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Fifteen novel immunoreactive proteins of Chinese virulent *Haemophilus parasuis* serotype 5 verified by an immunoproteomic assay

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Abstract *Haemophilus parasuis* (*H. parasuis*) is associated with meningitis, polyserositis, polyarthritis and bacterial pneumonia. At present, its prevention and control is difficult because of the lack of suitable subunit vaccines. Nowadays, high-throughput methods, immunoproteomics, are available to screen for more vaccine candidates. A protein extraction method for *H. parasuis* and two-dimensional electrophoresis (2-DE) were optimized to provide high-resolution profiles covering pH 3 to 10. Twenty immunoreactive spots were excised from gels after strict comparison between 2-DE Western blot membranes and the relevant gels. Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) and MALDI-TOF–TOF-MS successfully identified 16 different proteins. Fifteen of them were reported as immunoreactive proteins in *H. parasuis* for the first time. In addition, recombinant HP5-7 (ABC transporter, periplasmic-binding protein) showed immunoreactivity both with hyperimmune rabbit serum and convalescent swine serum. Four recombinants of the 14 successfully expressed genes showed immunoreactivity with hyperimmune rabbit serum.

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Introduction

H. parasuis is a Gram-negative bacterium that belongs to the *Pasteurellaceae* family. Its clinical infection signs are termed Glässer disease (Riley et al. 1977). There are 15 distinct serotypes, while 15–41 % of the isolates were reported as non-typable. Serotypes 1, 5, 10, 12, 13 and 14 are the most virulent (Angen et al. 2004; Oliveira et al. 2003). The most prevalent serotypes in China are 4 and 5 (Cai 2006; Zhou et al. 2010).

Abuse of antimicrobials in farmed animals is a hazard to humans, so vaccination is the preferred method. However, pigs immunized with monovalent vaccines are protected against challenge with the homologous serotype strains but not with other heterologous ones (Takahashi et al. 2001). Three ABC-type transporters (OppA, YfeA, and PlpA) and one curli protein assembly (CsgG) showed cross-reactivity when tested with sera raised against serovars 4 and 5 of *H. parasuis* (Hong et al. 2011). The purified recombinants of outer membrane protein (OMP) P2 and OmpP5 (also known as OmpA, PalA, *P2*, D15, HPS-06257) provided partial protection against *H. parasuis* infection in mice (Ahn et al. 2012; Sturgill et al. 2013; Tang et al. 2010; Zhou et al. 2009). However, some reports indicated that recombinant OmpP5 could not provide satisfactory protection in mice after bacterial challenge (Zhou et al. 2009). Recently, it was reported that recombinant virulence-associated trimeric autotransporters (VtaA) could provide partial protection against *H. parasuis* infection in colostrum-deprived piglets (Olvera et al. 2011). Thus, in contrast to other diseases of similar importance, there are few effective subunit vaccines.

The whole *H. parasuis* genome sequences SH0165 (serotype 5) (Yue et al. 2009) and 29755 (serotype 5) (Mullins et al. 2011) have been completed. In the post-genomics era, immunoproteomics has emerged as a high-throughput method

for screening novel vaccine candidates. All of these developments lay a solid foundation for our research.

In this paper, we used an immunoproteomic approach to identify immunogenic antigens according to the immune response in *H. parasuis* serotype 5-immunized rabbits.

Materials and methods

Bacterial strains and culture conditions

H. parasuis strain GD10 (serotype 5) was isolated from a diseased pig in Guangdong Province in China. It was kindly provided by Dr. Guiping Wang (Guangdong Modern Agriculture Research Institute) and cultured in tryptic soy broth (TSB; Sigma-Aldrich Co. LLC., MO, USA) with 5 % newborn calf serum (Tian Jin Hao Yang Biological Manufacture Co., Ltd., Tian Jin, China) and 20 μg/mL NAD (Sinopharm Chemical Reagent Co., Ltd., China). Cultures were incubated at 37 °C in a rotary incubator (180 rpm) until the late stage of the exponential phase (Cai 2006).

