al., 2012). Pigs can be colonized by different microorganisms before weaning (Pijoan and Trigo, 1990), but some of these microorganisms are potentially pathogenic. In the last years *H. parasuis*, *Actinobacillus suis and Streptococcus suis* have emerged as significant pathogens for the swine industry, associated with serious clinical conditions, especially in high health status herds. These bacteria are known as early colonizers. They are difficult to control and, under the appropriate conditions, can cause severe outbreaks (Pijoan et al., 1997). For that reason during the end of the 90s they received the name of "suis-ide diseases" by some researchers in the swine area (MacInnes and Desrosiers, 1999). Infection by *H. parasuis* is widespread in commercial farms and it is considered one of the most important pathogens causing economical losses in the swine industry, especially in the nursery piglets (Holtkamp et al., 2007). Control of infections by *H. parasuis* can be achieved by antimicrobial therapy or by ensuring the proper immunity of the animals. Nowadays, commercial vaccines consist of bacterines prepared with one or two of the most prevalent serovars. However, these commercial vaccines do not provide protection against all the virulent serovars of *H. parasuis*, and thus the need for an improved vaccine is clear.

1.1.2. Clinical signs and lesions

Glässer's disease is observed mainly in piglets at weaning, and associated with stress, with a morbidity of 10-15% and a mortality rate of 5-10%. The course of *H. parasuis* infection is particularly serious in specific-pathogen-free (SPF) herds and herds with a good health status, where outbreaks are associated with a high

morbidity and mortality. *H. parasuis* can be also involved in the porcine respiratory complex in conventional herds, acting as a secondary or opportunistic pathogen (Rodriguez Ferri et al., 2000).

Clinical infection by *H. parasuis* occurs primarily in two forms: acute (or Glässer's disease) and chronic.

1.1.2.1. Acute form

This form is typical of farms where there was no prior exposure; clinical symptoms occur quickly a few days after exposure. The clinical presentation depends on the location of lesions, which are characteristic of Glässer's disease (fibrinous polyserositis).

Usually, clinical signs are observed in piglets from 3 to 8 weeks of age but can be seen in growing and fattening pigs. First, the animals show signs of depression with high temperature (40-41ºC) and a reduced food uptake. In other words, pyrexia and inappetence followed by apathy and anorexia are observed in the animals (Rodriguez Ferri et al., 2000). Swollen joints (mainly in the tarsal and carpal joints), nervous signs (such as peddling or tremors) and dyspnea can also be seen. Death can occur around 2-5 days after onset of the disease. Weight loss and skin covered with curly hair can be seen in weaned piglets and fattening pigs. When pericarditis is produced, death can occur due to heart failure. It is also common to see higher incidence of cough, dyspnea and lameness.

Microscopically, the exudate from the lesions is composed of fibrin, neutrophils and macrophages (Vahle et al., 1995, 1997).

1.1.2.2. Chronic form

This clinical form is characterized by growth retardation with or without respiratory signs. It is the consequence of the recovery from a mild or moderate acute disease but with lesions that do not heal and become chronic and affect their growth.

When adult animals are affected, abortions, dyspnea, and incoordination can be observed in gilts and chronic lameness in boars (Rodriguez Ferri et al., 2000).

Similarly, a hyperacute disease manifestation can develop with sudden death in naïve healthy piglets. This form of disease can show high mortality (10 - 50%). In naïve animals that are infected for the first time, the organism can lead to a devastating effect on animals of any age and in sows produce anthrax-like illness with a high mortality rate (Rodriguez Ferri et al., 2000).

The characteristic macroscopic lesions in an acute infection by *Haemophilus parasuis* are fibrinous polyserositis, including pericarditis, peritonitis and fibrinopurulent meningitis and arthritis. In the arthritic joints turbid yellowish liquid with fibrin deposition can be observed. Normally, fibrinous pericarditis is associated with signs of chronic congestive heart failure (enlarged heart). Enlarged liver and spleen may also be observed. In hyperacute disease, no specific lesions are observed, but lesions similar to those of endotoxic shock, such as petechiae in kidneys can be seen.

Lesions observed in animals with chronic disease consist of fibrous polyserositis, where the bacteria cannot be found.

1.1.2.3. Respiratory form

Catarrhal-purulent bronchopneumonia is usually observed in the lungs of animals with respiratory distress.

1.1.3. Co-infection with other virus and bacteria

The co-infection of other bacterial agents with *H. parasuis* has been reported. *Streptococcus suis, Escherichia coli, Bordetella bronchiseptica, Mycoplasma hyopneumoniae, Mycoplasma hyorhinis* and *Pasteurella multocida* are the bacteria more frequently co-isolated with *H. parasuis* (Brockmeier, 2004; Cai et al., 2005; Fablet et al., 2011; Palzer et al., 2006; Zhao et al., 2011). Clinical co-

infection of *H. parasuis* with some viruses, such as the porcine reproductive and respiratory syndrome virus (PRRSV), the porcine circovirus type 2 (PCV2) and the swine influenza virus (SIV) has also been reported; and these viruses were frequently found associated with cases of Glasser's disease (Kim et al., 2002; Li et al., 2009; Palzer et al., 2007). As an example, the co-infection by *H. parasuis*, PRRSV and PCV2 was reported in China (Li et al., 2009). Multi-infections of *H. parasuis*, PRRSV, SIV and PCV2 are observed frequently in farms; however more studies are needed to determine the mechanism of interaction among the different infectious agents. It is worth mentioning the association observed at the farm level between *H. parasuis* and PRRSV. However, the interaction between the two microorganisms could not be demonstrated under experimental conditions (Segales et al., 1999).

1.1.4. *Haemophilus parasuis*

In 1910, Karl Glässer was the first who describe a small Gram-negative bacillus in serous exudates from pigs with serofibrinous pleurisy, pericarditis, peritonitis, arthritis, and meningitis (Glässer, 1910). The agent was probably isolated for the first time by Schermer and Ehrlich in 1922 (Little, 1970). Later, it was denominated *Haemophilus influenzae* variety *suis* by (Lewis and Shope, 1931). In the following years, several authors reproduced the disease described by Glässer with pure cultures of this microorganism (Bakos et al., 1952; Hjärre, 1958; Hjärre and Wramby, 1943).

Although, originally the agent of Glässer's disease was named *Haemophilus suis*, (Biberstein and White, 1969) demonstrated that the bacterium required factor V (NAD) for growth, but not factor X (hemin). After some reclassification the bacterium was renamed *Haemophilus parasuis*, based on the accepted nomenclature for *Haemophilus* genus, which uses the prefix "para"- for microorganisms that do not require factor X for growth.

H. parasuis belongs to the Gammaproteobacteria and is classified in the family *Pasteurellaceae*, which includes important pathogens and members of the microbiota of human and other mammalian species. In the last few years, new genera have been included into the family *Pasteurellaceae* and it is currently

constituted of 17 genera: *Pasteurella, Actinobacillus, Haemophilus, Lonepinella, Mannheimia, Phocoenobacter, Gallibacterium, Histophilus, Volucribacter, Avibacterium, Nicoletella, Aggregatibacter, Bibersteinia, Chelonobacter, Basfia, Bisgaardia* and *Necropsobacter* (Christensen, updated 2013; Dewhirst et al., 1992). In addition to *H. parasuis*, other NAD-dependent *Pasteurellaceae* can be isolated from domestic swine (Kielstein et al., 2001) and wild boars (Olvera et al., 2007a).

The actual position within the family *Pasteurellaceae* (Biberstein and White, 1969) remains uncertain, due to a lack of genomic homology with other species of the same genus (Olsen et al., 2005). In addition, Olvera et al. (2006a) and Angen et al. (2007) observed that, in fact, *H. parasuis* does not form a monophyletic cluster by 16S rRNA gene sequence, and two main clusters were defined within the species (Angen et al., 2007; Olvera et al., 2006a).

1.1.4.1. Morphology and cultivation characteristics

Microscopically, *H. parasuis* is a Gram-negative non-motile, small pleomorphic bacillus or cocco-bacillus, with occasional presence of filamentous chains. Its length, therefore, is extremely variable, from 1 to 7 μm of length by 0.2 to 2 μm of width. It has special requirements for growth in the laboratory and therefore it is classified as a fastidious bacterium (Kilian, 2005). Culture isolation of *H. parasuis* from clinical samples can be difficult. Various NAD-enriched media are used for the culture of *H. parasuis* and include chocolate agar, Levinthal agar or PPLO agar supplemented with NAD. *H. parasuis* also grows on blood agar with a *Staphylococcus* nurse streak, showing satellitic growth around the *Staphylococcus* growth. It does not produce haemolysis in blood agar and requires 24-48 hours to grow and to produce small brown to gray colonies on chocolate agar plates or small translucent non-hemolytic colonies on blood agar. Some strains produce colonies of different sizes, but the significance of this phenomenon is not known. *H. parasuis* grows under normal atmosphere at 37ºC, although added humidity and 5% CO_2 may improve growth (Aragon et al., 2012). Due to the poor survival of the bacterium outside the animal, samples have to be submitted to the laboratory fast and under refrigeration. The best transport medium for swab samples is the Amies

medium (del Rio et al., 2003; Olvera et al., 2007b).

The most notable biochemical characteristics of *H. parasuis* are: urease-negative, catalase-positive, reduces nitrates, does not produce indol, and causes fermentation of glucose, galactose, mannose, fructose, saccharose and maltose (Kielstein et al., 2001; Møller and Kilian, 1990). For these tests, a liquid culture is usually needed and PPLO or BHI broth supplemented with NAD can be used.

1.1.4.2. Heterogeneity among *H. parasuis* strains

The heterogeneity among the strains of *H. parasuis* is revealed at different genotypic and phenotypic levels, including virulence. The correct classification of the strains witin this species is important in order to perform an accurate diagnosis and control of the pathogen. An aspect of especial importance is the differentiation between colonizing non-virulent strains and pathogenic virulent strains. This heterogeneity is evident even at the 16S RNA gene sequence (Angen et al., 2007; Olvera et al., 2006a).

One of the first approaches to classify *H. parasuis* strains was the use of serotyping, which demonstrated high antigenic heterogeneity among *H. parasuis* strains. Bakos et al. (1952) reported the existence of four *H. parasuis* serovars designated A, B, C and D, based on a precipitation test with soluble antigen extracts. Later, Morozumi and Nicolet, (1986) defined 7 serovars (1-7) and Kielstein and Rabbach, (1991) added 6 additional serovars (Jena 6-Jena 12). This method underwent some modifications untill the current serotyping classification by gel diffusion (GD) , which defines a total of 15 serovars using a thermostable antigen that was resistant to proteolytic enzymes (Rapp-Gabrielson and Gabrielson, 1992). Using this classification, a high prevalence of serovar 4 and 5 was determined, with 15% of non-typeable isolates. However, a correlation between serovar and the site of isolation/clinical origin or pathogenic potential of the strains could not be determined (Rapp-Gabrielson and Gabrielson, 1992). The major drawback of this technique is that up to 45% of the strains are not typeable. In an attempt to reduce the number of non-typeable (NT) isolates, del Rio et al. (del Rio et al., 2003) modified the serotyping scheme using saline, boiled and

autoclaved extracts for serotyping *H. parasuis* and compared their use in the characterization of field isolates through a coagglutination technique (CA) and an indirect hemagglutination test (IHA). No specific results were obtained with CA due to the presence of numerous cross-reactions, while specific reactions, with minimal cross-reactions, were obtained for all serovars with IHA and saline extracts. The use of IHA was also supported by an additional study with 300 isolates from North-America (Tadjine et al., 2004a). Thus, the IHA test was recommended as an additional method for reliable serotyping of *H. parasuis*.

Later, the IHA and the GD tests were again compared with Australian and Chinese isolates and some discrepancies were observed between both tests, suggesting that the use of IHA is complementary to GD (Turni and Blackall, 2005). An additional study was performed with 281 isolates of *H. parasuis*, which were serotyped by both the GD and the indirect haemagglutination (IHA) tests. Again, serovar 4 (24.2%) and serovar 5 (19.2%) were the most prevalent serovars, while 12.1% of the isolates were nontypable. A comparison of the number of isolates obtained from the respiratory tract of swine without polyserositis with those obtained from swine with polyserositis revealed an increased frequency of serovar 4 and a significantly decreased frequency of serovar 13 among isolates from the respiratory tract of swine without polyserositis, whereas the frequency of serovars 5, 12, 14 and non-typable from swine with or without polyserositis were similar (Cai et al., 2005). Animal infections have also shown that there is no direct correlation between strain serovar and virulence (Aragon et al., 2010).

The initial serotyping studies already indicated a high prevalence of serovar 4 and 5, along with NT isolates, and this observation was confirmed in several reports with isolates from different countries (Angen et al., 2004; Blackall et al., 1996; Cai et al., 2005; del Rio et al., 2003; Morikoshi et al., 1990; Neil et al., 1969; Oliveira et al., 2003a; Rubies et al., 1999; Tadjine et al., 2004a; Turni and Blackall, 2005), with the exception of United Kingdom where serovar 10 presented a high prevalence (Morris et al., 2006). In Spain, Rubies et al. (1999), in a study with 174 isolates from 1993 to 1997, found that four serovars, 5 (18.4%), 4 (16%), 2 (9.2%) and 13 (8%) were the most frequently isolated. In addition, 29.3% of the 174 isolates were classified as NT isolates (Rubies et al., 1999). Similar results were

reported by del Rio et al., (2003), indicating that the distribution of serovars in Spain is very similar to that found in other countries. The non-typeability may indicate that some isolates failed to express sufficient amounts of type-specific antigen or the existence of unknown serovars.

Besides serotyping, other molecular methods have been used in the classification of *H. parasuis* strains, such as multilocus enzyme electrophoresis (MEE), restriction endonuclease fingerprinting (REF), PCR-Restriction Fragment Length Polymorphism (RFLP) and PCR based on repetitive intergenic consensus sequences (Enterobacterial Repetitive Sequence Consensus intergenic, ERIC-PCR). Some of these systems have advantages over the conventional serological typing method, allowing a better identification and classification of all isolates.

MEE is a method for the characterization of microorganisms by the relative electrophoretic mobility of a large number of intracellular enzymes. Blackall et al. (1997) described 34 electrophoretic patterns among strains of *H. parasuis* isolated in Australia. The analysis of the patterns allowed classifying all the strains in two groups, A and B, except one. Group A was constituted by two reference strains of the serovar 5 and 12 field isolates from serovars 4, 5 and 13, and by some NT strains. On the other hand, group B was constituted by field strains of serovars 1, 2, 7, 9, 10 and 13 and by the reference strains of serovars 1, 2, 3, 4, 8 and 9. No relationship was observed between MEE classification and virulence, confirming the existence of a great heterogeneity in this species.

REF is a method that involves the digestion of the bacterial genomic DNA by restriction endonucleases and the subsequent analysis of the fragments by electrophoresis. To study the distribution of *H. parasuis*, isolates of healthy pigs from conventional and SPF farms were compared using REF with the restriction endonuclease BamHI (Smart et al., 1988). Diseased pigs were also included in this study. It allowed identification of 34 unique REF patterns from a total of 69 isolates. The remaining 35 isolates were grouped into 13 REF groups. In most of the studied farms a large heterogeneity in serovars was observed indicating the possibility of other strains present in these farms which have not been identified. Therefore, this study was not able to demonstrate the presence of common *H. parasuis* strains when comparing the conventional farms and the SPF farms.

PCR-RFLP is a technique based on the amplification of a gene, digestion of the amplicon with restriction endonucleases and the analysis of the fragments obtained by agarose gel electrophoresis. De la Puente Redondo et al. (2003) and del Rio et al. (2006) conducted PCR-RFLP studies with the gens *tbpB* and *aroA*, respectively. A high rate of typeability was achieved with *tbpA* gene which allowed the development of a system for detecting and identifying *H. parasuis* by a PCR-DITPAR (detection, identification and typing of *H. parasuis*) (de la Puente Redondo et al., 2003). In the case of the *aroA* gene, common restriction patterns were observed between some serovars of *H. parasuis* reference strains and members of the genus *Actinobacillus* (del Rio et al., 2006).

ERIC-PCR is a PCR that uses primers whose targets are repetitive intergenic sequences. This technique was initially described in enterobacteria and was used for the first time in *H. parasuis* (Rafiee et al., 2000). The fifteen serovar reference strains of *H. parasuis* yielded specific ERIC-PCR patterns. The suitability of this technique was established for epidemiological studies, and after using this technique it is concluded tha a high variability of strains was present within the same serovar, indicating that ERIC-PCR is more discriminative than serotyping. The major problem of ERIC-PCR is the difficulty in comparing results from different laboratories (Oliveira et al., 2003a). To solve this problem, sequencing methods were established for *H. parasuis*.

Recent analysis of the *hps60* sequence in strains of *H. parasuis* has confirmed a great variability among different strains of this bacterium. This is a method that allows epidemiological and phylogenetic studies. Although a separate lineage of virulent strains was found, the relationship with virulence in the rest of the isolates was not clear. Also, the possible existence of lateral gene transfer between *H. parasuis* and some species of the genus *Actinobacillus* was observed (Olvera et al., 2006a).

To reduce the impact of recombination in *H. parasuis* genotyping, a multilocus sequence typing (MLST) scheme was designed and later optimized (Mullins et al., 2013; Olvera et al., 2006b). MLST grouped strains in 3 main clusters, one associated to nasal isolation from healthy animals from disesase-free farms, a second associated with isolation from systemic lesions and a third with no clear

association with the clinical origin of the strains (Aragon et al., 2010).

In summary, the heterogeneity of *H. parasuis* strains is very high and can be demonstrated by different methods. A typing method with a clear correlation with virulence is still needed.

1.1.5. Animal models

The pig can be considered the ideal animal model to reproduce Glässer's disease, since *H. parasuis* is a pathogen specific to swine. This model has been used historically from the beginning of the 40s (Bakos et al., 1952; Hjärre, 1958; Hjärre and Wramby, 1943). The main problem with the pig model is the availability of susceptible pigs since, as we have already indicated, *H. parasuis* is an early colonizer of piglets. Therefore, animal models have evolved to produce susceptible pigs for experimental infections in order to observe the classical symptoms of Glasser's disease and study the pathogenesis of the disease. Thus, to avoid prior colonization by *H. parasuis* and interference due to maternal antibodies, piglets obtained at birth by cesarean or natural birth, followed by artificial feeding (to avoid colostrum; i.e., antibody, uptake) have been used (Blanco et al., 2004; Martín de la Fuente et al., 2009d; Neil et al., 1969; Rapp-Gabrielson et al., 1997; Vahle et al., 1995, 1997).

Such investigations usually require high doses of challenge inoculum to mimick the characteristic lesions of *H. parasuis*. In fact, a positive relationship between the size of inoculum and the lesions in the animal model has been observed (Oliveira et al., 2003b). The interference of maternal antibodies in disease reproduction (and therefore, in protection) was demonstrated with colostrum-deprived versus sow-reared piglets, since only in colostrum-deprived animals the disease was observed after intratracheal inoculation (Blanco et al., 2004). As an alternative to cesarean-derived piglets, snatch-farrowed colostrum-deprived pigs and specific pathogen free (SPF) pigs have been used to reproduce Glässer's disease successfully (Amano et al., 1994; Amano et al., 1997; Aragon et al., 2010; Blanco et al., 2004; Oliveira et al., 2003b). The advantage of the snatch-farrowed colostrum-deprived pig model is the easy availability of animals even

from herds which are positive. However, the success of this model depends heavily on strict hygiene regimes and the use of antibiotics. Although the survival rate of these pigs has reached 80%, high pre-challenge mortality can still occur (Oliveira et al., 2003a; Oliveira et al., 2003b).

Alternatives to the use of pigs have been explored with different outcomes. Mouse models have been used unsuccessfully to reproduce disease (Martín de la Fuente et al., 2007a; Morozumi et al., 1982), but it is still a model that has been used for vaccine protection experiments (Ahn et al., 2012; Fu et al., 2013; Tadjine et al., 2004b; Tian et al., 2011). One of the most recent studies using the mouse model reported the infection of the animals by intranasal and intraperitoneal inoculation without deaths, clinical signs or macroscopic lesions after receiving several doses of bacteria (with the exception of a high dose used in the mice intraperitoneally). In addition, the microorganism was cleared rapidly from spleen and lungs (Martín de la Fuente et al., 2007a).

Several studies have confirmed the guinea pig to be a better animal model than the mouse to reproduce experimentally Glässer'disease, without the need of using a swine model, which is more expensive and laborious (Morozumi et al., 1982; Rapp-Gabrielson et al., 1992). Recently, the guinea pig model has also been used for vaccine efficacy studies (Huang et al., 2013).

1.2. Pathogenesis and virulence mechanisms

Møller and Kilian (1990) examined the diversity and ecology of the family *Pasteurellaceae* in the respiratory tract. *H. parasuis* was isolated from nasal cavities in higher proportion than from oral cavity in healthy pigs. So far, no other host besides swine, domestic and wild (Olvera et al., 2007a), has been identified and therefore *H. parasuis* is considered a pig-specific bacterium.

H. parasuis is commonly found in the upper respiratory tract of healthy piglets, and it is considered part of the normal respiratory microbiota of the animal. The initial acquisition of this bacterium takes place through contact with the sow after birth. Also, *H. parasuis* is one of the earliest isolated bacterium with

higher prevalence in nasal swabs from piglets at one week of age and reason by which it is commonly described as an early colonizer of the upper respiratory tract in pigs (Harris et al., 1969; MacInnes and Desrosiers, 1999; Pijoan, 1995). *H. parasuis* preferentially colonizes the nasal mucosa of swine, but the microorganism can also be isolated from tonsil and other areas of the upper respiratory tract, including the trachea (Møller et al., 1993). Our own results support these observations, but we only detected *H. parasuis* in the trachea when the animal was infected with a virulent strain (Bello et al. unpublished results). *In vitro* studies have demonstrated the ability of *H. parasuis* to adhere to epithelial cells and induce apoptosis on these cells (Bouchet et al., 2009). This ability could be involved in colonization and initiation of infection.

H. parasuis is also the etiological agent of Glässer's disease and this role of this bacterial species has been the main focus of research. Vahle et al. (1995, 1997) studied the pathogenesis and progression of disease after intranasal inoculation of colostrum-deprived pigs. Starting at 4 hours post-infection, necropsies revealed the presence of neutrophil infiltration in the nasal submucosa of the animals, which was progressively reduced in the following hours; leading to lymphocytic infiltration, loss of cilia and alteration of the mucosa. After, 12 hours of intranasal inoculation, the bacteria was isolated from the nasal cavity and the trachea of the animals, where purulent rhinitis and trachea injury were observed, respectivelly. Later, from 12 to 36 hours, bacteria were found in the bloodstream and subsequently in internal organs. Once *H. parasuis* establishes colonization of the upper respiratory tract, under some situations, some strains can spread to the lung, where they may cause pneumonia or procced to invade systemic sites. Several authors have reported the isolation of *H. parasuis* from pigs with pneumonia, but its association with this type of lesion is not clear, since it has been also isolated from healthy lungs (Kobisch and Desmettre, 1980; Little, 1970; Møller et al., 1993; Palzer et al., 2008). Resistance to phagocytosis by alveolar macrophages has been observed in virulent strains of *H. parasuis* and is expected to play an essential role in bacterial survival in the lung and, therfore to contribute to disease development (Olvera et al., 2009). Some bacterial factors have been descrived to be involved in phagocytosis resistance, such as the capsule (Olvera et al, 2009) and the virulent-associated trimeric autotransporters VtaA8 and Vta9

(Costa-Hurtado et al., 2012b). The interaction between *H. parasuis* strains and alveolar macrophages *in vivo* is also dependent on the virulence of the strains. Virulent strains produced an early inactivation of macrophages (low level of CD163, SLAII and CD172a) as compared to non-virulent strains (Costa-Hurtado et al., 2013). This early inactivation of the response by macrophages, together with their capacity to resist phagocytosis, allow virulent strains to multiply and spread, inducing a high, but late, activation of macrophages and inflammatory precursors (IL-8 and soluble CD163) that are responsible, at least partially, for the characteristic pathological lesions. In addition, virulent strains have been localized in non-phagocytic cells in the lung of infected animals (Bello-Orti et al., 2013). This localization in pneumocyte-like cells could be a mechanism to avoid the immune system and allow persistence.

Moreover, *H. parasuis* has the capability to produce systemic infections and strains isolated from systemic sites have been shown to be resistant to the bactericidal activity of the complement, which allows the survival of the microorganism in the bloodstream. This phenomenom is commonly known as serum-resistance and it was reported for the first time in *H. parasuis* by Cerdà-Cuéllar and Aragon (Cerdà-Cuéllar and Aragon, 2008). In the same report, the nasal strains of the healthy piglets were sensitive to the serum.

In addition, some cases of Glässer's disease proccedes with meningitis, whose production requires the ability of *H. parasuis* to cross the blood-brain barrier. Virulent strains of *H. parasuis* have been shown to be able to invade endothelial cells and this process of invasion is considered a virulence mechanism associated with the ability of the strain to cause meningitis (Vanier et al., 2006; Aragon et al., 2010). The cellular adhesion of *H. parasuis* to endothelial cells induced apoptosis and up-regulation of inflammatory mediators, contributing to the pathogenesis in a mechanism partially mediated by the LPS (Bouchet et al., 2008)

Studies carried out by Martín de la Fuente et al. (2009a) confirmed the production of proinflamatory cytokines (IL-1α) in animals with severe Glässer's disease after experimental inoculation (Martín de la Fuente et al., 2009a). Further analysis found significant increases in the proportions of monocytes and

granulocytes (SWC3+) and B cells (αIgM+), as well as a significant reduction in CD3+ cells, suggesting an increase of trafficking of inflammatory cells and the onset of the adaptive antibody response against *H. parasuis* infection (Martín de la Fuente et al., 2009c). Results from these series of experiments confirmed the role of antibodies and discarded the role of cellular immunity in protection against Glässer's disease (Martín de la Fuente et al., 2009c; 2009d).

Also, *in vitro* studies have shown that *H. parasuis* is capable of producing biofilm (Jin et al., 2006). Biofilm formation is commonly associated with pathogenic bacteria as a form to cause progressive and persistent infections. However, the role of biofilm in the infection by *H. parasuis* is not clear, since in the *in vitro* assays, nasal strains were more efficient in forming biofilm than virulent strains (Jin et al., 2006). Recently, the study of two *H. parasuis* mutants, Δ*galU* and Δ*galE*, indicated that GalU plays a role in autoagglutination and biofilm formation, while GalE may affect the biofilm production indirectly, since the production of biofilm in the Δ*galE* mutant was increased (Zou et al., 2013).

The pathogenic potential of the strains of *H. parasuis* in a herd is also considered a factor for severity and progression of disease. The serovars commonly isolated from the upper respiratory tract in pigs include serovars infrequently isolated from systemic sites (Bloch, 1985; Rapp-Gabrielson, 1993) Thus, it is believed that a subpopulation of *H. parasuis* in the upper respiratory tract, have the ability to systematically invade and cause disease (Rapp et al., 1986; Rapp-Gabrielson, 1993).

On a practical note, it is important to mention that the samples to be submitted to the laboratory for diagnosis of Glässe's disease will depend on the lesions observed in the animals. Samples from the acute fibrinous lesions observed in non-treated animals are the best options, and the samples vary with the pathology observed. Samples from the upper respiratory tract are of no diagnostic use, since the detection of the bacterium is common as colonizer and not associated to disease.

1.3. Virulence factors

Studies on *H. parasuis* pathogenicity have been limited and the virulence factors have not been unequivocally defined yet. Several putative virulence factors have been reported, but some of these apparent virulence factors can also be found in isolates recovered from healthy piglets.

The importance of the knowledge about the virulence factors resides in its use for clarifying the pathogenesis of the disease and developing strategies to combat it. The unequivocal definition of the virulence factors fom *H. parasuis* will allow the improvement of diagnostic techniques, as well as the development of effective treatments and preventive measures, including effective vaccines. The factors involved in the invasion of *H. parasuis* during infection are mostly unknown and recent studies about their role and regulation are just beginning to reveal potential components of disease-causing mechanisms.

In the recent years, quite a few groups have reported genomic and transcriptomic studies that have detected potential virulence factors of *H. parasuis*. An assessment of *H. parasuis* strains suggested that certain outer membrane protein profiles may be related with the virulence (Ruiz et al., 2001), although none of the proteins was further characterized. Additionally, a global search for *H. parasuis* virulence genes, using differential-display RT-PCR identified seven targets with a response to heat stress, including genes involved in heat shock response (Hill et al., 2003). While other investigations assessing gene expression *in vivo* (Jin et al., 2008) or under *in vitro* growth conditions designed to mimic those encountered *in vivo* (Melnikow et al., 2005; Metcalf and MacInnes, 2007) identified numerous potential virulence genes, including a variety of transporters, metabolic and biosynthetic enzymes, putative cell surface proteins, and some apparent related of virulence genes expressed by other members of the *Pasteurellaceae* family. However, in the absence of genetic tools, the precise roles of these potential virulence factors have not yet been confirmed.

The specific virulence factors described in the literature are presented below.

1.3.1. 6-phosphogluconate dehydrogenase (6PGD)

The purification and characterization of the cell wall protein 6-phosphogluconate dehydrogenase (6PGD) of *H. parasuis* was recently described (Fu et al., 2012). The 6PGD protein was also previously described in the swine pathogens *S. suis* as a protective antigen (Tan et al., 2008; Tan et al., 2009). This protein seems to be involved in adherence to swine alveolar epithelial cells (SJPLC), since recombinant 6PGD protein considerably inhibited the capacity of *H. parasuis* to adhere to SJPLC cells. In addition, immunogenicity and partial protection in mice suggested its role as a potential vaccine candidate (Fu et al., 2012).

1.3.2. Lipooligosaccharide (LOS)

The LPS in most Gram-negative bacteria has a specific O chain, and gives bacteria phenotypic characteristic, such as smooth colony morphology. *H. parasuis* has a short LPS by the absence of repeating O-antigen subunits, which is denominated lipooligosaccharide or LOS (Ogikubo et al., 1999), and it is probably responsible for the cross-reactions between serovars. In fact, the structure of the LOS core from two *H. parasuis* strains of different serovar has been determined and found to be identical (Perry et al., 2013).

The interaction of LOS with host defense mechanisms *in vivo* and *in vitro* revealed its role in the inflammatory response, producing thrombosis and disseminated intravascular coagulation (Amano et al., 1997). The action seems to be exerted on monocytes and macrophages, which secrete a high variety of proinflammatory mediators, playing an important role in the acute inflammation observed in Glässer's disease. The *in vitro* mitogenic capacity of the *H. parasuis* LOS in spleen cells of BALB/c mice has been also reported (Ogikubo et al., 1999). LOS from *H. parasuis* was able to induce the release of IL-8 and IL-6 by porcine brain microvascular endothelial cells (PBMEC) and newborn pig tracheal (NPTr) cells (REF). On the other hand, LOS seems to play a partial role in adhesion, since competitive assay with LOS did not completely eliminate the adhesion of *H.*

parasuis to endothelial or epithelial cells, suggesting the involvement of other adhesins (Bouchet et al., 2009; Bouchet et al., 2008). In addition, a monoclonal anti-LOS antibody showed a protective role in a mouse infection model (Tadjine et al., 2004b). However, there is certain controversy regarding the role of LOS in *H. parasuis* virulence, since both virulent and non-virulent strains share a LOS with a similar pattern (Zucker et al., 1996).

Recently, genomic information for *H. parasuis* strain SH0165 genes *opsX*, *rfaF* and *waaQ,* which encode three heptosyltransferases required for transferring heptoses I, II and III to the LOS, respectively, allowed the production of mutants to study the role of these genes in virulence (Xu et al., 2013). Their respective LOS heptoses mutants appear to have a role in complement-mediated serum resistance and the Δ*opsX* and Δ*rfaF* mutants, which caused severe truncation in the LOS, had reduced ability to adhere to and invade PK-15 and PUVEC cells. These results indicate that a full length LOS is required for interaction with host cells and therefore can bee associated with some properties of virulence in *H. parasuis* (Xu et al., 2013).

1.3.3. Neuraminidase (Sialidase) and sialic acid.

Bacterial neuraminidases are important factors in the virulence of some members of the family *Pasteurellaceae,* including *Haemophilus influenzae,* specifically with serum resistance (Hood et al., 1999; Inzana et al., 2002; Marti-Lliteras et al., 2011; Nakamura et al., 2011). This enzyme, in association with the permease and aldolase, can contribute to bacterial survival by scavenging carbohydrates from host cells. Furthermore, bacterial neuraminidases, in addition to its nutritional role, can serve as mediators in the removal of the sialic acid residues from glycoconjugates to unmask the necessary receptors for colonization or invasion of host cells or to use the released sialic acid to modify the bacterial surface and interfere with the host immune recognition. Also, neuraminidase activity can decrease the viscosity of mucine with a subsequent increase in cell adhesion (Corfield et al., 1990; Lichtensteiger and Vimr, 1997). Once internalized, sialic acid can be used as source of carbon and/or nitrogen or can be modified by

CMPNeu5Ac synthetases (such as NeuA and SiaB) to be incorporated into the lipopolysaccharide (LPS) by the sialyltransferase LsgB (Steenbergen et al., 2005).

A neuraminidase from the outer membrane of *H. parasuis* was identified and purified. The neuraminidase had a molecular weight of 82 kDa and was inhibited by Neu-Ac2 (specific inhibitor) but not by EDTA or EGTA, therefore it was not dependent on divalent cations. Supplementation of the culture medium with sialic acid improved the growth of *H. parasuis,* indicating that the acid is metabolized by the bacterium (Lichtensteiger and Vimr, 1997, 2003).

A recent study carried out by Martinez-Moliner et al. (Martinez-Moliner et al., 2012) reported the cloning of *nanH* (neuraminidase) and detection of the neuraminidase activity in *H. parasuis* strains of different clinical origins. In agreement with previous reports, neuraminidase activity was found in the majority of *H. parasuis* strains tested. This activity did not correlate with the clinical origin of the strains. On the other hand, *lsgB* (sialyltransferase) was predominantly present in the systemic isolates, and was not amplified from any of the nasal isolates tested. A correlation between the possibility to sialylate the lipooligosaccharide (LOS) molecule and serum resistance was established. In addition, using the reference strain Nagasaki (virulent, *lsgB*+) the presence of sialic acid in the LOS was demonstrated. The function of sialic acid in *H. parasuis* pathogenesis has been also suggested by other authors, who reported the transcription of *siaB*/*neuA* during infection (Jin et al., 2008).

1.3.4. Cytolethal distending toxin (CDT)

The cytolethal distending toxins (CDTs) are heat-labile toxins that produce a progressive cytoplasmic and nuclear enlargement in cultured mammalian cells. They are a class of heterotrimeric AB-type genotoxins produced by many Gram-negative pathogens of humans and animals (Smith and Bayles, 2006). The microorganisms that produce CDTs often persistently colonize their host, and usually the affected cells die by apoptosis (Jinadasa et al., 2011). The CDTs commonly comprises three subunits designated as CdtA, CdtB and CdtC, where CdtB is the active toxic unit and CdtA and CdtC are required for CDT binding to

target cells and for delivery of CdtB into the cell (Lara-Tejero and Galan, 2002). CdtB contains a pentapeptide motif that is present in all DNase I enzymes (Elwell and Dreyfus, 2000) and is necessary for the CDT-mediated cell cycle arrest at the G2/M phase, leading to the enlarged or distended cells, for which these toxins are named, and eventual cell death (Elwell and Dreyfus, 2000; Jinadasa et al., 2011; Smith and Bayles, 2006). With respect to *H. parasuis* it is noteworthy that two *cdt* gene cluster loci have been identified in the genomic sequence of *H. parasuis* strain SH0165 (Zhou et al., 2012). The recombinant proteins showed toxic activity and were able to arrest the cell cycle in cultures. CdtB protein was expressed by 109 clinical isolates and all the 15 reference strains of *H. parasuis*, independently of their virulence, indicating that CDT is a conserved molecule, probably not involved in the virulence of *H. parasuis*.

Recently a *H. parasuis* CDT-deficient mutant was studied, demonstrating that *H. parasuis* CDT is involved in serum resistance, adherence to and invasion of porcine umbilicus vein endothelial cells (PUVEC) and porcine kidney epithelial cells PK-15 (Zhang et al., 2012b).

