Burkholderia pseudomallei Outer Membrane Protein 85

1. Technical Field of the Invention

The present invention relates to a recombinant protein of *Burkholderia pseudomallei,* which is effective for the protection against *B. pseudomallei* infection.

2. Background of the Invention

The gram-negative soil saprophytic bacillus, *Burkholderia pseudomallei* is the causative agent of melioidosis, a potentially fatal disease infecting both human and animal. Melioidosis is exclusively endemic in subtropical regions such as Southeast Asia and Northern Australia. The disease is responsible for more than 20% of all community-acquired septicaemia and sepsis-related mortality in endemic areas (Dance 2000, Wuthiekanun & Peacock 2006). Inhalation or entry into wounds by bacterial contaminated environment is the major route of infection in human melioidosis (Cheng & Currie 2005). Melioidosis exhibits non-specific clinical manifestation, ranging from an acute or fulminate septicemic illness to an indolent local infection or chronic suppurative infection (Brett & Woods 2000, Currie et al. 2000).

Treatment of *B. pseudomallei* infection solely depends on prolonged antibiotic therapy. Nevertheless, the intrinsic resistance of *B. pseudomallei* to a wide range of antibiotics (Vorachit et al. 2000), concomitant with its facultative intracellular lifestyle (Jones et al. 1996, Pruksachartvuthi et al. 1990) ultimately leads to the relapse and recrudescence of the disease after cessation of antibiotic therapy (Currie et al. 2000). Immunologically mediated protection would therefore provide an alternative remedy in prevention of melioidosis rather than the complicated antimicrobial treatment after infection. Unfortunately, presently no vaccine is approved for human use to prevent *B. pseudomallei* infection and protection from repeated natural immunizations has been hampered by the intracellular nature and multi-virulence factors of the pathogen (Brett & Woods 2000, White 2003).

Several prior arts have reported on the attempts to utilize subunit vaccines (Harland et al. 2007, Nelson et al. 2004), DNA vaccines (Chen et al. 2006a, 2006b) and attenuated live vaccines (Atkins et al. 2002, Ulett et al. 2005) against *B. pseudomallei* infection. However, antigens that confer complete protection against the infection have yet to be discovered.

In the present invention, a novel subunit vaccine for the prevention of melioidosis was developed based on the outer membrane protein of *B. pseudomallei*. Bacterial outer membrane proteins constitute potential vaccine candidates for several reasons. The proteins primarily provide a crucial interface

for interaction with a host. Many of the proteins implicated in bacterial pathogenesis such as for adhesion and invasion to host cells, as well as in immune evasion i.e. resistance to phagocytosis and the killing of host cells, are harboured on the outer membrane (Cole et al. 2005, Hancock et al. 1990). The proteins are also involved in maintaining structural integrity of the bacterial cells while mediating pathogen adaptation in an *in vivo* environment within the host (Bos & Tommassen 2004, Lin et al. 2002). Surface exposed outer membrane proteins are primary targets recognized and attacked by the host's immune system. Thus, the outer membrane protein of *B. pseudomallei* is seen as the ideal vaccine candidate against *B. pseudomallei* infection.

3. Summary of the Invention

The first aspect of the present invention provides for a recombinant outer membrane protein of *Burkholderia pseudomallei* that is effective for the protection against *B. pseudomallei* infection. The recombinant protein can be used as a vaccine against *B. pseudomallei* infection prophylactically.

The second aspect of the present invention provides for the DNA sequence that encodes the recombinant outer membrane protein of *B. pseudomallei.* A vector harbouring the DNA sequence and a host cell comprising the vector are also provided.

Further, the present invention provides for primers and optimum conditions for the amplification of the outer membrane protein of *B. pseudomallei.*

These and other objects of the present invention are achieved by,

A recombinant outer membrane protein 85 (Omp85) of *Burkholderia pseudomallei* or a fragment or a homologue thereof for the protection against *B. pseudomallei* infection.

An isolated nucleic acid, which encodes a recombinant outer membrane protein 85 (Omp85) of *Burkholderia pseudomallei* or a fragment or a homologue thereof. A vector harbouring the isolated nucleic acid and a host cell of prokaryotic or eukaryotic origin comprising the vector.

Forward and reverse primers for amplifying an outer membrane protein 85 (Omp85) from *Burkholderia pseudomallei* genome. The optimum conditions for amplification comprise of an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 64.8°C for 1 minute and extension at 72°C for 1 minute.

Use of the recombinant outer membrane protein 85 (Omp85) of *Burkholderia pseudomallei* or a fragment or a homologue thereof as a vaccine, prophylactically against *B. pseudomallei* infection.

Use of the recombinant outer membrane protein 85 (Omp85) of *Burkholderia pseudomallei* or a fragment or a homologue thereof as a diagnostic molecule to detect for the presence of *B. pseudomallei* infection.

4. Brief Description of the Accompanying Drawings

Other aspects of the present invention and their advantages will be discerned after studying the detailed description in conjunction with the accompanying drawings in which:

Figure 1 shows the SDS-PAGE and western blot analysis of recombinant Omp85 (rOmp85) (~89 kDa) expressed in *E. coli* BL21 (DE3).

Figure 2 shows indirect western blotting of purified rOmp85 and *B. pseudomallei* D286 lysate proteins with mouse polyclonal anti-rOmp85 and sera from *B. pseudomallei* D286 infected mice.

