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Identification, Isolation, and Structural Studies of the Outer Membrane Lipopolysaccharide of *Caulobacter crescentus*

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The lipopolysaccharide (LPS) of the outer membrane of *Caulobacter crescentus* was purified and analyzed. Two distinct strains of the species, NA 1000 and CB2A, were examined; despite differences in other membrane-related polysaccharides, the two gave similar LPS composition profiles. The LPS was the equivalent of the rough LPS described for other bacteria in that it lacked the ladder of polysaccharide-containing species that results from addition of variable amounts of a repeated sequence of sugars, as detected by gel electrophoresis in smooth LPS strains. The purified LPS contained two definable regions: (i) an oligosaccharide region, consisting of an inner core of three residues of 2-keto-3-deoxyoctonate, two residues of α-L-glycero-D-mannoheptose, and one α-D-glycero-D-mannoheptose unit and an outer core region containing one residue each of α-D-mannose, α-D-galactose, and α-D-glucose, with the glucose likely phosphorylated and (ii) a region equivalent to the lipid A region of the archetype, consisting primarily of an esterified fatty acid, 3-OH-dodecanoate. The lipid A-like region was resistant to conclusive analysis; in particular, although a variety of analytical methods were used, no amino sugars were detected, as is found in the lipid A of the LPS of most bacteria.

Caulobacter crescentus is a stalked, gram-negative eubacterium which undergoes a simple cycle of cellular differentiation (45). Throughout the life cycle, caulobacters assemble a two-dimensional paracrystalline protein layer (S-layer) distal to the outer membrane (50). We are interested in the *C. crescentus* cell surface architecture, in particular, the interaction of the S-layer with the outer membrane. However, the outer membrane of *C. crescentus* is incompletely characterized, owing partly to the lack of a reliable method to separate the cell envelope into outer and cytoplasmic membrane fractions.

The outer membrane of characterized gram-negative bacteria is composed of phospholipids, proteins, lipopolysaccharide (LPS), and if present, extracellular polysaccharide, all of which interact to form a permeability barrier towards the aqueous milieu (40). Of these components, it is the LPS or extracellular polysaccharide that first meets the extracellular environment and, if an S-layer is present, must in some way accommodate its attachment.

We have isolated and chemically characterized the extracellular polysaccharides of two strains of *C. crescentus* (47), CB2A and NA1000, noting that there were different extracellular polysaccharides in each; both strains are in common use in our laboratory, and a description of the LPS structure would be valuable for a number of cell surface studies. Only one other study on the cell wall polysaccharides of *C. crescentus* has been published (13). Those researchers identified various amounts of mannose, galactose, glucose, D-glycero-D-mannoheptose, L-glycero-D-mannoheptose, and 2-keto-3-deoxyoctulosonate (KDO) in their preparations but did not detect D-glucosamine. Knowing the structure of the extracellular polysaccharide molecules, we were interested in determining the chemical structure of the LPS, in particular, to determine whether D-glucosamine is indeed absent from the lipid A of this organism. A dimer of D-glucosamine typically forms a key part of the amphipathic LPS molecule in that it attaches both the core sugars of the hydrophilic portion and the fatty acids of the hydrophobic region.

Agabian and Unger (2) examined the membrane fraction of disrupted cells of *C. crescentus* CB13B1a and NA1000. They found that KDO (typically present in LPS and therefore in outer membranes) was reduced by two-thirds to 10-fold, in comparison with rough-mutant or wild-type *Salmonella typhimurium*, respectively. They concluded that KDO was not a useful marker in caulobacter membrane fractionation experiments. Two other studies have cited this finding to account for the absence of KDO in their outer membrane fractions (17, 33). We were interested in determining whether *C. crescentus* is indeed deficient in KDO and thus has an atypical LPS or reduced amounts of LPS.

In this study, we report that the LPS of *C. crescentus* is of the rough type in that it does not contain a heterogeneous O antigen attached to the core sugars. Thus, the molecule may also be termed a lipooligosaccharide. However, owing to the pervasive use of the term LPS, this designation will be used in this report.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. crescentus* CB2A and NA1000 (also called CB15A in previous reports [51]) were grown in peptone-yeast extract (PYE) medium (44) at 30°C. For LPS isolation, 500-ml cultures were grown in 2-liter Erlenmeyer flasks shaken at 200 rpm and harvested during the late log phase (optical density at 600 nm, 0.6 to 0.7) and washed with 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. For some experiments, NA1000 was grown in a fermentor as a 60-liter batch culture. *Escherichia coli* B was grown in LB medium (49) at 37°C.

LPS isolation. LPS was isolated by using a modification of

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the procedure described by Darveau and Hancock (19). After nuclease digestion of the disrupted cells, the cell lysate was made to contain 0.1 M EDTA, 2% sodium dodecyl sulfate (SDS), and 10 mM Tris-HCl (pH 8.0) and then incubated at 37°C for 2 h. The extended incubation was necessary to dissociate the caulobacter cell membranes completely. The published procedure was followed until completion of the final ultracentrifugation step. The LPS pellet was suspended in 10 mM Tris-HCl (pH 8.0) and washed five times by ultracentrifugation at 200,000 × *g* for 2 h at 15°C and resuspension. The final pellet was considered to be the crude LPS fraction.

Lipid analysis revealed contamination from phospholipids, and therefore further fractionation of the LPS was done by the Sonesson et al. (52) modification of the Galanos et al. (23) procedure. The freeze-dried crude LPS preparation was extracted three times with 8 ml of phenol-chloroform-hexane (2:5:8) per g of the original dry weight of cells and centrifuged at 2,000 × *g*. The pooled supernatants were evaporated on a rotary evaporator, and the phenol was removed by dialysis against distilled, deionized water (4 × 2 liters). The LPS recovered by lyophilization was washed three times with 10 ml of chloroform-methanol (2:1) and precipitated by centrifugation (200,000 × *g* for 2 h). The precipitate was dried by a stream of nitrogen gas, dissolved in distilled, deionized water, and then freeze-dried to yield the pure LPS fraction.

LPS chromatography. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) by using the Laemmli (34) buffer system with a 4% stacking gel and a 12.5% separating gel. The gels were stained by using either Coomassie blue, the method of Tsai and Frasch (55), or the Bio-Rad silver stain kit (Bio-Rad Laboratories, Mississauga, Ontario, Canada) (39). Samples of cells were also prepared by the method of Hitchcock and Brown (27) for qualitative analysis of LPS by SDS-PAGE.

