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Identification of an Outer Membrane Protein from *Actinobacillus Pleuropneumoniae* Serotype 7

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Abstract

Actinobacillus pleuropneumoniae causes porcine pleuropneumonia, a disease which occurs world-wide and affects pigs of all ages. The study on the protective immune mechanism is not thoroughly clear. An outer membrane protein (OMP) was identified by screening a phage library of 3~8kb random DNA fragments of *A. pleuropneumonia* serotype 7. The inserted segment encoding the entire amino acid sequence of the OMP was determined by sequencing the rescued plasmids and BLAST analysis. Genes encoding the OMP from the isolate were amplified from genomic DNA template by PCR and cloned into a pET15b prokaryotic expression vector, generating the pET15b-OMP. *Escherichia coli* BL21 (DE3) competent cells were transformed with the construct followed by the induction of protein expression by the addition of IPTG. A band corresponding to the predicted size (48.766kDa) was seen on the SDS-PAGE. Polyclonal antibodies raised against recombinant OMP from Rabbits were reacted with bacterial proteins. Immunization with the recombinant OMP showed 40% mice were protected from the challenge. This result indicates that the recombinant OMP can induce immunological responses and might be useful as a candidate component for *A. pleuropneumoniae* vaccine.

Keywords: *A. pleuropneumoniae*; Outer membrane protein; Identification

Introduction

Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, a severe, contagious pulmonary disease that causes important economic losses in industrialized pig production worldwide. *A. pleuropneumoniae* is a Gram-negative bacterium of the Pasteurellaceae family [1]. The importance of the infection derives from the fact that it can result in acute, chronic, or subclinical infection, causing losses due to reduced production, and increasing costs of medication and vaccination [2]. There are two biotypes of App differentiated on the basis of their requirement for nicotinamideadenine dinucleotide (NAD). Biotype 1 strains require NAD, whereas biotype 2 strains can synthesise NAD in the presence of specific pyridine nucleotides or their precursors [3]. On the basis of their capsular and lipopolysaccharide antigens, 15 serotypes of *A. pleuropneumoniae* have been recognized, with variations in their virulence and regional distributions [4]. One of the most common pathogenic *A. Pleuropneumoniae* isolated from China is serotype 7 [5]. A variety of virulence factors of *A. pleuropneumoniae* have been found and their roles in pathogenesis and protective mechanisms need to be further clarified. Type 4 fimbriae were demonstrated to be involved in adherence on *A. pleuropneumoniae*. Capsular polysaccharides are probably not involved in adherence but rather mask, at least in part, the adhesive functions [6]. Pore forming exotoxins now named as ApxI, ApxII, ApxIII and ApxIV seem to be important in pathogenesis by inhibiting the immune defenses of the host to allow the bacterium to become established in the host. ApxI is strongly hemolytic and strongly cytotoxic for phagocytic cells. ApxII is weakly hemolytic and weakly cytotoxic. It is produced by all the serotype reference strains except serotype 10 [1,7]. ApxIII is not hemolytic, but is strongly cytotoxic for alveolar macrophages and neutrophils. ApxIVwas demonstrated in all *A.pleuropneumoniae* strains and recombinant ApxIVN alone showed little protection against challenge [8]. A great deal of effort was devoted to demonstrating the functions of the capsular polysaccharides, lipopolysaccharides, adhesion factors and exotoxins [3,6,9], but less is known on role of the outer membrane protein (OMP) of *A. pleuropneumoniae*. In this paper, OMP was identified by screening a genomic library of *A. pleuropneumoniae* serotype 7, BLAST analysis, and protection test. Studies demonstrated that OMP is relatively conservative among several serotypes whose sequences were available and recombinant OMP may be a potential candidate for subunit vaccine components of *A. pleuropneumoniae*.

Material and Methods

Bacterial strains and convalescent sera

A. pleuropneumoniae serotype 7(CVCC4217) and its convalescent sera were obtained from Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences. The bacterium was cultured in brain heart infusion broth (Difco, Becton–Dickinson,

Spark, MD, USA) supplemented with NAD (10 mg/L) (Sigma, St. Louis, MO, USA). *Escherichia coli* XL-l MRF' and SOLR (Stratagene, California, USA) were hosts for phage manipulation and plasmid excision. DH5α and BL21 (DE3) were used for cloning and expression of recombinant proteins and were cultured according to the manufacturer's protocol (Takara, Dalian, China).

