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The structure of the capsular polysaccharide from a swarming strain of pathogenic *Proteus vulgaris*

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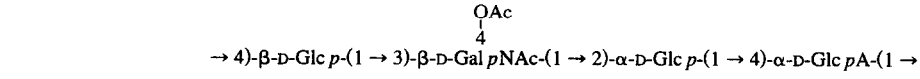
Abstract

The structure was determined for the capsular polysaccharide (CPS) isolated from a swarming strain of *Proteus vulgaris*, CP2-96, which was obtained from the spleen of an infected mouse. The CPS was extracted from the cell pellet by hot water, precipitated with ethanol, and further purified by gel-permeation chromatography. The structure was established by glycosyl composition and linkage analyses, and by NMR spectroscopy. The sequence of the glycosyl residues was determined by a NOESY experiment. The CPS is composed of a tetrasaccharide repeating unit with the following structure:



Keywords: *Proteus vulgaris*; Capsular polysaccharide; Lipopolysaccharide

1. Introduction

Bacteria of the genus *Proteus* are human opportunistic pathogens that frequently cause urinary tract and wound infections [1,2] and may also be involved in rheumatoidal diseases [3]. This genus includes three species: *Proteus mirabilis*, *Proteus vulgaris*, and *Proteus myxofaciens*. *P. mirabilis* and *P. vulgaris* are divided into 49 O-serogroups [4]; however, a number of smooth strains remain unclassified [4].

Recent genetic and compositional analyses of a capsular polysaccharide (CPS) of *P. mirabilis*, a species closely related to *P. vulgaris*, indicated that its capsule was required for virulence since capsule-minus mutants were impaired in their ability to migrate across solid surfaces and to cause urinary tract infections in mice [5,6]. In these studies, mutation of

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capsular genes did not affect the outer membrane flagella or hemolysin, suggesting that the capsule provides a physical milieu favorable to the functioning of flagella. Little is known about the structure of any of the *Proteus* spp. capsules other than that a few immunological differences are evident [7]. The previously reported structure of CPS from *P. mirabilis* [8] bore no resemblance to the CPS composition reported in a later genetic study of *P. mirabilis* [5]. This lack of information suggests that several capsules from various swarming *Proteus* spp. require characterization before it can be understood how capsular structure influences swarming migration. This report describes the structure of a CPS isolated from a swarming *P. vulgaris* isolate, strain CP2-96, that has not been passaged in the laboratory. This *P. vulgaris* isolate was more adept at undergoing swarm cell migration than the more characterized *P. mirabilis* strains used for genetic manipulations. It is not yet possible to tell if the structure of its capsule is directly responsible for this enhanced swarm migration. However, the selection of isolates by their ability to swarm on at least two different agar surfaces provides a strategy to examine the structural features of capsules that may be associated with swarming [9].

The lipopolysaccharides (LPSs) from a number of clinically isolated *Proteus* strains have been structurally and serologically characterized [10]. One other CPS structure from a clinical isolate of *P. vulgaris* has been reported; it has a trisaccharide repeating unit consisting of 2:1:1 D-galactose, *N*-acetyl-D-glucosamine, and pyruvic acid [11].

2. Results and discussion

Isolation and purification of CPS.—The crude CPS was characterized by PAGE analysis (Fig. 1). Previous work [12] has shown that acidic CPS can be visualized when the gel is fixed in the presence of Alcian blue prior to silver staining, while LPS stains with or without the use of Alcian blue. The result showed that the crude CPS contained some contaminating LPS. Crude CPS was separated into two fractions by a Sephadex G-150 column using buffer containing deoxycholate (DOC). The DOC–PAGE analysis of the fractions separated from the Sephadex G-150 column is shown in Fig. 1. The high-molecular-weight fraction (HMW) is stained only in the presence of Alcian blue (Fig. 1A) and showed a broad band of low mobility that is characteristic for CPS. The low-molecular-weight (LMW) fraction

