RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

Identification of a class B acid phosphatase in *Haemophilus parasuis*

Paula Manrique-Ramírez,[1] Núria Galofré-Milà,[1] Marta Serrano,[1] Virginia Aragon[1,2]*

[1]Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autonoma de Barcelona, Bellaterra, Barcelona, Spain. [2]Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Barcelona, Spain

Summary. An acid phosphatase activity was detected in the supernatant of *Haemophilus parasuis*, a Gram-negative pleomorphic bacillus and the causative agent of Glässer's disease in pigs. To identify the gene responsible for the secreted activity, a genomic library of *H. parasuis* strain ER-6P was produced in *Escherichia coli*. Screening of the library allowed identification of two homologs to known phosphatases: PgpB and AphA. PgpB was predicted to be located in the bacterial membrane through six transmembrane domains while AphA was predicted to have a signal peptide. The *aphA* gene was cloned and expressed in *E. coli*. Characterization of *H. parasuis* AphA indicated that this protein belongs to the class B nonspecific acid phosphatases. AphA contained sequence signatures characteristic of this family of phosphatases and its activity was inhibited by EDTA. The optimal pH of recombinant AphA differed from that of the phosphatase activity found in *H. parasuis* supernatants. In addition, the phosphatase activity from *H. parasuis* supernatants was not inhibited by EDTA, indicating that *H. parasuis* AphA does not account for the phosphatase activity observed in the supernatants. Our results demonstrate the presence of a class B acid phosphatase (AphA) in *H. parasuis* and suggest that the bacterium would also secrete another, as yet unidentified phosphatase. [**Int Microbiol** 2014; 17(3):141-147]

Keywords: *Haemophilus parasuis* · non-specific acid phosphatases · phosphatase activity · Glässer's disease

Introduction

Haemophilus parasuis is a Gram-negative pleomorphic bacillus and the etiological agent of Glässer's disease in pigs, a severe systemic disease characterized by fibrinous polyserositis, polyarthritis, and meningitis that is responsible for important economic losses in the swine industry worldwide. *H. parasuis* has been shown to produce a variety of potential mem-

brane-associated virulence factors [5], but little is known about the role of its acid phosphatases.

Acid phosphatases are ubiquitous in prokaryotes and eukaryotes. Based on criteria such as specificity and optimum pH, phosphatases can be classified into several families. Thus, bacterial non-specific acid phosphatases (NSAPs) are categorized into three types (classes A, B and C) [3,23]. Reports indicate that in Gram-negative bacteria these enzymes play a critical role in numerous processes, including pathogenesis [2,6,8,9,16,19,20]. Acid phosphatases catalyze the transfer of a phosphoryl group from phosphomonoesters to water at an acidic pH. The class B acid phosphatases are a group of homotetrameric secreted phosphohydrolases containing a 25-kDa polypeptide monomer. These enzymes are characterized by

*Corresponding author: Virginia Aragon
Centre de Recerca en Sanitat Animal (CReSA)
Universitat Autònoma de Barcelona
08193 Bellaterra, Barcelona, Spain
Tel. +34-935814494. Fax +34-935814490
E-mail: virginia.aragon@cresa.uab.es

conserved sequence motifs including four conserved aspartate residues, which are characteristic of the molecular super-family of phosphohydrolases referred to as "DDDD" [11,21, 22]. The class B signature sequence is divided into two domains, an N-terminal motif composed of FDIDDTVLFSSP and a C-terminal motif of YGD-(A/S)-DXD-[I/V] [18]. The aspartate nucleophile in this group is located in the N-terminal region of the signature sequence. These phosphatases are members of the haloacid dehalogenase (HAD) superfamily and include a large number of bacterial phosphatases. The first bacterial nonspecific class B acid phosphatase described was NapA from *Morganella morganii* [21], but others have been identified since then, such as the AphA phosphatase of *Escherichia coli* [14,22]. A *napA* homolog was also detected in the genome of *Haemophilus influenzae* [7]. Here we provide the first report of a class B acid phosphatase in *H. parasuis*.