Hyperimmune sera preparation

Polyclonal antibodies were raised in rabbits immunized with formaldehyde-inactivated GD10 bacteria after the rabbits were determined to be negative for GD10 antibodies by whole-cell enzyme-linked immunosorbent assay (ELISA). Three doses of 1.0×10^8 cells per rabbit were administered by subcutaneous injections at 2-week intervals (Cai 2006).

Convalescent sera screening

The sera of apparently healthy swine that had not been immunized with any *H. parasuis* vaccine were screened by ELISA using recombinant (OMP) P2, whose sequence is conserved among *H. parasuis* but specific to other bacteria, especially *Pasteurella multocida* (PM) and *Actinobacillus pleuropneumoniae* (APP). A swine that tested positive was considered to have been infected by *H. parasuis* and to have recovered.

Protein sample extraction

Proteins were extracted according to the approach of Zhang et al. with slight modifications (Zhai et al. 2012). Trichloroacetic acid (TCA, Sinopharm Chuan Kang Pharmaceutical Co., Ltd. China) was added to the sample preparation solution extract at a final concentration of 5 %.

Isoelectric focusing (IEF)

IEF was performed according to the approach of Zhang et al. with slight modifications (Zhai et al. 2012) and was carried out at 20 °C for 10.5 h (max. voltage 8,000 V; max. current 50 μA per IPG strip; total 28,000 V/h).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

After SDS-PAGE, one gel was stained with Coomassie Brilliant Blue (CBB-G250), while the duplicate gel was subjected to Western blot analysis. After blocked, the membrane was incubated with anti-GD10 sera from immunized rabbits (1:2,000 dilution) at 37 °C for 2 h. Three replicates were run for each sample.

Image processing

Imagemaster 7.0 (GE Healthcare) was used to analyze the images scanned from the PVDF membrane and the corresponding two-dimensional electrophoresis (2-DE) gels.

MALDI-TOF-MS and database searches

Spots that identified as immunoreactive were excised and analyzed by MALDI-TOF-MS/MALDI-TOF–TOF-MS (NanJing Steed BioTechnologies Co. Ltd., Nanjing, China). The MASCOT server (http://www.matrixscience.com) was used to analyze the peptide mass fingerprinting (PMF) data.

Bioinformatic analysis

The compute pI/Mw server (http://expasy.org/tools/pi_tool.html) was used to calculate isoelectric point (pI) and molar mass (MW). The TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict transmembrane helices. The PSORT server (http://www.psort.org/) was used to predict the subcellular location and the SignalP 3.0 server (http://www.cbs.dtu.dk/servicess/SignalIP) identified potential signal peptides.

Characterization of recombinant immunoreactive proteins

Primer Premier 5.0 and Oligo 6.24 were used to design PCR primers (Table 1). Gene sequences of the 16 identified proteins were cloned into the pET-32a vector and expressed in *Escherichia coli* BL21 (DE3). Whole-cell extracts were subjected to SDS-PAGE with CBB-R250 staining and Western blotting using the anti-GD10

