JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5663–5669 Vol. 179, No. 18
0021-9193/97/$04.00+0

Characterization of the Capsular Polysaccharide of *Burkholderia* (*Pseudomonas*) *pseudomallei* 304b

HUSSEIN MASOUD,[1] MAY HO,[2] TINEKE SCHOLLAARDT,[2] AND MALCOLM B. PERRY[1]*

Institute for Biological Sciences, National Research Council, Ottawa, Ontario, Canada K1A OR6,[1] *and Department of Microbiology and Infectious Diseases, Health Science Centre, Calgary, Alberta, Canada T2N 4NI*[2]

Received 4 April 1997/Accepted 1 July 1997

***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 $[\alpha]_D$ of +99° (water), was composed of D-galactose (D-Gal), 3-deoxy-D-*manno*-2-octulosonic acid (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-D-Gal*p*-(1-4)-α-D-Gal*p*-(1-3)-β-D-Gal*p*-(1-5)-β-D-KDO*p*-(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 L-6dTal*p* residues bear a 2-*O*-methyl substituent and the other L-6dTal*p* 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 anticapsular 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 D-galactose and 3-deoxy-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 of cells in 50 ml of 2% phenol) at 65°C was treated with 95% aqueous phenol (50 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

* Corresponding author. Mailing address: Institute for Biological Sciences, National Research Council, Ottawa, Ontario, Canada K1A OR6. Phone: (613) 990-0837. Fax: (613) 941-1327.

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 ultracentrifugation (105,000 × *g*, 10 h, 4°C), the centrifugate, removed from the precipitated LPS (ca. 25 mg), was treated with acetone (75 ml), and precipitated crude polysaccharide was collected by centrifugation (10,000 × *g*). The crude polysaccharide was dissolved 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 (K_{av}, 0.02 to 0.14; 0.188 mg) was collected and lyophilized.

Analytical methods. Neutral sugar was determined by gas-liquid chromatography (GLC) of its 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 glycose was reduced ($NaBH_4$) and acetylated (Ac_2O) 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 DB17 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 D-*manno* configuration was made by treating CPS and authentic D-KDO (1 to 2 mg) at 85°C for 16 h with (−)-2-butanol containing 30 μl of TFA, followed by concentration in vacuo over P_2O_5 (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 210°C at a rate of 2°C/min.

Methylation analysis was performed on CPS (~3 mg) with methyl iodide in excess of the potassium methylsulfinyl methanide 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 carboxymethyl ester group of methylated KDO residues were reduced with 1 M lithium triethylborohydride in tetrahydrofuran (Super Hydride; Aldrich; 0.5 ml) for 2 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 ketosidic KDO linkage in the methylated CPS was cleaved by 2% acetic acid (100°C, 2.5 h), the liberated oligosaccharide was reduced ($NaBH_4$) and acetylated (Ac_2O), and the product was extracted from the water solution with chloroform (2 × 1 ml). The partially methylated oligosaccharide was analyzed by fast-atom bombardment (FAB)-MS. The methylated oligosaccharide was further hydrolyzed with 2 M TFA (100°C, 1 h), reduced ($NaBH_4$), acetylated (Ac_2O), and analyzed by GLC. Partially methylated alditol acetate derivatives were separated and identified with a Varian Saturn II ion-trap GC-MS, fitted with a DB17 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 was analyzed in the positive-ion mode with a mass resolution of 1,500; its *m/z* scan range from 1 to 2300, 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 Xe atom beam 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) fitted with an electrospray (ES) ion source. Samples were 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-ion- and 2.5 kV for negative-ion-mode analyses; scan range, *m/z* 150 to 2,500; scan time, 10 s. Data were collected by the VG Masslynx software.

