◆❖ **Scientific**
❖❖ **Research**

A *wzt* Mutant *Burkholderia mallei* Is Attenuated and Partially Protects CD1 Mice against Glanders

Aloka B. Bandara

Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, USA.
Email: abandara@vt.edu

ABSTRACT

Burkholderia mallei is the etiologic agent of glanders in solipeds and humans. Lipopolysaccharide (LPS) is a major component of cell envelop of this pathogen. O-antigen, the most external component of LPS, is a virulence factor and a protective antigen in many pathogenic bacteria. Two putative proteins named *Wzm* (integral membrane protein) and *Wzt* (hydrophilic ATP-binding protein) are believed to make up an ABC-2 transporter of *B. mallei* that facilitates transport of components of O-antigen from cytosol to outer-membrane. We studied the importance of *wzt* (encoding Wzt) to growth, LPS O-antigen profile, and pathogenicity of *B. mallei*. A *wzt* mutant strain was generated by deleting a portion of the *wzt* in *B. mallei* wild type strain ATCC 23344 by gene replacement. Compared to the wild type strain, the *wzt* mutant displayed slower growth *in vitro* and less lethality in CD1 mice when inoculated intraperitoneally. The 50% lethal doses (LD$_{50}$) of the wild type and the *wzt* mutant strains were 5.9×10^5 and 9.1×10^5 cfu, respectively. CD1 mice inoculated with a non-lethal dose of the *wzt* mutant produced specific serum immunoglobulins IgG1 and IgG2a and were partially protected against challenge with 11.2 times LD$_{50}$ of the wild type strain. These findings suggest that the *wzt* is required for optimal *in vitro* growth and pathogenesis of *B. mallei*, and a *wzt* mutant protects CD1 mice against glanders.

Keywords: *Burkholderia mallei*; ABC-2 Transporter; *wzm* Integral Membrane Protein; *wzm* Hydrophilic ATP-Binding Protein; Glanders; CD1 Mice; Pathogenicity; Protection

1. Introduction

Burkholderia mallei, the causative agent of glanders, is a Gram-negative, aerobic bacillus. This bacterium is primarily responsible for disease in horses, mules, donkeys and occasionally humans [1-3]. Relatively little is known about the mechanisms of *B. mallei* pathogenesis [4,5]. In gram-negative bacteria, lipopolysaccharides (LPS), commonly referred to as endotoxins, are a major component of cell envelopes [6,7]. Bacterial outermembranes provide the "barrier function" largely due to the presence of LPS [8]. Bacterial strains expressing a smooth phenotype synthesize LPS molecules that are composed of three covalently linked domains: an O-polysaccharide antigen (O-antigen), a core region, and a lipid A moiety [9]. The O-antigen is the most external component of LPS, and it consists of a polymer of oligosaccharide repeating units. Chemical composition of O-antigens varies among different bacterial species, as a result of the genetic variation in the genes involved in O-antigen biosynthesis, designated the *wb* cluster. The genetics of O-antigen biosynthesis have been intensively studied in the Enterobacteriaceae, and it has been shown that the *wb*

clusters usually contain genes involved in biosynthesis of activated sugars, glycosyl transferases, O-antigen polymerases, and O-antigen export [10,11]. Burtnick *et al.* [12] identified the gene cluster responsible for O-antigen biosynthesis in *B. mallei* ATCC 23344, and determined the physical structure of the *B. mallei* ATCC 23344 O-antigen.

In bacteria, LPS O-antigen is exported to the cell surface using three distinctive pathways, as reviewed by Samuel and Reeves [13]. One of these pathways called ATP-binding cassette (ABC) transporter-dependent pathway, which has been found in *E. coli* O8 and O9 and *Klebsiella pneumoniae* O1 and O12 [14-17] is comprised of an integral membrane protein, *Wzm*, and a hydrophilic protein containing an ATP-binding motif, *Wzt*. The mechanism for the biosynthesis of LPS O-antigen in *B. mallei* is largely unknown. The genome of *B. mallei* strain ATCC 23344 carries a *wzm* gene encoding a putative *Wzm* integral membrane protein of an ABC-2 transporter complex, and a *wzt* gene encoding a putative *Wzt* hydrophilic protein of this complex [12]. In this communication, we report the influence of *wzt* on *in vitro* growth and *in vivo* pathogenicity of *B. mallei*, and the protective

efficacy of a *wzt* mutant as a vaccine candidate against glanders.

2. Materials and Methods

2.1. DNA and Protein Sequence Analyses

The nucleotide sequences of the *B. mallei wzm* and *wzt* genes encoding respectively the putative *Wzm* and the putative *Wzt* proteins were analyzed with DNASTAR software (DNASTAR, Inc., Madison, WI). The presence of any signal sequence of *Wzm* and *Wzt* proteins was predicted by using the SignalP 3.0 server (http://www.cbs.dtu.dk/) [18]. The destination of the *Wzm* and *Wzt* proteins upon translation and processing was predicted using the Subloc v1.0 server (http://www.bioinfo.tsinghua.edu.cn/). The identity of *B. mallei Wzm* and *Wzt* to proteins of the EMBL/GenBank/DDBJ databases was analyzed using the BLAST software [19].

2.2. Bacterial Strains, Plasmids, and Reagents

B. mallei strains ATCC 23344 and 23344Δ*sacB* [20]. were obtained from our culture collection. *B. mallei* strains were grown in trypticase soy broth or trypticase soy agar (Difco Laboratories, Sparks, MD) supplemented with 4% glycerol (TSB-G and TSA-G, respectively) at 37°C in the presence of 5% CO_2 as previously described [21]. *Escherichia coli* XL1Blue was used for general cloning, and *E. coli* S17-1 [22] was used as a mobilizing strain for constructing mutants. The suicide vector pGRV2 [23] that carries the counter-selectable marker *sacB* was employed in generating the mutant *B. mallei* strains. Bacteria containing plasmids were grown in the presence of polymyxin at 15 g/ml. Genomic DNA from *B. mallei* strain ATCC 23344 and plasmid DNA from recombinant *E. coli* strains were harvested by use of kits obtained from Qiagen (Qiagen Inc., Valencia, CA). Restriction digests, Klenow reactions, and ligations of DNA were performed using standard procedures [24]. All experiments with live *B. mallei* were performed in a Biosafety Level 3 facility in the Infectious Disease Unit of the Virginia-Maryland Regional College of Veterinary Medicine per CDC-approved standard operating procedures.

