Structural and Biological Diversity of Lipopolysaccharides from *Burkholderia pseudomallei* and *Burkholderia thailandensis* 

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*Burkholderia pseudomallei*, the etiological agent of melioidosis, is a facultative intracellular pathogen. As *B. pseudomallei* is a gram-negative bacterium, its outer membrane contains lipopolysaccharide (LPS) molecules, which have been shown to have low-level immunological activities in vitro. In this study, the biological activities of *B. pseudomallei* LPS were compared to those of *Burkholderia thailandensis* LPS, and it was found that both murine and human macrophages produced levels of tumor necrosis factor alpha, interleukin-6 (IL-6), and IL-10 in response to *B. pseudomallei* LPS that were lower than those in response to *B. thailandensis* LPS in vitro. In order to elucidate the molecular mechanisms underlying the low-level immunological activities of *B. pseudomallei* LPS, its lipid A moiety was characterized using mass spectrometry. The major lipid A species identified in *B. pseudomallei* consists of a biphosphorylated disaccharide backbone, which is modified with 4-amino-4-deoxy-arabinose (Ara4N) at both phosphates and penta-acylated with fatty acids (FA) C14:0(3-OH), C16:0(3-OH), and either C14:0 or C14:0(2-OH). In contrast, the major lipid A species identified in *B. thailandensis* was a mixture of tetra- and penta-acylated structures with differing amounts of Ara4N and FA C14:0(3-OH). Lipid A species acylated with FA C14:0(2-OH) were unique to *B. pseudomallei* and not found in *B. thailandensis*. Our data thus indicate that *B. pseudomallei* synthesizes lipid A species with long-chain FA C14:0(2-OH) and Ara4N-modified phosphate groups, allowing it to evade innate immune recognition.

*B. pseudomallei* is the etiological agent of melioidosis, a bacterial disease endemic in certain tropical regions, especially in Southeast Asia and northern Australia (9, 11, 12, 13), but with an expanding geographical distribution (10, 23, 43). Infection results in a spectrum of clinical syndromes, ranging from chronic abscesses to acute sepsis (28). Despite the availability of intensive treatment with appropriate antibiotics (57), the fatality rates in countries in which the disease is endemic remain high and recurrence of infection is common (27). In Singapore, the mortality rates average 23.7% (33), although this rate can be as high as 46.5% (30, 34).

Lipopolysaccharide (LPS) is an outer membrane molecule of gram-negative bacteria and is the most common bacterial component that is implicated in initiating sepsis (3). Structurally, LPS is composed of an outer O-antigen-specific polysaccharide and an inner core oligosaccharide that is covalently linked to a lipophilic moiety termed lipid A. Lipid A has been described as being responsible for the endotoxic activity associated with LPS (32, 42). Recognition of LPS by the innate immune system triggers the production of proinflammatory cytokines by host cells, which aids in the clearance of the pathogen (56). However, overstimulation of host cells by LPS can lead to sepsis (29). Sepsis is a major cause of death in patients with melioidosis, which accounts for almost 20% of all community-acquired septicemias in northeastern Thailand (7). The LPS of *B. pseudomallei* has been implicated in its pathogenesis, as high concentrations of antibodies to LPS are associated with improved survival in severe melioidosis (8, 21). The use of LPSs as subunit vaccines was protective in a murine model of experimental melioidosis (38).

Despite its apparent role in sepsis, the LPS of *B. pseudomallei* has been shown to have low-level macrophage-activating activity in vitro, which was attributed to a delay in nitric oxide and tumor necrosis factor alpha (TNF-α) production (31, 51, 52), thus enabling the pathogen to evade macrophage killing. As lipid A is the endotoxic center of LPS (32), elucidation of the primary structure of lipid A may shed light on the molecular basis of the low-level immunological activities associated with *B. pseudomallei* LPS (31, 44, 51). In this report, the ability of LPS from *B. pseudomallei* to activate macrophages was compared to this ability of LPS from *Burkholderia thailandensis*, a close relative of *B. pseudomallei* that rarely causes disease in humans (16, 47). In addition, by using a combination of chemical and mass-spectrometric methods, the structures of lipid A from the two pathogens were compared. Collectively, our results provide insight into the mechanisms of *B. pseudomallei* virulence.