Materials and methods

Bacterial strains and growth conditions. *Haemophilus parasuis* strains (Table 1) were grown on chocolate agar plates (BioMerieux, Madrid, Spain) at 37ºC in an atmosphere of 5% CO_2 or in brain heart infusion (BHI) broth when a liquid culture was needed (see below). All *H. parasuis* strains were stored frozen in BHI with 20% glycerol at –80ºC. The majority of the strains were stored after the minimum number of passages needed for isolation (usually two passages on plates). The passage numbers of strains IT29205, 9904108, and P015/96 and the reference strains Nagasaki and SW114 are not known. *Escherichia coli* BL21 was used as the host for recombinant plasmids and was grown in LB medium at 37ºC. LB medium was supplemented with ampicillin (100 μg/ml) or chloramphenicol (30 μg/ml) as required.

Preparation of supernatants. Culture supernatants were analyzed at different growth phases. Strains of *H. parasuis* were grown in BHI broth supplemented with isovitalex (BD, Madrid, Spain) at 37ºC in an orbital shaker (200 rpm). Bacterial growth was assessed by measuring the absorbance at 660 nm in a VIS7200 spectrophotometer (Dinko Instruments, Barcelona, Spain). Supernatants were obtained by centrifugation followed by filtration (0.22 μm). For some experiments; the supernatants were concentrated 100-fold through a Millipore YM10 ultrafiltration cell.

Phosphatase assays. Phosphatase activity was measured using the fluorescent substrate 4-methylumbelliferyl phosphate (MUP) [13,17]. Acid phosphatase activity was measured in 0.1 M sodium acetate buffer (pH 5.2) or in 0.1 M sodium citrate buffer (pH 5.2) and MUP at a final concentration of 0.6 mg/ml. The reaction was measured in a fluorometer (ASCENT Fluoroskan & FL; Thermo Labsystems). The fluorometer was programmed for incubation at 37ºC and for fluorescence measurements at different time points (usually, at 0, 10 min, 30 min, 1 h and 2 h). Fluorescence was measured at an excitation of 355 nm and an emission of 460 nm.

In standard phosphatase assays, 96-well microtiter plates were used with 50 μl of sample, 50 μl of 2× reaction buffer, and MUP. To measure phosphatase activity at different pHs, 0.1 M sodium citrate buffer was used for pH 4,

5.2, and 6, and 0.05 M Tris buffer used for pH 7, 8, and 9. The MUP concentration was the same as above. An acid phosphatase from potato (Sigma-Aldrich) was used in the assays as a positive control.

The effect of ethylenediaminetetraacetic acid (EDTA) on phosphatase activity was assessed by the addition of 20 mM EDTA to the reaction. The effects of tartrate and molybdate on phosphatase activity were assayed by adding sodium tartrate or sodium molybdate to the reaction at 1, 10, 100, 1000 and 10,000 μM.

Construction of a genomic library and screening. DNA from *H. parasuis* strain ER-6P (acid phosphatase positive) was extracted and partially digested with *Sau*3AI. The reaction was incubated at 37ºC for 2 min and then stopped with 40 mM EDTA. DNA fragments between 4 and 5 kb were selected and excised from an agarose gel and then purified using the Wizard SV gel and PCR clean-up system (Promega). Plasmid pACYC184 digested with *Bam*HI and then dephosphorylated was used to clone the 4- to 5-kb genomic fragments from strain ER-6P, which were then introduced into *E. coli* BL21. The genomic library consisted of 2,880 clones, which was sufficient to ensure the representation of the *H. parasuis* genome. To screen for acid phosphatase activity, the individual clones from the genomic library were cultured in 96-well plates with 200 μl of medium. After incubation at 37ºC, the complete culture and the supernatant from each well were analyzed for phosphatase activity. Plasmid DNA from the positive clones was purified and the insert was sequenced with primers pACYCseq-F (5′-ACTTGGAGCCACTATC-GACTAC-3′) and pACYCseq-R (5′-CGGTGATGTCGGCGATATAGG-3′). The complete sequence included in each clone was deduced by comparison with the *H. parasuis* genome sequence from strain SH0165 (GenBank accession number NC_011852.1).

