JOURNAL OF BACTERIOLOGY, Dec. 1975, p. 1168-1176

Vol. 124, No. 3
Printed in U.S.A.

Analysis of the Cell Wall and Lipopolysaccharide of *Spirillum serpens*

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Received for publication 2 May 1975

Isolated walls of *Spirillum serpens* VHA contained lipid, lipopolysaccharide, and protein in amounts similar to those of other gram-negative organisms. The loosely bound lipids consisted mainly of phosphatidylethanolamine, lyso-phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol. Lipopolysaccharide was tightly bound to the wall and could only be removed in a substantial amount after digestion of the wall with Pronase. The lipopolysaccharide contained L-glycero-D-mannoheptose, rhamnose, glucosamine, ethanolamine, and phosphate in common with many of the lipopolysaccharides isolated from the *Enterobacteriaceae*. However, 2-keto-3-deoxyoctonic acid was not detected. Several unidentified sugars were present. The fatty acid composition resembled that found in lipopolysaccharides isolated from various pseudomonads. Two major regions were identified in the polysaccharide moiety, one apparently corresponding to the core polysaccharide and the other corresponding to the side-chain polysaccharide as in enterobacterial and pseudomonad lipopolysaccharides. The side chains were obtained as low-molecular-weight material and their structure was partially elucidated by the isolation and partial characterization of *N*-acetylglucosaminyl-(1→4)-rhamnose.

Various species of *Spirillum* possess regular arrays of protein macromolecules on their outer surface (6, 25, 39, 43). The hexagonally arranged outer structure of *S. serpens* (6) was removed from the cell surface together with a backing layer, which was concluded to be a lipid-lipopolysaccharide (LPS)-protein complex. The outer, structured layer with its backing layer was attached by weak, noncovalent, and probably electrostatic, bonds to what appeared to be a typical gram-negative wall with a trilaminar appearance in section when viewed with an electron microscope.

We have attempted to clarify the relationship between the various components present in the wall of *S. serpens* and the role of the LPS. This paper describes the preparation and analysis of the walls of *S. serpens* and preliminary investigation of the structure of the LPS isolated from them.

MATERIALS AND METHODS

Growth of organism. *S. serpens* VHA was grown, harvested, and washed as described by Buckmire and Murray (6); the cells were used directly to prepare walls or were freeze dried. In some cases, cells were stirred with acetone (10 volumes for 1 g of cell wet weight) for 1 h, the treatment was repeated, and the residue was treated with chloroform-methanol, as described below, to remove the remaining lipid material.

Preparation of walls. Washed cells (100 to 130 g, wet weight) were suspended in 300 ml of water, and the suspension was extruded from a French pressure cell (capacity, 40 ml; diameter, 1-inch [ca. 2.54 cm] bore) using an automatic press (American Instrument Co., Inc., Silver Spring, Md.) at 7,000 to 8,000 lb/in^2. The product was diluted with 700 ml of water and centrifuged at 3,500 × *g* for 20 min to remove intact cells. The supernatant suspension was centrifuged at 30,000 × *g* for 20 min to deposit walls which were then suspended in 300 ml of 1 M NaCl and centrifuged at 30,000 × *g* for 20 min. The wash with NaCl was repeated four times, during which a white granular substance was deposited beneath the walls. Thin-layer chromatography showed this to be poly-3-hydroxybutyrate, presumably derived from cytoplasm (26, 40). The walls were separated from this material during the subsequent purification procedure but, as will be evident later, this did not result in its complete removal, considerable amounts being found in the final preparation. The washed walls were suspended in 300 ml of water containing 25 mg of beef pancreatic ribonuclease (BDH Chemicals Ltd., Poole, Dorset, United Kingdom), and the mixture was incubated for 6 h at 37 C with occasional shaking. Walls were deposited by centrifugation at 30,000 × *g* for 20

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min, suspended in 300 ml of water, and recentrifuged. The deposited walls were then rewashed with water until the supernatant liquid obtained after centrifugation of the walls was free from nucleic acids as determined by ultraviolet spectroscopy. The walls were then freeze dried.

