Molecular and immunological characterization of Lp1, the 34 kDa outer membrane lipoprotein of *Pseudomonas aeruginosa*

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Abstract: Polysaccharide-based vaccine for *Pseudomonas aeruginosa* has failed due to toxicity of lipid-A portion in liposaccharide. As a result, efforts to develop vaccine against *P. aeruginosa* have mostly focused on outer membrane protein (OMP). Vaccine candidates based on *P. aeruginosa* OMP have emerged in the form of purified recombinant protein, as alternative strategy for vaccine development. In the present study, we have determined the immunogenicity and protective capacity of Lp1 protein (as antigen). For this work we have cloned *lp1* gene (897 bp) into the pET32a expression vector and after expression a 34 kDa recombinant protein was obtained by induction with 0.5 mM isopropyl β -D-1-thiogalactopyranoside. The antigen was obtained as a recombinant protein in *E. coli*, purified and used to immunize mice. The antiserum developed against the lp1 antigen was capable to recognize the antigen by western blotting. After immunization, recombinant Lp1 induced bactericidal antibodies. These results suggest that Lp1 is a novel antigen worth to be included in a broadly protective *P. aeruginosa* vaccine.

Key words: *Pseudomonas aeruginosa*; lipoprotein; Lp1; vaccine; antigen; antibody.

Abbreviations: ELISA, enzyme linked immunosorbent assay; LPS, lipopolysaccharide; OMP, outer membrane protein; OD, optical density.

Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is a most important opportunistic pathogen and a notorious nosocomial infective agent in human and animal. This has low permeability of outer membrane and more drug efflux pumps. Therefore this bacterium has high intrinsic resistance to antibiotics (Stanislavsky & Lam 1997).

It infects immunocompromised persons, such as AIDS patients and people with severe burn wounds, and is a serious risk for intensive care unit patients (Lyczac et al. 2000). *P. aeruginosa* is also a predominant pathogen found in the airways of cystic fibrosis patients. In cystic fibrosis lungs, *P. aeruginosa* switches to a mucoid alginate exopolysaccharide producing phenotype, which causes chronic infections that correlate with high mortality rates in cystic fibrosis patients (Lyczack et al. 2002). A class of acylated proteins, called lipoproteins, has been shown to play a role in many fundamental cellular processes and in the pathogenesis of several bacterial infections. In *P. aeruginosa* microarray analysis has revealed that there is a prominent induction of lipoprotein-encoding genes during mucoid conversion (Firoved et al. 2004).

Outer membrane lipoproteins are capable of elic-

iting a strong host immune response. They are recognized by Toll-like receptors present on the surface of several antigen-presenting cells, which leads to the activation of a signal transduction cascade that initiates the expression of pro-inflammatory cytokines (Liang et al. 2005). The genome of *P. aeruginosa* comprises about 6.2 Mb and 3.3% of this genome is predicted to encode 185 different lipoproteins (Lewenza et al. 2005). However, despite the abundance and importance of this class of proteins, very little is known about the numerous *P. aeruginosa* lipoproteins. Almost 60% of the predicted lipoproteins are classified as hypothetical proteins in the *P. aeruginosa* genome database (Winsor et al. 2009). Lipopolysaccharides (LPS) and outer membrane proteins (OMP) are major antigenic surface-associated components of *P. aeruginosa*. Under clinical trial LPS-based vaccines has been successfully tested (Cryz et al. 1983, 1988; Roe et al. 1983; Schaad et al. 1991).

OMP of *P. aeruginosa*, molecular mass ranging from 20 to 100 kDa, has been used in clinical trials. These types of vaccines were efficacious in animal models against *P. aeruginosa* and induced high levels of specific antibodies in human volunteers. Serum from human volunteers containing anti-*P. aeruginosa* antibodies provided passive protection and helped the recovery

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of 87% of patients with severe forms of *P. aeruginosa* infection (Stanislavsky & Lam 1997).

