The Journal of Microbiology (2012) Vol. 50, No. 6, pp. 978–986
DOI 10.1007/s12275-012-2214-2

Identification of Conserved Surface Proteins as Novel Antigenic Vaccine Candidates of *Actinobacillus pleuropneumoniae*

Xiabing Chen, Zhuofei Xu, Lu Li, Huanchun Chen, and Rui Zhou*

Division of Animal Infectious Diseases in State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, P. R. China

(Received April 19, 2012 / Accepted July 6, 2012)

***Actinobacillus pleuropneumoniae* is an important swine respiratory pathogen causing great economic losses worldwide. Identification of conserved surface antigenic proteins is helpful for developing effective vaccines. In this study, a genome-wide strategy combined with bioinformatic and experimental approaches, was applied to discover and characterize surface-associated immunogenic proteins of *A. pleuropneumoniae*. Thirty nine genes encoding outer membrane proteins (OMPs) and lipoproteins were identified by comparative genomics and gene expression profiling as beinghighly conserved and stably transcribed in the different serotypes of *A. pleuropneumoniae* reference strains. Twelve of these conserved proteins were successfully expressed in *Escherichia coli* and their immunogenicity was estimated by homologous challenge in the mouse model, and then three of these proteins (APJL_0126, HbpA and OmpW) were further tested in the natural host (swine) by homologous and heterologous challenges. The results showed that these proteins could induce high titers of antibodies, but vaccination with each protein individually elicited low protective immunity against *A. pleuropneumoniae*. This study gives novel insights into immunogenicity of the conserved OMPs and lipoproteins of *A. pleuropneumoniae*. Although none of the surface proteins characterized in this study could individually induce effective protective immunity against *A. pleuropneumoniae*, they are potential candidates for subunit vaccines in combination with Apx toxins.**

***Keywords*:** *A. pleuropneumoniae*, outer membrane proteins, lipoproteins, vaccine

Introduction

The Gram-negative bacterium, *Actinobacillus pleuropneumoniae*, is an encapsulated coccobacillus which is the etiologic agent of a severe and contagious pleuropneumonia in swine, leading to great economic losses to the global pig industry (Bosse *et al.*, 2002). Based on differences in capsular polysaccharides, 15 serotypes of *A. pleuropneumoniae* have been recognized to date, with great diversity in virulence and interlocal distribution (Ward and Inzana, 1997).

Currently, vaccination against *A. pleuropneumoniae* is usually carried out by inoculating inactivated whole-cell bacterins, and/or several available subunit vaccines (e.g. exotoxins ApxI, ApxII, and ApxIII, and outer membrane proteins) (Ramjeet *et al.*, 2008). However, these vaccination strategies provide partial protection against challenges with homologous or heterologous serotypes of *A. pleuropneumoniae* (Liao *et al.*, 2009). Live attenuated vaccines are promising but have not been used in pig farms due to safety issues (Ramjeet *et al.*, 2008). Thus, novel vaccination antigens that are able to be expressed by various *A. pleuropneumoniae* serotypes need to be exploited to provide effective protection against this porcine pathogen. Antigens expressed by all serotypes may also trigger cross-protection against different *A. pleuropneumoniae* serotypes.

Surface-associated proteins are often involved in host-pathogen interactions and are also important targets for vaccine development (Grandi, 2010). Several outer membrane proteins (OMPs) and lipoproteins have been identified in *A. pleuropneumoniae*. Two recent studies have shown systematic outer membrane proteomic analyses for *A. pleuropneumoniae* serotypes 5b and 3 (Chung *et al.*, 2007). However, the distribution of these surface-associated proteins among *A. pleuropneumoniae* strains has not been well characterized, especially by empirical evidence from challenge experiments in animal models.

In this study, bioinformatic approaches were used to predict surface proteins of *A. pleuropneumoniae*. Together with the analyses of comparative genomics and transcriptional profiling from different serotype reference strains and field strains, genes found to be highly conserved and stably expressed were identified as potential subunit vaccine targets. Twelve of these candidates were evaluated for immunogenicity by homologous and/or heterologous challenges in the mouse model and in natural swine host.

Materials and Methods

Identification of surface proteins of *A. pleuropneumoniae*

To identify the candidate surface proteins of *A. pleuropneumoniae*, we used three strategies combining bioinformatic and experimental approaches. Firstly, a coding sequence (CDS) set of the complete genome of *A. pleuropneumoniae* serotype 3 strain JL03 (Xu *et al.*, 2008) was used to identify *in silico* possible surface proteins. The program PSORTb v2.0 was used to predict bacterial protein subcellular location

*For correspondence. E-mail: rzhou@mail.hzau.edu.cn; Tel.: +86-27-8728-1878; Fax: +86-27-8728-2608

Table 1. Bacterial strains used in this study

Strains	Characteristics	Sources
E. coli DH5α	*supE44 lacU169 Δ(φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 ΔluxS*	TaKaRa
E. coli BL21(DE3)	F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
A. pleuropneumoniae 4074	Serotype 1	Dr Blackall
A. pleuropneumoniae 4226	Serotype 2	Dr Blackall
A. pleuropneumoniae S1421	Serotype 3	Dr Blackall
A. pleuropneumoniae JL03	Serotype 3	Our lab
A. pleuropneumoniae M62	Serotype 4	Dr Blackall
A. pleuropneumoniae K17	Serotype 5a	Dr Blackall
A. pleuropneumoniae L20	Serotype 5b	Our lab
A. pleuropneumoniae Femφ	Serotype 6	Dr Blackall
A. pleuropneumoniae WF83	Serotype 7	Dr Blackall
A. pleuropneumoniae 405	Serotype 8	Dr Blackall
A. pleuropneumoniae CVJ13261	Serotype 9	Dr Blackall
A. pleuropneumoniae D13039	Serotype 10	Dr Blackall
A. pleuropneumoniae 56153	Serotype 11	Dr Blackall
A. pleuropneumoniae 1096	Serotype 12	Dr Blackall
A. pleuropneumoniae N-273	Serotype 13	Dr Blackall