Extraction of loosely bound lipids. Approximately 500 mg of freeze-dried walls was stirred with 100 ml of chloroform-methanol (2:1 vol/vol) under a nitrogen atmosphere for 3 h at room temperature. The walls were collected by filtration of the mixture through medium porosity sintered glass and were reextracted twice with chloroform-methanol. The combined filtrates were dried down in a rotary evaporator at 25 C, and the residue was dissolved in chloroform and filtered through medium-porosity sintered glass to remove traces of insoluble material. This solution was treated with 4 volumes of diethyl ether (64) to precipitate poly-3-hydroxybutyrate, which was then removed by filtration through sintered glass. The filtrate was evaporated to dryness in a rotary evaporator at 25 C, the residue was dissolved in chloroform, and the precipitation of poly-3-hydroxybutyrate was repeated. The resulting lipids were dried down and stored at −20 C under a nitrogen atmosphere. In some cases, lipids were stored under similar conditions in chloroform solution.

Treatment of walls with Pronase. After removal of loosely bound lipids, walls were suspended in 100 ml of water containing 20 mg of Pronase (grade B, Calbiochem, Los Angeles, Calif.), and the mixture was then stirred for 6 h at 37 C. The walls were deposited by centrifugation at 40,000 × *g* for 20 min, washed by resuspension and recentrifugation, and freeze dried.

Extraction of LPS from walls. Pronase-digested walls were stirred with 150 ml of the phenol-chloroform-petroleum ether solvent described by Galanos et al. (19). This was repeated twice and, after removal of chloroform and petroleum ether from the combined extracts, phenol was removed by dialysis. Insoluble material was removed, and the resulting solution was treated with 90% aqueous phenol as described by Westphal and Jann (62). Interfacial material and the aqueous phase were combined, dialyzed, and centrifuged at 150,000 × *g* for 4 h. The deposited pellet of LPS was resuspended, recentrifuged, and freeze dried.

Methods of quantitative analysis. Total carbohydrate was determined by the phenol-sulfuric acid method (11). It was recognized that there were variations in color development relative to a glucose standard (55). D-Glucose was determined by using Glucostat reagent (Worthington Biochemicals Corp., Freehold, N.J.), after hydrolysis with 1 M HCl for 4 h at 105 C and subsequent neutralization of the hydrolysate with ion-exchange resins (33). Heptose was determined by the cysteine-sulfuric acid method (9) as modified by Osborn (45); rhamnose was determined in the same experiment. Since no suitable heptose standards were available, the color yield given by L-glycero-D-mannoheptose under the same conditions as reported by Osborn was used. 2-Keto-3-deoxyoctonic acid (KDO) was determined by the method of Weissbach and Hurwitz (61) as modified by Osborn (45). Phosphorus was determined by the method of Bartlett (4). Protein was determined according to Lowry et al. (34). Amino sugars and fatty acids were released from samples by hydrolysis with constantly boiling HCl for 4 h at 105 C. Fatty acids were extracted from hydrolysates with chloroform (three times) and determined as described by Itayi and Ui (27). The amino sugars remaining in the aqueous phase were determined by the method of Strominger et al. (56). Ethanolamine present in the aqueous phase was determined with 1-fluoro-2,4-dinitrobenzene (20). Amino compounds were determined by using an automatic amino compound analyzer (Locarte, London, U.K.). The *O*-acetyl content was determined by the method of Hestrin (23).

Paper chromatography. For preparative purposes, water-washed Whatman 3MM paper was used. Otherwise, Whatman no. 1 paper was used. The following solvent systems were used: (A) pyridine-butan-1-ol-water (4:6:3, vol/vol/vol); (B) acetone-water (19:1, vol/vol); (C) 85% ethanol-28% (100:2, vol/vol); (D) ethyl acetate-pyridine-acetic acid-water (5:5:1:3, vol/vol/vol/vol); (E) butan-1-ol-acetic acid-water (4:1:5, vol/vol/vol), the upper layer being used as the solvent; and (F) ethyl acetate-acetic acid-water (3:1:3, vol/vol/vol), the upper layer being used as the solvent.

Sugars were detected using the silver nitrate reagent of Trevelyan et al. (57) or the aniline phosphate reagent described by Frahn and Mills (16). Ninhydrin-positive material was detected by Randerath (47), phosphorus-containing material was detected by Hanes and Isherwood (22), and polyhydroxy-containing material was detected with periodate-Schiff reagent (3). KDO was sought by using the reagent described by Warren (60).