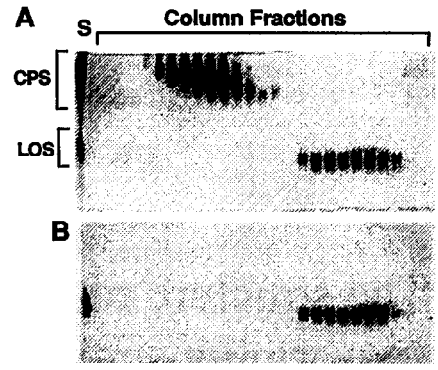

Fig. 1. DOC–PAGE analysis of the fractions from a Sephadex G-150 column of the crude CPS from *Proteus vulgaris*. Well 'S' contains crude CPS prior to a Sephadex G-150 column. Fractions (5 mL) were collected of which 10-μL samples of every other fraction starting with fraction 21 were applied to each well. The gel shown in Panel A was fixed in the presence of Alcian blue prior to silver staining, while that shown in Panel B was fixed without Alcian blue. Fractions 29–41 were combined and comprise CPS (CPS–DOC), and fractions 53–67 were combined and comprise pure low-molecular-weight lipopolysaccharide (LMW-LPS), i.e. the LOS.

stained even in the absence of Alcian blue and showed a broad band of high mobility-characteristic of LMW-LPS, i.e. lipooligosaccharide (LOS). The CPS purified by this method is referred to as CPS–DOC.

During the purification of CPS by Sephadex G-150 column chromatography using DOC buffer and during the removal of DOC from the separated fractions by dialysis, the CPS was exposed to alkaline conditions (pH 9.5) for 7 days. Due to the alkaline conditions, the O-acetyl substituents present in the CPS were hydrolyzed. In order to purify CPS without removal of O-acetyl groups, a portion of crude CPS (50 mg) was hydrolyzed with mild acid, and purified by Sephadex G-75 column chromatography. Two fractions were obtained; the high-molecular-weight CPS and LMW-oligosaccharides (OSs) derived from the LOS. The CPS eluted just after the void volume and the LMW-OS eluted at twice the void volume (not shown). The yields of CPS and LMW-OS were 35 and 5 mg, respectively. The CPS purified by this method is referred to as CPS–G75.

Composition analysis.—The results of glycosyl and fatty acid composition analyses of CPS–DOC, LOS, and CPS–G75 are shown in Table 1. Both CPS–DOC and CPS–G75 have very similar glycosyl compositions, i.e. glucose, glucuronic acid, and *N*-acetyl-D-glucosamine in the molar proportion of 2:1:1. The LOS contains glycosyl residues characteristic of LPS core oligosaccharides, i.e. glucose, galactose,

Table 1
Composition analysis of CPS–DOC, CPS–G75, and LOS isolated from *P. vulgaris* CP2-96 [a]

Residue	Relative mole-%		
	CPS–DOC	LOS	CPS–G75
Mannose	–	21	–
Glucose	57	48	55
Glucuronic acid	23	–	24
Galactose	–	10	–
Glucosamine	–	3	–
Galactosamine	20	–	21
D,D-Heptose	–	7	–
L,D-Heptose	–	10	–
$C_{14:0}$	–	+	–
$C_{16:0}$	–	+	–
3-OH–$C_{14:0}$	–	+	–

[a] –, absent; +, present in significant amounts but not quantified.

mannose, D,D-heptose, and L,D-heptose [13]. Fatty acid analysis showed that CPS did not contain any detectable fatty acyl residues, whereas the LOS contains myristic, palmitic, and β-hydroxy myristic acids, a result consistent with the presence of lipid A. Determination of the absolute configurations of the glycoses present in CPS revealed that all glycoses had the D-configuration.