Tandem ES-MS analysis of the oligosaccharide from the hydrolysis 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* 50 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 use of standard Bruker software. All measurements were made on native and O-deacetylated CPS at 32 and 23°C, respectively, dissolved in D_2O, subsequent to several lyophilizations with D_2O. Proton NMR spectra were recorded by use of a spectral width of 10.6 kHz and a 90° pulse. Broad-band proton-decoupled ^{13}C-NMR spectra were obtained at 125 MHz by use of a spectral width of 45 kHz, a 90° pulse, and WALTZ decoupling (38). Acetone (δ_H 2.225; δ_C 31.07) 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 ($t_1 \times t_2$) of 256 × 2,048 or 512 × 2,048 points, respectively. Spectra were processed in the magnitude mode with symmetrization about the diagonal. Two-dimensional (2D) total correlated spectroscopy (TOCSY) (40) and nuclear Overhauser effect spectroscopy (NOESY) (26) were performed in a phase-sensitive manner for the spectral width of 2.0 kHz by use of a data set of 256 × 2,048 points. Mixing times of 82.6 and 200 ms were used for the TOCSY and NOESY experiments, respectively. Heteronuclear 2D ^{13}C-^{1}H chemical-shift correlations were recorded in the ^{1}H detection mode via multiple-quantum coherence (HMQC) (5) with proton decoupling in the ^{13}C domain by use of data sets of 2,048 × 256 points and spectral widths of 5.1 and 15.1 kHz for the ^{1}H and ^{13}C domains, respectively. An HMQC experiment acquired without proton decoupling was used for determining one-bond ^{1}H-^{13}C coupling ($^1J_{C,H}$) 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 ^{1}H 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).

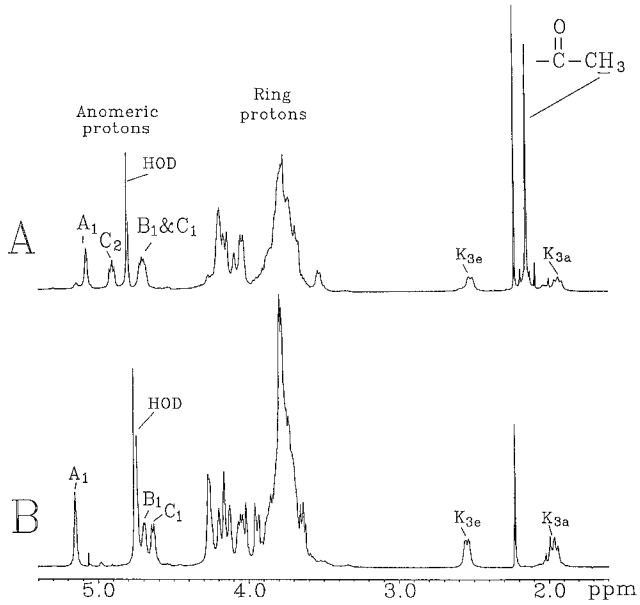


FIG. 1. ^{1}H-NMR spectrum of the native (A) and the O-deacetylated (B) CPS of *B. pseudomallei* 304b. The signals of the axial and equatorial methylene protons from the KDO residues and the methyl protons from the *O*-acetyl substituents are indicated.

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 37°C for 2 h. Sera from 15 patients with localized or septicemic melioidosis who had been admitted to Sappasitprasong Hospital, Ubon, Ratchatani, 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., Kiryat Weizman, Rehovot, Israel) at a 1:1,000 dilution was then added for 1 h at 37°C. After washing, the substrate 2-2′-azino-di-(3-ethyl-benzthiazoline sulfonate) was added for 30 min 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 $[\alpha]_D$ 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-(*S*)-butyl glycoside derivatives (21). The ^{1}H-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_1 at 5.08 ppm, B_1 at 4.70 ppm, and C_1 at 4.70 ppm, were attributed to resonances from anomeric protons, from direct correlation

TABLE 1. ^{1}H-NMR chemical shifts of native and O-deacetylated CPS of *B. pseudomallei*[a]

Residue	Glycose unit	Proton chemical shift (ppm)									
		H-1	H-2	H-3a[b]	H-3e[b]	H-4	H-5	H-6	H-6′	H-7	H-8	H-8′
Native CPS												
A	-4)-α-D-Gal*p*-(1-	5.08	3.68	4.04		4.16	4.20					
B	-3)-β-D-Gal*p*-(1-	4.70	3.74	3.78		4.14	3.74					
C	-3)-β-D-Gal*p*OAc-(1-	4.72	4.91	4.19		4.09	3.74					
K	-5)-β-D-KDO*p*-(2-			1.91	2.52	3.87	4.19	3.52		4.03	3.82	3.72
O-Deacetylated CPS												
A	-4)-α-D-Gal*p*-(1-	5.15	3.93	4.03		4.27	4.26	3.83	3.72			
B	-3)-β-D-Gal*p*-(1-	4.69	3.77	3.79		4.16	3.72					
C	-3)-β-D-Gal*p*-(1-	4.62	3.63	3.78		4.12	3.70					
K	-5)-β-D-KDO*p*-(2-			1.97	2.54	3.87	4.19	3.86		4.07	3.68	3.77