Thin-layer chromatography. Kieselgel G (E. Merck A.-G., Darmstadt, West Germany) was used with the following solvent systems: (a) chloroform-methanol-water (65:25:4, vol/vol/vol); (b) chloroform-methanol-7 M ammonia (65:25:4, vol/vol/vol); chloroform-methanol-17% ammonia (2:2:1, vol/vol/vol); (e) phenol-water (3:1, wt/vol).

Lipids were detected by treatment of the plates with iodine vapor, periodate-Schiff reagent (3), or ninhydrin reagent (47); the reagent described by Vaskorsky and Kostetsky (58) was used to detect phospholipids.

Column chromatography. Sephadex G-25 and G-50 (medium grades) were used with 0.05 M ammonium bicarbonate or 0.05 M pyridine acetate (pH 5.3) as eluant, which was removed from samples by rotary evaporation at 30 C. Anion-exchange column chromatography was carried out using Whatman DE 52 diethylaminoethyl-cellulose with pyridine acetate (pH 5.3) as the eluting solution applied as 0 to 0.3 M gradient.

Gas-liquid chromatography. Fatty acids were methylated (41) for identification by gas-liquid chromatography, using a solution of boron trifluoride in methanol (14%, wt/vol). Hydroxy acids were acetylated prior to methylation as described by Fritz and Schenk (18). Columns of polydiethyleneglycol succi-

nate and silicone gum (SE-30) were used.

Sugars were examined by gas-liquid chromatography of their alditol acetates, which had been prepared by the method of Sawardeker et al. (53) as modified by Björndal (5), using a column of a copolymer of ethyleneglycol succinate polyester and a nitrile silicone.

Electrophoresis. A Shandon electrophoresis apparatus (model U77; Shandon Scientific Co. Inc., Sewickley, Pa.) was used with Whatman no. 1 chromatography paper. For preparative purposes, we used prewashed Whatman 3MM chromatography paper. Pyridine acetate (pH 5.3) prepared from pyridine-acetic acid-water (5:2:43, vol/vol/vol) was used as buffer a, and buffer b was 0.05 M sodium tetraborate. A potential gradient of 12 to 15 V/cm was used.

Isolation of lipid material from LPS. After acidic hydrolysis of the LPS, the resulting mixture was cooled and treated with an equal volume of chloroform. The chloroform phase was separated by centrifugation at 5,000 × *g* for 15 min and removed. The procedure was repeated, and the chloroform solutions were combined, evaporated under N_2, and filtered for storage at −20 C.

The LPS was hydrolyzed with 1 M HCl at 100 C for 35 min, and both the lipid and polysaccharide moieties were collected. After centrifugation, the aqueous phase was removed and the remaining chloroform phase was washed twice with water. The aqueous phases were combined, centrifuged at 40,000 × *g* for 15 min, and freeze dried to remove HCl. The resulting material, degraded polysaccharide, was stored in an aqueous solution at −20 C. The chloroform solution was treated as above, the residue being lipid A.

De-O-acylation of lipid A. De-*O*-acylation was carried out by the procedure of Kasai (28) as described by Fensom and Gray (13).

Periodate oxidation and borohydride reduction. LPS was treated as described by Wheat and co-workers (63).

RESULTS

Appearance and composition of walls. The purified walls of *S. serpens* have the characteristic appearance of gram-negative walls when viewed in sections under an electron microscope (17).

Paper chromatography of hydrolysates (solvent systems A through F) showed that sugars were present (see below). Two-dimensional thin-layer chromatography and automatic amino compound analysis of hydrolysates showed that typical protein amino acids were present in addition to the peptidoglycan components, muramic acid, and 2.6-diaminopimelic acid. Glucosamine, a component of both peptidoglycan and LPS, was detected.

Loosely bound lipids accounted for 22.1 to 23.3% of the walls and were shown by thin-layer chromatography (solvent systems a through c) to contain a considerable amount of poly-3-3-hydroxybutyrate. When this was removed, the remaining lipid fraction accounted for 11.9 to 12.3% of the wall and contained 3.9 to 4.2% phosphorus. The major component was phosphatidylethanolamine, together with smaller amounts of diphosphatidylglycerol, phosphatidylglycerol, and lyso-phosphatidylethanolamine.

The fatty acid composition of the lipids was: C_{16} (18.3%), monoenoic C_{16} (53.2%), and monoenoic C_{18} (28.5%). Traces of saturated C_{14} were present, but no other acids (including hydroxy acids) could be detected.