(Xu *et al.*, 2008). Signal peptide cleavage sites and transmembrane helices (TMHs) of open reading frames (ORFs) were predicted using the packages SignalP 3.0 and TMHMM 2.0 respectively (Krogh *et al.*, 2001; Bendtsen *et al.*, 2004). Integral beta-barrel outer membrane proteins were identified by BOMP (Berven *et al.*, 2004). Potential lipoproteins were predicted by LipoP (Juncker *et al.*, 2003). Secondly, the conservation of the candidates was further investigated using public genomic information of 11 distinct serotypes of *A. pleuropneumoniae*, including two complete genomes of serotype 5b strain L20 (Foote *et al.*, 2008) and serotype 7 strain AP76, and nine draft genome sequences of reference strains of serotypes 1, 2, 4, 6, 9, 10, 11, 12, and 13 (Xu *et al.*, 2010). The *A. pleuropneumoniae* strains used in this study are listed in Table 1. Thirdly, gene expression profiling experiments for reference strains and Chinese field isolate JL03 of *A. pleuropneumoniae* were carried out to identify the genes stably transcribed *in vitro*, as follows. *A. pleuropneumoniae* strains were grown at 37°C in tryptic soy broth (TSB) supplemented with 10 μg/ml nicotinamide adenine dinucleotide (NAD) and 5% newborn bovine serum. The bacterial cells were harvested from middle exponential phase for RNA extraction and hybridization. The detailed protocols for Agilent microarray preparation and hybridization have been described in our previous work (Li *et al.*, 2011a). The raw microarray data have been deposited in the NCBI GEO database under the accession number GSE15545.

Preparation of recombinant proteins

The CDSs of candidate genes were amplified from the genomic DNA of *A. pleuropneumoniae* JL03 using the primers listed in Table 2. The PCR products were cloned into pET-28a and transformed into *E. coli* DH5α. Positive clones were transformed into *E. coli* BL21 (DE3) for expression. Transformants, which were grown to mid-log, were induced at 37°C by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma). Recombinant 6× His-tagged proteins were purified by Ni-NTA spin columns (QIAGEN, Germany) and dialyzed using D-Tube™ Dialyzers Midi (Novagen, Germany) according to the manufacturer's instructions. The purified proteins were analysed by SDS-PAGE and Western blot.

Table 2. Primers used in this study

Gene	Primer	Sequence (5′→3′)	Restriction site
APJL_0126	0126-1	GAAGATCTTATGAAACAGTTAAAATCTTATTGG	*Bgl*II
	0126-2	CGCGTCGACGTCATCGGTTACCACGTAAATAG	*Sal*I
APJL_0241	0241-1	GAAGATCTTATGAAAAAAATTGCTTTCATTGC	*Bgl*II
	0241-2	CGCGTCGACGTTATTTCACTTTGTGTAATACGTA	*Sal*I
APJL_0495	0495-1	CCGGAATTCATGAAAACAGCATTAGTGACC	*Eco*RI
	0495-2	CGCTAGAAGCTTGTTAACCTCTTGCCACCGCA	*Hin*dIII
APJL_1851	1851-1	CCGGAATTCATGAAACATAGCAAATTCAAATTA	*Eco*RI
	1851-2	CGCTAGAAGCTTGTTAGAACTTATAATAAAGGCTAAG	*Hin*dIII
APJL_1976	1976-1	CGGGATCCATGAAGAAATCATTTATTTTATTCC	*Bam*HI
	1976-2	CGCTAGAAGCTTGTTATCTTACACGCAGTATTTGAC	*Hin*dIII
atpF	atpF-1	GAAGATCTTGTGAATTTAAATGCAACACTAATC	*Bgl*II
	atpF-2	CGCGTCGACGTTATAGTTCTGCAACTAGCTTAT	*Sal*I
hbpA	hbpA-1	GAAGATCTTATGAAATTAGCCAATTTAACTAAA	*Bgl*II
	hbpA-2	CGCGTCGACGTTAGTCAGCAAGTTTTGTACCG	*Sal*I
mltA	mltA-1	CCGGAATTCATGAATTGGAAGGCGTATAAAC	*Eco*RI
	mltA-2	CGCTAGAAGCTTGTTAGTTTAATACCCAAACACGT	*Hin*dIII
ompP2	ompP-1	CCGGAATTCATGAAAAAAACTCTAGTTGCATT	*Eco*RI
	ompP-2	CGCTAGAAGCTTGTTACCAGAATACGCGCATAC	*Hin*dIII
ompW	ompW-1	CCGGAATTCATGAAAAAAGCAGTATTAGCGG	*Eco*RI
	ompW-2	CGCTAGAAGCTTGTTAGAATTTGTAGCTAATACCTG	*Hin*dIII
potD2	potD2-1	GAAGATCTTATGAAAAAATTAGCGGGTTTATT	*Bgl*II
	potD2-2	CGCGTCGACGTTATTTCGCCGCTTTTAACTCT	*Sal*I
smpA	smpA-1	GAAGATCTTATGAAAATGAAATCTCTGTTAGCT	*Bgl*II
	smpA-2	CGCGTCGACGTTATTGCGCTTCATTCGTAATA	*Sal*I