**MATERIALS AND METHODS**

Bacterial strains and culture conditions. *B. pseudomallei* strain KHW, obtained from the *B. pseudomallei* collection at the Defense Medical and Environmental Research Institute, DSO National Laboratories (Singapore), was isolated.
from a national serviceman who died of melioidosis in 1989. *B. thailandensis* (ATCC 700388) was obtained from the American Type Culture Collection. Both were grown on N-minimal medium (5 mM KCl, 0.5 mM K$_2$SO$_4$, 1 mM KH$_2$PO$_4$, 1.8 μM FeSO$_4$ \cdot 7H$_2$O, 2 μg/ml thiamine-HCl, 0.1 M Tris-HCl, pH 7.4, 22 mM glucose) (37). The basal N-minimal medium was supplemented with 10 mM MgSO$_4$.

To assess the LPS purification procedures, *B. pseudomallei* strain K926243 and a K926243 mutant strain (SB04/3518) (kindly provided by P. Atkins, Defence Science and Technology Laboratory, United Kingdom) were included as controls in this study. The mutant strain has been confirmed to be of a capsular polysaccharide 1 (CPS I)-negative phenotype (Timothy P. Atkins, personal communication).

**Isolation of LPS.** LPS was extracted using an LPS extraction kit (Intron Biotechnology, Korea) with some modifications. Briefly, cells were harvested and lysed in lysis buffer (50 mg of cells/ml of lysis buffer) and then subjected to a vigorous vortex to dissolve cell clumps. After the addition of chloroform, the sample was centrifuged for 45 min at 4°C. The upper aqueous layer was collected, and 2 volumes of purification buffer were added to 1 volume of aqueous layer. The mixture was then incubated at ~20°C for 2 h, before centrifugation at 20,000 x g for 10 min at 4°C. The resulting pellet was washed twice with 70% ethanol before being lyophilized overnight. Extracted LPS was stored at 4°C. LPS samples were further purified to remove contaminating proteins, nucleic acids, phospholipids, and Toll-like receptor 2 (TLR2) contaminating proteins as described previously (49).

To obtain the whole-cell LPS profile, one full loop of bacterial lawn was harvested from agar plates and 50 μl of solubilization buffer was added to every milliliter of bacteria. The bacterial cells were resuspended by use of a vortex to disperse cell clumps followed by heating at 90°C for 10 min to kill the bacteria. After the suspension was cooled, an equal volume of solubilization buffer containing 3 mg/ml of proteinase K was added and heated at 60°C for 2 h. Proteinase K was inactivated by boiling for 10 min, and the sample was stored at ~20°C until use.

**GC-MS analysis of FA derived from LPS.** Total fatty acids (FA) in the LPS were liberated and derivatized to FA methyl esters by methylation in 2 M methanolic HCl (48) at 90°C for 18 h. An equal volume of saturated NaCl solution was added, and the FA methyl esters were extracted with chloroform. Extracted methyl esters were characterized by gas chromatography-mass spectrometry (GC-MS). Pentadecanoic acid was used as an internal standard. GC-MS analysis was performed on a MAT95XL-T GC-MS spectrometer by using an Rtx-5MS column (30 m by 0.25 mm by 0.25 μm).

**Isolation of lipid A.** Lipid A was isolated from LPS by using modified mild acid hydrolysis (58). Briefly, 1 mg of LPS was dissolved in 500 μl of 1% sodium dodecyl sulfate in 10 mM sodium acetate, pH 4.5, and heated at 100°C for 1 h. The mixture was dried under a vacuum. For the removal of sodium dodecyl sulfate, the sample was resuspended in 100 μl of distilled water and sonicated in an ultrasonic bath for dispersal of the sample. Five hundred microliters of acidified ethanol was added to the suspension and centrifuged at 2,000 × g for 10 min. The pellet was washed twice with 500 μl of 0.1% sodium citrate and lyophilized to give fluffy white, solid lipid A.

