

Molecular and Physical Characterization of *Burkholderia mallei* O Antigens

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***Burkholderia mallei* lipopolysaccharide (LPS) has been previously shown to cross-react with polyclonal antibodies raised against *B. pseudomallei* LPS; however, we observed that *B. mallei* LPS does not react with a monoclonal antibody (Pp-PS-W) specific for *B. pseudomallei* O polysaccharide (O-PS). In this study, we identified the O-PS biosynthetic gene cluster from *B. mallei* ATCC 23344 and subsequently characterized the molecular structure of the O-PS produced by this organism.**

Burkholderia mallei is a gram-negative bacterium responsible for a disease known as glanders in solipeds and occasionally in humans (3, 8, 13). The factors involved in the pathogenesis of *B. mallei* infection remain relatively poorly defined at the molecular level. A previous study that identified a polysaccharide gene cluster in *B. mallei* showed that *B. mallei* lipopolysaccharide (LPS) cross-reacts with polyclonal antibodies raised against the LPS of *Burkholderia pseudomallei*, a closely related organism responsible for a disease known as melioidosis (6). In the present study, we investigated the LPS profiles of *B. mallei* strains, identified the gene cluster responsible for O polysaccharide (O-PS) biosynthesis in *B. mallei* ATCC 23344, and determined the physical structure of the *B. mallei* ATCC 23344 O-PS. Additionally, we showed that the O-PS moiety of *B. mallei* LPS is required for resistance to the bactericidal action of serum. Finally, we identified the presence of insertion sequences in two strains of *B. mallei* that disrupt the expression of O-PS.

Analysis of LPS profiles of *B. mallei* strains. The strains and plasmids used in this study are shown in Table 1. The first goal of this study was to assess the LPS profiles of *B. mallei* strains. Initially, we performed Western blot analysis of *B. mallei* ATCC 23344 whole-cell lysates with polyclonal rabbit sera raised against a *B. pseudomallei* bovine serum albumin (BSA)–O-PS conjugate as well as with a *B. pseudomallei* O-PS-specific MAb (Pp-PS-W) according to a previously described protocol (1, 2). As shown in Fig. 1A, *B. mallei* ATCC 23344 reacted with the anti-LPS polyclonal sera, resulting in a typical LPS banding pattern; however, the *B. pseudomallei* O-PS-specific MAb (Pp-PS-W) did not react. This indicated that differences exist between *B. mallei* and *B. pseudomallei* O-PS. We further assessed the LPS profiles of 10 different *B. mallei* strains (Fig. 1B). By using Western blot analysis, we showed that 8 of the 10 strains assessed bound the anti-LPS polyclonal sera and displayed typical LPS banding patterns. In contrast, however, two strains, NCTC 120 and ATCC 15310, did not bind

the anti-LPS polyclonal sera, as indicated by the absence of bands (Fig. 1B). In order to confirm that the O-PS moiety was absent rather than a different type of O-PS, silver stain analysis was employed. Figure 1C shows the silver stain results confirming that both of these strains lacked O-PS moieties.

Identification and characterization of *B. mallei* ATCC 23344 O-PS biosynthetic gene cluster. In order to investigate the genes responsible for O-PS biosynthesis in *B. mallei*, we constructed a cosmid library by using *B. mallei* ATCC 23344 genomic DNA and the cosmid pScosBC1 by using a previously described protocol (12). Colony hybridizations were then performed with a 1.1-kb DNA fragment containing the recently identified *B. mallei wbiA* gene (P. Brett, M. Burtneck, and D. Woods, unpublished data). Six positive cosmid clones were obtained. Based on the *Bam*HI-*Kpn*I restriction patterns obtained, two cosmid clones, 1C3 and 2B5, were predicted to harbor the entire *B. mallei* O-PS gene cluster. Sequence analysis resulted in 19,918 bp of contiguous sequence containing the entire *B. mallei* O-PS biosynthetic gene cluster with an IS407-like insertion sequence element at the 3' end.

The first 18,738 bp of the *B. mallei* DNA sequence contained 16 predicted ORFs that were identical to those previously defined as the O-PS biosynthetic gene cluster in *B. pseudomallei* (Fig. 2) (5). Sequence alignment of the *B. pseudomallei* and *B. mallei* O-PS biosynthetic regions revealed 99% identity at the nucleotide level. The genes comprising the *B. mallei* O-PS biosynthetic operon were named as per the identical genes found in *B. pseudomallei* (5).

