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

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Received April 23rd, 2012; revised May 25th, 2012; accepted June 27th, 2012

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 (LD50) of the wild type and the wzt mutant strains were $5.9 \times 10^5$ and $9.1 \times 10^5$ 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 LD50 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; wzt 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 proteins of the EMBL/GenBank/DDBJ databases was analyzed using the SignalP 3.0 server (http://www.cbs.dtu.dk/) [18].

2.2. Bacterial Strains, Plasmids, and Reagents

*B. mallei* strains ATCC 23344 and 23344ΔsacB [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% CO₂ 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 µg/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 *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 Δwzt 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, CGGGCATGATGTCCT CTAATTGCAG; *wzt*-2, CGGGGATCCCGCGACG TACATTGCCGCCTC; *wzt*-3 CGGGGATCCGCGTCC GAACGAGTGGAGT; and *wzt*-4 CGGGCATGTT CAGATTTCAATGCAGC. The 5’ end of the primers *wzt*-1 and *wzt*-4 carried *NcoI* sites (in bold case), whereas the 5’ ends of primers *wzt*-2 and *wzt*-3 carried *BamHI* (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 *NcoI* 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* 23344ΔsacB [20] via conjugation with S17-1 (pABwzt) by using a membrane filter mating technique, as described elsewhere [20]. Strain 23344ΔsacB was chosen as the platform strain, as its resistance to sucrose was useful as a non-antibiotic marker in selecting the recombinant strains [20]. Polymyxin was used to counterselect *E. coli*. One of the 23344ΔsacB: 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 23344ΔsacBΔwzt.

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

Extraction of RNA, treatment with DNase, and RT-PCR were performed as described elsewhere [20]. The primers *wbIA*-Forward (5’ TAGATTTCCATAGAGATGTC 3’) and *wbIA*-Reverse (5’ ATGTTGGCGCCTACGGTCAA 3’) were used for PCR amplification 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% CO₂. 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 dialyates were then clarified by centrifugation and concentrated by lyophilization. The crude preparations were solubilized in 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM CaCl₂, 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 electrophoresed 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 (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 10⁶ 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 a 1.7% lead citrate and 2% uranyl acetate, and viewed with a Zeiss 10C 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 × 10⁵, 6.6 × 10⁵, or 8.8 × 10⁵ 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 (LD₅₀) 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/stat2050/software/software_2050.html). The number of animals dying up to 6 d post-inoculation was used in LD₅₀ calculations. The animals that received wild type or sacB strains and survived were sacrificed by exposing to CO₂ 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 × 10⁶ cfu/mouse (11.2 times the LD₅₀) 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 CO₂ 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 to 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 23344ΔsacB [20], and designated as 23344ΔsacBΔwzt. A PCR assay with the primer pair wzt-1/wzt-4 (see Materials and Methods) produced a 1.4-kb amplicon from wild type *B. mallei*, and an approximately 0.9-kb amplicon from the wzt mutant strain 23344ΔsacBΔwzt (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 wild type strain 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. pseudomallei* 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 WptL*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

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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 23344ΔsacB (lanes-2 and -5), and wzt mutant 23344ΔsacBΔwzt (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% Tricine gel, and stained with silver.

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 × 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
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Figure 3. Survival of CD1 mice inoculated *B. mallei* strains wild (♦), *sacB* mutant (●), and *wzt* mutant (▲). Groups of five mice were injected intraperitoneally with $4.4 \times 10^5$ (a), $6.6 \times 10^5$ (b), or $8.8 \times 10^5$ (c) cfu/mouse (n = 5), and the number of survivors was recorded during a course of eight days post-inoculation.
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 × 10⁶ 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 LD50 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

The author thanks Anna Champion for assistance in LPS extraction and SDS-PAGE, Kathy Lowe for assistance in electron microscopy, Lynn Heffron and Dustin Lucas for expert handling of mice, and Kay Carlson and Nancy Tenpenny for technical assistance.

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