Western blot analysis

Proteins were electrophoretically separated using 10% SDS-PAGE and transferred to PVDF membrane (Invitrogen, USA). The membrane was blocked with 5% skimmed milk in phosphate buffered saline (PBS) for 2 h at 37°C and probed with a 1/1000 dilution of mouse monoclonal antibody against 6× His-Tag (Abcam, USA) or a 1/200 dilution of convalescent pig serum JL03 taken from a SPF-pig 14 days post experimental infection with the serotype 3 strain JL03. The membrane was washed three times with TBS-T buffer (0.05% Tween-20, 20 mM Tris-HCl, 150 mM NaCl) and incubated with a 1/5000 dilution of goat anti-mouse or anti-porcine IgG-horseradish peroxidase (HRP) conjugates (Southern Biotech, USA) for 1 h at 37°C. After washing with TBS-T buffer, the membrane was developed using DAB Horseradish Peroxidase Color Development Kit (Beyotime, China).

Vaccination and challenge of mice

All animal experiments were carried out according to the Regulation for Biomedical Research Involving Animals in China (1988). One hundred and forty 6-week old SPF BALB/c mice (Hubei CDC, China) were randomly allocated into 14 groups of 10 animals and immunized subcutaneously with 10 μg antigen in 100 μl of alhydrogel adjuvant (Wuhan Chopper Biotech, China) (groups 1–12), 100 μl of Porcine Contagious Pleuropneumonia Trivalent Bacterin (group 13), or alhydrogel adjuvant alone (group 14). The Trivalent Bacterin (Tri-Bacterin) is a killed vaccine made from 3 *A. pleuropneumoniae* strains of serotypes 1, 3, and 7 (Wuhan Keqian Biotech, China). Intraperitoneal injection was used for secondary immunization using the same dosage after two weeks. Each group was intraperitoneally challenged with 4.5×10^8 CFU log-phase *A. pleuropneumoniae* JL03 at 14 days post the second vaccination. Mouse survival numbers were recorded every day for 7 days post challenge.

Vaccination and challenge of pigs

Thirty 4-week old commercial pigs were confirmed to be *A. pleuropneumoniae*-free by bacteria isolation from nasal swabs and ApxIV-ELISA test using the pig serum. Then, the pigs were randomly allocated into 10 groups of 3 animals, pre-bled and immunized for intramuscular injection with 3 μg antigen per kg of body weight in 1 ml of alhydrogel adjuvant (groups I-VI) (Oldfield *et al.*, 2008), 2 ml of the Tri-Bacterin (groups VII and VIII), or alhydrogel adjuvant alone (groups IX and X). Secondary immunization was carried out after two weeks. All pigs were intratracheally challenged with approximately 5×10^3 CFU of log-phase *A. pleuropneumoniae* strain JL03 (groups I, III, V, VII, and IX) or strain 4074 (groups II, IV, VI, VIII, and X) two weeks post secondary immunization. Following challenge, pigs were monitored regularly and scored using the 0–4 scale as previously described (Oldfield *et al.*, 2008). Pigs showing no signs of diseases were scored as 0, and those showing very serious disease were given a clinical score of 4. Animals with a clinical score of 4 were humanely euthanized and autopsied immediately; those that survived the challenge were euthanized 7 days post challenge. Swabs were taken from the upper lobe of each lung and from the cut surface of a bronchial lymph node and swabbed onto TSA supplemented with 10 μg/ml NAD and 5% newborn bovine serum to confirm the presence of *A. pleuropneumoniae*. Lung lesions were scored 0–6 according to the area of the lung affected to give a score of up to 3 for each apical and intermediate and cardiac lobes, and up to 6 for each of the two larger diaphragmatic lobes.

Determination of antibody titers by enzyme-linked immunosorbent assay (ELISA)

The IgG antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). Polyvinylchloride 96-well plates were coated with 250 ng/100 μl of the purified recombinant protein which was diluted by sodium carbonate buffer (pH 9.6) and incubated at 4°C overnight. After washing the plate three times with PBS-Tween (0.05% Tween 20 in PBS) and blocking with 5% skimmed milk solution for 2 h at 37°C, 100 μl diluted sera (initially diluted 1:20 when testing mice serum and 1:40 when testing pig serum) was added and incubated for 1 h at 37°C. Following washing, 100 μl of HRP-conjugated goat anti-mouse or anti-swine IgG diluted 1/1000 was added to the plate, then incubated for 1 h at 37°C and 3,3′5,5′- tetramethylbenzidine (Tiangen, China) was used to start the reaction. Finally, 50 μl of 0.25% hydrofluoric acid was added to stop the reaction. The OD values were measured at 630 nm using an ELISA reader (Bio-Tek Instruments, USA). Antibody titers were represented as the highest serum dilution fold (log2 values) giving 2-fold higher than the negative control, which was the commercially *A. pleuropneumoniae* negative serum (Wuhan Keqian Biotech, China).

Neutrophil phagocytosis assay

Porcine neutrophils were isolated from freshly collected blood of healthy piglets as described previously (Benga *et al.*, 2008). Freshly collected heparinized blood from healthy piglets was mixed with equal volume of 0.9% NaCl, then layered on Ficoll-Hypaque (Haoyang Biological Manufacture Co. Ltd, China) and centrifuged at 400×g, at 20°C for 30 min. The phase containing polymorphonuclear leukocytes (PMNs) and erythrocytes was isolated and was depleted of erythrocytes by two consecutive hypotonic shocks with 0.155 M NH_4Cl. The PMNs were resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, China) to 5×10^6 cells/ml and immediately used for phagocytosis assay. The viability of the cells was > 95% as determined by trypan blue exclusion assay.

