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Immunoproteomic characterization of outer membrane vesicles from hyper-vesiculating *Actinobacillus pleuropneumoniae*

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ABSTRACT

Outer membrane vesicles (OMVs) are produced and secreted virtually by every known Gram-negative bacterium. Despite their non-live nature, they share antigenic characteristics with the bacteria they originate from. This, together with their relative ease of purification, casts the OMVs as a very promising and flexible tool in both human and veterinary vaccinology. The aim of the current work was to get an insight into the antigenic pattern of OMVs from the pig pathogen *Actinobacillus pleuropneumoniae* in the context of vaccine development. Accordingly, we designed a protocol combining 2D Western Blotting and mass spectrometric identification to robustly characterize the antigenic protein pattern of the vesicles. Our analysis revealed that *A. pleuropneumoniae* OMVs carry several immunoreactive virulence factors. Some of these proteins, LpoA, OsmY and MIDG2331_02184, have never previously been documented as antigenic in *A. pleuropneumoniae* or other pathogenic bacteria. Additionally, we showed that despite their relative abundance, proteins such as FrpB and DegQ do not contribute to the antigenic profile of *A. pleuropneumoniae* OMVs.

1. Introduction

Actinobacillus pleuropneumoniae is a Gram-negative bacterium belonging to the *Pasteurellaceae* family, able of infecting pigs as its sole host and reservoir. *A. pleuropneumoniae* is a widespread pathogen and it is currently classified into 18 serovars and 2 biovars varying in geographic distribution globally and over time (Bossé et al., 2018). The lesions induced by *A. pleuropneumoniae* include necrotizing pleuropneumonia with an up to 80% mortality rate, highly dependent on the serovar involved (Bossé et al., 2002). *A. pleuropneumoniae* outbreaks can severely affect pig production, causing an estimated loss of 6.4€ per affected pig in European herds ("Production diseases: The cost to pig producers |PROHEALTH, 2019,). Currently available *A. pleuropneumoniae* vaccines can be classified into two categories: (i) based on inactivated whole-cell bacterins; (ii) based on the Apx toxins (Apx I-III), cytotoxins expressed by all known *A. pleuropneumoniae* serovars (Ramjeet et al., 2008). Bacterin-based vaccines typically confer protection against one or a limited number of serovars and are often unable at preventing acute disease (Tarasiuk et al., 1994; Ramjeet et al., 2008). Vaccines based on inactivated Apx toxins (toxoids) in combination with other immunogens are effective at limiting the morbidity associated with infection (Sadilkova et al., 2012; Del Pozo Sacristán et al., 2014), but unable to prevent colonization of the lung in vaccinated animals, posing the risk of producing asymptomatic carriers (Tumamao et al., 2004; Ramjeet et al., 2008; Sadilkova et al., 2012). Thus, it is highly desirable to develop an alternative, reasonably cheap and effective vaccine that confers broad protection against *A. pleuropneumoniae* in a serovar-independent manner. To achieve this goal our approach focused on the production, analysis and utilization of the outer membrane vesicles (OMVs), as vehicles to deliver a selection of conserved antigens to the host immune system.

A. pleuropneumoniae, as many Gram-negative bacteria, commonly secrete OMVs, which partly share the antigenic pattern of the bacteria from which they have been generated (van der Pol et al., 2015). This feature characterizes the OMVs as promising immunogens for the development of vaccines against Gram-negative bacteria (van der Pol et al., 2015). Nonetheless, our data previously showed that native OMVs from an hyper-vesiculating *A. pleuropneumoniae* strain are unable

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to confer protection against *A. pleuropneumoniae* infection when employed as immunogens (Antenucci et al., 2018). Where native OMVs fall short though, engineered OMVs may represent an alternative. Several studies have in fact shown that engineered OMVs may be employed as a carrier, in order to expose native or recombinant bacterial antigens to the host immune system and confer protection against a natural infection (van der Pol et al., 2015). Prior to employment as an antigen carrier though, OMVs need to be extensively characterized, to avoid undesirable toxic effects in the host and detect the possible presence of super-antigens that could hamper the desired protective immune response in the host (Zariri et al., 2016). *A. pleuropneumoniae* OMVs have previously been demonstrated to carry virulence factors, in particular toxin determinants and proteases (Negrete-Abascal et al., 2000). Nonetheless, a thorough characterization of *A. pleuropneumoniae* OMVs antigenic properties has not been reported yet.