Analytical figures for a typical batch of walls were: phosphorus, 0.8%; total sugar, 5.4% (determined by the phenol-sulfuric acid method, with glucose as the standard); and protein, 70.9% (determined by the Lowry method, with bovine serum albumin as the standard).

Isolation of LPS from walls. A standard 45% aqueous phenol extraction (62) failed to release LPS. This release required pretreatment of walls with Pronase, after which most of the LPS was recovered at the phenol/water interface, but substantial amounts were in the aqueous phase. The amount of LPS isolated varied from batch to batch. Similarly, treatment of walls with Pronase was required from the release of LPS by phenol-chloroform-petroleum ether (19). After evaporation of the solvents, the LPS was only partially precipitated by the addition of water to the phenol, which was then removed by dialysis. A second treatment with phenol-chloroform-petroleum ether or 45% aqueous phenol removed small amounts of protein.

The scheme adopted for the preparation of LPS is shown in Fig. 1.

Composition of the LPS. Electrophoresis (buffer system i) and diethylaminoethyl-cellulose chromatography of the LPS did not indicate heterogeneity. Nucleic acids were not detected (ultraviolet spectroscopy).

The major amino components in hydrolysates were glucosamine, ethanolamine, and their phosphates. Small amounts of typical protein amino acids and 2,6-diaminopimelic acid indicated that traces of wall material were present. Typical amino compound analyses are shown in Table 1. The identity of glucosamine was confirmed (using ninhydrin) by its degradation to arabinose (55).

LPS was hydrolyzed with HCl (0.5 to 3.0 M) for various times (2 to 180 min) at 100 C, and the products were examined by paper chromatography (solvent systems A through F) after removal of charged material by deionization.

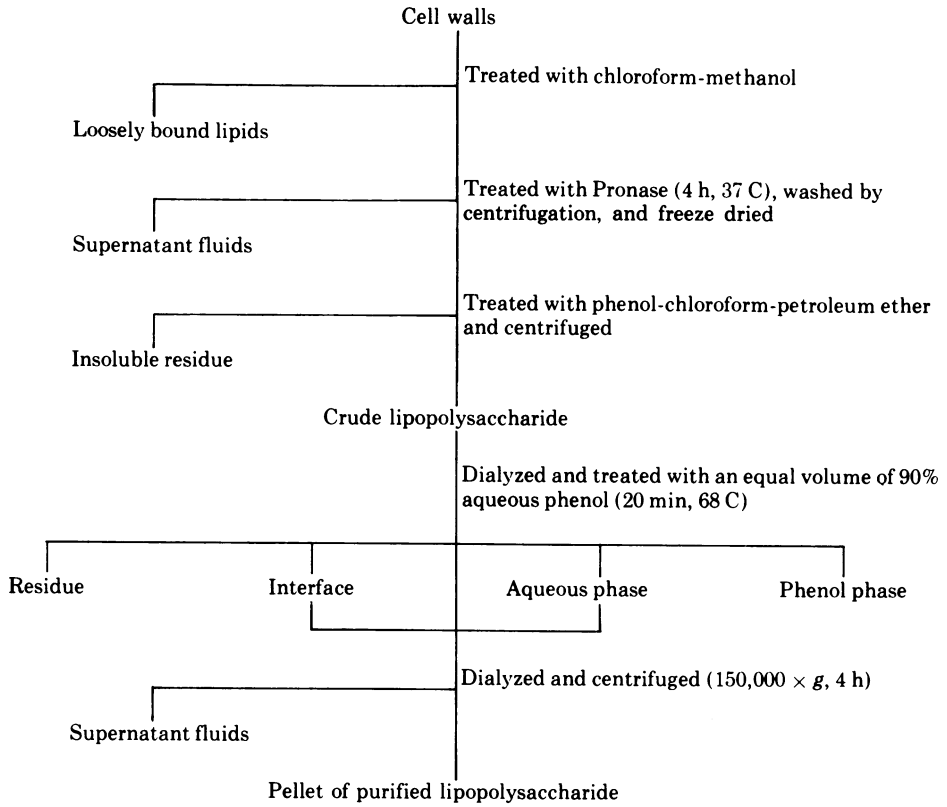

FIG. 1. *Flow diagram of the purification of the LPS from S. serpens.*

TABLE 1. *Analyses of the LPS*

Determination	% (wt/wt)
D-Glucose	0.9
Rhamnose	7.1
L-Glycero-D-mannoheptose	17.9
Glucosamine[a]	15.7
Ethanolamine[a]	4.1
Phosphate	6.1
O-acetyl content	1.2
Amino compounds[b]	1.9
Fatty acid[c]	22.9

[a] Determined by automatic amino compound analysis; values are corrected to include phosphorylated material present in hydrolysates.