Glycosyl linkage analysis.—Glycosyl linkage analysis was performed by methylation, followed by hydrolysis, reduction, and preparation of alditol acetates. Linkage analysis of the uronic acid was performed by methanolysis, followed by reduction of the permethylated sample prior to hydrolysis. The glycosyl linkages of the CPS are shown in Table 2. Prior to carboxyl group reduction, 2-linked glucose, 4-linked glucose, and 3-linked *N*-acetyl-D-galactosamine were present in a 1:1:1 ratio. After the carboxyl group reduction, an additional glucosyl residue was observed. The mass spectrum of its partially methylated alditol acetate was consistent with a 4,6-linked residue with two deuteride atoms at C-6, and thus was derived from 4-linked glucuronic acid.

NMR spectroscopic analysis.—The ^{1}H NMR spectrum of the CPS–DOC (Fig. 2) confirmed that galactosamine was N-acetylated, as indicated by a singlet at 2.05 ppm. The ^{1}H NMR spectrum of the CPS–G75 fraction (data not shown) showed that it has exactly the same chemical shifts as those of CPS–DOC, except that it contained an O-acetyl residue as indicated by an additional signal at δ 2.10. Integration of this spectrum showed that the N-acetyl to O-acetyl ratio is 1:1. The anomeric region shows two downfield α-anomeric proton signals (Table 3)

Table 2
Glycosyl linkage analysis of CPS–DOC isolated from *P. vulgaris* CP2-96

Glycosyl residue	Linkage	Relative mole-%	
		CPS–DOC	CR–CPS–DOC
Glucose	2-linked	40	33
Glucose	4-linked	37	28
Glucose	4,6-linked	–	22
Galactosamine	3-linked	23	17

[a] CPS–DOC = methylation analysis of CPS–DOC; CR–CPS–DOC = carboxyl reduction of CPS–DOC after permethylation.

at δ 5.63 and 5.32 ($J_{H-1,H-2}$ 3 Hz), and two β-anomeric proton signals at δ 4.72 and 4.53 ($J_{H-1,H-2}$ 8.0 Hz). With the aid of 2D COSY (spectrum not shown), TOCSY (Fig. 3, top panel), and broad-band decoupled HSQC (spectrum not shown) spectra, almost all of the ^{1}H and ^{13}C NMR signals could be assigned (Tables 3 and 4). The four glycosyl residues were designated **A–D** according to their increasing anomeric chemical shifts.

Residue **A** has an anomeric signal at δ 4.53 and a $J_{H-1,H-2}$ coupling constant of 8 Hz, indicating that it is a β-linked residue. The proton chemical signals (Table 3) from H-1 to H-4 for residue **A** were assigned from the COSY and TOCSY (Fig. 3, top panel) experiments, and the H-5, H-6, and H-6′ signals from the TOCSY experiment via subscan through its H-4 signal. A large $J_{H-3,H-4}$ coupling constant (> 5 Hz) was observed for **A**, supporting the conclusion it is a D-glucosyl residue. The carbon signals (Table 4) from C-1 to C-6 for residue **A** were determined from the HSQC spectrum. The downfield position of the C-4

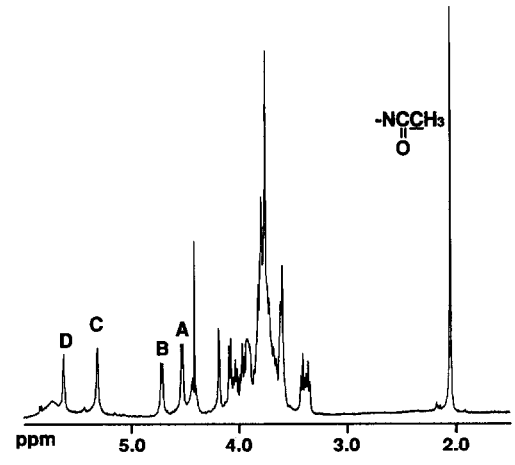

Fig. 2. The ^{1}H NMR spectrum at 500 MHz of *Proteus vulgaris* CPS–DOC.