The biogenesis of OMVs has been extensively investigated, showing that OMV secretion is negatively regulated by several proteins and other factors (McBroom et al., 2006). Accordingly, we previously demonstrated that the deletion of the *A. pleuropneumoniae* genes *degS* and *nlpI*, respectively, increased the amount of OMVs secreted by the mutants (Antenucci et al., 2017).

Natural characteristics including multi-antigenic nature, self-adjuvation and the possibility of genetic engineering of the content and yield of the OMVs makes them an attractive entity for vaccine development. Nevertheless, it is crucial to define their antigenic and toxic potential in order to estimate dosage and formulation for *in vivo* testing. While a comprehensive proteomic analysis of *A. pleuropneumoniae* membrane and whole extracellular fractions was previously published (Dom et al., 1992), proteomic and immunoproteomic studies have not been reported on *A. pleuropneumoniae* OMVs to date. Accordingly, the aim of this study was to provide an immunoproteomic characterization of OMVs from hyper-vesiculating *A. pleuropneumoniae* strains.

The traditional vaccinology approach would be to express and test separately OMV proteins after *a priori* proteomic definition of the content. This would represent a highly tedious task, considering that shotgun proteomics, when applied for OMVs profiling, regularly shows numbers of proteins higher than 100. Instead, we designed a more straightforward approach, where proteins from highly purified OMV fractions were separated by 2D electrophoresis and antigenic proteins where detected by Western Blotting (WB) with convalescent pig sera and identified by mass spectrometry (MS).

Poor detection of membrane proteins is a limitation of isoelectric-focusing (IEF) based workflows (see Section 4.4), as the one proposed in this work. Keeping this in mind, our approach offers the advantage of a rapid screening of many proteins according to their antigenic potential, avoiding lengthy cloning procedures that would be required for conventional protein expression. Moreover, with this approach, most of the OMV proteins are kept in their natural ratio and therefore, the most significant antigens can by estimated considering both abundance and antigenicity.

2. Materials and methods

2.1. OMV isolation and quantification

OMVs from *A. pleuropneumoniae* serovar 8 MIDG 2331 (MIDG 2331) Δ*degS* and MIDG 2331 Δ*nlpI*, and the MIDG 2331 wild type (wt) parent strain (courtesy of Janine Bossé; Imperial College London, UK) were produced and isolated in duplicates using the procedure described in Antenucci et al. (2017). Unless otherwise stated all data sets presented in this work originate from biological duplicate experiments. Briefly, the strains were cultured in brain-heart infusion (BHI) media supplemented with 5 μg/mL NAD (Sigma Aldrich) until late exponential phase (37 °C/200 rpm), and the OMVs were isolated from the filtered supernatants by hydrostatic filtration (Antenucci et al., 2017). OMVs were then quantified by Tunable Resistive Pulse Sensing (TRPS) using a qNano device (Izon Sciences Ltd) following a standard protocol (Blundell et al., 2016). Data were analyzed using the data capture and analysis software, Izon Control Suite V.3.3.2.2001.

2.2. 1D SDS-PAGE

The concentration of OMV samples from wt, Δ*degS* and Δ*nlpI* mutants was normalized to 1.9×10^{11} OMVs/ml and aliquots were loaded on 10% NuPAGE® Novex® Bis–Tris gels (Invitrogen) and run under reducing conditions. PageRuler™ Plus Prestained Protein Ladder (Thermofisher Scientific) was used for size determination. Protein gels were then stained with SimplyBlue™ SafeStain (Invitrogen).

2.3. Protein extraction and 2D PAGE

OMV proteins from an equal amount of vesicles from the wt and Δ*nlpI* mutants, respectively, were purified by Wessel-Flüge extraction (Wessel and Flügge, 1984) and resolved by 2D PAGE as described previously (Antenucci et al., 2017). Gels were then used for 2D WB or stained with SimplyBlue™ SafeStain or Sypro Ruby (Thermo Fisher Scientific) according to the manufacturer's instructions and imaged at 100 μm resolution by the Typhoon 9500 Variable Mode Imager (GE Life Sciences).

2.4. 2D WB

After 2D separation the OMVs proteins were analyzed by WB as described previously (Antenucci et al., 2017). Pig serum pooled from animals challenged with an *A. pleuropneumoniae* serovar 2 H K 361 (HK 361) strain (heterologous serovar) was used as primary antibody, while the commercial Anti-Swine IgG (H + L)-Alkaline Phosphatase antibody (Sigma Aldrich) was used as secondary antibody.