Library construction

The genome of *A. pleuropneumoniae* serotype 7 was extracted by the QIAamp genomic DNA Mini Kit (QIAGEN, Guangzhou, China) and randomly digested by Tsp5091. The recovered 3~8kb genome fragments were ligated with predigested A lambda ZAPII vector. After packaging and amplification, the library was titered and characterized according to the manufacturer's protocol (Stratagene, California, USA).

Library screening and plasmid rescue

Library screening and plasmid rescuing were conducted as previously reported [10,11]. The lambda ZAPII libraries containing 3 to 8 kb fragments of genomic DNA of *A. pleuropneumoniae* serotype 7 were screened by immunoblot with the convalescent sera (1:200) which were pre-absorbed with *E. coli* XL-l MRF', followed by HRP conjugated goat anti-pig IgG (H+L) (KPL, Gaithersberg, USA) with 4-chloro-l-naphthol as substrate. Positive plaques were selected, replated, and rescreened. Plasmids of positive plaques were rescued according to provided protocols (Stratagene, California, USA). For those positive plaques whose plasmids were not rescued, phage DNA was extracted by using Qia lambda mini kit. Primers T3 and T7 were used to amply the insert sequence from the phage DNA.

Gel electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using an X-Cell SureLock Mini-Cell for 2 h at 125 V, with Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS [pH 8.3]. Plaque samples to estimate protein size were mixed with l/5 volume of 5x gel loading buffer (100 mM Tris-Cl [pH 6.8], 10% SDS, 50% glycerol, 500 mM dithiothreitol, and 0.1% bromophenol blue) and boiled for 5 min before loading. The gels were rinsed twice in distilled water and stained with Protoblue Safe Stain (Sangon, Shanghai, China).Protein bands in gel were transferred to nitrocellulose (0.2 μM pore size) and blocked with 4% skim milk in Tris-buffered saline (20 mM Tris, 150 mM NaCl, 0.05% Tween 20 [pH 7.5]. The membranes were incubated with antiserum, followed by Protein G conjugated to horseradish peroxidase (Thermo, Rockford, USA). Bound conjugate was detected by using 4-chloro-l-naphthol.

DNA sequencing and analysis

Amplified fragments and plasmids excised from selected recombinant phages by using the ExAssist helper phage and *E. coli* SOLR were isolated with the QIAprep spin miniprep kit and sequenced. Sequencing was performed in a commercial DNA sequencing facility (Sangon, Shanghai, China), and editing was performed using Chromas 1.61. Nucleotide sequences were aligned and connected by using DNASIS. Analyses of nucleotide sequence and deduced amino acid sequences were performed with DNASIS and the DNAstar. Homologies were identified by a BLAST search with the National Center for Biotechnology Information server. Prediction of signal sequence was analyzed by Signal P 3.0 Server.

Protein expression

Primers OMPf (TTACTCGAGGGACGGTTCGCTTGAACAAG) and OMPr (CCCGGATCCCTATCTTCTATATTTACCCGCC), including an *XhoI* restriction enzyme site and a *BamHI* restriction site respectively, were designed by using DNASIS. The sequence encoding OMP was amplified by PCR from a rescued clone, which was denatured at 92 ^{0}C for 2 min followed by 30 cycles of 92 ^{0}C at 1 min, 56 ^{0}C at 50 sec, and 72 ^{0}C for 90 sec. The product and pET-15b vector were digested with *XhoI* and *BamHI* and then ligated together. The resulting construct was transformed into *E. coli* BL21. Expression of OMP was induced with 1mM IPTG when the culture reached an optical density of 0.6 at 600 nm, and cells were harvested after 3h.

Determination of LD50

The LD50 was determined by the method provided by [12]. Four groups of 6 mice each were intraperitoneally challenged with 10-fold increasing inoculations, ranging from 3.1×10^4 to 3.1×10^7 CFU of *A. pleuropneumoniae.* Deaths were recorded for 7 days after challenge.