Cloning of *aphA* homolog from *Haemophilus parasuis*. Screening of the 4- to 5-kb genomic library and BLAST searches of the genome of *H. parasuis* SHO165 (accession number YP002476402.1) detected an *aphA* homolog in *H. parasuis*. The *aphA* gene, with its putative promoter region (predicted by BProm), was PCR-amplified from the strains described in Table 1, using primers APha-Fw-prom (5′-ATGTTCCCTATAACCTATT-GTG-3′) and Apha-Rev (5′-ATTAGTAGCTTGAATTTATAATAAC-3′). The resulting 900-bp PCR fragment was purified, cloned into pGemT-easy, and electroporated into *E. coli* BL21. Transformants were selected and the acid phosphatase activity of the clones was evaluated in complete cultures and in the supernatants. Inserts in the clones were sequenced and the orientation of the gene in each clone was checked. The presence of a putative signal peptide in the predicted amino acid sequence was analyzed using SignalP 4.1 and PSORT. Transmembrane domains and cellular location were analyzed using TMHMM Server v.2.0 and PSORT.

Results and Discussion

Secreted acid phosphatase activity. An acid phosphatase activity was detected in the supernatant of *H. parasuis*, with a secretion peak in the early-stationary phase of bacterial growth and an optimal pH of 5–6 (Fig. 1). The activity of the supernatants could be preserved at –80ºC if 50% glycerol was added to the samples. Failure to add glycerol rendered the samples inactive after freezing at –80ºC. Although the activity may have been released into the supernatant after bacterial lysis, this was unlikely since the samples were obtained at the end of the

Table 1. Strains of *Haemophilus parasuis* used in this study and their main characteristics[a]

Strain	Serovar	Susceptibility to[b]		Disease status	Reference or source
		Serum	Phagocytosis		
Strains from lesions					
Nagasaki	5	R	R	Glässer's disease	Reference strain [10]
ER-6P	15	R	R	Glässer's disease	[4]
IT29205	4	R	R	Glässer's disease	[1]
373/03A	7	I	R	Glässer's disease	[1]
264/99	10	R	R	Glässer's disease	[1]
2725	10	S	R	Glässer's disease	[1]
228/04	5	R	R	Glässer's disease	[12]
PV1-12	15	S	R	Glässer's disease	[1]
CT175-L	15	S	ND	Glässer's disease	[4]
PC4-6P	12	R	R	Glässer's disease	[4]
9904108[c]	4	S	R	Glässer's disease	[1]
P015/96	5	R	R	Pneumonia	[12]
Nasal strains					
SW114	3	S	S	Healthy	Reference strain [10]
F9	6	S	S	Healthy	[12]
IQ1N-6	9	S	S	Healthy	[4]
ND14-1	7	S	S	Healthy	[4]
FL3-1	7	ND	ND	Healthy	[12]
FL1-3	10	S	I	Healthy	[12]
CA38-4	12	R	R	Glässer's disease	[12]
MU21-2	7	S	S	Healthy	[12]
VS6-2	15	I	S	Healthy	[12]
SC14-1	15	S	S	Healthy	[12]
SL3-2	10	S	S	Healthy	[4]

[a]All strains are *aphaA* positive.
[b]S, Sensitive; I, intermediate; R, resistant; ND, Not determined.
[c]This strain did not reproduce disease when inoculated intranasally in colostrum-deprived piglets [1].

exponential phase of growth, during which time no significant lysis occurred. Moreover, supernatants obtained after overnight incubation showed phosphatase activity at an alkaline pH (not shown), probably due to the release of an alkaline phosphatase after bacterial lysis.