Physical characterization of *B. mallei* O-PS moieties. In order to structurally analyze the *B. mallei* O-PS structure, it was necessary to construct a *B. mallei* strain unable to produce capsular polysaccharide (CPS), because CPS copurifies with LPS. The suicide vector pGSV3008 was employed as previously described to construct *B. mallei* PB100, a derivative of ATCC 23344 that does not produce CPS (6). The O-PS was purified as previously described for *B. pseudomallei*. Figure 3 shows ¹³C nuclear magnetic resonance (¹³C-NMR) analysis (Complex Carbohydrate Research Center, University of Georgia, Athens) results demonstrating that the *B. mallei* O-PS

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TABLE 1. Bacterial strains and cosmids or plasmids used in this study

Strain, cosmid, or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>E. coli</i>		
SM10	Mobilizing strain: transfer genes of RP4 integrated into the chromosome; Km ^r Sm ^s	11
TOP10	High-efficiency transformation strain with blue/white screening; Ap ^r Km ^r	Invitrogen
HB101	Serum-sensitive strain	7
<i>B. mallei</i>		
NCTC 120		USAMRIID ^a
NCTC 10248	Human isolate	USAMRIID
NCTC 10229		USAMRIID
NCTC 10260	Human isolate	USAMRIID
NCTC 10247		USAMRIID
ATCC 23344	Human isolate; Pm ^r Gm ^s	USAMRIID
NCTC 3708	Mule isolate	USAMRIID
NCTC 3709	Horse isolate	USAMRIID
ATCC 10399		USAMRIID
ATCC 15310		USAMRIID
PB100	ATCC 23344::pGSV3008; Pm ^r Gm ^r	This study
<i>B. pseudomallei</i>		
1026b	Clinical isolate; Gm ^r Km ^r Sm ^r Pm ^r Tp ^s	4
Cosmids		
pScosBC1	Broad-host-range cosmid cloning vector based on pSuperCos1; Ap ^r Tp ^r	12
p1C3	pScosBC1 from ATCC 23344 library with a 23-kb fragment containing the O-PS biosynthetic gene cluster	This study
p2B5	pScosBC1 from ATCC 23344 library with a 27-kb fragment containing the O-PS biosynthetic gene cluster	This study
Plasmids		
pUC19	Cloning vector with blue/white selection; Ap ^r	14
pGSV3008	pGSV containing a 379-bp <i>Eco</i> RI fragment from pDD3008, contains internal fragment from the <i>wcbB</i> gene; Gm ^r	6

^a U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md.

backbone is similar to that previously described for *B. pseudomallei* O-PS, a heteropolymer of repeating D-glucose and L-talose (9, 10). However, changes are apparent in the O-acetylation pattern of the *B. mallei* L-talose residue in comparison to that of *B. pseudomallei*. Similar to *B. pseudomallei* O-PS, *B. mallei* O-PS demonstrates the presence of O-acetyl or O-methyl substitutions at the 2' position of the talose residue. In contrast, *B. mallei* O-PS is devoid of an O-acetyl group at the 4' position of the talose residue. Thus, the structure of *B. mallei* O-PS is best described as 3)-β-D-glucopyranose-(1,3)-6-