Thin-layer chromatography (TLC) was performed on Merck aluminum sheets coated with Silica Gel 60F (0.2-mm thickness). Samples were dissolved in water at a concentration of 2 mg/ml, and 5 drops were applied. A solvent system of isobutyric acid-1 M NH_4OH (5:3) (14) was used, and the carbohydrate-containing spots were visualized by spraying with *p*-anisaldehyde–sulfuric acid–ethanol (1:1:18), followed by heating at 110°C for 5 to 10 min.

Colorimetric assays. An LKB Biochrom Ultrospec II UV/VIS spectrophotometer was used for all colorimetric assays. Protein levels were determined by the method of Markwell et al. (36), KDO was determined by the method of Karkhanis et al. (31), P_i was determined by the method of Ames and Dubin (3), and sugars were determined by the method of Dubois et al. (20).

For determination of total cellular KDO, 1 ml of 0.2 N H_2SO_4 was added to 5 to 10 mg of freeze-dried cells and heated at 100°C for 30 min. The tubes were centrifuged to pellet the cells, and the clear supernatant was saved. The cells were then suspended in 1 ml of 0.2 N H_2SO_4 and again pelleted by centrifugation. Both supernatants were then assayed for KDO. A third extraction of the cells yielded no thiobarbituric acid-positive material.

Purified LPS was subjected to a variety of hydrolysis conditions for KDO analysis. Samples were hydrolyzed by using 0.2, 0.5, and 1.0 N H_2SO_4 and heated at 100°C for 20, 30, or 60 min. LPS treated with HF was also assayed.

Cleavage of LPS into lipid A and polysaccharide. LPS was cleaved by 0.1 M acetate buffer (pH 4.4) at 100°C for 1 h (12). Hydrolysis was monitored by TLC. Lipid A and uncleaved LPS were pelleted by ultracentrifugation at 425,000 × *g* for 10 min by using a TLA 100.2 rotor (Beckman, Palo Alto, Calif.), redissolved in the buffer, and hydrolyzed for a further hour if this was shown to be necessary by TLC analysis. The precipitated lipid A was extracted twice with water and once with acetone and lyophilized. The polysaccharide was obtained by freeze-drying of the combined acetate solutions. The residual sodium acetate was removed as follows. The polysaccharide was dissolved in water and reduced by using $NaBD_4$. The solution was acidified by using glacial acetic acid and treated with Amberlite IR120 (H^+) ion-exchange resin before lyophilization. Residual borate was removed by coevaporation (five times) with methanol to yield the reduced core oligosaccharide.

Carbohydrate composition. The neutral and amino sugars of the samples were determined by gas chromatography (GC) of the alditol acetate derivatives on a Hewlett-Packard 5890A gas chromatograph fitted with dual flame ionization detectors and a 3392A recording integrator. Separations were performed on a fused-silica DB17 capillary column (12 m by 0.25 mm [inside diameter]; J & W Scientific, Rancho Cardova, Calif.) by using program A (180°C for 2 min and then a heating rate of 5°C/min to bring the temperature to 240°C), program B (160°C for 2 min and then 1.5°C/min to 240°C), or program C (170°C for 10 min and then 15°C/min to 240°C). When possible, identification and quantitation were done by comparison to authentic standards with inositol as the internal standard. Alditol acetate derivatives of the glycosyl residues were also identified by GC-mass spectrometry (MS) by using the DB17 capillary column on a Varian Vista 600 series GC coupled directly to a Selsi Nermag R10-10C quadrupole MS. The peaks eluted were ionized by electron impact and chemical ionization (using ammonia) MS to obtain both the detailed fragmentation patterns (30) and molecular weights (29) of the compounds, respectively.

A variety of hydrolytic procedures were used: A, 2 M trifluoroacetic acid (TFA) at 100°C for 18 h; B, 4 M TFA at 120°C for 1 h; C, methanolysis using 1, 2, or 4 M HCl in methanol at 100°C for 18 h; D, 4 M HCl at 100°C for 6 to 18 h. The acid was removed either by coevaporation with water (A and B) or *tert*-butyl alcohol (C) at temperatures less than 40°C or by neutralization achieved by trituration with Ag_2CO_3 (D).

The hydrolysates were reduced by using $NaBH_4$ in water at room temperature for at least 1 h. Excess $NaBH_4$ was destroyed by using glacial acetic acid, and the sample was coevaporated with 10% acetic acid–methanol (three times) and then with methanol (three times) to remove the borate. The sodium acetate generated was used as the catalyst in the acetylation reaction, which was achieved by using acetic anhydride at 100°C for 1 h. In some instances, pyridine was added to ensure acetylation. The derived alditol acetates were extracted into chloroform and washed sequentially with 10% sulfuric acid, water, saturated sodium hydrogen carbonate, and then water (five times). GC analysis was performed after drying the chloroform extract with anhydrous sodium sulfate.

Method C provided a method for simultaneous determination of acidic and neutral sugars by the procedure recommended by Dudman et al. (21). Samples were methanolysed and then neutralized by coevaporation by using *tert*-butyl alcohol. The residue was dissolved in ethanol and reduced overnight by using $NaBD_4$ at room temperature to effect reduction of the methyl-esterified carboxyl groups of uronic acid to 6,6-dideuterio-substituted glycosyl residues. Excess $NaBD_4$ was then decomposed by addition of glacial acetic

acid, and the samples were coevaporated with 10% acetic acid–methanol (three times) and then with methanol (three times). The methyl glycosides were then hydrolyzed at 120°C for 1 h in 2 M TFA to yield the free sugars which were converted to their alditol acetate derivatives as described above.

The absolute configurations of the sugars were assigned by comparison of the retention times of their trimethylsilylated (−)-2-butyl glycosides with those of standards (24). The polysaccharide samples (2 mg) were solubilized in methanolic 4 M HCl for 24 h at 85°C. HCl was removed by addition of *tert*-butyl alcohol, followed by evaporation under a stream of nitrogen gas. The methyl glycosides were vacuum dried overnight and then treated with (−)-2-butanolic 1 M HCl (saturated butanol in 1 M HCl) (1 ml) for 8 h at 80°C. After removal of the HCl as before, the residues were vacuum dried overnight and then treated with hexamethyldisilazane-chlorotrimethylsilane-pyridine (0.4 ml, 1:1:5) for 30 min at room temperature. Standards of the trimethylsilylated (+)- and (−)-2-butyl glycosides of D-mannose, D-glucose, and D-galactose were prepared as described above, except that the methanolysis step was not required. GC was performed by using the DB17 column and heating program C. (+)-Alkyl glycosides have the same elution profiles as their (−)-alkyl glycoside enantiomers on nonchiral stationary phases, thus both of the enantiomers of each sugar were accounted for.