Neutrophil phagocytosis assay was performed as in our previous study (Li *et al.*, 2011b). Log-phase of *A. pleuropneumoniae* JL03 was washed with sterile PBS and resuspended in DMEM to 1×10^9 CFU/ml. The bacteria were pre-incubated for 1 h at 37°C with the sera from vaccinated or control groups after the second vaccination at a ratio of bacteria : serum of 100:1 (v/v). Aliquots of 100 μl of bacteria were added to 1 ml of neutrophils at a ratio of 20:1 (bacteria : neutrophil) and 100 μl of healthy piglet serum was supplied as complement. The mixture was incubated for 30 min at 37°C with gentle shaking. Then it was washed with PBS three times and the extracellular bacteria were removed by repeatedly pelleting the cells four times at 250×g for 5 min followed by

resuspension in PBS. The neutrophils were lysed with 1 ml sterile distilled water. The number of viable bacteria was determined by the dilution plate count method. Percent opsonophagocytosis by the specific antibodies was presented as [(A–B)/B], where A equals the number of the bacteria recovered from the lysates of the co-cultures with the serum from vaccinated groups, and B equals that with the serum from adjuvant control group. Results presented are representative of three independent experiments with three sera in each group.

Results

Potential genes encoding conserved surface proteins

From the 2097 ORFs of *A. pleuropneumoniae* JL03, 501 proteins were predicted to reside at the cell surface and may be involved in the structural components of the cytoplasmic membrane, periplasm or outer membrane. Furthermore, 375 proteins were found to have signal peptide sequences, 262 were predicted to have one to four transmembrane alpha-helices with a Hidden Markov Model, and 52 were classified into beta-barrel outer membrane proteins. The number of genes encoding surface proteins in the intersection between the set of proteins identified by PSORTb and the set of proteins identified by at least one of the three topologies was 298. In addition, 75 lipoproteins were predicted by detecting a lipoprotein signal peptide.

In our previous work (Xu *et al.*, 2010), 1,709 core genes that are shared by 12 *A. pleuropneumoniae* strains were identified. The above data set was used to investigate the conservation of predicted surface proteins. We found 346 core genes that encoded putative surface-associated antigens, 12.7% of which were OMPs and 20.8% were lipoproteins.

Table 3. Genes encoding highly conserved and stably transcribed proteins located at the outer membrane of *A. pleuropneomoniae*

Locus	Class	Name	Product function
APJL_0050	outer membrane	-	hypothetical protein
APJL_0265	outermembrane	-	probable outer membrane protein
APJL_0286	outermembrane	*frpB*	iron-regulated outer membrane protein
APJL_0317	outermembrane	*pal*	peptidoglycan-associated outer membrane lipoprotein
APJL_0382	outermembrane	-	serotype-specific antigen 1 precursor
APJL_0435	outermembrane	*ompD*	outer membrane protein D-15
APJL_0487	outer membrane	*plpD*	lipoprotein
APJL_0569	outer membrane	*nlpI*	lipoprotein
APJL_0639	outer membrane	*ompP2*	outer membrane protein P2-like protein
APJL_0930	outer membrane	*irp*	iron-regulated outer membrane protein
APJL_0981	outer membrane	*imp*	organic solvent tolerance protein
APJL_1102	outer membrane	*ompW*	outer membrane protein ompW precursor
APJL_1380	outer membrane	-	hypothetical protein
APJL_1453	outer membrane	*momP1*	major outer membrane protein
APJL_1597	outer membrane	*tbpA1*	transferrin-binding protein 1 precursor
APJL_1729	outer membrane	-	hypothetical protein
APJL_1851	outer membrane	-	putative outer membrane protein
APJL_1894	outer membrane	*momP2*	major outer membrane protein
APJL_0037	lipoprotein	-	hypothetical protein
APJL_0117	lipoprotein	-	hypothetical protein
APJL_0126	lipoprotein	-	predicted periplasmic lipoprotein
APJL_0157	lipoprotein	*apbE*	thiamine biosynthesis lipoprotein
APJL_0228	lipoprotein	-	hypothetical protein
APJL_0239	lipoprotein	*lolB*	putative lipoprotein
APJL_0241	lipoprotein	-	putative lipoprotein
APJL_0347	lipoprotein	-	putative lipoprotein
APJL_0348	lipoprotein	*hlpB*	lipoprotein HlpB
APJL_0386	lipoprotein	*potD2*	spermidine/putrescine-binding periplasmic protein
APJL_0410	lipoprotein	*ompP4*	lipoprotein E precursor
APJL_0453	lipoprotein	*smpA*	small protein A
APJL_0495	lipoprotein	-	probable NADP-dependent dehydrogenase
APJL_0605	lipoprotein	-	hypothetical lipoprotein
APJL_0822	lipoprotein	*mltA*	lytic murein transglycosylase A
APJL_1429	lipoprotein	-	hypothetical protein
APJL_1467	lipoprotein	-	hypothetical protein
APJL_1683	lipoprotein	*atpF*	ATP synthase B chain
APJL_1777	lipoprotein	*mltC*	membrane-bound lytic murein transglycosylase C
APJL_1976	lipoprotein	-	lipoprotein
APJL_2060	lipoprotein	*hbpA*	heme-binding protein A