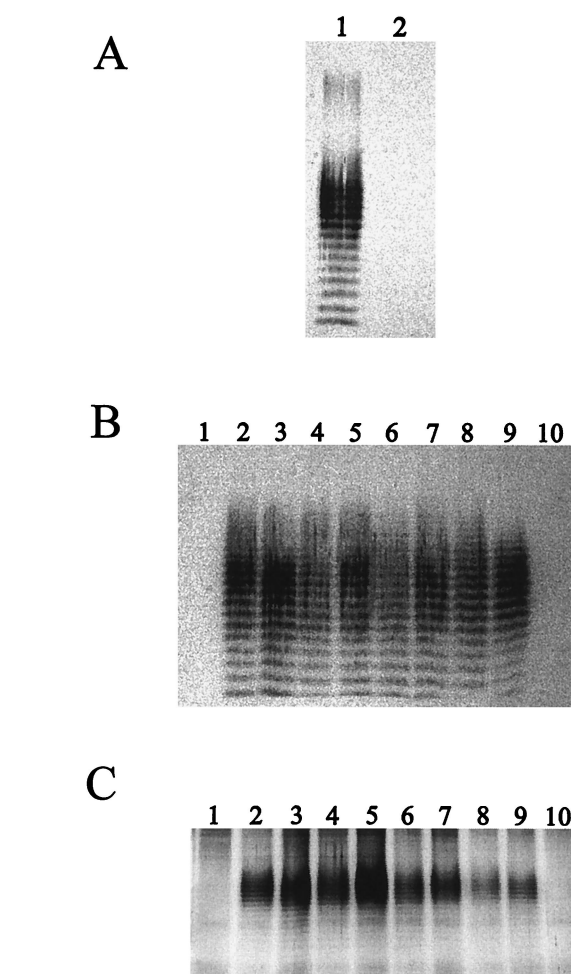


FIG. 1. (A) Western blot analysis of *B. mallei* ATCC 23344. Proteinase K-treated whole-cell lysates were used. In lane 1, the primary antibody used was a 1/2,000 dilution of polyclonal antisera raised against a *B. pseudomallei* BSA-O-PS conjugate, and in lane 2, the primary antibody used was a 1/2,000 dilution of the *B. pseudomallei* O-PS-specific MAb (Pp-PS-W). (B) Western blot profiles of proteinase K-treated whole-cell lysates of *B. mallei* strains. The primary antibody used was polyclonal sera raised against a *B. pseudomallei* BSA-O-PS conjugate. Lanes: 1, NCTC 120; 2, NCTC 10248; 3, NCTC 10229; 4, NCTC 10260; 5, NCTC 10247; 6, ATCC 23344; 7, NCTC 3708; 8, NCTC 3709; 9, ATCC 10399; and 10, ATCC 15310. (C) Silver stain analysis of proteinase K-treated whole-cell lysates of *B. mallei* strains. Lanes: 1, NCTC 120; 2, NCTC 10248; 3, NCTC 10229; 4, NCTC 10260; 5, NCTC 10247; 6, ATCC 23344; 7, NCTC 3708; 8, NCTC 3709; 9, ATCC 10399; and 10, ATCC 15310.

deoxy-α-L-talopyranose-(1-, in which the talose residue contains 2-O-methyl or 2-O-acetyl substituents. Recent studies indicate that the presence of 4-O-acetyl groups on the talose residues of *B. pseudomallei* O-PS is due to an O-acetylation locus unlinked to the previously described O-PS biosynthetic operon (Brett et al., unpublished). If this is the case, then the unlinked locus responsible for O-acetylation is either not present or is nonfunctional in *B. mallei* strains. The presence or absence of O-acetyl groups on the O-PS moieties may have consequences when O-PS is considered as a component of a vaccine that protects against both *B. mallei* and *B. pseudomallei*.