[b] Determined by automatic amino compound analysis; the figure includes protein amino acids and components of peptidoglycan but not glucosamine, ethanolamine, or their phosphates.

[c] Determined by weighing.

Milder hydrolysis demonstrated rhamnose, *N*-acetylglucosamine, and smaller amounts of material (designated U) which ran as a streak on chromatograms. The latter had a mobility and a color reaction with aniline phosphate identical to those of a standard mixture of L-glycero-D-mannoheptose and D-glucose isolated from the LPS of *Pseudomonas aeruginosa* (7). None of the solvent systems resolved the component sugars of the streak. Stronger hydrolysis conditions released greater amounts of U relative to rhamnose and lesser amounts of *N*-acetylglucosamine, presumably as a result of de-*N*-acetylation. The presence of heptose and rhamnose was confirmed by colorimetric estimation using the cysteine-sulfuric acid method (Table 1). Paper chromatography had indicated that glucose was a component of the LPS. However, only a small amount could be determined colorimetrically as D-glucose (Table 1), and no significant amounts of D- or L-glucose were shown by gas-liquid chromatography.

Repeated preparative paper chromatography of U (solvent systems A and F) led to isolation of a heptose (U1) and two other poorly separated and faster-moving fractions (U2 and U3), which remained unidentified. U2 was contaminated with heptose. The heptose was identified as L-glycero-D-mannoheptose as described previously (7).

Gas-liquid chromatography of the alditol ace-

tate derivatives of sugars in LPS hydrolysates (2 M HCl, 100 C, 120 min) showed that three other sugars were present in addition to rhamnose and a small amount of glucose. One of these (retention time 1.3 relative to hexa-*O*-acetyl glucitol) was present in an amount similar to glucose; the other two (retention times 1.9 and 2.2) were present in much larger amounts. Possibly, one of these corresponds to L-glycero-D-mannoheptose.

KDO, a common component of diverse bacterial LPS (12, 38), was not detected by paper chromatographic and electrophoretic techniques in our hydrolysates. Colorimetric determinations were also negative.

Fatty acids released from the LPS by hydrolysis accounted for 22.6 to 23.4% of the intact material. Colorimetric determinations using hexadecanoic acid as the standard gave values in the range 27.1 to 28.5%. The component acids were separated and estimated by gas-liquid chromatography of their methyl ester derivatives (Table 2). The unknown acids were not hydroxylated since, unlike those hydroxylated acids present, acetylation did not produce a change in retention time. Dodec-2-enoic acid was assumed to be a degradation product of 3-hydroxydodecanoic acid.

3-Hydroxydodecanoic acid and dodec-2-enoic acid were not split from the LPS by alcoholic alkali, indicating that these were *N*-acylating acids. The remaining acids were split and were probably *O*-acylating acids.

O-acetyl determinations indicated that small amounts of short-chain, ester-bound acids were present (Table 1). However, the values obtained in this way could include contributions from any longer-chain acids.

Those identifiable components of the LPS listed in Table 1 account for 77.8% of the total. If it is assumed that the glucosamine present in the polysaccharide moiety (see Table 4) is *N*-acetylated (since *N*-acetylglucosamine was identified as a component), then this can account for an additional 2.6% LPS. Therefore, unidentified components constitute 19.6% of the LPS.

Acid hydrolysis of the LPS. It was determined, using various conditions of hydrolysis to release chloroform-soluble material from the LPS (Table 3), that the mildest condition required to completely cleave lipid A was hydrolysis with 1.0 M HCl at 100 C for 35 min.

Lipid A. When dried down from chloroform solution, lipid A was a clear, waxy material. The major amino components were glucosamine, ethanolamine, and their phosphates (Table 4).