2.5. Protein identification by MALDI peptide mapping

Protein spots visualised by staining of 2D PAGE gels and/or detected by WB analysis were excised manually and subjected to in-gel digestion using trypsin (Sigma-Aldrich) following the manufacturer's instructions. Next, peptides were isolated from gel spots (by H_2O, trifluoroacetic acid (TFA) and acetonitrile (ACN) solutions) dried with centrifugal evaporator and purified by RP C18 chromatography (μZipTip, Millipore). The reduced and carboxamidomethylated tryptic peptides extracted from protein spots were subjected to positive ion MALDI MS, using a Bruker ULTRAFLEX TOF/TOF instrument. Peptide mass fingerprints were analysed using the MASCOT 2.6.00 search algorithm available on http://www.matrixscience.com (database: NCBInr LN908249.1; size: 2174 proteins; taxonomy: *Actinobacillus pleuropneumoniae* serovar 8 genome assembly MIDG2331). Search parameters were as follows: fixed modifications, carbamidomethyl (C); variable modifications: oxidation (M); cleavage by trypsin; mass tolerance, ± 100 ppm; missed cleavage, 1; significance threshold $p < 0.05$).

Individual peptides from gel spots not unequivocally identified were further analysed by MALDI-TOF/TOF-MS and the resulting daughter ions used for peptide identification using the corresponding MASCOT search algorithm. The BLASTp Sequence Analysis Tool was used to manually revise annotations for identified proteins (Madden, 2002).

3. Results

3.1. OMVs from wt, ΔdegS and ΔnlpI have a similar protein pattern

1D SDS-PAGE analysis indicated that proteins are similarly represented on the OMVs of wt, Δ*degS* and Δ*nlpI* mutants, with only slight difference in abundance of different bands (Fig. 1). We selected only the Δ*nlpI* mutant and wt strain for further analyses using 2D PAGE. The

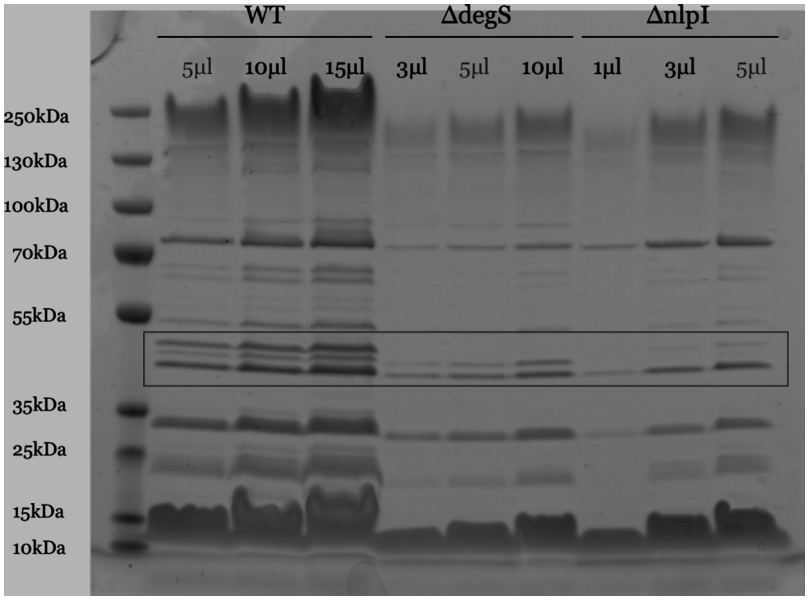

Fig. 1. SDS-PAGE analysis of *A. pleuropneumoniae* OMVs.
1: Protein Ladder, 2-4: wt OMVs, 5-7: *A. pleuropneumoniae* MIDG 2331 Δ*degS* OMVs, 8-10: *A. pleuropneumoniae* MIDG 2331 Δ*nlpI* OMVs. The box highlights an example for minor differences in the OMVs protein pattern in *A. pleuropneumoniae* serovar 8 MIDG 2331 wt and mutants Δ*degS* and Δ*nlpI*.

electrophoresis showed a very similar protein spot pattern for the OMVs for both wt and mutant. However, the same proteins were represented more abundantly in the case of the mutant (Additional file 1). For this reason, only the gel from the Δ*nlpI* mutant was selected for the identification of the proteins by MS.