[a] Measured at 23°C for the native and at 32°C for the O-deacetylated CPS in D_2O.
[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 (HMQC) 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-*O*-(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-*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, a significant upfield shift (0.10 ppm) of the anomeric signal of residue C_1, and a downfield shift (0.07 ppm) of residue A_1. The data described above indicate that the CPS is composed of regular repeating tetrasaccharide units composed of D-galactose, β-D-KDO, and *O*-acetyl (3:1:1).

Consistent with the ^{1}H-NMR data, the ^{13}C-NMR spectrum of the native CPS (Fig. 2A) showed four anomeric signals (A_1, B_1, C_1, and K_2; 96.38 to 105.22 ppm) together with acetyl signals at 21.66 ($\underline{C}H_3CO$) and 171.74 ppm ($CH_3\underline{C}O$), a methylene KDO signal (C-3) at 36.29 ppm, and a carboxyl KDO signal (C-1) at 174.52 ppm. The 1D ^{13}C-NMR spectrum of the O-deacetylated CPS showed a significant downfield shift (2.15 ppm) of the anomeric signal of the residue labeled C_1 (Table 2), while the acetyl signals were lost (Fig. 2B). The carboxyl and the anomeric ^{13}C signals of the KDO residue were not seen in the 1D spectrum (probably due to a relaxation time difference between the two spectra induced by removal of the O-acetyl substituent); however, a correlation between the H-3a and both C-1 and C-2 of the KDO residue was observed in the HMBC spectrum (data not shown).

Methylation analysis of the native CPS involving carboxyl reduction of the KDO methyl ester (with Super Hydride), subsequent methylation, mild acid hydrolysis, reduction ($NaBH_4$), and acetylation afforded a partially methylated tetrasaccharide product. The positive FAB-MS analysis of the product described above showed abundant ions of *m/z* 1015.6 and 1043.6 consistent with the product being a mixture of two permethylated 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

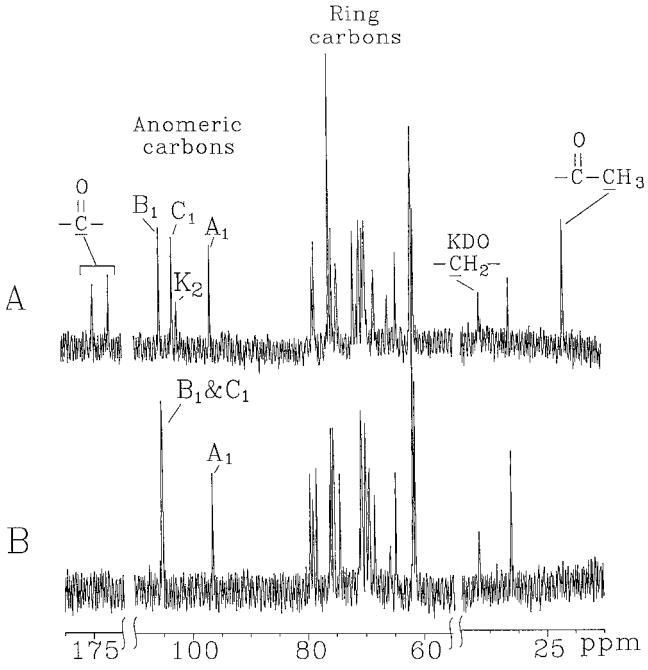


FIG. 2. ^{13}C-NMR spectrum of the native (A) and the O-deacetylated (B) CPS of *B. pseudomallei* 304b.