TABLE 2. *Composition of the fatty acids of LPS*

Fatty acid	% (wt/wt)
Unknown 1 (carbon no. 5.1)[a]	TR[b]
Dodecanoic acid	29.2
Dodec-2-enoic acid	10.2
Unknown 2 (carbon no. 14.5)[a]	1.9
Unknown 3 (carbon no. 15.4)[a]	1.6
3-Hydroxydecanoic acid	2.7
Hexadecanoic acid	1.3
3-hydroxydodecanoic acid	53.1

[a] Carbon numbers were determined by using a column of polydiethyleneglycol succinate.
[b] TR, Trace.

TABLE 3. *Release of chloroform-soluble material by hydrolysis of the LPS*[a]

Acid used	Release (%)							
	10[b]	20	30	35	40	60	90	120
1.0 M HCl	23.1	30.1	32.1	32.3	32.1	32.0	—[c]	—
0.5 M HCl	—	—	24.0	—	—	—	—	—
0.1 M HCl	—	—	17.1	—	—	23.2	—	—
0.5 M H_2SO_4	—	—	32.4	—	32.5	—	—	—
1% CH_3COOH	—	—	—	—	—	—	21.4	27.1

[a] Percentage (weight/weight) of the intact LPS.
[b] Time (minutes) of hydrolysis.
[c] Not done.

TABLE 4. *Analyses of lipid A and polysaccharide isolated from LPS*

Determination	% (wt/wt)					
	Rhamnose	Heptose[a]	Glucosamine[b]	Ethanolamine[c]	Phosphate	Fatty acid[d]
Lipid A	0.0	0.0	18.2	2.9	6.2	66.0
Polysaccharide	9.9	25.1	14.9	4.6	6.0	0.0

[a] Calculated as L-glycero-D-mannoheptose.
[b] Determined by the method of Strominger et al. (63).
[c] Determined with 1-fluoro-2,4-dinitrobenzene (23).
[d] Determined by weighing.

Traces of protein amino acids were detected, but no neutral sugars were found. The fatty acid composition was almost identical to that of the intact LPS (Table 2). Thin-layer chromatography with solvent system a revealed the presence of two major phosphorus-containing components, together with traces of free fatty acids. At least 12 different components could be detected with solvent system b; these same components were detected in chloroform-soluble material isolated from milder hydrolyses (1% acetic acid for 90 min at 100 C; and 0.1 M HCl for 30 min at 100 C), so they were unlikely to be products of gradual degradation of lipid A during extraction.

Polysaccharide. The freeze-dried product was a brown-yellow, granular, hygroscopic material (Table 4). Sephadex G-25 chromatography (Fig. 2) revealed one fraction (peak 1) that contained most of the heptose and another (peak 2) that contained most of the rhamnose and glucosamine (Table 5). The unknown components (U2 and U3) were mainly associated with peak 1.

Peak 1 was heterogeneous according to electrophoresis in buffer system i, and no clear separation of any components was achieved. Seven fractions were obtained by elution of peak 1 from diethylaminoethyl-cellulose with a gradient of pyridine acetate (0 to 0.3 M, pH 5.3); all were very similar analytically, except for the phosphate content, and treatment with sodium borohydride did not result in any significant decrease in sugar content. Electrophoresis (buffer system i) of peak 1 hydrolysates (2 M HCl, 100 C, 120 min) showed that most of the reducing material was neutral. A small amount of an unidentified acidic reducing component was present, which did not contain phosphorus and was not a uronic acid. Paper chromatography of the neutral sugars (solvent systems A, D, and F) showed that rhamnose, U, and a small amount of *N*-acetylglucosamine were present. Only one of several additional unknown components with a mobility less than that of U could be isolated and appeared to be homogeneous. Paper chromatography in solvent system D provided an $R_{glucose}$ of 0.33. Hydrolysates contained only heptose, 45% of which was destroyed when reacted with sodium borohydride. This indicated that the isolated material was a heptose disaccharide, but lack of material prevented confirmation.