1.3.5. Capsule

The majority of bacterial capsules consists of a polymer of carbohydrate with acids groups, which is consistent with the results of Morozumi and Nicolet (1986). The first study on the specific chemical composition of the capsule of *H. parasuis*, reported the presence of 1,1'-linked disaccharide of N-acetylglucosamine with galactose in the capsule from an undefined strain (Williamson and Zamenhof, 1964). Recently, a more detailed chemical structure of the surface polysaccharides of this microorganisms was described in two virulent strains (Nagasaki and ER-6P) of *H. parasuis* (Perry et al., 2013). The structure of the capsular polysaccharide in both strains had an identical main chain with a disaccharide repeating unit of β-glucose-6P and 2,4-diacetamido-2,4,6-trideoxy-D-galactopyranose, substituted with α-Neu5R-3-α-GalNAc-1-P- (serovar 15 strain) or α-Neu5R-3-α-Gal-1-P- (serovar 5 strain), where R is N-acetyl or N-glycolyl groups. The acetyl and the glycolyl were present in equivalent amounts in the ER-6P strain but only a 20% of

glycolyl substituent was found in the Nagasaki strain (Perry et al., 2013). However, the role of the capsule in the virulence of *H. parasuis* is not completely clear. Initial studies indicated that more than half of the isolates are noncapsulated and surprisingly this seemed to coincide with virulent strains (Morozumi and Nicolet, 1986; Nicolet et al., 1980). On the other hand, indications on the role of capsule in *H. parasuis* virulence can also be found in the literature. Capsule was observed in all strains after guinea pig passage, although it was inconsistent in some strains; interestingly, capsule production was reduced after in vitro passage (Rapp-Gabrielson et al., 1992), indicating a role in infection. In agreement to the later report, Olvera et al, (2009) found that virulent strains showed distinct capsule after incubation with alveolar macrophages, and the role of this surface structure in phagocytosis resistance was suggested (Olvera et al., 2009).

Recently, the *capD* gene, encoding a polysaccharide biosynthesis protein possibly involved in capsule production, was identified in *H. parasuis* virulent strain Nagasaki while was absent in the non-virulent strain SW114 (Zhou et al., 2010). Later, role of this gene in the pathogenesis of *H. parasuis* was demonstrated, since a deletion mutant Δ*capD* showed a significantly attenuated infectivity of the bacterium and, specifically, exhibited an extreme sensitivity to the complement-mediated killing (Wang et al., 2013).

1.3.6. Fimbriae

Fimbriae are adhesines that mediate attachment to host structures. Fimbria-like structures were observed in *H. parasuis* after *in vivo* passage in embryonated eggs (Munch et al., 1992). *H. parasuis* SH0165 possesses four type IV fimbrial genes encoding the major structural pili unit PilA (HAPS2013) and three biogenesis proteins PilBCD (HAPS2011-2009) (Xu et al., 2011). The implication of these molecules in bacterial adherence is expected, but it has not been ascertained. Interestingly, *pilA* was found to be up-regulated under iron-restriction conditions, suggesting that iron restriction could be a signal for colonization (Xie et al., 2009)

1.3.7. Proteins of the porin family.

Porins are an unusual class of membrane proteins that form hollow β-barrel structures with a hydrophobic outer surface. The barrel structure encompasses the transmembraneous pore that allows the passive diffusion of solutes across the outer membranes of Gram-negative bacteria and thus make this membrane semipermeable (Schirmer, 1998). Unlike other membrane transport proteins, porins are large enough to allow passive diffusion. There are four types of porins: general/non-specific porins, substrate-specific porins, gated porins, and efflux porins (also called channel-tunnels).

In *H. parasuis*, outer membrane protein P2 (OmpP2) and P5 (OmpP5) have been studied by different groups. Mullins et al. observed that the predicted amino acid sequences for both P2 and P5 proteins were significant heterogeneous, particularly the predicted extracellular loops (Mullins et al., 2009).

1.3.7.1. Outer membrane proteins P2 (OmpP2)

OmpP2 is the most abundant protein in the outer membrane of *H. parasuis* (Zhou et al., 2009). Omp P2 is highly conserved in *H. parasuis,* but some differences in sequence were reported, including insertion sequences that were found preferently in non-virulent strains (Li et al., 2012; Mullins et al., 2009).

The functional role of OmpP2 has been recently studied using knockout mutants. A deletion mutant of the SC096 strain was produced and the loss of OmpP2 resulted in increased sensitivity to complement killing, indicating the role in serum resistance of this protein (Zhang et al., 2012a). However, defective mutants showed growth defects and further alterations at protein composition level of the outer membrane, which may result in instability of the outer membrane. Thus, the defect in serum susceptibility of the OmpP2 mutant could be an indirect effect and not due directly from the functionality of P2.

Interestingly, when OmpP2 from different strains was studied, some virulent strains (including Nagasaki, 84-17975 and SC096) showed shorter sequences than non-virulent strains (including SW114, C5 or SC003) (Zhang et al.,

2012a). It was previously described that the longer sequences would include an extra loop in the predicted protein (Mullins et al., 2009), which might contribute to serum susceptibility in *H. parasuis*. OmpP2 has also been implicated in adherence to porcine alveolar macrophages (3D4/21 cell line) and resistance to phagocytosis. Mutant ΔompP2 showed reduced adherence to 3D4/21 cells and pre-incubation of macrophages with purified P2 resulted in an increase survival of wild–type SC096 (Zhang et al., 2012c).

1.3.7.2. Outer membrane proteins P5 (OmpP5).

A protein similar to *H. influenzae* P5 was purified and shown to have different adhesion attributes in *H. parasuis* (*H. parasuis* P5, or OmpA, did not bind carcinoembryonic antigen) (McVicker and Tabatabai, 2006). Later the corresponding gene was cloned (Zhang et al., 2009) and the analysis of the sequences from different strains displayed certain variability, with 4 hypervariable domains encoding the 4 putative surface-exposed loops (Mullins et al., 2009; Tang et al., 2010). Although P5 is involved in various pathogenic processes, including cell adhesion and invasion in other *Pasteurelleceae* (Dabo et al., 2003; Ojha et al., 2010), a *H. parasuis* Δ*ompP5* mutant did not show a defect in serum susceptibility or in adhesion and invasion to epithelial and endothelial cells. However, as it was observed with the Δ*ompP2* mutant, the Δ*ompP5* mutant showed growth defects and alterations in protein expression. At the same time, the immunogenicity of P5 was confirmed and its potential used as vaccine was suggested (Tian et al., 2011).

1.3.8. IgA protease activity

In order to colonize the respiratory mucosa, bacteria must overcome the protective effects of IgA, which participates in host defense by inhibiting microbial adherence and invasion, inactivating bacterial toxins, and mediating antibody-dependent cytotoxicity. The production of bacterial IgA extracellular proteases results in cleaving and elimination of the agglutination activity of the immunoglobulin (St. Geme III, 2001). Mullins et al. (Mullins et al., 2011)

demonstrated swine IgA protease activity in culture supernatants of *H. parasuis*, but no homologue of the *Haemophilus influenza iga* or *igaB* was detected in the genome of the strains. Recently, an *espP2* gene homologue (extracellular putative serine protease) was detected in *H. parasuis*, and this protease provided partial protection against a homologous challenge in guinea pigs (Zhang et al., 2012d). This protein identified by Zhang et al. (2012d) is a monomeric autotransporter (AT) and corresponds to the BmaA5 and BmaA6 described by Pina-Pedrero (Pina-Pedrero et al., 2012) The correlation between the IgA protease activity described in the supernatant of *H. parasuis* and the *espP2* gene has to be evaluated.

1.3.9. Transferrin binding protein.

Existence of transferrin receptor proteins in bacteria, which play important roles in microorganisms such as *A. pleuropneumoniae* and *H. influenza* has been reported (Beddek et al., 2004; Gray-Owen and Schryvers, 1995). Transferrin is a monomeric glycoprotein of 80 kDa molecule found in serum (Cornelissen, 2003). Its main function is to transport iron to different host cells and, thereby, it helps to reduce the concentration of free iron in serum (Baker et al., 2002). Binding of transferrin by bacteria is a mechanism for iron uptake that helps bacteria to survive inside the host. The existence of the genes *tbpA* and *tbpB* coding for transferrin binding proteins was detected in *H. parasuis*, together with other genes that are part of the *tonB* complex (exbB and exbD) (del Rio et al., 2005). As expected, *tbpA* and *tbpB* were upregulated under iron-restriction conditions (Xie et al., 2009). The use of TbpA and TbpB as vaccines is also being explored (Huang et al., 2013; Martín de la Fuente et al., 2009b).

1.3.10. Type V secretion system

The secretion of proteins by Gram-negative bacteria is an essential function for many metabolic and physiologic processes. There are numerous publications describing the type V secretion system (Cotter et al., 2005; Henderson et al., 2004; Kim et al., 2006; Linke et al., 2006). This secretion system does not require energy

for coupling or accessory factors for successful protein secretion and the proteins in this class are described as autotransporters. The IgA protease of *Neisseria gonorrhoeae* was the first bacterial autotransporter described (Pohlner et al., 1987a; Pohlner et al., 1987b), and subsequently, numerous autotransporters have been described for other Gram-negative bacteria (Desvaux et al., 2004; Henderson et al., 2000; Henderson et al., 2004).

This family of secreted proteins includes those secreted via the monomeric autotransporter system (Type Va or AT-1), the two partner secretion pathway (type Vb) and the type Vc system (also termed AT-2) (Annex 1) (Desvaux et al., 2004; Henderson et al., 2004). These surface exposed proteins seem to take part in diverse host-pathogen interactions associated with virulence; e.g. adhesion, invasion, autoagglutination, inhibition of the complement activation or IgA protease (Cotter et al., 2005; Mistry and Stockley, 2006). Furthermore, they can induce a good antibody bactericidal response (Giuliani et al., 2006). Recent, analysis of the SH0165 and Nagasaki genomic and proteomic studies have been established the presence of autotransporters in *H. parasuis* (Pina et al., 2009; Pina-Pedrero et al., 2012; Zhou et al., 2009); and up-regulation during lung infection has been reported (Jin et al., 2008).

1.3.10.1. Monomeric autotransporters (AT1)

The presence of six β-barrel monomeric autotransporters (Bma/AT-1) in *H. parasuis* was recently studied (Pina-Pedrero et al., 2012). A comparative genomic analysis of the AT-1 coding loci and their neighboring genes from three *H. parasuis* strains serovar 5 was performed. Using the recombinant passenger domains of *bmaA1*, *bmaA4*, *bmaA5* and *bmaA6* (*bmaA2* and *bmaA3* were predicted to be pseudogenes in at least one of the three *H. parasuis* strains), their *in vivo* expression and antigenicity was demonstrated (Pina-pedrero, 2012). As indicated above, a homolog to the extracellular serine protease EspP has been identified in *H. parasuis* (Zhang et al., 2012d), corresponding to BmaA5/6, but the functionality of

EspP has not yet been determined; although as mentioned before, it may correspond to the IgA protease activity found by Mullins et al. (2011).

1.3.10.2. Virulence associated trimeric autotransporters (VtaA)

The genes coding for VtaA constitute a multigene family of about 10 copies per genome in *H. parasuis*, subdivided into three groups (group 1, 2 and 3) by sequence similarity in the translocator domain (Pina et al., 2009). These *vtaA* genes encode for putative outer membrane proteins with characteristic adhesion domains. As several *vta*A copies per genome have been detected, it has been interpreted as a strategy to escape the immune system by antigenic switching. The presence of *vtaA* from group 3 is highly conserved in *H. parasuis*, while *vtaA* from group 1 and 2 is detected mainly in virulent strains. This differential presence of the *vtaA* genes was used for the identification of potentially virulent isolates by PCR (Olvera et al., 2012). Ten paralog *vtaA* genes were also found in SH0165.

Furthermore, the antigenicity of VtaA was examined using sera from colostrum deprived pigs challenged with a sub-lethal dose of Nagasaki (Olvera et al., 2010). This study revealed that VtaA1, 5, 6, 8, 9 and 10 are antigenic and expressed *in vivo*, but poorly expressed in *in vitro* growth conditions. The mixture of these six immunogenic passenger domains of VtaA1, 5, 6, 8, 9 and 10 were found to partially protect against a lethal challenge with the Nagasaki virulent strain (Olvera et al., 2011).

Costa-Hurtado et al. (2012a) demonstrated that VtaA8 and VtaA9 play a role in phagocytosis resistance of *H. parasuis*. The authors observed that these two proteins share epitopes, which are also present on the surface of the heterologous virulent strains and therefore they can be considered as vaccine candidates against Glässer´s disease (Costa-Hurtado et al., 2012a).

1.4. ENZYMES

Enzymes are protein molecules that have a three dimensional globular conformation. Their function is to facilitate chemical reactions, particularly within cells of organisms. Enzymes are therefore referred to as biological catalysts. These reactions take place in the distinct groove or "active site" of the enzyme and involve the binding of the reacting chemical or "substrate", and the breaking or making of chemical bonds. The outcome is a specific change to the substrate, which creates a new chemical molecule or "product". The enzyme molecule itself is unchanged by the event. Enzymes are classified into six groups according to the type of chemical reaction they catalyse: (1) transferases, (2) isomerases, (3) ligases, (4) oxidases, (5) lyases and (6) hydrolases (Bull et al., 2002).

Transferases catalyse the transfer of a small portion (or molecular group) of one substrate onto another, whereas isomerases will convert a molecule into its alternative structural form, called an isomer. Ligases promote bond formation between two substrates, creating one large molecule. Similarly, oxidases will combine oxygen with the substrate molecule, but they can also remove electrons. Lyases split the molecules and finally hydrolases act like a lyases but the water is always part of the process (Bull et al., 2002).

1.4.1. Phosphatases.

These enzymes are ubiquitous in nature, occurring in many animal tissues, plants and microorganisms. In humans, as well as other eukaryotes, phosphatases play critical roles in numerous processes and pathways, including signal transduction, cell cycle control, cell transformation, glycogen metabolism, muscle contraction, protein synthesis, T-cell and B-cell activation, insulin activation, transformation of cancer and cell adhesion (Vincent, 1995). There is a dependence on dephosphorylation events of important molecules within higher level organisms and it is not surprising that pathogenic bacteria have evolved phosphatases and kinases that alter host phosphorylation levels, thereby disrupting host signaling pathways and facilitating virulence. Because of their central importance in eukaryotic and prokaryotic biology, much attention has been

given to understanding the structures, functions, and catalytic mechanisms of phosphatases (Felts, 2007).

Phosphatases are phosphomonoesterases and belong to the transferase enzymes. It is an important and diverse group of enzymes that catalyze the transfer of phosphoryl from a wide variety of phosphate esters to water forming inorganic phosphate and one alcohol. They are classified as acid or alkaline phosphatases depending on their optimal pH (Figure 1).

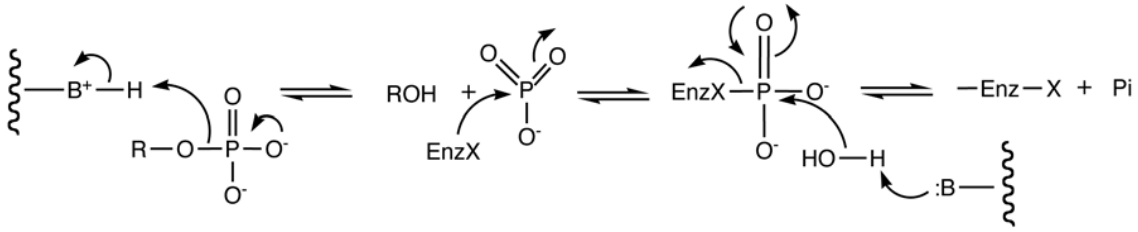


Figure 1. General mechanism for phosphomonoester hydrolysis. In this mechanism a general acid donates a proton forming the alcohol and the meta phosphate intermediate. Nucleophilic attack at the P atom then takes place forming the phosphoenzyme intermediate. The general base abstracts a proton from water forming the hydroxyl nucleophile that attacks the P atom forming inorganic phosphate and retuning the enzyme to its native form. Figure taken from Richard L. Felts, 2007. Structural studies of acid phosphatases from pathogenic bacteria, PhD Thesis, University of Missouri-Columbia, with permission.

Although the enzymatic hydrolysis of phosphate monoesters looks to be a well-defined and a simple chemical reaction, phosphatases are, in fact, a very diverse group of enzymes. The classification scheme of Taylor and Widlansk (Taylor and Widlanski, 1995) for phosphatases is based on substrate preference and leads to 3 general categories: (1)The non-specific phosphatases, (2) the phosphoprotein-specific phosphatases and, (3) the small molecule specific phosphatases (Figure 2).

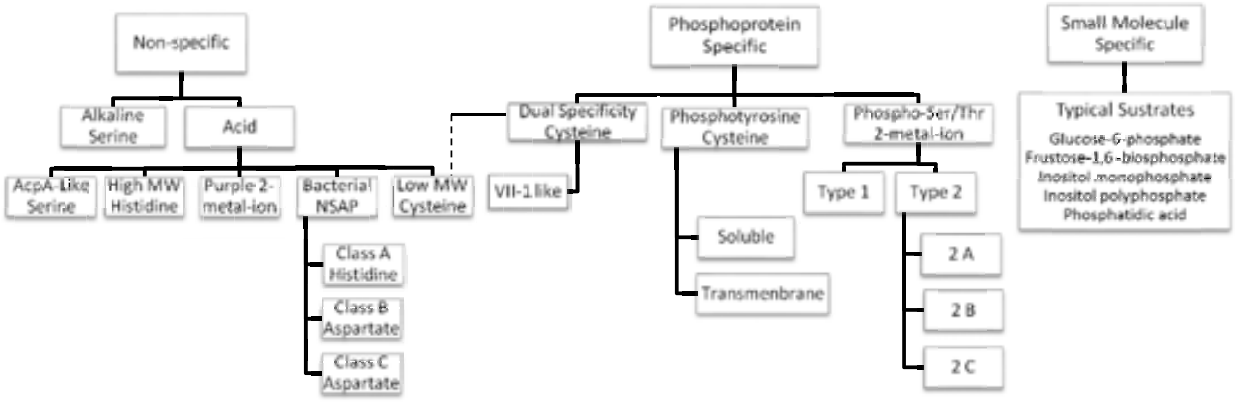


Figure 2. Classification of phosphatases. The initial classification is based on substrate specificity. The secondary classification is based on mechanism. Low molecular weight phosphatases have been found to have properties of both specific and non-specific phosphatises. Figure taken from Richard L. Felts, 2007. Structural studies of acid phosphatases from pathogenic bacteria, PhD Thesis, University of Missouri-Columbia, with permission.

The non-specific phosphatases have the ability to catalyze a variety of phosphorylated substrates. These phosphatases are further divided based on the optimal pH for catalysis and are classified as alkaline or acid phosphatases (ACPs). The phosphoprotein-specific phosphatases cleave phosphorylated proteins or peptides and include dual specificity phosphatases, phosphotyrosine phosphatases, phosphoserine phosphatases, and phospho-Ser/Thr phosphatases. The third category of phosphatases is the small molecule specific phosphatases, that hydrolyze substrates such as glucose-6-phosphate, inosotol monophosphate, and fructose 1,6-bisphosphate.

1.4.1.1. Acid phosphatases.

The acid phosphatases (APs) are a family of enzymes that are grouped together because of the shared ability to catalyze the hydrolysis of orthophosphate monoesters under acidic conditions (Bull et al., 2002). However, although they have a common functionality, different types of APs differ widely regarding

molecular weight, amino acid homology, sequence length, and resistance to L(+) tartrate and to fluoride. Figure 2 shows the classification of acid phosphatases into five categories: high molecular weight ACPs, low molecular weight ACPs, purple ACPs, bacterial non-specific acid phosphatases (NSAP), and AcpA-like ACPs. These enzymes play critical roles in numerous processes and pathways, including virulence (Felts, 2007).

ACPs with high molecular weight have been isolated and characterized from a variety of plant, bacterial, and mammalian sources. These phosphatases have subunits molecular masses of 40-60 kDA and broad substrate specificity (Ostanin et al., 1992; Saini et al., 1981). The signature sequence for this class of phosphatases is R**H**G*X*R*X*P, which is generally located near the N-terminus of the protein. The nucleophile in this ACP class is the histidine of the signature sequence (active site) and therefore this group of ACPs has been redefined as "histidine phosphatases" (Van Etten, 1982). The human prostatic ACP and lysosomal acid phosphatases with nucleophilic histidine at their active sites are the most studied phosphatases of this class (Kilsheimer and Axelrod, 1957; Porvari et al., 1994; Van Etten, 1982; Van Etten et al., 1991; Van Etten and McTigue, 1977). This highly conserved motif has been reported also in *Escherichia coli* (Ostanin et al., 1992).

Members of the low molecular weight (M_r) ACP class are known to be cytoplasmic phosphotyrosine phosphatases; also known as low M_r PTPases and are characterized by a molecular weight around 20 kDa. These enzymes are present in a high variety of organisms, form prokaryotes to yeast and mammals. The active site sequence motif CXXXXXRS/T, with the cysteine residue as the nucleophile, contains the Cys and Arg residues involved in enzyme catalysis (active site signature) found in all PTPases. The distribution of these enzymes in philogenetically distant unicellular and multicellular organisms supports their participation in important and conserved cell functions. The low *M*r PTPases are not inhibited by the classical inhibitors of acid phosphatases (tartrate) or phosphoserine/threonine protein phosphatases (EDTA, fluoride, okadaic acid); instead, they are specifically inhibited by micromolar Zn^{2+} and orthovanadate and show maximal activity in the presence of EDTA (Brautigan and Shriner, 1988; Cohen, 1989; Gordon, 1991). Low *M*r PTPases have been found also in prokaryotes.

The first microbial gene coding for a Tyr-specific low *M*r PTPase was identified in 1993 in the marine cyanobacterium *Synechococcus sp* WH8020 (Wilbanks and Glazer, 1993).

Purple ACPs contain a dinuclear metal center, either Fe(III)-Fe(II) as in the active form of mammalian enzymes, or Fe(III)-Zn(II) as found in plant enzymes. The distinctive purple color of these enzymes result from the tyrosinate to Fe(III) charge transfer transition.

The bacterial non-specific acid phosphohydrolases (NSAP) are secreted enzymes that are produced as soluble periplasmic proteins or as outer membrane-bound lipoproteins. Rossolini *et al* have categorized NSAPs in classes A, B, and C based on short conserved sequence motifs. These phosphatases are able to dephosphorylate a broad variety of structurally unrelated organic phosphoesters (nucleotides, sugar phosphates, phytic acid etc.) to acquire inorganic phosphate (Pi) and organic byproducts. They show optimal catalytic activity at acidic to neutral pH.

The class A acid phosphatases are oligomeric enzymes with four identical monomers of aproximately 25-27 kDa. Their short signature sequence is GSYPSG**H**, including the histidine nucleophile. Class A NSAPs require a metal co-factor for activity and are able to hydrolyze several phosphomonoesteres, as well as of catalyze phosphate transfer to hydroxyl groups of organic compounds. They are not inhibited by inorganic phosphate, fluoride and tartrate, but they are inhibited by EDTA, nucleosides, and Ca (II) (Thaller et al., 1997). Class A NSAP from *Escherichia blattae* is used as the model for this class (Ishikawa et al., 2000).

The class B acid phosphatases are a group of homotetrameric secreted phosphohydrolases consisting of subunits of approximately 25 kDa. These phosphatases include a large number of bacterial, archaeal and eukaryotic enzymes (Thaller et al., 1995). They are all characterized by conserved sequence motifs including four conserved aspartate residues, which are characteristic of the

molecular superfamily of phosphohydrolases indicated as "DDDD" (Leone et al., 2008; Thaller et al., 1998). The class B NSAP signature sequence is divided into two components, an N-terminal motif of F**D**IDDTVLFSSP and a C-terminal motif of YGD-[A/S]-DXD-[I/V] (Rossolini et al., 1998; Thaller et al., 1998). The aspartate nucleophile in this group is located in the N-terminal region of the signature sequence (shown in bold). These phosphatases are members of the haloacid dehalogenase (HAD) superfamily. AphA from *Escherichia coli* is the best characterized enzyme (27-kDa), of the molecular class B family NSAPs and is used as representative of this class of phosphatases (Calderone et al., 2004; Rossolini et al., 1994; Calderone et al., 2004; Passariello et al., 2003). According to Passariello et al. (2006), AphA can be considered a broad-spectrum nucleotidase highly active against 3'- and 5'- mononucleotides and monodeoxynucleotides, suggesting a role in scavenging nucleotides that enter the periplasmic space, to release nucleosides and inorganic phosphate (Passariello et al., 2006).

Class B bacterial acid phosphatases are found in some major pathogens (e.g. *Salmonella enterica, Schigella flexneri, Escherichia coli, Klebsiella pneumoniae, Actinobacillus actinomycetemcomitans, Haemophilus influenzae, Streptococcus pyogenes* and *Streptococcus agalactiae*) but are not ubiquitous among bacteria (Calderone et al., 2004).

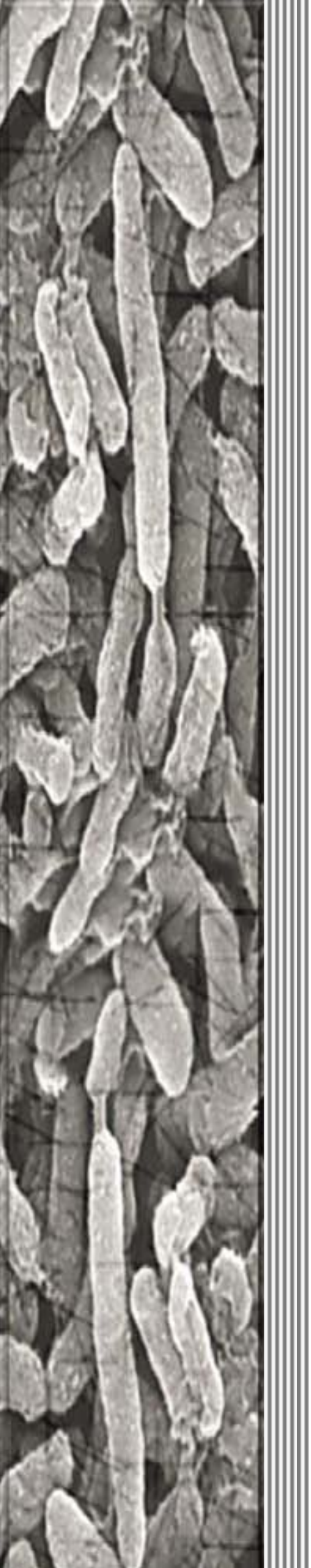
The class C acid phosphatases are membrane anchored lipoproteins with an average mass of 28 kDa and are also resistant to inorganic phosphate and tartrate. These enzymes are distinguished by the bipartite sequence motif [IV]-[VAL]-D-[IL]- DET-[VM]-LX-[NT]-X(2)-Y in the N-terminus and [IV]-[LM]-X(2)-GD-[NT]- LXDF in the C-terminus (Thaller et al., 1998). Class C NSAPs are similar to class B NSAPs in that they have an aspartate nucleophile located in the N-terminal signature sequence motif and are members of the DDDD and HAD superfamilies. Genes encoding class C NSAPs have been identified in *Chryseobacterium meningosepticum* (OlpA) (Passariello et al., 2003), *Streptococcus equisimilis* (LppC) (Malke, 1998), *H. influenzae* (Reilly et al., 1999; Reilly and Smith, 1999), *Clostridium perfringens* (Reilly et al., 2009; Wang et al., 2010), *Helicobacter pylori* (HP1285) (Godlewska et al., 2002; Reilly and Calcutt, 2004), *Elizabethkingia*

meningoseptica (Passariello et al., 2003), *Staphylococcus aureus* (du Plessis et al., 2002), *Pasteurella multocida* (Singh et al., 2009; Singh et al., 2011a), *Bacillus anthracis* (Felts et al., 2006a) and *Mycoplasma bovis* (Singh et al., 2011b).

There are some ACPs that do not fit into any of the previously described phosphatase categories. AcpA from *F. tularensis* is one such example. AcpA is structurally similar to human alkaline phosphatase (AlkP), has a serine nucleophile, and is a mononuclear metallo-enzyme. Thus, AcpA is the prototype member of the AcpA-like ACPs (Felts et al., 2006b). It has been proposed that this phosphatase is involved in intracellular survival (Reilly et al., 1996). Nevertheless, recent studies by Child et al. (Child et al., 2010) demonstrated that most of the acid-phosphatases-encoding genes of AcpA, AcpB and AcpC, are disrupted in virulent strains and that the most conserved loci are not required for phagosomal escape and therefore they do not play a main role in the virulence of type A *Francisella* strains. On the other hand, Dai et al. (2012), demonstrate that AcpA is secreted *in vitro* and *in vivo* by *F. novicida* and the highly virulent and pathogenic strain of *F. tularensis* subsp. *tularensis* (Dai et al., 2012).

However there are some cases of bacteria and parasites with acid phosphatase activity whose classification is unknown and does not fit within the types of phosphatases reported and outlined above. Such is the case of *Bordetella bronchiseptica* (Jungnitz et al., 1998), *Leishmania donovani* (Remaley et al., 1985; Katakura and Kobayashi, 1988), *Legionella micdadei* (Saha et al., 1985) and *Legionella pneumophila* (Aragon et al., 2001; Aragon et al., 2000; Aragon et al., 2002).

Previous studies by our group had detected an acid phosphatase activity in supernatants of *H. parasuis* (Serrano, 2008; Serrano and Aragon, 2009), but the proteins producing this activity and the role in the physiology and virulence of the bacterium are unknown and have been the focus of this thesis.



2. HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES.

Control of the disease caused by *H. parasuis* is performed mainly by antibiotics or commercial bacterins. However, commercial bacterins have a limited use due to the deficient efficacy against serovars different than those included in the vaccine. The need for an effective vaccine is clear, especially when the reduction in the use of antibiotics in animal production is also a goal. To improve current vaccines in a rational way, it is essential to understand the pathogenesis of the disease caused by *H. parasuis*.

As explained in Introduction, *H. parasuis* includes virulent and non-virulent strains; the latter being part of the microbiota of the pigs. Previous results showed the presence of a secreted acid phosphatase in the culture supernatant of some strains of *H. parasuis*. Bacterial acid phosphatases have been shown to play important roles as mediators of infection and therefore these enzymatic activities could be linked with survival and the development of disease in the host. Based on these evidences, we formulated the following **hypothesis**: *H. parasuis* is capable of expressing an acid phosphatase activity whose role in infection is relevant for host colonization and/or pathogenesis of disease.

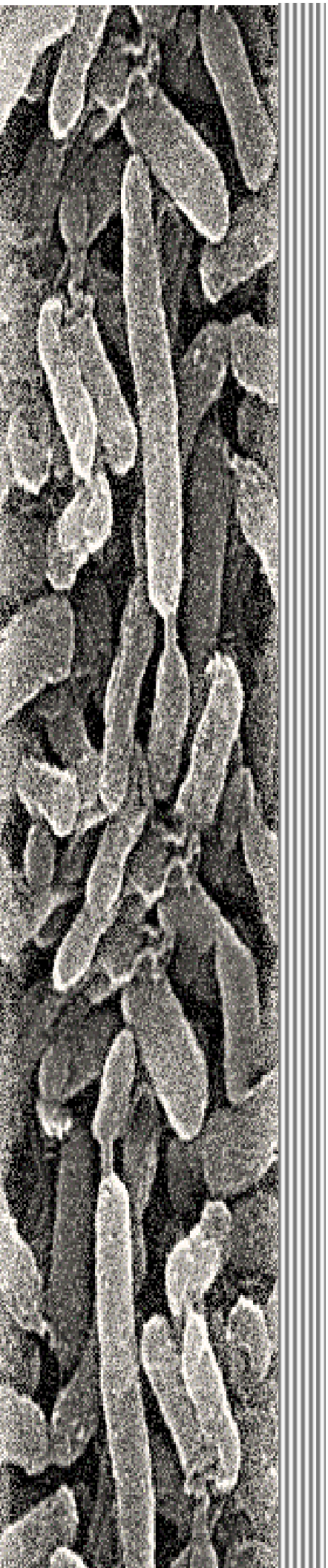
This proposed hypothesis was approached through the following objectives in this thesis:

General objective:

- Study the role of the acid phosphatase activity in the pathogenesis of the disease by *H. parasuis*.

Specific objectives:

- Identify the gene responsible for the secreted acid phosphatase activity of *H. parasuis*.
- Identify and express the acid phosphatase to produce monoclonal antibodies to utilize in the development of new tools for *H. parasuis* research.



3. MATERIALS AND METHODS

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3.1. Bacteria and culture conditions

Bacterial strains and plasmids used in this study and their relevant characteristics are described in Annex 2 and Annex 3.

H. parasuis strains were grown on chocolate agar plates (Biomerieux), In pleuropneumonia-like organism (PPLO) broth (DIFCO) supplemented with isovitalex (BD) (*supplemented,* sPPLO) or brain hearth infusion (BHI) broth (OXOID) supplemented with isovitalex (*supplemented,* sBHI). In all cases, bacteria were grown at 37ºC. Liquid cultures were performed in an orbital shaker at 200 rpm. Agar plates were incubated in an atmosphere of 5% CO_2. All *H. parasuis* strains were stored frozen in BHI with 20% glycerol at -80ºC.

Growth curves were performed to analyze culture supernatants at different growth phases. Strains of *H. parasuis* were grown in broth at 37ºC and with continuous agitation (220 rpm) until cultures reached the stationary phase. Bacterial growth was assessed by measuring the absorbance (Abs) hourly at 660 nm, until the culture reached the stationary growth phase. The absorbance was measured in a spectrophotometer VIS7200 (DINKO).

Escherichia coli BL21 was used as a host for recombinant plasmids, and was grown in Luria-Bertani (LB) medium at 37ºC. LB was supplemented with ampicillin (100 µg/ml), chloramphenicol (30 µg/ml) or spectinomycin (10 µg/ml), as required.

3.1.1. Culture supernatants

H. parasuis was cultured in liquid medium, as described above, for measuring acid phosphatase activity in the supernatant. Each strain to be evaluated was streaked separately on chocolate agar plates and allowed to grow for approximately 20 hours at 37ºC in an atmosphere of 5% CO2. After overnight incubation, liquid cultures in sBHI or sPPLO were prepared. Preliminary experiments with several strains were performed with 4 ml cultures in 10 ml tubes. Later, for further analysis of the activity, 50 ml cultures in 500 ml flasks were used. Liquid cultures

were inoculated to achieve an Abs_{660} of 0.2-0.3. Bacteria were grown at 37ºC and shaking (220 rpm). Abs_{660} was measured hourly, covering a period of about 10 hours, until the culture reached the stationary growth phase. To obtain the supernatant, cultures were centrifuged for 20 min at 2000 x *g* and 4ºC to pellet the bacteria. The supernatant was then transferred to a different tube and filtered through a 0.2 micron filter to ensure completely absence of bacteria. Supernatant samples were directly analyzed or stored at -80ºC with 50% glycerol until analysis.

For some experiments culture supernatants were concentrated by ultrafiltration**.** Bacteria were eliminated from the cultures by centrifugation (2000 x *g*, for 20 min) and filtered as described before. The cell-free supernatant was concentrated by ultrafiltration (UF) using Amicon Ultra-15 centrifugal filter with a filter of 10 KDa of nominal molecular weight cutoff (Millipore Corp., Bedford, M.A, USA). The filters were centrifuged at 4,000 x *g* for 30 min at 4ºC. All the process was carried out on ice to maintain the activity of the enzyme. Concentrated supernatants were directly used or stored at -80ºC with 50% of glycerol until analysis.

For detailed analysis, we mainly used strain *H. parasuis* ER-6P (ER-6P), which is a virulent strain isolated from the pericardium of an animal with Glässer's disease. This strain was subjected to several passes on agar plates and the growth obtained after 6, 10 and 14 passes was kept and named ER-6Pp6, p10 and p14, respectively. A second strain used in our analysis was strain CT175-L, isolated from the lung of an animal from a farm with pneumonia and Glässer's disease.

3.1.2. Phosphatase activity assay.

Phosphatase activity was measured using fluorescence (Omene et al., 1981; Robinson and Willcox, 1969).

Acid phosphatase activity was measured in 0.1 M sodium acetate buffer (pH 5.2) or in 0.1 M sodium tribasic citrate buffer (pH 5.2). As control, alkaline phosphate activity was measured in 0.05 M Tris buffer (pH 8.0). The fluorescent substrate 4-metilumbeliferyl phosphate (MUP) at a final concentration of 0.6 mg/ml was used to measure the reaction. This substrate produces fluorescence when the phosphate

is released by a phosphatase. The phosphatase cleaves the phosphate from the fluorogenic substrate (MUP) forming 4-methylumbelliferone, which is fluorescent. The reaction was measured in a fluorimeter (ASCENT Fluoroskan & FL; Thermo labsystems). The fluorimeter was programmed for incubation at 37ºC and fluorescence measurements at different time points (commonly, at 0, 10 min, 30 min, 1h and 2h). Fluorescence was measured with an excitation of 355 and an emission of 460.

For a standard phosphatase assay, 96-well microtitre plates were used with 50 µl of sample and 50 µl of 2X reaction buffer with MUP. When the phosphatase activity was measured at different pHs, 0.1 M sodium tribasic citrate buffer was used for pH 4, 5.2 and 6, and 0.05 M Tris buffer was used for pH 7, 8 and 9. Concentration of MUP was the same as above. The effect of ethylenediaminetetraacetic acid (EDTA) on the phosphatase activity was assessed by addition of 20 mM EDTA to the reaction. An acid phosphatase from potato (Sigma-Aldrich) was used in the assays as positive control.