A gradual loss of the acid phosphatase activity was observed after several in vitro passes of strain ER-6P on agar plates. This was the case after 6, 10, and 14 passes (ER-6Pp6, ER-6Pp10, and ER-6Pp14). After 14 passes, acid phosphatase activity in the supernatant of ER-6Pp14 was no longer detectable, even though

the growth rate of this strain in the laboratory was similar to that of the original ER-6P strain (not shown), recovered from the pericardium of a diseased pig and maintained frozen with no additional passages. We assume that, under standard laboratory conditions, *H. parasuis* does not require this acid phosphatase and therefore blocks its production. Similarly, a previous report had demonstrated the reduced expression of the *H. parasuis* capsule after in vitro passage of the bacterium [15]. The role of the acid phosphatase activity in infection, whether to obtain nutrients or as a virulence factor, remains to be determined.
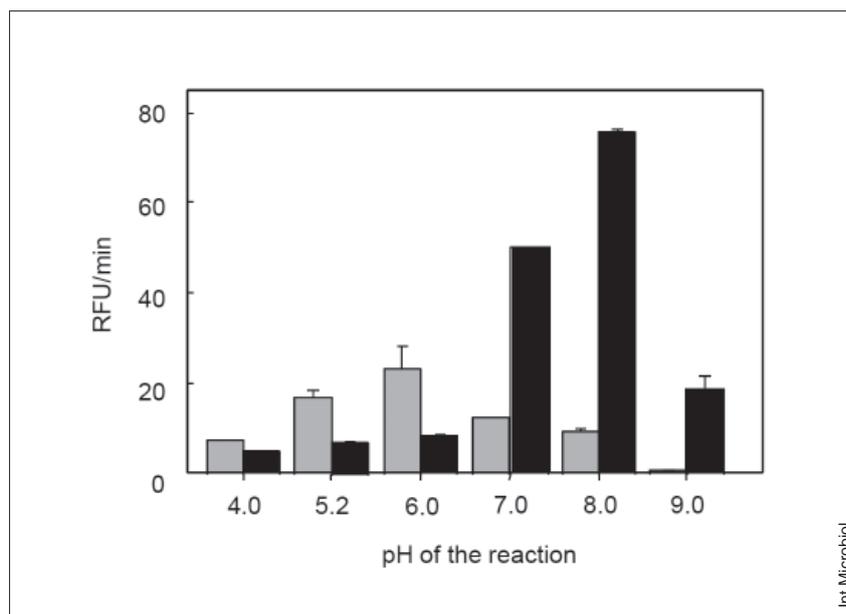
Fig. 1. Phosphatase activity in the supernatant prepared from *Haemophilus parasuis* strain ER-6P harvested at the beginning of the stationary phase of growth (gray bars) and in the supernatant of clone *E. coli* BL21 pGem-T Easy-*aphA* FL3-1#19 (black bars). Phosphatase activity was measured at different pH values, with MUP as the substrate. Phosphatase activity is represented as relative fluorescence units (RFU) per min. (Average of duplicate samples ± standard deviation.)

Screening of the genomic library. To identify the gene responsible for the phosphatase activity detected in *H. parasuis* supernatants, individual clones of the genomic library *H. parasuis* ER-6P were tested for the production of phosphatase activity in the presence of MUP. Sequencing of the phosphatase-positive clones yielded four clones with a class B acid phosphatase (*aphA* homolog gene) and one clone with a phosphatidylglycerophosphate phosphatase (*pgpB* homolog gene). The *pgpB* and *aphA* genes were PCR-amplified and cloned to subsequently determine their activity. Acid phosphatase activity was demonstrated in the *pgpB* clones (not shown). The predicted sequence of PgpB contained six transmembrane domains probably located in the cytoplasmic membrane. These data are in agreement with the location of other bacterial PgpB proteins in the cytoplasmic membrane. Thus, in the following, we focus our attention on the *aphA* homolog as a possible secreted phosphatase.