3.2. MS identification of OMV proteins

Approximately 50 protein spots in total were visible on 2D PAGE gels and after excision proteins were detected in 21 spots. Some of the proteins were represented by more than one spot on the gel. A total of 15 individual proteins were annotated (depicted on Fig. 2) and their characteristics are listed in Table 1 (and Additional file 2 with detailed information regarding MS identification) and described in the discussion section.

3.3. OMVs immunological profiling

2D WB testing with convalescent pig sera, revealed that wt and Δ*nlpI* OMVs exhibit a very similar antigenic pattern, with slightly more intense spotting pattern in the case of the mutant (Additional file 1). The three most abundant proteins identified by MS (ApxII, ApxIII and LpoA) all appeared to be strongly antigenic (Fig. 2). Several others of the MS-detected proteins were also recognised by the convalescent pig sera, This assay allowed us to detect several proteins for which the concentration was too low to allow visualisation on the 2D gel by SyproRuby (Fig. 2, Additional file 1). Table 1 presents the complete summary of the immunoreactive proteins detected in this study, cross-referenced to published data regarding their antigenic potential.

4. Discussion

4.1. ΔnlpI OMV proteins

A considerable fraction of the 15 individual proteins identified was represented by well-known *A. pleuropneumoniae* virulence factors, including the RTX (repeats-in-toxin) toxin determinants ApxII, ApxIII and ApxIVA. Other proteins, including MomP and OsmY, have been

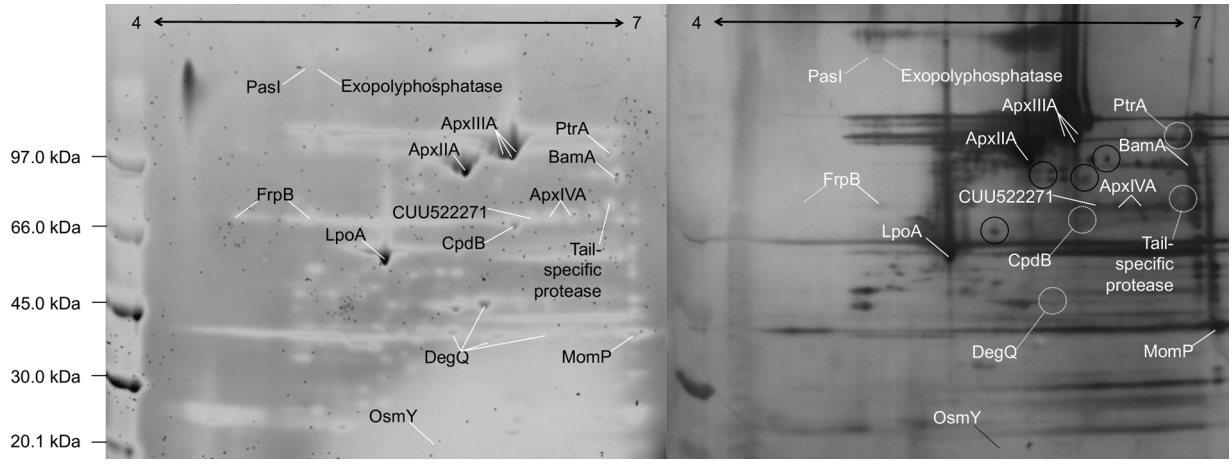

Fig. 2. Protein identification and WB characterisation of Δ*nlpI* OMVs.
Δ*nlpI* OMV proteins separated by 2D PAGE and stained with Sypro Ruby. Arrows highlight proteins detected by MS. (B) 2D WB analysis using sera from animals challenged with live *A. pleuropneumoniae* HK361 and Anti-pig secondary antibody. Sharp lines indicate immunoreactive protein spots. Dashed lines indicate the lack of immunoreaction for some of the proteins visible in (A). Circles indicate spots with no signal from non-immunoreactive proteins appearing on a smear of other immunoreactive proteins. Black circles indicate few exemplary spots of clearly immunoreactive proteins that could not be visualized by SyproRuby staining. Band size in kilodaltons (kDa) of the protein marker used in both gels is provided on the right side of the figure.

Table 1
Proteins identified from OMV isolates by Mass Spectrometry analysis.

