Characterization of the Capsular Polysaccharide of *Burkholderia pseudomallei* 304b

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**Burkholderia (Pseudomonas) pseudomallei** is the causative agent of melioidosis, a bacterial infection of considerable morbidity in areas of endemicity of Southeast Asia and northern Australia. Clinical isolates of *B. pseudomallei* have been demonstrated to produce a lipopolysaccharide (LPS) containing two separate and chemically distinct antigenic O polysaccharides against which infected patients produced antibodies. A putative capsular polysaccharide (CPS) has also been reported and is thought to be antigenically conserved based on results of serological studies with clinical *B. pseudomallei* isolates. In the present study, the CPS isolated from *B. pseudomallei* 304b from northeastern Thailand was found to have an [α1,3:1,1] β-D-glucopyranosiduronic acid (KDO), containing an O-acetyl group (KDO), and O-acetyl 3:1:1, and was a linear unbranched polymer of repeating tetrasaccharide units having the following structure: -3)-2-O-Ac-β-p-Galp-(1-4)-α-D-Galp-(1-3)-β-D-Galp-(1-5)-β-D-KDOp-(2-). Sera from 13 of 15 patients with different clinical manifestations of melioidosis but not normal controls recognize the CPS, which suggests that it is immunogenic and raises the possibility that it may have a role as a vaccine candidate and/or diagnostic agent.

Melioidosis or *Burkholderia pseudomallei* (formerly *Pseudomonas pseudomallei*) [42] infection is a disease of animals and humans which is endemic primarily in rice-farming communities in Southeast Asia and northern Australia (18). In recent years, it has been recognized as a major cause of community-acquired septicemia, resulting in significant mortality (28). Radical cure of infection requires prolonged and expensive antibiotic regimens, which most of the populations at risk can ill afford. No effective methods of prevention of melioidosis currently exist. However, it is reasonable to predict that a vaccine inducing protective antibodies would be efficacious because of the septicemic nature of the severe disease and that specific lipopolysaccharide (LPS) components of *B. pseudomallei* could be a potential source of effective macromolecular immunogens.

In previous studies, we have shown that clinical strains of *B. pseudomallei* produce an LPS which is unusual in having two distinct antigenic O polysaccharides (O-PS) (31). One polysaccharide (O-PS I) is an unbranched high-molecular-weight polymer of 1,3-linked 2-O-acetyl-6-deoxy-β-D-manno-heptopyranoside residues. The second O antigen (O-PS II) is an unbranched polymer of repeating disaccharide units having the structure -3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-1-, in which ca. 33% of the 1-6Talp residues bear a 2-O-methyl substituent and the other 1-6Talp residues carry only 2-O-acetyl substituents. Analysis of patient sera indicates that O-PS II is the more potent immunogen (23a). Indeed, passive immunization with an anti-O-PS II monoclonal antibody produced a 4- to 5-log reduction in the 50% lethal dose in a diabetic rat model (12).

Less is known about the structure and immunogenicity of the capsular polysaccharide (CPS) of *B. pseudomallei*, which was first described by Chambon and Fournier (14–16) and later partially characterized by other investigators (11, 19, 34, 35), than of its LPS. More recently, Steinmetz et al. (39) reported on a putative specific exopolysaccharide of *B. pseudomallei* which was strongly reactive with sera from patients with melioidosis. However, no chemical or physical data on this material were recorded.

Many bacterial pathogens, such as *Neisseria*, *Streptococcus*, *Haemophilus*, and *Klebsiella* spp. and *Escherichia coli* (22, 27, 36), are able to combat the bactericidal activity of complement and phagocytes by encapsulation with structurally diverse polysaccharides, and it is only in the late phase of infections, when specific antcapsular antibodies are produced by the host, that the protective encapsulation activity is overcome. Because of the important role of bacterial capsules as virulence factors, their analysis has been undertaken to provide at a structural molecular level an understanding of their immunobiological properties and serological specificities and their use as protective vaccines, either as native purified polysaccharides or synthesized polysaccharide-protein conjugates (36).