Analysis of the *aphA* gene from the available *H. parasuis* sequence demonstrated that the predicted protein had the features of a NSAP class B phosphatase, including the two signature motifs characteristic of these enzymes (Fig. 2). Also, a signal peptide was detected by SignalP in the predicted protein. The predicted molecular mass of 24.33 kDa for the monomer is characteristic of class B phosphatases [18].

The complete *aphA* gene, with the putative promotor region, from virulent and non-virulent strains of *H. parasuis* was PCR-amplified and sequenced (Table 1). No association between *aphA* and the putative virulence of the strains was observed, since the gene was detected by PCR in all of the analyzed strains (Table 1). Clones were produced with *aphA* from strains ER-6P, FL3-1, IQ1N-6, and ND14–1. All of the clones showed phosphatase activity, but only one *aphA*-containing clone, from strain FL3-1 (clone pGem-T Easy *aphA* FL3-1#19), secreted the activity into the culture supernatant, (Fig. 1). Sequencing revealed that the only difference between this clone and others from the same strain was the orientation of the insert in the plasmid, as the orientation of the insert in clone FL3-1#19 was opposite to that of the other inserts. Thus, why the activity was secreted by this clone but not by the others is unclear.

Characterization of the AphA homolog from *Haemophilus parasuis*. The supernatant from BL21 pGem-T Easy *aphA* FL3-1#19 was analyzed at different pHs and the activities were compared to the activity in *H. parasuis* supernatants (Fig. 1). The activity produced by the *H. parasuis* AphA homolog had a different optimal pH than the activity of the *H. parasuis* supernatants, indicating that the two
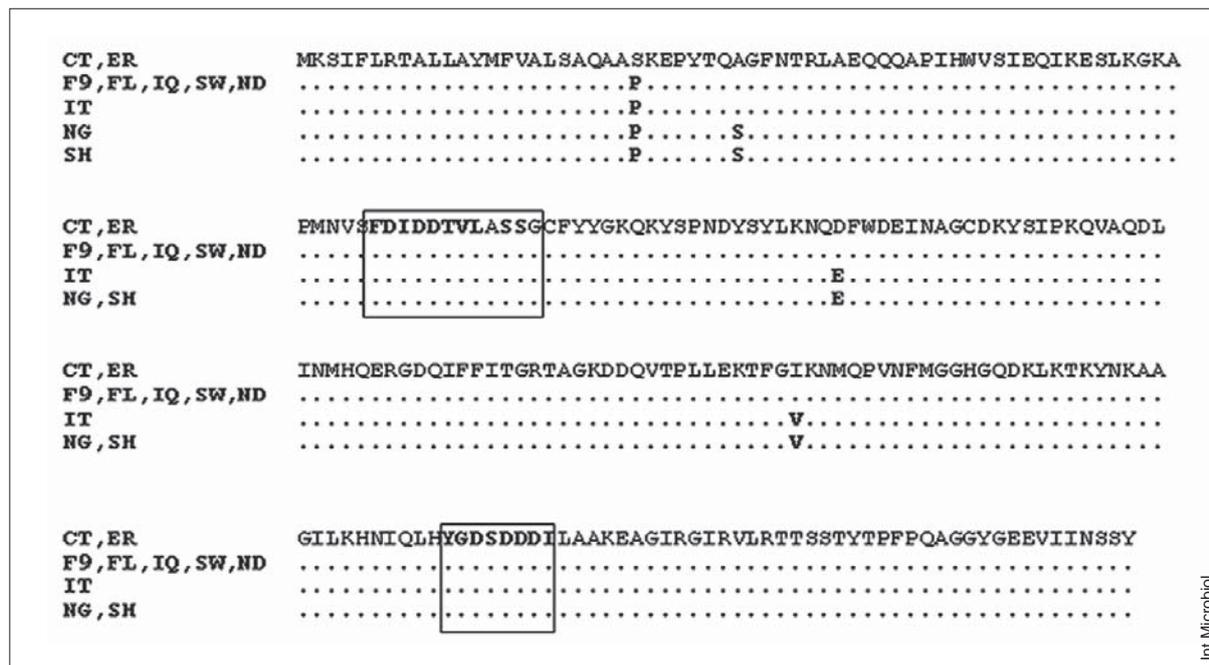
Fig. 2. Alignment of the predicted amino acid sequence of acid phosphatase AphA from different strains of *Haemophilus parasuis* (CT: CT175-L; ER: ER-6P; F9; FL: FL3-1; IQ: IQ1N-6; SW: SW114; ND: ND14-1; IT: IT29205; NG: Nagasaki; SH: SH0165). The catalytic domain characteristic of class B acid phosphatases is presented in two boxes; motif F-D-I-D-D-T-V-L-F-S-S-P, located in the N-terminal moiety, and motif Y-G-D-(A/S)-D-X-D-(IV), located near the C-terminus [23].