Table 3
^{1}H NMR data at 60 °C for the CPS–DOC isolated from *P. vulgaris* CP2-96

Glycosyl residue	Chemical shifts [a]						
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→ 4)-β-D-Glc*p*-(1 → **A**	4.53 [8]	3.36	3.77	3.61	3.60	3.73	3.91
→ 3)-β-D-GalNAc*p*-(1 → **B**	4.72 [8]	4.03	3.92	4.19	3.72	3.76	
→ 4)-α-D-GlcA *p*-(1 → **C**	5.32 [3]	3.67	3.98	3.81	4.08		
→ 2)-α-D-Glc*p*-(1 → **D**	5.63 [3]	3.61	3.76	3.41	3.71	3.85	3.80

[a] $J_{H-1,H-2}$ values are given in square brackets.

carbon signal (δ 81.8) indicates that residue **A** is linked at this position. Thus, **A** is identified as the 4-linked glucosyl residue since this is the only 4-linked hexose present in the CPS. The carbon chemical shifts from C-1 to C-6 for residue **A** (Table 4) are also similar to those previously reported for a 4-linked glucosyl residue [14].

The anomeric signal for residue **B** is δ 4.72 ($J_{H-1,H-2}$ 8.0 Hz), showing that it is β-linked. The proton chemical shifts (Table 3) from H-1 to H-5 protons were assigned from COSY and TOCSY (Fig. 3, top panel) spectra, and an H-6 proton could be assigned from the TOCSY spectrum via a subscan through its H-5 signal. A relatively small $J_{H-3,H-4}$ coupling constant (< 5 Hz) for residue **B** indicates that it has a *galacto* configuration. The carbon chemical shifts (Table 4) from C-1 to C-6 for residue **B** were assigned from HSQC spectrum. The C-2 chemical shift of residue **B** is δ 52.8, typical of a nitrogen-bearing carbon. The downfield shift of C-3 (δ 81.2) indicates that residue **B** is 3-linked. Glycosyl linkage analysis (Table 2) showed that galactosamine is the only 3-linked aminoglycosyl residue found in the CPS. Therefore, residue **B** is identified as the 3-linked *N*-acetyl-β-galactosaminosyl residue. The carbon chemical shifts for residue **B** (Table 4) are also very similar to published values [14].

Residue **C** has an anomeric proton chemical shift at δ 5.32 ($J_{H-1,H-2}$ 3 Hz), indicating that it is α-linked. The proton chemical shifts (Table 3) from H-1 to H-5 for residue **C** were assigned from the COSY and TOCSY (Fig. 3, top panel) spectra. The large $J_{H-2,H-3}$ and $J_{H-3,H-4}$ coupling constants (> 5 Hz) indicate that the residue **C** has a *gluco* configuration. The carbon chemical shifts (Table 4) from C-1 to C-5 were assigned from HSQC spectrum. The downfield chemical shift of C-4 (δ 77.6) indicates that residue **C** is 4-linked. Glycosyl linkage analysis (Table 2) showed one 4-linked glucosyl residue and one 4-lin-