2.3. Construction of a Δ*wzt* Mutant Strain of *B. mallei*

PCR primers were designed to amplify the 5' and 3' ends of the *wzt* gene (BMA1985) on chromosome I of strain ATCC 23344 (NC_006348). The primer sequences (5' to 3') were as follows: *wzt*-1, CGGCCATGGATGTCCTCTGAATTGCCG; *wzt*-2, CGGGGATCCCCGCACGTACATGCCGCTCG; *wzt*-3 CGGGGATCCGCTGCGCAACGACGTGGAGT; and *wzt*-4 CGGCCATGGTTCAGATTTCATTGCGCC. The 5' end of the primers

wzt-1 and *wzt*-4 carried *Nco*I sites (in bold case), whereas the 5' ends of primers *wzt*-2 and *wzt*-3 carried *BamH*I (in bold case). The PCR amplifications were performed using nearly 100 ng of ATCC 23344 genomic DNA and 20 pmoles oligodeoxyribonucleotide primers.

The fragment-1 was amplified using primers *wzt*-1 and *wzt*-2, and the fragment-2 was amplified using *wzt*-3 and *wzt*-4. Each fragment was restricted digested with *Nco*I and *BamHI*. The digested two fragments were then cloned into the *NcoI* digested plasmid pGRV2 [23] to produce *pABwzt*.

The plasmid pABwzt was harvested from XL1Blue, and introduced into competent *E. coli* S17-1 cells by electroporation to produce S17-1 (*pABwzt*). The plasmid was then delivered to *B. mallei* 23344Δ*sacB* [20] via conjugation with S17-1 (*pABwzt*) by using a membrane filter mating technique, as described elsewhere [20]. Strain 23344Δ*sacB* was chosen as the platform strain, as its resistance to sucrose was useful as a non-antibiotic marker in selecting the recombinant stains [20]. Polymyxin was used to counterselect *E. coli*. One of the 23344Δ*sacB*: *pABwzt* colonies was used to inoculate TSB-G. Ten-fold dilutions of the overnight culture were spread onto TSA-G + 5% sucrose. Six sucrose-resistant colonies were screened by PCR for the deletion in the *wzt* gene (data not shown). One of the colonies carrying the deletion was chosen for further work and designated 23344Δ*sacB*Δ*wzt*.

2.4. RNA Isolation and Reverse Transcription-PCR (RT-PCR)

Extraction of RNA, treatment with DNase, and RT-PCR were perfomed as described elsewhere [20]. The primers *wbiA*-Forward (5' TAGATTCCATACGAGTAGTC 3') and *wbiA*-Reverse (5' ATGTGGCGCCTGACGCTCAA 3') were used for PCR amplifycation of *wbiA*, whereas, primers *wzt*-1 and *wzt*-4 (see section 2.3) were used for amplification of *wzt*.

2.5. Extraction and Analysis of LPS and Other Cellular Components

Single colonies of the wild type, the *sacB* mutant, and the *wzt* mutant were patched on TSA-G, and incubated at 37°C for 96 h in 5% CO_2. The cells were harvested and treated with 0.5% phenol for 72 h at 4°C. The lysate was used for extraction of LPS using a modified hot aqueous-phenol procedure [25,26]. Following extraction, the resulting phenol and aqueous phases were combined and dialyzed in distilled water to remove phenol. The dialysates were then clarified by centrifugation and concentrated by lyophilization. The crude preparations were solubilized in 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 50 µg·ml^{-1} RNase A and 50 µg·ml^{-1} DNase I,

and incubated for 3 h with shaking at 37°C. Proteinase K was then added to a final concentration of 50 μg·ml^{-1} and the digests were incubated for an additional 3 h at 60°C. The enzymatic digests were clarified by centrifugation, and LPS was isolated from the supernatants as precipitated gels following three rounds of ultracentrifugation at 100,000 *g* and 4°C. After the final spin, the gel-like pellets were resuspended in pyrogen-free water and lyophilized. The purified LPS were electrophorased using 16% Tricine gels (Invitrogen) as described elsewhere [27], and the products were stained with silver using the procedure of Tsai and Frasch [28].

Western blotting was performed using standard procedures [24]. Briefly, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane by using a Trans-blot semidry system (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with a solution of 1.5% non-fat milk powder plus 1.5% bovine serum albumin. For analysis of O-antigen profile, the membranes were incubated with mouse monoclonal antibody 3D11 that is specific to B. pseudomallei LPS O-antigen (Research Diagnostics, Inc.) overnight and subsequently developed with goat anti-mouse IgG (whole molecule) conjugated with horseradish peroxidase (Sigma Chemical Co).

2.6. Thin-Section Immune-Electron Microscopy

Bacteria were grown for 3 days on TSA-G and gently suspended in PBS to 10^9 colony forming units (CFU) ml^{-1}. Mouse monoclonal antibody 3D11 was diluted 1:40 in PBS and incubated with the cells for 1 h at 37°C. The bacteria were washed with PBS, suspended in 0.5 ml of a 1:20 dilution of protein A-20 nm gold particles (Polysciences, Warrington, PA), incubated at 37°C for 1 hour, and washed in PBS at 2000 × g for 15 min. The final pellet was suspended in 0.1 M phosphate buffer (pH 7.3), mixed with molten agar, solidified, and cut into small blocks. The cubes were fixed in 2.5% gluteraldehyde/0.1 M L-lysine for 25 min, then 2.5% gluteraldehyde for 90 minutes at room temperature, and stored in 0.1 M phosphate buffer at 4°C. Samples were dehydrated with a series of ethanol washes at 30, 50, 70, and 80% ethanol in 0.1 M phosphate buffer (pH 7.4) for 15 minutes each at room temperature. Samples were dehydrated once more with 2 parts LR White, 1 part 80% ethanol for 15 minutes at RT, infiltrated with LR white for 39 hours, and polymerized at 60°C for 20 hours. Thin sections on copper grids were stained with 1.7% lead citrate and 2% uranyl acetate, and viewed with a JEOL 100 CX-II transmission electron microscope (Zeiss 10C; Carl Zeiss Inc., New York, NY) with ×25,000 magnification.