Protective Immunity

Mice was immuned respectively with 10μg and 20μg of purified recombinant His of recombinant protein and complete Freund's adjuvant (Sigma, St. Louis, USA). On day 14 the mice were challenged intraperitoneally with the indicated number of *A. pleuropneumoniae* that had been grown to early log-phase in brain heart infusion broth with NAD. Deaths were recorded twice daily for 7 days.

Opsonization of OMP-specific antiserum

Phagocytosis of *A. pleuropneumoniae* by neutrophils was performed using a modification of a previously described procedure [13]. *A. pleuropneumoniae* were grown to early exponential phase and opsonized with OMP-specific rabbit antiserum (1:50) for 1 h at 37 °C. Several aliquots of neutrophils (2×10^6) and opsonized *A. pleuropneumoniae* (1×10^7 CFU) were prepared with a final volume of 1 ml for each aliquot, and control bacterial suspensions were opsonized *A. pleuropneumoniae* (1×10^7 CFU) with naïve rabbit serum (1:50). Mixtures were rotated for 1 h at 37 °C and phagocytosis was terminated by placing the suspensions on ice. Aliquots of each suspension taken at 0 and 60 min were plated on agar plates and colonies were counted after overnight incubation. Bacterial survival was expressed as the percentage of viable *A. pleuropneumoniae* remaining at 60 min.

Results

Isolation and characterization of OMP gene

Thirty-one clones strongly reactive with the pool of convalescent sera were isolated. Those clones were purified and evenly reacted with the convalescent sera. One clone strongly reacted with the convalescent sera was isolated, expressing a protein of about 48 kDa. A fragment with about 1.4 kb neucleotides was amplified from the phage DNA. Sequence analysis showed that each of these colonies contained the same 1395-bp open reading frame encoding a 465aa protein and a 23-aa signal sequence. The molecular weight of the mature protein was calculated as 48,766 kDa.

Amplification of OMP gene and construction of recombinant plasmid

The OMP gene was successfully amplified. The gene and pET15b were ligated after digestion by restriction enzymes and the recombinant plasmids were verified with the same digestion.

Phylogentic analysis

OMP was compared by DNAstar with homologous proteins obtained from the Genebank, which included OMP1, OMP of *A. pleuropneumoniae* serovar 1 (ZP_00135398); OMP3, OMP of *A. pleuropneumoniae* serovar 3 (YP_001651308); OMP5, OMP of *A. pleuropneumoniae* L20 (YP_001052970); OMP6, OMP of *A. pleuropneumoniae* serovar 6 (ZP_07335793); and OMP7, OMP of *A. pleuropneumoniae* serovar 7 (YP_001968053). Those amino acid sequences were aligned with DNAstar by ClustalW method to analyze phylogenetic relationship. Figure 1 showed that OMP was relatively conservative among the different serotypes because OMP shared a 99.8%, 99.6%, 99.5%, and 99.3% homology with OMP3, OMP7, OMP5, and OMP6, and 98.5% with OMP1 that was the most distant sequence available (Figure 1).