The presence of amino acids and amino sugars was investigated by using an 11BBL amino acid analyzer (Beckman), following hydrolysis under vacuum in 6 N HCl at 100°C for 18 h by standard procedures by the University of Victoria Biochemistry Department.

An LPS sample (5 mg) was also treated with 0.1 M sodium periodate (2 ml) in the dark at room temperature (1). After 3 days, the excess periodate was decomposed and the oxidized material was reduced by $NaBH_4$. Following decomposition of the hydride with acetic acid, the solution was freeze-dried and methanol was added to the residue and removed by evaporation (three times). The sugar composition was investigated by hydrolysis using method B.

Methylations of both the intact LPS and the reduced core oligosaccharide were attempted by using the Hakomori method (25, 26), as modified by Phillips and Fraser (43) or the NaOH-dimethyl sulfoxide procedure of Ciucanu and Kerek (16). In both cases, further methylation was carried out by the Purdie and Irvine method (46). Methylated samples were recovered by extraction into chloroform and analyzed by GC-MS after hydrolysis (method B) and conversion of the aldoses into partially O-methylated alditol acetates.

Dephosphorylation of LPS samples was performed either by using 48% HF at 4°C for 72 h or by achieving complete solvolysis in anhydrous HF with stirring overnight at room temperature. HF was removed under a stream of nitrogen gas.

Lipid analysis. Total fatty acids were determined by GC after release by hydrolysis with 4 M HCl in methanol at 100°C for 18 h, followed by extraction into hexane (52). The fatty acid methyl esters were identified by retention times and their chemical ionization (ammonia) and electron impact MS spectra (42, 60). GC-MS was performed on a DB17 capillary column as previously described, by using heating program A or D (160°C for 2 min and then heating of 5°C/min to 240°C).

NMR studies. The reduced core oligosaccharide (20 mg) was deuterium exchanged by freeze-drying in D_2O (three times) and then dissolved in D_2O (0.45 ml) containing a trace of acetone as an internal reference (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded at 30°C on a Bruker WH-400 or AMX-500 spectrometer equipped with an Aspect 3000 computer and an array processor with standard Bruker software. Two-dimensional homonuclear chemical shift-correlated (coherence transfer NMR spectroscopy [COSY] and relay COSY) experiments were done by using conventional pulse sequences (5, 6, 56), while the ^{13}C-^1H chemical shift-correlated spectrum (heteronuclear ^1H-^{13}C shift-correlated spectroscopy) was ^1H detected (7). The ^{31}P nuclear magnetic resonance (NMR) spectrum was recorded at 30°C on a Varian XL-300 spectrometer operating at 121.51 MHz. The chemical shift was recorded relative to an external standard of 85% phosphoric acid.

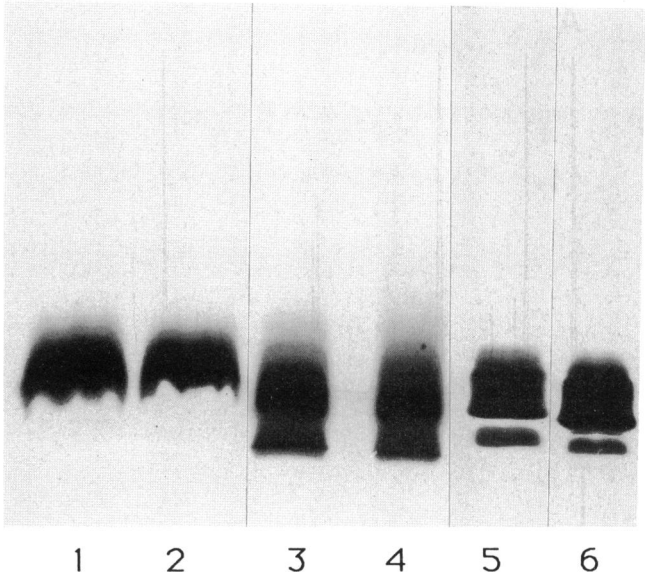


FIG. 1. SDS-PAGE of LPS prepared by the methods of Hitchcock and Brown (27) (lanes 1 and 2) and Darveau and Hancock (19) (lanes 3 to 6). Gels were stained by the method of Tsai and Frasch (55) (lanes 1 to 4) or by using the Bio-Rad silver stain kit (lanes 5 and 6). Odd-numbered lanes contained CB2A; even-numbered lanes contained NA1000. All lanes were loaded with samples containing 1 μg of KDO.

RESULTS

Electrophoretic analysis. SDS-PAGE of the purified LPS and subsequent staining of the gels, by the method of Tsai and Frasch (55) or by using the Bio-Rad silver stain kit, revealed that the *C. crescentus* strains produce a rough LPS (Fig. 1). Only two bands of similar mobility resulted from LPS preparations, and none of the extensive morphological heterogeneity typical of smooth LPS was noted. LPS profiles of these strains prepared by the method of Hitchcock and Brown (27) showed the same banding pattern (Fig. 1), indicating that the isolation method did not select against recovery of smooth LPS species. The broader and more condensed bands resulting from the samples prepared by the Hitchcock and Brown method (Fig. 1, lanes 1 and 2) are most likely due to the presence in the sample of bulk cellular components, such as undigested protein, peptidoglycan, and nucleic acids, which slightly alter the mobility of the LPS. The doublet of LPS is typical of the microheterogeneity found in rough LPS species, as detected by SDS-PAGE or

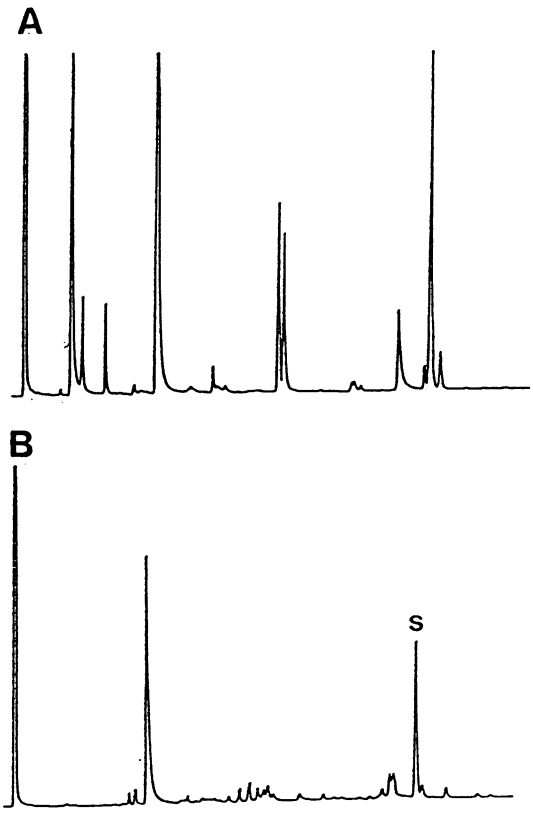


FIG. 2. GC trace of fatty acid methyl esters from the LPS of strain NA1000. (A) Crude preparation. (B) Pure LPS. S, methyl ester of octadecanoic acid, an internal standard.