Protein Name	pBLAST protein Homology	Accession number	Protein Function	Immuno-reactive in this study	Immuno-reactive in other studies
ApxIIIA	RTX-III toxin	CUU52669.1	virulence, hemolysis, cytotoxicity	+	(Cruijsen et al., 1992; Shin et al., 2011; Hur et al., 2016)
ApxIIA	hemolysin	CUU52271.1	virulence, hemolysis, cytotoxicity	+	(Shin et al., 2011; Hur et al., 2016)
PtrA	pitrilysin	CUU53326.1	stress tolerance, virulence	–	–
BamA	outer membrane protein assembly factor BamA	CUU51734.1	outer membrane protein assembly	?	(Loosmore et al., 1997; Zielke et al., 2016)
Tsp	carboxy-terminal protease	CUU51438.1	stress tolerance, virulence	–	–
ApxIVA	RTX toxin	CUU52321.1	virulence, weak hemolysis, cytotoxicity	+	(Wang et al., 2009; Sadilkova et al., 2012)
–	acetone carboxylase	CUU52271.1	–	+	–
CpdB	2′,3′-cyclic-nucleotide 2′-phosphodiesterase	CUU51932.1	nutrient mobilization	–	–
Frpb	ligand-gated channel	CUU51594.1	iron acquisition	+	(Pettersson et al., 1990; Kortekaas et al., 2006)
lpoA	penicillin-binding protein activator	CUU52771.1	peptidoglycan synthesis, antibiotic resistance	+	-*
PasI	hypothetical protein APL_0258	CUU51576.1	stress resistance, colonization	?	–
–	exopolyphosphatase	CUU52019.1	–	?	–
MomP	porin OmpA	CUU52846.1	structural and ion-permeable porin roles	+	-
DegQ	serine endoprotease DegQ	CUU52629.1	Stress tolerance, virulence	–	(Hirono et al., 1997; Hu and Sun, 2011)
OsmY	osmotically-inducible protein OsmY	CUU52774.1	osmoregulation, virulence	+	–

* antigenicity shown only for MomP from other bacterial species with very low homology to *A. pleuropneumoniae* MomP.

described previously as virulence factors in other bacterial species (Bader et al., 2003; Dong and Schellhorn, 2009). Some of the proteins were found to be metabolic regulators and/or hypothetical proteins. They will only be mentioned briefly below.

It is important to note that: (I) a number of the identified proteins were represented by more than one spot on the gel, possibly due to post-translational modifications altering their molecular weight and/or isoelectric point; (II) Although the focus of this work was to specifically identify antigenic proteins present on *A. pleuropneumoniae* OMVs, non-antigenic proteins identified will also be mentioned for the sake of providing a more comprehensive characterization of the general content of *A. pleuropneumoniae* OMVs; (III) Despite the relative number of protein identified here may seem low when compared with LC–MS-based studies, the validity of our approach is supported by a recently published work on *Moraxella catharalis* OMVs (Augustyniak et al., 2018), where the authors presented a comparable 2D PAGE protein separation with a similar number of proteins identified.

4.2. Toxins

The RTX toxins, also called cytolysins, are a family of calcium-dependent, pore-forming, secreted toxins found in several of Gram-negative bacteria and centrally involved in the pathogenicity of the organisms expressing them (Linhartová et al., 2010). RTX toxins target specifically leukocytes, and are able to induce inflammation and apoptosis, resulting in generalised necrosis of the host tissues (Frey, 2011). Four different Apx toxins have been described for *A. pleuropneumoniae*, Apx I, II, III and IV; and are expressed in various combinations by different serovars (Bossé et al., 2002).

All the Apx toxins produced by *A. pleuropneumoniae* MIDG 2331 (ApxII, III and IV) were identified in the isolated OMVs (Fig. 2). ApxII and III in particular were highly abundant on the 2D gel. These two toxins have lethal effects on neutrophils and macrophages and thus play a role at impairing the host defences (Dom et al., 1992; Cullen and Rycroft, 1994). ApxIV exhibits instead only weak haemolytic and cohemolytic activity (Schaller et al., 1999a), although notably the expression *in vivo* of ApxIVA has been reported as necessary for the achievement of full virulence in *A. pleuropneumoniae* (Liu et al., 2009).