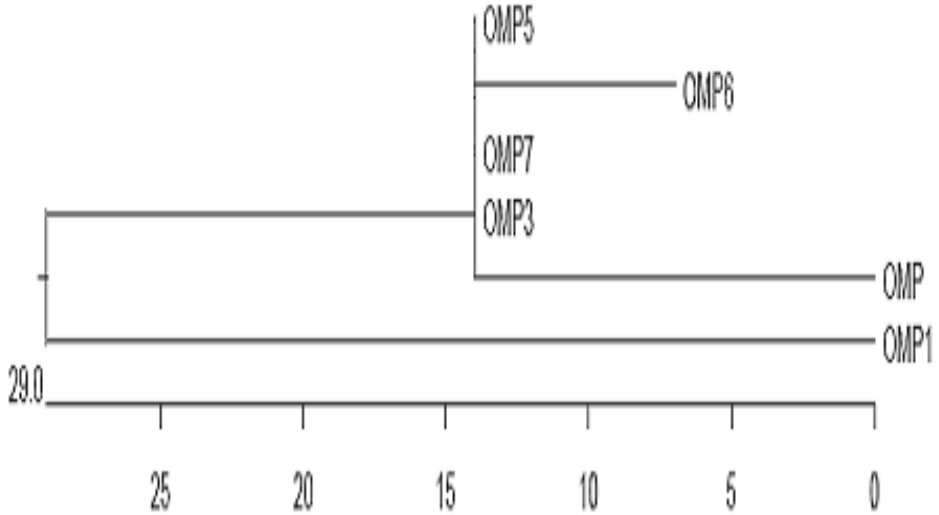

Figure 1: Phylogenetic analysis of OMP with homologous proteins. The amino acid of OMP and related proteins were aligned by DNAstar. The phylogenetic tree was constructed by the ClustalW method based on the alignment. Abbreviation and Gene Bank accession number for each sequence are: OMP1, OMP of *A. pleuropneumoniae* serovar 1 (ZP_00135398); OMP3, OMP of *A. pleuropneumoniae* serovar 3 (YP_001651308); OMP5, OMP of *A. pleuropneumoniae* L20 (YP_001052970); OMP6, OMP of *A. pleuropneumoniae* serovar 6 (ZP_07335793); and OMP7, OMP of *A. pleuropneumoniae* serovar 7 (YP_001968053)

Expression and purification of recombinant OMP

SDS-PAGE of a lysate of *Escherichia coli* expressing recombinant OMP was shown on Figure 2d with control *E. coli* (Figure 2c). Recombinant OMP was purified by nickel exchange chromatography (Figure 2e). Reactivity of the recombinant protein with the convalescent sera was shown on Figure 2b (Figure 2).