Table 1 Specific primers for sequences encoding the identified proteins

Spot no.	Specific primers (5′–3′)	Tm (°C)
HP5-1	CGCGGATCCCGCCTTGCGAATGGAATG CCGCTCGAGAATGGCCTGCTGATAGAA	53.3
HP5-2	CGCGGATCCGAAGGTACGGTGCTTGCAGAG CCGCTCGAGACCTTTCACGAATGGCTTAAC	53.6
HP5-3	CGCGGATCCGAAGGTACGGTGCTTGCAGAG CCGCTCGAGACCTTTCACGAATGGCTTAAC	53.7
HP5-4	CGCGGATCCCAATTTAATCGTGGCGATAAA CCGCTCGAGACCTTCACGGATAGCGAAACG	54.2
HP5-5	CGCGGATCCGTTAGCCGTTCACCAACAGGT CCGCTCGAGAACCGCTTTGTGTAACAACGC	54.8
HP5-6	CGCGGATCCATCGTAAAATCCGCTAACTCT CCGCTCGAGTACCGCTTTCGCTTCTGGAGA	53.4
HP5-7	CGCGGATCCCATTTTGAACACAATGAATCT CCGCTCGAGTACACCAATGAATGACATACC	52.0
HP5-9	CGCGGATCCACGAGTTAACACAAGCGGATA CCGCTCGAGGTGCTTAACCAAGCTGGATTA	54.0
HP5-11	CGCGGATCCTTCTAATTTTAATGCATCGGT CCGCTCGAGTTAAAAGACTTTGAAAAACAA	51.1
HP5-12	CGCGGATCCGTGATGTCGCACGTGCGCCTG CCGCTCGAGCACCTGGTCCACAAAAGCCTT	62.6
HP5-13	CGCGGATCCAATTTGCACGCTGATGAATTA CCGCTCGAGAAAGCCTTTTGGCGTACATCG	54.4
HP5-14	CGCGGATCCTTTTTGTAACACTTCAGAGGT CCGCTCGAGAGTGCATTAGCAAATGACACT	50.3
HP5-16	CGCGGATCCAAACTCCTTGAATTTTTTGTG CCGCTCGAGACACCATTTTCTCAACGTTTT	51.6
HP5-17	CGCGGATCCGCGGATCGCATTGGTGTGACA CCGCTCGAGATCTACGCCAACATAAGGAAT	52.3
HP5-18	CGCGGATCCATGACTCCACATATTAACGCA CCGCTCGAGTTAACCTTGCTGATCACCAAT	52.9
HP5-19	CGCGGATCCATTCAATCTCACGTCGTGTAT CCGCTCGAGAATTTTTACCGCTTGATAATG	52.0

hyperimmune rabbit sera, swine convalescent sera and their corresponding negative sera.

Results

2-DE profiles of H. parasuis bacterial proteins and Western blot analysis

IEF separated over a pH range of 3–10 and 13-cm length in the first direction and a separation using SDS-PAGE in the vertical dimension identified 375 spots. Most proteins had a molar mass between 10 and 150 kDa (Fig. 1a). The repeat gels showed the corresponding immunoreactive spots (Fig. 1b).

Identification of immunoreactive spots

Twenty immunoreactive spots (HP5-1 to HP5-20) were excised from repeated 2-DE gels and analyzed by MALDI-TOF-MS/MALDI-TOF–TOF-MS followed by PMF searches. Finally, the 20 protein spots were identified as belonging to 16 different proteins (Table 2).