3.1.3. Effect of inhibitors on enzyme activity.

The following compounds were tested for their inhibitory effect in the standard assay at the concentrations indicated and at a pH scale of 4.0, 5.2, 6.0, 7.0, 8.0 and 9.0. The effect of ethylenediaminetetraacetic acid (EDTA) on the phosphatase activity was assessed by addition of 20 mM EDTA to the reaction. The effect of tartrate and molybdate on phosphatase activity was assayed by adding sodium tartrate or sodium molybdate to the reaction at 1, 10, 100, 1000 and 10000 µM. An acid phosphatase from potato (Sigma-Aldrich) was used in the assays as positive control.

3.2. Generation and screening of a genomic library of 4-5 kb of size from the genome of the strain ER-6P of *H. parasuis*.

DNA from strain ER-6P was extracted following the protocol of the NucleoSpin blood kit (Macherey-Nagel). DNA was concentrated in a concentrator 5301(Eppendorf). Bacterial DNA was partially digested with Sau3AI (Biolabs). The reaction was incubated for 2 minutes at 37ºC and then the reaction was stopped with 40mM EDTA. The reaction was analyzed by electrophoresis in 1% agarose.DNA fragments between 4 and 5 Kb were selected and excised from the gel and were purified with the Wizard SV gel and PCR clean-up system (Promega).

In parallel, the plasmid pACYC184 was digested with BamHI overnight and dephosphorylated with shrimp alkaline phosphatase (New England Biolabs) at 37ºC for 15 min. The digested plasmid was ligated with the 4-5 Kb genomic fragments from strain ER-6P with T4 ligase (Promega) at 16ºC overnight. Then, we proceeded to the electrotransformation of the product of ligation using an electrocompetent strain (*Escherichia coli* BL21). As controls, a pACYC184 previously digested with BamHI and dephosphorylated with an alkaline phosphatase (Biolabs) was used. This control was electroporated directly or after the ligase reaction, to ensure that all vector was digested and that the dephosphorylation was equally efficient. The number of colonies selected, and required for the small library (4-5 Kb), was calculated taking into account the length of the original fragment and the number of fragments generated after complete digestion with *Sau3AI*, using the equation:

$$N = \ln(1-P) / \ln(1-f)$$

f: proportion of the genome contained in a single clone = size of insert/size of genome.

P: desired probabilility, expressed as a fraction.

Each colony was cultured in a 96-well microtitre plate with 100 ul of LB broth supplemented with chloramphenicol (30 µg/ml) in each well, incubated overnight (O/N) at 37ºC and freeze with 50% glycerol at -80ºC. The genomic library consisted of 2880 clones, which was sufficient to ensure the representation of the *H. parasuis* genome.

In order to screen for acid phosphatase activity, the genomic library was cultured in deep 96-well plate with 200 µl of medium. After incubation at 37ºC, the pellet and the supernatant from each well was analyzed for phosphatase activity. Subsequently, plasmid DNA from the positive clones was purified and the insert was sequenced with primers pACYCseq-F (5' ACTTGGAGCCACTATCGACTAC 3') and pACYCseq-R (5' CGGTGATGTCGGCGATATAGG 3').

3.3. PCR, cloning and protein expression

Molecular techniques used in this study were performed following standard protocols (Sambrook and Russell, 2001) or commercial kits. Plasmid DNA was purified using the Wizard Plus SV minipreps DNA purification system (Promega). Genomic DNA from *H. parasuis* was purified with the InstaGene matrix (Bio Rad). PCR reactions were performed using the enzyme GoTaq ® Flexi DNA polymerase (Promega). Amplicon purification was performed with Wizard SV Gel and PCR clean-up system (Promega).

All primers used in this work were designed from the sequenced genome of *H. parasuis* Nagasaki strain (Pina et al., 2009) or strain SH0165 (GenBank accession number CP001321 (Yue et al., 2009).

E. coli BL21 was used as a host for recombinant plasmids and the transformation was performed by electroporation. For preparation of electrocompetent cells, *E. coli* BL21 was grown to exponential phase and washed with 10% ice-cold glycerol, following a standard protocol (Sambrook and Russell, 2001). Electrocompetent bacteria were kept in aliquots of 50 µl at -80ºC until used. The electroporation was done using the Gene Pulser electroporator with the Xcell II PC Module (Bio-Rad), 1

mm cuvette and the following conditions: 2.5 KV, 25 µF and 200 Ω. After the pulse, bacteria were quickly resuspended in 1 ml of SOC medium and incubated at 37ºC for 1 hour. Finally, the bacteria were plated on LB agar plates containing the appropriate antibiotics for selection of the transformants, and incubated at 37ºC for 18-24 h.

3.3.1. Cloning of an *aphA* homolog from *H. parasuis*

Screening of the 4-5 kb genomic library and BLAST searches of the genome of *H. parasuis* SHO165 (accesion number YP002476402.1) detected an *aphA* homolog in *H. parasuis*. The protein sequence databases in GenBank revealed a high level of similarity of *H. parasuis* AphA with prokaryotic acid phosphatases.

AphA belongs to the typeB phosphatase family, previously described as the "class B non-specific bacterial acid phosphatase" family. The *apha* gene with its putative promoter region was *a*mplified by PCR from *H. parasuis* strains Nagasaki, IT29205, CT175-L, IQ1N-6, F9, SW114, ND14-1, FL3-1 and ER-6P, using primers APha-Fw-prom (5'- ATGTTCCCTATAACCTATTGTG-3') and Apha-Rev (5'-ATTAGTAGCTTGAATTTATAATAAC-3') (Annex 4). The PCR fragment (900 bp) was purified from an agarose gel and cloned into pGemT-easy and electroporated into *E. coli* BL21. Transformants were selected on LB supplemented with 100 µg/ml ampicillin and acid phosphatase activity was evaluated in the clones. Activity was examined in complete cultures, in bacterial pellets and in supernatants from the clones. Inserts in the clones were sequenced and the orientation of the gene in each clone was checked.

3.3.2. Cloning of a *pgpB* homolog from *H. parasuis*

The *H. parasuis* phosphatidylglycerophosphatase B (*pgp*) gene was identified in the screening of the genomic library of 4-5 kb as a possible candidate for having acid phosphatase activity. To confirm if the phosphatase activity was due to this gene,

the *pgp* gene, together with the putative promoter sequence, was amplified by PCR, using as template DNA from strains Nagasaki and ER-6P with primers pgp-F and pgp-R (pgpF: 5' TATTCATTATATTACAGAAACATTC 3'; pgpR: 5' CCAAACCGTAATAAACCATAG 3'; (Annex 4).

The PCR fragment (838 bp) was purified from an agarose gel, cloned into vector PCRII Topo and electroporated into *E. coli* BL21. Transformants were selected with ampicillin and the acid phosphatase activity was evaluated in the bacterial pellet and the supernatant of cultures after overnight incubation.

3.3.3. Cloning of gene HPS_05483 from *H. parasuis*

Gene HPS-05483 was identified in the analysis of the supernatant of *H. parasuis* by size chromatography and Mass Spectrometry (MS) (see below). Gene HPS-05483 was amplified by PCR using specific primers (Annex 4). Virulent Nagasaki and ER6P strains and non-virulent SW114 strains were evaluated. The PCR fragment containing the open reading frame and the putative promoter region (639 bp) was purified from an agarose gel and cloned into pGem-T Easy and electroporated into *E. coli* BL21. In addition, primers HPS05483-Fw (5' TTAACTATTCGGAGTATTCTATG 3') and HPS05483-Rev-non-stop (5' TTTCTTGGTAATCTGTTTTACATC 3') were used for cloning in the Gateway system for gene expression and protein purification.

3.4. SDS-PAGE, semi-native gel electrophoresis and zymogram.

Standard SDS-PAGE was carried out according to the method described by (Laemmli, 1970), in slab gels consisting of a 12% acrylamide separating gel and a 4% stacking gel. Seminative-PAGE was performed using basically the same protocol as SDS-PAGE but not including SDS in the gels and using a running buffer with 0.17% SDS (1/3 of the percentage used for SDS-PAGE) . Also, loading buffer did not contain β-mercaptoethanol and the samples were no boiled for seminative-PAGE.

Standard SDS-PAGE was carried out at room temperature, but the seminative-PAGE was run in an iced water bath, to prevent loss of phosphatase activity.

Running conditions for semi-native PAGE were 100 Volts for 3 hours. An acid phosphatase from potato (Sigma-Aldrich) was used in the assays as positive control.

In respect to the zymogram, the acid phosphatase activity was detected in the semi-native gels using the same fluorogenic substrate (MUP) used before in the assays of activity in the 96-well microtitre plates (Kameshita et al., 2010). The electrophoresed seminative gel was washed three times for 15 min with 20 ml of acetate buffer (pH 5.2). Then the gel was directly soaked in 20 ml of acetate buffer with MUP (0.6 mg/ml) and incubated at 37ºC for up to 1 hour. The fluorescent bands were observed on a UV transilluminator (Chemi Genius-Bio imagine system, Syngene).

3.5. Analysis of *H. parasuis* culture supernatant by Matrix-assisted laser desorption/ionization (MALDI)- Time-Of-Flight (TOF)

Chromatography and MALDI-TOF analyses were carried out in the Proteomics facility from UAB, a member of ProteoRed-ISCIII network.

3.5.1. Gel-filtration chromatography

A size-exclusion chromatography was carried out with a superdex_75, 26/60 High Load column (GE Healthcare) in an AKTA Purifier (GE healthcare). Supernatant of *H. parasuis* ER-6P, previously concentrated as described before, was used in the analysis. About 2.5 ml of sample were injected through a loop of 5 ml. The chromatography was performed in 50 mM trisodium citrate buffer (pH 6.0) at a flow of 2.5 ml/min. Prior to use, the column was washed with 1 column volume (CV) of H_2O and allowed to equilibrate with 1.5 CV of running buffer.

From each chromatography, we collected an average of 72 fractions of 5ml. Each fraction was analyzed by fluorimetry to measure the acid phosphatase activity on MUP, as described previously. Samples with high enzyme activity were further analyzed by polyacrylamide gel electrophoresis (PAGE) with SDS (SDS-PAGE).

Negative fractions (no activity), eluted before and after the phosphatase activity were used as controls. The bands observed in the positive fractions (with high enzymatic activity) were excised with a scalpel, diced into 1-mm 3 pieces, introduced into an eppendorf tube and analyze by MALDI- TOF-TOF.

3.5.2. Protein identification by MALDI-TOF

The peptides resulting from the digestion of the selected proteins were deposited on a MALDI plate with a matrix (α-cyano-4-hydroxycinnamic acid).

Tryptic digestion: Protein bands of SDS-PAGE, corresponding to an approximate weight of ~15 kDa were excised with a scalpel, diced into 1-mm^3 pieces and introduced into the wells, treated with a destaining solution buffer of 50 mM ammonium bicarbonate (NH_4 HCO_3)/Acetonitrile (ACN) 50%, followed by a reduction of the proteins with 20 mM DTT in buffer 25 mM $NH4HCO_3$ for 20 minutes at 60ºC. Then the Cys sulphydryls were alkylated with 25 mM iodoacetamide in 25 mM $NH4HCO_3$ for 15 min at 37ºC. The washing/destaining solution buffer was used for additional washing and finally the gel pieces were dehydrated with pure ACN, and dried. The digestion of the sample was carried with trypsin: 25 ng/sample (sequencing grade, Promega) for 3h at 37ºC. Finally, the peptides were eluted with H_2O:ACN (1:1)+ 0.2% TFA.

Mass spectrometry (MS): MALDI-TOF MS was used for protein identification by peptide mass fingerprinting (PMF). The preparation of the samples to be analysed by MALDI-TOF, was performed as follows. The plate was from ground steel, the matrix was made with α-cyano-4-hydroxycinnamic acid (HCCA). The sample was mixed in a proportion of 1:1 with the matrix (0.5 μL: 0.5 μL) and 1 μL of this mixture was deposited on a ground steel plate. Then the sample was analyzed by MALDI-TOF in an UltrafleXtreme MALDI-TOF machine (Bruker) using a reflectron mode method and an accelerating voltage of 25kV. The analyzes were calibrated using external calibrators (Peptide Calibration Standard, Bruker Daltonik).

The mass spectrum (MS) or "peptide fingerprint" obtained from the samples were acquired and compared. Comprehensive peak assignments were accomplished

using the FlexAnalysis and BioTools software packages (Bruker Daltonics). Protein identification was performed automatically using GPS (Global Protein Server) coupled to TOF-TOF data, combining both types of spectra (MS-MS/MS) obtained for each sample and using as search tool MASCOT (MatrixScience, UK) on public databases of protein sequences.

3.6. Production of monoclonal antibodies

Four 4-week-old BALB/c mice (Harlan Interfauna) were used for immunization. All the procedures involving animals were performed in accordance with the regulations required by the Ethics Commission in Animal Experimentation of the Generalitat de Catalunya (Approved Protocol Number 5767). Animals were housed in specific-pathogen free conditions and were used in accordance with the animal care and use guidelines of the animal house, an accredited facility at the UAB (Universidad Autonoma de Barcelona). Concentrated supernatant obtained at the beginning of the stationary phase of strain CT175-L grown in sBHI (with high phosphatase activity) was used for immunization of mice.

The animals were immunized intraperitoneally with 400 ng of the concentrated supernatant and Complete Freund Adyuvant (Difco), followed by a second immunization 19 days later with the same dose of protein with Incomplete Freund´s Adjuvant (Difco). Finally, 15 days later, a third intraperitoneal boost, was perform with 400 ng of the concentrated supernatant in 400 µl of saline solution. Antibody response was measured by ELISA using a concentrated supernatant of strain CT175-L produced with sPPLO, in order to minimize the interference of the culture medium in the analysis. A 96-well microtitre plate (High Binding, CULTEK SL) was coated with 50 ng of supernatant produced in PPLO of the protein (50 µl per well), diluted in carbonate bicarbonate buffer (pH 9.6) and incubated at 4ºC overnight. The content of the plate was discarded wringing out the excesses vigorously and 100ul of the blocking solution was added to the plate (PBS 1%-Tween 20/albumin from bovine serum 1%). After 1 hour incubation at 37ºC, the solution was discarded without washing the plate and 100 µl of the sample (hybridoma culture supernatants) were added to the plate. As positive and

negative controls sera from immunized and non-immunized mice were used. The plate was incubated for 2 hour at 37ºC. Then the plate was washed three times with 200µl of washing solution (0.05% of PBS/Tween 0.1 %.); followed by the addition of 50 µl per each well of a 1/5000 diluted secondary anti-mouse antibody conjugated to horseradish peroxidase (Sigma) and incubated for 40 min at 37ºC. Afterwards, wells were washed six times with 200µl of washing solution. TMB color development reagent (50 µl) was added thereafter an incubated for 8 minutes. This step is performed in the dark. Positive wells turn to green and the reaction was stopped with 50 ul of 1N sulfuric acid. The absorbance of the peroxidase reaction product in the enzyme-linked immunosorbent assay (ELISA) was read on an automated microplate reader (Bio-Rad model 450) at 450 nm. All the hybridomas showing at least 30% of the O.D value of the positive control were considered as positive and selected for further characterization.

The mouse with the highest antibody response was selected and injected subcutaneously in either the left hind footpad or in the left hind hock with 400 ng of the protein in saline solution. The boost was carried 1 days before the fusion. Sera collected from non-immunized and immunized mice served as negative and positive controls in the ELISA.

***Cell fusion*:** X63AG8 myeloma cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% heatinactivated bovine fetal serum, 100 U gentamicin ml21 and 2 mM L-glutamine (Gibco). The fusion of spleen cells from the selected mouse with X63AG8 myeloma cells followed a standard method previously described (Miller et al., 1986), by using 50% (w/v) of polyethylene glycol (molecular mass, 3000–3700 Da; Sigma), and incubated at 37ºC in a humid atmosphere of 5% CO2.

The specificity of the positive monoclonal antibodies was determined by ELISA for the selection of the subclones and further characterization by western blotting. Selected MAbs were purified using a protein G-agarose column according to the manufacturer's instructions (GE Healthcare, Barcelona, Spain). The antibodies were dialyzed against phosphate buffer (PB: 10 mM phosphate buffer, pH 7.3), and the antibody concentration was adjusted to 1 mg/ml. Also, the supernatant of the selected and specific hybridoms were tested by western blotting following

standard methods and nitrocellulose membranes (Sambrook and Russell, 2001). Coomassie brilliant blue (Acid violet 17-Aldrich) was used to detect electrophoresed proteins.

3.7. Analysis of alveolar macrophages by flow cytometry

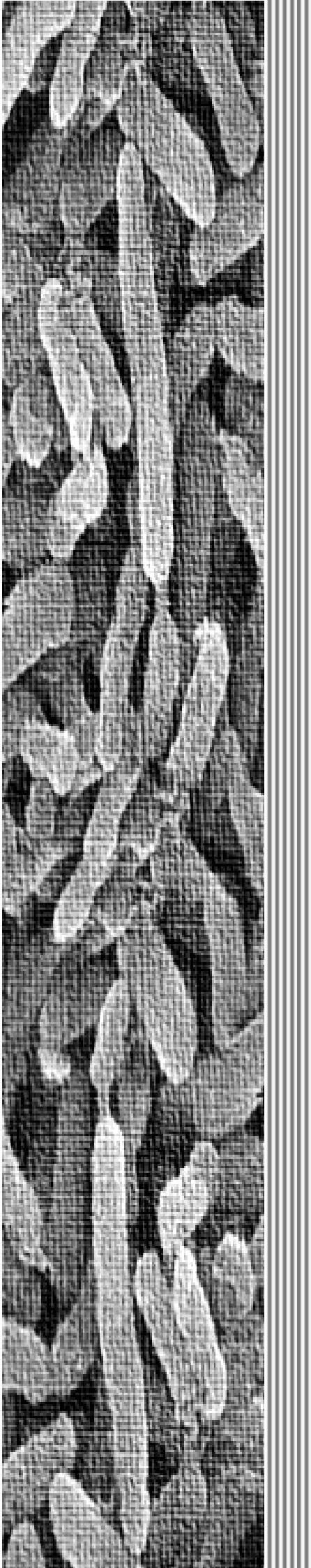
Porcine alveolar macrophages (PAM) were obtained from healthy pigs by bronchoalveolar lavage (BAL) with sterile PBS supplemented with 1 µg/ml gentamicin (Sigma). The cells in the BAL fluid (BALF) were pelleted at 300 x *g* for 10 min and stored at -150ºC at a concentration of $5x10^7$cells/ml in 10% dimethyl sulfoxide (DMSO)-fetal bovine serum (FBS).

PAMs were seeded in 6-well plates at a concentration of 5×10^5 cells in 3 ml per well of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% l-glutamine (CDMEM). After attachment of the cells to the wells, 300 ul of ER-6P supernatant, heat- inactivated supernatant (100ºC, 10 min) or PPLO broth were added to each well and incubated for 2 hours or overnight at 37ºC with 5% CO_2. After the corresponding incubation, plates were transferred onto ice, wells were washed with cold PBS. PAMs were scraped and collected. PAMs were processed for the detection of selected markers with previously characterized specific monoclonal antibodies (MAb): anti-CD163 (clone 2H12/BM), anti-SLA II (clone DQB14H2), anti-SLA I (clone 4B7/8), and anti-sialoadhesin/CD169 (clone 1F1/CR4) and anti -SWC3 (clone BA1CII). An irrelevant MAb was used as a negative control. The incubation with the mAb was performed for 40 min on ice. Then, the reaction of the monoclonal antibodies with PAMs was detected with a FITC-conjugated goat anti-mouse IgG, F(ab')2 (Jackson ImmunoResearch Europe Ltd., United Kingdom), incubated for 40 min on ice. Label intensity in the samples (identified by FL1 x-mean in the population with SS and FS characteristic of macrophages) was determined by flow cytometry in a BD FACSAria I flow cytometer (Becton, Dickinson, Madrid, Spain). Results were compared using Student's *t* test ($P<0.05$).

3.8. Statistical and sequence analysis

The statistical analysis of the data and graphics for this study was performed using the SigmaPlot 10.0 program.

For the sequences analysis, it has been used different bioinformatics tools available online: Basical Local Alignment Search Tool (BLAST), the SignalP 4.1, the ExPASy-ProtParam tool and the BProm tool using SoftBerry for the prediction of bacterial promoters.



4. RESULTS

4. RESULTS

4.1. Acid phosphatase production

As already mentioned in the introduction, acid phosphatase activity has been reported widely in other pathogens (section 2.1). For *Haemophilus parasuis*, acid phosphatase activity was reported for the first time by our group (Serrano and Aragon, 2009). Those studies indicated that some strains of *H. parasuis* were able to secrete into the culture medium an acid phosphatase activity. In order to find maximum levels of secreted acid phosphatase activity, cultures of strains ER-6P (virulent) and SW114 (non-virulent) were performed and samples were taken at different points of growth to measure the amount of acid phosphatase by fluorescence. A faster growth by the non-virulent strain SW114 was observed, which was expected, since non-virulent strains show better growth in the laboratory than virulent strains (Figure 3). However, the acid phosphatase activity was higher in ER-6P as compared to the activity in SW114, which can be considered negative (Figure 4). The acid phosphatase activity showed different level at different stages of growth of ER-6P, being highest at the beginning of the stationary phase.

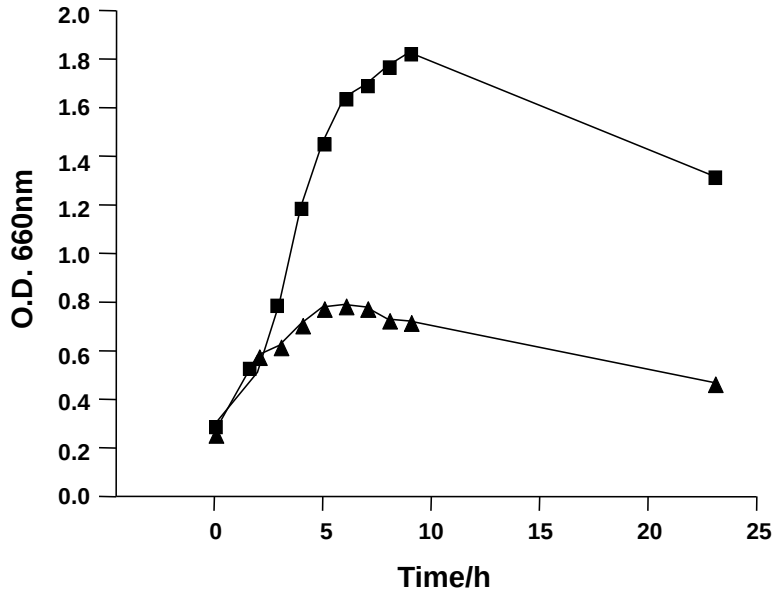


Figure 3. Growth curve of SW114 (■) and ER-6P (▲) strains in BHI broth.

To rule out the dephosphorylation of MUP due to interference by bacterial alkaline phosphatase, samples were subjected to the same reaction but using TRIS buffer (pH=8.0). In these tests, some level of phosphatase activity was detected in the supernatant of ER-6P, but always lower than that observed at pH 5.2 (Figure 4). However, it was observed that a greater activity was detected at pH 8.0 in the culture supernatant of SW114 after 23 hours of growth, indicating that this activity could be due to the bacterial alkaline phosphatase originated from bacterial cell lysis at this late growth phase. This alkaline phosphatase can interfere with the test at pH 5.2, and it is probably responsible for the slight increase of activity in the supernatant of SW114 at this time point (Figure 4).

These experiments were repeated 4 times to confirm that the point of maximum production of acid phosphatase activity was at the beginning of the stationary phase. Together, the data presented in Figure 4, show that the virulent strain ER-6P efficiently secreted an acid phosphatase activity at the beginning of the stationary phase of the bacterial growth. Furthermore, the non-virulent strain SW114 did not produce acid phosphatase, and the level of activity observed in the culture supernatant at 23h is likely due to alkaline phosphatase from bacterial lysis, because this enzyme is not secreted and is associated with the bacteria.

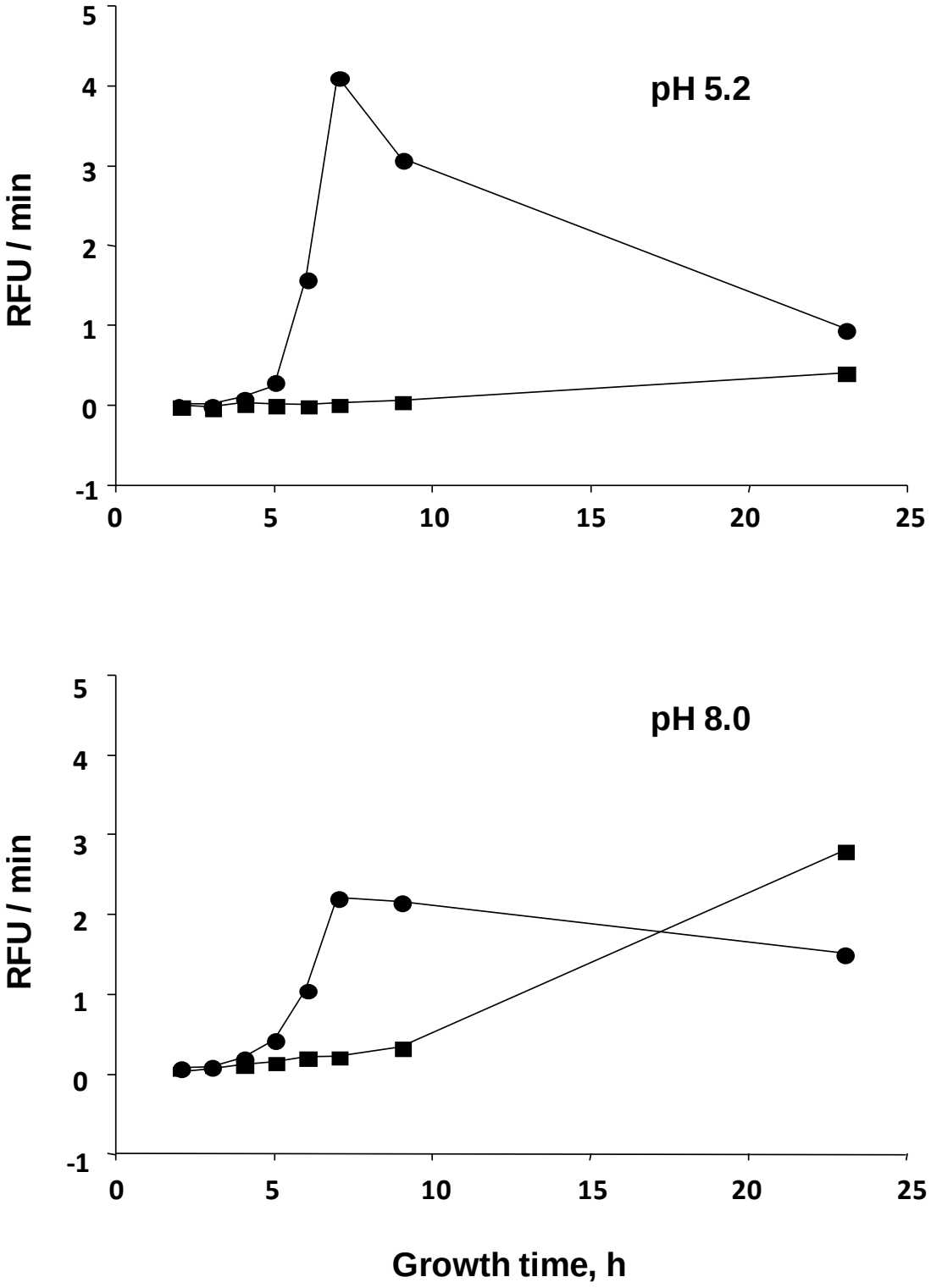


Figure 4. Phosphatase activity in the supernatant of the strain SW114 (■) and ER-6P (●) at pH 5.2 and pH 8.0 during the growth curve, with MUP as substrate. Activity is expressed as relative fluorescence units (RFU).

Our next goal was to make a trial of phosphatase activity at different pHs to establish the optimal pH of the enzyme. Figure 5 shows the results obtained with ER-6P supernatant taken at the beginning of the stationary phase of growth and different pHs. The maximum activity in the supernatant of the strain ER-6P was observed at pH 5.2, but the activity could also be observed at different higher pHs.

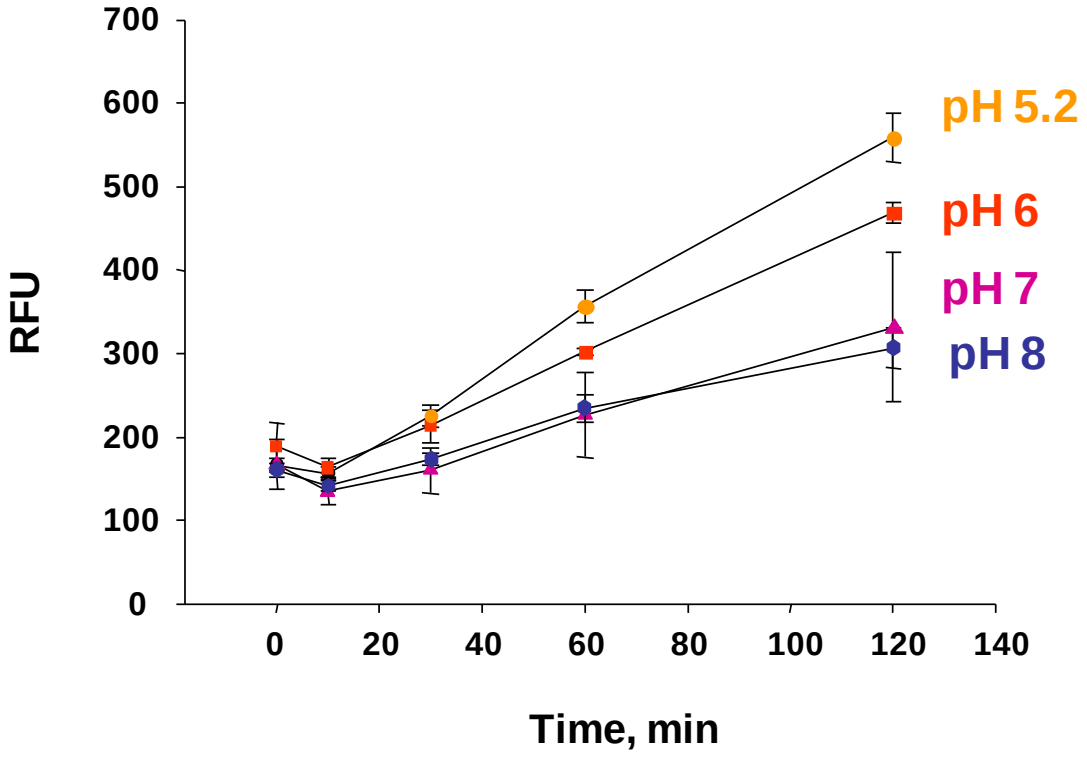


Figure 5. Phosphatase activity in ER-6P supernatant taken at the beginning of stationary phase and measured at pH 5.2, 6, 7 and 8, with MUP as substrate. Phosphatase activity is presented as the mean relative fluorescence units (RFU) ± SD.

These first results suggested a certain relationship between the secretion of an acid phosphatase activity and the virulence of the strain, since SW114 is a reference non-virulent strain and ER-6P is a systemic isolate, serum and

phagocytosis resistant.

Subsequently, we examined the activity secreted by the strain ER-6P after 14 passages in agar (ER-6Pp14). As observed before, ER-6P efficiently secreted acid phosphatase at the beginning of stationary phase. However, ER-6P p14 did not secrete this activity at any phase of growth (Figure 6). The growth of the original ER-6P strain and ER-6Pp14 in the laboratory was equivalent (Figure 7).

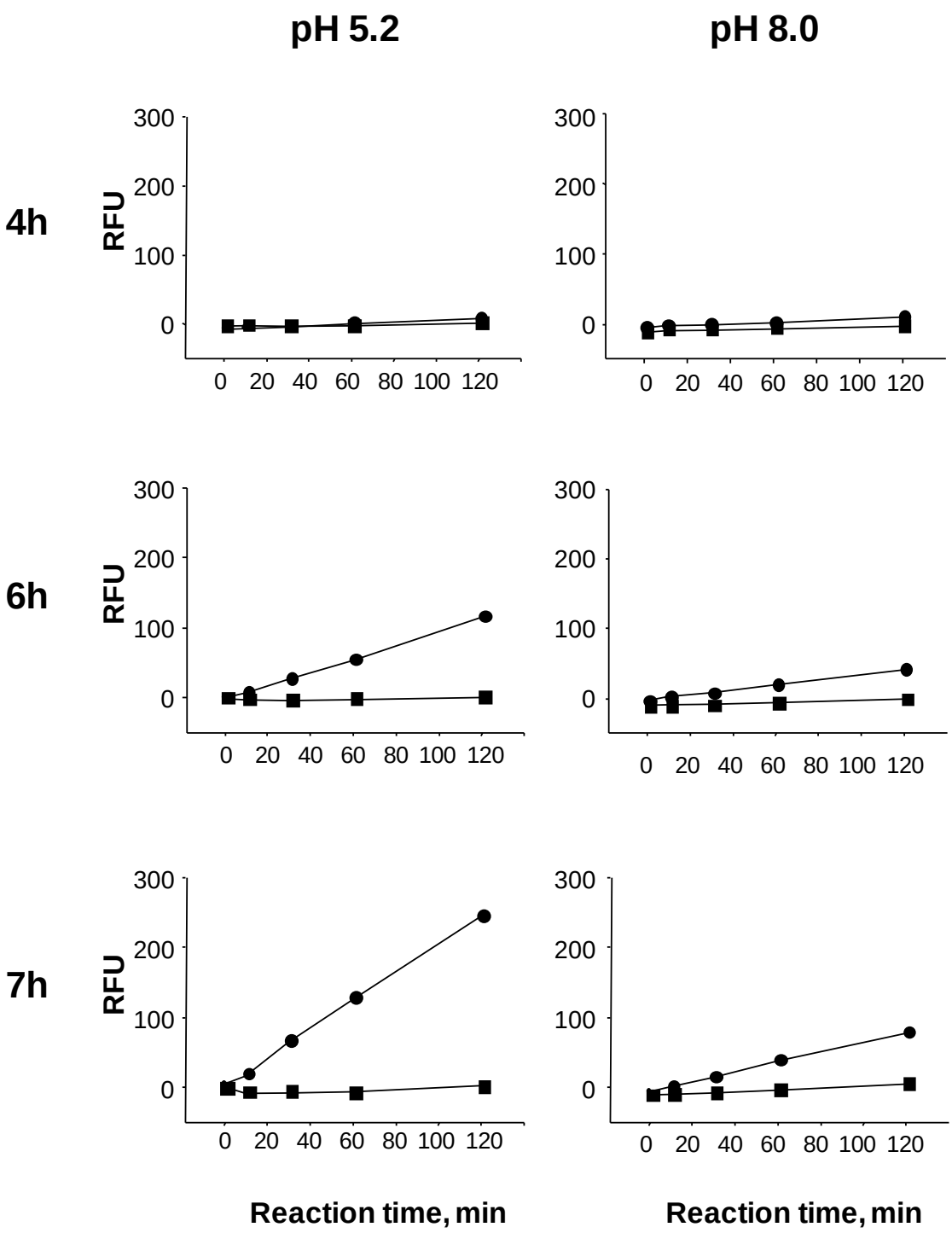


Figure 6. Phosphatase activity measured with MUP in supernatants of strain ER-6P (●) and the same strain after 14 passages on agar (ER-6Pp14;■) at different time points of the growth curve (4h, 6h and 7h) and different pH (pH 5.2 and pH 8.0). Activity is expressed as relative fluorescence units (RFU)

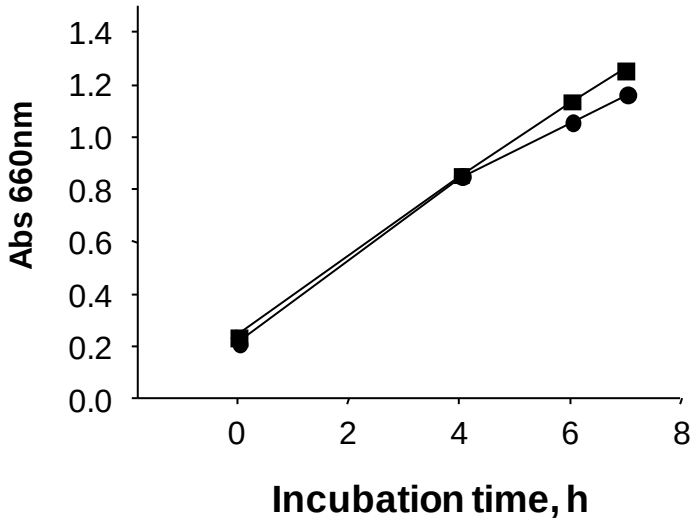


Figure 7. Growth curve of strain ER-6P (●) and the same strain after 14 passages on agar (ER-6Pp14; ■). Bacterial growth was measured as turbidity at 660 nm.