activities were produced by different enzymes. The optimal pH for *H. parasuis* Apha with MUP was around 7–8. This may seem high for an acid phosphatase, but the optimal pH of these enzymes varies with the substrate [18]; thus, the activity of AphA with its natural substrate may be maximal at an acidic pH.

Since class B NSAPs require Mg^{2+}, we evaluated the inhibitory effects of EDTA. The activity produced by clone pGem-T Easy *aphA* FL3-1#19 was indeed inhibited by EDTA (Fig. 3), in agreement with its class B phosphatase structure [18]. However, the activity in the supernatant of *H. parasuis* strain ER-6P was not affected by the addition of 20 mM EDTA to the reaction (not shown). Thus, besides having the characteristic domains of a type B acid phosphatase, the *H. parasuis* AphA homolog is inhibited by EDTA, in agreement with its classification within this group of phosphatases. Therefore, the inhibition of Apha activity by EDTA is compatible with the requirement of divalent cations by class B phosphatases.

Tartrate was also tested as phosphatase inhibitor but it had no effect on the secreted activity in the supernatants of either *H. parasuis* ER-6P or the recombinant AphA, up to a concentration of 10 mM sodium tartrate. When molybdate concentrations up to 10 mM were similarly tested, the activity in the supernatant of strain ER-6P was only partially inhibited at 10 mM whereas there was no effect on the recombinant AphA phosphatase (Fig. 4).
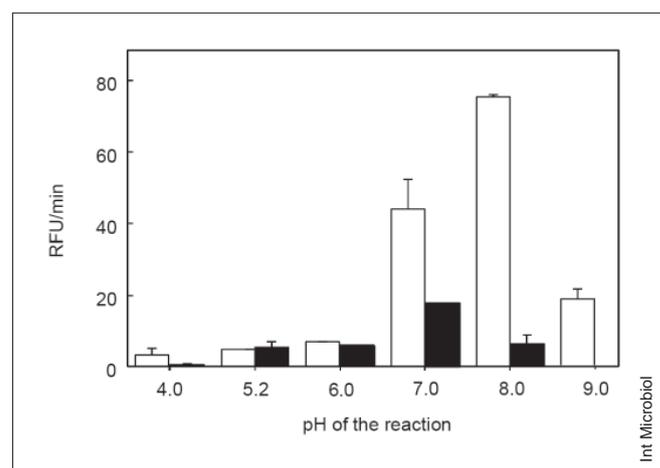