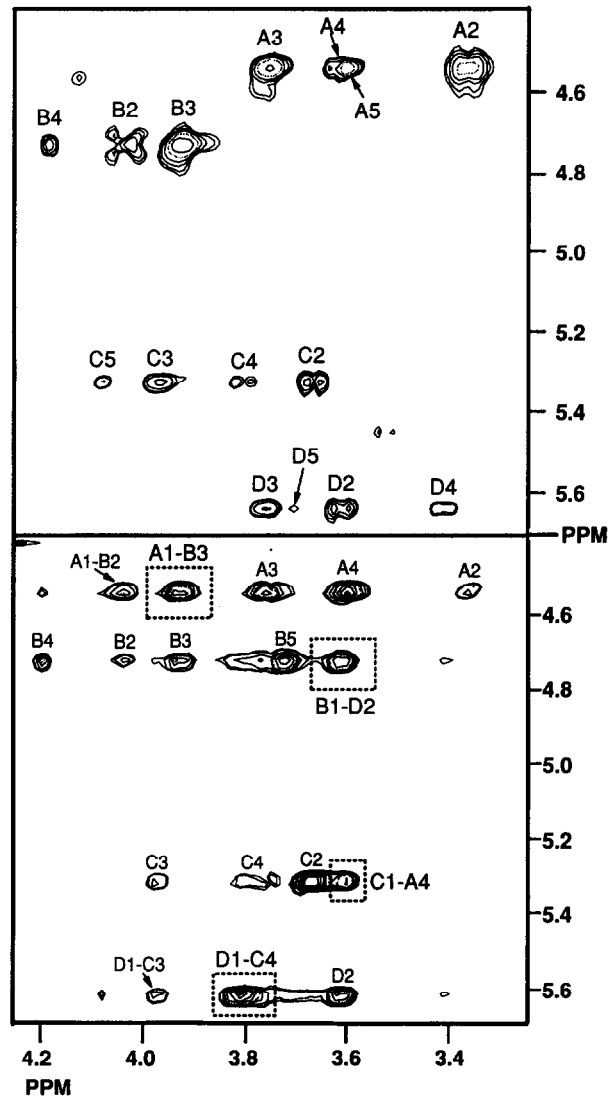

Fig. 3. The ^{1}H–^{1}H TOCSY (top panel) and ^{1}H–^{1}H NOESY (bottom panel) spectra of *Proteus vulgaris* CPS–DOC. The 'boxed' signals shown in the NOESY spectrum indicate the strong interresidue NOE contacts from which the glycosyl sequence was deduced. The mixing time for the TOCSY spectrum shown was 120 ms. Complete assignment required several TOCSY experiments requiring several mixing times ranging from 60 to 320 ms.

Table 4
^{13}C NMR data at 60 °C for the CPS–DOC isolated from *P. vulgaris* CP2-96

Glycosyl residue	Chemical shifts					
	C-1	C-2	C-3	C-4	C-5	C-6
→ 4)-β-D-Glc*p*-(1 → **A**	105.2	73.9	77.1	81.8	75.7	61.9
→ 3)-β-D-GalNAc*p*-(1 → **B**	104.3	52.8	81.2	69.2	76.1	61.7
→ 4)-α-D-GlcA *p*-(1 → **C**	101.2	72.6	74.8	77.6	74.6	
→ 2)-α-D-Glc*p*-(1 → **D**	99.3	79.3	72.8	70.9	72.6	61.9

ked glucuronosyl residue. Since residue **A** is the 4-linked glucosyl residue, residue **C** is identified as the 4-linked α-glucuronosyl residue.

The anomeric proton chemical shift for residue **D** was observed at δ 5.63 ($J_{H-1,H-2}$ 3 Hz), indicating that it is α-linked. The proton chemical shifts (Table 3) from H-1 to H-5 for residue **D** were assigned from the COSY and TOCSY (Fig. 3, top panel) spectra, and the chemical shifts of H-5, H-6, and H-6′ were assigned from a TOCSY subscan through its H-4 signal. The $J_{H-3,H-4}$ and $J_{H-4,H-5}$ coupling constants for residue **D** are similar to those for residue **A** (i.e. > 5 Hz), indicating that it has a *gluco* configuration. The carbon chemical shifts (Table 4) from C-1 to C-6 carbon for residue **D** were determined from the HSQC spectrum. The downfield chemical shift for C-2 (δ 79.3) indicates that residue **D** is 2-linked. The only 2-linked hexosyl residue observed in the glycosyl linkage analysis of the CPS (Table 2) was 2-linked glucose. Thus, residue **D** is identified as the α-2-linked glucosyl residue.