Concerning ApxIV, its identification in *A. pleuropneumoniae* OMVs is likely the result of misidentification by the MASCOT algorithm employed of ApxII or ApxIII degradation products. MALDI-TOF coverage for the spot corresponding to the putative ApxIV toxin was in fact just below significance (Additional file 2), while the molecular mass of the spot on the 2D gel was significantly lower than the theoretical 202 kDa expected (Fig. 2). This interpretation is also supported by the available literature on ApxIV expression, showing that the expression of ApxIV seems to occur only under *in vivo* conditions (Schaller et al., 1999b; Liao et al., 2009).

The presence of some Apx toxins in *A. pleuropneumoniae* OMVs has been described before (Negrete-Abascal et al., 2000), but the relatively high concentration of Apx toxins in the OMVs by *A. pleuropneumoniae* MIDG 2331 suggests that the OMVs may represent a central determinant in the *A. pleuropneumoniae* pathogenesis.

4.3. Periplasmic proteases

The second group of proteins found in the isolated OMVs consisted of periplasmic proteases; serine endoprotease DegQ, pitrilysin PtrA, and the carboxy-terminal protease Tsp, all involved in protein maturation in the periplasm (Fig. 2). DegQ, belonging to the high-temperature requirement A (HtrA) protease family, was reported as a virulence factor in several pathogens (Elzer et al., 1994; Lewis et al., 2009). HtrA was shown to be required for the virulence of *Haemophilus parasuis* in a murine model of infection, where it also appeared to contribute to the resistance against complement-mediated killing and the suppression of biofilm formation by the host immune system (Zhang et al., 2016).

Other examples of the role of HtrA in pathogenesis include E-cadherin degradation in tight junctions by *Helicobater pylori* and manipulation of the host signalling pathways in *Chlamydia trachomatis* infections (Hoy et al., 2010; Zhong, 2011). Interestingly, HtrA was concentrated both in secreted inclusions and in the cytoplasm of cells infected by *Chlamydia trachomatis* (Wu et al., 2011). Similarly, the possibility of OMV internalisation into mammalian cells was previously reported (Furuta et al., 2009). We thus hypothesise that *A. pleuropneumoniae* OMVs could be used by the bacterium as carriers to deliver DegQ and other virulence factors directly into the cytosol of target host cells.

4.4. Membrane proteins

A previously reported *in silico* prediction of OM proteins in *A. pleuropneumoniae* produced a list of 93 proteins, accounting for 45 integral membrane proteins and 48 lipoproteins. 47 of these proteins, representing roughly 50% of the predicted OM proteome, could be identified by LC–MS/MS (Chung et al., 2007). In a separate study, 110 spots were detected by 2D PAGE in the *A. pleuropneumoniae* OM protein enriched fraction (Liao et al., 2009). However, only antigenic spots were analysed in aforementioned studies and 17 proteins from 28 spots were identified, of which seven were predicted as OM proteins.

The relatively smaller number of OM proteins routinely identified by 2D PAGE as compared to gel-free based approaches can be attributed to the limitations of handling and separation of hydrophobic proteins. The main limitations are: (1) use of SDS-free sample buffers compatible with IEF, leading to the loss of a significant portion of hydrophobic proteins during standard sample centrifugation before loading onto IPG strip; and (2) tendency of solubilised hydrophobic proteins to precipitate once they reach their isoelectric point that obstruct them to enter SDS-PAGE gels.

These factors could explain why only four OM proteins were detected in the OMV fraction analysed in our study; the iron-regulated FrpB protein, the major OM protein MomP, BamA and the penicillin-binding protein LpoA (Fig. 2). However, it cannot be excluded the presence of a native sorting mechanism, allowing only some of *A. pleuropneumoniae* OM proteins to be shed and incorporated into the vesicles, similarly to what has been suggested for *Porphyromonas gingivalis* (Haurat et al., 2011).

FrpB is a well-described virulence factor belonging to the TonB-dependent receptor family. In *A. pleuropneumoniae*, FrpB was proven to be involved in iron uptake under anaerobic conditions and ferric deprivation (Buettner et al., 2009). MomP is an abundant OM protein belonging to the OmpA family, a family of proteins with both structural and ion-permeability roles and often suggested as a virulence factor (Nikaido, 2003). BamA is an essential outer membrane protein that is part of the large multi-protein BAM complex and responsible for the assembly and insertion of β-barrel proteins in the OM (Wu et al., 2005).