2.7. Serum Bactericidal Assay

The bactericidal activity of 20% guinea pig serum (PCS; which contains no antibody) for *B. mallei* was determined as previously described [29] Control tubes contained heat-inactivated serum.

2.8. Pathogenicity of *B. mallei* Strains in Mice

The cultures of the wild type, the *sacB* mutant, and the *wzt* mutant strains were grown in TSB-G for 24 h at 37°C with shaking (200 rpm). The cells were harvested by centrifugation at 2000 × g for 20 min, washed with PBS, and resuspended in 10 ml of PBS. The dilutions of cultures were plated on TSA-G plates to determine the cfu/ml.

Seven-week-old female CD1 mice (Charles River Laboratories, Wilmington, MA) were allowed 1 week of acclimatization. Groups of five mice each were intraperitoneally injected with saline or three different doses (4.4 × 10^5, 6.6 × 10^5, or 8.8 × 10^5 cfu/mouse) of each strain: wild type, *sacB* mutant, and *wzt* mutant. Survival of animals for 36 days post-inoculation was monitored, and abnormal animal behaviors of surviving animals (any huddling or fur ruffling) were recorded. The 50% lethal dose (LD$_{50}$) of the treatment groups was calculated using the Probit.exe program of STAT 2050 server of the University of Guelph, Canada (http://www.uoguelph.ca/~jhubert/stat 2050/software/software_2050.html). The number of animals dying up to 6 d post-inoculation was used in LD$_{50}$ calculations. The animals that received wild type or *sacB* strains and survived were sacrificed by exposing to CO$_2$ on day 36 post-inoculation. Their spleens and livers were homogenized and cultured to determine the presence of the inoculated strain [21].

2.9. Immune and Protective Responses in CD1 Mice Inoculated with the *B. mallei wzt* Mutant

Blood samples were collected by retro-orbital bleeding from mice injected/inoculated with saline or the *wzt* mutant, on day 30 post-inoculation/injection. Sera were collected by centrifugation at 3,000 *g* for 5 min, and serum IgG1 and IgG2a levels were determined by enzyme-linked immunosorbent assay as described elsewhere [20]. The heat-killed wild type strain ATCC 23344 suspended in 0.06 M sodium carbonate buffer (pH 9.6) was used as the antigen to coat polystyrene plates.

At day 36 post-inoculation, those mice that were injected with saline (5 mice) or the *wzt* mutant (8 mice) and survived were challenged intraperitoneally with 6.6 × 10^6 cfu/mouse (11.2 times the LD$_{50}$) of wild type strain ATCC 23344. Survival of the mice for 15 days post-challenge was monitored, and abnormal animal behaviors were recorded. On day 15 post-challenge, the surviving animals were killed by CO$_2$ asphyxiation. Their spleens and livers were homogenized and cultured to determine the presence of *B. mallei*.

3. Results

3.1. Organization of LPS O-Antigen Biosynthetic Gene Cluster, and Nucleotide and Protein Sequences of *wzm* and *wzt*

The goals of this study were to elucidate the influence of the *wzt* gene encoding the putative hydrophilic ATP-binding protein of the polysaccharide ABC transporter system (also called *Wzt*) on LPS O-antigen biosynthesis and pathogenicity of *B. mallei*, and evaluate the protective efficacy of a *wzt* mutant against glanders infection in mice. The gene cluster believed to encode O-antigen biosynthesis is comprised of at least 15 individual genes, and is located in Chromosome I of *B. mallei* strain ATCC 23344 (GenBank accession AY028370 and NC006348) [12].

The *wzm* gene (BMA1986) encoding the putative permease protein of the polysaccharide ABC transporter system (also called *Wzm*) is 833-bp long. The DNA sequence analyses predicted that *Wzm* is a non-secretory protein without a clear N-terminal signal sequence (Signal peptide probability: 0.043). The predicted subcellular localization of *Wzm* is cytoplasmic (Reliability Index: RI = 1; Expected Accuracy = 63%). The *wzt* gene (BMA1985) encoding the putative ATP-binding protein of the polysaccharide ABC transporter system (also called *Wzt*) is 1397-bp long. The ATG starting codon of *wzt* is located just 2-bp downstream the stop codon of *wzm*. The DNA sequence analyses predicted that *Wzt* is also a non-secretory protein without a clear N-terminal signal sequence (Signal peptide probability: 0.00). The predicted subcellular localization of *Wzt* is cytoplasmic (Reliability Index: RI = 6; Expected Accuracy = 98%).

3.2. Genomic and Transcriptomic Characterization of the *wzt* Mutant

A *wzt* mutant strain of *B. mallei* was constructed by disrupting the *wzt* gene of the *sacB* mutant 23344Δ*sacB* [20], and designated as 23344Δ*sacB*Δ*wzt*. The sucrose resistance of strain 23344Δ*sacB* was used as the non-antibiotic marker in selecting the *wzt* mutant. A PCR assay with the primer pair wzt-1/wzt-4 (see Materials and Methods) produced a predicted 1.4-kb amplicon from wild type *B. mallei*, and an approximately 0.9-kb amplicon from the *wzt* mutant strain 23344Δ*sacB*Δ*wzt* (data not shown), indicating that due to homologous recombination event, a 484-bp region was deleted from the *wzt* gene.