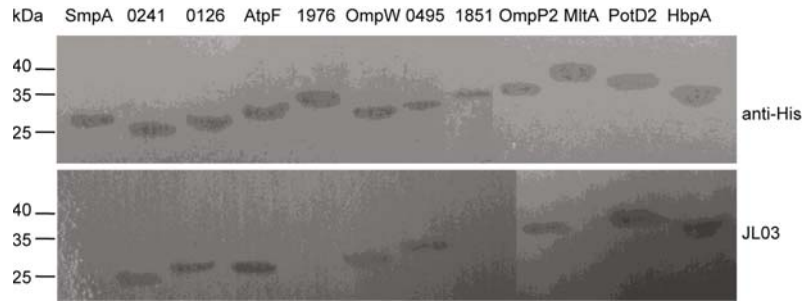

Fig. 1. Western blot analysis of the recombinant proteins. One microgram of each N-terminally 6× His-tagged recombinant protein was separated by SDS-AGE, transferred onto PVDF membrane and probed with anti-His antibody (upper panel) or convalescent pig serum JL03 (lower panel). Except for SmpA, APJL_976, APJL_1851 and MltA, the remaining 8 recombinant proteins were recognized by the pig anti-serum JL03.

Transcriptional profiling analyses

The transcriptional profile of protein coding genes was estimated at the genome wide level using microarray-based analyses for 15 *A. pleuropneumoniae* strains. The results showed that the fluorescence intensities of Cy3 ranged from 3.73 to 19.40 (log base 2 of normalized signals), and the median value of signal intensities was 14.74. Genes with intensity greater than this median were defined as genes with stable or high transcriptional levels. We found that 656 genes satisfied the above criteria and were predicted to be stably expressed *in vitro* in all strains tested. On the other hand, in combination with the analyses of subcellular location and comparative genomics, 83 conserved genes encoding surface-associated proteins were identified. After subtracting periplasmic proteins and cytoplasmic membrane proteins from these, 39 ubiquitous surface proteins (18 OMPs and 21 lipoproteins) with stable transcription were suggested to be potential vaccine targets against *A. pleuropneumoniae* (Table 3).

Expression and purification of recombinant immunoreactive proteins

Among the 39 surface proteins, 12 of them were successfully expressed in *E. coli*. The His-tagged recombinant proteins were purified using the Ni-NTA spin columns, which recognised the anti-pentahistidine antibodies. Except for SmpA, APJL_1976, APJL_1851 and MltA, the remaining 8 recombinant proteins were shown to be detectable by the porcine convalescent serum JL03 (Fig. 1). None of the recombinant proteins was detectable using the pre-immune pig sera. The homologous and/or heterologous protection of these recombinant proteins against challenge was further evaluated in mice and pigs.

Homologous protection in mice

The homologous protection trials were carried out in a mouse vaccination-challenge model. Fourteen groups of SPF mice were vaccinated twice with the 12 recombinant proteins, Tri-Bacterin or adjuvant alone, followed by challenge with *A. pleuropneumoniae* JL03. As shown in Fig. 2, the 12 recombinant proteins had different immunogenicities in mice but all induced high antibody titers after second immunization. Proteins APJL_0126, HbpA, and OmpW induced relatively higher levels of antibody titers compared with the other proteins. After challenge, all 10 mice in the adjuvant group died within 7 days. While all mice were protected in the Tri-Bacterin group, the 12 recombinant proteins displayed different protection rates (0–40%), with APJL_0126, OmpW and HbpA reaching 40% protection (Table 4). The homologous and heterologous protection of these three recombinant proteins was further tested in pigs.

Homologous and heterologous protection in pigs

To evaluate the homologous and heterologous protection of the recombinant proteins, APJL_0126, OmpW and HbpA, five groups of piglets were inoculated intramuscularly with

Table 4. Survival of mice after immunization with recombinant proteins, Tri-Bacterin or adjuvant alone followed by homologous challenge with strain JL03

Protein	Survival rate (survival/total) at different days post challenge							
	0	1	2	3	4	5	6	7
APJL_0126	10/10	4/10	4/10	4/10	4/10	4/10	4/10	4/10
APJL_0241	10/10	3/10	3/10	3/10	3/10	3/10	3/10	3/10
APJL_0495	10/10	2/10	2/10	2/10	2/10	2/10	2/10	2/10
APJL_1851	4/10	3/10	3/10	3/10	3/10	3/10	3/10	3/10
APJL_1976	10/10	1/10	1/10	1/10	1/10	1/10	1/10	1/10
AtpF	10/10	3/10	3/10	3/10	3/10	3/10	3/10	3/10
HbpA	10/10	4/10	4/10	4/10	4/10	4/10	4/10	4/10
MltA	10/10	3/10	3/10	3/10	3/10	3/10	3/10	3/10
OmpP2	10/10	3/10	3/10	3/10	3/10	3/10	3/10	3/10
OmpW	10/10	4/10	4/10	4/10	4/10	4/10	4/10	4/10
PotD2	10/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10
SmpA	10/10	2/10	2/10	2/10	2/10	2/10	2/10	2/10
Adjuvant	10/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Tri-bacterin	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10