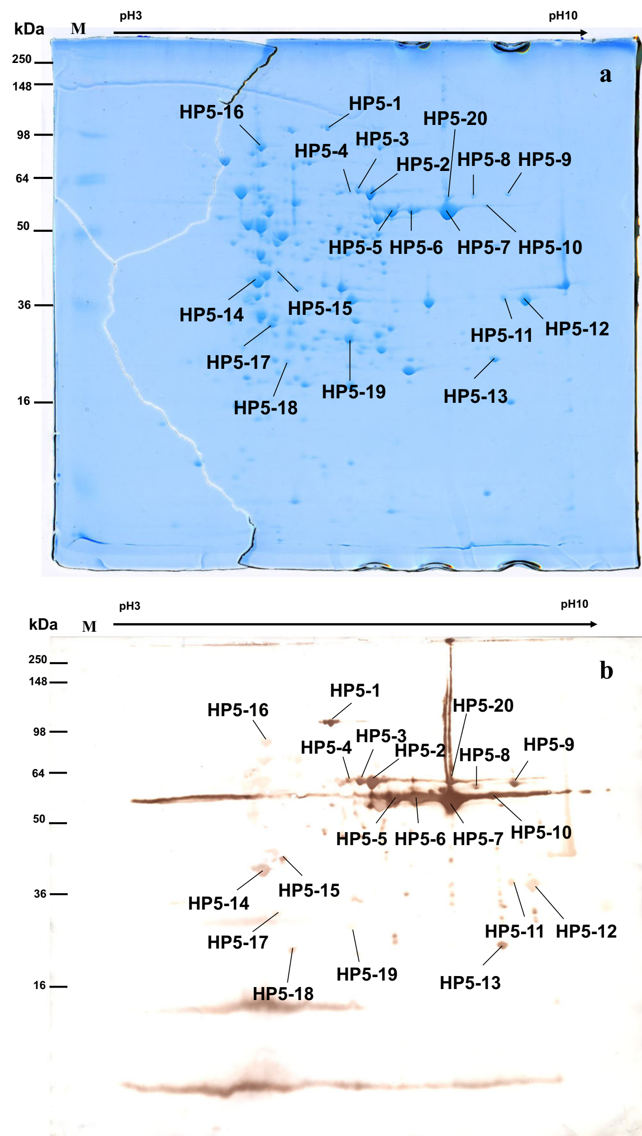

Fig. 1 The *numbers on the left* (M) indicate the molar mass of standards (kDa). The *numbers in the figure* represent different independent spots. *Arrows* indicate the corresponding proteins. 2-DE and Western blotting of whole-cell proteins from GD10. **a** CBB-G250-stained 2-DE gel of whole-cell proteins from GD10 (pH 3–10, 13 cm). **b** Western blot of corresponding gels of whole-cell proteins from GD10 using immunized anti-GD10 hyperimmune sera

Bioinformatics analysis

The prediction results of subcellular locations, transmembrane helices and signal peptide cleavage sites of all identified proteins are listed in Table 3.

Characterization of recombinant immunoreactive proteins gene

Without HP5-3 and HP5-14/HP5-15, 4 out of the 14 successfully expressed proteins could react with hyperimmune rabbit sera raised against GD10 (Fig. 2). The four proteins were a

Table 2 Analysis of protein spots identified by MALDI-TOF-MS/MALDI-TOF–TOF-MS

Spot no.[a]	Top score of Mascot search results (%)[b]	Protein	Theoretical (pI/MW)[c]	Experimental (pI/MW)
HP5-1	292 (43)	dsDNA-mimic protein	5.93/109,942.20	6.0/100,000
HP5-2	206 (33)	Holliday junction DNA helicase B	6.51/61,005.96	6.5/60,000
HP5-3	139 (32)	Oligopeptide permease ABC transporter membrane protein	6.71/60,948.95	6.4/61,000
HP5-4	156 (42)	Elongation factor-Tu	5.23/43,414.46	6.2/60,000
HP5-5	102 (31)	Heme-binding protein A	7.06/59,316.97	6.7/57,000
HP5-6	208 (39)	Periplasmic-binding protein/LacI transcriptional regulator	6.44/32,976.60	6.8/57,000
HP5-7	98 (37)	ABC transporter, periplasmic-binding protein	8.23/57,887.17	8.0/57,000
HP5-8	254 (54)	ABC transporter, periplasmic-binding protein	8.23/57,887.17	8.2/58,000
HP5-9	171 (46)	DNA-directed RNA polymerase subunit omega	5.09/23,056.13	8.8/59,000
HP5-10	216 (46)	ABC transporter, periplasmic-binding protein	8.23/57,887.17	8.3/57,500
HP5-11	135 (55)	FbpA	8.80/37,665.04	8.8/37,000
HP5-12	200 (64)	Indolepyruvate ferredoxin oxidoreductase alpha/beta subunit	8.81/27,474.14	8.9/34,000
HP5-13	175 (27)	AraC family transcriptional regulator	6.22/33,481.01	8.4/31,000
HP5-14	184 (59)	Hypothetical protein HPS_10240	5.26/38,464.01	5.2/38,000
HP5-15	92 (41)	Hypothetical protein HPS_10240	5.26/38,464.01	5.5/39,000
HP5-16	178 (67)	Putative solute/DNA competence effector	9.62/25,201.23	5.3/80,000
HP5-17	375 (55)	Galactose ABC transporter periplasmic-binding protein/LacI transcriptional regulator	5.72/35,436.72	5.4/33,000
HP5-18	604 (99)	Purine nucleoside phosphorylase	5.30/26,012.87	5.6/28,000
HP5-19	344 (52)	Pyridoxine kinase	5.92/31,338.13	6.2/32,000
HP5-20	460 (41)	ABC transporter, periplasmic-binding protein	8.23/57,887.17	8.1/58,000