Reverse-transcription (RT)-PCR with the primer pair *wzt*-1/*wzt*-4 produced a 1.4-kb product from the wild type and the *sacB* mutant strains (lanes 1 and 2 of **Figure 1**), but no product from the *wzt* mutant (lane 3 of **Figure 1**). These results suggest that both the wild type and the *sacB* mutant expressed a full-length *wzt* mRNA, whereas, the *wzt* mutant failed to express a *wzt* mRNA as a result

of the deletion event in the *wzt* gene. In order to characterize any polar effect induced by this deletion event, the expression of mRNA of *wbiA* was analyzed by RT-PCR using the primer pair wbiA-Forward/wbiA-Reverse (see Materials and Methods). The *wbiA* gene was chosen for this assay since it is located immediately downstream of *wzt* in LPS O-antigen cluster. Just like the wild type and the *sacB* mutant strains, the *wzt* mutant produced an approximately 0.8-kb amplicon (lanes 4, 5, and 6 of **Figure 1**), suggesting that expression of *wbiA* mRNA was not affected due to the deletion in *wzt*.

3.3. Growth Characteristics of *B. mallei* Strains

The growth rates of *B. mallei* strains in trypticase soy broth supplemented with 4% glycerol (TSB-G) was measured. During log phase, the growth of the *wzt* mutant was slower (approximately 4.5 h doubling time) than that of the wild type or the *sacB* mutant (approximately 2 h doubling time).

3.4. LPS Profiles of *B. Mallei* Strains

LPS extracts were electrophoresed in 16% Tricine gels, and stained with silver to analyze any visible differences among strains with regard to expression of cellular components at translational level (**Figure 2**). No major differences were visible between strains with regard to products in the range of 20 to 200-kDa. When the LPS extracts were analyzed by western immunoblot procedure using the mouse monoclonal antibody 3D11 that is specific to *B. pseudomalle*i LPS O-antigen, no differences were seen between the wild type and the *wzt* mutant (data not shown). The results suggest that the mutation in *wzt* did not affect O-antigen biosynthesis.

3.5. Transport of O-Antigen to the Cell Surface of *B. mallei*

In order to find the effect of mutation in *wzt* on O-antigen export, the presence of this carbohydrate on cell surface of strains was determined by immune-electron microscopy. The gold particles were found attached to the cell surface of both the wild type and the *wzt* mutant (data not shown). The results suggest that the O-antigen was transported to the cell surface of the *wzt* mutant similar to that of the wild type.

3.6. Resistance of *B. mallei* Strains to Serum Bactericidal Killing

The wild type and the *wzt* mutant strains were completely resistant to the bactericidal action of guinea pig sera at 20% (v/v) (data not shown). The serum sensitive *Francisella tularensis* mutant strain WptI$_{G191V}$ [30] that was used as the control was completely killed by 20% guinea pig sera. The results suggest that disruption of *wzt* did not
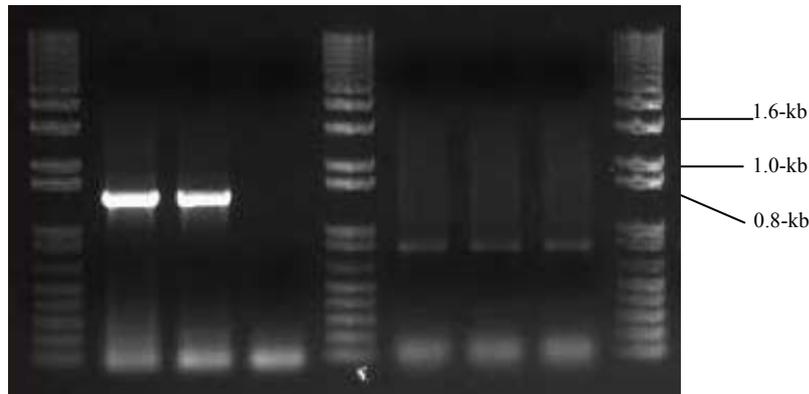
Figure 1. Expression of *wzt* and *wbiA* mRNA by *B. mallei* strains as determined by RT-PCR. For PCR assays, cDNA from the wild type strain ATCC 23344 (lanes-1 and -4), *sacB* mutant 23344Δ*sacB* (lanes-2 and -5), and *wzt* mutant 23344Δ*sacB*Δ*wzt* (lanes-3 and -6) was used. PCR with forward and reverse primers for *wzt* produced a 1.4-kb ampicon from the wild type (lane-1) and *sacB* mutant (lane-2), but not from *wzt* mutant (lane-3). PCR with forward and reverse primers for *wbiA* produced 0.8-kb ampicons from all three strains; wild type (lane-4), *sacB* mutant (lane-5), and *wzt* mutant (lane-6). Lane-M represents 1-kb molecular size markers.
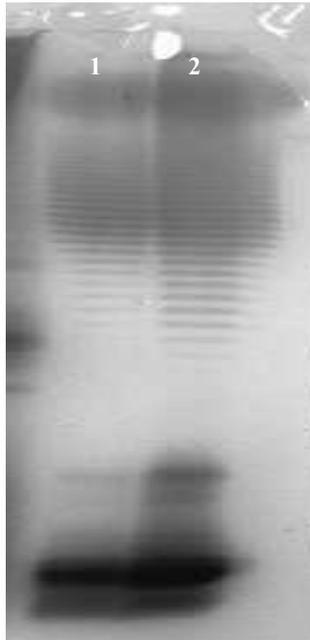
Figure 2. LPS profiles of *B. mallei* as observed by SDS-PAGE and silver staining. LPS extracts harvested from the wild type ATCC 23344 (lane 1), and the *wzt* mutant (lanes 2) were electrophoresed using 16% Tricin gel, and stained with silver.

influence resistance of B. mallei to bactericidal killing.