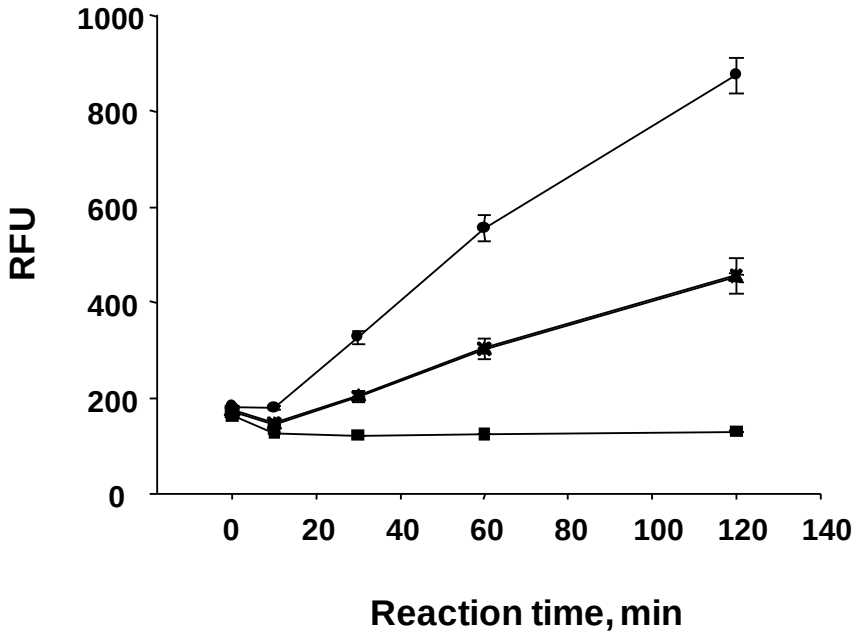


Figure 8. Phosphatase activity measured at pH 5.2 with MUP in early stationary phase of supernatants after different passages on agar. Without passages (ER-6P; ●); after 6 passages (ER-6Pp6; **X**); after 10 passages (ER-6Pp10; ▲), and after 14 passages ER-6Pp14; ■). The activity is expressed as mean relative fluorescence units (RFU) ± SD.

The loss of phosphatase activity after several passages on agar plates was gradual, as it was observed in passes 6 and 10 of ER-6P (ER-6Pp6 and ER-6Pp10), which showed an intermediate quantity of activity (Figure 8).

Thus, ER-6P lost acid phosphatase activity after *in vitro* passes on agar plates. Probably under laboratory conditions, *H. parasuis* does not need this acid phosphatase and stops producing it. Our results indicate that acid phosphatase activity may be required for *in vivo* infection, either to obtain nutrients or as a virulence factor. When *H. parasuis* is cultured for several passages in agar, the bacteria lose some essentials characteristics that are useful *in vivo*, as it was already observed with the capsule (Rapp-Gabrielson et al., 1992). This would also be the case of the acid phosphatase, since it is lost *in vitro*, indicating that the bacteria do not have the selective pressure to produce it.

4.2. Storage conditions of the acid phosphatase activity

Storage of the samples for later analysis was examined and the best option was determined to be the addition of glycerol to give a final concentration of 50% and storage at -80ºC. Actually, samples stored at -20ºC lost some phosphatase activity, but at -80ºC without glycerol the activity was completely lost, probably because it was denatured and could not be refolded again. Thus, throughout this thesis, all the samples that needed further analysis were stored with 50% glycerol at -80ºC.

Even keeping the samples under these standardized conditions, a decrease in the enzymatic activity was observed over time (Figure 9) and many assays had to be performed with fresh supernatants. The activity of the supernatant was lower after 10 days of storage in respect to the day of production (day 1). This results suggest that every time we freeze and thaw the samples some activity is lost, although the

standardized conditions (50% glycerol at -80ºC) were used (Figure 9).

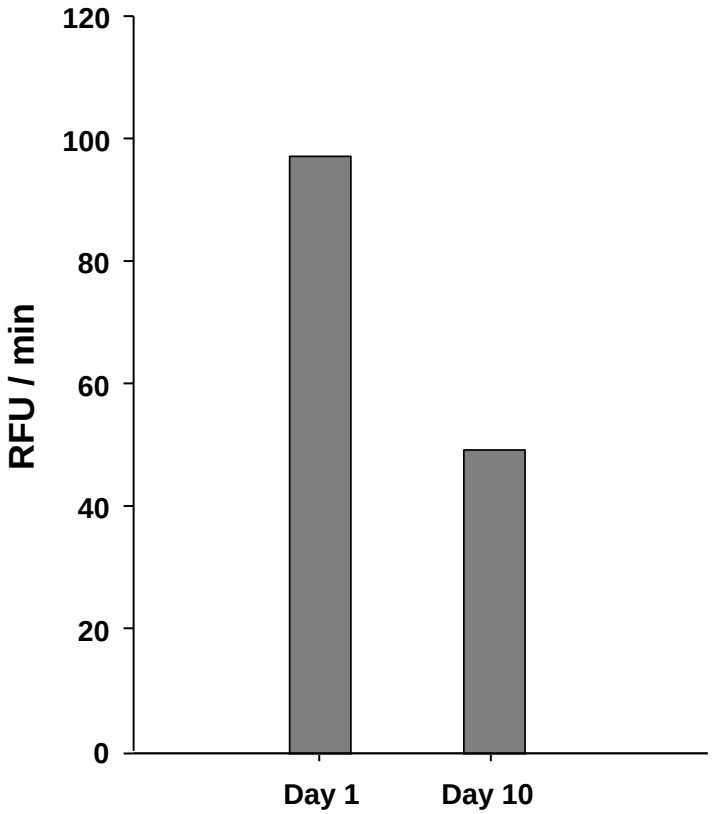


Figure 9. Effect of storage at -80ºC with 50% glycerol on the phosphatase activity in a supernatant from strain CT175-L. Phosphatase activity was measured with MUP reagent the day of production of thesupernatant (Day 1) and after 10 days at -80ºC with 50% glycerol (Day 10). Phosphatase activity is expressed as relative fluorescence units (RFU).

4.3. Determination and analysis of acid phosphatase activity in virulent and non-virulent strains from the CReSA collection.

In order to determine if the acid phosphatase activity was present and specific to virulent strains, we analyzed the activity in various *H. parasuis* strains of the research group collection. Different strains of *H. parasuis* from different sources were selected for this assay (Annex 2) and were grown in broth BHI, in glass tubes, until they reached the end of the exponential growth phase and the acid

phosphatase activity was measured by fluorescence in the supernatants before and after being conserved at -80ºC with 50% glycerol. Strains showing higher acid phosphatase activity at pH 5.2 were CT175-L, ND14-1 and ER-6P. It is noteworthy to indicate that strain ND14-1 is a nasal isolate, serum and phagocytosis sensitive. We expanded these results with more nasal strains (F9, MU21-2 and IQ1N-6). Figure 10 shows that the majority of strains tested secreted phosphatase activity, although in different quantities. Strain F9 was considered negative in the assay. Differences in activity level among strains of *H. parasuis* were also observed in neuraminidase assays (Martinez-Moliner et al., 2012). These results confirm the existence of phenotypic variability within *H. parasuis.*

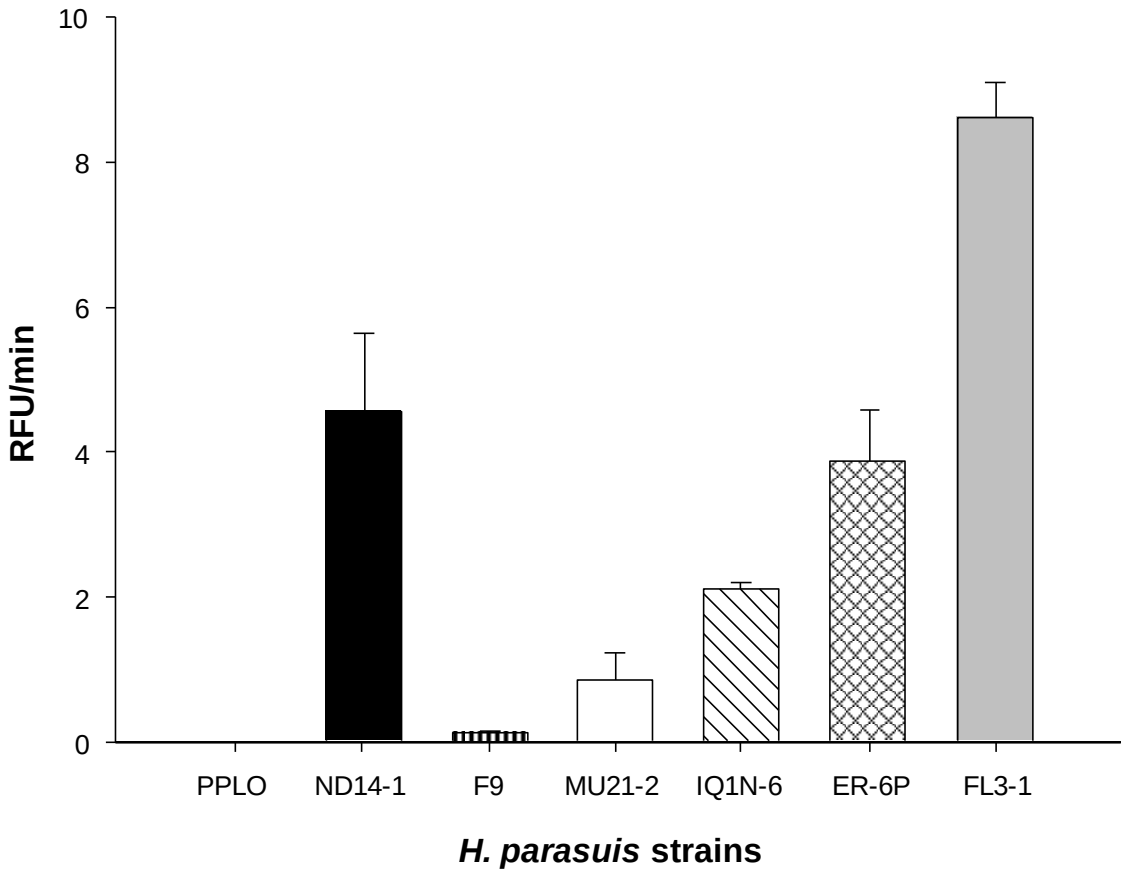


Figure 10. Phosphatase activity in culture supernatant from nasal strains ND14-1, F9, MU21-2, IQ1N-6 and FL3-1, evaluated at pH 5.2 with MUP. Supernatant from the virulent strain ER-6P was used as positive control and PPLO broth was used as negative control. Phosphatase activity was measured with MUP and expressed as mean relative fluorescence units (RFU) ± SD.

4.4. Screening genomic library of 4-5 kb of size from the genome of the strain ER-6P of *H. parasuis.*

In order to identify the gene responsible of the phosphatase activity observed in *H. parasuis*, a genomic library of strain ER-6P was screened, using both supernatants and the corresponding complete cultures. The results showed acid phosphatase activity (pH 5.2) only in the cultures of some clones but not in the supernatants. Table 1 and Figure 11 show the results of phosphatase activity in the culture of selected clones after the screening.

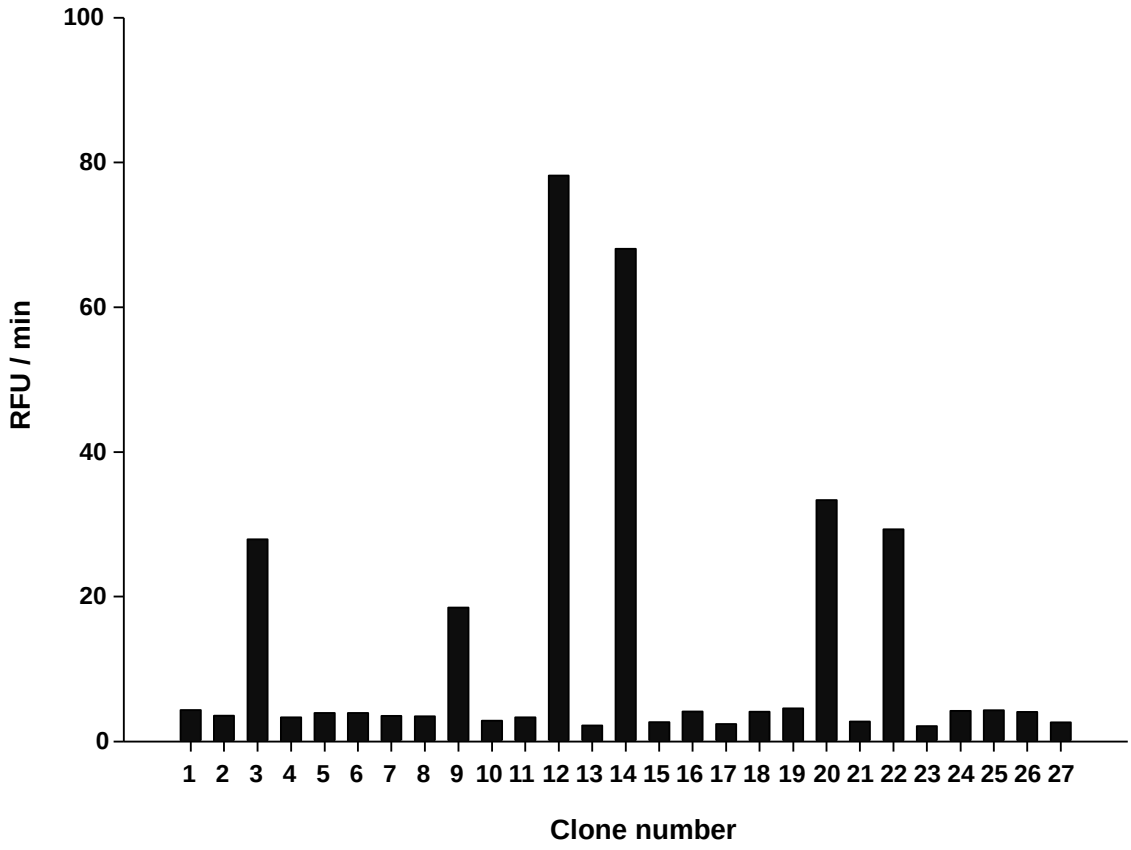


Figure 11. Acid phosphatase activity in culture of the selected clones from the genomic library (insert size of 4-5 Kb) of strain ER-6P. Phosphatase activity was measured with MUP and expressed as relative fluorescence units (RFU). Identification of the clones is presented in Table 1.

Table 1. Summary of the screening of the genomic library from strain ER-6P.

Clone of the library		Acid phosphatase activity	Homology of the inserted sequence
1	**P5G1**	-	Undecaprenyl pyrophosphate synthetase
2	**P6H1**	-	nd
3	**P7F4**	**+**	nd
4	**P7A5**	-	nd
5	**P7C1**	-	nd
6	**P7B10**	-	nd
7	**P7H4**	-	nd
8	**P8H9**	-	nd
9	**P9B12**	**+**	*pgpB**
10	**P9H7**	-	nd
11	**P9H9**	-	nd
12	**P9F9**	**+**	AphA
13	**P10H9**	-	nd
14	**P11C1**	**+**	AphA
15	**P13G10**	-	nd
16	**P14H11**	-	nd
17	**P15H1**	-	nd
18	**P16B3**	-	nd
19	**P18H6**	-	nd
20	**P19C3**	**+**	AphA
21	**P20H11**	-	nd
22	**P24A2**	**+**	AphA
23	**P25C8**	-	nd
24	**P26D4**	-	nd
25	**P26H2**	-	nd
26	**P26H6**	-	nd
27	**P29H3**	-	nd

nd: not determined
*pgpB: phosphatidylglycerophosphatase B

Clones with genes *pgpB* and *ahpA* (with homology to phosphatases) were selected for further analysis.

4.4.1. Characterization of the AphA homolog from *H. parasuis*.

Analysis of the AphA homolog from the sequence of the strain Nagasaki demonstrated that the predicted protein had the features of a type B non-specific acid phosphatase (Figure 12). The predicted AphA protein had also a signal peptide, as predicted with SignalP (Figure 12) and a molecular weight of 24.33 kilodaltons.

MKSIFLRTALLAYMFVALSAQAAPKEPYTQAGFNTRLAEQQQAPIHWVSIEQI
KESLKGKAPMNVSFDIDDTVLASSGCFYYGKQKYSPNDYSYLKNQEFWDEI
NAGCDKYSIPKQVAQDLINMHQERGDQIFFITGRTAGKDDQVTPLLEKTFGIK
NMQPVNFMGGHGQDKLKTKYNKAAGILKHNIQLHYGDSDDDILAAKEAGIRG
IRVLRTTSSTYTPFPQAGGYGEEVIINSSY

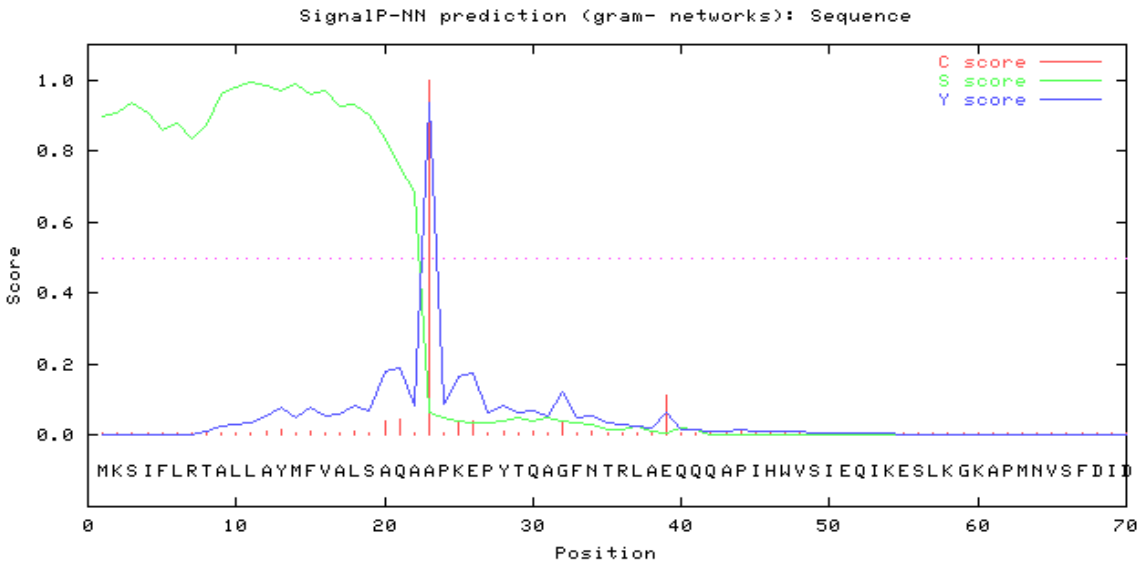


Figure 12. Predicted sequence of the *H. parasuis* AphA homolog (top panel). Catalytic domain characteristic of type B acid phosphatases are presented in red (motif F-D-I-D-D-TV-L-F-S-S-P, located in the N-terminal moiety, and Y-G-D-[AS]-D-X-D-[IV] located near the C-terminus). In blue are shown amino acid identities with all type B acid phosphatases, and underlined are shown additional identities to NapA from *Haemophilus influenzae*. Prediction of signal peptide by SignalP (bottom panel). Predicted signal peptide is shown underlined in the aminoacid sequence (top).

The *aphA* homolog with the putative promoter region was PCR amplified from strain ER-6P and cloned into pGem-T Easy. Different clones were cultured in LB broth overnight and the total culture and supernatant from each clone were evaluated for acid phosphatase activity (Figure 13). Clones carrying the *aphA* gene (clones 1, 2, 3, 6 and 7) showed phosphatase activity in the culture, but none of them secreted the activity to the supernatant. Clones 5 and 10 consisted of empty pGem-T Easy vector and therefore showed no phosphatase activity (Figure 13). Thus, clones 3 and 6 served as positive controls for further cloning experiments, and clone 10 served as negative control.

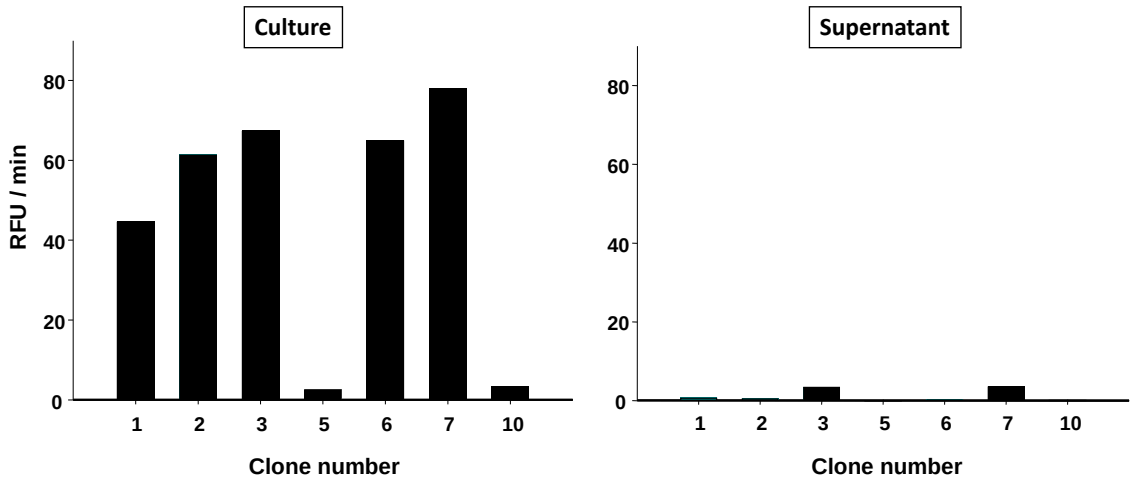


Figure 13. Acid phosphatase activity (pH 5.2) of the clones *E. coli* BL21 pGem-T Easy *aphA* gene from *H. parasuis* ER-6P. Left panel shows the activity of the cultures and the right panel shows the activity in the culture supernatants. Phosphatase activity was measured with MUP and is expressed as relative fluorescence units (RFU).

The complete *aphA* gene with the putative promotor sequence was PCR amplified and sequenced from several strains of *H. parasuis*, in order to examine any difference. We checked several virulent and non-virulent strains from our collection for the presence of the gene encoding for the type B AphA homolog. We did not observe any association with the putative virulence of the strains, since the gene was detected in virulent and non-virulent strains (Figure 14).

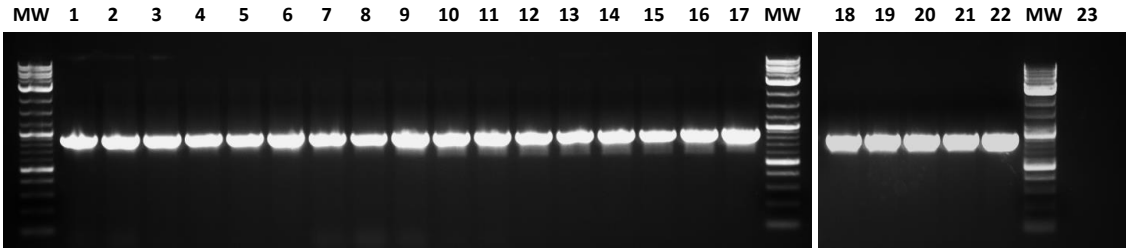


Figure 14. PCR amplification of the *aphA* gene from strains isolated from lesions 9904108 (lane 1), Nagasaki (lane 2), 373/03A(lane 3), PV1-12 (lane 4), 264/99 (lane 5), 228/04 (lane 6), P015/96 (lane 7), ER-6P (lane 8), PC4-6P (lane 9), IT29205 (lane 10) and 2725 (lane 11); and from nasal strains IQ1N-6 (lane 12), SL3-2 (lane 13), VS6-2 (lane 14), ND14-1 (lane 15), SC14-1 (lane 16), MU21-2 (lane 17), CA38-4 (lane 18), FL1-3 (lane 19), F9 (lane 20), SW114 (lane 21) and FL3-1 (lane 22). In lane 23, a negative control consisting of water was included. MW: DNA ladder.

Then, we extended the cloning to other strains of *H. parasuis*, IQIN-6 and FL3-1 in order to observe any difference. The results of the activity found in the clones are presented in Figure 15. Again, activity was detected in the cultures (pellet) but not in the culture supernatants, indicating that the acid phosphatase activity from these clones was not secreted.

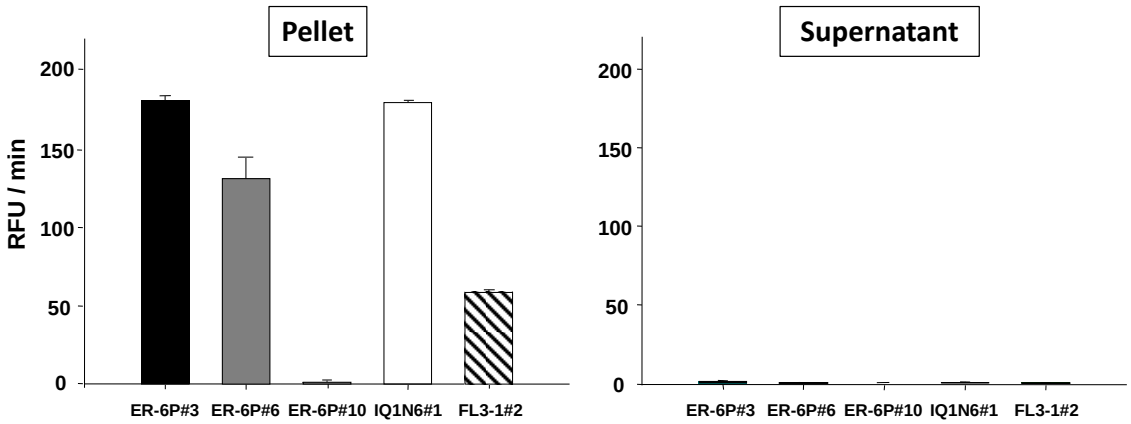


Figure 15. Acid phosphatase activity observed in different clones of *aphA* homolog from different strains of *H. parasuis*. The clones ER-6P#3 and ER-6P#6 were included as positive controls of activity in culture and the ER-6P#10 as negative control. Phosphatase activity was measured with MUP and is expressed as mean relative fluorescence units (RFU) ± SD.

We examined in more detail the clones produced from strain FL3-1. From the 19 different clones obtained, 16 were positive and 3 were found negative for phosphatase activity in culture. Clones 1 (FL3-1#1) and 19 (FL3-1#19) were selected for further activity analysis. Thus, clones FL3-1#1 and FL3-1#19 were grown overnight and the activity was measured in culture and culture supernatant (Figure 16). To our surprise, clone FL3-1#19 secreted the phosphatase activity into the supernatant.

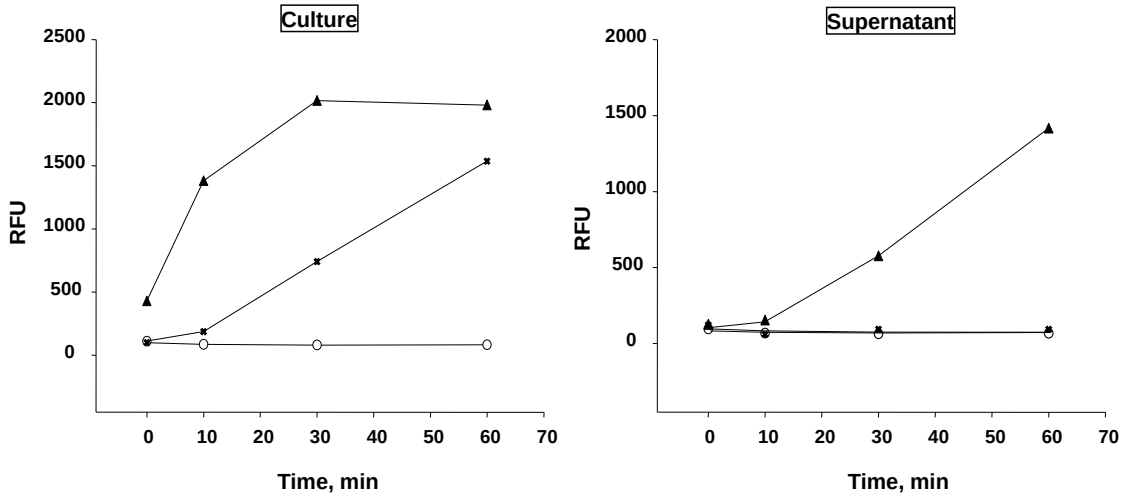


Figure 16. Phosphatase activity in cultures and supernatants of BL21 pGem-T Easy with *aphA* homolog from the strain FL3-1 (clones FL3-1#1, **x**; FL3-1#19, ▲). BL21 with pGem-T Easy (○) was used as negative control. Phosphatase activity was measured with MUP and is expressed as relative fluorescence units (RFU).

In addition, the *aphA* gene was sequenced and the predicted protein sequence from each strain was obtained (Figure 17) and the hypothetic promoter region was compared (Figure 18).

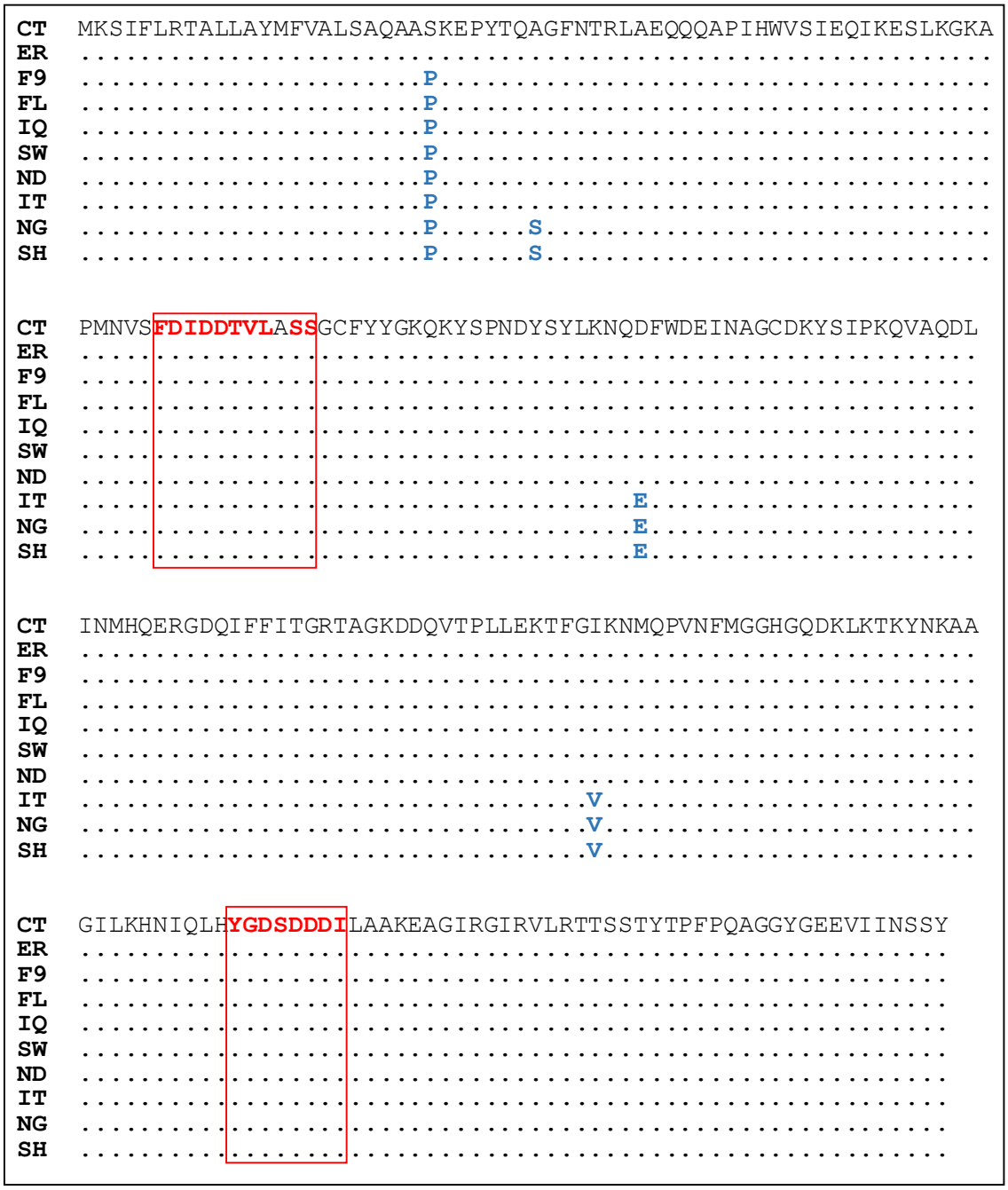


Figure 17. Alignment of the predicted amino acid sequence of acid phosphatase AphA from different strains of *H. parasuis* (CT: CT175-L; ER: ER-6P; F9; FL: FL3-1; IQ: IQ1N-6; SW: SW114; ND: ND14-1; IT: IT29205; NG: Nagasaki; SH: SH0165). The catalytic domain characteristic of type B acid phosphatases are presented in red in two red boxes (motif F-D-I-D-D-TV-L-F-S-S-P, located in the N-terminal moiety, and Y-G-D-[A/S]-D-X-D-[IV] located near the C-terminus). Differences in amino acids are presented in blue.

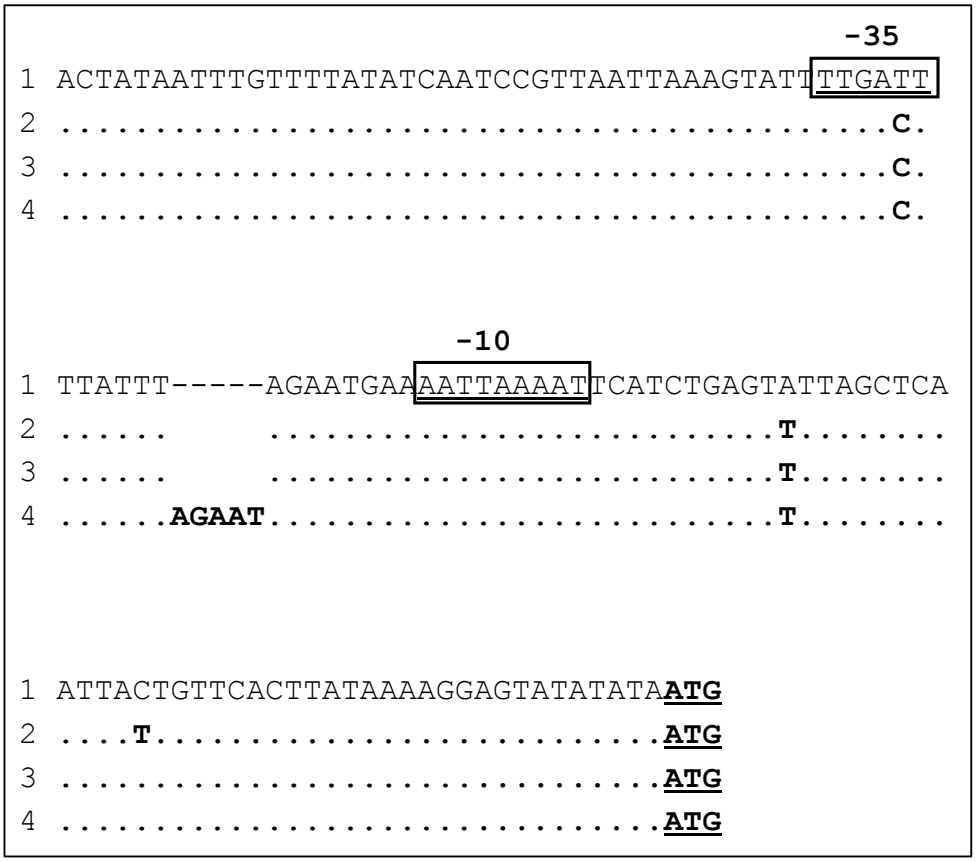


Figure 18. Analysis of the hypothetic promoter of the *aphA* gene in different strains of *H. parasuis*: Nagasaki, IT29205 and CT175-L (line 1); IQ1N-6 and F9 (line 2); SW114 and ND14-1 (line 3); FL3-1 and ER-6P (line 4).

Minor differences were observed in the protein and promoter sequences, but none that may explain our previous negative results with SW114 and F9, for instance.