Figure 3 shows survival curves of rOmp85 immunized BALB/c after intra-peritoneal challenge with $10LD_{50}$ CFU of *B. pseudomallei* D286. Data represent the sum of three independent experiments. Survival curves were significant at $P<0.0001$ by LogRank test.

Figure 4 shows the 10 days bacterial load of *B. pseudomallei* in multiple organs of immunized mice following intra-peritoneal infection with $10xLD_{50}$ CFU *B. pseudomallei* D286. Data represents the mean of counts ± standard deviation (SD) of bacterial CFU per organ or CFU/mL blood from two independent experiments (three mice in each experiment). Differences in bacterial load between immunized and control groups were statistically significant at $P<0.05$ for all organs except heart ($P=0.073$).

Figure 5 shows percentage opsonophagocytosis of *B. pseudomallei* by polymorphonuclear cells (PMNs) in the presence of complement and specific antibody. Data represents the mean ± SD from three independent experiments. One asterisk indicates a significant increase ($P<0.05$) in opsonophagocytosis in the experimental group when compared to the control group (bact+GHBSS). Double asterisks indicate difference at $P=0.06$ when compared with the bact+ab+cp+PMN data.

Figure 6 shows the binding reaction between Omp85 and sera samples from melioidosis clinical patients and normal sera as the control. Data represents the mean ± SD from three independent experiments. A total of 23 clinical samples, 9 normal sera (NS1 – 10) and 5 samples from Legionella positive patients were utilized in an ELISA based detection system. All sera samples were also tested against total *B. pseudomallei* lysate (positive control) and *E. coli* BL21 lysate (negative control).

Figure 7 shows a representative dot blot of the Omp85 (OMAg) antigen against melioidosis confirmed patient sera (a, b) or control sera (c).

5. Detailed Description of the Invention

In accordance with the present invention, a recombinant outer membrane protein of *Burkholderia pseudomallei*, which is effective for the protection against *B. pseudomallei* infection, is provided. The recombinant protein is a recombinant outer membrane protein 85 (Omp85) of *B. pseudomallei* and include fragments and homologues of the recombinant protein that are capable of providing a protective immune response against *B. pseudomallei* infection.

In the context of the present invention, the term "fragment" refers to any portion of the given amino acid sequence of a polypeptide which has the same activity as the complete amino acid sequence. Fragments will suitably comprise at least five and preferably at least ten consecutive amino acids from the basic sequence, or combinations of these fragments. In order to retain activity, fragments will suitably comprise at least one epitopic region. Meanwhile, the term "homologue" is defined as a gene related to a second gene by descent from a common ancestral DNA sequence. The term may apply to the relationship between genes separated by the event of speciation ("ortholog") or to the relationship between genes separated by the event of genetic duplication ("paralog").

Outer membrane protein 85 (locus tag: BPSL2151) was initially identified in a study (Su et al. 2008) as one of *B. pseudomallei* immunogenic proteins expressed during human infection. The Omp85 is believed to be expressed in an *in vivo* environment during human melioidosis and immunogenic enough to induce the host's humoral immune response, making it suitable as a subunit vaccine for the prevention of *B. pseudomallei* infection.

In general, the method used for this invention involved molecular cloning of the gene encoding for Omp85 from the *B. pseudomallei* genome into an expression vector for subsequent recombinant protein production using the *Escherichia coli* heterologous expression system. The polypeptide sequence of the recombinant Omp85 is as shown in SEQ ID NO: 1 having approximately 769 amino acid residues with a molecular weight of approximately 89 kDa. While the nucleic acid sequence that encodes the recombinant Omp85 of *B. pseudomallei* or a fragment or a homologue thereof is as shown in SEQ ID NO: 2 or a fragment or a homologue thereof.

The sequence of BPSL2151 locus (Omp85) was first identified from the *B. pseudomallei* strain K96243 whole genome sequence available at The Wellcome Trust Sanger Institute. Primers for the polymerase chain reaction (PCR) were then designed based on the predicted sequence in order to amplify the full open reading frame (ORF) of the BPSL2151 homologue in a *B. pseudomallei* isolate. The forward and reverse primers of the present invention are as depicted in SEQ

ID NO: 3 and SEQ ID NO: 4 respectively. The optimum condition in which amplification was carried out comprises of an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 64.8°C for 1 minute and extension at 72°C for 1 minute. The purified amplicon were then cloned into expression vectors to produce recombinant plasmids, which were then transformed into host cells. Host cells may be of prokaryotic or eukaryotic origin, preferably prokaryotic cell, more preferably expression host *Escherichia coli* is used in this invention.

The expression of His-tagged protein was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and confirmed with western blotting (Towbin et al. 1979) using a horseradish peroxidase (HRP)-conjugated mouse-anti-HisG (Invitrogen, USA) (1:5000 dilution) and captured with Supersignal® West Pico (Pierce, USA) substrate on an X-ray film (Kodak, USA). Over-expression of the Omp85 ORF resulted in the production of a polypeptide with a molecular weight of approximately 89 kDa (Figure 1), which is in good agreement with the predicted molecular weight of His_6Gly-tagged recombinant protein. The expressed recombinant protein was also subjected to immunoblotting with pooled melioidosis clinical serum and bound antibody was detected using an alkaline phosphatase (AP)-conjugated protein A/G (Pierce) and BCIP/NBT phosphatase as substrate (Pierce, USA). The recombinant Omp85 reacted with pooled patients' sera as shown in Figure 1 but not to serum from an uninfected individual, confirming the protein is

immunogenic with melioidosis patients' sera.

