Polysaccharides and virulence of *Burkholderia pseudomallei*

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Burkholderia pseudomallei is the causative agent of melioidosis, an infectious disease of humans and animals. Gene clusters which encode capsular polysaccharide (type I O-PS) and LPS (type II O-PS), both of which play roles in virulence, have previously been identified. Here, the identification of two further putative clusters, type III O-PS and type IV O-PS, is reported. Mice challenged with type III O-PS or type IV O-PS mutants showed increased mean times to death (7.8 and 11.6 days) compared to those challenged with wild-type *B. pseudomallei* (3 days). To investigate the possible roles of polysaccharides in protection, mice were immunized with killed cells of wild-type *B. pseudomallei* or killed cells of *B. pseudomallei* with mutations in the O antigen, capsular polysaccharide, type III O-PS or type IV O-PS gene clusters. Immunization with all polysaccharide mutant strains resulted in delayed time to death compared to the naïve controls, following challenge with wild-type *B. pseudomallei* strain K96243. However, immunization with killed polysaccharide mutant strains conferred different degrees of protection, demonstrating the immunological importance of the polysaccharide clusters on the surface of *B. pseudomallei*.

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INTRODUCTION

Burkholderia pseudomallei is a Gram-negative saprophytic rod which is particularly prevalent in the soil and standing water in Southeast Asia and Northern Australia (Dance, 1990; Currie, 2003). It is the causative agent of melioidosis, an infectious disease of both humans and animals. Melioidosis can present itself in a number of forms including acute septicaemia, acute pulmonary infection, and sub-acute and chronic diseases (Currie, 2003; White, 2003; Cheng & Currie, 2005). In some cases, a latent infection is established which may reactivate to cause disease in later life. The factors that influence the presentation of disease are not known, but differences in the virulence of strains might contribute to the outcome of infection.

Currently there is no vaccine to protect against *B. pseudomallei* and little is known about the virulence mechanisms of the pathogen. However, *B. pseudomallei* expresses surface polysaccharides, two of which have been well characterized: the capsular polysaccharide and LPS, also known as type I O-PS and type II O-PS, respectively (Deshazer *et al.*, 1998; Reckseidler *et al.*, 2001; Perry *et al.*, 1995). Previous studies have shown that LPS O antigen and capsular polysaccharide mutants of *B. pseudomallei* are markedly attenuated in animal models of disease (Deshazer *et al.*, 1998; Atkins *et al.*, 2002; Reckseidler *et al.*, 2001). The

B. pseudomallei O antigen appears to have properties typical of bacterial O antigens, for example by providing resistance to serum killing (Deshazer *et al.*, 1998). The function of the capsular polysaccharide is less clear, but it may provide resistance to phagocytosis by reducing levels of complement C3 deposition (Reckseidler-Zenteno *et al.*, 2005). The role of the capsular polysaccharide in virulence is supported by the observation that the encoding gene cluster is present in the related human pathogen *Burkholderia mallei* (Deshazer *et al.*, 2001). In contrast, the gene cluster is absent in *Burkholderia thailandensis*, a non-pathogenic environmental saprophyte that is genetically closely related to *B. pseudomallei* (Ong *et al.*, 2004; Kespichayawattana *et al.*, 2004; Brett *et al.*, 1998; Yu *et al.*, 2006).

The potential immunological importance of these polysaccharides has also been demonstrated by the reduction in the lethality of infection in mice and diabetic rats when passively immunized with antibodies to the LPS or capsular polysaccharide (Bryan *et al.*, 1994; Jones *et al.*, 2002). Furthermore, immunization of mice with LPS or capsular polysaccharide provides protection against a subsequent challenge with *B. pseudomallei*, suggesting that these polysaccharides have potential as vaccine candidates (Nelson *et al.*, 2004). The relative roles of the polysaccharides in *B. pseudomallei* virulence remain unclear, and have been further complicated by the identification of two further putative polysaccharide clusters, encoding type III O-PS

Abbreviations: i.p., intraperitoneal; MTTD, mean time to death.

and type IV O-PS, in the *B. pseudomallei* strain K96243 genome sequence (Holden *et al.*, 2004). In support of the possibility that *B. pseudomallei* expresses polysaccharides other than LPS and capsular, a study conducted with 1327 strains has shown that 3% of strains have either another LPS serotype which has a different O-antigen ladder profile, or a rough form of LPS that lacks an O-antigen ladder (Anuntagool *et al.*, 2006). Another exopolysaccharide has been reported (Nimtz *et al.*, 1997); however, the operon encoding this polysaccharide has not been identified.