TABLE 2. ^{13}C chemical shifts and coupling constants of the native and O-deacetylated CPS of *B. pseudomallei*[a]

Residue	Glycose unit	^{13}C chemical shifts (ppm) (coupling constants [Hz])[b]							
		C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
Native CPS									
A	-4)-α-D-Gal*p*-(1-	96.38	69.33	69.80a	78.59	71.02	61.82b		
B	-3)-β-D-Gal*p*-(1-	105.22	70.57	78.36	65.74	75.26c	61.52b		
C	-3)-β-D-Gal*p*-(1-	103.01	71.68	75.84	69.62	75.84c	61.20		
K	-3)-β-D-KDO*p*-(2-	174.52	102.22	36.29	68.00	75.84	74.43	69.99a	64.35
O-Deacetylated CPS									
A	-4)-α-D-Gal*p*-(1-	96.32 (171.3)	~69.80	~70.80	~79.45	71.05	61.82f		
B	-3)-β-D-Gal*p*-(1-	105.16 (163.0)	70.59d	78.40	65.72	75.78e	61.67f		
C	-3)-β-D-Gal*p*-(1-	105.16 (162.0)	70.79	79.04	69.30	75.39e	61.41f		
K	-5)-β-D-KDO*p*-(2-	175.30	104.70	36.74	68.30	76.07	74.28	69.94	64.70

[a] Measured at 23°C for native and 32°C for *O*-deacetylated CPS in D_2O.
[b] Values followed by the same lowercase letters may have their assignments reversed.

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*-substituted D-Gal*p*, two 3-*O*-substituted D-Gal*p*, and one 1,5-*O*-substituted 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 HMQC 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 substitution of *O*-acetyl at C-2 of residue C. Similar ^{13}C chemical shifts were reported for C-2 *O*-acetyl substitution in β-D-mannose residues (17). The anomeric configurations of the three D-galactopyranosyl residues were established from the magnitude of the heteronuclear one-bond ^1H-^{13}C coupling constants. In the O-deacetylated CPS spectra, the coupling constant ($^1J_{C\text{-}1,H\text{-}1}$, 171.3 Hz) for the D-Gal*p* residue A established its α configuration, while the D-Gal*p* residues B and C had coupling constants ($^1J_{C\text{-}1,H\text{-}1}$, 163.0 and 162.0 Hz, respectively) assigned to the β-D configurations (8). In agreement with the assigned configurations described above, strong intraresidue nuclear Overhauser effects (NOEs) were observed between H-1 and H-2 of residue A and between H-1 and both H-3 and H-5 of residues B and C.

The sequence 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 (β-D-Gal*p*) and H-4 of residue A (α-D-Gal*p*), H-1 of residue B (β-D-Gal*p*) and H-5 of residue K (β-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 D-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 β-D

TABLE 3. Methylation analysis of the CPS of *B. pseudomallei*

Methylated derivative	T_G[a]	Relative detector response[a]
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactitol	0.82	0.52
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol	1.00	1.00
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol	1.05	1.60
2,5,6-Tri-*O*-acetyl-3-deoxy-1,4,7,8-tetra-*O*-methyl-octitol	1.45	0.22
2,5,6-Tri-*O*-acetyl-3-deoxy-1,4,7,8-tetra-*O*-methyloctitol	1.45	0.28

[a] Retention times (T_Gs) and detector responses are given relative to that of 1,4,5-tetra-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol (=1.00).

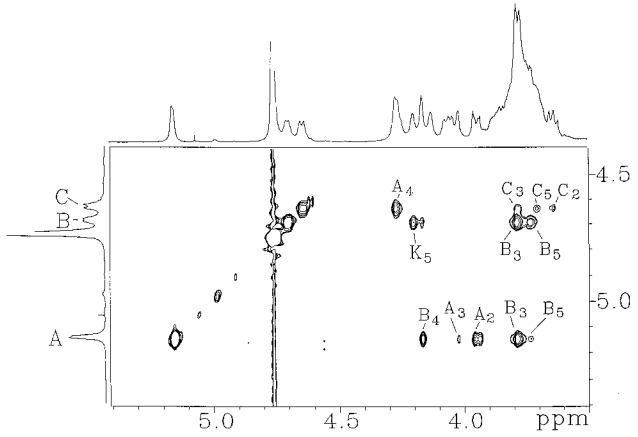


FIG. 3. Partial 2D NOESY NMR spectrum of the O-deacetylated CPS of *B. pseudomallei* 304b showing NOE connectivities relating anomeric proton resonances of the three D-galactopyranose residues (A, B, and C).