TLC (41). In other studies (57), we learned that longer periodate oxidation (15 to 20 min instead of the 5 min stipulated by Tsai and Frasch [55]) revealed a second polysaccharide-containing species that runs with significantly slower electrophoretic mobility than the LPS. We are currently isolating and purifying this molecule to determine chemically whether it constitutes a second LPS species or is a novel carbohydrate. This additional carbohydrate was not isolated by using the method of Darveau and Hancock (19) (Fig. 1, lanes 5 and 6).

Isolation of LPS, monitored by lipid analysis. The cold ethanol extraction procedure of Darveau and Hancock (19) yielded the crude LPS fraction. Methanolysis, followed by GC analysis of the fatty acid methyl esters (Fig. 2A), gave the results displayed in Table 1. The presence of saturated and monounsaturated 16- and 18-carbon fatty acids, together with the 3-OH-dodecanoic acid, suggested contamination by phospholipids (35). Subsequent purification yielded an LPS product largely free of these fatty acids (Fig. 2B and Table 1). Lipid analysis of the PCH-insoluble and chloroform-methanol washes revealed negligible amounts of 3-OH-dodecanoic acid, indicating that little LPS was lost during these purification steps. The material extracted from the final pure LPS was examined by SDS-PAGE and staining with the silver stain method and Coomassie blue. The gels showed that residual protein and not carbohydrates had been extracted. Coomassie blue-stained gels showed that a pronase-resistant protein of approximately 31 kDa was the major protein contaminant after the ultracentrifugation steps (data not shown). Typically, phenol-chloroform-hexane extraction resulted in a 10% decrease in the dry weight of the crude LPS whereas the chloroform-methanol wash decreased the weight by 30%.

Lipid analysis. Methanolysis of the pure LPS samples, followed by GC-MS analysis of the hexane-soluble fraction, showed that the major fatty acid component of both strains was 3-OH-dodecanoic acid (3-OH-C12:0). The assignment was made on the basis of GC retention time and MS analysis by chemical ionization using ammonia [Fig. 3A; $(M + 18)^+$ and $(M + 1)^+$ at *m/z* 248 and 231, respectively] and electron impact (Fig. 3B; major peak at *m/z* 103 due to $CHOH\ CH_2CO_2CH_3$).

Colorimetric analysis. The pure LPS was analyzed for protein, phosphate, and KDO. No protein was detected, and phosphate and KDO were found to account for 0.5 and 12% of the LPS dry weight, respectively. The molar ratio of phosphate to KDO, determined colorimetrically, was approximately 1:3. Given the direct demonstration of phosphate in the core region by ^{31}P NMR (see below), this suggests three KDO residues per LPS molecule. More

TABLE 1. Lipid analysis of NA1000 crude LPS and pure LPS

T[a]	Peak area (%)[b]		Assignment	Diagnostic ions (MS)	
	Crude LPS	Pure LPS		EI[c] (70 eV)	CI[d] (NH_3)
0.17	40		Hydrocarbon	55, 69, 83, 97, 111	NR[e]
0.20	2		C12:1	74, 87	213, 230
0.26	2		Hydrocarbon	55, 69, 83, 97, 111, 168	229, 246
0.37	28	82	3-OH-C12:0	74, 103	231, 248
0.51	1		C15:0	74, 87, 213, 225, 256	257, 274
0.66	5	5	C16:0	74, 87, 227, 239, 270	271, 288
0.67	5		C16:1	74, 87	269, 286
0.94	4	9	2-OH-C16:1	90, 225, 284	285, 302
1.00	1		C18:0	74, 87	NR
1.01	11	4	C18:1	74, 87, 180, 222, 264, 296	297, 314
1.04	1		C19:1	194, 279, 310	311, 328

[a] *T*, retention time relative to *methyl-n*-octadecanoate (C18:0).
[b] Analysis of fatty acid methyl esters by GC-MS on a DB17 capillary column (heating program D). The data are from Fig. 2A (crude LPS) and B (pure LPS).
[c] EI, electron impact.
[d] CI, chemical ionization.
[e] NR, not resolved.

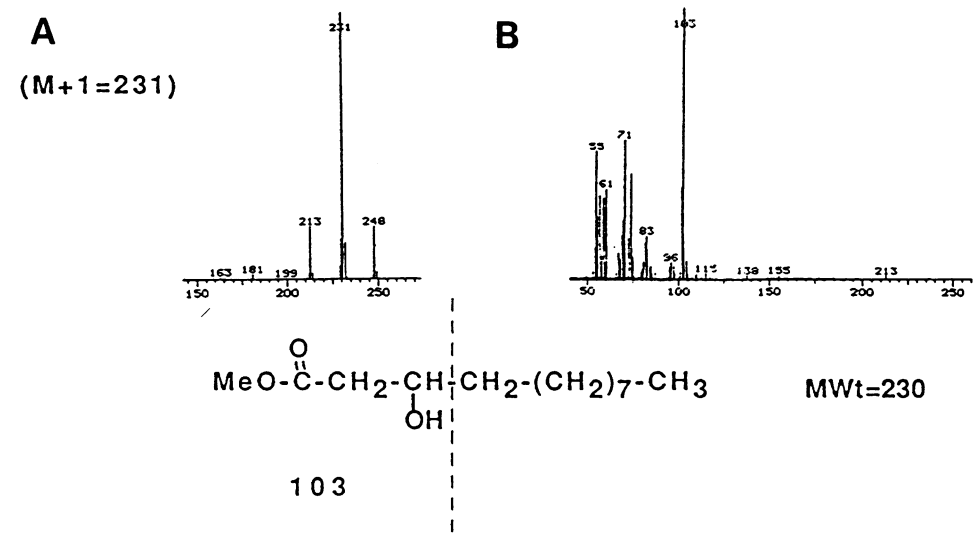


FIG. 3. MS analysis of the major fatty acid component of strain NA1000. (A) Chemical ionization (ammonia). (B) Electron impact. MWt, molecular weight.

severe hydrolysis conditions than those recommended by Karkhanis et al. (31) did not result in an increase in the amount of KDO detected, indicating that the KDO was likely not phosphorylated.