In this study, the role of each polysaccharide gene cluster in virulence and protection was investigated by insertionally inactivating genes within each operon.

METHODS

Bacterial strains. *B. pseudomallei* strain K96243 was grown in Luria-Bertani (LB) broth at 37 °C with shaking for 18 h. For viable counts, media were solidified with 15 g Bacto agar l⁻¹. *Escherichia coli* strains DH5 α λ pir and E19851 were grown in LB broth, supplemented with 50 μ g kanamycin ml⁻¹ (Sigma) as required, with shaking for 18 h.

PCR. Primers used to amplify partial sequences from *wbiA*, *wcbH*, *BPSS0421* and *BPSS1833* genes of *B. pseudomallei* are described in Table 1. The partial genes were amplified under the following conditions: denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension for 1 min, followed by final extension at 72 °C for 10 min. Amplified PCR products were initially cloned into pCR-Blunt II-TOPO (Invitrogen) and subsequently cloned into the *SmaI* or *EcoRV* site of the pKNOCK vector (Alexeyev, 1999). Restriction sites were engineered onto both the forward and reverse primers for subsequent digestion with either *SmaI* or *EcoRV*, creating blunt products for cloning into pKNOCK. The recombinant pKNOCK plasmids were transformed into *E. coli* strain E19851.

Conjugation of *B. pseudomallei* strain K96243. Overnight cultures of recombinant *E. coli* E19851 or *B. pseudomallei* were centrifuged at 3000 *g* for 10 min at room temperature. Supernatants were discarded, and the pellets were resuspended in half the original volume. A 10 μ l aliquot of each culture was pipetted onto a sterile nitrocellulose membrane on an LB agar plate and incubated for 6–8 h at 37 °C. The nitrocellulose membrane was vortexed in 1 ml LB medium, and 100 μ l aliquots were plated onto LB agar plates supplemented with 100 μ g ampicillin and 700 μ g kanamycin. The plates were incubated for 2 days at 37 °C.

Southern blotting. Genomic DNA was prepared from 100 ml cultures of *B. pseudomallei* in LB broth by a lysozyme, phenol/chloroform extraction method (Mack & Titball, 1998). *B. pseudomallei* genomic DNA (5 μ g) was digested with the selected restriction endonuclease, separated by gel electrophoresis using a 0.8% (w/v) agarose gel, and transferred to a positively charged nylon membrane (Hybond-N⁺; Amersham Biosciences). Southern hybridization was performed using the Roche DIG labelling Southern kit following the manufacturer's instructions.

Animals. Female BALB/c age-matched mice, approximately 6 weeks old, were used in this study. The mice were grouped together in cages of five with free access to food and water, and subjected to a 12 h light/dark cycle. For challenge with viable *B. pseudomallei*, the animals were handled under biosafety level III containment conditions within a half-suit isolator, compliant with British Standard BS5726. All investigations involving animals were carried out according to the requirements of the Animal (Scientific Procedures) Act 1986. Humane end points were strictly observed, and animals deemed incapable of survival were humanely killed by cervical dislocation.

Virulence studies. Groups of five mice were challenged with 1×10^4 c.f.u. of wild-type *B. pseudomallei* strain K96243, *B. pseudomallei* K96243 Δ BPSS0421::kan (type III O-PS) or *B. pseudomallei* K96243 Δ BPSS1833::kan (type IV O-PS) by the intraperitoneal (i.p.) route, and the infection was monitored for 5 weeks.

Determination of bacterial hydrophobicity. The hydrophobicity of bacterial strains was measured using the bacterial adhesion to hydrocarbon (BAH) test (Beck *et al.*, 1988; Rosenberg, 1984). All experiments were carried out using overnight bacterial cell suspensions, washed twice in sodium phosphate buffer (pH 6.8), and resuspended to OD₄₄₀ 1.0. Bacterial suspensions (3 ml), prepared in sodium phosphate buffer (pH 6.8), were overlaid with 1 ml n-hexadecane (Sigma). After 1 min of vigorous agitation, the phases were allowed to separate for 15 min at room temperature. Partitioning in the hydrocarbon phase was calculated as follows:

$$\frac{\{OD_{440} \text{ (original bacterial suspension)} - OD_{440} \text{ (aqueous phase)}\}}{OD_{440} \text{ (original bacterial suspension)}} \times 100$$

From each bacterial strain, the percentage hydrophobicity was obtained from the mean values of three separate experiments; the error bars on Fig. 3 represent the mean $\pm$ SD.