The recombinant Omp85 produced according to the present invention was further purified from crude inclusion bodies. The recombinant Omp85 in both crude and purified forms are able to induce strong immune response in animal and therefore are used as prophylaxis agent against *B. pseudomallei* infection. The crude and/or purified form of the recombinant protein can be incorporated into a composition with suitable carrier or excipient known in the art. The composition preferably comprises the crude and/or purified recombinant Omp85 and an adjuvant such as Freund's complete/incomplete adjuvant.

Sera harvested from animals immunized with recombinant Omp85 showed strong serum anti- recombinant Omp85 antibody response for all the antibodies isotypes compared to the control group as shown in Table 1. The predominant antibody isotype induced by recombinant Omp85 immunization was IgG1 with the antibody titer of 81920 as compared to the IgG2a isotype with a titer of 640. The IgG2a/IgG1 ratio was 0.008, indicating the induction of Th2-type immune response triggered by the recombinant Omp85 immunization.

TABLE 1. Antibody isotype responses in immunized mice after second immunization boost

Antigen	*B. pseudomallei* D286 specific Omp85 antibody titer				
	Serum dilution				
	IgM	IgG	IgG1	IgG2a	IgG2b
rOmp85	1280	40960	81920	640	640
PBS (control)	<20	<20	<20	<20	<20

Immunoblots performed using both crude and purified recombinant Omp85 as the antigen proved the specificity of the antibody response. As shown in Figure 2, polyclonal sera derived from the recombinant Omp85 immunization group of mice reacted predominantly with the recombinant Omp85 at the protein band of ~89 kDa in both the crude and purified protein sample. The sera did not detect any *E. coli* BL21 (DE3) proteins, confirming the antigen specificity of the mouse anti- recombinant Omp85. When the mouse anti- recombinant Omp85 was probed against the *B. pseudomallei* D286 lysate, a ~82 kDa protein band was detected which is smaller than the estimated size of recombinant Omp85. Based on the predicted molecular weight of Omp85 after cleavage of signal peptide, this protein might represent the mature form of Omp85 when expressed in *B. pseudomallei*. This result demonstrates the expression of wild type Omp85 in *B. pseudomallei*, confirming the presence of Omp85 ORF predicted on the *B. pseudomallei* genome. Control mice receiving PBS plus adjuvant had no antibodies reactive with the recombinant Omp85 and any of the *B. pseudomallei* components. Sera from experimentally infected mice detected only the recombinant Omp85 and *B. pseudomallei* D286 proteins but not *E. coli* BL21 (DE3) proteins, demonstrating the expression of *B. pseudomallei* Omp85 *in vivo* and is immunogenic during infection in BALB/c mice. Uninfected mice had no anti-*B. pseudomallei* nor anti-Omp85 antibodies.

The protection efficacy of the present vaccine against melioidosis was evaluated by exposing recombinant Omp85 immunized mice to a lethal dose of *B.*

pseudomallei infection. Table 2 shows that mice immunized with recombinant Omp85 demonstrated better survival rates than control mice.

TABLE 2. Prophylactic effect of recombinant Omp85 immunization against *B. pseudomallei* D286 infection in mice

Group	No. (%) of surviving animals/group			
	Expt 1	Expt 2	Expt 3	Total
rOmp85	7/10 (70)	8/10 (80)	6/10 (60)	21/30 (70)
PBS (Control)	1/10 (10)	0/10 (0)	2/10 (20)	3/30 (10)
Significance	P=0.0027	P<0.0001	P=0.0635 (NS)	P<0.0001

Combined experiment results showed that the survival rate in recombinant Omp85 immunized mice was 70% as compared to 10% in the control group (Table 2 and Figure 3). Moreover, recombinant Omp85 immunized animals did not present with organ tropism (abscessed and enlarged liver and spleen, data not shown) that was observed in infected control mice.

Meanwhile, Figure 4 shows the sterilizing immunity conferred by the recombinant Omp85 immunization. Throughout the 10-day infection, significantly less bacterial loads in all organs were observed in recombinant Omp85 immunized animals as compared to the control group. This indicated that the growth of bacteria was restricted in recombinant Omp85 immunized mice. While *in vivo* bacterial colonization continued to increase on day 6 in the control group, recombinant Omp85 immunized mice began to show significant decrease in bacterial loads after day 5 post-infection, indicating a better clearance of bacteria from the organs and blood at the end of the experiment. No bacterial

count from the organs of control group could be documented after day 6 due to the progressive death of the animal after infection. In general, the significantly reduced bacterial loads in recombinant Omp85 immunized animals are directly correlated with the survival data described above. The inventors suggest that one clearance mechanism responsible for the removal of *B. pseudomallei* in the immunized animal model may involve antibody-mediated bacterial opsonization and subsequent phagocytosis and killing by phagocytes.

Figure 5 demonstrates that the mouse anti-rOmp85 was required for maximum *in vitro* opsonophagocytosis of *B. pseudomallei* by polymorphonuclear cells (PMNs). These observations demonstrate the importance of identifying target antigens of opsonic antibody. Omp85 exposed on the *B. pseudomallei* surface might serve as a potential opsonic antibody-targeted antigen and mediates the bacterial clearance from *in vivo* environment.