In the case of clone FL3-1#19, the sequencing found that the orientation of the insert was opposite to clone FL3-1#1, but the inserted sequence was identical. No explanation could be found to the fact that the activity was secreted by this clone but not the others.

4.4.2. Characterization of the PgpB homolog from *H. parasuis.*

The *pgpB* gen was also selected from the screening of the 4-5 Kb library, and the distribution of the complete gene in several strains of *H. parasuis* was studied by PCR (Figure 19).

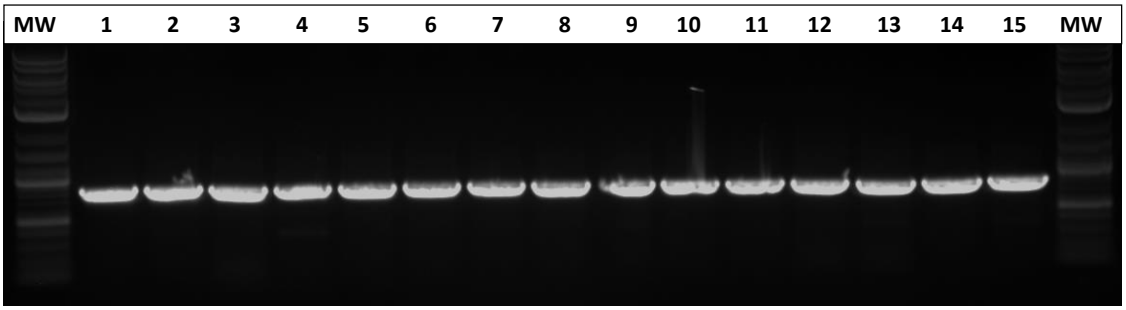


Figure 19. PCR amplification of gene *pgpB* from strains of *H. parasuis* isolated in different location. From systemic site **1**. 373/03A, **2.** Nagasaki, **3**. ER-6P, **4**. P555/04, **5**. 2725, **6**. 264/99. From pulmonary site, **7**.230/03, **8**. 228/04, **9**. 19/04, **10**. 279/03 and from nasal site, **11**. SW114, **12**. F9, **13**. MU21-2, **14**. VS7-6, **15**. IQIN6. MW: DNA ladder.

Again, the gene was detected in all the strains tested independently of their clinical origin.

After cloning of the gen, acid phosphatase activity was observed in the cultures (Figure 20), but none of the clones had activity in the supernatant (data not shown). This is not surprising, since PgpB did not have a signal peptide, as determined by SignalP and six transmembrane domains were detected by TMHMM Server v. 2.0 (Prediction of transmembrane helices in proteins) (Figure 21).

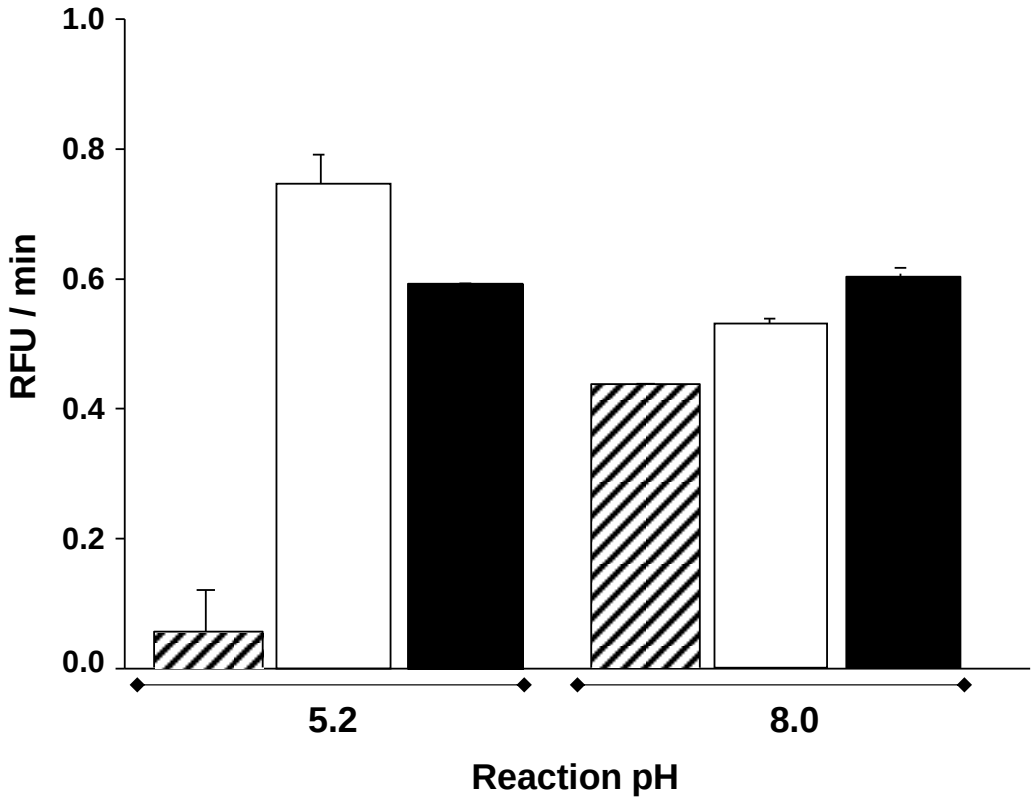


Figure 20. Acid phosphatase activity in cultures of BL21 pGem-T Easy with gene *pgpB* from strains ER-6P (white bars) and Nagasaki (black bars). BL21 with pGem-T Easy (diagonal bars) was used as negative control. Phosphatase activity was measured with MUP at pH 5.2 and pH 8.0; and it is expressed as mean relative fluorescence units (RFU) ± SD.

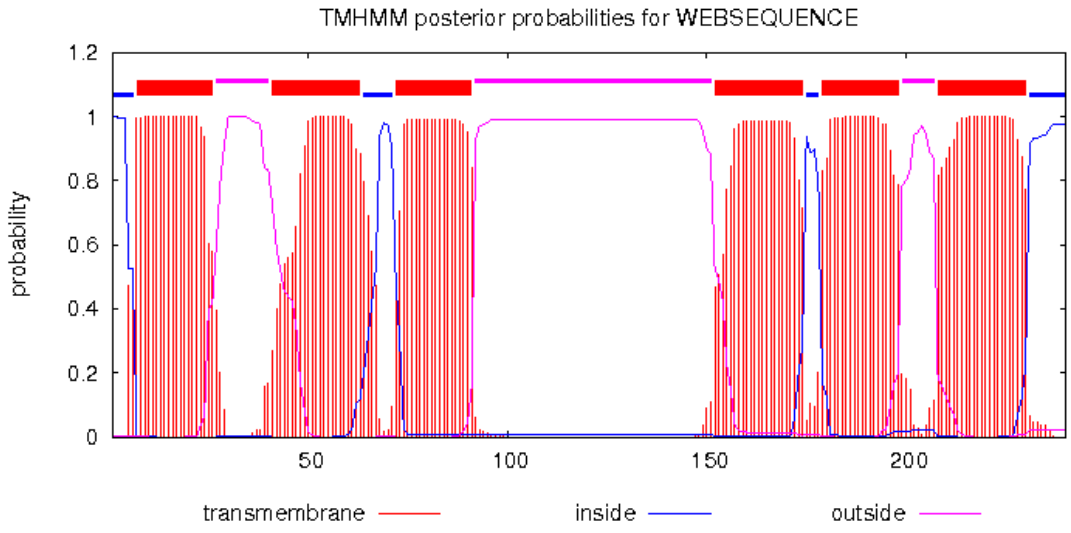


Figure 21. Predicted transmembrane domains in the *H. parasuis* PgpB (Inside: 1- 6; TMhelix: 7-26; outside:27-40; TMhelix: 41-63; inside: 64-71; TMhelix: 72- 91; outside: 92-151; TMhelix: 152-174; inside: 175-178; TMhelix: 179-198; outside: 199-207; TMhelix: 208-230; inside: 231-240). Numbers indicate the aminoacid position.

4.5. Phosphatase activity at different pH

Figure 22 shows the phosphatase activity in ER-6P supernatants through a wide range of pH, from pH 4.0 to pH 9.0. From this Figure, it can be inferred that the optimal pH of the activity found in *H. parasuis* supernatants is between 5.2 and 6.

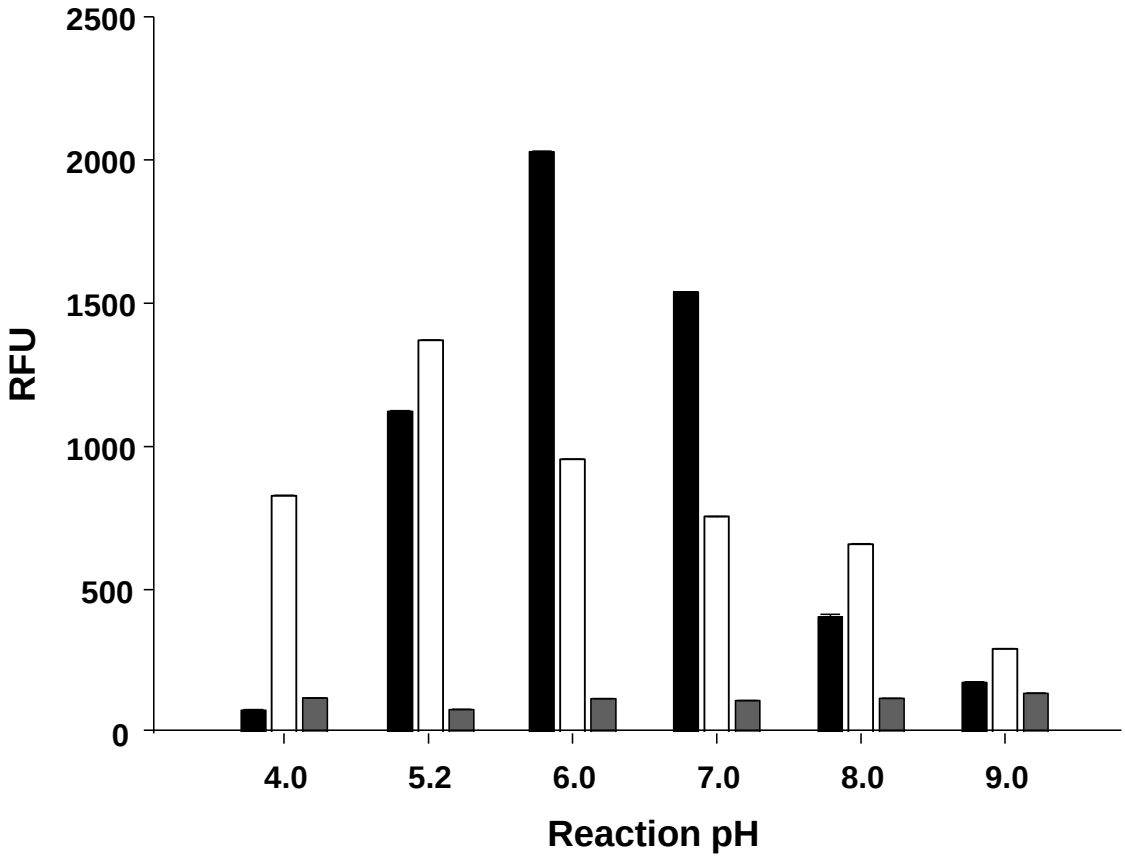


Figure 22. Measurement of the phosphatase activity in the supernant of *H. parasuis* at different pH (4.0, 5.2, 6.0, 7.0, 8.0 and 9.0). The black and white bars represent two different concentrated supernatants of ER-6P. PPLO broth (gray bars) was used as control. Phosphatase activity was measured with MUP and is represented as relative fluorescence units (RFU).

The activity found in the supernatant of clone BL21 pGem-T Easy *aphA* FL3-1#19 was also analyzed in the same range of pH to explore if this activity could correspond to the secreted activity. Figure 23 shows that the activity produced by the *H. parasuis* AphA homolog presented a different optimal pH than the activity

found in *H. parasuis* supernatants, indicating that both activities were produced by different enzymes. The optimal pH for *H. parasuis* Apha was around 8.0.

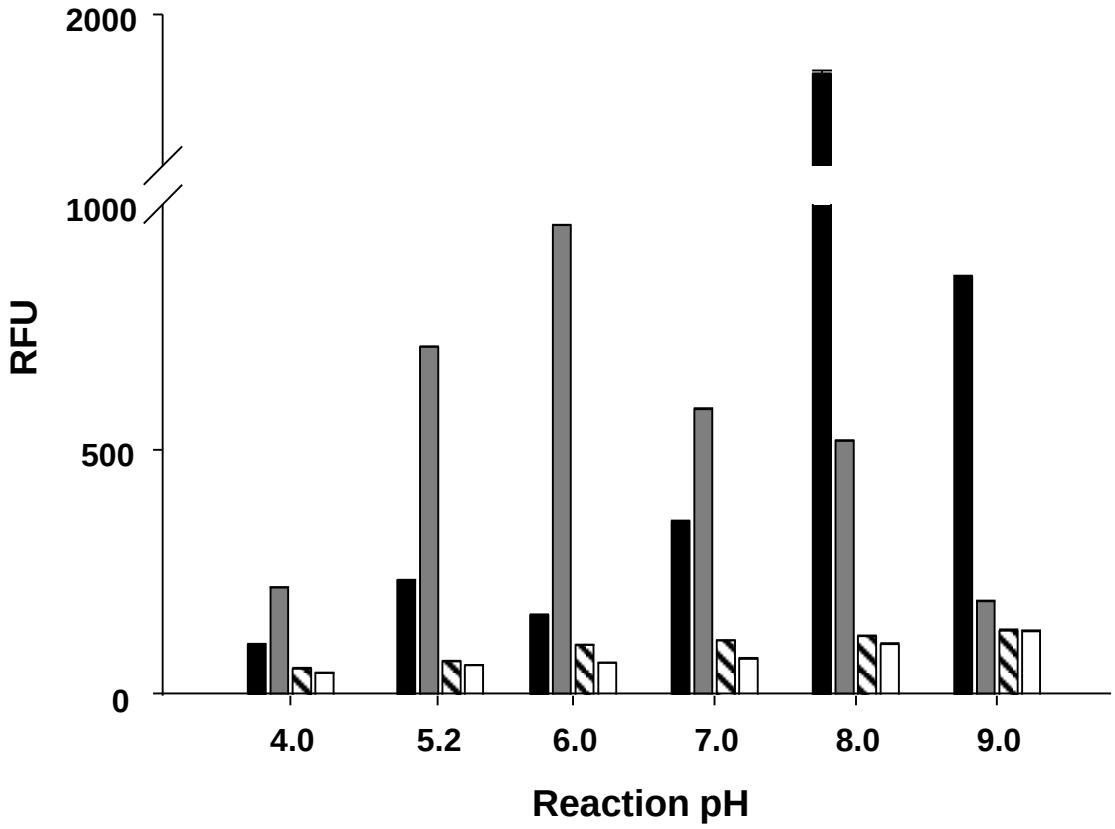


Figure 23. Measurement of the phosphatase activity by the AphA homolog of *H. parasuis* at different pH (4.0, 5.2, 6.0, 7.0, 8.0 and 9.0). The supernatant of the clone BL21 pGem-T Easy-*aphA* FL3-1#19 (black bars) was tested at different pH with MUP. Activity in a supernatant from ER-6P culture (gray bars) is also shown. *E. coli* BL21 pGEM-T Easy (white bars) and LB broth (dashed bars) were used as control. Phosphatase activity was measured with MUP and is represented as relative fluorescence units (RFU).

In the case of the PgpB protein, the activity had to be measured with the complete cultures, since none of the clones secreted the activity to the culture supernatant. The optimal pH of PgpB was comparable to the optimal pH of the activity in the supernatants of *H. parasuis* (Figure 24).

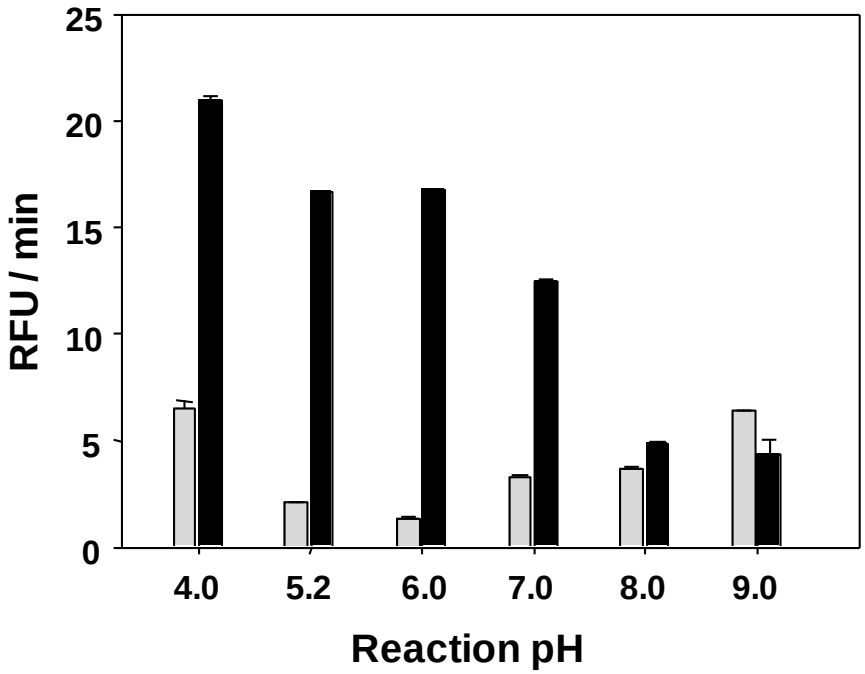


Figure 24. Measurement of the phosphatase activity in the gen *pgpB* at different pH (4.0, 5.2, 6.0, 7.0, 8.0 and 9.0). Phosphatase activity in whole culture of BL21-PCRII Topo *pgpB* ER-6P#1 (black bars) was examined at different pH. *E. coli* BL21 (gray bars) was used as control. Phosphatase activity was measured with MUP and is represented as the mean relative fluorescence units (RFU) ± SD.

The Figure 25 presents all the controls, positive and negative, used in the phosphatase activity assays included in this thesis. It is not uncommon that acid phosphatases present also activity at higher pH, as it was observed with the control acid phosphatase from potato.

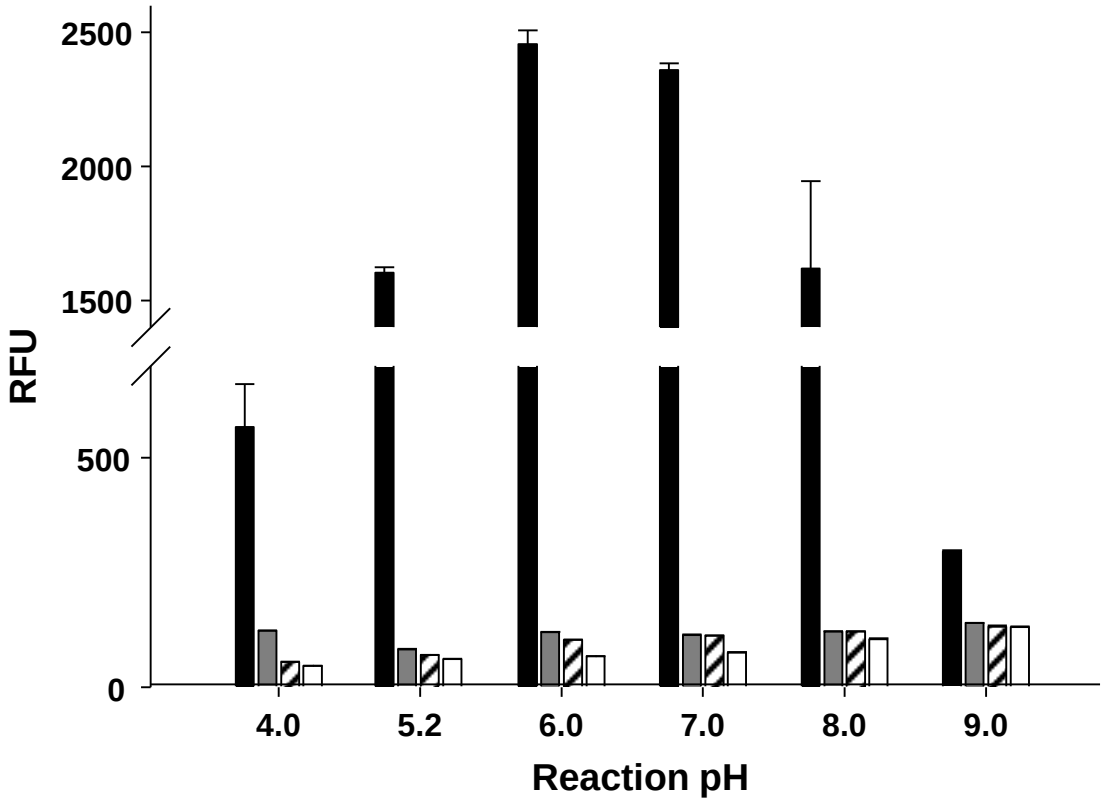


Figure 25. Measurement of the phosphatase activity of the controls at different pH (4.0, 5.2, 6.0, 7.0, 8.0 and 9.0). Acid phosphatase of potato (black bars), PPLO broth (gray bars), LB broth (dashed bars) and *E. coli* BL21 pGEM-T Easy (white bars). Phosphatase activity was measured with MUP and is represented as relative fluorescence units (RFU).

Since it seemed that these phosphatases could be cell associated, we decided to do an analysis of phosphatase activity in the bacterial pellet from *H. parasuis*. Bacterial pellets of strains CT175-L and FL3-1 were analyzed with MUP at different pHs and 2 maximum peaks of activity were detected at around pH 4 and pH 6-7 (Figure 26).

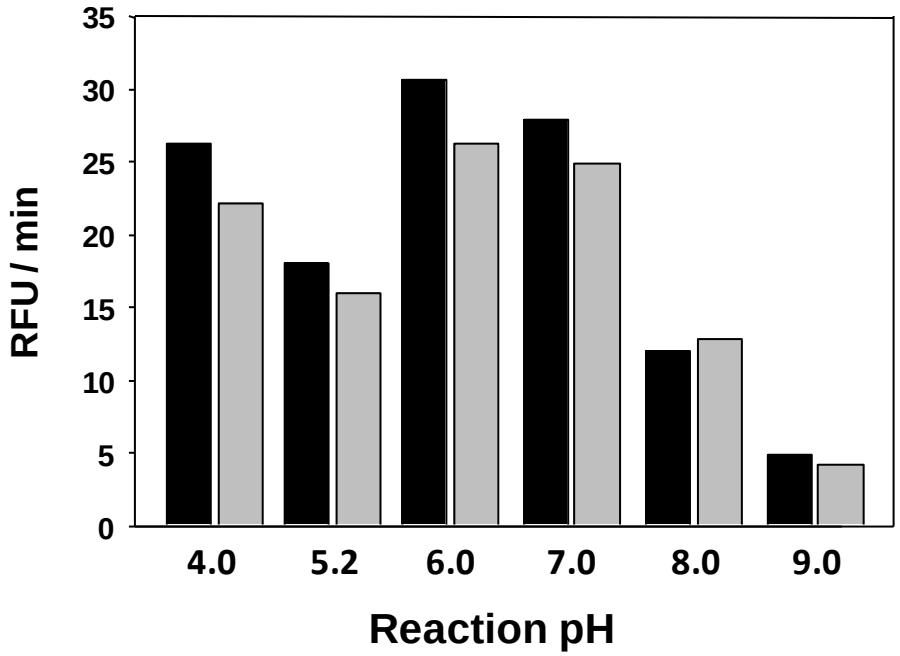


Figure 26. Phosphatase activity in bacterial pellet from *H. parasuis* strains CT175-L (black bars) and FL3-1 (gray bars) at the pH indicated. Phosphatase activity was measured with MUP and is represented as relative fluorescence units (RFU).

4.6. Effects of several inhibitors on the phosphatase activities

Another characteristic that differentiates among various types of phosphatases is the requirement of different metal cations, and therefore their inhibition by EDTA at 20 mM. Therefore, we decided to analyze the effect of the chelating agent EDTA on the *H. parasuis* phosphatase activities (Figure 27). The activity in the supernatant of the strain ER-6P was not affected by addition of EDTA to the reaction. However, the activity due to AphA was inhibited by EDTA, as it is common for phosphatases within the type B group (Felts, 2007). Thus, besides having the characteristic domains of type B, *H. parasuis* AphA homolog requires cations for full activity, supporting its classification within this group of phosphatases.

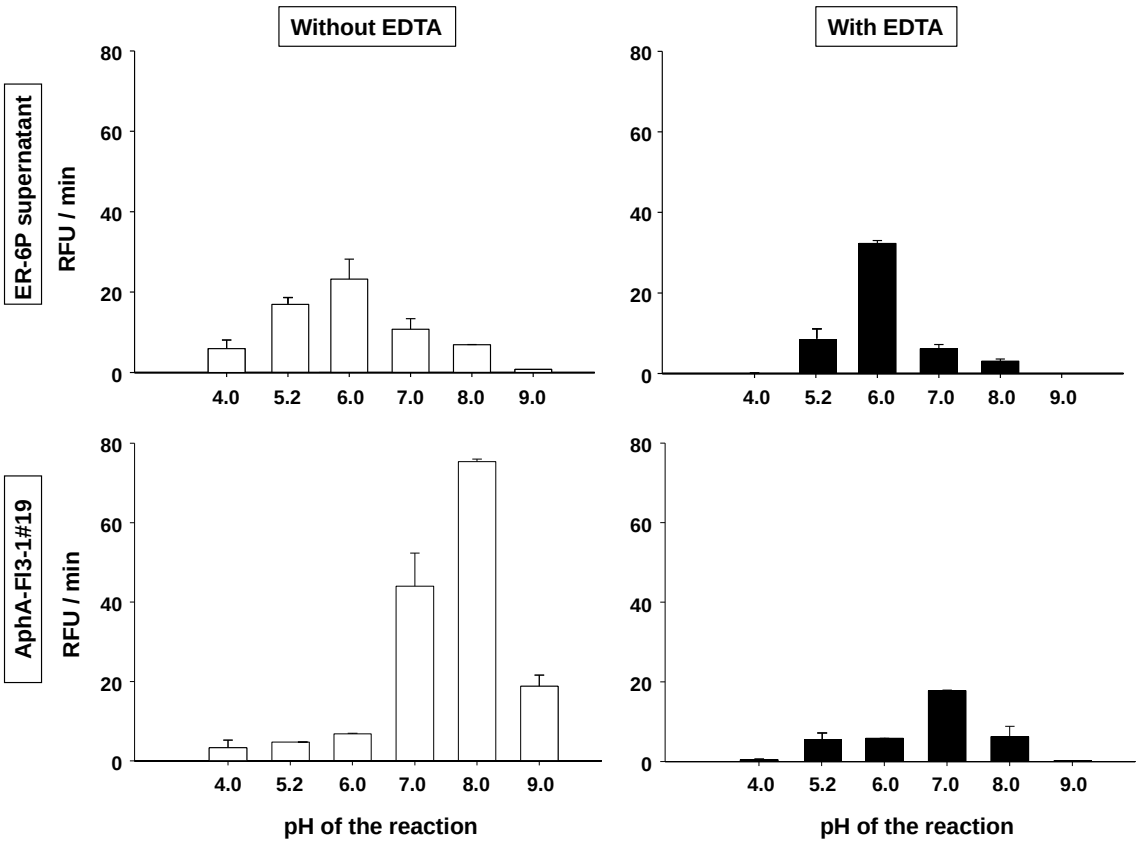


Figure 27. Phosphatase activity in supernatants of strain ER-6P and BL21 pGem-T Easy *aphA* FL3-1#19 with and without EDTA 20 mM, at different pH (4.0, 5.2, 6.0, 7.0, 8.0 and 9.0). Phosphatase activity was measured with MUP and is represented as relative fluorescence units (RFU).

Tartrate was also tested as phosphatase inhibitor and we did not see any effect on the secreted activity by *H. parasuis* ER-6P and neither on the AphA expressed by clone BL21 pGem-T Easy *aphA* FL3-1#19 up to a concentration of 10 mM sodium tatrate. When we used molybdate, only a partial inhibition was observed in the activity from strain ER-6P at 10 mM and no effect on the recombinant AphA phosphatase (Figure 28).

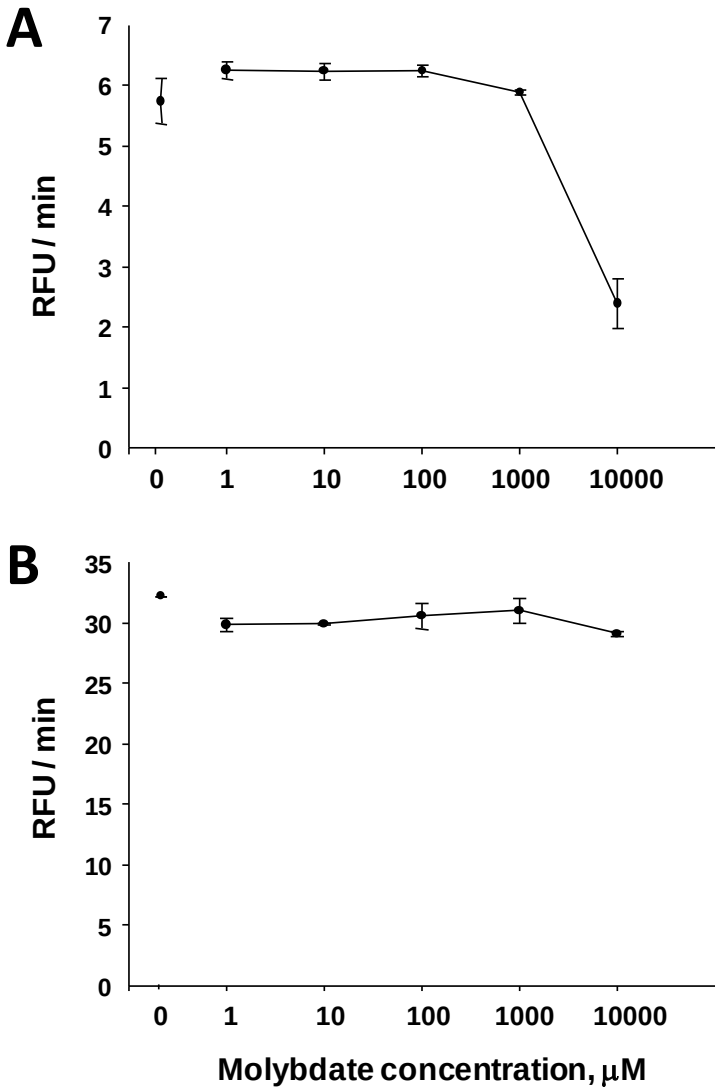


Figure 28. Effect of sodium molybdate on the phosphatase activity from supernatants of strain ER-6P (A) and BL21 pGem-T Easy *aphA* FL3-1#19 (B) at pH 5.2. Phosphatase activity was measured with MUP and is represented as relative fluorescence units (RFU).

4.7. Determination of the acid phosphatase activity by a fluorescent zymogram and MALDI-TOF

As an alternative to identify the phosphatase activity secreted by *H. parasuis*, zymograms were performed. In order to characterized the acid phosphatase without compromising its original structure and thus to conserve its activity, semi-native gel electrophoresis were performed. Virulent strains ER-6P and CT175-L were grown and tested for the secretion of the phosphatase activity (Figure 29).

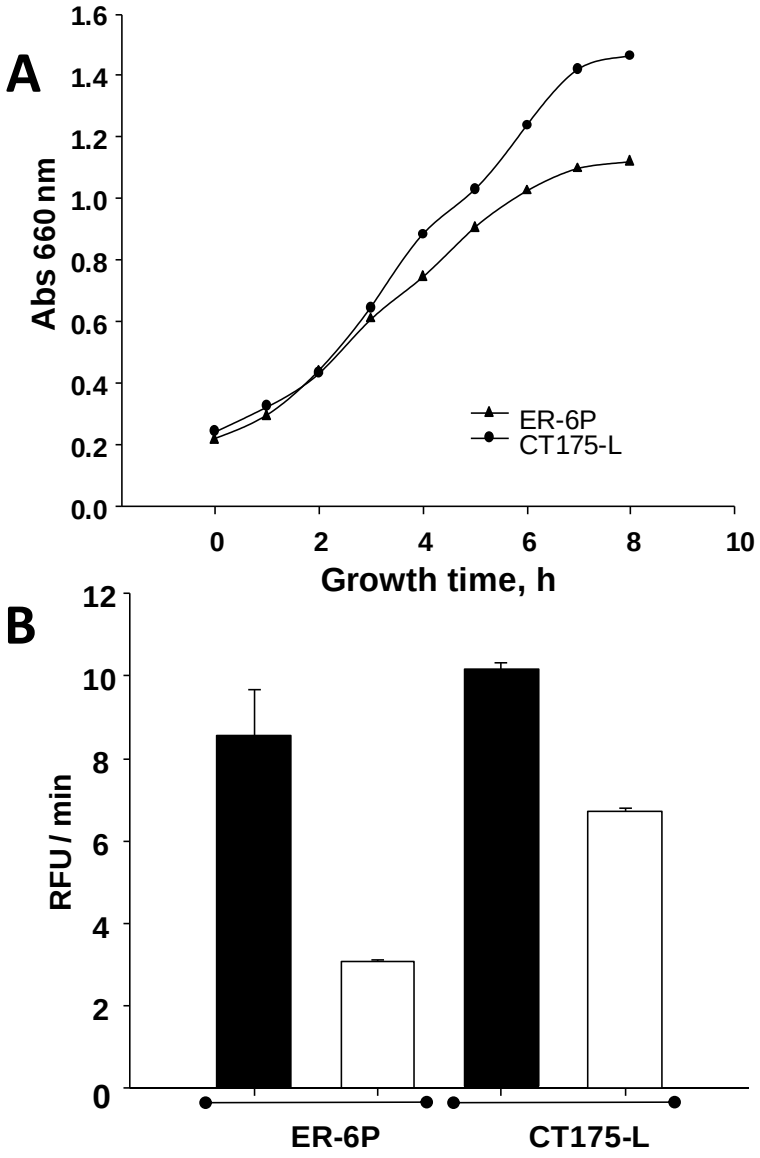


Figure 29. Growth curve (A) and phosphatase activity (B) of supernatants obtained at the beginning of the stationary phase (last time point shown in the growth curve in A) from strains CT175-L and ER-6P. Phosphatase activity (B) was measured with MUP at pH 5.2 (black bars) and at pH 8.0 (white bars) and is represented as relative fluorescence units (RFU).

After confirmation of activity, supernatant was concentrated and evaluated in a zymogram with MUP as substrate. Supernatant from strain ER-6P was discarded due to its weaker activity in the zymograms, and analysis was continued with supernatant samples from the strain CT175-L.

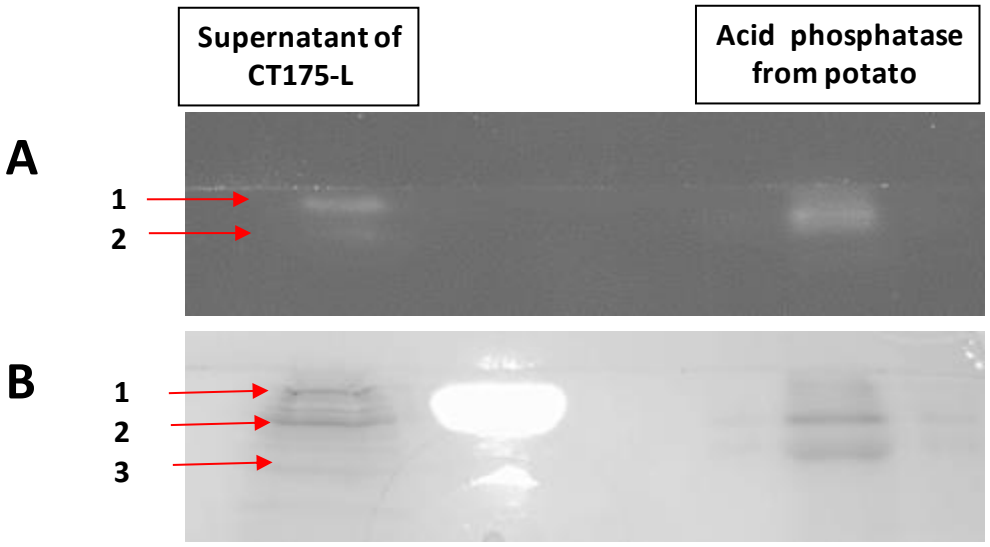


Figure 30. Determination of the acid phosphatase activity in a native-gel assay. Zymogram with MUP as substrate in acetate buffer (pH5.2) (A) and coomassie blue staining of the same gel after observation of the activity (B) were performed with a concentrated supernatant from strain of *H. parasuis* CT175-L. A commercial acid phosphatase from potato was used as positive control. Bands 1 and 2 were considered to have acid phosphatase activity, while band 3 was used as negative control for further analysis.