**ESI-quadrupole time of flight (QTOF) MS of lipid A.** Electrospray ionization-MS (ESI-MS) was performed on a Micromass Q-ToF micro-mass spectrometer (Waters Corp., Milford, MA). The capillary voltage and sample cone voltage were maintained at 3.0 kV and 55 V, respectively. The source temperature was 80°C, and the desolvation temperature was set at 250°C. Mass spectra were acquired in the negative-ion mode. Isolated lipid A was resuspended in 1:1 (vol/vol) chloroform-methanol containing 3% of 300 mM piperidine as an ion signal enhancer (45). The sample was directly infused into the mass spectrometer at a flow rate of 10 μl/min.

Negative ESI-tandem MS (MS-MS) analyses were carried out under similar conditions as described above except for collision energies. Most of the major ion peaks, including ions at m/z of 965.60, 900.08, 852.52, and 786.99, were further investigated using MS-MS, either through ESI-MS-MS or high-performance liquid chromatography-ESI-MS-MS, with collision energies ranging from 35 to 80 V. Argon was used as the collision gas.

**In vitro biological assays.** Mouse macrophage cells (RAW 264.7 cells) were seeded in 24-well plates at a density of 2.5 × 10⁵ cells/well and stimulated with increasing concentrations of LPS from *Escherichia coli* strain 055:B5 (Sigma, Singapore), *B. pseudomallei* strains KHW, K926243, and SB04/3518, and *B. thailandensis* strain ATCC 700388. The cell culture supernatant was collected at various time points and was analyzed for TNF-α, interleukin-6 (IL-6), and IL-10 release by using the Quantikine enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Results given are from 18 h poststimulation.

**RESULTS**

LPs from *B. pseudomallei* and *B. thailandensis* are differentially recognized. To determine whether antigen-presenting cells could differentially recognize *B. pseudomallei* LPS from *B. thailandensis* LPS, murine macrophage cell line RAW 264.7 was exposed to increasing concentrations of LPS and the supernatant was analyzed for TNF-α, IL-6, and IL-10 (Fig. 1). RAW 264.7 cells produced less TNF-α in response to *B. pseudomallei* LPS than they did in response to *B. thailandensis* LPS at concentrations above 1 ng/ml (*P < 0.05). LPS from *E. coli* strain 055:B5, a potent stimulator of macrophage cells, was used as a control. LPs from both *B. thailandensis* (*P < 0.05*) and *B. pseudomallei* (*P < 0.05*) were less potent in stimulating the murine macrophages than *E. coli* LPS. The patterns of IL-6 and IL-10 production were comparable to the pattern of TNF-α production. Overall, LPS from *B. pseudomallei* was approximately two times less potent in stimulating cytokine release in RAW 264.7 cells than *B. thailandensis* LPS.

**THP-1 human monocytes, which were differentiated into macrophage-like cells, were stimulated with LPS from *B. pseudomallei* or *B. thailandensis* to determine if the differences in murine macrophage activation would be similarly observed in human macrophages. Differentiated THP-1 cells produced lower levels of TNF-α, IL-6, and IL-10 in response to *B. pseudomallei* LPS than they did in response to LPs from both *B. thailandensis* (*P < 0.05*) and *E. coli* (*P < 0.05*) (Fig. 2). In contrast to the case for IL-6, *E. coli* LPS and *B. thailandensis* LPS induced similar levels of TNF-α and IL-10 release from the human macrophage-like cells.**
I-negative mutant, SB04/3518. No significant difference between the abilities of the two LPSs to induce TNF-α (Fig. 3), IL-6, and IL-10 (data not shown) production from THP-1 and RAW 264.7 cells was observed, indicating that the amount of LPS in the wild-type strain of K96243 was similar to that of the CPS I-negative strain, SB04/3518, prepared using the purification procedures. B. thailandensis LPS was similarly found to be a more potent LPS than K96243 and SB04/3518 LPSs (P < 0.05). Collectively, the results indicate that both murine and human macrophages respond differently to B. pseudomallei LPS relative to B. thailandensis LPS.