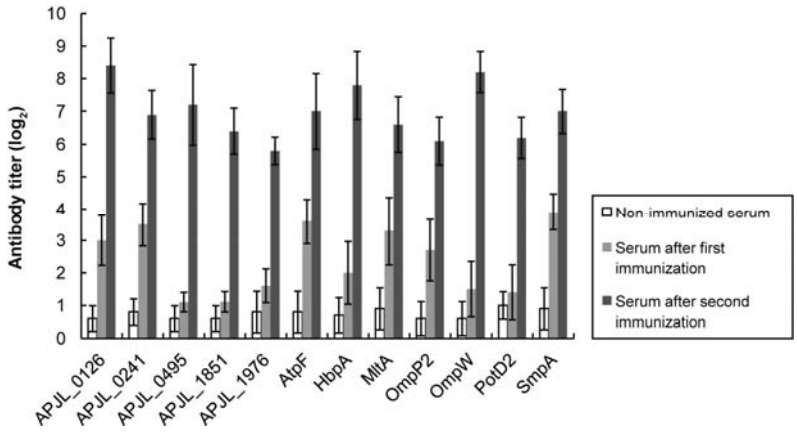

Fig. 2. Antibody levels induced by the recombinant proteins in mice. Antibodytiters were determined by ELISA and were represented as the logarithm (log_2) values of the highest dilution of the mice serum initially diluted 1:20. Antibody titers of the non-immunized serum, serum after first and second immunization are shown as means±SD from 10 mouse sera in each group. All proteins elicited high levels of antibody titers. Protein APJL_0126, HbpA and OmpW induced higher level of antibody titers compared with other proteins.

APJL_0126, OmpW, HbpA, Tri-Bacterin, or adjuvant alone. All of the recombinant proteins elicited high levels of antibody in pigs (Fig. 3A). Following booster inoculations, all groups were intratracheally challenged with *A. pleuropneumoniae* JL03 (serotype 3) or 4074 (serotype 1). No piglets showed disease signs before or at the time of challenge. The clinical scores, lung scores and bacteria recovery results of all the groups after challenge are summarized in Table 5.

In the adjuvant group, one pig (no. 30) was euthanized within 24 h of challenge with strain 4074, while the remaining 5 animals survived to the end of the trial period. Pigs challenged with strain 4074 developed more serious symptoms of infection than those challenged with strain JL03. Post mortems revealed hemorrhagic lung lesions and fibrinous pleurisy, the typical symptoms of *A. pleuropneumoniae* infection (lung lesion score greater than 5). *A. pleuropneumoniae* could be consistently re-isolated from the lungs in each case and from the bronchial lymph nodes in 5 of the 6 piglets. The results indicated that the *A. pleuropneumoniae* challenge resulted in typical signs of severe porcine pleuropneumonia under these experimental conditions.

In the Tri-Bacteringroup, all animals survived the duration of the study. The pigs challenged with JL03 developed very mild or no disease (clinical score ≤1), and the animals challenged with 4074 developed mild disease (clinical score ≤2). Post mortems revealed very small areas of hemorrhagic lesions in the apical, intermediate or cardiac lobes of all the three pigs challenged with 4074 and one pig (no. 21) challenged with JL03 (lung lesion score ≤3). The remaining 2 pigs (no. 19 and 20) challenged with JL03 showed no obvious lung lesions. *A. pleuropneumoniae* could be only re-isolated from the lung of pig no. 24.

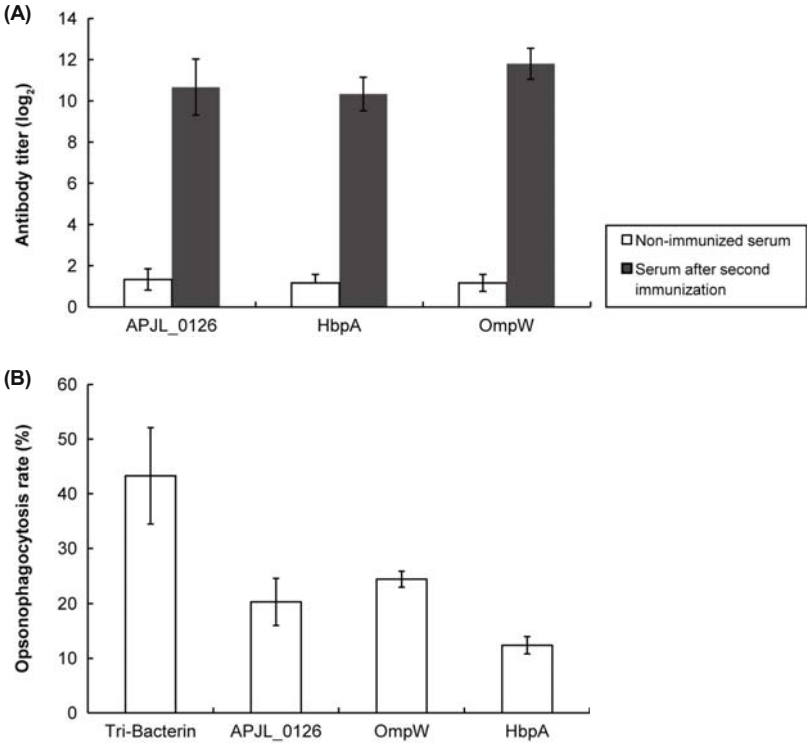

Fig. 3. Antibody levels induced by APJL_126, OmpW and HbpA in pigs (A) and effect of antibodies on opsonophagocytosis of *A. pleuropneumoniae* (B). (A) Antibody titers were determined by ELISA and were represented as the logarithm (log_2) values of the highest dilution of the pig serum initially diluted 1:40. Antibody titers of the non-immunized serum and serum after second immunization are shown as means±SD from six pig sera in each group. All of the three proteins elicited high levels of antibody in pigs. (B) Neutrophil phagocytosis assay was carried out as described in Materials and Methods. Data are shown as means±SD from three independent experiments with six pig sera in each vaccinated group normalized with the data from the adjuvant control group after the second immunization. Opsonophagocytosis, mediated by the anti-sera against the recombinant proteins, was weaker than that mediated by the sera from the Tri-Bacterin vaccinated pigs ($P<0.05$ as indicated by an asterisk).