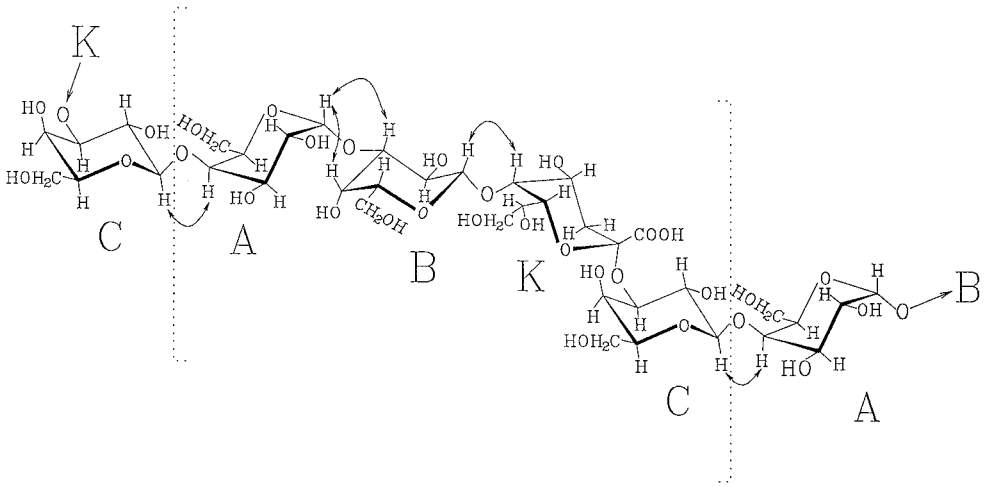


FIG. 4. Structure of the O-deacetylated repeating tetrasaccharide unit of *B. pseudomallei* 304b CPS showing the network of transglycosidic NOE connectivities (dotted lines) used to establish the glycose sequence.

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 β-D-Gal*p* (residue B) and KDO protons were in accord with the calculated minimum energy structural study of the β-D-Gal*p*-(1-5)-β-D-KDO*p* disaccharide.

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

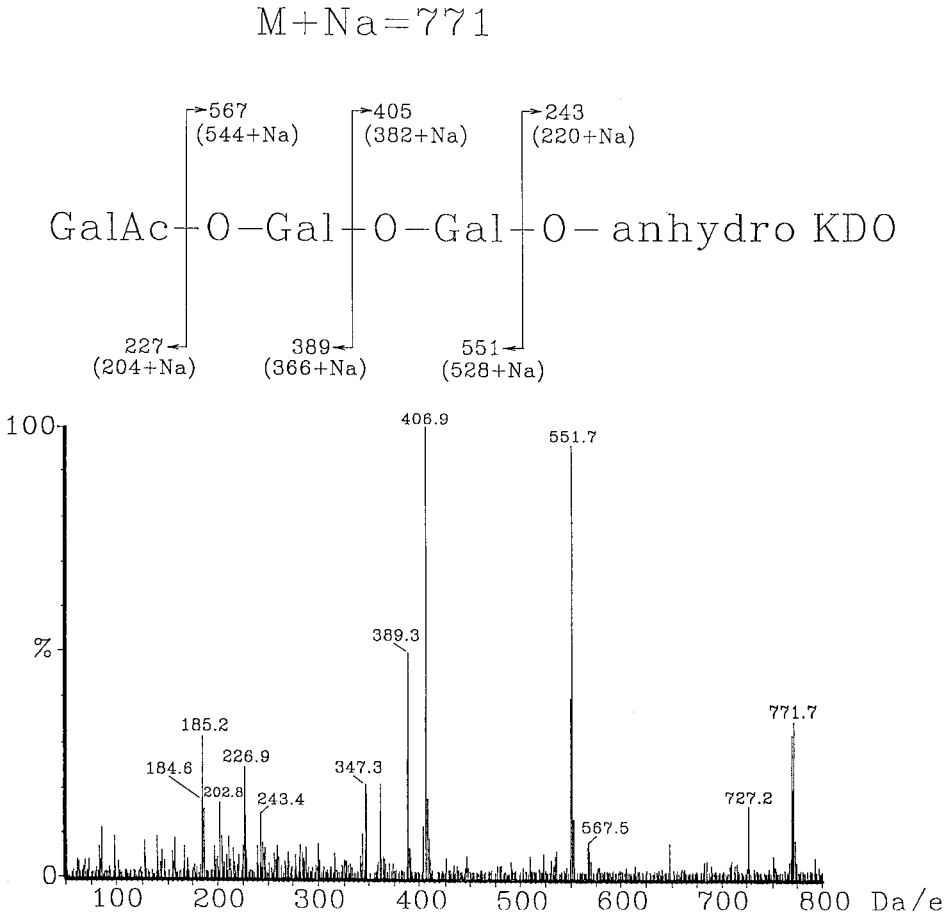
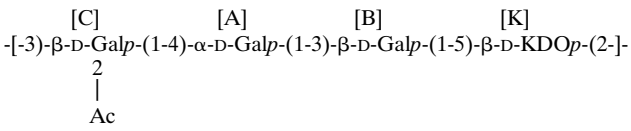