LpoA and LpoB are OM lipoprotein involved in the control of peptidoglycan (PG) synthesis outside a sacculus. These penicillin-binding protein (PBP) activators bind specifically to their cognate PBPs and stimulate their transpeptidase activity (Paradis-Bleau et al., 2010). LpoB is recruited to the divisome but also to the lateral wall, whereas LpoA concentrates more at the sidewall of elongating cells (Typas et al., 2010). Interestingly, our data show LpoA to represent the third most abundant protein in *A. pleuropneumoniae* vesicles. This suggests that OMV biosynthesis in *A. pleuropneumoniae* could rely on pathways involved in cell elongation rather than cell division.

4.5. Other proteins detected in OMVs

Another protein detected by MS was the osmo-inducible protein OsmY (Fig. 2). OsmY plays a role in the bacterial resistance to osmotic stress, and was shown to be directly involved in the pathogenicity of several bacterial species (Bader et al., 2003; Dong and Schellhorn, 2009). The same is true for PasI, a member of a toxin-antitoxin system that was demonstrated to play a role in colonization and stress resistance (Norton and Mulvey, 2012).

The detected 5′-nucleotidase CpdB is a bifunctional enzyme that catalyses two consecutive reactions during ribonucleic acid degradation, first converting a 2′,3′-cyclic nucleotide to a 3′-nucleotide and then the 3′-nucleotide to the corresponding nucleoside and phosphate (Liu et al., 1986). The deletion of this gene in *Salmonella enterica* was shown to decrease the colonization rate in the cecum and internal organs of chickens (Liu et al., 2015). Furthermore, CpdB was also determined to likely represent a *Streptococcus sanguinis* virulence factor (Fan et al., 2012) and found in the extracellular polymeric matrix of *Shewanella* (Pinchuk et al., 2008). Its presence in the extracellular matrix suggests that CpdB could be involved in the extracellular degradation of DNA under phosphorus-limited conditions, and that CpdB may be shed *via* vesicles to mobilise nutrients for bacterial use. A similar role may be ascribed to the exopolyphosphatase identified in this analysis (Table 1).

4.6. Antigenicity of OMV proteins

Δ*nlpI* and wt OMVs showed a highly similar antigenic pattern when challenged with immune sera (Additional file 1), suggesting that the antigenicity of Δ*nlpI* OMVs were conserved despite the lack of the *nlpI* gene product. This, together with the general high immunogenicity we previously reported for *A. pleuropneumoniae* Δ*nlpI* OMVs (Antenucci et al., 2017), confirmed the validity of these OMVs as antigen carriers. Among the individual antigens identified, we were able to demonstrate for the first time that LpoA, OsmY, MomP and the hypothetical protein MIDG2331_02184 all elicit an antibody response during an experimental challenge *in vivo* (Fig. 2).

Surprisingly, three of the identified proteins with a predicted proteolytic activity (Table 1) did not contributed to the antigenic pattern of *A. pleuropneumoniae* OMVs (Fig. 2). The same was true for DegQ, which our results indicate to be scarcely antigenic in pigs (Fig. 2), despite what was previously reported for several of the homologs (Hirono et al., 1997; Hu and Sun, 2011).

Finally, we were able to demonstrate for the first time that LpoA, OsmY, MomP and the hypothetical protein MIDG2331_02184 all elicit an antibody response during an experimental challenge *in vivo* (Fig. 2).

5. Conclusions

MS analysis showed that *A. pleuropneumoniae* OMVs carry a broad array of virulence factors, including at least two of the three Apx toxins encoded by the serovar 8 strain characterized and proteins involved in nutrient mobilization. This suggests that the OMVs could represent a ‘‘long-range’’ weapon for *A. pleuropneumoniae*; able of modifying the surrounding environment of the bacterium to satisfy its nutritional needs, and hamper the effectiveness of the host immune response by targeting lymphocytes.

The identification of LpoA as the third most abundant protein in the OMVs, without its cognate LpoB, suggests that OMVs biosynthesis in *A. pleuropneumoniae* may be dependent on mechanisms involved in cell elongation.

2D gel analysis revealed a unique antigenic pattern for *A. pleuropneumoniae* OMVs, leading to the identification of four new antigens that had never been described in other pathogenic bacteria: LpoA, OsmY, MomP and the hypothetical protein MIDG2331_02184.

In conclusion, these data cast new light on the OMVs antigenic profile, immunogenic potential and biological role in the *A. pleuropneumoniae* pathogenesis, supporting the use of *A. pleuropneumoniae* OMVs as a potential scaffold for *A. pleuropneumoniae* antigen presentation to the porcine immune system.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.07.001.

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