Table 5. Clinical scores, lung scores and bacterial recovery from pigs after intramuscular immunization with recombinant proteins, Tri-Bacterin or adjuvant alone followed by homologous (strain JL03) and heterologous (strain 4074) intratracheal challenge

Group	Vaccination	Challenge	Pig No.	Clinical score		Lung lesion score	Bacterial recovery	
				24 hpc	48 hpc		Lung	Bronchial lymph node
I	APJL_0126	JL03	1	1	1	5	+	+
			2	1	0	3	+	-
			3	1	0	0	+	-
II		4074	4	1	1	2	-	-
			5	4			+	+
			6	2	2	6	+	+
III	OmpW	JL03	7	1	0	2	+	+
			8	1	1	3	-	-
			9	1	0	0	+	-
IV		4074	10	1	1	3	-	-
			11	2	1	2	+	-
			12	3	2	3	+	+
V	HbpA	JL03	13	1	0	3	-	-
			14	2	1	3	+	+
			15	1	1	1	-	-
VI		4074	16	2	2	6	+	+
			17	4			+	+
			18	2	2	3	+	-
VII	Tri-Bacterin	JL03	19	0	0	0	-	-
			20	0	0	0	-	-
			21	1	0	1	-	-
VIII		4074	22	1	1	1	-	-
			23	1	1	2	-	-
			24	2	1	3	+	-
IX	Adjuvant	JL03	25	1	1	5	+	-
			26	2	1	6	+	+
			27	2	1	6	+	+
X		4074	28	3	3	6	+	+
			29	2	2	6	+	+
			30	4			+	+

Hpc, hours post challenge

In the piglets vaccinated with APJL_0126, OmpW or HbpA, pigs from the same group displayed some variations in disease outcome, but the majority of animals developed a mild disease and survivedto the end of the trial period, with one APJL_0126-vaccinated pig (no. 5) and one HbpA-vaccinated pig (no. 17) being euthanized within 24 h of challenge because of heavy disease. All OmpW-vaccinated pigs except pig no. 12 developed very mild disease (clinical score ≤2). Post mortems revealed small areas of hemorrhagic lung lesions in all six OmpW-vaccinated pigs (lung lesion score ≤3). *A. pleuropneumoniae* could be re-isolated from the majority of the animals vaccinated with recombinant protein. Opsonophagocytosis assays revealed that the antibodies induced by APJL_0126, OmpW and HbpA could mediate opsonophagocytosis against *A. pleuropneumoniae*, but the opsonophagocytosis rates were significantly lower ($P<0.05$) than those induced by the Tri-Bacterin (Fig. 3B). Overall, of the three recombinant proteins, OmpW provided higher protection against homologous and heterologous *A. pleuropneumoniae* challenge based on clinical score, lung lesion score and bacterial counts.

Discussion

A. pleuropneumoniae can be divided into 2 biotypes and at least 15 serotypes. Inactivated whole-cell bacterins or subunit vaccines targeting Apx toxins expressed by different serotypes can provide clinical protection and reduce mortality after challenge with the homologous serotypes of *A. pleuropneumoniae* (Perry and MacLean, 2004; Chung *et al.*, 2007). However, lack of cross-protection against heterologous serotypes hinders the prevention and control of disease caused by *A. pleuropneumoniae* (Ramjeet *et al.*, 2008). As for other pathogenic bacteria of multiple serotype (Yang *et al.*, 2006; He *et al.*, 2007), a major task in the prevention of porcine contagious pleuropneumoniais to identify conserved antigens or proteins involved in immunogenicity, which might provide cross-protection against distinct *A. pleuropneumoniae* serotypes.

Together with the analysis of comparative genomics and transcriptional profiling, the results of the *in silico* identification of protein subcellular location showed that 39 OMPs and/or lipoproteins were highly conserved and stably transcribed in the *A. pleuropneumoniae* strains tested in this study (Table 3). Previous researchers have confirmed that several of the subunit vaccine candidates inferred in this study are able to induce a protective immune response, such as major outer membrane protein P2 MomP2 (also named OmpA) encoded by APJL_1894 and lipoprotein LolB encoded by APJL_0239 (Oldfield *et al.*, 2008). The protein OmpP2, which was predicted to be a beta barrel porin, has been experimentally shown to be an immunogenic surface antigen (Chung *et al.*, 2007). In addition, it is noteworthy that the deleterious

effect of peptidoglycan-associated lipoprotein Pal (APJL_0317) in vaccination has been reported (van den Bosch and Frey, 2003). Therefore, we believe that the inferred surface-exposed proteins could serve as potential antigenic vaccine candidates. Therefore, twelve of the 39 conserved OMPs and/or lipoproteins, whose antigenicity has not been tested in animals previously, were successfully expressed and purified, and were investigated for their ability to provide immunity against challenges with *A. pleuropneumoniae* following vaccination of mice and/or pigs.