3.7. Pathogenicity of *B. mallei* Strains in Mice

The pathogenicity of the *B. mallei* strains in CD1 mice was evaluated by inoculating groups of five mice intraperitoneally with doses of *B. mallei* strains, and recording mortality of animals over a course of eight days (**Figure 3**). When nearly 4.4×10^5 colony forming units (cfu) of strains were used for inoculations, 20% of mice inoculated with the wild type strain died, but no mice inoculated with the *sacB* or *wzt* mutants died (**Figure 3(a)**). When a dose of 6.6×10^5 cfu was used in inoculations, 60% of animals inoculated with the wild type strain and 40% inoculated with the *sacB* mutant died within 6 days post-inoculation, but only 20% of animals inoculated with the same dose of *wzt* mutant died (**Figure 3(b)**). When the mice were inoculated with a dose of 8.8×10^5 cfu, all the mice injected with the wild type strain or the *sacB* mutant died within 4 days post-inoculation, but only 40% of animals inoculated with the *wzt* mutant died (**Figure 3(c)**). The LD_{50} dose of the wild type strain, the *sacB* mutant, and the *wzt* mutant were 5.9×10^5, 6.6×10^5, and 9.1 v 10^5 cfu, respectively. None of the inoculated mice died after day 6 post-inoculation. Those mice that survived the *B. mallei* inoculations exhibited clinical manifestations including huddling during the first 4 days following inoculations, but remained clinically normal throughout the rest of the 36-day observation period.

3.8. Immune and Protective Responses of Mice Inoculated *wzt* Mutant

Specific serum immunoglobulins IgG1 and IgG2a of CD1 mice were measured by enzyme-linked immunosorbent assay. Serum IgG titers of mice inoculated with the *wzt* mutant were 34 to 43-fold higher than naïve controls at 30 days post infection (data not shown). The two IgG isotypes were present in sera in almost equal amounts.

The protective efficacy of the *wzt* mutant in CD1 mice against challenge with the wild type *B. mallei* (ATCC 23344) was determined. At day 36 post-inoculation, those mice that were injected with saline (n = 5) or the *wzt* mutant (n = 8) and survived were challenged intraperitoneally with 6.6×10^6 cfu (11.2 times the LD_{50}) of the wild type strain, and behaviors and survival of mice were
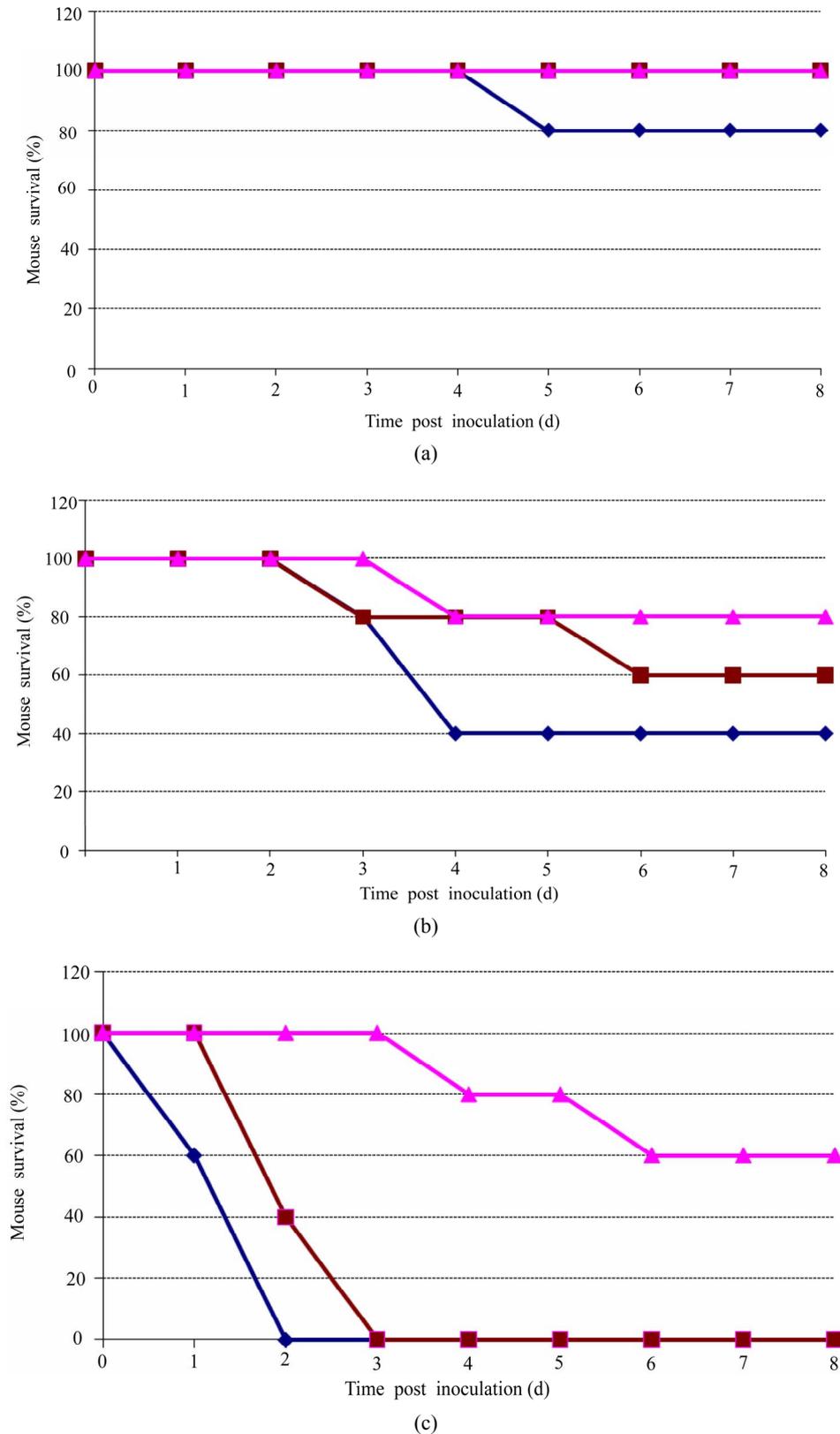
Figure 3. Survival of CD1 mice inoculated *B. mallei* strains wild (♦), *sacB* mutant (▪), and *wzt* mutant (▲). Groups of five mice were injected intraperitoneally with 4.4×10^5 (a), 6.6×10^5 (b), or 8.8×10^5 (c) cfu/mouse (n = 5), and the number of survivors was recorded during a course of eight days post-inoculation.