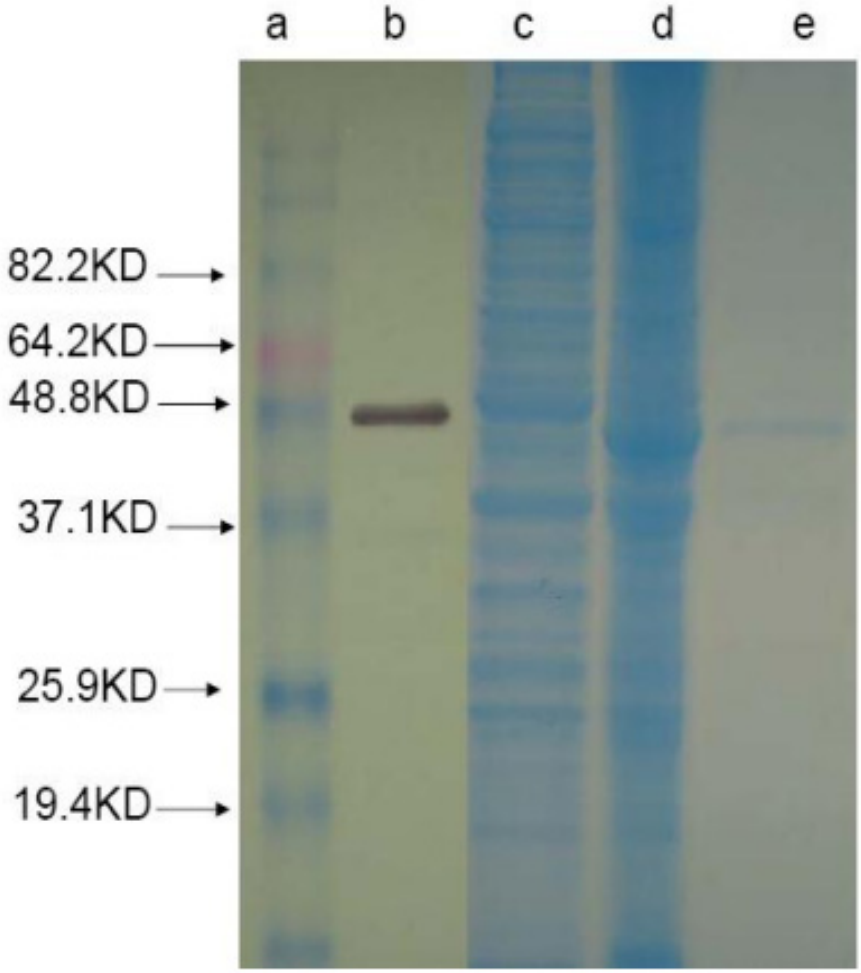

Figure 2: SDS-PAGE of a purified recombinant OMP. Lanes: (a) protein marker, (b) immunoblot of purified OMP reacted with convalescent sera, (c) *E. coli*, (d) *E. coli* containing recombinant OMP, (e) purified OMP stained by Protoblue Safe Stain

Mouse protection test

Mouse protection examination was performed to demonstrate the protective role of OMP. All the mice were handled in accordance with China Animal Welfare Draft (2009). The lethal dose of *A. pleuropneumoniae* for KM mice was calculated as 4.5×10^5 CFU. Results demonstrated that all the mice in the control group died from the challenge with 4.5×10^7 CFU (Table 1). However, only 4 of 10 mice vaccinated with 10μg or 20μg recombinant OMP survived from the challenge, and these were different from the control group ($p\leq0.05$). Taken together, these results demonstrated the protective role of OMP.

Groups	Inoculated item	Challenged CFU	Survival
Control	Phosphate buffered saline	4.5×10^7	0/10
Test A	10 μg rOMP with Freund's adjuvant	4.5×10^7	4/10
Test B	20 μg rOMP with Freund's adjuvant	4.5×10^7	4/10

Mice of different groups were vaccinated with indicated above by subcutaneous injection. After one month, all the mice were challenged intraperitoneally with the indicated number of *A.pleuropneumoniae* that had been grown to early log-phase in brain heart infusion broth with NAD. All mice died in the control group. There was no different between Test A and Test B vaccinated with different amount of rOMP, and these were statistically different from the control group ($p\leq0.05$)

Table 1: Mouse protection test with recombinant OMP (rOMP)

Opsonization of OMP-specific antiserum

ELISA showed that the OD value of OMP specific rabbit antiserum (1:100) was up to 2.0, compared with OD 0.15 of naïve rabbit serum, indicating that rabbits produced significantly high titer to OMP after 3 immunizations. Neutrophils in swine plasma were incubated with *A. pleuropneumoniae* which is opsonized with either OMP specific rabbit antiserum or rabbit naïve serum. Bacterial survival of neutrophils for each treatment is expressed as a percentage of numbers (CFU) of *A. pleuropneumoniae* following incubation for 60 min in the naïve rabbit serum. The data from Figure 3 showed OMP-specific antiserum was opsonic (Figure 3).