FIG. 5. Tandem MS analysis of the native CPS repeating unit of *B. pseudomallei.*



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 CPS. The oligosaccharide from the O-deacetylated CPS showed a molecular ion at 706.1 $(M\text{-}1H)^{1-}$ 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 $(M+Ac\text{-}1H)^{1-}$ (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 $(M+Ac+Na)^{1+}$ 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 tetrasaccharide repeating unit. The fragment at 226.9 (204+Na) unambiguously confirmed that the *O*-acetyl group is carried by the terminal nonreducing D-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 *B. pseudomallei* 304b cells revealed that in addition to the previously characterized species-common LPS found in the phenol phase of extracted cells, a CPS was located in the water-phase extraction product. The yields of this CPS were greater from plates showing a high density of spontaneous mucoid cells than from those plates producing nonmucoid cells. The relative CPS yields were not unexpected, considering earlier microscopically observed heavy polysaccharide encapsulation of cells selected from mucoid colonies of *B. pseudomallei* (14–16).

The structure of the CPS is unique and to our knowledge does not show any close relationship to that of any presently reported bacterial polysaccharide. KDO was previously thought to be a characteristic LPS constituent, providing the linkage glycose between lipid A and the carbohydrate moiety of LPSs. It is now known that KDO is a major constituent of a number of bacterial CPSs. Thus, KDO has been characterized in the CPSs (K antigens) of *E. coli* serotypes K-6, K-12, K-13, K-14, K-15, K-16, K-19, K-20, K-23, K-24, K-74, K-95, and K-97 (24), of *Actinobacillus pleuropneumoniae* serotypes 5 (1), 5b (2), and 10 (6), and of *Neisseria meningitidis* serotype 29e (7). Considering the successful use of human vaccines based on the CPS and CPS conjugates derived from the specific serogroup polysaccharides of *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* type b (22, 27, 36), there is good reason to believe that the CPS of *B. pseudomallei* could form the basis of a protective human vaccine.

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 *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

We thank the Medical Research Council of Canada, the Canadian Bacterial Diseases Network Centres of Excellence, and the Alberta Heritage Foundation for Medical Research for support, D. W. Griffith for the growth of bacterial cells, and J.-R. Brisson for helpful advice.