LPSs from B. pseudomallei and B. thailandensis are TLR4 agonists. In order to determine the receptor specificity of B. pseudomallei LPS, HEK293 cells expressing either hTLR2-CD14 or hTLR4-MD2-CD14 were stimulated with LPS and the level of NF-κB activation was determined using a NF-κB-dependent luciferase reporter (Fig. 4). TLR4-dependent activation of NF-κB was identified in response to B. pseudomallei LPS (P < 0.001). LPS from B. thailandensis similarly elicited a robust TLR4-dependent activation of NF-κB (P = 2.8 × 10⁻⁴). The level of NF-κB induced by B. pseudomallei LPS was significantly lower than that induced by B. thailandensis LPS (P = 0.007). TLR2-dependent activation of NF-κB was not observed when the TLR2-transfected cells were stimulated with LPS from either B. pseudomallei or B. thailandensis. These results indicate that both B. pseudomallei and B. thailandensis LPSs specifically activate the TLR4 complex but not TLR2.

Compositional analysis of lipid A. To investigate if differences in lipid A structure may contribute to the differential recognition by RAW 264.7 cells, the LPSs from B. pseudomallei and B. thailandensis were hydrolyzed and their lipid A moieties were subjected to a combination of structural analyses. FA analysis of B. pseudomallei LPS revealed the presence of tetradecanoic acid (C₁₄:0), 2-hydroxytetradecanoic [C₁₄:0(2-OH)], 3-hydroxytetradecanoic acid [C₁₄:0(3-OH)], hexadecanoic acid (C₁₆:0), and 3-hydroxyhexadecanoic acid [C₁₆:0(3-OH)] in its lipid A (data not shown). The LPS of B. thailandensis contained all the above FA with the exception of 2-hydroxytetradecanoic acid [C₁₄:0(2-OH)].

To further characterize the differences detected by FA compositional analysis and to investigate the heterogeneity of purified lipid A from B. pseudomallei and B. thailandensis, lipid A was analyzed by ESI-QTOF MS. Isolated lipid A was successfully detected by using piperidine as a signal enhancer. The negative-ion ESI-QTOF mass spectra of purified lipid A from B. pseudomallei revealed a heterogeneous mixture with major doubly charged ions at m/z of 973.59 and 965.60 (Fig. 5A), indicative of penta-acylated species that were most likely...
diphosphorylated. Minor peaks of doubly charged ions at m/z of 908.07 and 900.08 were detected as well. The mass differences of 131 units between doubly charged ions at m/z of 965.60 and 900.08 and between ions at m/z of 973.59 and 908.07 indicate a likely difference of a 4-amino-4-deoxy-arabinose (Ara4N) residue for the corresponding lipid A pairs (5, 35, 46). The doubly charged ion at an m/z of 973.59 possessed one more hydroxyl (—OH) group than the corresponding lipid A with a doubly charged ion at an m/z of 965.60. Based on the accurate masses of the doubly charged species, the ions at m/z of 973.59, 965.60, 908.07, and 900.08 were assigned to lipid A species with molecular formulae of C_{96}H_{182}N_{4}O_{31}P_{2}, C_{96}H_{182}N_{4}O_{30}P_{2}, C_{91}H_{173}N_{3}O_{28}P_{2}, and C_{91}H_{173}N_{3}O_{27}P_{2}, respectively, based on the accurate masses of the doubly charged species.

The negative-ion ESI-QTOF mass spectra of purified lipid A from *B. thailandensis* revealed a complex pattern as well (Fig. 5B). In addition to the doubly charged ions at m/z of 965.60 and 900.08 detected in *B. pseudomallei*, the mass spectra of *B. thailandensis* showed major doubly charged ions at m/z of 852.52 and 786.99 and a minor doubly charged ion at an m/z of 834.55 (not labeled), which was indicative of a heterogeneous mixture of penta- and tetra-acylated species. The lipid A species represented by the doubly charged ion at an m/z of 973.59 was unique to *B. pseudomallei* and was not detected in *B. thailandensis*. The mass difference of 226 units between doubly charged ions at m/z of 965.60 and 852.52 and between ions at m/z of 900.08 and 786.99 could likely be attributed to a difference of one FA C_{14:0}(—OH) for the corresponding lipid A pairs. The doubly charged ions at m/z of 834.55 and 786.99 represent the sequential loss of Ara4N (fragmen B) from the doubly charged parental ion.