       AID

monitored. Eighty percent of mice injected with saline and subsequently challenged with the wild type strain died within 24 h post-challenge (**Figure 4**). Among the mice inoculated intraperitoneally with the *wzt* mutant and subsequently challenged intraperitoneally with the wild type strain, 87.5% survived longer than 15 days post challenge (**Figure 4**). The surviving mice did not exhibit any clinical symptoms during the 15-day post-challenge period.

4. Discussion

The O-antigen is a key component of LPS in the outer membrane of many gram-negative bacteria. It consists of repeats of an oligosaccharide unit (O unit), which usually contains two to eight residues of a broad range of both common and rarely occurring sugars and their derivatives. Sugar nucleotides are the activated precursors for cell surface polysaccharides. Tosynthesize O-antigens, monomers are assembled on a lipid carrier (undecaprenol phosphate) by enzymes encoded in the *wb* gene cluster before their incorporation into the LPS molecule. The ABC-2 transporters consist of an integral membrane protein, Wzm, and a hydrophilic protein containing an ATP-binding motif, Wzt. Involvement of the transporter with the translocation of the polymer has not been proven experimentally and details of the process are not clear at this stage [9].

The O-antigen biosynthetic cluster of *B. mallei* is comprised of a *wzm* gene encoding a protein identical to membrane component of the ABC transporter of other bacteria, and a *wzt* gene encoding a protein identical to ATP-binding component of the ABC transporter. Accordingly, it can be speculated that O-antigen in *B. mallei* is exported by an ABC transporter pathway just like in *E. coli* O8 and O9 [15], and *K. pneumoniae* O1 and O12 [14, 16,17]. The *B. mallei wzm* and *wzt* shared substantial identity with *wzm* and *wzt* proteins of a large number of other bacterial species suggesting that these proteins are conserved and are likely important for the O-antigen biosynthesis, growth and/or persistence of many bacteria. Both *wzm* and *wzt* were predicted to localize in the cytoplasm of the cell, an observation consistent with the predicted functions of these two proteins.

Western immunoblotting assays revealed that disruption of *wzt* did not influence O-antigen biosynthesis. Burtnick *et al.*, [12] reported that the O-antigen moiety is required for resistance of *B. mallei* to the bactericidal action of serum. Our bactericidal assays revealed that the *wzt* mutant and the wild type *B. mallei* were similarly resistant to serum bactericidal killing. These observations suggest that the *wzt* mutant strain produced O-antigen uninterruptedly. Electron micrographs suggested that disruption of *wzt* did not prevent transport of O-antigen to the cell surface.
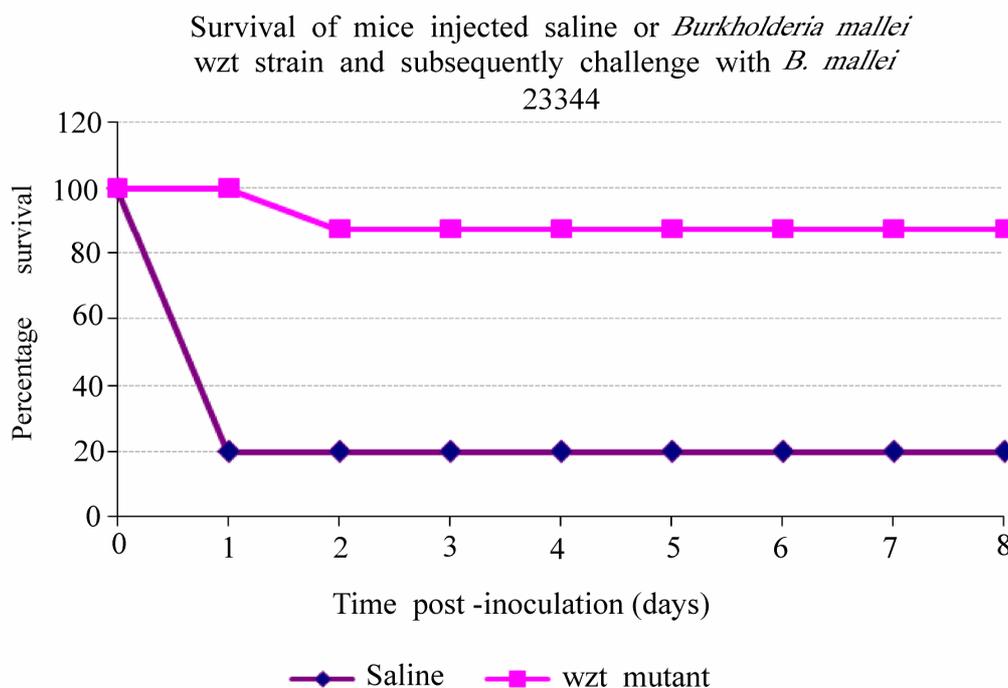
Figure 4. Protective efficacy of the *wzt* mutant against challenge with virulent wild type *B. mallei*. Thirty-six days after injection with saline (n = 5) (♦) or inoculation with the *wzt* mutant (n = 8) (▪), the mice that survived were challenged intraperitoneally with 6.6×10^6 cfu/mouse of the wild type strain ATCC 23344. The number of survivors was recorded during a course of seven days post-challenge.