REFERENCES

1. **Altman, E., J.-R. Brisson, and M. B. Perry.** 1987. Structure of the capsular polysaccharide of *Haemophilus pleuropneumoniae* serotype 5. Eur. J. Biochem. **170:**185–192.
2. **Altman, E., J.-R. Brisson, S. M. Gagné, and M. B. Perry.** 1992. Structure of the capsular polysaccharide of *Actinobacillus pleuropneumoniae* serotype 5b. Eur. J. Biochem. **204:**225–230.
3. **Bax, A., and M. F. Summers.** 1986. 1H and 13C assignments from sensitive-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. **108:**2093–2094.
4. **Bax, A., R. Freeman, and G. Morris.** 1981. Correlation of proton chemical shifts by two-dimensional Fourier transform NMR. J. Magn. Reson. **42:**164–168.
5. **Bax, A., R. H. Griffey, and B. L. Hawkins.** 1983. Correlation of proton and nitrogen-15 chemical shifts by multiple quantum NMR. J. Magn. Reson. **55:**301–315.
6. **Beynon, L. M., J. C. Richards, and M. B. Perry.** 1991. Structure of the capsular polysaccharide of *Actinobacillus pleuropneumoniae* serotype 10. Carbohydr. Res. **220:**185–193.
7. **Bhattacharjee, A. K., H. J. Jennings, and C. P. Kenney.** 1978. Structural elucidation of the 3-deoxy-D-*manno*-octulosonic acid containing meningococcal 29e capsular polysaccharide using carbon-13 nuclear magnetic resonance. Biochemistry **17:**645–651.
8. **Bock, K., and C. Pedersen.** 1974. A study of ^{13}CH coupling constants in hexopyranoses. J. Chem. Soc. Perkin Trans. II 293–297.
9. **Bock, K., and C. Pedersen.** 1983. Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides. Adv. Carbohydr. Chem. Biochem. **41:**27–66.
10. **Bock, K., C. Pedersen, and H. Pedersen.** 1984. Carbon-13 nuclear magnetic resonance data for oligosaccharides. Adv. Carbohydr. Chem. Biochem. **42:**193–225.
11. **Bozhko, V. G., I. I. Denisov, V. S. Rybkin, S. F. Popov, and V. P. Vasiliev.** 1992. Influence of acid exopolysaccharide produced by the melioidosis pathogen on functional macrophage activity. Zh. Mikrobiol. (Kiev) **54(6):**54–58.
12. **Bryan, L. E., S. Wong, D. E. Woods, A. B. Dance, and W. Chaowagul.** 1994. Passive protection of diabetic rats with antisera specific for the polysaccharide portion of the lipopolysaccharide isolated from *Pseudomonas pseudomallei*. Can. J. Infect. Dis. **5(4):**170–178.
13. **Carlson, R. W., R. L. Hollingsworth, and F. B. Dazzo.** 1988. A core oligosaccharide component from the lipopolysaccharide of *Rhizobium trifolii* ANU 483. Carbohydr. Res. **176:**127–135.
14. **Chambon, L., and J. Fournier.** 1956. Constitution antigenique de *Malleomyces pseudo-mallei*. I. Caracteres morphologiques, culturaux, biochimiques et variations de type immunologique. Ann. Inst. Pasteur **91:**355–362.
15. **Chambon, L., and J. Fournier.** 1956. Constitution antigenique de *Malleomyces pseudo-mallei*. II. Demonstration de l'existence des antigenes M, K, et O et etudes de leurs proprietes immunologiques. Ann. Inst. Pasteur **91:**472–485.
16. **Chambon, L., A. M. Staub, and M. Bourdet.** 1957. Constitution antigenique de *Malleomyces pseudo-mallei*. III. Mode d'extraction et premiers essais de purification de l'antigene d'enveloppe K. Ann. Inst. Pasteur **93:**376–389.
17. **Cox, A. D., and S. G. Wilkinson.** 1990. Structure of the O-specific polymer from *Pseudomonas cepacia* serogroup O7. Carbohydr. Res. **198:**153–156.
18. **Dance, D. A. B.** 1991. Melioidosis: tip of the iceberg. Clin. Microbiol. Rev. **4:**52–60.
19. **Denisov, I. I., V. I. Iliukhin, N. P. Khrapova, B. I. Melinkov, S. M. Farber, V. G. Bozhko, V. I. Sorokin, and V. I. Kapliev.** 1995. The acid exopolysaccharide of the causative agent of melioidosis. Zh. Mikrobiol. Epidemiol. Immunobiol. **6:**16–17.
20. **Gerwig, G. J., J. P. Kamerling, and J. F. G. Vliegenthart.** 1978. Determination of the D and L configuration of neutral monosaccharides by high-resolution capillary G.L.C. Carbohydr. Res. **62:**349–357.