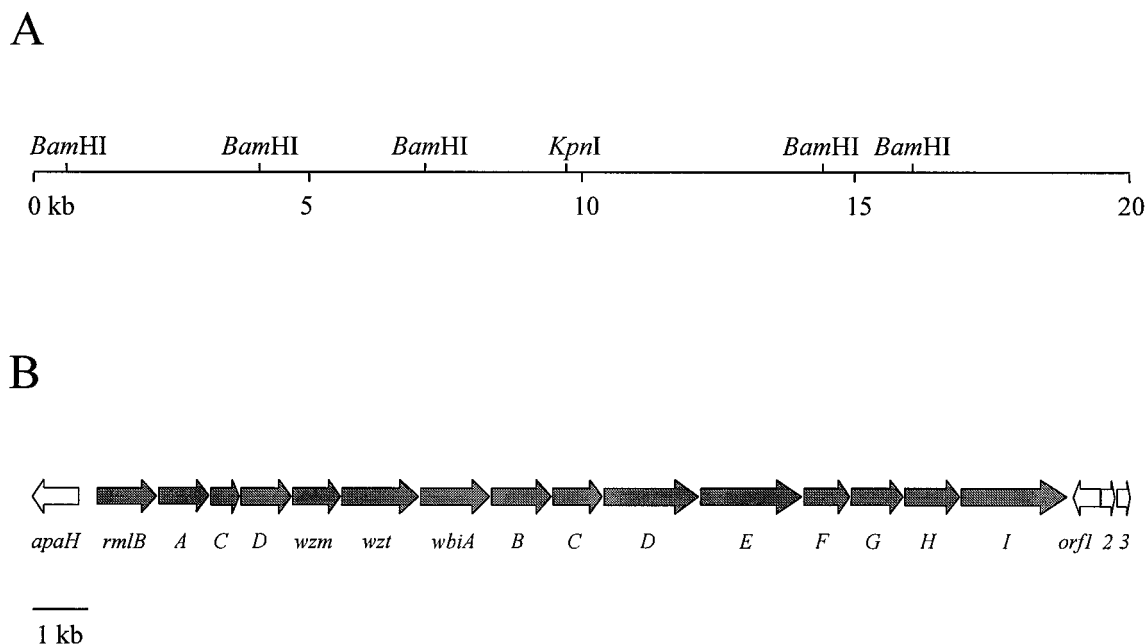


FIG. 2. Restriction and genetic maps of the *B. mallei* O-PS biosynthetic gene cluster. (A) Restriction map. The horizontal line represents the *B. mallei* DNA insert of cosmid 2B5. The locations of *Bam*HI and *Kpn*I cleavage sites used for subcloning are shown. Two additional *Bam*HI sites at the 5' and 3' ends of 2B5, which were part of the pScosBC1 vector, are not shown. (B) Genetic map. The location and direction of transcription of the genes are represented by arrows, and the gene names are shown below. The gray arrows indicate the genes involved in O-PS biosynthesis based on homology to the *B. pseudomallei* O-PS biosynthetic operon.

***B. mallei* survives in 30% NHS, and serum-sensitive strains lack the O-PS moiety of LPS.** The ability of *B. mallei* ATCC 23344 to grow in the presence of 30% normal human serum (NHS) was initially assessed with a serum bactericidal assay (5) in which viable counts were determined at 2, 4, 8, and 18 h. *B. mallei* ATCC 23344 was shown to survive in the presence of 30% NHS over the course of 18 h (Fig. 4A). Serum-resistant *B. pseudomallei* 1026b and serum-sensitive *Escherichia coli* HB101 were employed as controls.

In order to assess the role of *B. mallei* O-PS in serum resistance, NHS bactericidal assays (5) were performed with *B. mallei* ATCC 23344 and *B. mallei* NCTC 120 and ATCC 15310, the two strains lacking O-PS. *B. mallei* ATCC 23344 remained resistant to the killing action of 30% NHS, while NCTC 120 and ATCC 15310 were completely killed following a 2-h incubation in 30% NHS (Fig. 4B). The other seven *B. mallei* strains used in this study possessed intact O-PS moieties and were resistant to the bactericidal action of 30% NHS (data not shown). These results suggested that *B. mallei* O-PS moieties play a crucial role in the serum resistance of this organism: this correlates well with previous studies demonstrating that *B. pseudomallei* O-PS is required for serum resistance (5).

Identification of insertion sequence IS407 in the O-PS biosynthetic gene clusters of *B. mallei* NCTC 120 and ATCC 15310. In order to determine if the O-PS biosynthetic gene clusters of NCTC 120 and ATCC 15310 had been disrupted, we chose to individually PCR amplify each gene present in this cluster. Deoxyoligonucleotide primers were designed outside of the 5' and 3' ends of each gene. *B. mallei* ATCC 23344

chromosomal DNA was used as a control as an indicator of the size of a wild-type copy of each gene. Alterations were observed in the *wbiE* PCR product from NCTC 120 and in the *wbiG* PCR product from ATCC 15310. The PCR products obtained in both cases were approximately 1.5 kb larger than those obtained with ATCC 23344 genomic DNA (data not shown). Cloning and sequence analysis of the NCTC 120 *wbiE* and ATCC 15310 *wbiG* PCR products revealed the presence of insertion sequences within these two genes. In NCTC 120, an IS407-like element was located after nucleotide 13615 of the O-PS operon in the *wbiE* gene. In ATCC 15310, an IS407-like element was located following nucleotide 15107 of the O-PS operon in the *wbiG* gene. It is likely that the presence of insertion elements in the O-PS biosynthetic gene clusters of *B. mallei* NCTC 120 and ATCC 15310 is responsible for the loss of expression of O-PS in these two strains. DeShazer et al. have previously demonstrated the presence of an IS407-like element (termed "IS407A") at the 3' end of the CPS gene cluster and have shown that this element is active in *B. mallei* (6). The data presented in this paper certainly support the view that IS407 is functionally active in *B. mallei*.