An ideal antigen used for immunization basically should meet the following criteria: first, it should have a virulence factor expressed by all pathogenic strains of the microorganism; second, it should be located on the pathogen's surface, and third, it should be immunogenic i.e. capable of inducing an effective, protective response, as verified in an animal model (André et al. 1994). The inventors have demonstrated that *B. pseudomallei* Omp85 meets the second and third criteria as an ideal prophylactic candidate against *B. pseudomallei* infection, i.e. Omp85 represents a target antigen of opsonic antibodies, and is an immune-protective

antigen that is be useful as a putative vaccine candidate for melioidosis.

In addition, the recombinant Omp85 was also evaluated against a panel of confirmed melioidosis sera samples for its utility as a diagnostic antigen. The Omp85 was equally effective in detecting antibodies in the patients' sera as was *B. pseudomallei* whole lysate in an ELISA-based detection system (Figure 6). A markedly low sensitivity was observed for sera from normal individuals as well as sera from Legionella infected (melioidosis negative) patients. The ELISA based hybridization data was further verified by a simple dot blot analysis with patient and control sera (Figure 7) and the data once again indicated that Omp85 can be further developed as a potential diagnostic agent for melioidosis suspected sera samples.

The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention, which is defined by the claims.

Cloning and sequence analysis of gene Omp85 from *B. pseudomallei* 286

The full open reading frame (ORF) of the BPSL2151 homologue in *B. pseudomallei* isolate D286 was amplified using forward and reverse primers of SEQ ID NO: 3 and SEQ ID NO: 4 respectively from 200 ng of *B. pseudomallei* D286 genomic DNA. The conditions for amplification using the Expand High Fidelity PCR System (Roche Diagnostics, Germany) consist of an initial

denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 64.8°C for 1 min and extension at 72°C for 1 min. The purified amplicon was cloned into an expression vector pET200/D-TOPO® (Invitrogen, USA) for expression of recombinant protein with N-terminus-associated His_6Gly tag, in accordance with the manufacturer's instructions. The validity of the cloned DNA and polyhistidine tag was confirmed by nucleotide sequencing with vector primers T7 and reverse T7 (Invitrogen, USA) on the PRISM DNA Analyzer 3730 (Applied Biosystems, USA). A BLAST search against the GenBank non-redundant protein database revealed that Omp85 in *B. pseudomallei* D286 is highly conserved among the strains of *B. pseudomallei* as shown in Table 3 and the antigenic domains in Omp85 have limited distribution in the bacterial genome.

TABLE 3. BLAST analysis and sequence conservation of *B. pseudomallei* D286 BPSL2151 homologue with strains of *B. pseudomallei* and other bacterial species

Organism	Protein	ORF Length	Molecular weight (kDa)	Amino acid sequence conservation (%)			
				Identity	Similarity	E-value	Accession No.
B. pseudomallei K96243	putative outer membrane protein	769	84.776	100	100	0.0	YP_10876
B. pseudomallei 1710b	Omp85	769	84.806	99	99	0.0	YP_333967
B. pseudomallei 1106a	Omp85	768	84.663	99	100	0.0	YP_001066743
B. pseudomallei Pasteur 52237	Omp85	768	84.693	99	99	0.0	YP_002023340
B. pseudomallei 305	Omp85	738	81.642	99	99	0.0	ZP_01764420
B. mallei ATCC 23344	Omp85	769	84.718	99	99	0	YP_103187
Ralstonia metallidurans CH34	D15	791	83.167	62	78	0.0	YP_583595
Bordetella petrii DSM 12804	Omp85	791	86.098	52	69	0.0	YP_001631137
Neisseria meningitidis 053442	Omp85	797	88.460	38	58	1e-161	YP_001600055
Neisseria gonorrhoeae NCCP11945	Omp85	792	87.809	38	58	5e-161	YP_002003098
Haemophilus influenzae strain Rd KW20	D15	782	89.170	31	45	2e-108	F64102
Pasteurella multocida	Omp87	778	87.571	30	40	3e-	CAD20126

From the deduced amino acid sequence, Omp85 protein was predicted to have a molecular weight of 84,906.7 Da whereas recombinant Omp85 expressed with N-terminal associated His_6Gly tag have a molecular weight of 89,032.2 Da. Signal peptide analysis with SignalP 3.0 predicted a signal peptidase cleavage site between Ala-27 and Thr-28 of the protein sequence, and cleavage on this site will produce a mature protein with a molecular weight of 82,127.4 Da.

Production of recombinant Omp85

The recombinant plasmid of pET 200/D-TOPO® carrying the Omp85 ORF was transformed into the expression host *Escherichia coli* BL21 (DE3) in accordance with the manufacturer's instruction (Invitrogen, USA). Recombinant protein expression in *E. coli* BL21 (DE3) was performed at 200 rpm and induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) (Promega, USA) for 3 hours at 30°C. The expression cells were harvested by centrifugation at 10, 000 x g for 15 min at 4°C. The cell pellet was resuspended in phosphate buffer (50 mM NaH_2PO_4, 0.5 M NaCl, 10 mM imidazole and adjusted to pH 8.0) and subsequently disrupted by alternate freeze-thawing and sonication five times at 10 s each (Vibracell, Sonics and Materials, USA). Cell lysate was separated by centrifugation into supernatant (soluble) and pellet (inclusion bodies) fraction. The inclusion bodies was solubilized in denaturation buffer (6M urea, 20 mM NaH_2PO_4, 0.5 M NaCl and adjusted to pH 7.8).