The sequence of glycosyl residues was determined from a NOESY experiment (Fig. 3, bottom panel; Table 5). Residue **A** has an NOE contact from H-1 to H-3 of residue **B**, in addition to intraresidue NOE contacts to H-3 and H-5, and a weak interresidue contact to H-2 of residue **B**. Since residue **B** is 3-linked, the following sequence was established:

→ 4)-β-D-Glc *p*-(1 → 3)-β-D-Gal *p*NAc-(1 →
A B
1

Residue **B** has a strong NOE contact from H-1 to H-2 of residue **D**, in addition to intraresidue contacts to H-3, H-5, and H-6, indicating that residue **B** is

Table 5
NOE data for the CPS–DOC isolated from *P. vulgaris* CP2-96

Anomeric proton		NOE contact to proton		
Glycosyl residue	δ	δ	Intensity [a]	Residue, atom
→ 4)-β-D-Glc*p*-(1 → **A**	4.53	3.36	m	**A** H-2
		3.60	m	**A** H-5
		3.75	w	**A** H-3
		3.92	s	**B** H-3
		4.03	w	**B** H-2
→ 3)-β-D-GalNAc*p*-(1 → **B**	4.72	3.61	s	**D** H-2
		3.72	m	**B** H-5
		3.92	w	**B** H-3
		4.03	w	**B** H-2
		4.19	w	**B** H-4
→ 4)-α-D-GlcA *p*-(1 → **C**	5.32	3.60	s	**A** H-4
		3.66	s	**C** H-2
		3.97	w	**C** H-3
		4.08	w	**C** H-5
→ 2)-α-D-Glc*p*-(1 → **D**	5.63	3.61	s	**D** H-2
		3.81	s	**C** H-4

[a] The intensities are estimated from visual inspection of the NOESY spectrum shown in Fig. 3 and are given as: s = strong, m = medium, and w = weak.

linked to the 2-position of residue **D**. Thus, the trisaccharide element **A–B–D** was established,

→ 4)-β-D-Glc*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → 2)-α-D-Glc*p*-(1 →
A B D

2

Residue **D** has a strong NOE contact of H-1 to H-4 of residue **C** and a weak contact to H-3 of residue **C**. Since residue **C** is 4-linked, residue **D** is linked to the 4-position of residue **C**. In addition, residue **C** has a strong NOE contact from H-1 to H-4 of residue **A**, showing that residue **C** is linked to the 4-position of residue **A**. Therefore the tetrasaccharide repeating unit **–(A–B–D–C)–** was established,

→ 4-β-D-Glc*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → 2)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*A-(1 →
A B D C

3

The COSY and TOCSY experiments were also performed on the CPS–G75 fraction, the CPS fraction which still contains the O-acetyl substituent (data not shown), and showed that all the proton chemical shifts for the residues **A–D** were identical to those for CPS–DOC (shown in Table 3), except that CPS–G75 shows a downfield shift for the H-4 proton of residue **B**; i.e. the proton chemical shift at H-4 for residue **B** in the CPS–G75 is δ 4.25 while it is δ 4.19 in CPS–DOC (Table 3). This result indicates that the O-acetyl group present in CPS–G75 is attached at the O-4 position of residue **B**.

From the combined results it is concluded that the CPS from *P. vulgaris* CP2-96 is composed of a tetrasaccharide repeating unit with the following structure:

OAc
4
→ 4-β-D-Glc*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → 2)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*A-(1 →
A B D C