Zymogram showed 2 bands with activity on MUP (Figure 30A). The corresponding proteins, detected by Coomasie stainning (Figure 30B) were excised from the gel, together with an extra band for control, and analyzed by MALDI-TOF (Annex 5). MASCOT analysis of the spectra revealed no hit with any protein, under the search parameters used. One explanation for this lack of identification of our target proteins is that each band was actually constituted by a mixture of proteins (the gel was run under semi-native conditions) and not one single individual protein, impeding the correct identification of the proteins.

4.8. Analysis of acid phosphatase activity in seminative-PAGE and electroelution.

In an effort to purify the phosphatase activity, we attempted to perform a zymogram for electroelution of the active proteins, and subsequent separation by other methods (such as SDS-PAGE). Two different samples of concentrated supernatants from strain CT175-L were used for this purpose. A zymogram assay showed active bands at different positions in the two samples (Figure 31A). The bands showing activity, including the positive control AphA, were excised from the gel, electroeluted and phosphatase activity was tested in the electroeluted proteins. However, none of the electroeluted proteins showed phosphatase activity (Figure 31B).

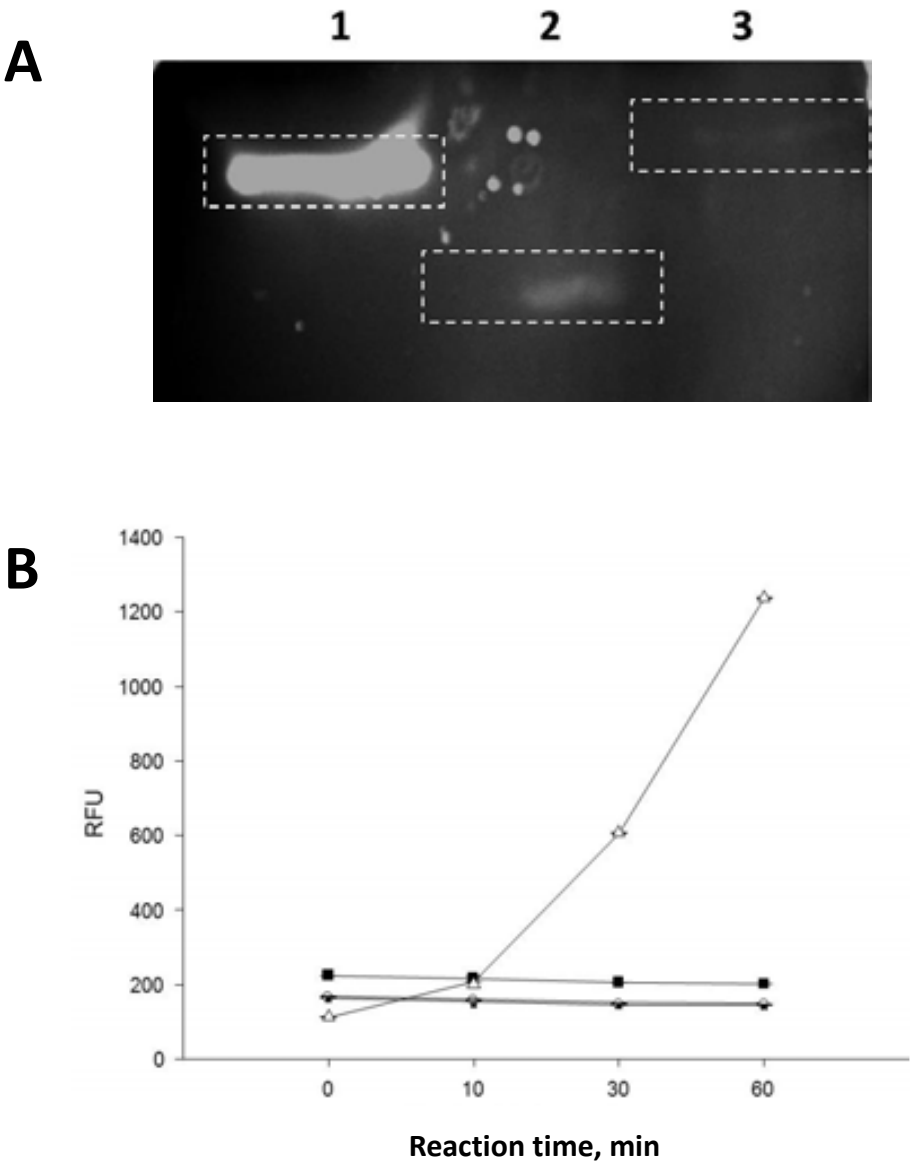


Figure 31. Acid phosphatase activity of supernatants from strain CT175-L observed in a semi-native gel with MUP as substrate (A) and after electroelution of the proteins with activity (B). Supernatant from clone BL21 FL3-1#19-*aphA* was used as control of activity (lane 1 in A; ■ in B); supernatant of CT175-L (sample 1: lane 2 in A; x in B) and supernatant of CT175-L (sample 2: lane 3 in A; ○ in B). Untreated supernatant from clone FL3-1#19 was used as positive control of the activity in B [Δ].

The presence of different proteins showing activity in the two samples analyzed from CT175-L could be explained by the formation of dimers or other complexes under the conditions of the assay. The negative results obtained after the electroelution prevented us from further characterization of these samples and we decided to change our strategy.

4.9. Determination of the acid phosphatase activity by chromatography and MALDI-TOF mass spectrometer analysis.

To enhance the probabilities of identifying the correct protein, we decided to perform a chromatography to separate the proteins present in the supernatant with acid phosphatase activity, before performing SDS-PAGE and MALDI-TOF. Therefore a new fresh concentrated supernatant of *H. parasuis* strain ER-6P was produced, and high values of activity were confirmed. A size exclusion chromatography was done and 5 ml fractions were collected for phosphatase detection. The samples were kept in ice during the process to maintain the integrity of the enzyme. After chromatography, phosphatase activity was detected at high level in 3 fractions (fractions 7, 8 and 9 in Figure 32A). Fractions were precipitated for concentration and analyzed in SDS-PAGE (Figure 32B).

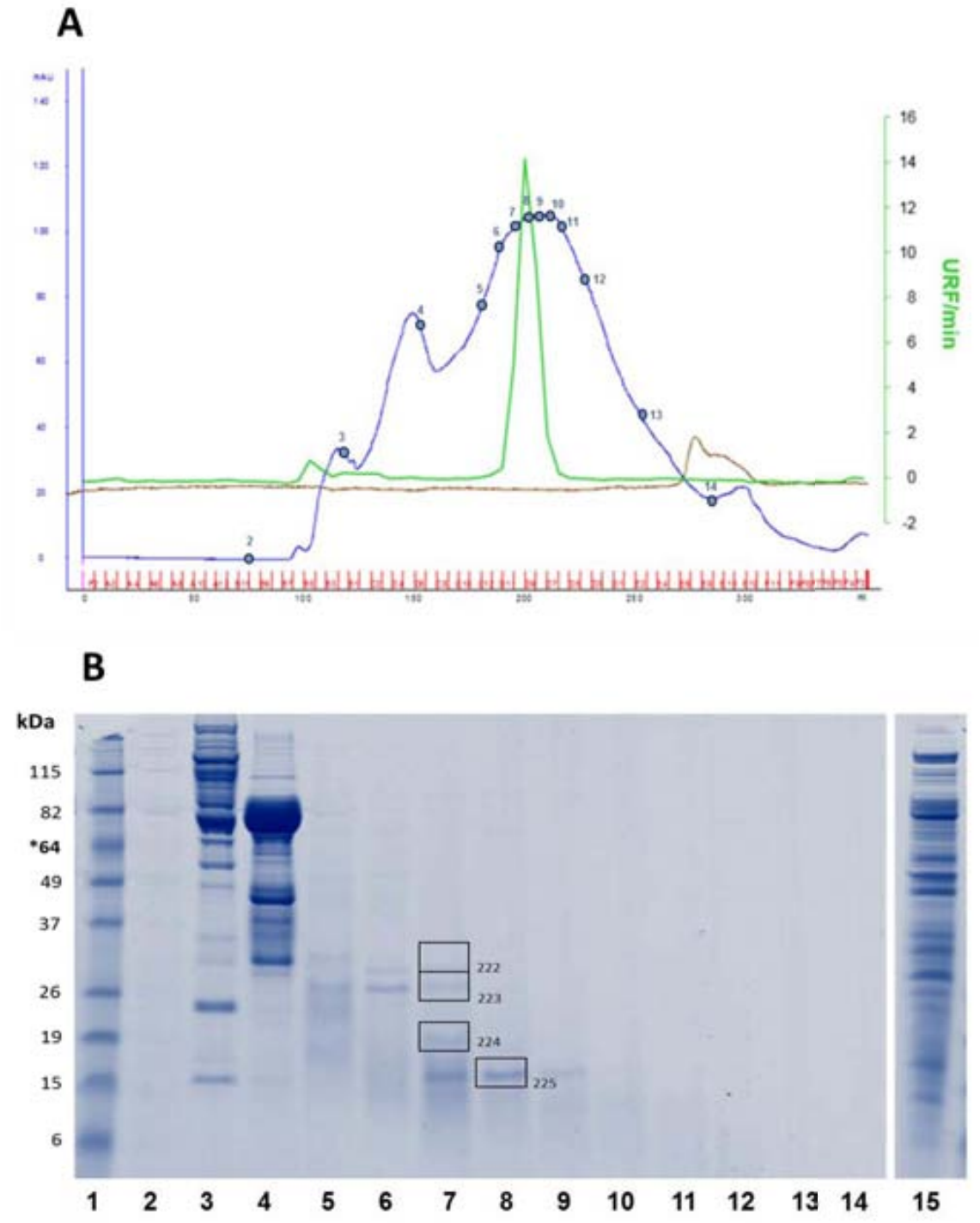


Figure 32. Size exclusion chromatogram (A) of a supernatant from strain CT175-L with phosphatase activity and SDS-PAGE analysis (B) of the fractions collected from the chromatography (2 to 14 in A and B). Elution of the proteins from the column was detected by Abs at 280 nm (blue line in A). The phosphatase activity of the fractions was measured with MUP at pH 5.2 and is represented as relative fluorescence units (RFU) (green line in A). A complete supernatant sample was included in the SDS-PAGE (lane 15 in B) and a protein marker was added in lane 1 (B). Boxes indicate the selected proteins for mass spectrometry analysis with the labeling used for this analysis (222, 223, 224 and 225).

Fractions in lanes 7, 8 and 9 showed the highest phosphatase activity and a few number of proteins in SDS-PAGE (Figure 32). There was only one common protein that was found in all 3 fractions (labeled 225, Figure 32B), which was selected as the most likely candidate for the enzyme responsible for the secreted phosphatase activity. Nevertheless, other proteins were also analyzed (labeled 222, 223 and 224, Figure 32B). The analysis of protein 222 by MALDI-TOF-TOF followed by MASCOT (Annex 6) identified the protein as a thiol:disulfide interchange protein dsbA from *H. parasuis* SH0165 (gi|219871890), with a score of 450 (Protein scores greater than 85 are significant [p<0.05]). Protein 223 matched with a thiol peroxidase from *H. parasuis* SH0165 (gi|219872113) with a score 75 (Individual ions scores >57 indicate identity or extensive homology [p<0.05]). Protein 224 did not match with any protein; the best hit was a hypothetical protein Sinac_6370 from *Singuilisphaera acidiphila*, isolated from an acidic wetland, with a score of 39 (individual ions scores >56 indicate identity or extensive homology [p<0.05]). Finally, band 225 was analyzed by MALDI-TOF (Figure 33). Two peaks from this spectrum were selected (indicated in Figure 31) to complete the analysis by MALDI-TOF-TOF. The MS spectra of the selected peaks are shown in Figures 34 and 35.

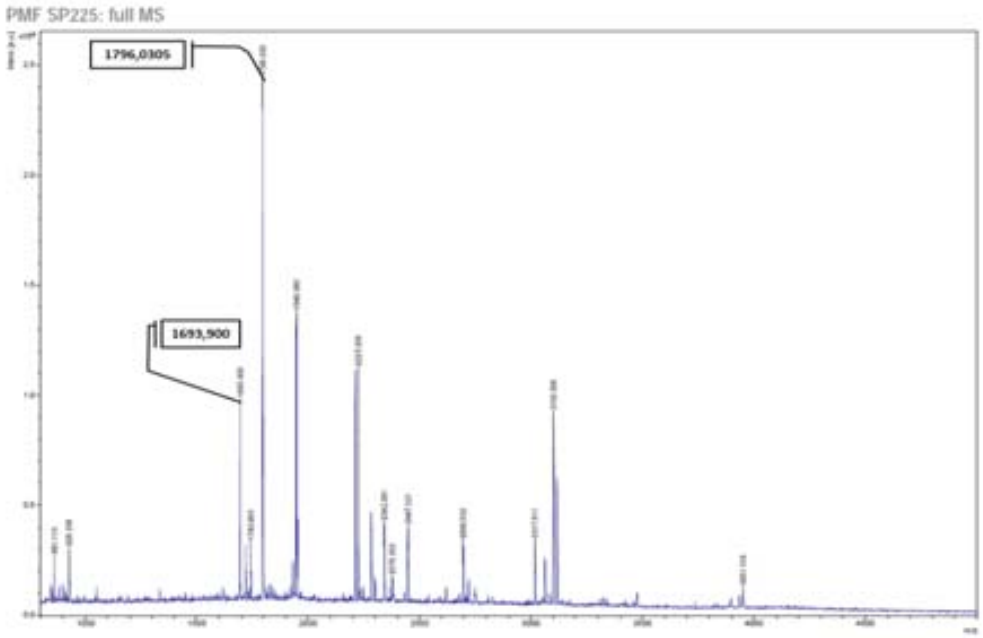


Figure 33. Chromatograph of the full MS spectrum of the band 225. In boxes are the two peaks selected for the full MS-MS analysis.

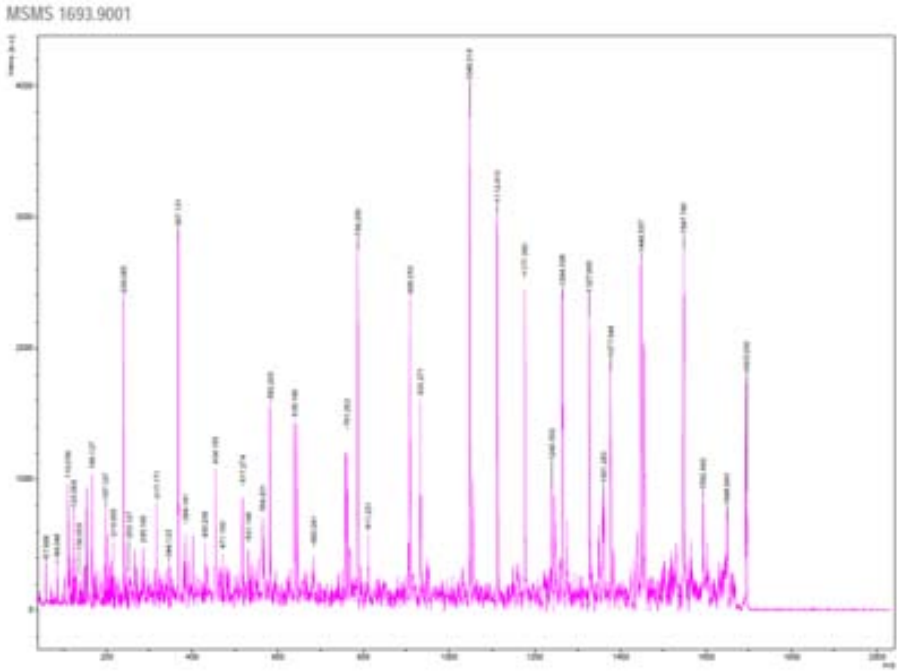


Figure 34. Analysis of the spectrum MS225 and the MS-MS peak corresponding to the fraction 1693.900.

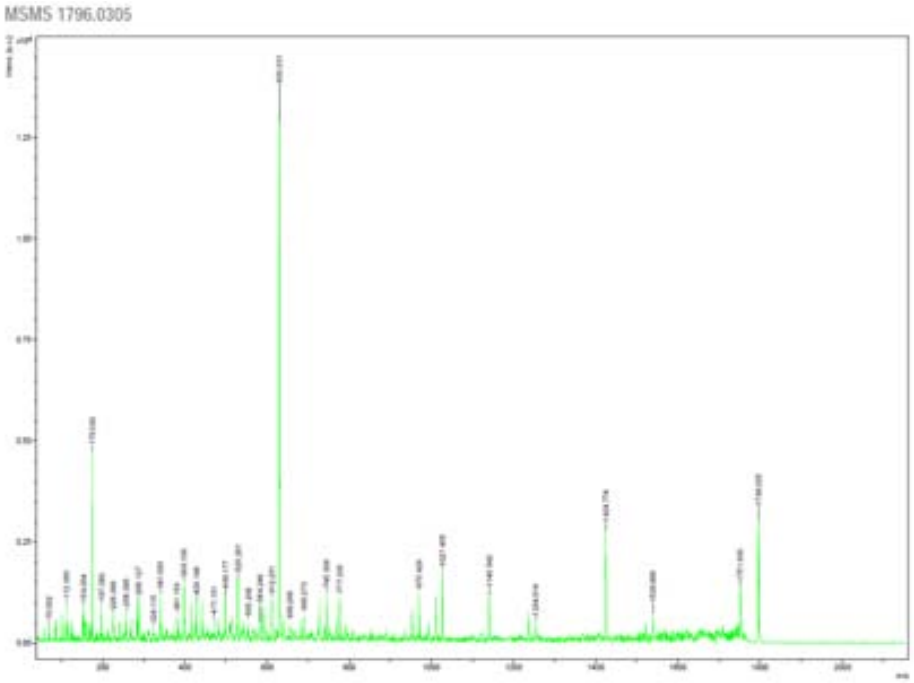


Figure 35. Analysis of the spectrum MS225 and the MS-MS peak corresponding to the fraction 1796.0305

MASCOT analysis of the spectra from protein 225 identified the protein as a hypothetical protein from *H. parasuis* SH0165 (YP_002475997.1) with a score of 335 and HPS_05483, a putative outer membrane protein of *H. parasuis* 29755 (EDS25200.1), with a score of 315 (protein scores greater than 85 are significant [$p<0.05$]).

4.9.1. Identification and analysis of the hypothetical protein HPS_05483.

The sequence of the hypothetical protein of *H. parasuis* HPS_05483 (Figure 36) was analyzed for phosphatase domains, but no hit was detected. BLASTP found that the protein belongs to the superfamily cl01196 and the putative conserved domain BOF (Bacterial [oligonucleotide/oligosaccharide binding] OB fold) was detected, with unknown function. Analysis of the predicted binding site of BOF family proteins implies that they lack nucleic acid-binding properties. They contain a predicted N-terminal signal peptide which indicates that they localize in the periplasm where they may function to bind proteins, small molecules, or other typical OB-fold ligands. As hypothesized for the distantly related OB-fold containing bacterial enterotoxins, the loss of nucleotide-binding function and the rapid evolution of the BOF ligand-binding site may be associated with the presence of BOF proteins in mobile genetic elements and their potential role in bacterial pathogenicity. In addition, Delta-BLASTP identified homology to YgiW from various bacterial species.

MKKAIILTTLLAVSTVSMAKGDDDYRHGGYHDGKARTHQSQGFFDESIAVKS
VADALKAADDTPALLEGQIVKQIDKDEFIFKDATGEIEIDVSKRAWNGLNIGPQ
DTIQIRGKVDNDWNKVQVDVKQITKK

Bacterial OB fold (BOF) protein

E-value: 1.6e-31

```
#HMM     TEAVQGGFQGPEAKKTTVKAAKEAADDASVVLEGNIVKQIDDDEYVFRDASGEIKVDIDDKVFNGQEVQPKDKVKIEGEVDKDLEKAEIDVKAIEK
#MATCH             ++ GF +  + +V+ A++AADD++ LEG+IVKQID+DE++F+DA+GEI++D+++++++NG ++ P+D+++I+G+VD+D++K++DVK+I+K
#SEQ       RTHQSQGFFDESIAVKSVADALKAADDTPALLEGQIVKQIDKDEFIFKDATGEIEIDVSKRAWNGLNIGPQDTIQIRGKVDNDWNKVQVDVKQITK
```

Delta-BLAST

Expect: 2e-63

```
HPS   1   MKKAIILTTLLAVSTVSMAKGDDDYRHGGYHDGKARTHQSQGFFDESIAVKSVADALKAA  60
          K A I+ +  ST +A     GG+   A  Q  GF  + +  +V  A
YgiW  2   KKLAAIVAVMALCSTPVLAAQ-----QGGFSGPSATQTQGGGFVGPNGSSTTVESAKSLR  56

HPS   61  DDTPALLEGQIVKQIDKDEFIFKDATGEIEIDVSKRAWNGLNIGPQDTIQIRGKVDNDWN  120
          DD   L G IV++I  D ++FKDA+G I +D+  + WNGL + PQDT++I+G+VD DWN
YqiW  57  DDAWVTLRGNIVERISDDLYVFKDASGTINVDIDHKRWNGLTVTPQDTVEIQGEVDKDWN  116

HPS   121 KVQVDVKQITKK  132
          V++DVKQI K
YgiW  117 SVEIDVKQIRKV  128
```

Figure 36. Predicted sequence of the hypothetical protein HPS_05483 (top), homology with the BOF motif (center) and delta-blast homology with YgiW from *Salmonella* . HMM: Hidden Markov Models (motif definition). HPS: sequence HPS_05483. YqiW: sequence YqiW from *Salmonella enterica*.

Sequence analysis by SignalP detected a predicted signal peptide in the protein (Figure 37).

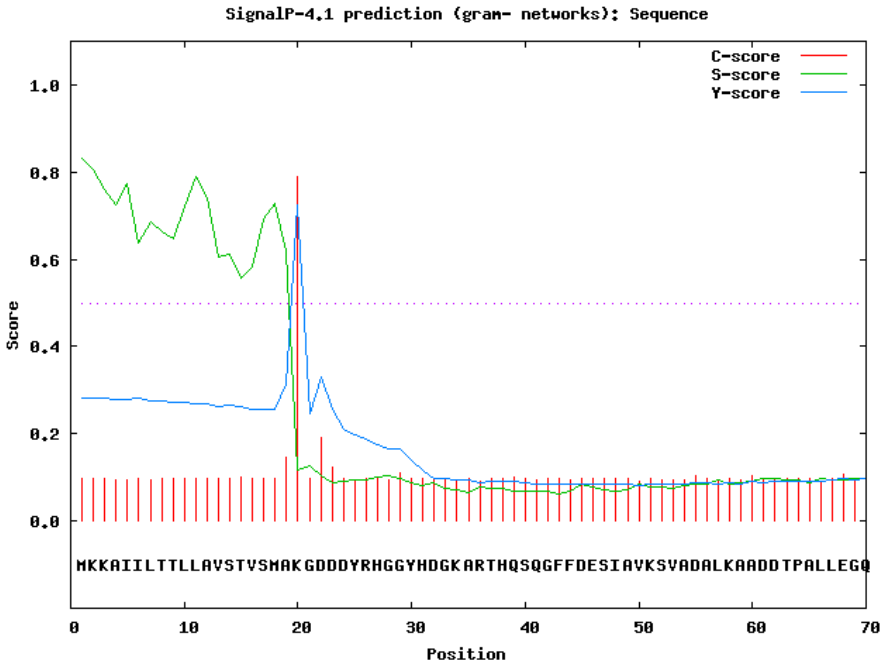


Figure 37: Detection by SignalP of the predicted signal peptide of the hypotetical protein HPS_05483 of *H. parasuis*.

From the gene sequence, primers were designed for the amplification of the gene, with or without the putative promoter region (Annex 4). Cloning of the gene without promoter into pGem-T Easy was possible, but no activity was observed. When the amplicon included the putative promoter region it was not possible to clone the fragment.

Cloning in the Gateway system was not problematic, since the first clone is done with no expression of the insert and once in the destination vector the expression is controlled. This way, a His-tagged HPS_05483 protein was purified and used in further analysis.

4.10. Production and analysis of mAbs against the acid phosphatase.

Mice were immunized with a supernatant of *H. parasuis* grown in BHI with high acid phosphatase activity. Initially, mouse sera were checked against a supernatant of *H. parasuis* produced in PPLO, in order to eliminate background reactions against the proteins in the medium. ELISA results of the four immunized mice are presented in Figure 38.

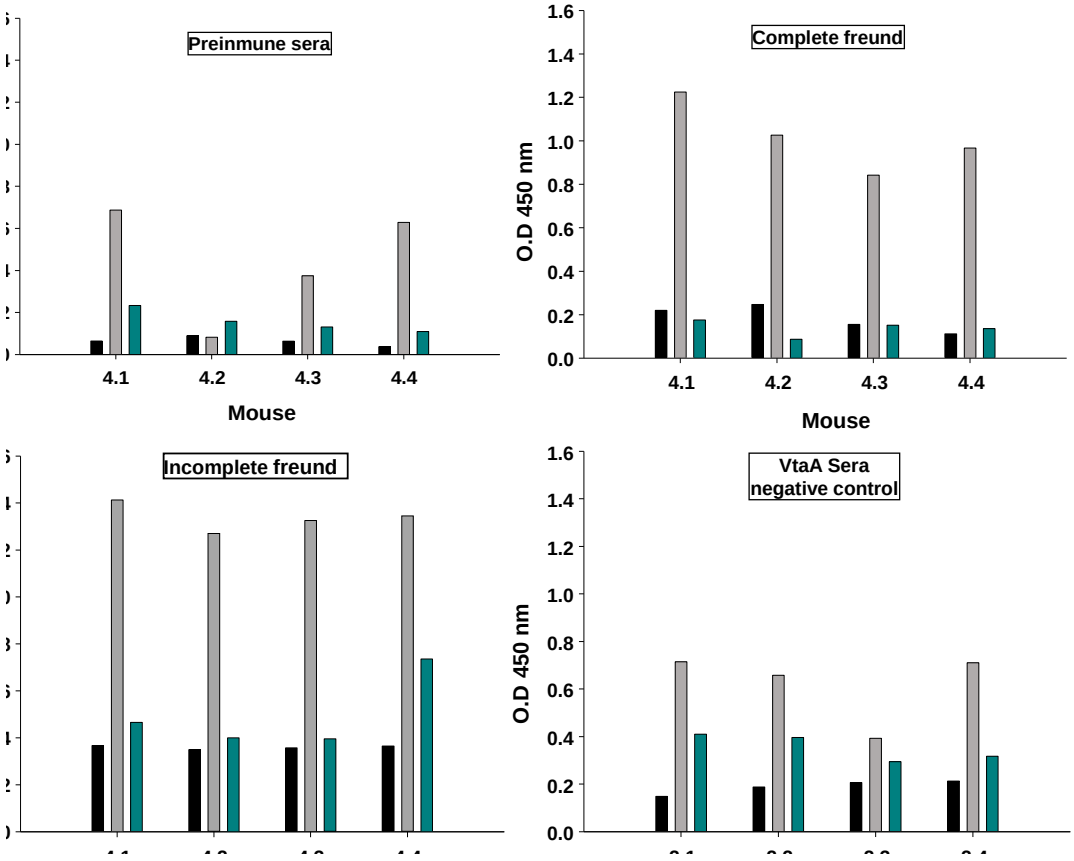


Figure 38. Evaluation of the reactivity of the sera from immunized mice with an acid phosphatase positive supernatant of the strain CT175-L produced in BHI. The mouse serum was checked in ELISA with the same supernatant (gray bars) or supernatant produced in PPLO (also positive to acid phosphatase; black bars). As an additional control, fresh BHI was also used (blue bars). Pre-immune sera, sera after the first immunization (complete Freund) and after the second immunization (incomplete Freund) were tested. The sera from mice immunized with an unrelated antigen (VtaA serum) were also included for comparison.

The four mice reacted against the proteins present in the BHI broth used in the inoculum, but a reaction against secreted proteins was also detected (Figure 38). Due to the higher difference between the reaction against the BHI culture supernatant and the reaction to fresh BHI of mouse 4.1, this mouse was selected to produce monoclonal antibodies (mAb). In order to avoid the background due to the medium used to produce the supernatant, a culture supernatant produced in PPLO was used in the selection of the mAb. Thus, two fusions were performed from the spleen of mouse 4.1 to obtain the hybridromas. The first fusion yielded nine positive hybridromas and the second fusion yielded eighteen positive hybridromas (Table 2).

Table 2. Selected hybridoma clones and sub-clones obtained in the first and second *fusion.*

First fusion		**Second fusion**	
Clones mAbs	**Sub-clones**	**Clones mAbs**	
B54F	B54C-9G*	P11P5B6	P11P5H6
B54C*	B54C-9F*	B2P3C6	P11P5E7
C5F5	B54C-9B	F7P5H8	P11P5E6
B5C5*	B54C-9C	F7P5D8	P11P5C7
C5B5	B54C-9D	P11P5C6	P11P5B6
C5B7	B54C-9E	P11P5D7	P11P5B7
C5C5	B54C-10C	P11P5E8	P11P5A7
B5B7	B54C-10D	P11P5D9	P11P5G6
B54G*	B54C-10E	C3P3F8	P11P5P6
* Purified mAbs			

4.10.1. Specificity of selected mAbs against AphA

After selection of hybridomas with PPLO supernatant, a second screening with protein AphA was performed. Lysates of clones BL21 pGem-T Easy *aphA* ER-6P#3 and ER-6P#6 (see section 4.4.1) were used as antigen in ELISA. No differences in reaction with respect to the negative control ER-6P#10 (clone with empty pGem-T Easy) were observed with mAbs C5B5, C5B7, B5B4, B54C, C5 and B54F. In a second screening, supernatant from clone BL21 pGem-T Easy *aphA* FL3-1#19 was used as antigen in ELISA, since this clone secreted the acid phosphatase activity to the culture supernatant (Figure 39). In addition, this supernatant was used in western blot (Figure 40).

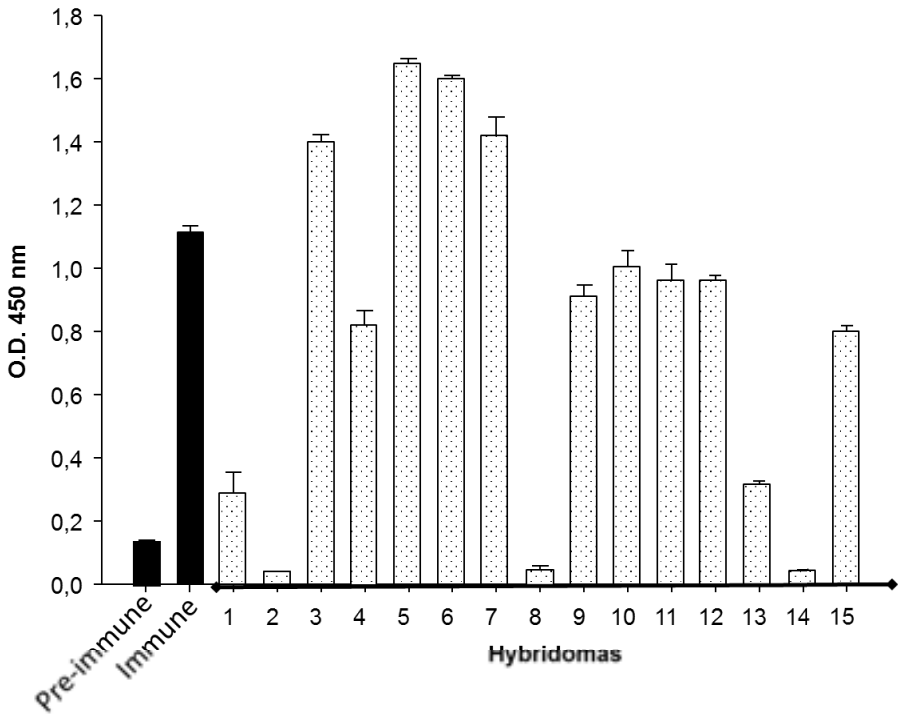


Figure 39. ELISA results of selected hybridomas with supernatant of clone BL21 pGem-T Easy *aphA* FL3-1#19. Pre-immune and immune sera from mouse 4.1 were included as control. Hybridomas tested were: C5 (1), B54F (2), C5B7 (3), B5E5 (4), B5B4 (5), B54C (6), C5C5 (7), B57B (8), C5B5 (9), B5B7 (10), B54G (11), C5F5 (12), B54G (13), B55E (14) and B5C5 (15).

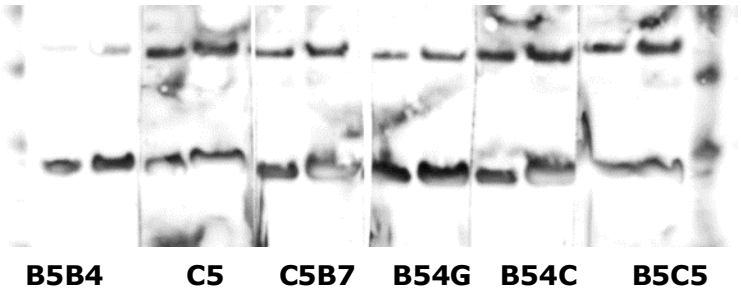


Figure 40. Western blot with supernatant of clone BL21 pGem-T Easy AphA FL3-1#19 and hybridomas B5B4, C5, C5B7, B54G, B54C and B5C5.

Antibodies produced by hybridomas C5, C5B7, B54G, B54C and B5C5 detected two protein bands in the western blot. The apparent molecular weights of the proteins were compatible with the detection of the monomeric and dimeric form of AphA. Actually, class B acid phosphatases are known to form tetramers, so this finding is in agreement with the current knowledge about this type of phosphatases.

4.10.2. Specificity of selected mAbs against PgpB

Selected hybridomas were tested in ELISA and western blot with whole bacteria of clone BL21 pCR2.1-TOPO TA *pgpB* ER-6P#1, as well as BL21 carrying the empty pGem-T Easy as negative control. The results from ELISA (Table 4) and western blot (not shown) demonstrated that the selected mAbs are not specific for epitopes of PgpB.

Table 3. ELISA results with some supernatants of hybridomas against the PgpB clone (Topo+pgpB). Background reaction was observed with an empty clone (pGem-T Easy). Pre-immune and immune sera of the mice 4.1 were included as controls. *Purified antibodies.

PgpB		
mAb	**Topo+ *pgpB***	**pGem-T Easy**
*B54G**	0.090	0.114
*B54C9F**	0.053	0.063
*B5C5**	0.06	0.071
*B54C9G**	0.069	0.087
B54C10E	0.166	0.168
B54C9E	0.255	0.264
B54C10D	0.145	0.147
B54G	0.245	0.260
B54C9C	0.218	0.227
B54C10C	0.199	0.216
B54C	0.171	0.189
B54C9D	0.125	0.130
B55C	0.189	0.227
Pre-immune	0.054	0.073
Immune	0.057	0.069

4.10.3. Specificity of selected mAbs against HPS_05483

Selected MAb were also checked for reaction against the protein HPS_05483, identified by chromatography and MALDI-TOF-TOF. Fractions 8 and 9, with phosphatase activity (Figure 32), were used as antigen in ELISA. The ELISA data in the Table 3 show that the selected mAbs could not specifically recognize the hypothetical protein HPS_05483 present in the fractions with activity. A fraction from the chromatography without activity was used as negative control in this ELISA. Also, the pre-immune and immune sera from mouse 4.1. were used as control, but no reaction was observed either.

Table 4. ELISA data of some supernatants of hybridomas against fraction 8 and 9 from the chromatography in Figure 30, containing the hypothetical protein HPS_05483. As controls pre-immune and immune serum of the mouse 4.1 were included. Reaction against a fraction from the chromatography with no phosphatase actiovity (negative fraction) was also checked as control. *Purified mAbs.

Hypothetical protein Hps-05483		
mAb	**Fractions 8 and 9**	**Negative fraction**
*B54G**	0.049	0.049
*B54C9F**	0.046	0.046
*B5C5**	0.047	0.050
*B54C9G**	0.049	0.052
B54C10E	0.081	0,076
B54C9E	0.162	0.126
B54C10D	0.071	0.070
B54G	0.175	0.149
B54C9C	0.137	0.106
B54C10C	0.113	0.103
B54C	0.084	0.075
B54C9D	0.059	0.061
B55C	0.102	0.088
Pre-immune	0.084	0.100
Immune	0.142	0.193

Then a western blot was performed with recombinant His-Tagged HPS_05483. The results from the western blot show that none of the purified mAbs nor the supernatants from growing hybridomas recognized the protein (Figure 41). Nevertheless the serum from the immunized mouse 4.1. showed a positive reaction against the hypothetical protein HPS_05483 of *H. parasuis*, indicating that the protein is probably present in the supernatant used in the production of the mAbs.