When CD1 mice were inoculated intraperitoneally, the *B. mallei wzt* mutant caused less murine mortality than the wild-type strain or the *sacB* mutant. These observations suggest that functions of *wzt* are critical for virulence of *B. mallei in vivo*. The *wzt* mutant displayed slower growth *in vitro*, suggesting that *wzt* functions are important for normal growth. The less pathogenicity of this mutant can partly be attributed to its slower growth rate. The slow growing mutant may be less capable of evading the effects of host immune system.

Since *B. mallei* is a facultative intracellular pathogen, a live attenuated vaccine may be the best strategy to induce protective cell-mediated and antibody-mediated immune responses. When the CD1 mice were inoculated with a non-lethal dose of *wzt* mutant, both IgG1 and IgG2a were induced. When those mice were challenged with a 11.2 time LD_{50} of the wild type, a greater proportion survived relative to uninoculated controls suggesting that the mutant induces protection in mice against glanders. The mice that survived after *wzt* inoculation and subsequent challenge with the wild type strain did not exhibit prolonged clinical symptoms such as huddling or fur ruffling. In contrast, the *B. mallei* mutant strains generated by disrupting the capsule biosynthesis [21], type III secretion system [31], branched-chain amino acid biosynthesis [23], and quorum-sensing network [32] failed to induce reasonable protection against glanders in mice or Syrian Hamsters. Taking all these observations into account, we speculate that the *wzt* mutant has potential as a vaccine candidate against glanders.

5. Conclusion

The functions of *wzt* were found important for *B. mallei* in its growth in culture medium and pathogenicity in CD1 mice. The attenuated *wzt* mutant induced partial protection in mice against *B. mallei* challenge, and therefore, has potential as a vaccine candidate against glanders.

6. Acknowledgements

REFERENCES

[1] E. Yabuuchi, Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki and M. Arakawa, "Proposal of Burkholderia gen. nov. and Transfer of Seven Species of the Genus Pseudomonas Homology Group II to the New Genus, with the Type Species Burkholderia cepacia (Palleroni and Holmes 1981) comb. nov.," *Microbiol Immunol*, Vol. 36, No. 12, 1992, pp. 1251-1275.

[2] A. Srinivasan, C. N. Kraus, D. DeShazer, P. M. Becker, J. D. Dick, L. Spacek, J. G. Bartlett, W. R. Byrne and D. L. Thomas, "Glanders in a Military Research Microbiologist," *The New England Journal of Medicine*, Vol. 345, No. 4, 2001, pp. 256-258. doi:10.1056/NEJM200107263450404

[3] M. S. Redfearn, N. J. Palleroni and R. Y. Stanier, "A Comparative Study of *Pseudomonas pseudomallei* and *Bacillus mallei*," *Journal of General Microbiology*, Vol. 43, No. 2, 1966, pp. 293-313. doi:10.1099/00221287-43-2-293

[4] L. Wilkinson, "Glanders: Medicine and Veterinary Medicine in Common Pursuit of a Contagious Disease," *Medical History*, Vol. 25, No. 4, 1981, pp. 363-384.

[5] G. C. Whitlock, D. M. Estes and A. G. Torres, "Glanders: Off to the Races with *Burkholderia mallei*," *FEMS Microbiology Letters*, Vol. 277, No. 2, 2007, pp. 115-122. doi:10.1111/j.1574-6968.2007.00949.x

[6] M. R. Burns, S. A. Jenkins, N. M. Vermeulen, R. Balakrishna, T. B. Nguyen, M. R. Kimbrell and S. A. David, "Structural Correlation between Lipophilicity and Lipopolysaccharide-Sequestering Activity in Spermine-Sulfonamide Analogs," *Bioorganic & Medicinal Chemistry Letters*, Vol. 16, No. 24, 2006, pp. 6209-6212. doi:10.1016/j.bmcl.2006.09.026

[7] M. R. Burns, S. A. Jenkins, S. J. Wood, K. Miller and S. A. David, "Structure-Activity Relationships in Lipopolysaccharide Neutralizers: Design, Synthesis, and Biological Evaluation of a 540-Membered Amphipathic Bisamide Library," *Journal of Combinatorial Chemistry*, Vol. 8, No. 1, 2006, pp. 32-43. doi:10.1021/cc0500755

[8] H. Nikaido, "Molecular Basis of Bacterial Outer Membrane Permeability Revisited," *Microbiology and Molecular Biology Reviews*, Vol. 67, No. 4, 2003, pp. 593-656. doi:10.1128/MMBR.67.4.593-656.2003

[9] C. R. Raetz and C. Whitfield, "Lipopolysaccharide Endotoxins," *Annual Review of Biochemistry*, Vol. 71, 2002, pp. 635-700.

[10] C. A. Schnaitman and J. D. Klena, "Genetics of Lipopolysaccharide Biosynthesis in Enteric Bacteria," *Microbiology Reviews*, Vol. 57, No. 3, 1993, pp. 655-682.

[11] C. Whitfield, "Biosynthesis of Lipopolysaccharide O Antigens," *Trends in Microbiology*, Vol. 3, No. 5, 1995, pp. 178-185. doi:10.1016/S0966-842X(00)88917-9

[12] M. N. Burtnick, P. J. Brett and D. E. Woods, "Molecular and Physical Characterization of *Burkholderia mallei* O Antigens," *Journal of Bacteriology*, Vol. 184, No. 3, 2002, pp. 849-852. doi:10.1128/JB.184.3.849-852.2002

[13] G. Samuel and P. Reeves, "Biosynthesis of O-Antigens: Genes and Pathways Involved in Nucleotide Sugar Precursor Synthesis and O-Antigen Assembly," *Carbohydrate Research*, Vol. 338, No. 23, 2003, pp. 2503-2519. doi:10.1016/j.carres.2003.07.009

[14] B. R. Clarke and C. Whitfield, "Molecular Cloning of the rfb Region of *Klebsiella pneumoniae* Serotype O1:K20: the rfb Gene Cluster Is Responsible for Synthesis of the D-Galactan I O Polysaccharide," *Journal of Bacteriology*, Vol. 174, No. 14, 1992, pp. 4614-4621.