21. **Gerwig, G. J., J. P. Kamerling, and J. F. G. Vliegenthart.** 1979. Determination of the absolute configuration of monosaccharides in complex carbohydrates by capillary G.L.C. Carbohydr. Res. **77:**1–7.
22. **Griffiss, J. M., M. A. Apicella, B. Greenwood, and P. H. Makela.** 1987. Vaccines against encapsulated bacteria: a global agenda. Rev. Infect. Dis. **9:** 176–188.
23. **Hakomori, S.** 1964. Rapid permethylation of glycolipids and polysaccharides by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem (Tokyo) **55:** 205–208.
23a. **Ho, M., et al.** Unpublished data.
24. **Jann, B., and K. Jann.** 1990. Structure and biosynthesis of the capsular polysaccharide of *Escherichia coli*. Curr. Top. Microbiol. Immunol. **150:**19–42.
25. **Johnson, K. G., and M. B. Perry.** 1976. Improved techniques for the preparation of bacterial lipopolysaccharides. Can. J. Microbiol. **22:**29–34.
26. **Kumar, A., R. R. Ernst, and K. Wuthich.** 1980. A two-dimensional nuclear Overhauser enhancement (2D NOE) experiment for the elucidation of complete proton-proton cross-relaxation networks in biological macromolecules. Biochem. Biophys. Res. Commun. **95:**1–6.
27. **Lee, C.-J.** 1987. Bacterial capsular polysaccharides—biochemistry, immunity and vaccine. Mol. Immunol. **24(10):**1005–1019.
28. **Leelarasmee, A., and S. Bovornkitti.** 1989. Meliodiosis: review and update. Rev. Infect. Dis. **11:**413–425.
29. **Masoud, H., M. B. Perry, J.-R. Brisson, D. Uhrin, and J. C. Richards.** 1994. Structural elucidation of the backbone oligosaccharide from the lipopolysaccharide of Moraxella catarrhalis serotype A. Can. J. Chem. **72:**1466–1477.
30. **Mort, A. J., S. Parker, and M.-S. Kuo.** 1983. Recovery of methylated saccharides from methylation reaction mixtures using Sep-Pak C-18 cartridge. Anal. Biochem. **133:**380–384.
31. **Perry, M. B., L. L. MacLean, T. Schollaardt, L. E. Bryan, and M. Ho.** 1995. Structural characterization of the lipopolysaccharide O-antigens of *Burkholderia pseudomallei*. Infect. Immun. **63:**3348–3352.
32. **Perry, M. B., and D. T. Williams.** 1969. The structure, analysis, and occurrence of 3-deoxy-D-manno-octulosonic acid. Can. J. Biochem. **47:**691–695.
33. **Peters, T., B. Meyer, R. Stuike-Prill, R. Somorjai, and J.-R. Brisson.** 1993. A. Monte Carlo method for conformational analysis of saccharides. Carbohydr. Res. **238:**49–73.
34. **Popov, S. F., V. Y. Kurilov, and A. T. Iakovlev.** 1995. *Pseudomonas pseudomallei* and *Pseudomonas mallei* are capsule-forming bacteria. Zh. Mikrobiol. (Moscow) **5:**32–36.
35. **Popov, S. F., N. G. Tikhonov, and V. Y. Kurilov.** 1994. Influence of capsule formation in *Pseudomonas pseudomallei* and *Pseudomonas mallei* on their relations with host cells. Immunol. Infect. Dis. **4(3):**142–145.
36. **Robbins, J. B.** 1978. Vaccines for the prevention of encapsulated bacterial diseases: current status, problems and prospects for the future. Immunochemistry **15:**839–854.
37. **Schmidt, M. A., and K. Jann.** 1983. Structure of the 2-keto-3-deoxy-D-manno-octonic acid containing capsular polysaccharide (K12 antigen) of the urinary-tract-infective Escherichia coli O4:K12:H^-. Eur. J. Biochem. **131:** 509–517.
38. **Shaka, A. J., J. Keeler, F. Frenkiel, and R. J. H. Freeman.** 1983. An improved sequence for broadband decoupling: WALTZ-16. J. Magn. Reson. **52:**335–338.
39. **Steinmetz, I., M. Rohde, and B. Brenneke.** 1995. Purification and characterization of an exopolysaccharide of *Burkholderia* (*Pseudomonas*) *pseudomallei*. Infect. Immun. **63:**3959–3965.
40. **Subramanian, S., and A. Bax.** 1987. Generation of pure phase subspectra for measurement of homonuclear coupling constants. J. Magn. Reson. **71:**325–330.
41. **Unger, F. M.** 1981. The chemistry and biological significance of 3-deoxy-D-*manno*-2-octulosonic acid (KDO). Adv. Carbohydr. Chem. Biochem. **38:** 323–388.
42. **Yabuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki, and M. Arakawa.** 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni & Holmes 1981) comb. nov. Microbiol. Immunol. **36:**1251–1275.
43. **York, W. S., A. G. Darvill, M. McNeil, and P. Albersheim.** 1985. 3-Deoxy-D-*manno*-2-octulosonic acid (KDO) is a component of rhamnogalacturonan II, a pectic polysaccharide in the primary cell walls of plants. Carbohydr. Res. **138:**109–126.
44. **York, W. S., A. G. Darvill, M. McNeil, T. T. Stevenson, and P. Albersheim.** 1985. Isolation and characterization of plant cell wall components. Methods Enzymol. **118:**3–40.