This paper describes the structural analysis of the CPS produced by *B. pseudomallei* (strain 304B) which revealed it to be an unbranched polymer of a repeating tetrasaccharide unit composed of β-D-galactose and 3-deoxy-β-D-manno-octulosonic acid (KDO), containing an O-acetyl substituent. Evidence that this CPS functions as an immunogen in the course of melioidosis infections was demonstrated by the high titers of antibody found in sera from infected patients as compared with those of control human sera.

**MATERIALS AND METHODS**

**Growth of *B. pseudomallei* and isolation of CPS.** *B. pseudomallei* 304b, a clinical isolate from a patient in northeastern Thailand, was kindly supplied by D. A. B. Dance, Wellcome-Mahidol University, Oxford Tropical Medicine Research Programme, Bangkok, Thailand. It was grown on agar plates containing 3.7% brain heart infusion broth (Difco) for 20 h, and the mucoid cells were removed with a rubber spatula and suspended in 2% phenol solution. The cell suspension (1.8 g in 0.3 ml) at 70°C, and the mixture was stirred vigorously for 10 min at 65 to 70°C (25). The cooled mixture (4°C) was centrifuged (10,000 × g, 30 min), and the top water phase was collected by aspiration and dialyzed against running tap water until...
free from phenol. Following lyophilization, the residual material was dissolved in water (15 ml) and sequentially treated with RNase, DNase, and proteinase K. The solution was subjected to ultra-centrifugation (105,000 g, 10 h, 4°C), the centrifugate, removed from the precipitated LPS (ca. 25 mg), was treated with acetic anhydride, and precipitated crude polysaccharide was collected by centrifugation (10,000 g). The crude polysaccharide was desalted in 0.05 M pyridinium acetate (5 ml, pH 4.8) and subjected to Sephadex G-50 column chromatography (2 by 90 cm). The high-molecular-weight CPS that was eluted in a broad band (K1, 0.02 to 0.14; K2, 0.21) was collected and lyophilized.

Analytical methods. Neutral sugar was determined by gas-liquid chromatography (GLC) of the alditol acetate derivative (44). CPS was hydrolyzed with 2 M trifluoroacetic acid (TFA) for 90 min at 125°C and evaporated to dryness under a stream of dry nitrogen. The liberated glucose was reduced (NaBH4) and acetylated (Ac2O) as described previously by York et al. (44). GLC was performed with a Hewlett-Packard 5890A gas chromatograph (GC), fitted with a hydrogen flame ionization detector and a DB1 fused-silica capillary column (0.25 mm by 30 m), and a program to increase the temperature from 180°C (delay 2 min) to 240°C at a rate of 2°C/min. The identification of KDO and its ω-manno configuration was made by treating CPS and authentic LKDO (1 to 2 mg) at 85°C for 16 h with (—) -2-butanol containing 30 μl of TFA, followed by concentration in vacuo over P2O5 (20). The residues were dissolved in acetonitrile (20 μl) and treated with bis(trimethylsilyl)trifluoroacetamide (20 μl) at 80°C for 20 min, and the derivatives were analyzed by GLC-mass spectrometry (GLC-MS) as described above with a program to increase the temperature from 150 to 220°C at a rate of 2°C/min.

Methylation analysis was performed on CPS (—3 mg) with methyl iodide in excess of the potassium methylsulfinate methide in dimethyl sulfoxide (23). Excess methyl iodide was removed in a stream of nitrogen, and the methylated CPS was purified on a Sep-Pak C-18 cartridge as described previously (30). The excess methyl iodide was removed in a stream of nitrogen, and the methylated CPS was desalted with Dowex 50(H+) (evaporated from 10% acetic acid in methanol (three times) and from methanol acid, and following evaporation to dryness under nitrogen, the residue was for 24 h at 22°C (29). Excess reagent was destroyed by acidification with acetic acid, and following evaporation to dryness under nitrogen, the residue was evaporated from 10% acetic acid in methanol (three times) and from methanol (three times). The methylated CPS was desalted with Dowex 50 (H+), eluted with aqueous 50% methanol, and remethylated and purified as described above.