Heat-inactivation of bacteria. *B. pseudomallei* strains were grown in LB broth at 37 °C overnight with agitation. The cultures were adjusted with PBS to the same OD₅₉₀ value and harvested by centrifugation at 5000 *g* for 15 min. Bacteria were washed once in the original culture volume of PBS, harvested again, and then

Table 1. Primers used in this study to construct mutant strains

Strain	Primer
<i>B. pseudomallei</i> K96243 Δ wbiA::kan	wbiA.KO.For – ggactagtggatgcgttcagtttcgt wbiA.KO.Rev – gaagcttgatgccaactgaacatgcg
<i>B. pseudomallei</i> K96243 Δ wcbA::kan	wcbH.KO.For – ggggatcccgatggacatggagcctg wcbH.KO.Rev – ggaagcttcaactctccgcatagtcg
<i>B. pseudomallei</i> K96243 Δ BPSS0421::kan	BPSS0421.KO.For – gatatcggaactgcagcacatggtgg BPSS0421.KO.Rev – gatatcggtagaactcagcgtcgcg
<i>B. pseudomallei</i> K96243 Δ BPSS1833::kan	BPSS1833.KO.For – cccgggcgatcggtagtactcgatg BPSS1833.KO.Rev – cccgggcgagttcacgaagtatgcgg

resuspended in the original volume of PBS. Bacteria were heat-inactivated by incubating in a water bath maintained at 80 °C for 3 h. Inactivated bacteria were then placed at 4 °C for at least 16 h. Prior to heat-inactivation, a viable count was performed to determine the number of heat-killed bacteria in c.f.u. ml⁻¹. Inactivation was confirmed by culturing 10% of each inactivated culture in LB broth for 7 days at 37 °C, then plating out the broth on LB agar plates for 7 days at 37 °C to confirm that no viable bacteria remained.

Immunization and challenge. Groups of 10 BALB/c mice were immunized by the i.p. route with 10⁸ c.f.u. bacteria on day 0, followed by boosts administered on days 14 and 28. A period of 5 weeks elapsed prior to i.p. challenge with 6.05 × 10⁴ c.f.u. wild-type bacteria, and the animals were monitored for a further 5 weeks.

RESULTS AND DISCUSSION

Organization of polysaccharide gene clusters in *B. pseudomallei* strain K96243

Previously, gene clusters encoding the capsular polysaccharide (type I O-PS) and O antigen (type II O-PS) have been identified, and mutations in these gene clusters shown to attenuate *B. pseudomallei* (Atkins *et al.*, 2002; Deshazer *et al.*, 1998; Reckseidler *et al.*, 2001). Two additional polysaccharide clusters of unknown function have also been identified in the genome sequence of *B. pseudomallei* strain K96243 (Holden *et al.*, 2004). To correspond with the existing nomenclature of the polysaccharides in *B.*

pseudomallei, these novel clusters would encode type III O-PS and type IV O-PS. The putative type III O-PS polysaccharide would be encoded by a cluster of 13 genes located between *BPSS0417* and *BPSS0429*. The genes have been assigned putative functions related to polysaccharide biosynthesis and transport (Holden *et al.*, 2004). Genes *BPSS0419–BPSS0421* show homology to the *rfb* cluster of *Salmonella typhimurium* and the *asc* gene cluster of *Yersinia pseudotuberculosis*, both of which are involved in O-antigen biosynthesis (Brahmbhatt *et al.*, 1988; Thorson *et al.*, 1994). The gene cluster which encodes polysaccharide O-PS IV consists of eight genes from *BPSS1825* to *BPSS1832* with putative functions of capsular polysaccharide biosynthesis and polysaccharide transport proteins. The type III O-PS and type IV O-PS clusters are not present in *B. mallei*, but part of the clusters is conserved in *B. thailandensis*, indicating that these putative polysaccharide clusters have a possible role in environmental survival.