Database of complete genome sequence of many pathogenic microorganisms accelerates the development of vaccine candidate (Scarselli et al. 2005). Classical proteomics and immuno-proteomics also play an important role in the identification of the full set of antigens targeted by immune system (Klade et al. 2002). Comparative proteome analysis has been applied to deduced protein as a vaccine candidate for bacterial pathogens (Vytvytska et al. 2002; Mollenkopf et al. 2004; Ying et al. 2005). The genomics and proteomics approaches to develop vaccine against pathogenic microorganism represent a robust methodology (Cordwell et al. 2004). In this study our attention was towards cloning, expression and immunization of lipoprotein (Lp1) in mice. Here we present the evidence regarding the comparison of antibody titers against *P. aeruginosa* and recombinant lipoprotein (Lp1) of *P. aeruginosa*. Antibody titer study could prove that the lipoprotein (Lp1) of *P. aeruginosa* can be used as a successful vaccine.

Material and methods

Growth conditions of bacterial strains

P. aeruginosa glycerol stock was revived in Luria-Bertani broth medium (HiMedia) at 37°C temperature and the same procedure was followed for the DH5 α and BL-21, required for cloning and expression purpose, respectively.

PCR amplification and cloning of sequence

Genomic DNA from *P. aeruginosa* was isolated by using Nucleo-pore gDNA Fungal and Bacterial mini kit. This genomic DNA was used as a template for the amplification of outer membrane lipoprotein (*lp1*) gene with the following primers: 5'-ATGCTGGATCCGGGTGGGCCAGCGATA TCTACG-3' (forward) and 5'-ATGCTAAGCTTGCGCTT AGCGAACCAGGCGG-3' (reverse). Primers were designed for amplification of segment (*lp1* gene) from genomic DNA of UCBPP-PA14 AK strain (GenBank accession No. LC075476.1). PCR amplified product of *lp1* gene sequence was digested with the restriction enzyme Bam HI and Hind III and ligated into the same restriction sites Bam HI and Hind III sites of pET32a vector, which were created by digesting with the same restriction enzyme. The vector pET32a provides 6 $\times$ His tag at the C-terminal of the expressed protein. The vector contains T7 promoter required isopropyl β -D-1-thiogalactopyranoside (co-inducer) to express recombinant protein. The ligation mixture was transformed into DH5 α competent cells. The recombinant clones were selected on ampicillin containing Luria-Bertani agar plate and by digesting with Bam HI and Hind III restriction enzyme. Then the recombinant plasmid was transformed into expression host BL-21.

Expression and purification of recombinant protein

The expression of lipoprotein (Lp1) was achieved using 0.5 mM isopropyl β -D-1-thiogalactopyranoside at 0.6 optical density. The recombinant lipoprotein (Lp1) was expressed with 6 $\times$ His tag to enable immobilized metal affinity chromatography to be used in the purification process. Recombinant cells harbouring the lipoprotein (Lp1) were centrifuged to achieve in pellet form. Then the pellet was suspended in

TE buffer (10 mM Tris, 1.0 mM ethylenediaminetetraacetic acid; pH-8.0). The suspension of cells was subjected to sonication for 5 cycles, 50 s each with 2 min of intervals for the incubation period on ice, and then centrifuged for 30 min at 4,000 rpm. The pellet was again suspended in TE buffer containing 10 mM MgCl₂, 0.7 M NaCl and 0.1% triton X-100 and centrifuged at 6,000 rpm. The pellet was suspended in TE buffer, centrifuged, and solubilised by overnight agitation on ice in loading buffer (60 mM phosphate, 0.3 M NaCl, 2.0 M urea; pH-8.0). The preparation was again centrifuged at 10,000 rpm. The final supernatant was applied on to a column with Ni²⁺ nitrilotriacetic acid agarose (Qiagen) and washed with 10 mL TE buffer containing 20 mM imidazole, eluted with 3 mL of TE buffer containing 200 mM imidazole.

Immunization of mice

Freund's Complete, Incomplete adjuvant (Thermo Scientific) and aluminum hydroxide (Sigma-Aldrich) were used in the immunization study. According to the manufacturer's instructions, vaccine formulations were prepared.

The group of 8 BALB/c mice, 5-6 week old was used in this study. Mice were immunized subcutaneously with 25 μ g of Lp1 antigen according to vaccine formulations of manufacturer's instructions. The control mice were treated with the same quantity of 25 μ g of Freund's adjuvant and aluminium hydroxide. The mice were boosted on 7, 14, 21, and 28 day according to immunization schedule. The mice were bled on days 0 for pre-immune and 35 day for immune. Sera were tested by immunoassay.