The His-tagged recombinant Omp85 was purified from the crude inclusion bodies using the immobilized metal affinity chromatography-based ProBond™ Purification System (Invitrogen, USA). Protein purification was performed under denaturing conditions as per the supplier's instruction manual. SDS-PAGE and western blotting were used to examine the success of the purification procedure. The protein concentration was determined using a bicinchoninic acid assay (Sigma, USA). Urea was removed from the purified protein by dialysis as previously described (Ou et al. 2006) with some modifications. Briefly, purified fractions were pooled, protein concentration was adjusted to 50 μg/mL and 1 mM ditiothreitol (DTT) was added. The protein sample was then dialyzed for 6 hr at 4°C against 50 volume of renaturation buffer (6M urea, 10% (v/v) glycerol, 0.1 mM DTT , 20 mM Tris-Cl, 0.2 M NaCl, 0.3 M L-arginine and adjusted to pH 9.5). The dialysis steps were repeated with renaturation buffer containing gradually decreasing concentrations of urea from 4 M to 0 M and finally with phosphate buffered saline (PBS). The dialyzed product was concentrated with Amicon® Ultra Centrifugal Filter Devices (10, 000 MWCO) (Millipore, USA).

Immunization with recombinant Omp85 in an animal model

The animal model used in this invention is female BALB/c mice, aged 6-8 weeks old. The animals were provided by the Animal House, Universiti Kebangsaan Malaysia. Mice were maintained in a positive pressure environment at 20°C to 25°C, subjected to a 12 hr light/dark cycle and fed with a protein-enriched diet and water *ad libitum*. Purified recombinant Omp85 was used to immunize naïve

mice prior to immunoprotection assay against the lethal dose challenge by *B. pseudomallei* D286. A group of 10 mice was immunized via intraperitoneal (i.p.) route with heat treated purified recombinant Omp85 (20 μg resuspended in 100 μL PBS) emulsified in Freund's complete adjuvant (Pierce, Rockford, USA) on day 0 and boosts were administered with the antigen in Freund's incomplete adjuvant on day 21 and day 42. Control mice were injected with PBS plus adjuvant as per the above schedule. Mice were tail-bled two weeks after each immunization. Blood was pooled for each immunization group, and sera were recovered according to the procedure of Harlow and Lane (Harlow & Lane 1988). Prior to infectious challenge with *B. pseudomallei* D286, sera harvested from the immunized animals were characterized by enzyme-linked immunosorbent assay (ELISA) to evaluate the presence of specific antibodies to recombinant Omp85 in the animals following immunization. End-point antibody titer of the harvested animal sera was verified by ELISA as previously described (Engvall & Perlmann 1972) with some modifications. Briefly, 96-well plates were coated overnight with 1 μg of the recombinant Omp85. Nonspecific binding was blocked with 5% (w/v) skimmed milk in PBS. The plates were then washed using 0.05% (v/v) Tween 20 (Sigma, USA) in PBS. Serum samples were serially diluted two-fold (1:20 to 1:327680) across the microtiter plate in 5% skimmed milk and incubated for 2 hr at 37°C. Antibody-antigen binding was detected with HRP conjugated goat anti-mouse IgG (Promega, USA) (1:2500 dilution); rabbit anti-mouse IgM (μ chain) (1:2500 dilution), IgG1 (gamma 1) (1:500 dilution), IgG2a (gamma 2a) (1:1000 dilution) or IgG2b (gamma 2b) (1:1000 dilution) (Zymed® Laboratories,

USA) and ABTS peroxidase/peroxide solution B as the substrate (KPL, Kirkegaard & Perry Laboratories, USA). The reaction was monitored by optical density at 405 nm on an ELISA reader (Dynex MRX). All experiments were performed in triplicate.

For immunoblotting of *in vivo*-expressed antigen during the course of infection, a group of 10 naïve mice was experimentally infected with *B. pseudomallei* via i.p. inoculation of $0.1LD_{50}$ CFU *B. pseudomallei* D286 ($1x10^4$ CFU, unpublished). Mice sera were collected on day 7, 14 and 21 post-infection. The procedures to determine the end point antibody titer of mouse polyclonal anti-*B. pseudomallei* against *B. pseudomallei* lysate were as described above.

Immunoblots using crude and purified recombinant Omp85 as the antigen were performed to test the specificity of the antibody response. 20 μg of purified recombinant Omp85 and *B. pseudomallei* D286 lysate were separated by 12% SDS-PAGE and electrotransfered onto a 0.45 μm nitrocellulose membrane. The blot was subsequently blocked with 5% skimmed milk in TBS (100 mM Tris-HCl, pH8.0 and 150 mM NaCl) overnight at 4°C. The membrane was washed with TBST (TBS and 0.05% Tween 20) and probed with immunized mouse sera (1:640 dilution) or sera from experimentally infected mice (1:160 dilution) diluted in 5% skimmed milk in TBS for 1 hr at 37°C and washed with TBST. Reactive antibodies from immunized mice were visualized with AP-conjugated goat anti-mouse IgG (Novagen, Germany) (1:5000 dilution) and BCIP/NBT

phosphatase substrate. Antibodies from infected mice were detected with HRP-conjugated rabbit anti-mouse IgM (μ chain) (1:2500 dilution) and Supersignal® West Pico (Pierce, USA) substrate on an X-ray film.