The possibility that *B. pseudomallei* expresses polysaccharides other than type I O-PS and type II O-PS has been raised by several previous studies (Kawahara *et al.*, 1998; Masoud *et al.*, 1997; Nimtz *et al.*, 1997; Steinmetz *et al.*, 1995, 2000). For example, one polysaccharide distinct from type I O-PS and type II O-PS has been reported to be a linear tetrasaccharide repeating unit consisting of three galactose residues, one bearing a 2-linked O-acetyl group and a 3-deoxy-D-manno-2-2-octulosonic acid residue

Fig. 1. Southern blotting confirms the disruption of the polysaccharide clusters in the *B. pseudomallei* mutant strains. (a) Capsule operon, *wbiA* gene; (b) LPS operon, *wcbH* gene; (c) PS-III, *BPSS0421*; and (d) PS-IV, *BPSS1833*.

Fig. 2. Survival of mice infected with 10^4 c.f.u. wild-type (◆), *B. pseudomallei* ΔBPSS0421 (▲) and *B. pseudomallei* ΔBPSS1833 (■).

(Masoud *et al.*, 1997; Nimtz *et al.*, 1997). Screening of this polysaccharide using a monoclonal antibody has shown expression in all *B. pseudomallei* strains tested, and also in *B. mallei* (Steinmetz *et al.*, 1995). The absence of the putative polysaccharide clusters type III O-PS and type IV O-PS in *B. mallei* indicates that these clusters do not encode this polysaccharide.

Construction of mutants

B. pseudomallei mutants were constructed by insertionally inactivating a gene located in each of the polysaccharide gene clusters. Homologous regions from the *trans*-acylase gene *wbiA*, the mannosyltransferase gene *wcbH*, BPSS0421 and the BPSS1833 gene were cloned into the suicide vector pKNOCK and transformed into *E. coli* (Alexeyev, 1999). The suicide plasmid constructs were introduced into wild-type *B. pseudomallei* strain K96243 by conjugation, creating four mutants: *B. pseudomallei* K96243Δ*wbiA*::*kan* (type I O-PS), *B. pseudomallei* K96243Δ*wcbH*::*kan* (type II O-PS), *B. pseudomallei* K96243ΔBPSS0421::*kan* (type III O-PS) and *B. pseudomallei* K96243ΔBPSS1833::*kan* (type IV O-PS). Correct integration of the plasmid into each

polysaccharide biosynthesis operon was confirmed by Southern blotting (Fig. 1).

Disruption of the type IV O-PS but not the type III O-PS gene cluster significantly attenuates *B. pseudomallei*

The attenuation of type I O-PS or type II O-PS mutants of *B. pseudomallei* in mice is well documented (Atkins *et al.*, 2002; Deshazer *et al.*, 1998; Reckseidler *et al.*, 2001). To investigate the roles of the putative type III O-PS or type IV O-PS polysaccharides in virulence, mice were challenged by the i.p. route with 10^4 c.f.u. *B. pseudomallei* K96243ΔBPSS0421::*kan*, *B. pseudomallei* K96243ΔBPSS1833::*kan* or with wild-type *B. pseudomallei* (Fig. 2). Animals in the group which were challenged with wild-type bacteria died with a mean time to death (MTTD) of 3 days. There was a delayed time to death in the groups challenged with the mutant strain *B. pseudomallei* K96243ΔBPSS1833::*kan* with a MTTD of 11.6 days. The difference in survival between wild-type and *B. pseudomallei* K96243ΔBPSS1833::*kan* was statistically significant using the Mantel–Haenszel log rank test ($P=0.0027$), indicating that this polysaccharide cluster plays a role in the virulence of *B. pseudomallei*. Although we could not demonstrate a role in virulence for *B. pseudomallei* K96243ΔBPSS0421::*kan* in this experiment, the group challenged with this mutant strain showed survival for up to 15 days compared to the group challenged with wild-type bacteria, in which death occurred within 3 days.

The type III O-PS and type IV O-PS genes are absent in *B. mallei* but partially present in *B. thailandensis*, which may reflect the different lifestyles of these three related species. The *B. pseudomallei* K96243 genome comprises two chromosomes of 4.07 and 3.17 Mb in size (Holden *et al.*, 2004). The gene clusters encoding the LPS and capsular polysaccharides are present on chromosome 1. The genes on chromosome 1 are generally associated with cell growth and metabolism. Chromosome 2 is thought to encode functions associated with adaptation; both of the gene clusters which encode the putative type III O-PS and type IV O-PS are present on chromosome 2.