For economic and ethical reasons, the 12 recombinant proteins were first tested in the mouse vaccination-challenge model. All the proteins could induce high titers of antibodies against the corresponding protein, and protective immunity against homologous challenge to various degrees. In most cases, especially for extracellular bacteria, the antibody level of a recombinant antigen is usually coordinated with its protection/survival rate. In our study, the recombinant proteins APJL_0126, HbpA and OmpW, which induced higher levels of antibody than other proteins (Fig. 2), showed a higher protection rate (40%) in the mouse vaccination-challenge experiments (Table 4). So these 3 proteins were further evaluated in piglets. However, this coordination is not always present, depending on the characteristics of the proteins/antigens. For example, PotD2 induced a similar level of antibody as OmpP2, but their protection rates were different (PotD2: 0/10; OmpP2: 3/10). The possible reason is that some of the OMPs and/or lipoproteins are essential proteins involved in *A. pleuropneumoniae* infection, so these proteins could trigger effective antibodies to protect against the infection. APJL_0126, HbpA and OmpW might belong to this class of antigens. Some OMPs and/or lipoproteins may not be essential virulence factors or may not be involved in *A. pleuropneumoniae* survival *in vivo*, so they showed lower or no protection against infection, even though they induced high levels of antibodies. These antibodies may not be involved in the bacterial clearance including opsonophagocytosis. PodD2 might belong to this class of antigens. The antibodies against APJL_1976, APJL_1851, MltA and SmpA were not detectable in the convalescent serum from pigs infected by *A. pleuropneumoniae*, at least by strain JL03 (Fig. 1, lower panel), but could be detected in the sera of mice vaccinated with the corresponding recombinant proteins (Fig. 2). Consequently they showed some protective potential (Table 4). This may be due to the following reasons or facts: (1) Not all proteins of a bacterial pathogen could induce detectable antibody during its infection, depending on the levels and time course of its *in vivo* expression; (2) A purified (recombinant) protein can usually induce antibody when it is used to immunize an animal, and these antibodies may have some protective potential.

Based on induced antibody levels and protection in mice, the recombinant proteins APJL_0126, HbpA, and OmpW were selected for further investigation in pigs. APJL_0126 was predicted to be a periplasmic lipoprotein (Conserved Domain Database accession no. COG5633) consisting of 124 amino acid residues. The function of this protein family is unknown. This protein was found to be highly conserved among *A. pleuropneumoniae* serovars (identity of 96%–100%). The 215-aa OmpW protein was predicted to be an outer membrane protein, belonging to Surface Antigen 2 superfamily. This family includes a number of bacterial surface antigens expressed on the surface of Gram negative bacteria. Their functions are unknown, but recent data suggest that they could be involved in the protection of bacteria against various forms of environmental stress (Bosse *et al*., 2002; Kao *et al*., 2009). OmpW has been demonstrated to be a vaccine candidate in *Vibrio cholera* (Das *et al*., 1998) and *Klebsiella pneumoniae* (Kurupati *et al*., 2006). In *A. pleuropneumoniae*, this protein is highly conserved among different serovars (identity of 99%–100%). The 545-aa HbpA is a heme-binding lipoprotein. It has a high degree of identity with HbpA of *Haemophilus influenzae*. HbpA protein contains a substrate-binding domain of an ABC-type dipeptide import system, which is responsible for the uptake of a variety of nutrients such as heme and glutathione in *H. influenzae* (Morton *et al*., 2005; Vergauwen *et al*., 2010). This protein has also been shown to be a virulence determinant in *H. influenzae* (Morton *et al*., 2009). According to the vaccination-challenge results, the pigs from the same group displayed some variations in clinical scores, lung lesion scores and bacterial recovery (Table 5). This phenomenon is also reported by the previous study (Oldfield *et al*., 2008), and mainly due to the differences among individual animals which cannot be avoided in animal experiments.

The recombinant proteins APJL_0126, OmpW and HbpA induced high titers of antibodies which could mediate opsonophagocytosis against *A. pleuropneumoniae* (Fig. 3). The convalescent pig serum JL03 recognized the three recombinant proteins (Fig. 1), suggesting that *A. pleuropneumoniae* infection could induce immune responses against these proteins. Unfortunately, none of the recombinant proteins provided sufficient protection in pigs compared to adjuvant alone, although most vaccinated piglets displayed milder disease and fewer lung lesions, especially the OmpW-vaccinated pigs. Similar results were reported in a previous study on other four conserved OMPs of *A. pleuropneumoniae* (Oldfield *et al*., 2008). An important reason for these findings is possibly that Apx toxins are the most important virulence factors of *A. pleuropneumoniae*. Apx toxins are essential components giving efficient protection when they are used in combination with other bacterial components. Nearly all commercially available *A. pleuropneumoniae* subunit vaccines contain Apx toxins, such as Porcilis APP (Intervet) and Pleurostar™ (Novartis) (Ramjeet *et al*., 2008). Another efficient *A. pleuropneumoniae* subunit vaccine, which is extracted from the cultures of three *apxIIA*-mutants of serotypes 1, 2, and 5 grown under iron restriction, contains abundant outer membrane lipoproteins as well as ApxIA, ApxIIIA, and ApxIVA (Goethe *et al*., 2000; Buettner *et al*., 2011). Therefore, further investigations into protection provided by the three proteins identified in this study will be done by testing these proteins used as subunit vaccines combined with Apx toxins.

In summary, 39 conserved outer membrane proteins and lipoproteins with stable transcription have been identified among different serovars of *A. pleuropneumoniae*. Twelve of them were evaluated for their immunogenicity in mice and/or in pigs. Our results of challenge experiments gave novel insights into OMPs and lipoproteins of *A. pleurop-*

neumoniae. We suggest that any conserved surface protein alone could not induce effective protective immunity against *A. pleuropneumoniae*, but that the conserved surface proteins identified in this study may be used as subunit vaccines in combination with Apx toxins.

Acknowledgements

We are grateful to Dr Tracy Wang from the University of Cambridge for the manuscript modification. This work was supported by the National Basic Research Program of China (973 Program, 2012CB518802), and National Natural Science Foundation of China (31070113).

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