**Fig. 4.** hTLR4, but not hTLR2, confers responsiveness to LPSs from *B. pseudomallei* and *B. thailandensis*. HEK293 cells stably transfected with either hTLR2-CD14 complex (A) or hTLR4-MD2-CD14 complex (B) were stimulated with *Staphylococcus aureus* lipoteichoic acid (LTA), *E. coli* LPS (EC), *B. pseudomallei* strain KHW LPS (BP), or *B. thailandensis* strain ATCC 700388 LPS (BT) for 7 h. Control cells were stimulated with media alone. The concentration of LTA used was 1,000 ng/ml. EC, BP, and BT were used at 1,000 ng/ml in the TLR2-transfected cells and at 10 ng/ml in the TLR4-transfected cells. NF-κB activation was determined using pNifty2-luc and pRL-TK. NF-κB-directed firefly luciferase expression was normalized to thymidine kinase-directed constitutive renilla luciferase expression and expressed as relative light units. * indicates a P value of <0.05 and § indicates a P value of <0.01 in comparison to cells stimulated with media alone. Data represent the means ± standard errors of the means for triplicate samples. Results shown are representative of two independent experiments.

**Fig. 3.** Comparison of stimulatory activities of LPSs extracted from the wild-type strain and the CPS I-negative strain of *B. pseudomallei*, RAW 264.7 (A) and predifferentiated THP-1 (B) cells were stimulated with the indicated concentrations of LPS from *B. pseudomallei* strain KHW, K96243, or SB04/3518 or *B. thailandensis* strain ATCC 700388 for 24 h. The cell culture supernatant was measured for TNF-α. Results shown are representative of two independent experiments.
at an m/z of 965.60 (Fig. 6A). The subsequent loss of ions with m/z of 131 and 80 indicated that the Ara4N residue was attached through a phosphate group (35). Losses of phosphorylated Ara4N (fragment C) were commonly observed among parental ions at m/z of 965.60, 900.08, 852.52, and 786.99 (Fig. 6A to D). The fragmented ion at an m/z of 210.21 (Fig. 6A) refers to a loss of one water molecule (H2O) from phosphorylated Ara4N (fragment A). The doubly charged daughter ions at m/z of 852.48 and 739.5 (not labeled) arose from the sequential loss of FAC14:0(3-OH) from the doubly charged parental ion at an m/z of 965.60 (Fig. 6A). Similar results were observed in the sequential MS-MS pattern of doubly charged parental ion from m/z of 900.08 to 786.99 and subsequently to 673.93 (not labeled) (Fig. 6B). This indicates that FA C14:0(3-OH) is the major acyl chain attached to the disaccharide backbone. The presence of fragmented ion at an m/z of 243.31 further confirmed the presence of FA C14:0(3-OH) in the lipid A structure (Fig. 6A).

Using the information above, we hypothesized the structure for the major lipid A fraction at an m/z of 973.59, which was present only in B. pseudomallei (Fig. 7). This peak was consistent with a lipid A structure of a biphosphorylated disaccharide backbone modified with Ara4N at both phosphate groups and penta-acylated with a combination of FA C14:0(2-OH), FA C14:0(3-OH), and FA C16:0(3-OH). The substitution of FA in the lipid A backbone was not determined in this study and is not represented in any particular order in our hypothesised structure, as further nuclear magnetic resonance studies will be needed to confirm the positions of these FA. In contrast, the major lipid A species from B. thailandensis with a doubly charged ion at an m/z of 965.60 is likely to have the same structure as the ion at an m/z of 973.59 but with the substitution of C14:0 for FA C14:0(2-OH). This is consistent with the FA compositional analysis, which revealed the presence of C14:0(2-OH) only in the lipid A of B. pseudomallei.