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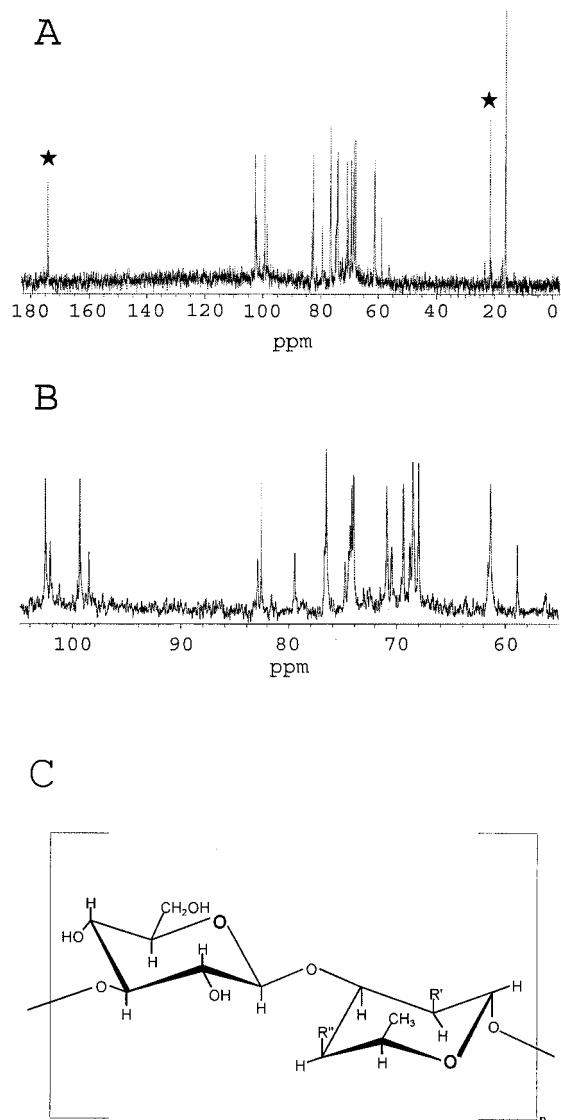


FIG. 3. ^{13}C -NMR analysis of *B. mallei* PB100 O-PS. (A) *O*-Acetyl peaks are indicated by stars. (B) Expanded view of the region running from 100 to 60 ppm. (C) Structure of *B. pseudomallei* and *B. mallei* O-PS. In *B. pseudomallei*, in 33% of the talose residues, $\text{R}' = \text{O}$ -methyl and $\text{R}'' = \text{O}$ -acetyl, and in 66% of the talose residues, $\text{R}' = \text{O}$ -acetyl and $\text{R}'' = \text{OH}$. In *B. mallei*, $\text{R}' = \text{O}$ -acetyl or *O*-methyl and $\text{R}'' = \text{OH}$.

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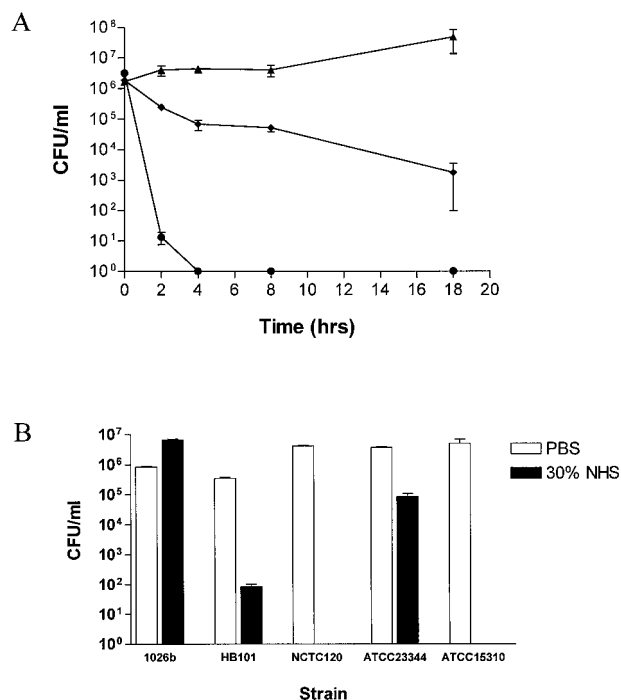


FIG. 4. Serum bactericidal assays with *B. mallei* strains. (A) Thirty percent NHS killing assay in which viable counts were determined at the 2-, 4-, 8-, and 18-h time points. *B. pseudomallei* 1026b (\blacktriangle), *B. mallei* ATCC 23344 (\blacklozenge), and *E. coli* HB101 (\bullet). (B) Thirty percent NHS killing assay in which viable counts were determined following a 2-h incubation at 37°C. Control tubes containing phosphate-buffered saline (PBS) are shown as white bars, and experimental tubes containing 30% NHS are shown as gray bars. Error bars indicate standard deviations.

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