The ketoamic KDO linkage in the methylated CPS was cleaved by 2% acetic acid (100°C, 2.5 h), the liberated oligosaccharide was reduced (NaBH4) and acetylated (Ac2O), and the product was extracted from the water solution with chloroform (2 x 1 ml). The partially methylated oligosaccharide was analyzed by fast atom bombardment mass spectrometry (FAB-MS). The methylated oligosaccharide was further hydrolyzed with 2 M TFA (100°C, 1 h), reduced (NaBH4), acetylated (Ac2O), and analyzed by GLC. Partly methylated aldol acetate derivatives were separated and identified with a Varian Saturn II ion-trap GC-MS, fitted with a DB1 fused-silica capillary column (0.25 mm by 30 m), by use of a program to increase the temperature from 180°C (delay 2 min) to 290°C at 5°C/min.

FAB-MS. FAB-MS analysis was done with a JEOL AX 505H double-focusing MS operating at an accelerating voltage of 3 kV. Partially methylated oligosaccharide that was analyzed in the positive-ion mode with a mass resolution of 1,500; m/z scan range from 1 to 2,300, with 3-nitrobenzyl alcohol (98%; Aldrich) as the supporting matrix. The matrix was added to the stainless-steel probe tip, and then the sample dissolved in methylene chloride was added prior to FAB analysis. A 25-μl stream of 6 kV was used to sputter and ionize the sample, and spectra were calibrated with Ultramark 1621.

ES-MS. Oligosaccharides derived from native and O-deacetylated CPS by mild acid hydrolysis (2% acetic acid, 100°C, 2.5 h) were analyzed with a VG Quattro (Fisons Instruments) mass spectrometer fitted with an electrospray (ES) ion source. The sample was further dissolved in 50% acetonitrile containing 1% (vol/vol) acetic acid for both the positive- and negative-ion modes. Flow injection of 4 μl/min was performed with a Harvard syringe pump 22, and the samples were analyzed under the following conditions: tip voltage, 3.5 kV for positive- and 2.5 kV for negative-ion mode analyses; scan range, m/z 250 to 2,500; scan time, 10 s. Data were collected by the VG Masslynx software.

Tandem ES-MS analysis of the oligosaccharide from the hydrolysate of the native CPS was made under the same conditions described above (unless otherwise stated). The MS-MS analysis was done with a positive-ion mode with a scan range of m/z 250 to 1500 and a scan time of 6 s. Argon was used as the collision gas, and a collision energy of 45 eV was used for positive-mode MS-MS analysis.

NMR. Nuclear magnetic resonance spectroscopy (NMR) spectra were obtained with a Bruker AMX-500 spectrometer by standard Bruker software. All measurements were made on native and O-deacetylated CPS at 32 and 23°C, respectively, dissolved in D2O, subsequent to several lyophilizations with D2O. Proton NMR spectra were recorded by use of a spectral width of 10.6 kHz and a 90° pulse. Broad-band proton-decoupled 13C-NMR spectra were obtained at 125 MHz by use of a spectral width of 45 kHz, a 90° pulse, and WALTZ decoupling (38). Acetone (δ2 2.22; δ2 3.07) was used as an internal standard. Two-dimensional homonuclear chemical-shift correlation experiments (COSY) (4) were recorded over a spectral width of 2.0 or 1.2 kHz, with data sets (τ1 x τ2) of 256 x 2048 or 512 x 2048 points, respectively. Spectra were processed in the magnetic mode with symmetrization about the diagonal. Two-dimensional (2D) total correlated spectroscopy (TOCSY) (40) and nuclear Overhauser effect spectroscopy (NOEY) (26) were performed in a phase-sensitive manner for the spectral width of 2.0 kHz by use of a data set of 256 x 2048 points. Mixing times of 82.6 and 200 ms were used for the TOCSY and NOEY experiments, respectively. Heteronuclear 2D 13C-1H chemical-shift correlations were recorded in the 1H detection mode via multiple-quantum coherence (HMQC) (5) with proton decoupling in the 13C domain by use of data sets of 2,048 x 256 points and spectral widths of 5.1 and 15.1 kHz for the 1H and 13C domains, respectively. An HMOC experiment acquired without proton decoupling was used for determining one-bond 1H-13C coupling (J13C) values on O-deacetylated samples by use of a digital resolution of 1.1 Hz/point in the proton dimension. The 2D heteronuclear multiple-correlation (HMBC) experiments were run in the 1H detection mode with the pulse sequence described by Bax and Summers (3). The NMR conformation analysis of the KDO residue was established with Monte Carlo methodologies as described by Peters et al. (33).