Likewise, the lipid A with an m/z of 900.08, which was present in both B. pseudomallei (minor species) and B. thailandensis (major species), was assigned to be a penta-acylated lipid A with only one Ara4N group. The other lipid A species present in only B. thailandensis were assigned as follows: lipid A with an m/z of 852.52 is a tetra-acylated lipid A containing two Ara4N groups; lipid A with an m/z of 786.99 is a tetra-acylated lipid A containing one Ara4N group; and lipid A with an m/z of 834.55 is a penta-acylated lipid A containing no Ara4N group.

In addition to FA C14:0(3-OH), FA C14:0 and FA C16:0(3-OH), depicted in our hypothesised structure, fatty acyls C16:0 and C18:0 were also identified in the MS-MS spectra of the lipid A species (not labeled for clarity), signifying the existence of other structural combinations with different fatty acyl numbers.

**DISCUSSION**

B. pseudomallei is a facultative intracellular pathogen that stimulates macrophages weakly, enabling the pathogen to evade macrophage killing (1, 31, 51). It has been postulated that unique features of B. pseudomallei LPS may contribute to the low-level immunological activities and thus facilitate the intracellular survival of the pathogen (51). However, the structure of B. pseudomallei LPS, specifically its lipid A, has not been determined until now. Structural information for B. pseudomallei lipid A, in comparison with that of the closely related but barely pathogenic B. thailandensis, may be an es-
sential component in the overall understanding of the molecular mechanisms underlying pathogenesis.

In this study, we observed that the LPS of *B. pseudomallei* stimulated both human and murine macrophages to produce levels of TNF-α, IL-6, and IL-10 lower than those in response to the LPS of *B. thailandensis* in vitro. In order to dissect potential molecular features contributing to this difference, the lipid A structure of *B. pseudomallei* LPS was characterized and compared to that of *B. thailandensis* LPS. The major lipid A species in *B. pseudomallei* consists of a biphosphorylated disaccharide backbone, which was modified with Ara4N at both phosphate groups and penta-acylated with FA C14:0(3-OH), FA C16:0(3-OH), and either FA C14:0 or FA C14:0(2-OH). On the contrary, the major lipid A species identified in *B. thailandensis* was a heterogeneous mixture of penta- and tetra-acylated structures varying in Ara4N substitutions and acylation of C14:0(3-OH). In addition, the substitution of FA C14:0(2-OH) into the lipid A backbone was unique to *B. pseudomallei* and was not found in *B. thailandensis*.

Modification of LPS is a strategy used by various gram-negative bacteria to evade antibacterial mechanisms initiated by the host innate immune system (36). The endotoxic activity of lipid A varies strongly with its primary structure, namely, the FA, polar head groups, and carbohydrate components that constitute it (44). The hexa-acylated lipid A of *Escherichia coli*, *Neisseria meningitidis*, and *Vibrio cholerae* with side chains of 12 to 14 carbons represents the most biologically active form of the molecule (6, 61). Any deviation from this structure, such as a difference in the number or length of fatty acyl chains, will reduce the magnitude of the signal (2, 6, 40, 41). The lipid A structures of closely related species *Burkholderia cepacia* and *Burkholderia mallei* have recently been characterized (5, 46). Similar to our data for *B. thailandensis*, *B. cepacia* and *B. mallei* contain a mixture of tetra- and penta-acylated lipid A struc-
Pathogenic bacteria also evade the immune system through capping phosphate groups at the terminal ends of lipid A with Ara4N and phosphoethanolamine. These modifications confer resistance to the bactericidal effects of endogenously produced host cationic antimicrobial peptides (CAMPs) (14, 17, 50). In Salmonella serovar Typhimurium, the terminal phosphate groups of lipid A are modified with Ara4N residues when grown under magnesium-deficient conditions (18, 59). This decreases the overall negative charge on the pathogen’s cell surface and thus lowers the affinity for CAMPs and cationic antibiotics (25). In vitro, B. pseudomallei has been shown to be resistant to the cationic peptides protamine sulfate and purified human defensin HNP-1 (24). In support of this, our structural analysis revealed that both phosphate groups in the major lipid A species identified in B. pseudomallei are capped with Ara4N residues. Modification with Ara4N may increase the resistance of B. pseudomallei to CAMPs and allow the bacterium to survive and replicate within host cells.