Ability of recombinant Omp85 to protect against *B. pseudomallei* infection in an animal model

Recombinant Omp85 immunized mice described above were subsequently exposed to a lethal dose infection by *B. pseudomallei* and the immune-protection conferred by the recombinant Omp85 was further evaluated. In this invention, i*n vivo* bacterial loads (in multiple organs), animal survival rate and activity of bacterial opsonophagocytosis with in-house generated anti-immunogenic proteins were the criteria used to evaluate the protection efficacy of a novel vaccine against melioidosis.

Groups of immunized mice were given a 200 μL suspension containing $1x10^6$ CFU (10 x $LD_{50,}$ unpublished) of *B. pseudomallei* D286 by the i.p. route. Following inoculation, mice were monitored daily over 15 days for signs of morbidity and mortality. Immunization and challenge experiments were performed in three independent experiments to confirm reproducibility.

Sterilizing immunity conferred by recombinant Omp85 immunization was also examined by the assessment of bacterial loads daily for 10 days post-infection in the lung, kidney, heart, liver, spleen and blood of the immunized animal. Groups

of immunized mice were inoculated with *B. pseudomallei* D286 as described above and euthanized at indicated times after inoculation. The organs were aseptically removed and 100 μL of blood was collected aseptically from each mouse in 10 mM EDTA, for enumeration of viable bacteria. Organs were individually homogenized using a Handheld Motorized Homogeniser (IKA® T10 Basic ULTRA-TURRAX®, Germany) in 10 mL of PBS. Blood and organ homogenates were diluted ten-fold serially with PBS and 50 μL of each dilution was plated on Ashdown agar. The numbers of bacteria were counted as CFU per organ or CFU/mL blood. The determination of bacterial loads in each organ was obtained from six individual animals (three mice for each experiment).

Test antisera opsonophagocytic activities were determined as described previously (Egan & Gordon 1996, Ho et al. 1997) with some modifications. Briefly, polymorphonuclear cells (PMN) were isolated aseptically from freshly obtained healthy human blood using a Polymorphprep™ PMN separation medium (Axis-Shield, Norway) according to the manufacturer's instructions. The cells were washed once in Hanks' balanced salt solution (HBSS) (Gibco BRL, USA) containing 0.1% gelatin (GHBSS) and resuspended in an opsonization buffer (GHBSS with 0.15 mM Ca^{2+} and 1 mM Mg^{2+}) at a concentration of $5x10^6$ cells/mL. Purity and viability were assessed by trypan blue exclusion and were greater than 90%. Approximately $1x10^5$ CFU *B. pseudomallei* D286 were pre-opsonized with 10 μL test sera (10% in final volume) in the opsonization buffer for 30 min at 37°C and followed by mixing with ~$1x10^5$ PMNs (1:1 effector target

ratio) and 20% normal human serum (complement source) in a sterile 96-well round bottom plate in a final volume of 100 μL. The mixtures were subsequently incubated for 2 hr at 37°C in a shaking incubator. All test sera used were heated at 56°C for 30 min prior to reaction to inactivate complement proteins. At 0 min (T_0 sample) and 120 min (T_{120} sample), 40 μL of cell suspensions were removed from the reaction into a second microtiter plate and lysed with 1.5% saponin at 37°C for 10 min. Lysed suspensions were serially diluted and plated on Ashdown agar. Preliminary treatment of bacterial cells with 1.5% saponin as above did not interrupt their viability while the PMN underwent complete lysis. The bacterial killing activity was expressed using the following formula: ((T_0 CFU -T_{120} CFU)/ T_0 CFU) x 100%. The opsonic killing assay was repeated in three individual experiments each performed in triplicate.

Omp85 as a potential diagnostic tool

A total of 23 melioidosis confirmed patients' sera, 5 non-melioidosis Legionella confirmed sera and 9 normal (non-melioidosis) control sera were used in a standard ELISA (as described above). The reactive antibodies from human samples were visualized with AP-conjugated goat anti-human IgG (Novagen, Germany) (1:5000 dilution) and BCIP/NBT phosphatase substrate. The dot blot was performed by spotting on 1 μg of selected proteins (Omp85, *E. coli* lysate and *B. pseudomallei* lysate) on nitrocellulose membranes and probing with selected sera samples.

While particular embodiments of the subject invention have been described, it will be obvious to those skilled in the art that various changes and modifications to the subject invention can be made without departing from the scope of the invention. It is intended to cover, in the appended claims, all such modifications that are within the scope of this invention.

SEQUENCE LISTING

1. GENERAL INFORMATION

(i) APPLICANT

A) NAME : Universiti Kebangsaan Malaysia

B) STREET : -

C) CITY: Selangor

D) COUNTRY : Malaysia

E) POSTAL CODE : 43600

(ii) TITLE OF INVENTION:

Burkholderia pseudomallei Outer Membrane Protein 85

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

A) MEDIUM TYPE: Floppy disk

B) COMPUTER: PC compatible

C) OPERATING SYSTEM: PC-DOS/MS-DOS

D) SOFTWARE: Microsoft Word

(v) CURRENT APPLICATION DATA:

Application Number:

2. INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH : 769 amino acids

(B) TYPE : -

(C) STRANDEDNESS : -

(D) TOPOLOGY : -

(ii) MOLECULE TYPE: Polypeptide

(iii) SEQUENCE DESCRIPTION: **SEQ ID NO: 1:**

MLFKPHRFVPKTVAAAALAAHGLAAHATAPFVVQDIKIEGLQRVE
AGSVFAYLPIKQGDTFTDDKASEAIRALYATGFFNDVRIATQGGV
VIVQVQERPAIASIDFTGIKEFDKDNLNKALKAVGLSQGRYYDKA
LVDKAEQELKRQYLTRGFYAAEVSTTVTPVDANRVSILFAVAEGP
SAKIRQINFIGNKAFKTSTLRDEMQLSTPNWFSWYTKNDLYSKEK
LTGDLENVRSYYLNRGYLEFNIESTQVSISPDKKDMYLTVALHEG
EPYTVSSVKLAGNLLDRQAELEKLVKIKPGDRFSAEKLQQTTKAIV
DKLGQYGYAFATVNAQPEIDQATHKVGLTLVVDPSRRVYVRRINI
VGNTRTRDEVVRREMRQLESSWFDSSRLALSKDRVNRLGYFTDV
DVTTVPVEGTNDQVDVNVKVAEKPTGAITLGAGFSSTDKVVLSA
GISQDNVFGSGTSLAVNVNTAKSYRTLTVTQVDPYFTVDGIKRITD
VFYRTYQPLYYSTNSSFRIITAGGNLKFGIPFSETDTVYFGAGFEQN
RLDVDSNTPQSYQDYVNEFGRVSNTVPLTIGWSRDARDSALIPSR
GYFTQANAEYGVPVGKIQYYKMDVQGQYYYSFARGFILGLNFQA
GYGNGIGNPYPIFKNYYAGGIGSVRGYEPSSLGPRDTKTNDPIGGS
KMVVGNIELTFPLPGTGYDRTLRVFTFLDGGNVWGNAPGGTSTG
ANGLRYGYGIGLAWISPIGPLKLSLGFPLQKHEGDQYQKFQFQIGT
AF

3. INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

A) LENGTH : 2310 base pairs

B) TYPE : -

C) STRANDEDNESS : - Double stranded

D) TOPOLOGY : -

(ii) MOLECULE TYPE: Nucleic acid

(iii) SEQUENCE DESCRIPTION: **SEQ ID NO: 2:**

ATGTTGTTCAAACCTCATCGCTTCGTACCTAAGACGGTTGCTGCC
GCGGCGCTCGCCGCGCATGGTCTCGCGGCACACGCAACGGCGCC
CTTCGTGGTGCAGGACATCAAGATCGAAGGCTTGCAGCGCGTCG
AAGCGGGTTCCGTGTTCGCCTACCTGCCGATCAAGCAGGGCGAT
ACGTTCACCGACGACAAGGCATCCGAGGCGATTCGCGCGCTGTA
CGCGACGGGCTTCTTCAACGACGTGCGGATCGCGACGCAGGGC
GGCGTCGTGATCGTCCAGGTGCAGGAGCGTCCGGCGATCGCGTC
GATCGACTTCACCGGCATCAAGGAATTCGACAAGGACAACCTG
AACAAGGCGCTGAAGGCCGTGGGCCTGTCGCAGGGGCGTTACT
ACGACAAGGCGCTCGTCGACAAGGCGGAGCAGGAGCTCAAGCG
CCAGTACCTGACGCGCGGCTTCTACGCGGCGGAAGTGTCGACGA

CGGTCACGCCGGTCGACGCGAACCGCGTGTCGATCCTGTTCGCG
GTCGCCGAAGGCCCGAGCGCGAAGATTCGCCAGATCAACTTCAT
CGGCAACAAGGCGTTCAAGACGAGCACGCTGCGCGACGAGATG
CAGCTGTCCACGCCGAACTGGTTCTCGTGGTACACGAAGAACGA
CCTGTACTCGAAGGAAAAGCTGACGGGCGACCTCGAGAACGTG
CGCTCGTACTACCTGAACCGCGGGTACCTGGAGTTCAACATCGA
GTCGACGCAGGTGTCGATCTCGCCGGACAAGAAGGACATGTAC
CTGACGGTGGCGCTGCACGAAGGCGAGCCGTACACGGTATCGA
GCGTGAAGCTCGCCGGCAATCTGCTGGATCGCCAGGCCGAGCTC
GAGAAGCTCGTCAAGATCAAGCCGGGCGACCGCTTCTCGGCCG
AGAAGCTGCAGCAGACGACGAAGGCGATCGTCGACAAGCTCGG
CCAGTACGGCTACGCGTTCGCGACGGTGAACGCGCAGCCGGAA
ATCGATCAGGCGACGCACAAGGTGGGCCTCACGCTCGTCGTCGA
TCCGAGCCGGCGCGTGTACGTGCGGCGGATCAACATCGTCGGCA
ACACGCGCACGCGTGACGAGGTGGTGCGTCGCGAGATGCGCCA
GCTCGAGAGCTCGTGGTTCGATTCGAGCCGTCTCGCGCTGTCCA
AGGACCGCGTGAACCGTCTCGGCTACTTCACCGACGTCGACGTG
ACGACGGTGCCCGTCGAGGGCACGAACGACCAGGTCGACGTGA
ACGTGAAGGTCGCCGAAAAGCCGACGGGCGCGATCACGCTCGG
CGCGGGCTTCTCGTCGACGGACAAGGTCGTGCTGTCGGCCGGCA
TCTCGCAGGACAACGTGTTCGGCTCCGGCACGAGCCTCGCGGTG
AACGTGAACACCGCGAAGAGCTACCGCACGCTGACCGTCACGC
AGGTCGACCCGTACTTCACCGTCGACGGCATCAAGCGGATCACC
GACGTGTTCTACCGCACGTATCAGCCGCTCTATTACTCGACGAA
CTCGAGCTTCCGGATCATCACGGCGGGCGGCAACCTGAAGTTCG
GCATTCCGTTCTCGGAAACCGACACGGTCTATTTCGGCGCGGGC
TTCGAGCAGAACCGCCTCGACGTCGATTCGAACACGCCGCAGTC
GTATCAGGATTACGTGAACGAGTTCGGCCGCGTGTCGAACACGG
TGCCGCTCACGATCGGCTGGTCGCGCGACGCGCGCGACAGCGCG
CTGATCCCGAGCCGCGGCTACTTCACGCAGGCGAACGCCGAATA
CGGCGTGCCGGTGGGCAAGATCCAGTACTACAAGATGGACGTG
CAGGGCCAGTACTACTACTCGTTCGCGCGCGGCTTCATCCTCGG
CCTGAACTTCCAGGCGGGCTACGGCAACGGCATCGGCAATCCGT
ACCCGATCTTCAAGAATTACTACGCGGGCGGTATCGGCTCCGTG
CGCGGCTACGAGCCGAGCTCGCTCGGCCCGCGCGACACGAAGA
CGAACGATCCGATCGGCGGCTCGAAGATGGTCGTCGGCAACATC
GAGCTGACGTTCCCGCTGCCGGGCACGGGCTACGACCGCACGCT
GCGCGTGTTCACGTTCCTCGACGGCGGCAACGTGTGGGGCAATG
CGCCGGGCGGCACGAGCACGGGCGCGAACGGTCTGCGCTACGG
CTACGGTATCGGCCTCGCGTGGATCTCGCCGATCGGGCCGCTCA
AGCTGAGCTTGGGCTTCCCGCTGCAGAAGCACGAAGGCGATCA
GTATCAGAAATTCCAGTTCCAGATCGGGACGGCGTTCTGA

4. INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

A) LENGTH : 25 base pairs

B) TYPE : -

C) STRANDEDNESS : -

D) TOPOLOGY : -

(ii) MOLECULE TYPE: Nucleic Acid

(iii) SEQUENCE DESCRIPTION: **SEQ ID NO: 3:**

CAC CAT GTT GTT CAA ACC TCA TCG C

5. INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

A) LENGTH : 24 base pairs

B) TYPE : -

C) STRANDEDNESS : -

D) TOPOLOGY : -

MOLECULE TYPE: Nucleic Acid

SEQUENCE DESCRIPTION: **SEQ ID NO: 4:**

TCA GAA CGC CGT CCC GAT CTG GA

What is claimed is:

1. A recombinant outer membrane protein 85 (Omp85) of *Burkholderia pseudomallei* or a fragment or a homologue thereof for the protection against *Burkholderia pseudomallei* infection.

2. A recombinant outer membrane protein 85 (Omp85) as claimed in claim 1, wherein said recombinant Omp85 is a BPSL2151 protein.

3. A recombinant outer membrane protein 85 (Omp85) as claimed in claim 2, wherein the sequence of said recombinant Omp85 is as shown in SEQ ID NO: 1 or a fragment or a homologue thereof.

4. A recombinant outer membrane protein 85 (Omp85) as claimed in any one of claims 1 to 3, wherein the molecular size of said recombinant Omp85 is approximately 89 kDa.

5. An isolated nucleic acid, which encodes a recombinant outer membrane protein 85 (Omp85) of *Burkholderia pseudomallei* or a fragment or a homologue thereof, wherein the sequence is as shown in SEQ ID NO: 2 or a fragment or a homologue thereof.

6. A forward primer comprising a sequence as set forth in SEQ ID NO: 3 and a reverse primer comprising a sequence as set forth in SEQ ID NO: 4 for amplifying an outer membrane protein 85 (Omp85) from *Burkholderia pseudomallei* genome.

7. A method of amplifying an outer membrane protein 85 (Omp85) from *Burkholderia pseudomallei* genome by using said forward and reverse primers as claimed in claim 6, wherein the conditions for amplification comprise of an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 64.8°C for 1 minute and extension at 72°C for 1 minute.

8. A recombinant clone comprising said isolated nucleic acid as claimed in claim 5.

9. A host cell of prokaryotic or eukaryotic origin comprising said recombinant clone as claimed in claim 8.

10. A host cell as claimed in claim 9, wherein said prokaryotic cell is *E. coli.*

11. A composition comprising a recombinant outer membrane protein 85 (Omp85) of *Burkholderia pseudomallei* or a fragment or a homologue thereof as claimed in any one of claims 1 to 4 for the protection against *Burkholderia*

pseudomallei infection.

12. Use of a recombinant outer membrane protein 85 (Omp85) of *Burkholderia pseudomallei* or a fragment or a homologue thereof as claimed in any one of claims 1 to 4 as a vaccine against *Burkholderia pseudomallei* infection.

13. Use of a recombinant outer membrane protein 85 (Omp85) of *Burkholderia pseudomallei* or a fragment or a homologue thereof as claimed in any one of claims 1 to 4 as a prophylaxis molecule against *Burkholderia pseudomallei* infection.

14. Use of a recombinant outer membrane protein 85 (Omp85) of *Burkholderia pseudomallei* or a fragment or a homologue thereof as claimed in any one of claims 1 to 4 as a diagnostic molecule for *Burkholderia pseudomallei* infection.