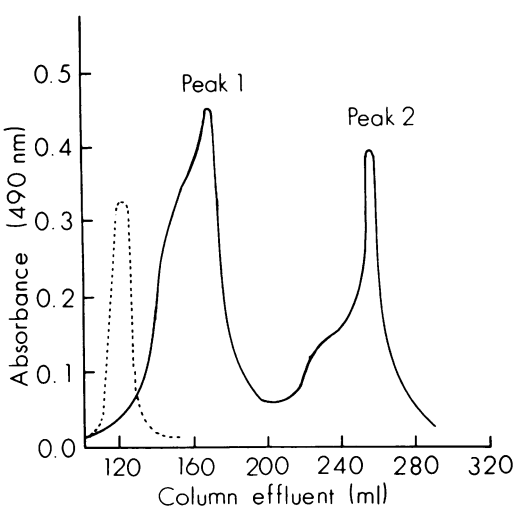

FIG. 2. *Fractionation of degraded polysaccharide by Sephadex G-25 gel permeation chromatography. Solid line represents degraded polysaccharide; dotted line represents blue dextran 2,000. Fractions were eluted with 0.5 M pyridine acetate (pH 5.3) and were screened by the phenol-sulfuric acid method (13).*

TABLE 5. *Analyses of peaks 1 and 2 isolated from the polysaccharide by Sephadex G-25 gel permeation chromatography*

Peak	% (wt/wt)				
	Rhamnose	Heptose[a]	Glucosamine[b]	Ethanolamine[c]	Phosphate
1	2.0	49.9	3.1	4.4	2.6
2	14.5	1.7	24.6	4.6	7.7

[a] Calculated as L-glycero-D-mannoheptose.
[b] Determined by the method of Strominger et al. (63).
[c] Determined with 1-fluoro-2,4-dinitrobenzene (23).

Paper chromatography of peak 2 detected (solvent system D) a reducing component of $R_{glucose}$ = 1.06. It was isolated by preparative paper chromatography and proved to be homogeneous (paper chromatography, solvent system A-F, and paper electrophoresis [buffer system ii]). Hydrolysis yielded rhamnose, glucosamine, and *N*-acetylglucosamine. However, the original material did not react with ninhydrin, indicating that all of the glucosamine was *N*-acetylated. Rhamnose and *N*-acetylglucosamine were present in a molar ratio of 0.89:1.00. Treatment with sodium borohydride did not result in the destruction of any significant amount of *N*-acetylglucosamine, but 93% of the rhamnose was destroyed. These results indicated that the isolated material was disaccharide in which *N*-acetylglucosamine was linked, through its reducing end, to rhamnose. Sodium borohydride did not destroy rhamnose in the intact lipopolysaccaride, indicating that in this case the reducing end of rhamnose was not free. However, periodate oxidation of the intact LPS resulted in almost complete destruction of rhamnose, showing that the C3 position of the rhamnose must be unsubstituted and that *N*-acetylglucosamine must be linked via C2 or C4. The correct linkage was determined by the method of Simmons (54), i.e., by measuring the ratio of color developed by the disaccharide before hydrolysis (according to Reissig et al. [49]) to that obtained after hydrolysis (using the method of Strominger et al. [56]). The results showed that the disaccharide was *N*-acetylglucosaminyl-(1→4)-rhamnose. An authentic sam-

ple of this disaccharide was not available for comparison.

DISCUSSION

The chemical composition of *S. serpens* walls is similar to that of walls from other gram-negative bacteria (52). The lipid fraction contains an appreciable amount of poly-3-hydroxybutyrate, but this is a common cytoplasmic component of members of the genus *Spirillum* (26) and a contaminant of fractions such as peptidoglycan (15, 26). The remaining lipid material contains phosphatidylethanolamine, lyso-phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol, all of which have been identified previously as component of gram-negative walls (17). A previous report (P.D. Steed-Glaister Ph.D. thesis, Univ. of Western Ontario, London, Ontario, Canada, 1967) that phosphatidylcholine is a component of *S. serpens* has not been confirmed.

LPS is closely associated with protein in the wall since it could not be isolated without prior digestion of the wall with Pronase. It is lipophilic in character and, after treatment of Pronase-digested walls with 45% aqueous phenol, was recovered at the interface. A similar situation was encountered by Kasai and Nowotny (29) in working with a heptoseless mutant of *Salmonella minnesota*, although Lüderitz et al. (35), in a parallel experiment, found LPS in the aqueous phase.

The LPS of *S. serpens* resembles that of other gram-negative bacteria (38). Most of lipid A can be accounted for as identifiable components, but several unidentified components were present in the polysaccharide. The major identifiable components of the polysaccharide (rhamnose, heptose, and glucosamine) are common components of LPS (38) as is glucose, which has been reported to be a component of the wall of *S. serpens* (51). However, only small amounts of glucose could be detected in our extracts. The heptose of the polysaccharide was isolated and identified as L-glycero-D-mannoheptose, the most common heptose of LPSs (38).