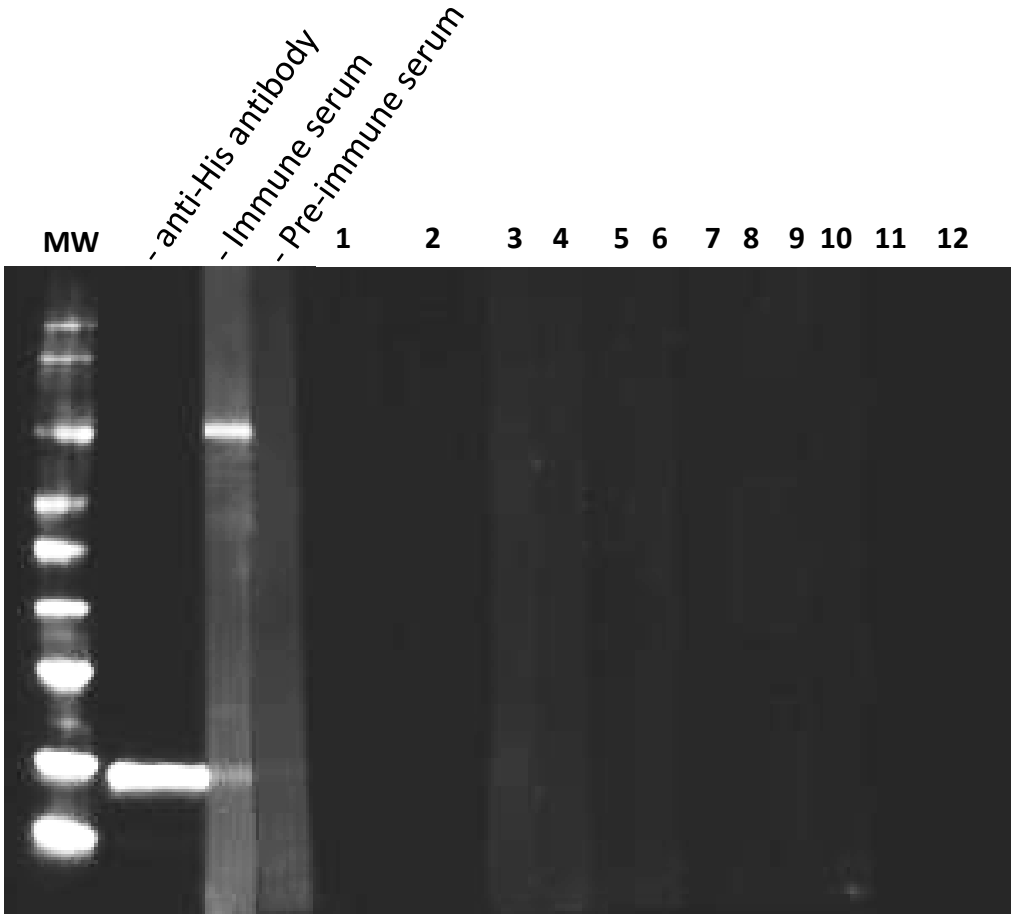


Figure 41. Western blot with the hypothetical protein HPS_05483 and different mAbs. Recombinant His-tagged HPS_05483 was analyzed in SDS-PAGE and transferred to nitrocellulose to check the reaction of purified mAbs (100 ng/µl) B54G (lane 1), B54C9F (lane 2), B5C5 (lane 3), B54C9G (lane 4); Supernatant of hybridomas**,** B54C10C (line 5), B54C9C (line 6), B54C9D (line 7), B54C10E (line 8), B54G (line 9), B54C10D (line 10), B54C (line 11), B54C9E (line 12). Pre-immune and immune sera were included as control. As an additional control, an anti-His antibody was also used. MW. BenchMark™ His-tagged Protein Standard.

In addition, the reaction of pig sera was evaluated in western blot. Infected pigs showed a reaction against HPS_05483 protein (Figure 42), indicating that this proteins is expressed during infection.

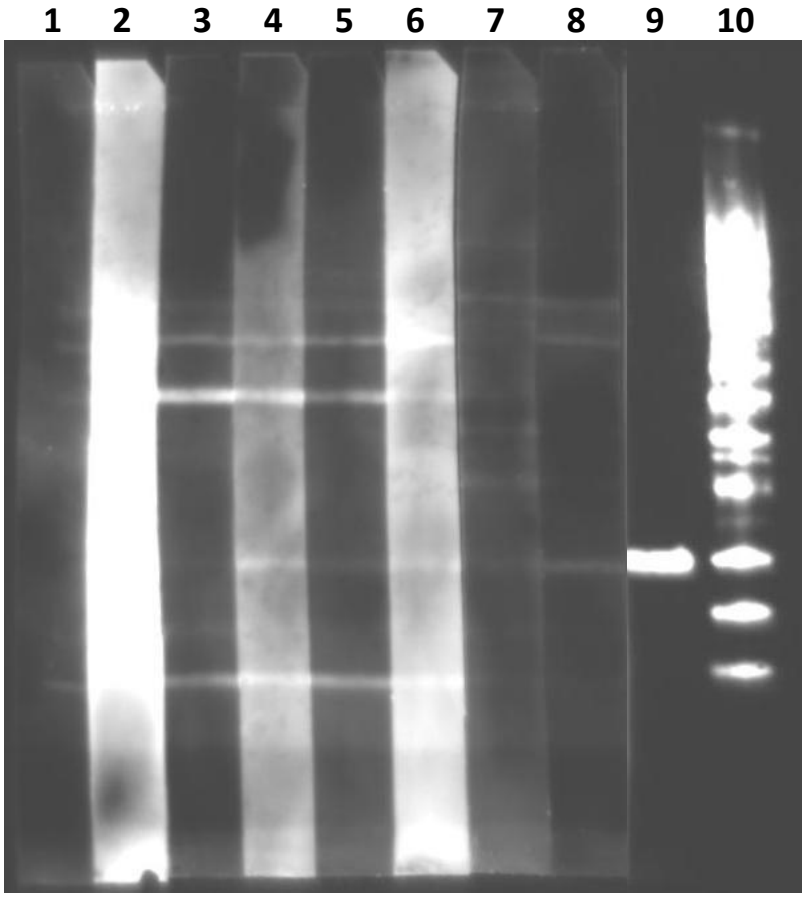


Figure 42. Western blot with porcine sera from animals infected with Nagasaki. Pre-infected sera were included (lanes 1, 3, 5 and 7), together with the sera from 15 days post-infection (lanes 2, 4, 6 and 8). As a control, an anti-His antibody was also used (lane 9). Lane 10, BenchMark™ His-tagged Protein Standard.

4.11. Effect of the secreted phosphatase activity of *H. parasuis* on alveolar macrophages.

In order to get an insight into the function of the secreted acid phosphatase in the biology of *H. parasuis*, the effect of supernatants on porcine alveolar macrophages was examined. Macrophages were examined in selected surface marker levels by flow cytometry. The incubation of the macrophages with active supernatant from strain ER-6P during 4h or overnight did not produce a significative effect on the levels of CD163, SLAI, SLAII, sialoadhesine or SWC3 (Figure 43).

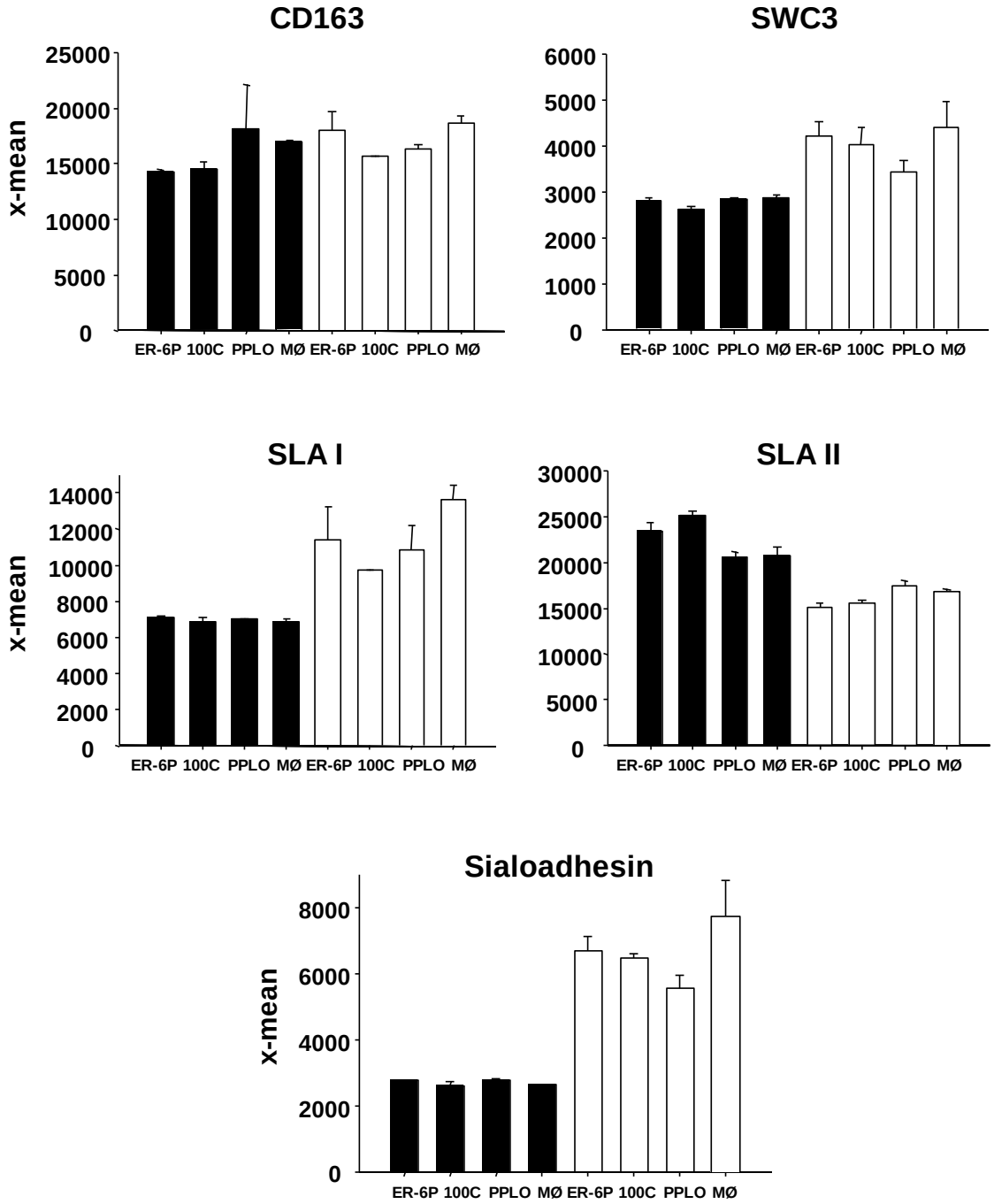


Figure 43. Levels of surface markers CD163, SWC3, SLAI, SLAII and sialoadhesin (expressed as x-mean) in alveolar macrophages after incubation with supernatant from strain ER-6P (ER-6P), heat inactivated supernatant (100C) or fresh PPLO medium (PPLO) for 2h (black bars) or overnight (white bars). The levels of the markers in macrophages without treatment (MØ) are included as reference.

Finally, the same experiment was performed but using the supernatant from clone BL21 pGem-T Easy *aphA* FL3-1#19, and again no effect on the macrophage surface markers was observed (Figure 44)

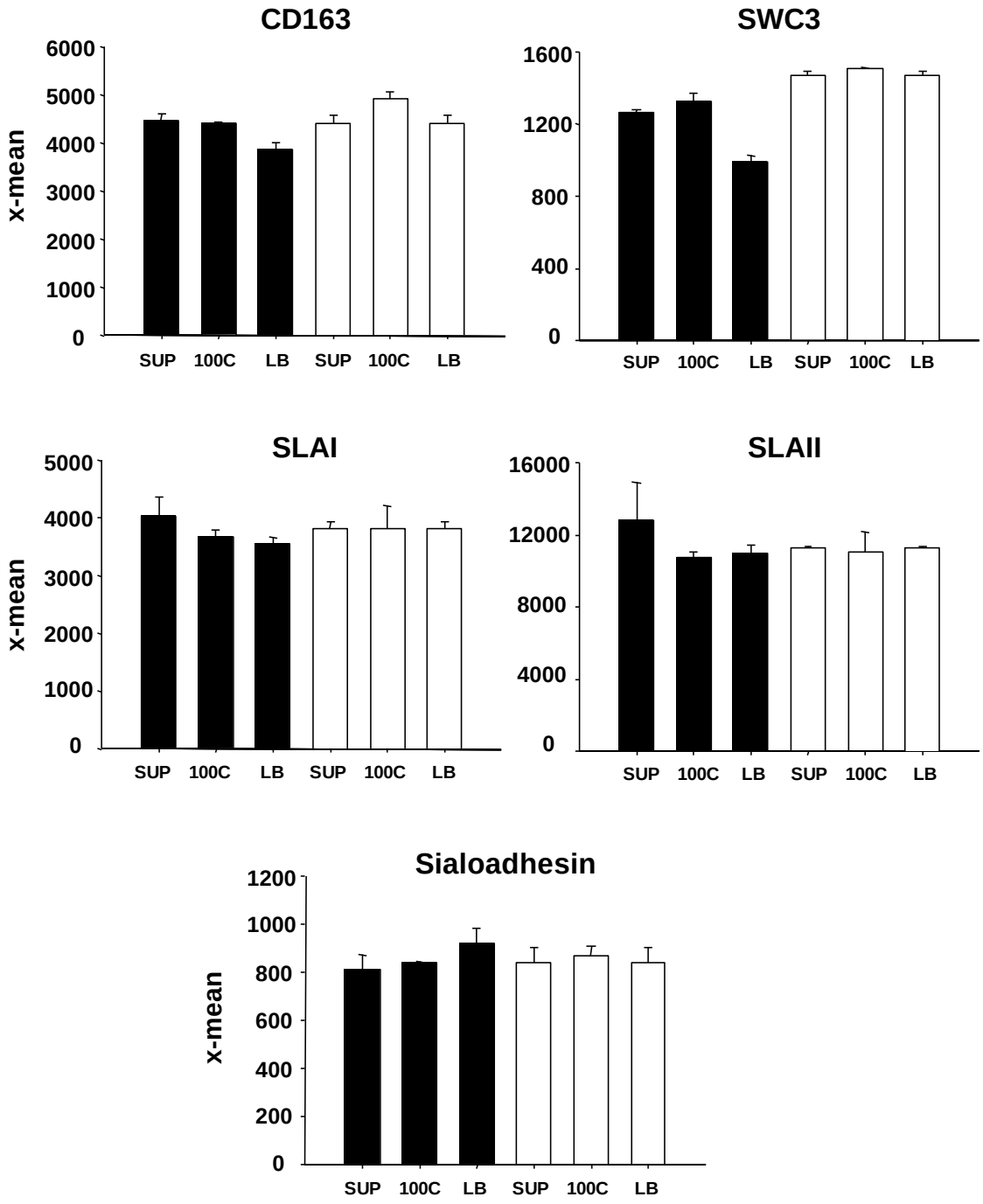


Figure 44. Levels of surface markers CD163, SWC3, SLAI, SLAII and sialoadhesin (expressed as x-mean) in alveolar macrophages after overnight incubation with 300 µl (black bars) or 30 µl (white bars) of supernatant from clone BL21 pGen-T Easy FL3-1#19 (SUP), heat inactivated supernatant (100C) or fresh LB medium (LB).

5. DISCUSSION

5. DISCUSSION.

Acid phosphatases are a family of enzymes that have been shown to play an important role as mediators of infection and therefore these enzymatic activities could be linked with disease development or survival inside the host. Several reports of bacterial acid phosphatases can be found in the literature, including acid phosphatases of the pathogens *Legionella micdadei* (Saha et al., 1985), *Bordetella bronchiseptica* (Jungnitz et al., 1998), *Francisella tularensis* (Reilly et al., 1996), *Moraxella catarrhalis* (Hoopman et al., 2008), *Legionella pneumophila* (Aragon et al., 2001), *Mycobacterium tuberculosis* (Saleh and Belisle, 2000) and *Staphylococcus aureus* (du Plessis et al., 2002). Also, previous results in our group showed the presence of a secreted acid phosphatase in the culture supernatant of some strains of *H. parasuis*.

H. parasuis secretes an acid phosphatase activity at the beginning of the stationary phase of growth in the laboratory and was found both in virulent and non-virulent strains, indicating that this is not directly related to virulence. Interestingly, this activity was lost after several passages in agar plates, indicating a probable role in survival inside the host. Thus, our goal was to examine the specific nature of the activity; i.e., identify the molecule responsible for the phosphatase activity to better define its role in infection. Different approaches were used to unveil the acid phosphatase gene (screening of a genomic library and subsequent study of the positive clones) or the acid phosphatase protein (zymogram or chromatography, followed by MALDI-TOF-TOF). The first approach allowed us to identify the genes phosphatidylglycerol phosphate (*pgpB*) phosphatase and the type B non-specific acid phosphatase *aphA*.

PgpB is a phosphatidyl glycerophosphate phosphatase, but it also shows phosphatidic acid and lysophosphatidic acid phosphatase activity. This family of enzymes is widely distributed and diverse in function. Distant members include the Gram-negative lipid A phosphatases (Wang et al., 2006) and the eukaryotic sphingosine phosphate and lyso-phosphatidic acid phosphatases, which are located in the plasma membrane and modulate signal transduction (Sigal et al., 2005). After cloning the *H. parasuis pgpB*, phosphatase activity was observed only

in the bacterial cells but not in the supernatant, in agreement with the detection of transmembrane domains, lack of signal peptide and similarity to PgpB in other bacteria (Icho, 1988). The localization of the enzymatic activity in the bacterial cell, together with the optimal pH observed, make very unlikely that this gene is the responsible for the secreted activity observed in the culture supernatants of *H. parasuis*.

With respect to Apha, we were able to demonstrate phosphatase activity by cloning the *H. parasuis aphA* in the heterologous host *E. coli*. Analysis of the predicted protein sequence also supported the identification of the protein as a phosphatase, since it showed the catalytic domain characteristic of type B acid phosphatases. In addition, the inhibition of the Apha activity by EDTA is compatible with the requirement of divalent cations by type B phosphatases. A signal peptide was detected in the predicted protein, but the protein was not secreted by *E. coli* with the exception of one clone (pGEM-T Easy *aphA* FL3-1#19) and AphA was not detected in supernatants of *H. parasuis* after size exclusion chromatography. In addition, the different optimal pH and different susceptibility to EDTA and molybdate, with respect to those observed for the activity in *H. parasuis* supernatant, indicate that probably Apha is not the protein of interest. These results are consistent with the localization of AphA in the periplasm of the bacteria, as it has been described in *E. coli* (Calderone et al., 2006; Passariello et al., 2006). However, mAb produced by immunization of mice with supernatant of *H. parasuis* showed reaction against AphA. These results could be explained by release of Apha to the supernatant by partial lysis of *H. parasuis* during growth in the supernatant used for immunization, or by cross-reaction of AphA with the secreted phosphatase present in the supernatant. Alternatively, in *H. parasuis* Apha is secreted to the medium but losses activity in the process of purification by chromatography and therefore it cannot be detected in the eluted fractions. The *E. coli* strain used in the cloning experiments may not have the necessary machinery for complete secretion of specific effectors. Further experiments are required to identify the mechanisms by which AphA is secreted in *H. parasuis*. The results of clone pGEM-T Easy *aphA* FL3-1#19 do not have a clear explanation, since the only difference with other clones of the same gene was the direction of the insert in the vector.

Both genes, *pgpB* and *aphA*, were expressed in *E. coli* demonstrating phosphatase activity, but the determination of their optimal pH and their characterization indicated that they cannot be accounted for the activity found in the supernatant of *H. parasuis*. Supporting this conclusion, the cell-bound phosphatase activity observed in *H. parasuis* presented two peaks of maximal activity at pHs that approximately corresponded to the optimal pH of AphA and PgpB.

Characterization of the phosphatase activities in this work was performed mainly by examining the activity at different pH, since pH is known to change the ionization of amino-acids and the enzymatic activity usually varies with pH changes (Dixon and Purdom, 1954). In this work, the optimal pH for *H. parasuis* Apha was higher than expected for an acid phosphatase and further studies need to be performed to confirm this finding with a natural substrate. Also PgpB presented an optimal pH lower than that reported by other authors (Touzé et al., 2008). However it is necessary to be cautious in the interpretation of the pH results and the direct correlation to the identity of the corresponding phosphatase, unless confirmed by other methods. It is known that, for example, the microenvironment in which a particular amino-acid residue exists, as a part of an enzyme, may affect the pH values as much as 3 pH units from that found in free solution. Therefore the pH conditions of the *in vitro* assay are not comparable with the conditions found by the enzyme inside of the host. Nevertheless, such studies are useful since they are easy to perform and can give the first characterization of the protein and indicate which amino-acid residues should be considered as essential component of a particular active site.

The *apha* and *pgpB* gens were detected in *H. parasuis* strains of different clinical origin and therefore they may be partially associated with survival inside the host but not necessarily with virulence.

The second approach to identify the protein responsible for the phosphatase activity (purification by chromatography and identification by MALDI-TOF-TOF) yielded the detection of a hypothetical protein, HPS_05483, in the fractions with high acid phosphatase activity. This protein is expressed by *H. parasuis* during infection, as demonstrated by the presence of antibodies in pigs

infected with the virulent strain Nagasaki. However, with the data obtained in this thesis, we cannot assure that this protein is the one responsible for the activity found in *H. parasuis* supernatants, since we have not been able to prove so far that this protein has phosphatase activity. Further analyses are required to support that this hypothetical protein is an acid phosphatase. In addition, we did not select any mAb specific of HPS_05483, but this can be explained by the need to re-screen the original hybridomas with the purified protein. It is important to mention that the pre-screening of the monoclonal antibodies was performed with Apha and therefore the selection of mAbs against HPS_05483 and PgpB was impeded. It would be appropriate to conduct further assays starting from new fusions and a new screening of the hybridomas for each particular protein.

Dai et al. (2012) reported for first time an acid phosphatase secreted by *Francisella spp in vivo* and also its ability to translocate this enzyme into the host macrophage cytosol during a macrophage infection. Our experiment in vitro with alveolar macrophages of pigs did not show significative effect of phosphatase-positive supernatants of *H. parasuis* on the levels of CD163, SLAI, SLAII, sialoadhesine or SWC3 and likewise no effect on the surface markers was observed in the supernatant from clone BL21 pGem-T Easy *aphA* FL3-1#19. These phosphatases may not play any role in the interaction with macrophages, but also it may be necessary the contact with the whole bacteria for translocation of the enzyme. To examine the latter situation, mAb against the specific enzyme would be essential.

Thus, the combined evidence suggests that the phosphatases discovered in this work are not fundamental in *H. parasuis* virulence, although, at least the secreted phosphatase, may have a role in survival during infection. The selection of specific mAb against each enzyme could help determining the expression of these enzymes by *H. parasuis* at different steps of the infection. Although much remains to be determined, the results of this study advance our understanding of acid phosphatases in *H. parasuis* for the first time and bring us closer to understanding *H. parasuis* biology.

6. CONCLUSIONS

6. CONCLUSIONS.

1. An acid phosphatase activity is secreted by some *H. parasuis* strains with a secretion peak at early-stationary phase of growth.

2. The production of a genomic library containing small fragments (4-5 Kb) of *H. parasuis* genome provides a useful tool to identify genes with phosphatase activity.

3. *H. parasuis* phosphatidylglycerophosphatase B (PgpB) is an acid phosphatase with six predicted transmembrane domains, which explains the location of this phosphatase in the bacterial cell.

4. *H. parasuis* Apha is a phosphatase with the characteristic catalytic domains of type B bacterial acid phosphatases.

5. The secreted acid phosphatase activity, PgpB and Apha are present in virulent and non-virulent strains and therefore they may not be related to the virulence of *H. parasuis*.

6. The hypothetical protein HPS_05483 was the only protein detected in samples with acid phosphatase activity, but further studies are needed to define its function.

7. Supernatants of *H. parasuis* with phosphatase activity did not have any effect on surface markers CD163, SLAI, SLAII, sialoadhesin or SWC3 of alveolar macrophages.

8. Supernatant of *E. coli* expressing *H. parasuis* AphA did not have any effect on surface markers CD163, SLAI, SLAII, sialoadhesin or SWC3 of alveolar macrophages.

7. REFERENCES

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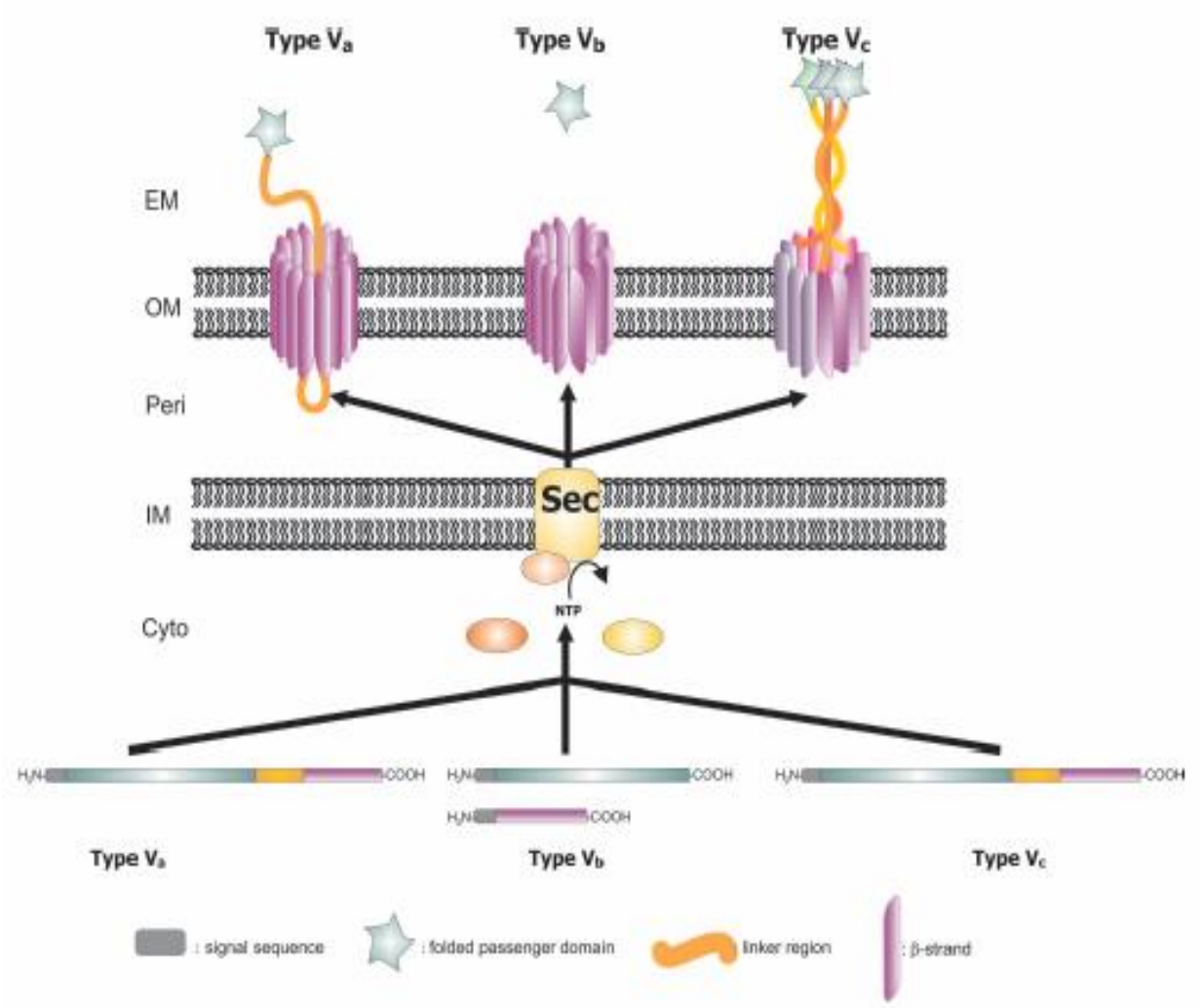
8. ANNEXES

8. ANNEXES.

ANNEX 1. Figure of the schematic representation of the type V secretion system



Schematic representation of the type V secretion system.

Cyto: cytoplasm; IM: inner membrane; Peri: periplasma; OM: outer membrane; EM: extracellular milieu. From: (Henderson et al., 2004).

ANNEX 2. Summary of different strains used of *Haemophilus parasuis* in this study and their main characteristics.

Strain	Serovar	Susceptibility* to:		Disease status	Reference or source
		Serum	Phagocytosis		
Strains from lesions					
SHO165	5	nd	nd	Glässer's disease	Sequence available in GenBank accession No. CP001321 (Xu et al., 2011)
Nagasaki	5	R	R	Glässer's disease	Reference strain (Kielstein & Rapp-Gabrielswon, 1992; Kielstein P, 1992)
ER-6P	15	R	R	Glässer's disease	Cerdà-Cuéllar & Aragon (2008)
IT29205	4	R	R	Glässer's disease	Aragon *et al.* (2010)
373/03A	7	I	R	Glässer's disease	Aragon *et al.* (2010)
279/03	7	nd	nd		
264/99	10	R	R	Glässer's disease	Aragon *et al.* (2010)
2725	10	S	R	Glässer's disease	Aragon *et al.* (2010)
228/04	5	R	R	Glässer's disease	Olvera *et al.* (2006)
230/03	15	nd	nd		
CT175-L	15	nd	nd		
PV1-12	15	S	R	Glässer's disease	Aragon *et al.* (2010)
P015/96	5	R	R	Pneumonia	Olvera *et al.* (2006)
PC4-6P	12	R	R	Glässer's disease	Cerdà-Cuéllar & Aragon (2008)
9904108**	4	S	R	Glässer's disease	Aragon *et al.* (2010)
Nasal strains					
SW114	3	S	S	Healthy	Reference strain
F9	6	S	S	Healthy	Olvera *et al.* (2006)
IQ1N-6	9	S	S	Healthy	Cerdà-Cuéllar & Aragon (2008)
ND14-1	7	S	S	Healthy	Cerdà-Cuéllar & Aragon (2008)
FL3-1	7	nd	nd		
FL1-3	10	S	I	Healthy	Olvera *et al.* (2006)

CA38-4	12	R	R	Glässer's disease	Aragon *et al.* (2010)
MU21-2	7	S	S	Healthy	Olvera *et al.* (2006)
VS6-2	15	I	S	Healthy	Olvera *et al.* (2006)
SC14-1	15	S	S	Healthy	Olvera *et al.* (2006)
SL3-2	10	S	S	Healthy	Cerdà-Cuéllar & Aragon (2008)

- * S, Sensitive; I, intermediate; R, resistant.
- ** Did not reproduce disease when inoculated intranasally in colostrum-deprived piglets (Aragon *et al.*, 2010).
- nd: Not determined

ANNEX 3. Vectors used in this study

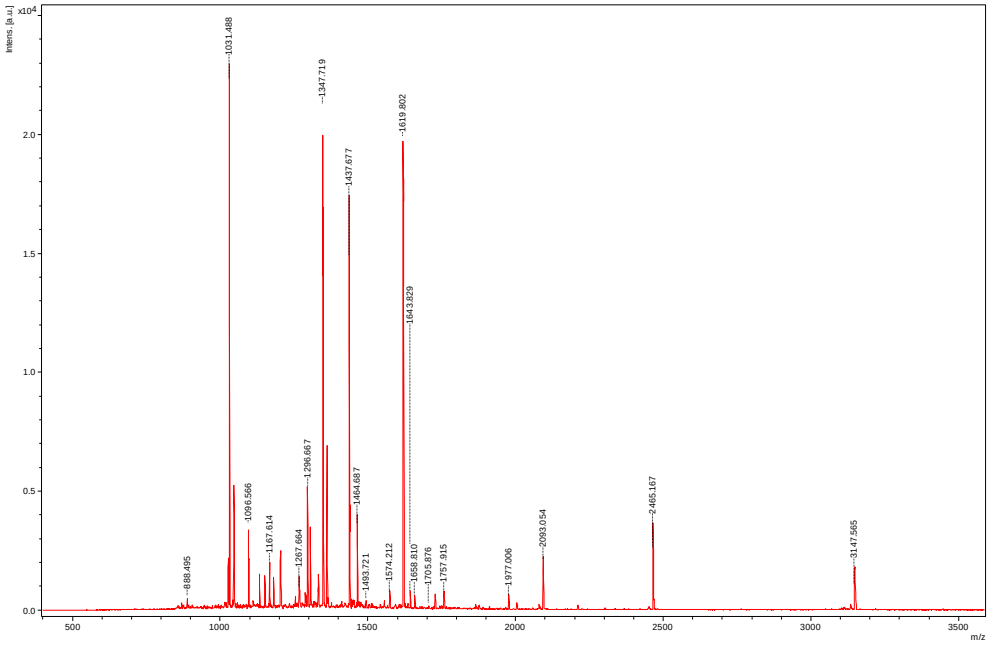
Vectors	Comments	Reference
pGEM-T Easy	Plasmid to clone fragments obtained by PCR with Taq polymerase.Ap^R	Promega
PCRII Topo 4.0 Kb	Vector used to clone fragments obtained by PCR	Invitrogen
pCR 8/GW/TOPO TA	Vector to clone PCR fragment. Entry vector for Gateway system	Invitrogen
pBAD-DEST49	Vector for regulated expression under Ara promoter. Destination vector	Invitrogen

ANNEX 4. Primers used in this study

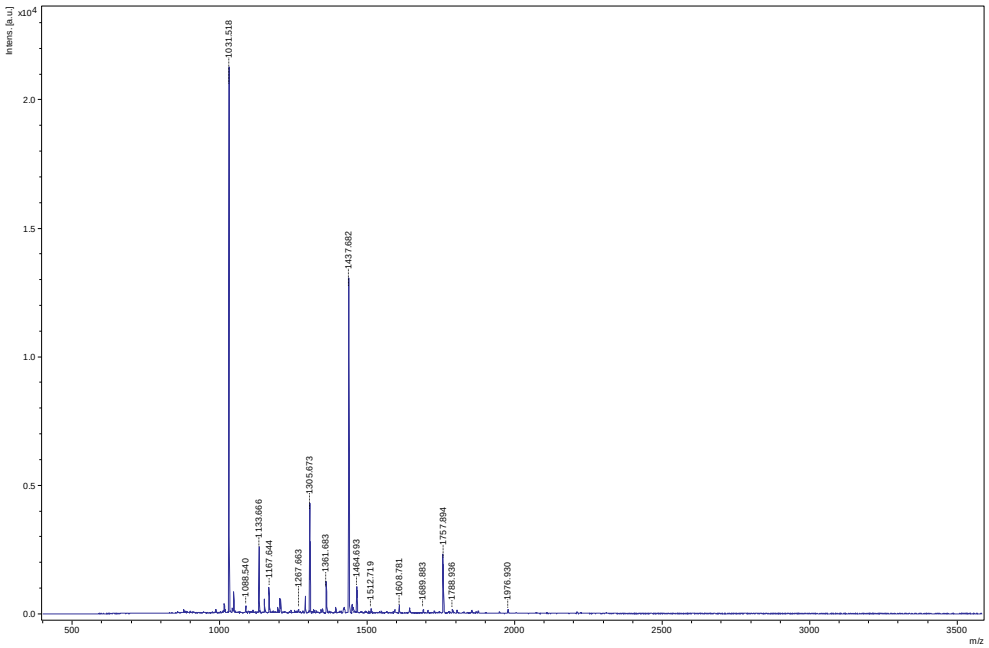
Primer name	Sequence (5´→ 3´)	Use
APho-Fw-prom	ATGTTCCCTATAACCTATTGTG	Forward primer for amplification *aphA* with promoter region
Apho-R	ATTAGTAGCTTGAATTTATAATAAC	Reverse primer for amplification of *aphA*
Pgp-F	TATTCATTATATTACAGAAACATTC	Forward primer for amplification *pgpB* with the promoter region
Pgp-R	CCAAACCGTAATAAACCATAG	Reverse primer for amplifycation of *pgpB*.
Hps 05483-F	TTAACTATTCGCAGTATTCTATG	Forward primer for amplification of HPS_05483 gene
Hps 05483-R	CAGACCGCTAGTGAGGATA	Reverse primer for amplifycation of HPS_05483 gene
Hps 05483-R-Non Stop	TTTCTTGGTAATCTGTTTTACATC	Reverse primer for amplifycation of HPS_05483 gene for expression in pBAD-DEST49
M13-F	GTAAAAGGACGGCCAG	Sequencing inserts in pGem-T Easy
M13-R	CAGGAAACAGCTATGAC	Sequencing inserts in pGem-T Easy

ANNEX 5. Figures of the different peaks obtained from the MALDI-TOF analysis performed to three protein bands obtained from zymogram.