Fig. 3. Surface hydrophobicity of *B. pseudomallei* strain K96243 wild-type (WT) and the polysaccharide mutant strains. An ANOVA table with Bonferroni's multiple comparison test showed that the difference between *B. pseudomallei* wild-type and the capsule mutant strain was significant ($P<0.001$).

Table 2. Survival of BALB/c mice, vaccinated with heat-killed strains and subsequently challenged with 6.05×10^4 c.f.u. wild-type *B. pseudomallei*

Vaccination	Polysaccharide cluster disrupted	Survivors (%)	MTTD (days)
<i>B. pseudomallei</i> K96243	None	40	26.6
<i>B. pseudomallei</i> K96243 Δ wcbH::kan	Type I O-PS (capsule)	70	32.5
<i>B. pseudomallei</i> K96243 Δ wbiA::kan	Type II O-PS (LPS)	80	33
<i>B. pseudomallei</i> K96243 Δ BPSS0421::kan	Type III O-PS	50	29.7
<i>B. pseudomallei</i> K96243 Δ BPSS1833::kan	Type IV O-PS	50	28.3
PBS control		10	9.2

Only the capsular polysaccharide affects the hydrophobic surface of *B. pseudomallei*

The ability of the polysaccharides to modify the surface hydrophobicity of *B. pseudomallei* was investigated using the BAH method, based on the degree to which bacterial cells can partition between water (aqueous phase) and hexadecane (organic phase) (Fig. 3). Only the capsule mutant strain *B. pseudomallei* K96243 Δ wcbH::kan showed a different hydrophobicity to that of the wild-type strain. There are reports of a link between the surface hydrophobicity of Gram-negative and Gram-positive bacteria and the potential to cause disease (Vercauteren *et al.*, 1993; Ofek, 1994). It is thought that a hydrophobic cell surface may be an advantage to pathogenic bacteria *in vivo* by promoting interactions with host cells. *B. thailandensis* does not produce capsular polysaccharide, and it has been reported that the ability of *B. pseudomallei* to adhere to and invade human epithelial cells is significantly higher than that of *B. thailandensis* (Ong *et al.*, 2004; Kespichayawattana *et al.*, 2004; Brett *et al.*, 1998).

Immunization with the LPS- or capsule-negative strains results in the highest levels of protection against wild-type *B. pseudomallei*

The relative level of protection contributed by each polysaccharide was evaluated using heat-killed whole cells. Heat-inactivated wild-type or mutant cells were used to immunize groups of 10 mice three times at 2-weekly intervals, followed by i.p. challenge with $10 \times$ MLD of wild-type *B. pseudomallei* K96243, and the animals were monitored for 35 days (Table 2). Nine of 10 control mice challenged with wild-type bacteria died, and the MTTD of these animals was 9.2 days. One mouse survived to day 35 but demonstrated signs of disease. The MTTD of mice in the immunized groups was increased approximately three-fold. There was 40 % survival in the group vaccinated with wild-type *B. pseudomallei*, and groups immunized with the mutant strains *B. pseudomallei* K96243 Δ BPSS0421::kan and *B. pseudomallei* K96243 Δ BPSS1833::kan also showed similar survival of 50 % in each group. Interestingly, groups vaccinated with the mutant strains *B. pseudomallei* K96243 Δ wbiA::kan and *B. pseudomallei* K96243 Δ wcbH::kan had higher levels of survival with 80 and

70 %, respectively, of mice still remaining alive at day 35. All the survivors in the vaccinated groups demonstrated no signs of disease. The observed increase in protection levels could be due to the exposure of other protective antigens on the bacterial cell surface, or increased expression of the remaining polysaccharides.

We aimed to investigate the roles of these polysaccharides in protection using heat-killed cells. Heat-killed cells have previously been shown to offer protection against a *B. pseudomallei* challenge by the i.p. route (Razak, 1986). The differences in the levels of protection afforded by each group may be an indication of the relative importance of each polysaccharide in protection. Surprisingly, although the capsule and LPS have previously been shown to be protective antigens, immunization with heat-killed cells with mutations in the type I O-PS or type II O-PS gene clusters provides enhanced protection compared to that with heat-killed wild-type cells (Nelson *et al.*, 2004). This could reflect the exposure of other protective surface antigens due to removal of the highly abundant polysaccharide, or it may be due to upregulation of one or more of the remaining polysaccharides. This finding merits additional investigation.

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