Fig. 3. Inhibition of AphA by EDTA. Phosphatase activity in the supernatant of clone *E. coli* BL21 pGem-T Easy *aphA* FL3-1#19 with (black bars) and without (white bars) 20 mM EDTA, at different pH values. Phosphatase activity was measured with MUP as the substrate and is represented as relative fluorescence units (RFU) per min (average of duplicate samples ± standard deviation).
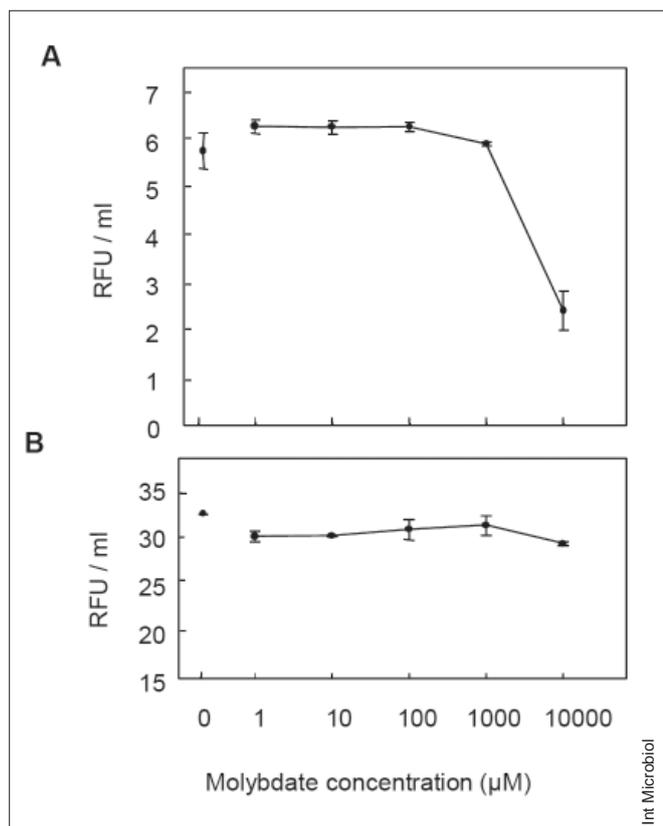
Fig. 4. Effect of molybdate on the phosphatase activity found in (**A**) the supernatant of *Haemophilus parasuis* ER-6P and (**B**) the supernatant from clone *E. coli* BL21 pGem-T Easy *aphA* FL3-1#19. Phosphatase activity is expressed as relative fluorescence units (RFU) per min (average of duplicate samples ± standard deviation). Sodium molybdate was used at different concentrations up to 10 mM.

The presence of the *aphA* gene in strains from different clinical backgrounds, including non-virulent nasal isolates, indicated that this enzyme was probably not a virulence factor. In addition, we did not detect any effect on the activation/inhibition of alveolar macrophages (cell-surface CD163, SLAI, SLAII, sialoadhesin and SWC3) after incubation of the cells with supernatant containing the AphA activity (not shown).

In summary, the different optimal pH and the different EDTA and molybdate susceptibilities between Apha and the activity found in *H. parasuis* supernatant suggested that Apha was not the activity found in the *H. parasuis* supernatant. These results are consistent with the periplasmic location of AphA, as described in *E. coli* [14], or with the secretion of the Apha enzyme into the supernatant together with another, as yet unidentified phosphatase activity. Further experiments are required to identify the enzyme responsible for the phosphatase activity found in *H. parasuis* supernatants. Although ac-

cording to our results, phosphatase activity is not be associated with virulence, a role for this activity in bacterial survival during infection cannot be ruled out.

Competing interest: None declared.