The total amounts of thiobarbiturate-positive material contained in freeze-dried cells of CB2A, NA1000, and *E. coli* B were also examined. For these studies, identical weights of the three cell types were analyzed simultaneously with KDO standards. The caulobacter strains contained less KDO per unit of dry weight than did *E. coli* B, the *E. coli* B-CB2A-NA1000 ratio being 1:0.83:0.68.

The thiobarbituric acid assay relies on formation of β-4-formylpyruvate, which can be derived from compounds other than KDO (e.g., 3-deoxyhexulosonic acid) (32). For this reason, chemical proof for the presence of KDO was also sought.

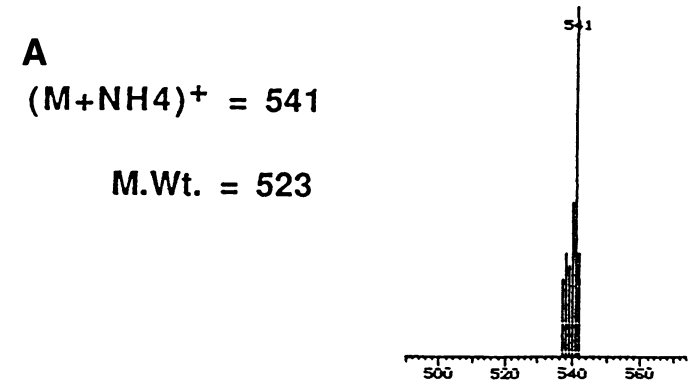
Chemical demonstration of KDO. Methanolysis (1 M HCl for 18 h at 100°C), followed by deuterium labelling of the acidic residues by reduction with $NaBD_4$ prior to conversion to alditol acetate derivatives, yielded trace amounts of a slowly moving component upon GC analysis (retention time, 1.75 relative to inositol hexaacetate; heating program A). MS analysis by chemical ionization using ammonia (Fig. 4A) indicated a molecular weight of 523, and a more detailed fragmentation by electron impact (Fig. 4B) confirmed the presence of 1,1,2-trideuterio-3-deoxyoctitol-heptaacetate (61); thus, the presence of KDO was unambiguously determined.

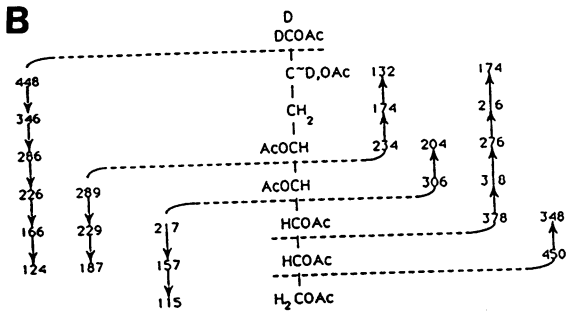
Carbohydrate composition. Results of carbohydrate analysis of the pure LPS fractions isolated from NA1000 (Fig. 5A) and CB2A (Fig. 5B) are displayed in Table 2. Sugar constituents were identified by their retention times and MS fragmentations. The peak at the relative retention time of 1.02 was assigned to the acetylated derivative of 1,6-anhydroheptopyranose on the basis of MS analysis by electron impact (15) and chemical ionization [using ammonia, $(M + 18)^+ = 378$]. Formation of 1,6-anhydro-L-glycero-D-*manno*-heptopyranose during acidic hydrolysis or methanolysis of LPS samples has been described by Chaby and Szabó (15). Assignment of the heptose derivatives was made by unambiguous identification of L,D-heptose by coinjection with a standard obtained from hydrolysis of *E. coli* O127 LPS (Difco Laboratories, Detroit, Mich.) and that of D,D-heptose on the basis of published retention times (28). Both heptoses are commonly found in the cores of gram-negative bacteria (38) and have been previously detected in the cell wall polysaccharides of *C. crescentus* (13). The small amount of glucose detected (0.4 relative to mannose) for CB2A LPS was attributed to phosphorylation of this residue, leading to incomplete release of glucose under the hydrolysis conditions used. After dephosphorylation of CB2A LPS with 48% HF, an increased amount of glucose was detected by GC analysis (0.8 relative to mannose), whereas after solvolysis with 100% HF, the glucose increased to 1.7 relative to mannose (Table 2).

No uronic acid derivatives (apart from KDO) were detected by GC-MS after deuterium labelling by acidic residues, prior to conversion to alditol acetate derivatives. No amino sugars were detected by GC-MS analysis after hydrolysis with TFA (A and B), HF, or HCl (either in methanol [C] or under aqueous conditions [D]). Similarly, unsuccessful results were obtained by using an amino acid analyzer.

Chemical analysis of LPS and lipid A. Mild acid hydrolysis of NA1000 LPS yielded the degraded polysaccharide, which gave a single carbohydrate-containing spot on TLC analysis (data not shown), indicating an oligosaccharide. Sugar analysis by GC gave the same results as found for the parent LPS molecule (Table 2), while the absolute configuration of the hexoses was found to be D by GC analysis of their (−)-2-butyl glycosides. The lipid A fraction still contained LPS according to TLC analysis and was therefore subjected to further mild acid hydrolysis. The final lipid A precipitate contained the 3-OH-dodecanoic fatty acid and negligible amounts of carbohydrate. Once again, a variety of hydrolytic procedures were used but no amino sugars were detected.