[a] Protein spots corresponding to position on gel and blot (Fig. 1)

[b] From PMF data, proteins scoring greater than 85 are significant ($p<0.05$). Data in parentheses indicate the extent of sequence coverage

[c] The threshold of significance was greater than 95 % for all values in this study

Table 3 Bioinformatics analysis of identified proteins

Spot no.	PSORT prediction (scores)					TMHMM	SignalP 3.0
	Outer membrane	Extracellular	Periplasmic	Cytoplasmic membrane	Cytoplasmic	Prediction	Prediction
HP5-1	0.06	0.11	9.76	0.06	0.00	+	+
HP5-2	0.00	0.00	10.00	0.00	0.00	−	+
HP5-3	0.00	0.00	10.00	0.00	0.00	−	+
HP5-4	0.00	0.00	0.01	0.01	9.97	−	−
HP5-5	0.01	0.00	9.99	0.00	0.00	−	+
HP5-6	0.00	0.01	6.58	3.41	0.00	−	+
HP5-7	0.06	0.11	9.76	0.06	0.00	+	−
HP5-9	2.00	2.00	2.00	2.00	2.00	−	−
HP5-11	0.00	0.00	10.00	0.00	0.00	−	+
HP5-12	2.00	2.00	2.00	2.00	2.00	−	−
HP5-13	0.00	0.00	0.01	0.01	9.97	−	−
HP5-14	0.00	0.00	10.00	0.00	0.00	−	+
HP5-16	0.01	0.26	0.26	0.51	8.96	−	−
HP5-17	0.06	0.11	9.76	0.06	0.00	−	+
HP5-18	0.00	0.00	0.01	0.01	9.97	−	−
HP5-19	0.00	0.00	0.01	0.01	9.97	−	−