ELISA determination of antibodies against CPS. An enzyme-linked immunosorbent assay (ELISA) was used to measure immunoglobulin G antibodies against CPS. Polyvinyl chloride plates (Falcon 3911; Becton Dickinson, Oxnard, Calif.) were coated with the CPS at an optimal concentration of 1 μg/ml in carbonate buffer (pH 9.6) at 2°C for 2 h. Sera from 15 patients with localized or septicemic melioidosis who had been admitted to Sappasitprasong Hospital, Ubon, Ratchathani, Thailand, and 10 uninfected Thai controls, kindly provided by M. D. Smith, Wellcome-Mahidol University, Oxford Tropical Medicine Research Programme, were studied at a 1:100 dilution. The controls and patients participated in clinical studies which were approved by the Ministry of Health, Bangkok, Thailand.

The plates were inoculated with the samples at 37°C for 1 h and then washed with phosphate-buffered saline-Tween. Peroxidase labeled goat anti-human immunoglobulin G (Miles-Yeda Ltd., Kirkat Weizman, Rehovot, Israel) at a 1: 1,000 dilution was then added for 1 h at 37°C. After washing, the substrate 2-azino-di-(3-ethyl-benzthiazoline sulfonate) was added for 30 mm at room temperature. The absorbance at 405 nm was read on a spectrometer. The mean absorbance of duplicate readings was determined.

RESULTS

The CPS had a δ1H of +99.0° (c 0.4, water) and upon hydrolysis yielded d-galactose (ca. 67% yield) characterized by capillary GLC-MS as hexa-O-acetylgalactitol and as its acetylated (2)O-butyrylgalactitol derivatives (21). The 1H-NMR spectrum of the native CPS showed three signals in the low-field region (5.08, 4.91, and 4.70 ppm) in a relative intensity of 1:1:2, respectively (Fig. 1A), of which three signals, labeled A, at 5.08 ppm, B, at 4.70 ppm, and C, at 4.70 ppm, were attributed to resonances from anomic protons, from direct correlation...
TABLE 1. $^1$H-NMR chemical shifts of native and O-deacetylated CPS of B. pseudomallei$^a$

<table>
<thead>
<tr>
<th>Residue</th>
<th>Glucose unit</th>
<th>Proton chemical shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H-1</td>
</tr>
<tr>
<td>Native CPS</td>
<td>A</td>
<td>-4α-D-Galp-(1-4)β-D-Galp-(1-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-3β-D-Galp-(1-3)β-D-Galp-(1-</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-3β-D-GalpOAc-(1-3)β-D-Galp-(1-</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>-5β-D-KDop-(2-)</td>
</tr>
<tr>
<td>O-Deacetylated CPS</td>
<td>A</td>
<td>-4α-D-Galp-(1-4)β-D-Galp-(1-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-3β-D-Galp-(1-3)β-D-Galp-(1-</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>-3β-D-Galp-(1-3)β-D-Galp-(1-</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>-5β-D-KDop-(2-)</td>
</tr>
</tbody>
</table>