SDS-PAGE and western blotting

SDS-PAGE was performed under reducing conditions. Separation process of recombinant lipoprotein (Lp1) was performed on 12% SDS-PAGE, and gel was stained with coomassie brilliant blue R-250.

In the western blotting, 10 μ L samples were separated by SDS-PAGE and transferred on polyvinylidene difluoride membrane (Thermo Scientific) (Towbin et al. 1979).

Analysis of immune response

Concentration of antibody in sera was determined by the enzyme linked immunosorbent assay (ELISA). A 96-well polystyrene plate was used to detect anti-Lp1 antibodies. Plates were coated with 80 μ L/well of Lp1 (5 μ g/mL) diluted in carbonate buffer (0.05 M Na₂CO₃; pH 9.6). After overnight incubation at 4°C, plates were washed three-times with phosphate buffer saline containing 0.05% (v/v) Tween 20 prior to blocking for 1 h at 37°C with 2% (w/v) skimmed milk powder, dissolved in phosphate buffer saline-T, and washed three times. Serum dilution (two times) was prepared in phosphate buffer saline-T by incubating for 45 min at 37°C in pre-coated plates, then plates were washed three times. Horseradish peroxidase-conjugate anti-mouse IgG (Promega) were used to detect antibody binding. After washing, substrate solution containing O-phenylenediamine (Promega) was added for colour development. After 15 min, 50 μ L of 0.5 M H₂SO₄ was added to stop the reaction. The absorbance was measured at 492 nm. In this study, titers were expressed as the reciprocal of the dilution showing two times increase in absorbance over that obtained with a negative control.

Whole cell ELISA

The antibodies against the Lp1 antigen exposed on the surface of bacteria were detected by whole cell ELISA as described previously by Rosenqvist et al. (1990).

Table 1. Titers of anti-sera obtained after immunization of mice with recombinant Lp1 protein.

Formulation	Geometric mean of ELISA titer ^a	Bactericidal titer ^b
Lp1-Freund's	115188	1:32
Lp1-Al(OH) ₃	198797	1:64
<i>E. coli</i> lysate-control	10232	—
Control – Freund's	12876	—
Control – Al(OH) ₃	15433	—

^aTiter measured against Lp1. Data are geometric means of titers from group of 8 mice.

^bBactericidal titers are as dilution of antiserum that reduced the viable cell count by 50%. “—” indicates that no reactivity was detected. Pre-immune sera showed no bactericidal activity (1:4).

Statistical analysis

For analysis of data obtained by immunization studies, the GraphPad Prism Version 6.00 (GraphPad Software, San Diego) was used for analysis (Priebe et al. 2003). Statistical significance was evaluated using a one-way analysis of variance and a *P*-value below 0.05 was considered statistically significant.

Results

PCR amplification and cloning of *lp1* gene sequence

The *lp1* gene sequence was amplified using the primers and a band of 897 bp (Fig. 1) was obtained in the 1% agarose gel. The *pfu* was used as a DNA polymerase enzyme for this reaction due to 3'-5' exonuclease activity (proofreading activity) to avoid misincorporation of nucleotides, which may lead to premature termination of translation resulted in truncated protein. After PCR amplification gene was subjected to cloning in the pET32a expression vector. To confirm cloning, gene was sequenced and gene data was submitted to the DNA Data Bank of Japan under the accession No. LC075476.

Expression and purification of recombinant Lp1 protein

A recombinant Lp1 protein was obtained from *E. coli* in purified form with the fused tag of 6× histidine. A whole cell lysate of *E. coli* expressing the Lp1 protein was separated by SDS-PAGE and due to T7 promoter in the pET32a vector it was over-expressed, approximately 25% of total cellular proteins. It was in the form of inclusion bodies, so buffer containing 2 M urea was used to solubilise the inclusion bodies. The protein Lp1 was obtained at the position of 34 kDa in the 12% SDS-PAGE. As shown in Figure 2, it was slightly larger than the theoretically calculated molecular mass (31.4 kDa), due to addition of 6× His tail and N-terminal fusion portion. The recombinant protein Lp1 was achieved in the purified form by Ni nitrilotriacetic acid agarose (Fig. 2).