3. Experimental

Bacterial strains and conditions of growth.—*Proteus vulgaris* CP2-96 was isolated by culturing the spleen of a naturally infected mouse (Musmuscalis), which was caught as part of a larger study of spleen-invasive enteric organisms present in the environment of laying hens and in resident mice. The spleen was removed and a cellular suspension was made as previously reported [15] by adding 200 μL brain–heart infusion (BHI) broth (Difco, Detroit, MI) to an O-ring capped 2-mL microcentrifuge tube and vortexing the spleens for 2 min with 1.0-mm sterilized glass beads in a mini-bead beater apparatus (Biospec). Fifty μL of spleen suspension was spread on to the surface of brilliant green (BG) agar (BBL, Becton–Dickenson, Cockeysville, MD). The culture was grown overnight at 37 °C, and colony morphology was observed the next day. After observing that this culture produced terraced, spreading colonies that extended across 50% of the agar surface by the next day, cells at the edge of the swarm colony were transferred to BG agar again to assess if other contaminants might be present. After determining that no contaminants were present, initial biochemical characterization was made by inoculating a commercially packaged battery of diagnostic reagents (Enterotube II, Becton–Dickenson, Cockeysville, MD). After initial characterization indicated that this swarm-proficient strain appeared to be a *Proteus* spp., a culture was sent to National Veterinary Services Laboratories, Ames, IA, where it was definitively characterized as *P. vulgaris*, although it failed to ferment two sugars, maltose and xylose.

Isolation and purification of CPS.—Bacterial cells were grown in BHI-broth, harvested by centrifugation, and washed once in PBS as described by Lee and Cherniak (1974) [16]. One hundred grams (wet weight) of bacterial cells were suspended in 300 mL of water and vigorously stirred in boiling water for 30 min. The suspension was cooled in an ice bath, and stirring was continued for 90 min. The cell residue was removed by centrifugation (10,000 × *g*, 30 min, 4 °C), the supernatant was adjusted to 1% CH_3COOH, and crude CPS was precipitated by the addition of EtOH (2.5 vol, 24 h, −20 °C). The CPS precipitate was collected by centrifugation (10,000 × *g*, 30 min, 4 °C), washed in EtOH, followed by acetone, dried, dissolved in water, and lyophilized. This crude CPS was suspended in a soln containing 6 mL of EDTA (0.05 M Na_2HPO_4–0.005 M EDTA, pH 7.0), 3 mL of DNase (4 mg in 0.04 M $MgCl_2$), and 3 mL of RNase (20 mg in 0.04 M $MgCl_2$). This soln was incubated for 16 h at 37 °C, followed by the addition of proteinase K (4 μg), and incubated again for 16 h at 37 °C. The resulting soln was dialyzed against distilled water for 48 h, centrifuged at 5000 × *g* for 20 min, and the supernatant was lyophilized. The final yield was 800 mg of crude CPS. Crude CPS was further purified by fractionation on a Sephadex G-150 column (90 × 1.6 cm) using a buffer soln consisting of

0.2 M NaCl, 1 mM EDTA, 50 mM Tris base, and 0.25% deoxycholic acid (DOC), pH 9.25. The content of each fraction was identified by analytical DOC–PAGE using 18% acrylamide [17]. Gels were fixed either with or without Alcian blue [18] and silver stained for LPS [19].

The CPS was also purified by mild acid hydrolysis in aq 1% CH_3COOH (25 mL) at 100 °C for 2 h, cooled, and centrifuged (10,000 × *g*). The supernatant was extracted with diethyl ether (3 × 10 mL), and the aq layer was fractionated on a Sephadex G-75 column (90 × 1.6 cm). The fractions were assayed for hexose with phenol–H_2SO_4. The resulting CPS and oligosaccharide (OS) fractions were lyophilized.

Nuclear magnetic resonance (NMR) spectroscopy.—Samples were prepared for NMR analysis by a two-fold lyophilization from D_2O, dissolved in D_2O, and analyzed. Spectra were recorded at 40 °C. Chemical shifts are reported in ppm, using sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) and acetone (δ_C 31.00) as internal references. All NMR spectra were recorded on Bruker AMX 500 or DRX 600 MHz spectrometers. Two-dimensional DQF–COSY [20], TOCSY [21,22], and NOESY [23] data sets were collected in phase-sensitive mode using the States–TPPI [24] method. In these experiments, low-power presaturation was applied to the residual HDO signal. Typically, data sets of 2048(t_2) × 512(t_1) complex points were collected with 16 scans per FID, and a sweep width in both dimensions of 6 ppm. The TOCSY experiments contained MLEV17 [25] mixing sequences ranging from 60 to 320 ms, and the NOESY mixing delay was 200 ms.