Heptose constitutes the backbone of the core polysaccharide of enterobacterial LPS (38, 46), and its presence in the LPS of *S. serpens* suggests the existence of a similar core polysaccharide. Removal of lipid A from enterobacterial (38, 42) and pseudomonad (8, 14) LPS by mild acid hydrolysis allows separation of low-molecular-weight core polysaccharide and high-molecular-weight side-chain polysaccharide by Sephadex gel filtration. Peak 1, derived in this way from the polysaccharide of *S. serpens*, contained the major part of the heptose of the polysaccharide and was a molecular weight similar to that of other core polysaccharides. The mixture of unknown components (U) was mainly associated with peak 1 and could constitute part of the core polysaccharide. No high-molecular-weight material was isolated. A very-low-molecular-weight fraction (peak 2) in the hydrolysate contained the major part of the rhamnose and glucosamine of the polysaccharide, together with *N*-acetylglucosamine and a disaccharide (partially characterized as *N*-acetylglucosaminyl-(1→4)-rhamnose). Presumably, the disaccharide is part of the side-chain polysaccharide linked to the heptose-containing core and, if so, the side chains must be of low molecular weight or extensively degraded. Recent results (I. R. Chester and R. G. E. Murray, unpublished data) suggested that the former situation is the case. The molar ratio of glucosamine/rhamnose in peak 2 and of the polysaccharide was 1.55:1.00 and 1.38:1.00, respectively, suggesting that some of the glucosamine present in the side-chain polysaccharide has some associations other than with rhamnose.

Most LPSs have been found to contain KDO (12, 38), although several cases have been reported in which this sugar is absent (1, 2, 24). The role of this material is to provide a link (usually acid labile) between the lipid A and polysaccharide moieties (45). However, KDO appeared to be absent from the LPS of *S. serpens* since this sugar could not be detected by using the thiobarbituric acid reagent and could not be identified as a component of any hydrolysates of the LPS or peaks 1 and 2. It must be borne in mind that if KDO is substituted in such a way that formylpyruvic acid is not produced on reaction with periodate, then no reaction will take place with the thiobarbituric acid reagent (10). In addition, under conditions of mild acidic hydrolysis, KDO is converted to anhydro-derivatives that do not react with the thiobarbituric acid reagent (59). If KDO is not present, then some other compound must link lipid A to the polysaccharide; possibly this could be the unidentified sugar acid detected in hydrolysates of peak 1. The lack of any KDO could explain the vigorous conditions required to cleave lipid A from the polysaccharide. It is interesting to note that LPS isolated from *S. itersonii* and *S. perigrinum* contains KDO in amounts similar to those found in the LPS of many enterobacteria (I. R. Chester and R. G. E. Murray, unpublished data). In addition, it was possible to cleave lipid A from these LPSs using much milder condi-

tions than those required to cleave lipid A from the LPS of *S. serpens*.

The lipid A of *S. serpens* is similar to those isolated from other LPSs (37, 38). However, in contrast to enterobacterial lipid A, where 3-hydroxytetradecanoic acid is the *N*-acylating acid, the corresponding acid in *S. serpens* is 3-hydroxydodecanoic acid, as in *P. aeruginosa* (7, 13, 21) and *P. alcaligenes* (30). Also, 3-hydroxydecanoic acid and 2-hydroxydodecanoic acid were present in the lipid A of *P. aeruginosa*, but only traces of 3-hydroxydecanoic acid were found in *S. serpens*. Thus, the acids present more closely resemble those of *P. alcaligenes*, in which the only detectable acids apart from 3-hydroxydodecanoic acid were 3-hydroxydecanoic, dodecanoic, and dodec-2-enoic acids (the latter probably being a degradation product of 3-hydroxydodecanoic acid).

Ion-exchange chromatography and paper electrophoresis provided no evidence of heterogeneity in the LPS of *S. serpens*. This was sought because of increasing evidence of heterogeneity in LPS fractions from other bacteria (31, 44, 48, 50). More recently, we have found evidence (I. R. Chester and R. G. E. Murray, unpublished data) of at least two LPS components in the wall of this organism.

ACKNOWLEDGMENTS

Thanks are due to Hanna Koppenhoffer, Myrtle Hall, and John Marak for expert technical assistance and to the Medical Research Council of Canada, which was generous in the provision of grants for the support of this research.

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