Immunization analysis by ELISA and western blotting

The Lp1 protein with 6× His was used to immunize mice in combination with Freund's adjuvants and in an attempt to induce the antibodies. Anti-sera from immunized mice were analyzed by ELISA against recombinant Lp1 protein (Table 1). The recombinant protein Lp1 exhibited the immunogenicity in mice, and found

Fig. 1. PCR amplification of Lp1 gene. Lane M, 1 kb marker; lanes 1 and 2, PCR product; lane c, control.

eliciting the IgG antibodies titer (Table 1). According to the geometric mean of ELISA, titer value of Lp1 with Al(OH)₃ is more effective in comparison to Lp1 with Freund's adjuvant. As shown in Figure 3, Freund's adjuvant and Al(OH)₃ showed very low titer. *E. coli* cell lysates were used as a control. The western blotting confirms renaturation of Lp1 protein in the native form after purification. The band at the same position (~ 34 kDa) was found due to binding of antibody at the Lp1 antigen (Fig. 4).

Discussion

In this research article, we described the immunogenic properties of outer membrane lipoprotein (Lp1) of *P. aeruginosa* for the development of vaccine. Numbers of different vaccines have been developed but not economically feasible, due to toxicity and low efficacy that are

Fig. 2. SDS-PAGE gel (12%) showing the separation of Lp1 recombinant antigen and purified recombinant Lp1 antigen by Ni NTA. Lane Lp1, purified Lp1 antigen; lanes 1 and 2, lysate of recombinant cells.

Fig. 3. Recognition of antigens (as titer values taken from mice) present in whole *P. aeruginosa* shown by antisera elicited against Lp1.

major problems about the vaccine development.

The expression of OMP genes in *E. coli* from other Gram-negative bacteria has been confirmed in several reports, including *Haemophilus influenza* (Deich et al. 1988), *Legionella pneumophila* (Engleberg et al. 1991) and *Brucella abortus* (Tibor et al. 1994).

The LPS vaccine of *P. aeruginosa* has been used successfully both in the animals and human, but the drawback of these vaccines are toxicity due to lipid-A portion of LPS (Pennington et al. 1981). The outer membrane lipoprotein vaccines may thus play an important role to resolve this problem. In *P. aeruginosa*, 80% of total lipoproteins are outer membrane lipoproteins (Remans et al. 2010) and the rest are the inner membrane lipoproteins. So the probability of outer membrane lipoproteins is more in the number of 185

Fig. 4. Western blotting of Lp1 antigen. The 34 kDa band obtained due to antigen and antibody reaction.

lipoproteins. The lipoprotein of *E. coli* and *P. aeruginosa* are not closely related; this is due to differences in amino acid composition (Mizuno et al. 1979) and immunological response (Halegoua et al. 1974). The geometric mean titer value of ELISA for the Lp1 with adjuvants is thus more in comparison to *E. coli* as control. The 34 kDa mass of the lipoprotein is sufficient to elicit immune response because the 18 kDa OMP (Lim et al. 1997) was confirmed by ELISA and western blotting to elicit the immune response.

In this study, inclusion body formation took place due to over-expression of recombinant Lp1 protein in *E. coli*. Then during isolation and purification of recombinant Lp1, it was necessary to include denaturation and renaturation steps. The results of whole-cell ELISA and bactericidal activity indicated that the recombinant Lp1 antigen is a surface-exposed protein and this antigen was probably folded like a native protein. It suggests that additional protective epitopes of the Lp1 antigen are better represented in the conformation of the protein resembling that present in the outer membrane. In the present study, we have also studied that Lp1 formulated with two adjuvants was able to elicit bactericidal antibodies against the *P. aeruginosa* (Table 1) and we have demonstrated that the Lp1 is a surface-exposed antigen. Therefore, previously uncharacterized Lp1 antigen has several attributes that make it an attractive vaccine candidate against *P. aeruginosa*.

The use of recombinant outer membrane lipoprotein of *P. aeruginosa* for mouse protection experiments will permit testing of whether this antigen is protective without any contamination of *P. aeruginosa* LPS. Since both antigens are cross-reactive between the different international antigenic typing scheme serotypes (Mutharia et al. 1982), cross-protectivity of recombinant OMPs from one strain towards challenge with another strain might be possible.

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