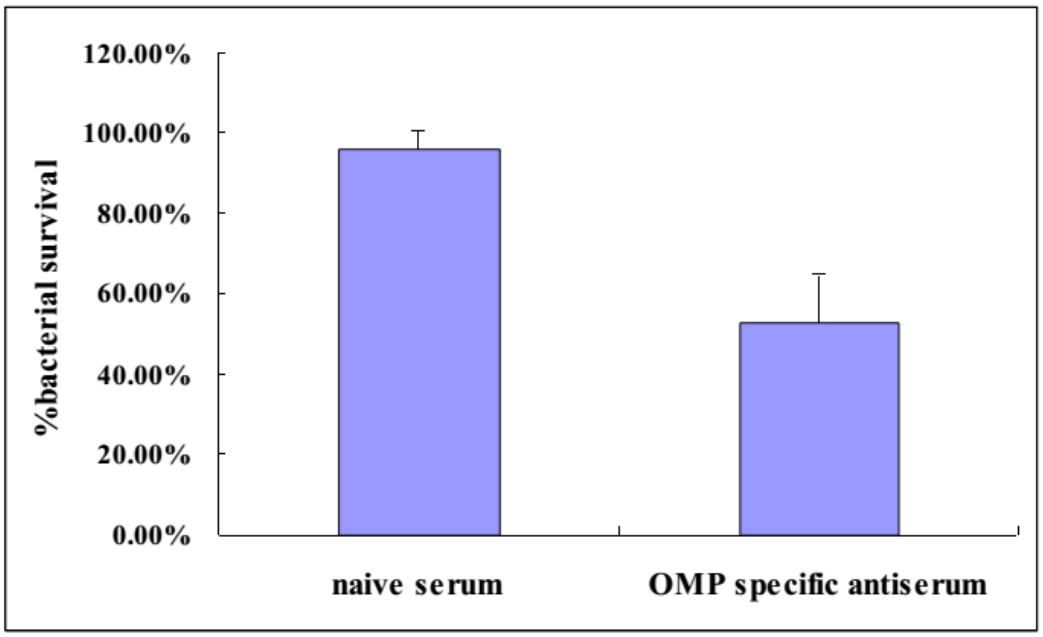

Figure 3: Opsonization of OMP-specific antiserum. Neutrophils in swine plasma were incubated with *A. pleuropneumoniae* opsonized with OMP specific rabbit antiserum and with rabbit naïve serum. Bacterial survival of neutrophils for each treatment is expressed as a percentage of numbers (CFU) of *A. pleuropneumoniae* following incubation for 60 min in the naïve rabbit serum. Vertical bars are standard errors of the mean calculated from the three time results of experiments and OMP specific antiserum was statistically different from the naïve serum ($p \leq 0.01$)

Discussion

A. pleuropneumoniae is the causative agent of porcine contagious pleuropneumonia, a severe and often fatal disease that affects swine of all ages. The pathogen has serious impact on economy, ecology and animal welfare in the pig rearing industry [3]. Infections with *A. pleuropneumoniae* are responsible for extensive economic losses due to mortality and treatment costs in acute outbreaks of the disease, as well as to an increased feed conversion and decreased growth rate in chronically infected animals. The significance of the disease and vaccination deficiency with available vaccines highlights the study on pathogenesis and protective immune responses. Biofilm formation in some field isolates has been found to be relevance to the colonization, pathogenesis and transmission of this bacterium except for virulence factors [14]. *A. pleuropneumoniae* is well adapted to survive and replicate in the host respiratory tract. Its survival and replication requires the expression of genes encoding proteins that protect the bacterium from the host immune response and help it to acquire nutrients.

It is generally believed that RTX (repeats in toxin) toxins, lipopolysaccharide, capsule, and various amino acid and iron transport systems of the bacterium are essential to cause acute disease [2,6,15,16]. In this study, we identified a new outer membrane protein from a genomic library of *A. pleuropneumoniae* serotype 7. The mature OMP was predicted to 48,766 kDa with a 23-aa signal sequence from the whole 1395 bp open reading frame. The OMP is very conservative among the surrogates from different isolates of serotype 3, 5, 6, and 7 by protein analysis, NCBI BLAST, and phylogenetic analysis. Complete genomic sequences now available will facilitate studies on pathogenesis and protective responses of the virulence factors [5,17]. The availability of the OMP sequence now makes it possible to study its role in virulence and pathogenesis, and further clarifying the epitopes and domains relevant to opsonic and protective responses.

In our studies, recombinant expression plasmid pET15b-OMP was constructed. The predicted protein was expressed, purified, and detected by Western blot. The recombinant OMP provides partial protection of mice challenged by the *A. pleuropneumoniae* serotype 7. We also found the recombinant OMP did not provide the protective effect with increasing dose probably because OMP had limited role, and protection may be involved in many virulence factors. Shao et al (2010) domonstrated that multicomponent recombinant subunit vaccine composed of rApxI, rApxII, rApxIII and rOMP can provide effective cross-protection against challenge with different serotype of *A. pleuropneumoniae* [18]. Ruiz et al (2001) investigated that the virulence of *Haemophilus parasuis* was attributed to the presence of certain membrane proteins, suggesting there may be more than one membrane proteins [19]. The complex virulence factors of various serotypes may implicate that complete protection from *A. pleuropneumoniae* serotypes could not be easily achieved only by vaccination with one or a few virulence factors. The protection of the OMP has implications for the important candidate of subunit vaccine components. Possibly the close relationship of OMP to other surrogates may provide partial protection against different serotype isolates of *A. pleuropneumoniae.*

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