In a study that compared the cellular FA profiles of B. pseudomallei and B. thailandensis, the two pathogens were described as sharing the same FA profile, with the exception of FA C_{14:0}(2-OH), which was detected only in B. pseudomallei (22). When comparing the lipid A structures of the two species, we similarly observed the presence of FA C_{14:0}(2-OH) only in the lipid A of B. pseudomallei. FA C_{14:0}(2-OH) appears to be unique to B. pseudomallei, as lipid A species of other closely related species, such as B. cepacia and B. mallei, do not synthesize this FA (5, 46). Based on these findings, the major doubly charged ion at an m/z of 973.59 in B. pseudomallei, which differs by the presence of one —OH group from the corresponding lipid A at an m/z of 965.60 present in both B. pseudomallei and B. thailandensis, was attributed to the substitution of FA C_{14:0} for FA C_{14:0}(2-OH). In Salmonella serovar Typhimurium, hydroxylation of FA C_{14:0} to FA C_{14:0}(2-OH) occurs in response to stimuli from host microenvironments, such as the reduced level of magnesium in the phagosome. This modifies the lipid A and has been described to confer resistance to CAMPs (18), permitting a prolonged survival of the bacteria inside the host cell (15). Our data thus indicate that the FA C_{14:0}(2-OH) in B. pseudomallei may enable the bacterium to subvert host cellular responses and survive within the host cell.

LPS is recognized by the innate immune system through interactions with the TLR4 complex present on immune cells (61). However, some LPSs have been shown to activate immune cells via TLR2 instead (4, 20). In this study, we observed that the LPSs from both B. pseudomallei and B. thailandensis specifically activated hTLR4. West and coworkers similarly characterized B. pseudomallei LPS as a TLR4 ligand (53). These findings are contrary to the results of Wiersinga and coworkers, who described B. pseudomallei LPS as acting as a TLR2 agonist (55). The discrepancy observed could be due to the presence of contaminants in LPS, which activate TLR2. A recent study has demonstrated that the ability of some LPSs to activate TLR2 is attributed to the presence of lipoproteins in the preparations (26). B. pseudomallei LPS is typically extracted using the modified hot aqueous-phenol extraction method (39, 55). However, in the case of B. pseudomallei, the LPS is closely associated with the proteins and partitions into the phenol phase together with the proteins (39). This isolation may result in an LPS preparation which is contaminated with proteins (49). Extensive purification of the LPS fraction is therefore required to remove the contaminating proteins, especially for biological assays in which the presence of contaminants may confound results (19). In accordance with these studies, we also observed that B. pseudomallei LPS extracted using the traditional method potently activates both TLR2 and TLR4. Thus, extensive purification of LPS is required to re-

FIG. 7. Hypothetical structure of the major lipid A species present in B. pseudomallei. The lipid A species at an m/z of 965.60 present in both B. pseudomallei strain KHW and B. thailandensis strain ATCC 700388 do not contain the unique 2-OH group indicated by the arrow. The substitution of FA in the lipid A backbone was not determined in this study and is not represented in any particular order in our proposed structure. The assignment of fragments A to C is as described in the text and the legend to Fig. 6.
move contaminating proteins and to eliminate recognition by TLR2.

Multiple factors probably contribute to the pathogenesis of melioidosis, with the LPS of the bacterium being one of the important factors (1, 51, 54). Based on the structural data determined in this study, it appears that the lipid A of B. pseudomallei, with its different fatty acyl chains, induces weak immunological activities and thus evades early host defenses. The presence of Ara4N-modified phosphate groups and C14:0 (2-OH) in the lipid A may confer resistance to the effects of CAMPS and allow the pathogen to survive intracellularly. In contrast, the more potent LPS synthesized by B. thailandensis may activate the innate immune system more strongly. Consequently, B. thailandensis becomes more susceptible to the bactericidal effects of host innate immune responses, resulting in efficient clearance of the pathogen. The significance of these modifications has to be determined in vivo in order to fully comprehend the role of LPS in the pathogenesis of B. pseudomallei. Further studies to determine the complete structure of lipid A and the relevance of the minor lipid A species in B. pseudomallei to establish a specific structure-function relationship are ongoing.

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