$^a$ Measured at 23°C for the native and at 32°C for the O-deacetylated CPS in D$_2$O.
$^b$ Suffixes a and e indicate axial and equatorial H-3s of KDO, respectively.

with $^{13}$C resonances seen in a 2D heteronuclear $^{13}$C-$^1$H correlation (HMOC) spectrum. Two signals observed in the high-field region, a triplet at 1.91 ppm and a doublet of a doublet at 2.52 ppm, were identified from their signal patterns and their chemical shifts as the axial and equatorial methylene protons (H-3a and H-3e, respectively) from a KDO ketopyranosyl residue with a β configuration (1, 13, 37, 41). The characterization of the octulosonic acid as KDO was confirmed by the GLC-MS production of two characteristic peaks from (−)-2-butyl-3-deoxy-4,5,7,8-tetra-oxy-(trimethylsilyl)-(α and β)-D-manno-octulosonate-(−)-2-butyl esters (21) with retention times and MS identical to those of reference samples. Further characterization of the KDO component was made by the GLC identification of the derivatives of 3-deoxy-D-galactose, 3-deoxy-D-galacto-octitol and 3-deoxy-D-talo-octitol as the reduction product of KDO (32) (see below). A third sharp resonance (2.13 ppm, 3H) in the one-dimensional (1D) $^1$H-NMR spectrum was due to an O-acetyl substituent as evidenced by its absence from the spectrum of the O-deacetylated CPS (Fig. 1; Table 1) and a coincidental shift of the signal at 4.91 ppm in the spectrum of the native CPS (H-3a and H-3e, respectively) from a KDO ketopyranosyl residue with a β configuration resulting from cleavage of the glycosidic bond of the terminal D-galactose residue in the tetrasaccharide ed tetrasaccharides (Gal-Gal-Gal-3-deoxyoctitol derivatives), one derivative substituted with 14 methyl and 2 acetyl groups [m/z 1015.6; (M+Na)$^+$] and the other substituted with 13 methyl and 3 acetyl groups [m/z 1043.6; (M'+Na)$^+$]. In addition, a major fragment ion was observed at m/z 797.6 arising from cleavage of the glycosidic bond of the terminal D-galactose residue from M and M' species. These data suggest that part of the terminal D-galactose residue in the tetrasaccharide derivatives is fully methylated, which probably resulted from alkaline hydrolysis of the KDO ketosidic linkage by the strongly basic conditions of the methylation procedure.

GLC-MS analysis of the acid-hydrolyzed (2 M TFA, 100°C, 1 h), methylated tetrasaccharide by the alditol acetate method (44) established the linkage positions of the glycosyl residues (Table 3). The GLC-MS analysis is in agreement with the FAB-MS data, showing that the 2,3,4,6-tetra-O-methyl-D-galactose was derived from the exposed D-galactose residue resulting

FIG. 2. $^{13}$C-NMR spectrum of the native (A) and the O-deacetylated (B) CPS of B. pseudomallei 304b.
from basic hydrolysis during methylation. Double GLC peaks with close retention times (Table 3) were observed for KDO derivatives and are characteristics for the two diasterioisomeric products formed by reduction of the 2-keto group (43). The combined 1D NMR data showed that the CPS contains one 4-O-acetylated D-Galp, two 3-O-acetylated D-Galp, and one 1,5-O-acetylated KDO residue.

The \( ^1H \) NMR spectra of the native and O-deacetylated CPS were assigned by 2D homonuclear proton (COSY) and total correlated (TOCSY) spectra. Subspectra corresponding to the ring system for each of the four glycopyranosyl residues were identified (Table 1). The triplet signal at 4.91 ppm (H-2 of C) in the native CPS was shifted upfield to 3.63 ppm after O-deacetylation (Table 1), due to the shielding effect caused by the removal of the O-acetyl group, indicating the location of the O-acetyl substituent in the CPS.