Structural studies of the oligosaccharide fraction. Periodate oxidation of the NA1000 LPS indicated that none of the sugar units were resistant to oxidation. However, determination of the linkage assignments of the reduced core oligosaccharides by methylation analysis was unsuccessful





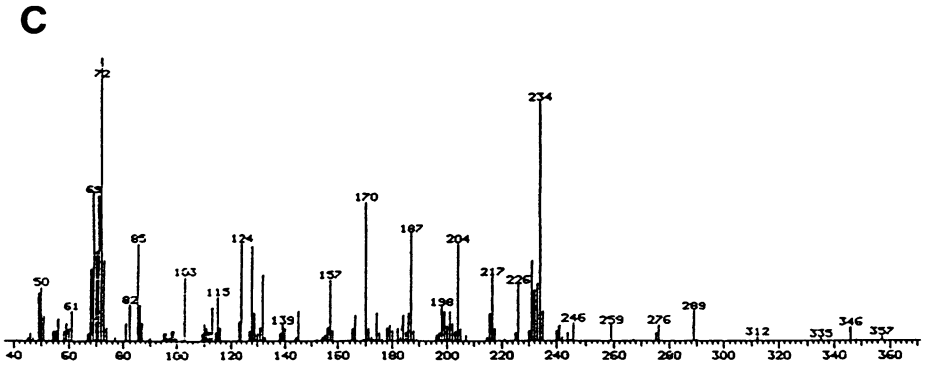


FIG. 4. MS analysis of KDO from strain NA1000. (A) Chemical ionization (ammonia). (B) Electron impact fragmentation pattern. (C) Actual electron impact spectrum. M.Wt., molecular weight.

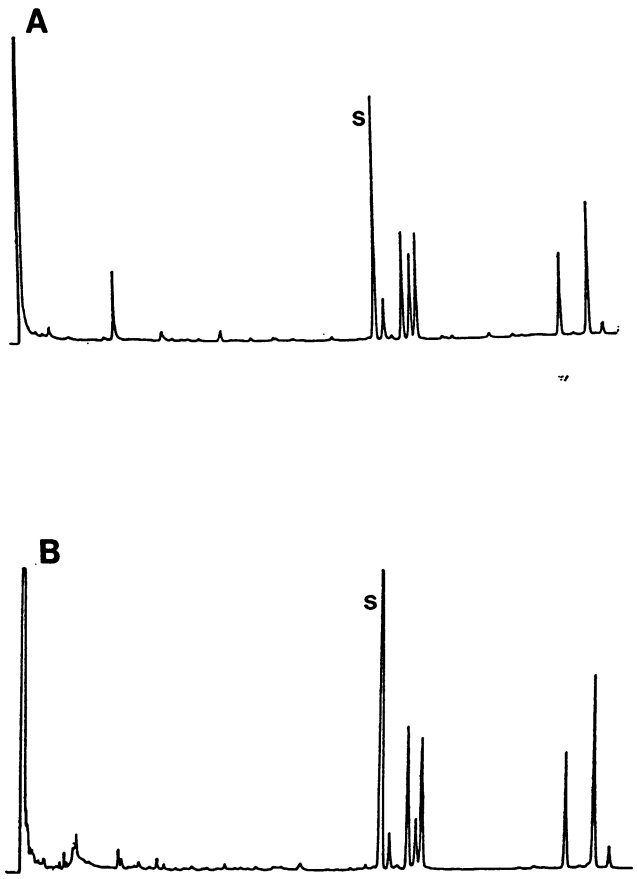


FIG. 5. GC trace of alditol acetates from the LPS of strains NA1000 (A) and CB2A (B). S, inositol hexaacetate standard.

owing to incomplete methylation, despite the variety of conditions applied.

The presence of phosphate in the core fraction was suggested by the presence of a single peak at δ 0.41 in the ^{31}P NMR spectrum. This indicates the presence of a phosphodiester group, although an unambiguous assignment would require spectra to be recorded at different pH values (4).

The 500-MHz ^{1}H NMR spectrum of the reduced core oligosaccharide (Fig. 6) showed the presence of six anomeric signals in the region from δ 5 to 5.6, which is characteristic of α anomers. The anomeric assignments were confirmed by inspection of the ^{13}C NMR spectrum (Fig. 7), which showed signals between 98 and 103 ppm, the connectivities being established by using heteronuclear ^{1}H-^{13}C shift-correlated spectroscopy (Fig. 8). The anomeric proton signals were designated a to f in order of decreasing δ values, the coupling constants of which were determined by a two-dimensional *J*-resolved experiment. Thus, a and b have $^{3}J_{1,2}$ = 4 Hz, typical of the α-*gluco* configuration (glucose and galactose), while c to f have $^{3}J_{1,2}$ = 2 Hz, characteristic of the α-*manno* configuration (mannose and heptose). The NMR data are presented in Table 3 and are in conformity with literature values for sugar residues having the α-*gluco* (2) and α-*manno* (4) configurations (9, 18).

The anomeric proton signals served as the starting point for determination of the scalar coupling connectivities by homonuclear ^{1}H-^{1}H COSY (Fig. 9). The COSY and relay COSY experiments (data not shown) aided assignment of some of the H-2 and H-3 resonances of the sugar residues; however, further identifications could not be made with confidence owing to spectral complexity and overlaps. The presence of KDO molecules indicated by H-3 axial and equatorial signals between δ 1 and 3 (11) exacerbated the complexity of the spectra. The high-field region also contained a doublet at δ 1.42 which could not be assigned.

TABLE 2. Sugar composition of the LPS fractions isolated from strains NA1000 and CB2A

Sugar[a]	*T*[b]	NA1000[c]	CB2A[c]	NA1000[d] core	CB2A[d] (100% HF)
Anhydroheptose (L, D)	1.02	0.4[e]	0.3	0.4	0.5
Mannose	1.07	1.0	1.0	1.0	1.0
Glucose	1.09	0.8	0.4	0.5	1.7
Galactose	1.10	1.0	0.9	0.9	1.2
D-Glycero-D-*manno*-heptose	1.48	1.0	0.9	0.8	1.0
L-Glycero-D-*manno*-heptose	1.55	1.3	1.4	1.4	1.5

[a] Analysis of alditol acetate derivatives by GC-MS on a DB17 capillary column using heating program A (180°C for 2 min and then 5°C/min to 240°C).
[b] Retention times relative to the internal standard (inositol hexaacetate).
[c] Hydrolysis with TFA (18 h, 100°C, 2 M TFA).
[d] Hydrolysis with TFA (1 h, 120°C, 4 M TFA).
[e] Peak area expressed relative to that of mannose.

DISCUSSION

In summary, the LPS produced by *C. crescentus* consists of an inner core region containing three KDO molecules, two α-L-glycero-D-mannoheptose and one α-D-glycero-D-mannoheptose; an outer core region of α-D-mannose, α-D-galactose, and α-D-glucose (probably phosphorylated); and a lipid A moiety containing 3-OH-dodecanoic acid attached to a backbone with an undetermined structure.

Use of the Bio-Rad silver stain kit to analyze the purity of the LPS was of significant benefit. The Bio-Rad silver stain kit allowed visualization of a pronase-resistant protein and contaminating nucleic acids in the crude LPS preparation (39). Most previous studies used the method of Tsai and Frasch (55) to stain purified LPS, which when used in concert with the elevated development temperature procedure of Hitchcock and Brown (27), is selective, revealing only easily oxidized carbohydrates. As a result, other contaminating materials are not visualized.