A gradient HSQC [26] data set was collected using the echo–antiecho method for pure absorption data. A data set of 2048(t_2) × 512(t_1) complex points was acquired, with 32 and 64 scans per FID. The sweep width was 7 ppm for proton (F2) and 60 ppm for carbon (F1). The GARP [27] sequence was used for ^{13}C decoupling during acquisition. Data were processed typically with a lorentzian-to-gaussian weighting function applied to t_2 and a shifted squared sinebell function and zero-filling applied to t_1. Data shown were processed with Felix software (Molecular Simulations, Inc.).

Glycosyl composition analyses.—Glycosyl composition of CPS (0.5 mg each) was performed by hydrolysis in 2 M CH_3COOH (0.5 mL) in a closed vial at 120 °C for 3 h. The glycoses in the hydrolysate were reduced with $NaBH_4$, acetylated, and analyzed by GLC and combined GLC–MS [28]. For the determination of uronic acid, the CPS sample (0.5 mg) was dried in vacuum, methanolyzed in 1 mL of MeOH–2 N HCl at 80 °C for 16 h. The resulting methyl glycosides were either trimethylsilylated, and the mixture was analyzed by GLC–MS [28], or reduced with $NaBH_4$ (10 mg) in water (100 μL), acetylated, and analyzed by GLC–MS. The absolute configurations of the glycoses present were determined by GLC–MS analysis of the trimethylsilylated (*S*)-(+)-2-butyl and (*S*)-(−)-2-butyl glycosides [29,30].

Glycosyl linkage analyses.—Glycosyl linkage analysis was carried out using a modified NaOH method [31,32]. The sample (1 mg) was dissolved in Me_2SO (100 μL), powdered NaOH (100 mg) was added, and the reaction mixture was stirred rapidly at room temperature for 30 min. Methylation was performed by the sequential additions of iodomethane (10, 10, and 20 μL) at 10-min intervals. After an additional 20 min stirring, 1 mL of 1 M $Na_2S_2O_3$ was added, and the methylated glycans were recovered in the organic phase by extraction with $CHCl_3$ (0.5 mL × 3). The permethylated product was further purified by reversed-phase chromatography using a Sep-Pak C_{18} cartridge [33]. The methylated glycan was hydrolyzed with 2 M CH_3COOH (120 °C, 3 h), reduced with NaB^2H_4, acetylated, and analyzed by GLC and GLC–MS [28]. For the linkage determination of the uronic acid, the permethylated CPS sample (0.5 mg) was dried in vacuum, methanolyzed in 1 mL of MeOH–2 N HCl at 80 °C for 16 h. Released partially methylated methyl glycosides were N-acetylated with a addition of 200 μL of MeOH, 20 μL of pyridine, and 20 μL of Ac_2O at room temperature for 5 h, and dried in air. The methyl ester of the uronic acid was reduced with NaB^2H_4 (10 mg) in water (100 μL), neutralized with CH_3COOH, and dried in air with the addition of MeOH. The carboxyl-reduced products were hydrolyzed with 2 M CH_3COOH (120 °C, 3 h), reduced with NaB^2H_4, acetylated, and analyzed by GLC and GLC–MS [28].

GLC and GLC–MS analyses were performed using capillary columns (length, 30 m; inner diameter, 0.32 mm) with helium as the carrier. A DB-1 column (J&W Scientific) was used for aminoglycosyl derivatives, and an SP2330 column (Supelco, Bellefonte, PA) was used for the neutral glycosyl derivatives. GLC equipment consisted of HP5890 gas chromatograph equipped with a flame-ionization detector (Hewlett–Packard). GLC–MS (EI) was performed using a Hewlett–Packard 5970 MSD.

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