References

1. Aragon V, Cerdà-Cuéllar M, Fraile L, Mombarg M, Nofrarias M, Olvera A, Sibila M, Solanes D, Segales J (2010) Correlation between clinico-pathological outcome and typing of *Haemophilus parasuis* field strains. Vet Microbiol 142:387-393
2. Aragon V, Kurtz S, Cianciotto NP (2001) *Legionella pneumophila* major acid phosphatase and its role in intracellular infection. Infect Immun 69:177-185
3. Bouchet B, Vanier G, Jacques M, Gottschalk M (2008) Interactions of *Haemophilus parasuis* and its LOS with porcine brain microvascular endothelial cells. Vet Res 39:42
4. Cerdà-Cuéllar M, Aragon V (2008) Serum-resistance in *Haemophilus parasuis* is associated with systemic disease in swine. Vet J 175:384-389
5. Costa-Hurtado M, Aragon V (2013) Advances in the quest for virulence factors of *Haemophilus parasuis*. Vet J 198:571-576
6. du Plessis EM, Theron J, Joubert L, Lotter T, Watson TG (2002) Characterization of a phosphatase secreted by *Staphylococcus aureus* strain 154, a new member of the bacterial class C family of nonspecific acid phosphatases. Syst Appl Microbiol 25:21-30
7. Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 269:496-512
8. Hoopman TC, Wang W, Brautigam CA, Sedillo JL, Reilly TJ, Hansen EJ (2008) *Moraxella catarrhalis* synthesizes an autotransporter that is an acid phosphatase. J Bacteriol 190:1459-1472
9. Jungnitz H, West NP, Walker MJ, Chhatwal GS, Guzmán CA (1998) A second two-component regulatory system of *Bordetella bronchiseptica* required for bacterial resistance to oxidative stress, production of acid phosphatase, and *in vivo* persistence. Infect Immun 66:4640-4650
10. Kielstein P, Rapp-Gabrielson VJ (1992) Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts. J Clin Microbiol 30:862-865
11. Leone R, Cappelletti E, Benvenuti M, Lentini G, Thaller MC, Mangani S (2008) Structural insights into the catalytic mechanism of the bacterial class B phosphatase AphA belonging to the DDDD superfamily of phosphohydrolases. J Mol Biol 384:478-488
12. Olvera A, Calsamiglia M, Aragon V (2006) Genotypic diversity of *Haemophilus parasuis* field strains. Appl Environ Microbiol 72:3984-3992
13. Omene JA, Glew RH, Baig HA, Robinson DB, Brock W, Chambers JP (1981) Determination of serum acid and alkaline phosphatase using 4-methylumbelliferyl phosphate. Afr J Med Med Sci 10:9-18
14. Passariello C, Forleo C, Micheli V, Schippa S, Leone R, Mangani S, Thaller MC, Rossolini GM (2006) Biochemical characterization of the class B acid phosphatase (AphA) of *Escherichia coli* MG1655. Biochim Biophys Acta 1764:13-19

15. Rapp-Gabrielson VJ, Gabrielson DA, Schamber GJ (1992) Comparative virulence of *Haemophilus parasuis* serovars 1 to 7 in guinea pigs. Am J Vet Res 53:987-994

16. Reilly TJ, Baron GS, Nano FE, Kuhlenschmidt MS (1996). Characterization and sequencing of a respiratory burst-inhibiting acid phosphatase from *Francisella tularensis*. J Biol Chem 271:10973-10983

17. Robinson D, Willcox P. 1969. 4-Methylumbelliferyl phosphate as a substrate for lysosomal acid phosphatase. Biochim Biophys Acta 191:183-186

18. Rossolini GM, Schippa S, Riccio ML, Berlutti F, Macaskie LE, Thaller MC (1998) Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology. Cell Mol Life Sci 54:833-850

19. Saha AK, Dowling JN, LaMarco KL, Das S, Remaley AT, Olomu N, Pope MT, Glew RH (1985) Properties of an acid phosphatase from *Legionella micdadei* which blocks superoxide anion production by human neutrophils. Arch Biochem Biophys 243:150-160

20. Saleh MT, Belisle JT (2000) Secretion of an acid phosphatase (SapM) by *Mycobacterium tuberculosis* that is similar to eukaryotic acid phosphatases. J Bacteriol 182:6850-6853

21. Thaller MC, Lombardi G, Berlutti F, Schippa S, Rossolini GM (1995) Cloning and characterization of the NapA acid phosphatase/phosphotransferase of *Morganella morganii*: identification of a new family of bacterial acid-phosphatase-encoding genes. Microbiology 141:147-154

22. Thaller MC, Schippa S, Bonci A, Cresti S, Rossolini GM (1997) Identification of the gene (*aphA*) encoding the class B acid phosphatase/phosphotransferase of *Escherichia coli* MG1655 and characterization of its product. FEMS Microbiol Lett 146:191-198

23. Thaller MC, Schippa S, Rossolini GM (1998) Conserved sequence motifs among bacterial, eukaryotic, and archaeal phosphatases that define a new phosphohydrolase superfamily. Protein Sci 7:1647-1652