Assignment of the \( ^{13}C \) resonances (Table 2) was made by direct correlation of the \( ^1H \) resonances with the \( ^{13}C \) resonances in HMOC experiments and by comparison of \( ^{13}C \) resonances with chemical shift data from the literature (1, 9, 10). A small upfield shift (0.89 ppm) was observed for the C-2 signal of residue C, and downfield shifts (2.15 and 3.20 ppm) were observed for C-1 and C-3 signals, respectively, after removal of the O-acetyl substituent, a result in accord with the resonance of unsubstituted residues (10). A similar downfield shift was also observed for C-4 of residue A. Since the methylation data indicated that only one \( \alpha \)-galactose is substituted at the O-4 position (residue A), two residues (B and C) must be substituted at the O-3 position, a conclusion supported by the relative deshielding of the carbon resonances observed for the C-3s of residues B and C, causing significant downfield chemical shifts when compared with the resonances of unsubstituted residues (10). A similar downfield shift was also observed for C-4 of residue A.

The NOE connectivities and the linkage positions of the glycosyl residues within the repeating tetrasaccharide units in both the native and O-deacetylated CPS polymers was established from the observed transglycosidic NOE connectivities between protons on adjacent glycosyl residues (Fig. 3). NOE measurements were made in the 2D mode by a NOESY experiment. Transglycosidic NOEs were observed between H-1 of residue C (\( \beta \)-D-Galp) and H-4 of residue A (\( \alpha \)-D-Galp), H-1 of residue B (\( \beta \)-D-Galp) and H-5 of residue K (\( \beta \)-D-KDO), and H-1 of residue A and both H-3 and H-4 of residue B. Since the methylation data indicated that only one \( \alpha \)-galactose is substituted at the O-4 position (residue A), two residues (B and C) must be substituted at the O-3 position, a conclusion supported by the relative deshielding of the carbon resonances observed for the C-3s of residues B and C, causing significant downfield chemical shifts when compared with the resonances of unsubstituted residues (10). A similar downfield shift was also observed for C-4 of residue A.

The NOE connectivities and the linkage positions of the glycosyl residues of the tetrasaccharide repeating unit deduced from NOE connectivities are illustrated in Fig. 4. The \( \beta \)-D configurations were assigned to the glycosyl residues within the repeating tetrasaccharide units in both the native and O-deacetylated CPS polymers was established from the observed transglycosidic NOE connectivities between protons on adjacent glycosyl residues (Fig. 3). NOE measurements were made in the 2D mode by a NOESY experiment. Transglycosidic NOEs were observed between H-1 of residue C (\( \beta \)-D-Galp) and H-4 of residue A (\( \alpha \)-D-Galp), H-1 of residue B (\( \beta \)-D-Galp) and H-5 of residue K (\( \beta \)-D-KDO), and H-1 of residue A and both H-3 and H-4 of residue B.
The configuration of the KDO was confirmed by use of the Monte
Carlo methods for conformation analysis (33). The observed
interresidue NOE connectivities between $^1$H-1 and the $\beta$-D-Galp (residue B) and KDO protons were in accord with the
calculated minimum energy structural study of the $\beta$-D-Galp-
(1-5)-$\beta$-D-KDOp disaccharide.

On the basis of combined NMR and methylation data, the
tetrasaccharide CPS repeating unit is established as follows:

\[
\text{M+Na = 771}
\]

\[
\text{GalAc-O-Gal-O-Gal-O-anhydro KDO}
\]

FIG. 4. Structure of the O-deacetylated repeating tetrasaccharide unit of \textit{B. pseudomallei} 304b CPS showing the network of transglycosidic NOE connectivities (dotted lines) used to establish the glycoside sequence.