The presence of LPS in the CB2A and NA1000 strains of *C. crescentus* was initially revealed by SDS-PAGE analysis of washed whole cells treated with proteinase K. The electrophoretic mobility of the band sensitive to the Tsai and Frasch stain and the absence of bands of higher molecular weight (Fig. 1) indicated that the LPS species was rough and therefore could be described as a lipooligosaccharide. The isolated and purified LPSs from the two strains yielded similar electrophoretic profiles (Fig. 1), suggesting that they have the same composition; this was confirmed by colorimetric and chemical analyses performed on these samples.

The colorimetric assay for KDO, a characteristic component of all bacterial LPS molecules so far investigated (48), was positive for both strains and was confirmed by detection of the acetylated derivative of KDO by GC-MS following methanolysis and reduction (Fig. 4). The positive response for this inner core constituent increased during the isolation procedure, with the most purified samples containing three KDO residues per molecule of LPS. This ratio is not unusual

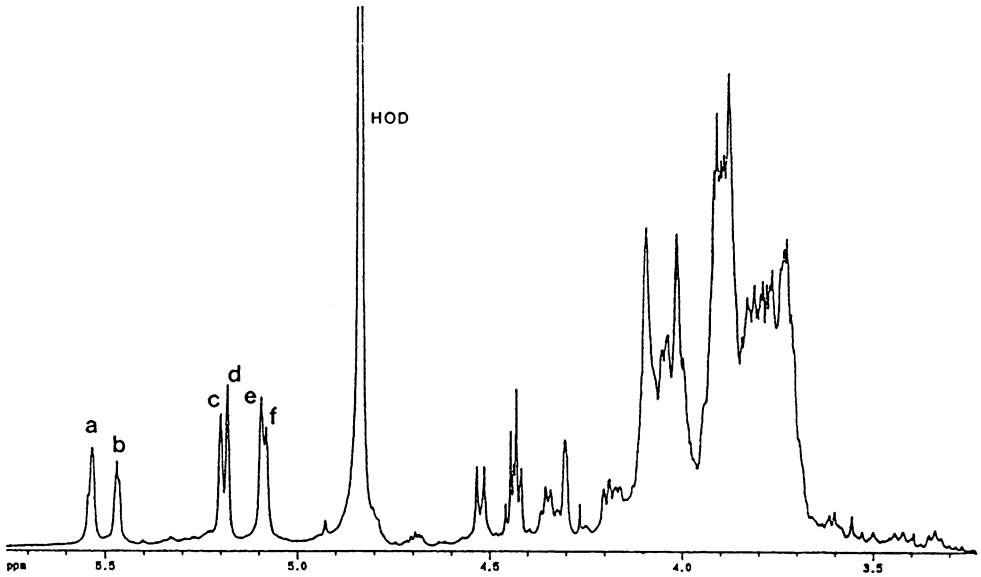


FIG. 6. One-dimensional 500-MHz ^{1}H NMR spectrum of the reduced core oligosaccharide from strain NA1000. The anomeric signals are labelled a to f in order of decreasing chemical shift values. HOD, monodeuterated water.

for enterobacterial LPS and has been found, for example, in the much-studied *S. minnesota* inner core region (10).

It has been reported that *C. crescentus* whole membranes contain from two-thirds to 10-fold less KDO than that reported for rough-mutant and wild-type *S. typhimurium*, respectively (2). This study showed that the total amount of KDO in NA1000 and CB2A is less than that found in *E. coli* B, but only by 20 to 30%. This indicates that KDO could be used as an outer membrane marker in procedures used to separate membrane fractions. The published methods for membrane separation and isolation in *C. crescentus* do not account for the missing KDO in the outer membrane fractions (2, 17, 33). Two of the protocols (17, 33) used phosphate-buffered saline in the procedure. We have since shown that phosphate-buffered saline disrupts the envelope of *C. crescentus* (22); the absence of KDO might be explainable on that basis.

In addition to KDO, the inner core region of enterobacte-

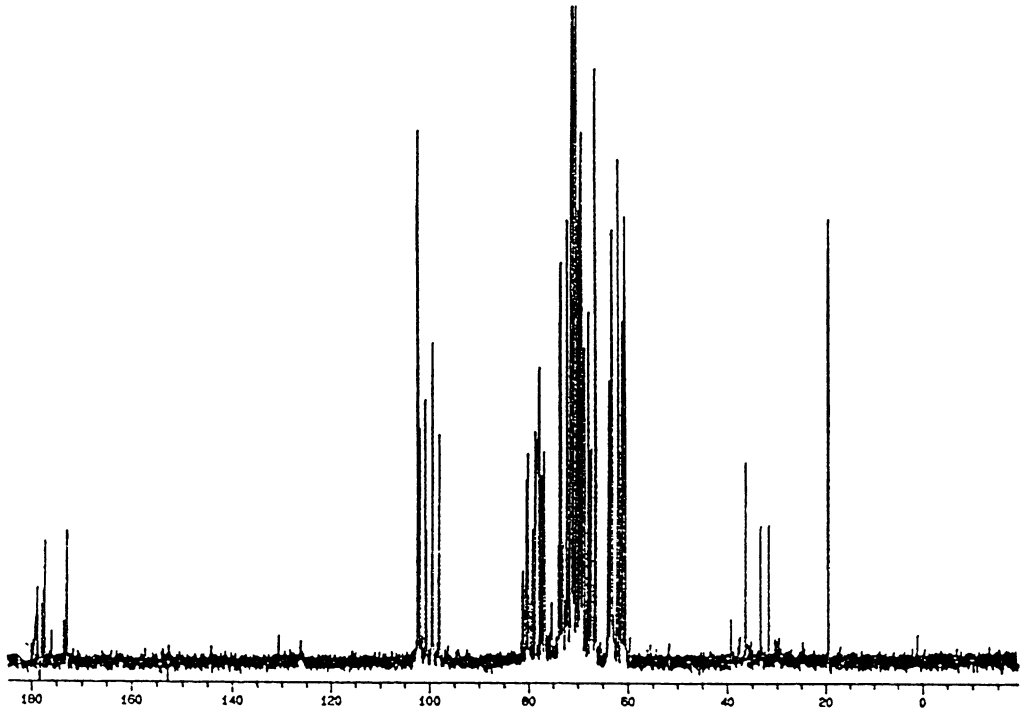


FIG. 7. One-dimensional 125-MHz ^{13}C NMR spectrum of the reduced core oligosaccharide from strain NA1000.