[15] H. C. Flemming and K. Jann, "Biosynthesis of the O9 Antigen of *Escherichia coli*. Growth of the Polysaccharide Chain," *European Journal of Biochemistry*, Vol. 83, No. 1, 1978, pp. 47-52. doi:10.1111/j.1432-1033.1978.tb12066.x

[16] L. Izquierdo, N. Coderch, N. Pique, E. Bedini, M. M. Corsaro, S. Merino, S. Fresno, J. M. Tomas and M. Regue, "The *Klebsiella pneumoniae* wabG Gene: Role in Biosynthesis of the Core Lipopolysaccharide and Virulence," *Journal of Bacteriology*, Vol. 185, No. 24, 2003, pp. 7213-7221. doi:10.1128/JB.185.24.7213-7221.2003

[17] L. Izquierdo, S. Merino, M. Regue, F. Rodriguez and J. M. Tomas, "Synthesis of a *Klebsiella pneumoniae* O-Antigen Heteropolysaccharide (O12) Requires an ABC 2 Transporter," *Journal of Bacteriology*, Vol. 185, No. 5, 2003, pp. 1634-1641. doi:10.1128/JB.185.5.1634-1641.2003

[18] J. D. Bendtsen, H. Nielsen, G. von Heijne and S. Brunak, "Improved Prediction of Signal Peptides: SignalP 3.0," *Journal of Molecular Biology*, Vol. 340, No. 4, 2004, pp. 783-795. doi:10.1016/j.jmb.2004.05.028

[19] S. F. Altschul, W. Gish, W. Miller, E. W. Myers and D. J. Lipman, "Basic Local Alignment Search Tool," *Journal of Molecular Biology*, Vol. 215, No. 3, 1990, pp. 403-410.

[20] A. B. Bandara, D. DeShazer, T. J. Inzana, N. Sriranganathan, G. G. Schurig and S. M. Boyle, "A Disruption of ctpA Encoding carboxy-Terminal Protease Attenuates *Burkholderia mallei* and Induces Partial Protection in CD1 Mice," *Microbial Pathogenesis*, Vol. 45, No. 3, 2008, pp. 207-216. doi:10.1016/j.micpath.2008.05.005

[21] D. DeShazer, D. M. Waag, D. L. Fritz and D. E. Woods, "Identification of a *Burkholderia mallei* Polysaccharide Gene Cluster by Subtractive Hybridization and Demonstration That the Encoded Capsule Is An Essential Virulence Determinant," *Microbial Pathogenesis*, Vol. 30, No. 5, 2001, pp. 253-269. doi:10.1006/mpat.2000.0430

[22] R. Simon, U. Priefer and A. Puhler, "A Broad Host Range Mobilization System for *in Vivo* Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria," *Bio/Technology*, Vol. 1, No. 9, 1983, pp. 784-791.

[23] R. L. Ulrich, K. Amemiya, D. M. Waag, C. J. Roy and D. DeShazer, "Aerogenic Vaccination with a *Burkholderia mallei* Auxotroph Protects against Aerosol-Initiated Glanders in Mice," *Vaccine*, Vol. 23, No. 16, 2005, pp. 1986-1992. doi:10.1016/j.vaccine.2004.10.017

[24] J. F. Sambrook and E. F. Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.

[25] P. J. Brett, M. N. Burtnick, D. S. Snyder, J. G. Shannon, P. Azadi and F. C. Gherardini, "*Burkholderia mallei* Expresses a Unique Lipopolysaccharide Mixture That Is a Potent Activator of Human Toll-Like Receptor 4 Complexes," *Molecular Microbiology*, Vol. 63, No. 2, 2007, pp. 379-390. doi:10.1111/j.1365-2958.2006.05519.x

[26] M. B. Perry, L. L. MacLean, T. Schollaardt, L. E. Bryan and M. Ho, "Structural Characterization of the Lipopolysaccharide O Antigens of *Burkholderia pseudomallei*," *Infection and Immunity*, Vol. 63, No. 9, 1995, pp. 3348-3352.

[27] U. K. Laemmli, "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4," *Nature*, Vol. 227, No. 5259, 1970, pp. 680-685. doi:10.1038/227680a0

[28] C. M. Tsai, R. Boykins and C. E. Frasch, "Heterogeneity and Variation among *Neisseria meningitidis* Lipopolysaccharides," *Journal of Bacteriology*, Vol. 155, No. 2, 1983, pp. 498-504.

[29] T. J. Inzana and P. Anderson, "Serum Factor-Dependent Resistance of *Haemophilus influenzae* Type b to Antibody to Lipopolysaccharide," *The Journal of Infectious Diseases*, Vol. 151, No. 5, 1985, pp. 869-877.

[30] J. Li, C. Ryder, M. Mandal, F. Ahmed, P. Azadi, D. S. Snyder, R. D. Pechous, T. Zahrt and T. J. Inzana, "Attenuation and Protective Efficacy of an O-antigen-Deficient Mutant of *Francisella tularensis* LVS," *Microbiology*, Vol. 153, No. Pt 9, 2007, pp. 3141-3153. doi:10.1099/mic.0.2007/006460-0

[31] R. L. Ulrich and D. DeShazer, "Type III Secretion: A Virulence Factor Delivery System Essential for the Pathogenicity of B*urkholderia mallei*," *Infection and Immunity*, Vol. 72, No. 2, 2004, pp. 1150-1154. doi:10.1128/IAI.72.2.1150-1154.2004

[32] R. L. Ulrich, D. Deshazer, H. B. Hines and J. A. Jeddeloh, "Quorum Sensing: A Transcriptional Regulatory System Involved in the Pathogenicity of *Burkholderia mallei*," *Infection and Immunity*, Vol. 72, No. 11, 2004, pp. 6589-6596.

AID