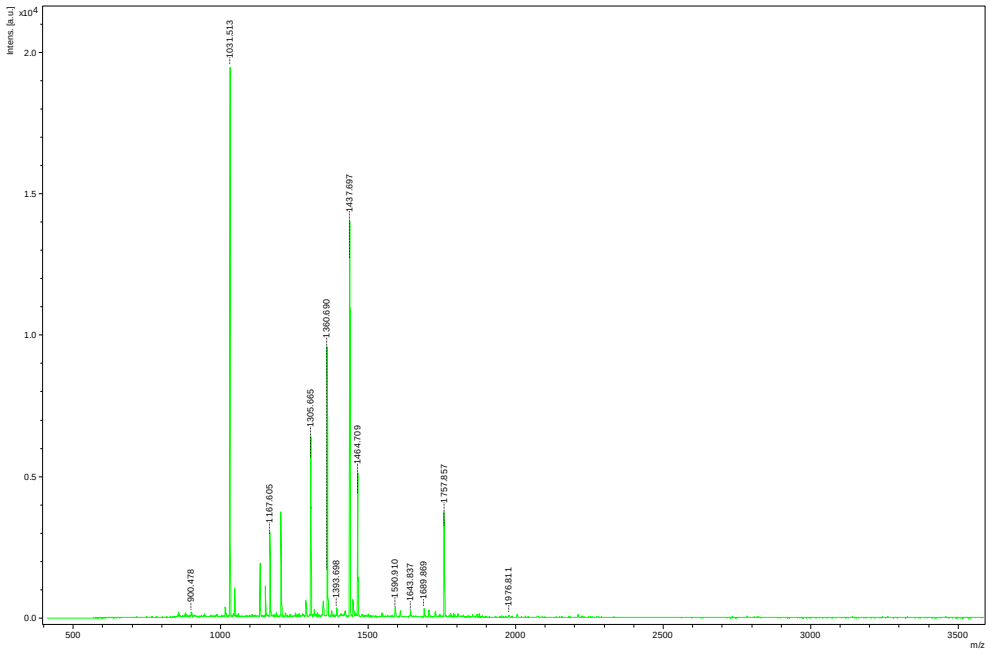
Band 1



Band 2



Band 3



- **The searching paramers used were:**

Database	**SwissProt 2010x (522019 sequences; 184241293 residues)**
Taxonomy	**Bacteria (Eubacteria) (324077 sequences)**
Type of search	**Peptide Mass Fingerprint**
Enzyme	**Trypsin**
Variable modifications	**Carbamidomethyl (C),Oxidation (M)**
Mass values	**Monoisotopic**
Protein Mass	**Unrestricted**
Peptide Mass Tolerance	**± 100 ppm**
Peptide Charge State	**1+**
Max Missed Cleavages	**3**

ANNEX 6. Report of the MALDI-TOF analysis performed to proteins 222, 223 and 224

SP-005B	Informe Resultados Espectrometria de masas: MALDI	Servei de Proteomica
UAB Universitat Autònoma de Barcelona / sePBioEs Servei de Proteòmica i Biologia Estructural	CODIGO INFORME:	PManrique_240513
	REALIZADO POR:	Sebastian trejo

MUESTRAS

CÓDIGO SERVICIO	CÓDIGO CLIENTE	CÓDIGO SERVICIO	CÓDIGO CLIENTE
SP222	Banda SDS-PAGE	SP223	Banda SDS-PAGE
SP224	Banda SDS-PAGE	SP225	Banda SDS-PAGE
SP226	Gel SDS-PAGE		

CONDICIONES EXPERIMENTALES

1- Digestión tríptica de las bandas de gel:

a) *Destinción: Bicarbonato amónico 50mM / ACN 50%*
b) *Reducción: DTT 20 mM /20 min /60ºC*
c) *Alquilación: Iodoacetamida 25 mM / 15 min / 37ºC*
d) *Digestión con tripsina: 25 ng/muestra (sequencing grade-Promega) 3h /37ºC*
e) *Elución: H_2O:ACN (1:1)+0.2%TFA*

2- Preparación muestra para su análisis por espectrometría de masas MALDI-TOF:

Placa: *Ground steel*
Matriz: *Ácido α-4-hidroxicinámico (hcca)*
Equipo: *MALDI-TOF UltrafleXtreme (Bruker Daltonics)*

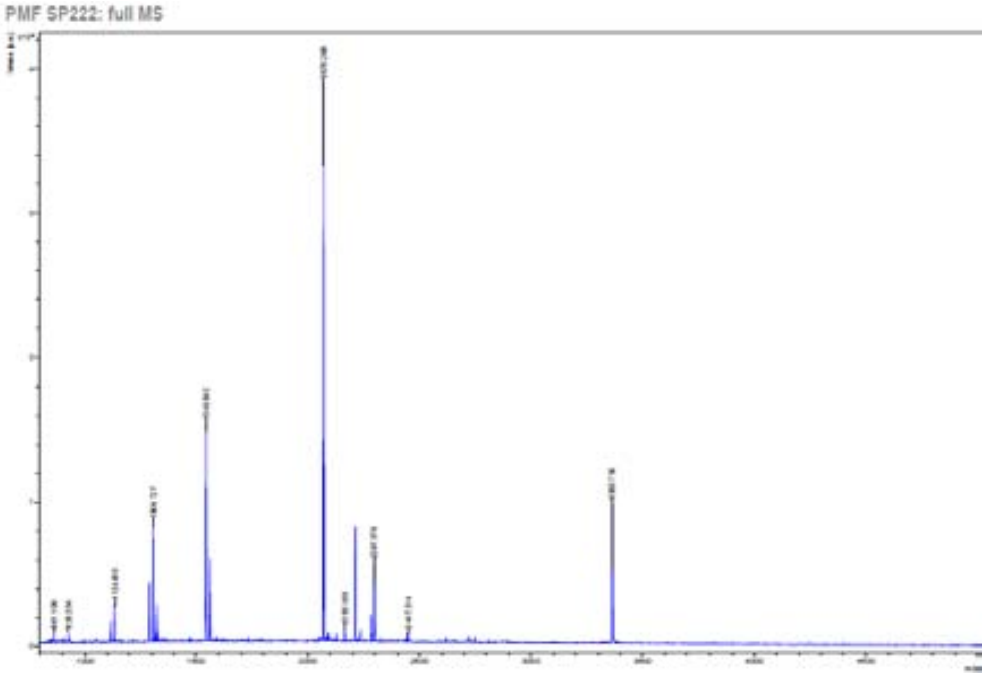
Se mezcla 1:1 muestra:matriz (0.5µL:0.5µL) y se deposita 1 µL sobre una placa ground steel. Se analiza la muestra usando un método en modo reflectrón y un voltaje de aceleración de 25kv. Los análisis se calibran usando calibradores externos

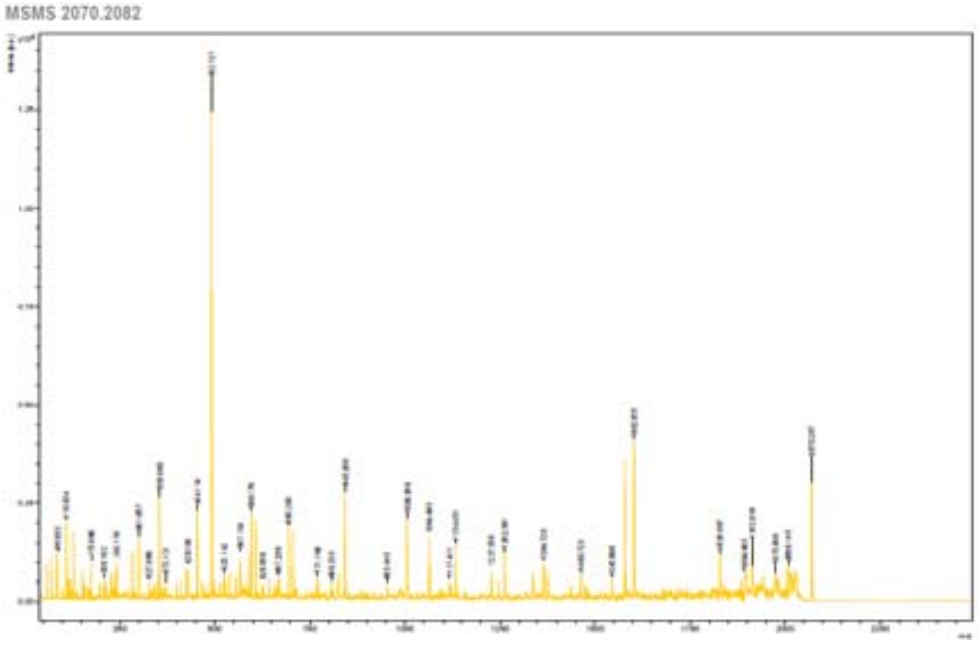
AGRADECIMIENTOS

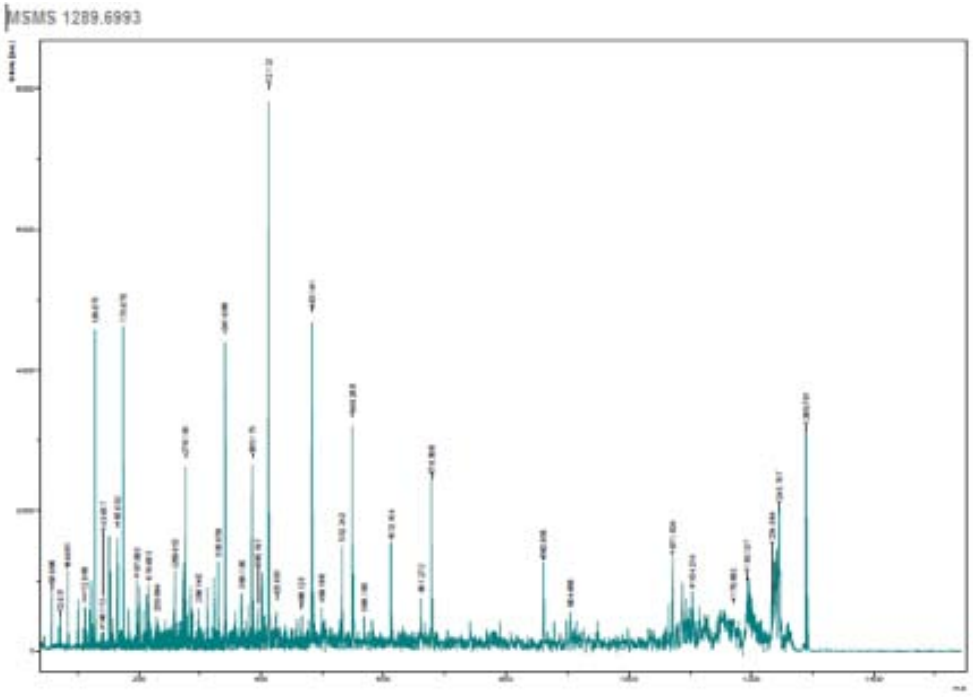
Se agradecería a los usuarios que en las publicaciones o documentos dónde se haga referencia a los resultados aquí descritos, se haga constar que los análisis se han realizado en el Servei de Proteòmica de la UAB, el cual pertenece a Proteored, el Insituto Nacional de Proteómica-Instituto de Salud Carlos III, mediante una frase similar a la siguiente:

"The MALDI-TOF MS analyses were carried out in the Proteomics facility from UAB, a member of ProteoRed-ISCIII network"

Analysis protein 222







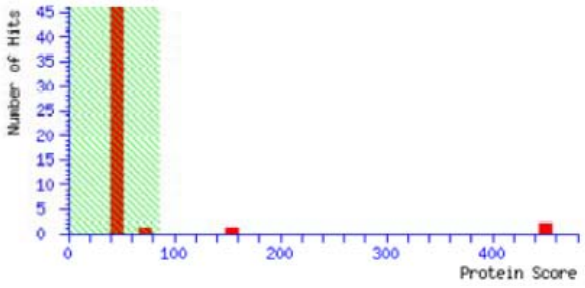
MASCOT Report protein 222

MATRIX SCIENCE Mascot Search Results

```
User            : SAT
Email           : sebastian.trejo@bioinf.uab.es
Search title    : SP222
MS data file    : DATA.TXT
Database        : NCBInr 20130519 (25779625 sequences; 8900870272 residues)
Taxonomy        : Bacteria (Eubacteria) (16187970 sequences)
Timestamp       : 24 May 2013 at 07:16:32 GMT
Warning         : A Peptide summary report will usually give a much clearer picture of MS/MS search results.
Top Score       : 450 for gi|219871890, Thiol:disulfide interchange protein dsbA [Haemophilus parasuis SH0165]
```

Mascot Score Histogram

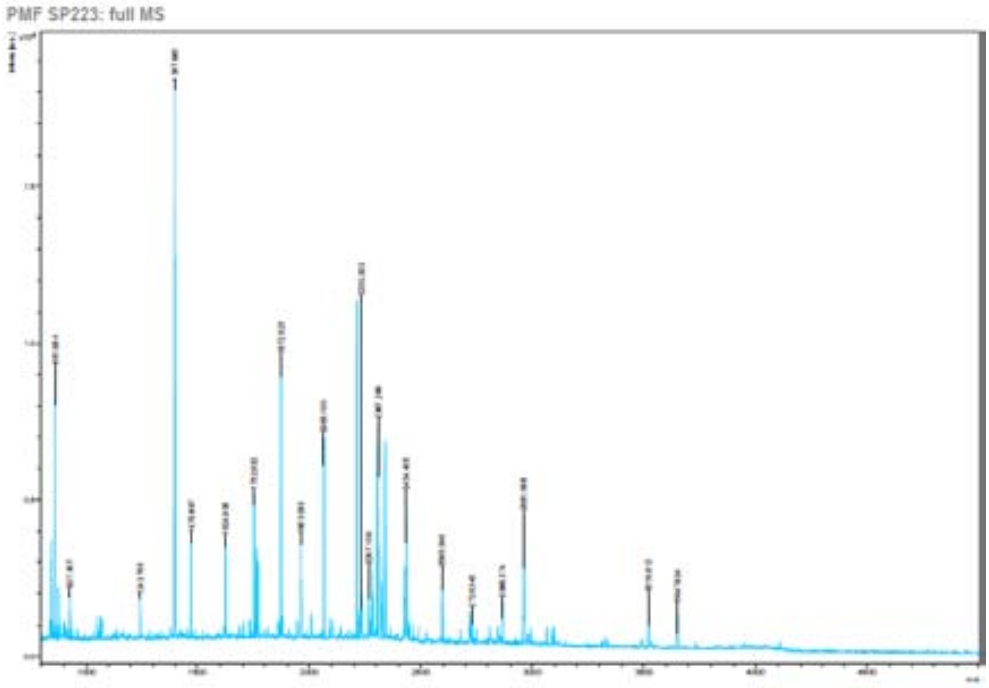
Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 85 are significant (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

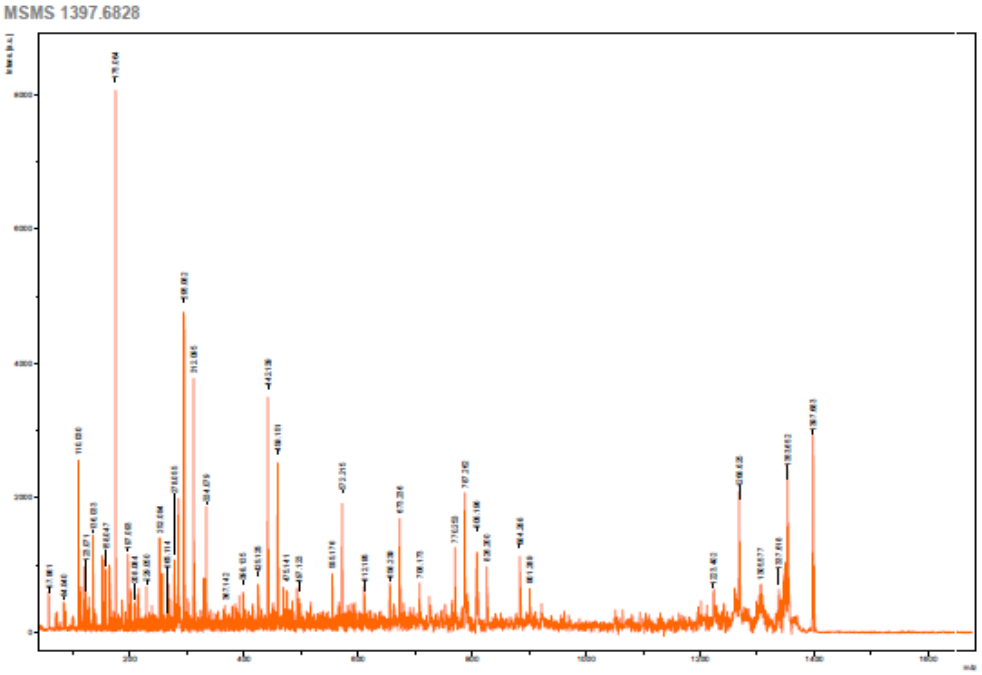


INDEX

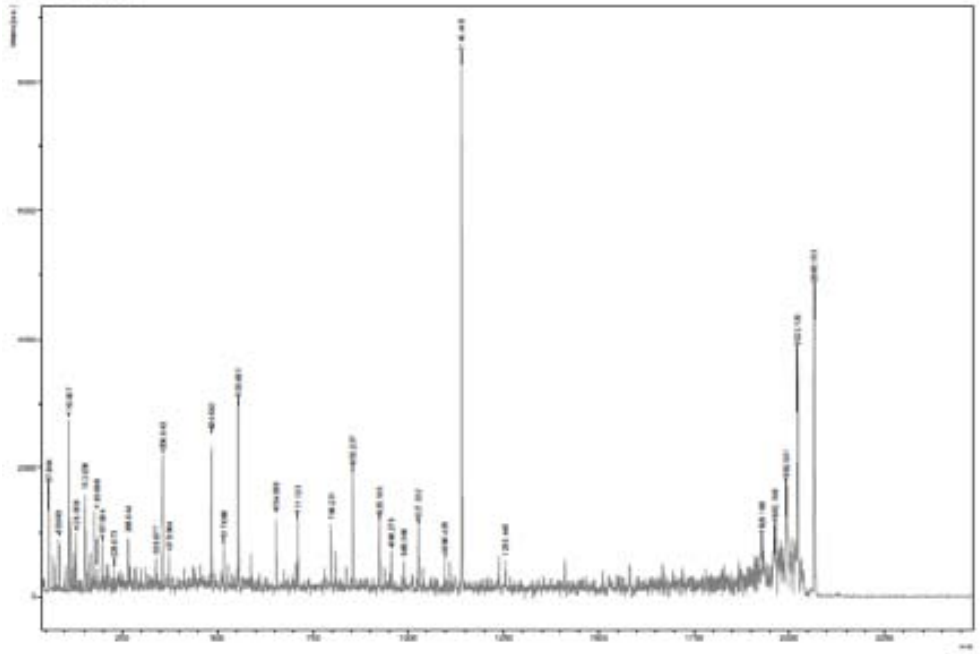
Accession	Mass	Score	Description
gi|219871890	21163	450	Thiol:disulfide interchange protein dsbA [*Haemophilus parasuis* SH0165]
gi|491997501	23560	445	Thiol:disulfide interchange protein dsbA precursor [*Haemophilus parasuis*]
gi|470166278	23741	142	Thiol:disulfide interchange protein DsbA [*Bibersteinia trehalosi* USDA-ARS-USMARC-192]
gi|162145963	15917	66	hypothetical protein GDI_0129 [*Gluconacetobacter diazotrophicus* PAl 5]
gi|15890740	12161	57	hypothetical protein Atu4242 [*Agrobacterium fabrum* str. C58]

Analysis protein 223

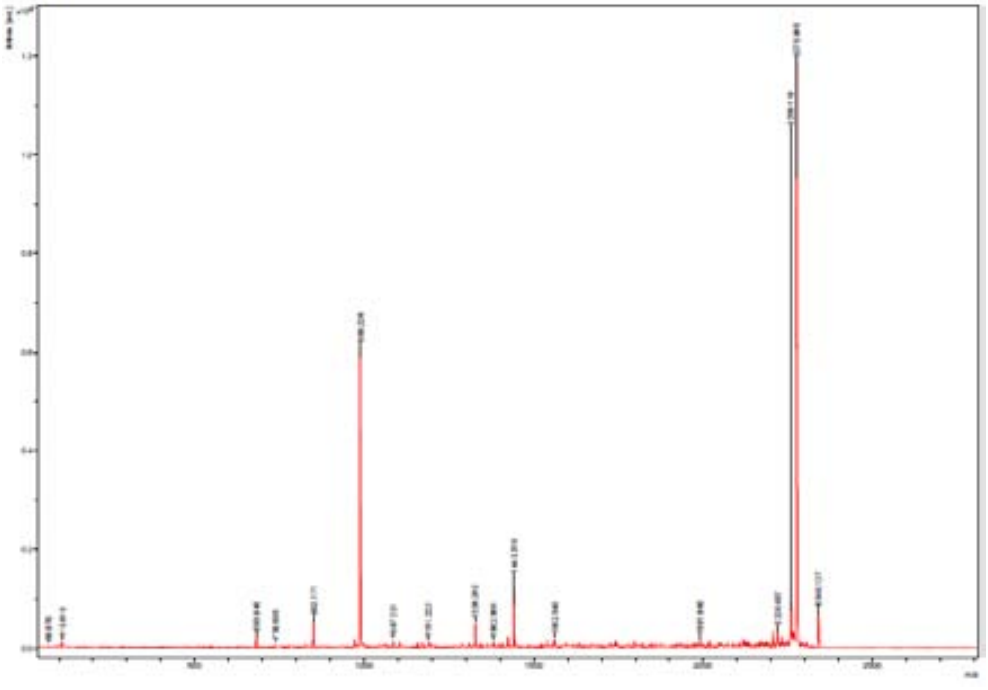




MSMS 2065.1654



MSMS 2340.1252



MASCOT Report Protein 223

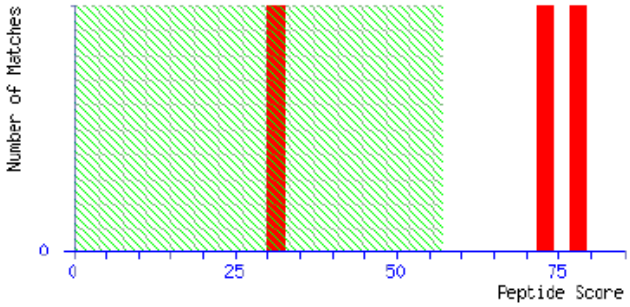
User : SAT
E-mail : sebastian.trejo@uab.cat
Search title : PMF
MS data file : DATA.TXT
Database : NCBInr 20130524 (25,805,290 sequences; 8,915,431,356 residues)
Taxonomy : Bacteria (Eubacteria) (16,188,144 sequences)
Timestamp : 27 May 2013 at 09:54:13 GMT

Not what you expected? Try the peptide summary.

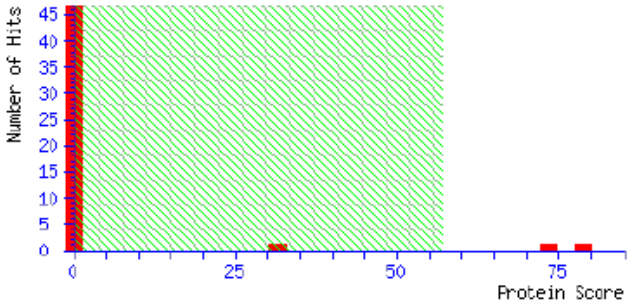
Search parameters

Type of search : MS/MS Ion Search
Enzyme : Trypsin
Variable modifications : Carbamidomethyl (C), Oxidation (M)
Mass values : Monoisotopic
Protein mass : Unrestricted
Peptide mass tolerance : ± 100 ppm
Fragment mass tolerance : ± 0.6 Da
Max missed cleavages : 2
Instrument type : MALDI-TOF-TOF
Number of queries : 21

Score distribution

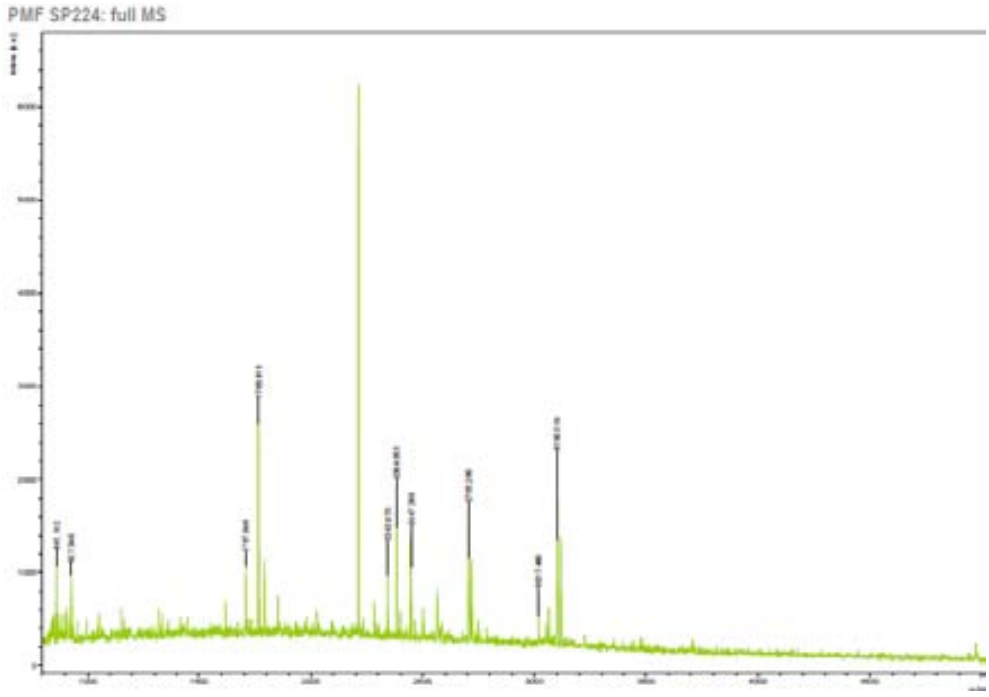


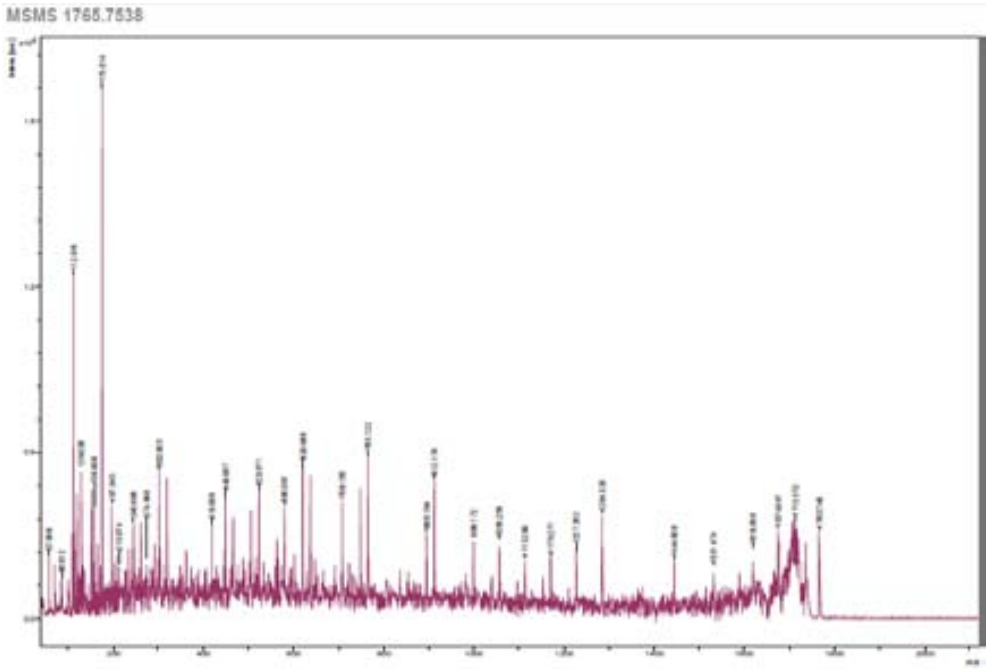
Peptide score distribution. Ions score is $-10\log(P)$, where P is the probability that the observed match is a random event. Individual ions scores **> 57** indicate **identity** or **extensive homology** ($p<0.05$).

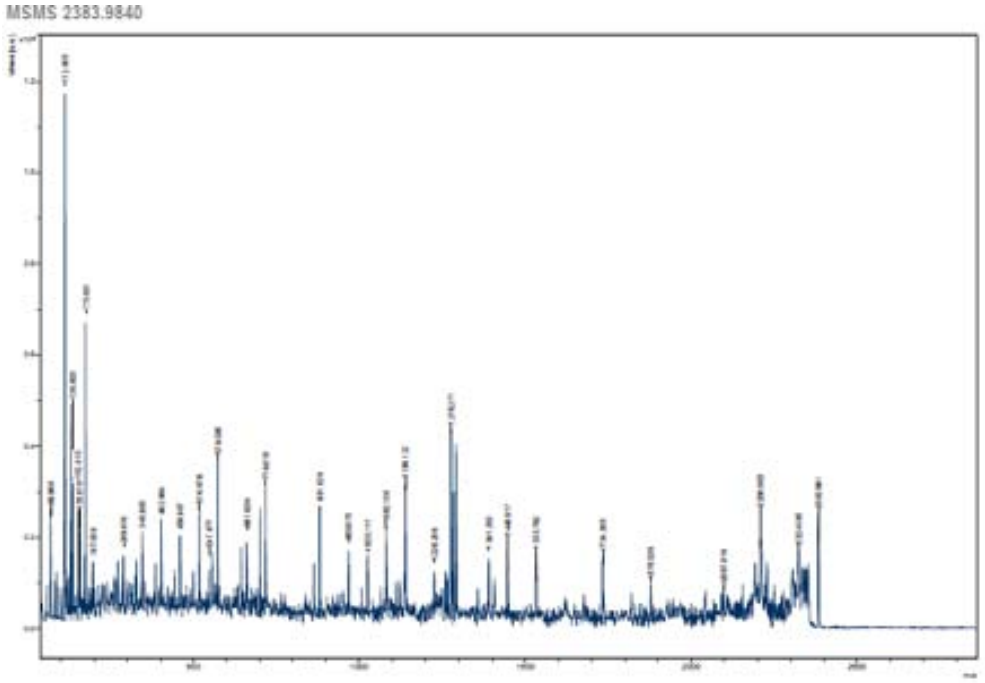


↑Family	M	DB	Accession	Score	Mass	Matches	Pep (sig)	Sequences	Seq (sig)	Description
1	1	NCBInr	gi|41205	79	7791	1	1	1	1	unnamed protein product [Escherichia coli K-12]
2	1	NCBInr	gi|219872113	75	17506	1	1	1	1	thiol peroxidase [Haemophilus parasuis SH0165]
3	1	NCBInr	gi|219870973	33	18992	1	1	1	1	superoxide dismutase [Haemophilus parasuis SH0165]

Analysis protein 224



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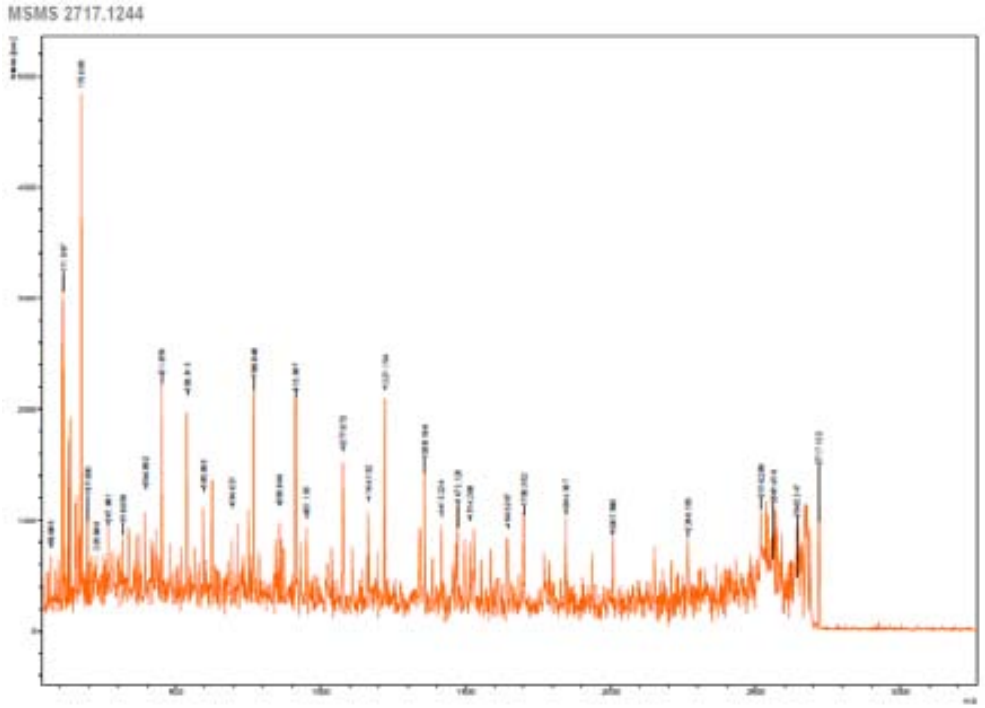
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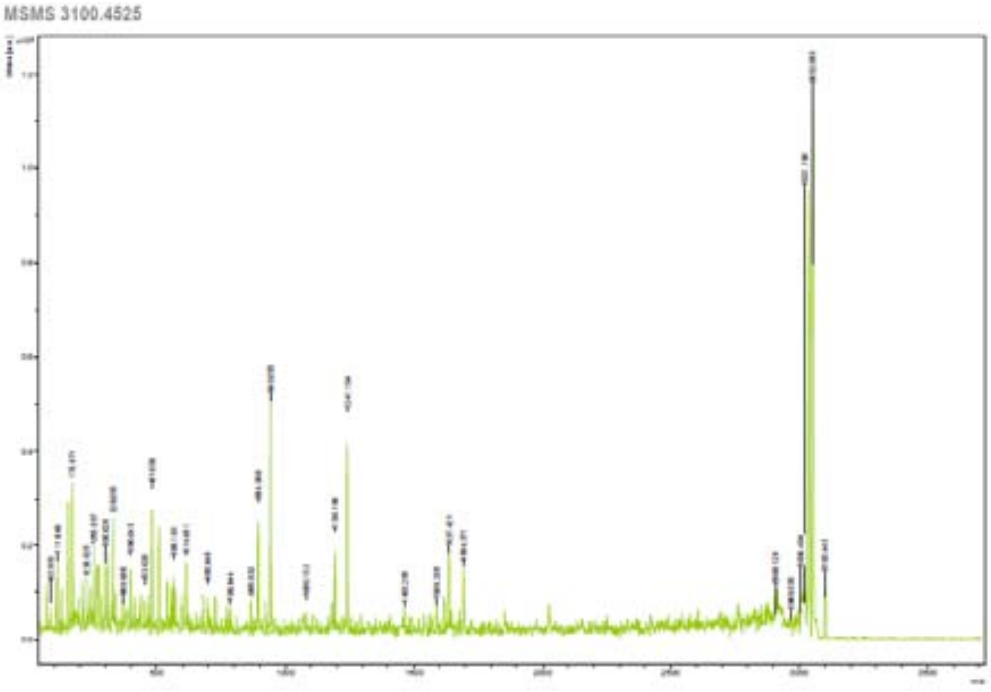

MASCOT Report Protein 224

MATRIX SCIENCE Mascot Search Results

```
User           : SAT
Email          : sebastian.trejo@bioinf.uab.es
Search title   : SP224
MS data file   : DATA.TXT
Database       : NCBInr 20130524 (25805290 sequences; 8915431356 residues)
Taxonomy       : Bacteria (Eubacteria) (16188144 sequences)
Timestamp      : 27 May 2013 at 10:59:57 GMT
Protein hits   : gi|430747016 hypothetical protein Sinac_6370 [Singulisphaera acidiphila DSM 18658]
                 gi|495625915 hypothetical protein [Thiovulum sp. ES]
                 gi|497438719 hypothetical protein [Selenomonas sp. oral taxon 137]
                 gi|496818645 OsmC-like protein [Parvimonas sp. oral taxon 110]
                 gi|495535988 single-strand binding protein [Brevundimonas sp. BAL3]
                 gi|341820372 trmA family tRNA (Uracil-5-)-methyltransferase [Weissella thailandensis fsh4-2]
                 gi|498799393 sensor kinase [Rhodococcus rhodnii]
                 gi|491175774 uroporphyrinogen decarboxylase (URO-D) [Holophaga foetida]
                 gi|261405675 sporulation protein, YlmC/YmxH family [Paenibacillus sp. Y412MC10]
                 gi|6939958   DNA gyrase subunit B [Sphingobacterium spiritivorum]
```

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 56 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.