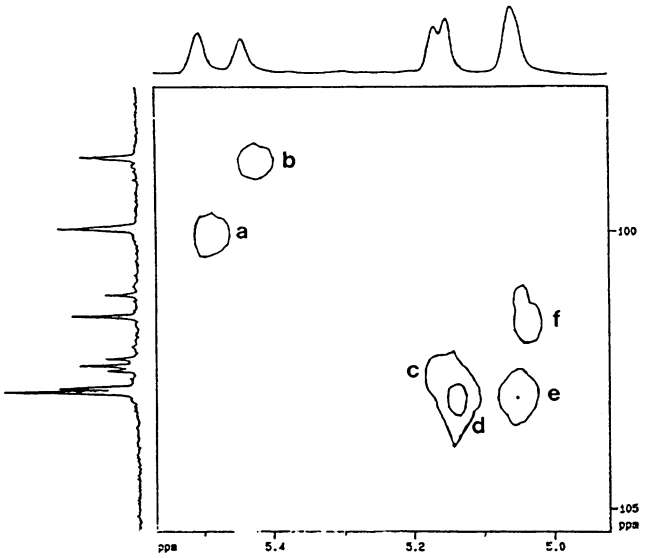


FIG. 8. Heteronuclear shift-correlated NMR spectrum of the anomeric region of the reduced core oligosaccharide from strain NA1000 ($J_{C,H}$ = 140 Hz). The correlated resonances are labelled a to f.

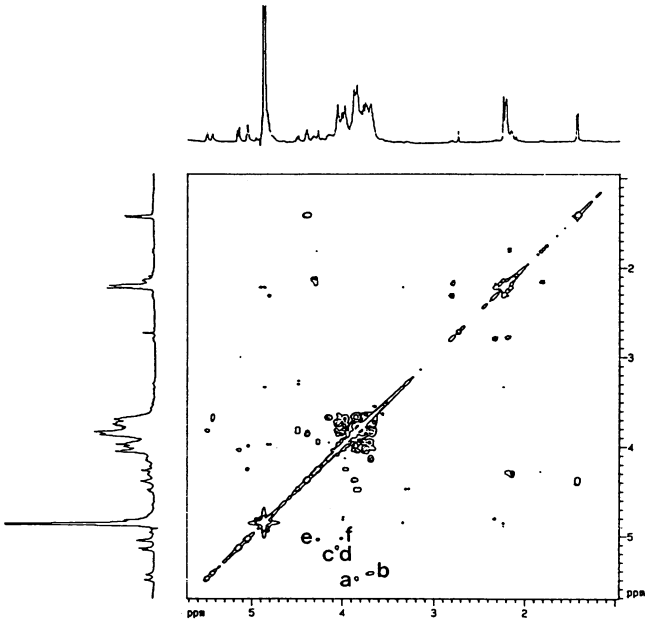


FIG. 9. ^{1}H-^{1}H COSY spectrum of the reduced core oligosaccharide from strain NA1000 at 30°C. H-1 to H-2 cross-peaks are indicated a to f.

rial LPS is also characterized by heptose residues (48), which were also observed for the LPSs of both caulobacter strains (Table 2). Both the common L-glycero-D-*manno* configuration and the less typical D-glycero-D-*manno* configuration of the heptoses were detected. Carbohydrate analysis also showed the presence of D-mannose, D-glucose (less than stoichiometric amounts), and D-galactose, which were attributed to the outer core hexose region. As detailed, the glucose is probably phosphorylated, as is found for the LPS of *Legionella pneumophila* (52). NMR analysis of the core oligosaccharide showed the presence of six anomeric signals, while the chemical shifts of the H-1 and C-1 resonances (Table 3) are characteristic of the α configuration. This is consistent with a sugar composition of three hexose and three heptose residues.

TABLE 3. Some ^{1}H and ^{13}C NMR data for the reduced core oligosaccharide

Residue and method	Shift (ppm) of proton or carbon no.[a]:		
	1	2	3
a			
^{1}H	5.50	3.86	4.03
^{13}C	99.3		
b			
^{1}H	5.44	3.71	NR[b]
^{13}C	98.0		
c			
^{1}H	5.16	4.08	3.86
^{13}C	102.2		
d			
^{1}H	5.14	4.08	3.73
^{13}C	102.3		
e			
^{1}H	5.05	4.28	3.98
^{13}C	102.2		
f			
^{1}H	5.04	4.01	3.88
^{13}C	100.8		

[a] Chemical shifts downfield from the signal for acetone at δ 2.23 and 31.07 for ^{1}H and ^{13}C, respectively.
[b] NR, not resolved.

The lipid A equivalent of the caulobacter LPS resisted complete analysis. The major or only fatty acid component, 3-OH-dodecanoic acid (Fig. 3 and Table 1), was readily cleaved by standard methods. It is less commonly found in LPS than is 3-OH-tetradecanoic acid but has been found, for example, in the lipid A from *Pseudomonas aeruginosa* (8). Mild acid hydrolysis readily cleaved the LPS into lipid A and core oligosaccharide fractions. Carbohydrate analysis of the core oligosaccharide gave results similar to those found for the intact LPS (Table 2), confirming that these residues are associated with the core region and not the lipid A. However, despite extensive efforts, no amino or diamino sugars, typical of the backbone region of other lipid A moieties (48), were detected by multiple approaches for amino sugar analysis. The effort included an amino acid analysis, a strong hydrolysis procedure which also detects amino sugars.

We do not have a clear explanation for the inability to cleave the lipid A into assayable sugars. It is notable, however, that the caulobacters may be members of a group of bacteria in which unusual lipid A structures are found. Caulobacters are members of the alpha subdivision of the proteobacteria, as defined by 16S rRNA sequence analysis (53, 54). Although there were differences in the algorithms for comparative analysis, the caulobacters are closely aligned with *P. diminuta* (54), which in turn has been identified as a member of the α-2 subgroup of the alpha proteobacteria (59). It is in this group that bacteria with the unusual amino sugar 2,3-diamino-2,3-dideoxy-D-glucose in their lipid A backbone structures appear to cluster (58). It

may be, then, that a variation of the 2,3-diamino-2,3-dideoxy-D-glucose type of lipid A is present in *C. crescentus*. High-voltage paper electrophoresis has proven useful for detection and separation of such unusual lipid A sugars in other systems (37) and may be appropriate for studies with caulobacter lipid A.

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