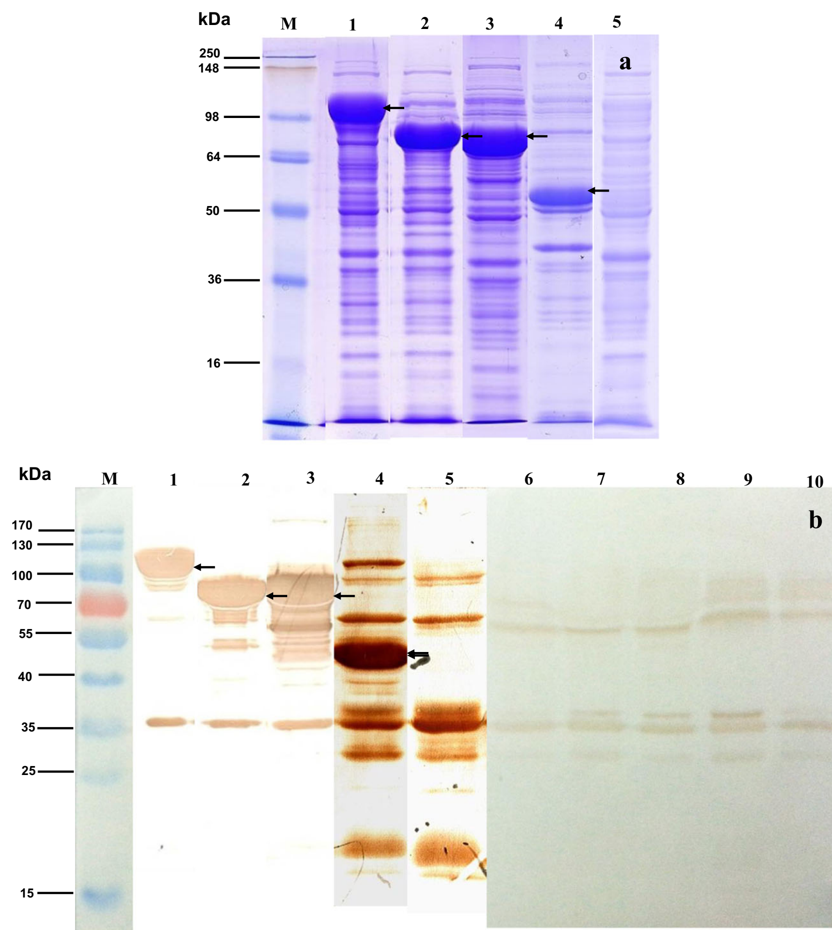

Fig. 2 The *numbers on the left* (M) indicate the molar mass of standards (kDa). The *numbers in the figure* represent different independent spots. *Arrows* indicate the corresponding proteins. CBB-R250-stained gel and Western blotting of the four recombinant immunoreactive proteins from cell lysates. **a** CBB-R250-stained gel of the four recombinant immunoreactive proteins in cell lysates CBB-R250-stained gel of induced *1* recombinant HP5-1, *2* recombinant HP5-2, *3* recombinant HP5-7, *4* recombinant HP5-17, and *5* pET-32a vector. **b** Western blot analysis of the four recombinant immunoreactive proteins in cell lysates. Western blot of induced *1* recombinant HP5-1, *2* recombinant HP5-2, *3* recombinant HP5-7, *4* recombinant HP5-17, and *5* pET-32a vector reacted with anti-GD10 hyperimmune sera. Western blot of induced *6* recombinant HP5-1, *7* recombinant HP5-2, *8* recombinant HP5-7, *9* recombinant HP5-17, and *10* pET-32a vector reacted with negative rabbit sera

double-stranded DNA (dsDNA)-mimic protein, Holliday junction DNA helicase B, an ABC transporter and periplasmic-binding protein/LacI regulator. The ABC transporter also reacted with the swine convalescent sera (Fig. 3).

Discussion

We obtained 2-DE profiles with better resolution, over a broader pH range and produced clearer Western blots with the modification of protein sample preparation, compared with other proteomics studies in *H. parasuis* (Zhou et al. 2009).

The 16 identified immunoreactive proteins could be divided into three categories. The first category comprised proteins that have already been studied as subunit vaccines in *H. parasuis*. HP5-7, HP5-8, HP5-10, and HP5-20 were identified as the same protein: ABC transporter, a ubiquitous membrane protein (Schmitt and Tampe 2002) in all species, mediating the uptake and efflux of a diverse array of compounds (Grote et al. 2009; Locher 2004), including the nonclassical secretion of signaling molecules and toxins. Three other ABC-type transporters (OppA, YfeA, and PlpA), which show amino acid identity of 34, 47, and 32 %, respectively, with the protein represented by HP5-7, showed cross-reactivity when tested with sera raised against serotypes 4 and 5 of *H. parasuis* (Hong et al. 2011).

The second category was proteins whose homologues in other bacteria were reported to be immunogenic. HI1450 may function as a dsDNA-mimic (HP5-1) to recognize, inhibit, or regulate an as yet unidentified dsDNA-binding protein. It is an immunoreactive protein against hyperimmune rabbit sera when expressed in *E. coli* (Parsons et al. 2004, 2005).

Elongation factor-Tu (EF-Tu, HP5-4) plays a central role during the selection of the correct amino acids during the elongation phase of translation (Kavaliauskas and Knudsen

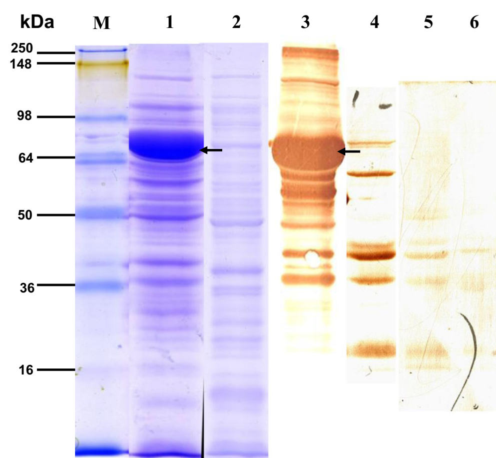

Fig. 3 The *numbers on the left* (M) indicate the molar mass of standards (kDa). The *numbers in the figure* represent different independent spots. *Arrows* indicate the corresponding proteins. CBB-R250-stained gel and Western blot analysis of the recombinant immunoreactive proteins from cell lysates. CBB-R250-stained gel of induced *1* recombinant HP5-7 and *2* pET-32a vector. Western blot analysis of induced *3* recombinant HP5-7 and *4* pET-32a vector reacted with convalescent swine serum. Western blot analysis of induced *5* recombinant of HP5-7 and *6* pET-32a vector reacted with negative swine sera