FIG. 5. Tandem MS analysis of the native CPS repeating unit of \textit{B. pseudomallei}.
saccharides of and CPS conjugates derived from the specific serogroup polymers. The successful use of human vaccines based on the CPS of \textit{B. pseudomallei} good reason to believe that the CPS of \textit{Haemophilus influenzae} disease localized or septicemic melioidosis were tested against the mild acid hydrolysis of the native and O-deacetylated CPS. It is now known that KDO is a major constituent of a number of bacterial CPSs. Thus, KDO has been characterized in the parent molecular ion at 706.1 (M+H)\(^+\) in the negative-ion mode, corresponding to a composition of three D-galactose residues and anhydro-KDO (molecular mass, 707 Da), while the corresponding oligosaccharide from the native CPS showed a molecular ion at 747.4 (M+Ac-H\(^+\)) in (molecular mass, 748 Da), confirming the presence of only one acetyl group substituent in each repeating unit of the native CPS. Positive-ion tandem mass spectrometry was used to confirm the sequence of the residues in the repeating unit derived from the native CPS. A parent molecular ion at 771.7 (M+Ac+Na)\(^+\) was produced in the positive-ion mode, which gave rise to the fragment spectrum shown in Fig. 5. The fragments at 551.7, 389.3, and 226.9 were produced from the nonreducing terminal end of the oligosaccharide, while fragments at 567.5, 406.9, and 243.3 were produced from the reducing terminal end of the native tetrascarharide repeating unit. The fragment at 226.9 (204+Na) unambiguously confirmed that the \(\alpha\)-acetyl group is carried by the terminal nonreducing \(\beta\)-galactopyranosyl residue.

To determine whether an antibody response develops to the CPS during an acute infection, the sera from 15 patients with localized or septicemic melioidosis were tested against the polysaccharide by ELISA. The results indicated that the CPS is immunogenic in vivo and that 13 of 15 patients were seropositive if the mean + 3 standard deviations of the normal controls were used as the cutoff point.

### DISCUSSION

Our studies of the hot aqueous phenol extraction products of \textit{B. pseudomallei} 304b cells revealed that in addition to the previously characterized species-common LPS found in the water-phase extraction product. The yields of this CPS were previously characterized species-common LPS found in the water-phase extraction product. The yields of this CPS were reported bacterial polysaccharide. KDO was previously thought does not show any close relationship to that of any presently recognized bacterial polysaccharides. KDO was previously thought does not show any close relationship to that of any presently recognized bacterial polysaccharides.

Further evidence for the proposed structure was obtained from an ES-MS analysis of the tetrasaccharides obtained from the mild acid hydrolysis of the native and O-deacetylated CPSs. The tetrasaccharide from the O-deacetylated CPS showed a molecular ion at 706.1 (M+H)\(^+\) in the negative-ion mode, corresponding to a composition of three D-galactose residues and anhydro-KDO (molecular mass, 707 Da), while the corresponding oligosaccharide from the native CPS showed a molecular ion at 747.4 (M+Ac-H\(^+\)) in (molecular mass, 748 Da), confirming the presence of only one acetyl group substituent in each repeating unit of the native CPS. Positive-ion tandem mass spectrometry was used to confirm the sequence of the residues in the repeating unit derived from the native CPS. A parent molecular ion at 771.7 (M+Ac+Na)\(^+\) was produced in the positive-ion mode, which gave rise to the fragment spectrum shown in Fig. 5. The fragments at 551.7, 389.3, and 226.9 were produced from the nonreducing terminal end of the oligosaccharide, while fragments at 567.5, 406.9, and 243.3 were produced from the reducing terminal end of the native tetrascarharide repeating unit. The fragment at 226.9 (204+Na) unambiguously confirmed that the \(\alpha\)-acetyl group is carried by the terminal nonreducing \(\beta\)-galactopyranosyl residue.

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In agreement with Steinmetz et al. (39), we also found that sera from a number of patients had antibodies against CPS, suggesting that the polysaccharide is immunogenic and could be a common constituent of \textit{B. pseudomallei} strains. Thus, it has potential use in serological diagnosis as well as vaccine development. However, the limited serology results reported here need to be confirmed in a larger patient and control population.

### ACKNOWLEDGMENTS

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