2012). Many studies have verified the immunoreactivity of EF-Tu (Vergauwen et al. 2010).

Heme-binding protein A (HbpA, HP5-5) was identified as a virulence determinant in a model of *Haemophilus influenzae* invasive disease constructed by an insertional mutation of *hbpA* in a type b and a non-typable *H. influenzae* strain (Morton et al. 2005, 2009). HbpA of APP grown under iron-restricted conditions generated immunoreactivity in an immunoproteomic analysis (Chung et al. 2012).

The acquisition of iron from transferrin by Gram-negative bacterial pathogens is dependent on a periplasmic ferric-ion-binding protein, FbpA (HP5-11). It was highly antigenic in mice and showed intraspecies and interspecies antigenic homogenicity and specific anti-FbpA antibodies are fully cross-reactive (Ferreiros et al. 1999).

The third category included proteins with no previous report of immunogenicity. The smallest subunit of *E. coli* RNA polymerase is termed omega (HP5-9) (Gentry and Burgess 1986). A deletion mutation in this gene of *Mycobacterium smegmatis* (*M. smegmatis*) caused reduced sliding motility and defective biofilm formation. This resulted from a deficiency in generation of the extracellular matrix and the mutant bacterium failed to synthesize the short-chain mycolic acids that are characteristic of biofilm growth in *M. smegmatis* (Mathew et al. 2006).

Indolepyruvate ferredoxin oxidoreductase (IOR, HP5-12) from hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1 catalyzes the oxidative decarboxylation of arylpyruvates by forming a heterooligomeric complex (alpha2beta2) (Siddiqui et al. 1998).

Most of the AraC family (HP5-13) are thought to be transcriptional activators that regulate genes related to carbon metabolism, stress responses, or pathogenesis (Egan 2002; Zeng and Spiro 2013). The *Mycobacterium tuberculosis* mutant strain disrupted in the AraC homologue *Rv1931c* exhibited reduced survival both in macrophages and in a mouse infection model (Frota et al. 2004).

Purine nucleoside phosphorylase (PNP, HP5-18) is a key enzyme to transfer glycosyl residues to acceptor bases. It has potential applications in the synthesis of nucleoside analogs used in the treatment of antiviral infections and in anticancer chemotherapy (Martins et al. 2011).

Pyridoxine kinase (HP5-19), which also phosphorylates pyridoxal (PL) and pyridoxamine (PM) in vitro, functions solely in the vitamin B6 salvage pathway. In *E. coli*, it contains an additional PL kinase associated with biosynthesis of pyridoxal 5′-phosphate (Yang and Winkler 1996).

The crystal structure of the Holliday junction DNA helicase B (HP5-2) bound to a single *E. coli* RuvA tetramer at 3.1-Å resolution has been solved.

Periplasmic-binding proteins (PBPs, HP5-6) are essential components of bacterial transport systems and are necessary for bacterial growth and survival (Shi et al. 2009).

The LacI regulator (HP5-17) is involved in the adaptive response of *Streptococcus pneumoniae* via its control of competence, adherence, and virulence (Chapuy-Regaud et al. 2003).

No details of oligopeptide permease ABC transporter membrane protein (HP5-3), hypothetical protein HPS_10240 (HP5-14/HP5-15), or putative solute/DNA competence effector (HP5-16) are available.

In conclusion, we obtained clear 2-DE and Western blot profiles of immunogenic proteins from *H. parasuis*. We identified 16 immunoreactive proteins, 15 of which are novel in *H. parasuis*. These data represent the basis for developing promising subunit vaccines.

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