

Isolation and partial chemical analysis of firmly bound exopolysaccharide from adherent cells of a freshwater sediment bacterium¹

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Cells of a freshwater sediment bacterium produced firmly bound extracellular polymers in laboratory cultures which, at the ultrastructural level, resembled those produced by natural sediment bacterial populations. Production of the exopolymers during subculture was maintained by using as a source of inoculum the population of cells which adhered to each other and to the wall of the glass culture vessel. The exopolymers were selectively released from the cells by blending and centrifugation in the presence of EDTA. Evaluation of glucose-6-phosphate dehydrogenase activity and 2-keto-3-deoxyoctonate concentration indicated that only small amounts of intracellular and cell wall components were released from the cells during exopolymer removal. Chemical analysis of the isolated crude exopolymer material indicated that it contained protein, polysaccharide, and DNA. The treatment promoted the selective isolation of firmly bound polymers from the surface of adherent cells.

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Des cellules bactériennes de sédiments d'eau fraîche cultivées en laboratoire ont produit des polymères extracellulaires fermement liés qui, au niveau ultrastructural, ressemblent à ceux produits par les populations bactériennes de sédiments naturels. Le maintien de la production d'exopolymères a été assuré au cours des repiquages en utilisant comme source d'inoculum la population de cellules qui adhéraient les unes aux autres et aux parois en verre des flacons de culture. Les exopolymères ont été libérés de façon sélective suite au mélange et à la centrifugation des cellules en présence de EDTA. L'évaluation de l'activité glucose-6-phosphate déshydrogénase et de la concentration du 2-céto-3-désoxyoctonate a révélé qu'au cours de l'extraction des polymères, seules de faibles quantités de composés intracellulaires et pariétaux étaient libérées. L'analyse chimique du matériel brut d'exopolymères a indiqué que ce matériel contenait des protéines, des polysaccharides et de l'ADN. Le traitement a favorisé l'isolation sélective des polymères fermement liés à la surface des cellules qui présentaient de l'adhérence.

[Traduit par le journal]

Introduction

Recent interest in the ecology of sessile microbial populations has often focused on the extracellular polymers elaborated by the cells (Corpe 1970; Costerton et al. 1981; Uhlinger and White 1983). In aquatic habitats, microbial exopolymers commonly occur as discrete capsules firmly attached to the cell surface or as slime fibers loosely associated with or dissociated from the cells. While it is now believed that many of the capsular polymers may serve as holdfasts, anchoring cells to each other and to inert surfaces, the extent to which they facilitate other interactions between sessile bacteria and their environment is less well understood.

The existence of relatively few procedures to release firmly bound capsular polymers from bacterial cell surfaces, free of intracellular polymers has made it difficult to define specific interactions between exopolymer components and substances in the environment. Most procedures currently in use do not achieve selective release of exopolymers from the cells (Pazur and Forsberg 1980). Except in a few instances (Farrah and Unz 1976; Brown and Lester 1980), the extent of contamination by

other cell polymers during isolation is not assessed (Mayer et al. 1975; Kang et al. 1982). While these factors may not be a problem with bacteria that produce copious amounts of easily released capsular material, they are important in instances where only small quantities of firmly bound capsular polymers are produced by cells. Characterization of the latter requires a procedure that will release sufficient amounts of exopolymer material for analysis with little or no disruption of the cells.

Recently, Brown and Lester (1980) compared the efficiency of various methods for the extraction of capsular polymers from cells of *Klebsiella aerogenes*. Using protein and DNA as indicators of exopolymer contamination by intracellular material, they found that high-speed centrifugation was the most effective method of exopolymer isolation from this bacterium.

We have found, however, that the techniques developed for the selective isolation of capsules from *K. aerogenes* and other bacteria (Corpe 1970; Parsons and Dugan 1971; Williams and Wimpenny 1978; Sutherland 1979) are not suitable for the isolation of firmly bound exopolymers surrounding some freshwater sediment bacteria. One isolate, which exhibited tendencies to aggregate in suspension and to attach to submerged surfaces, produced a thin capsule which could not be removed by centrifugation alone. To obtain sufficient amounts of the capsular layer for evaluation of its adhesive and metal-binding capacity, a procedure was developed to release selectively the firmly bound capsule from the cells. The procedure provided sufficient amounts of material for partial chemical character-

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ization and metal-binding capacity (M. W. Mittelman and G. G. Geesey, to be published) of the exopolymers elaborated by this sediment bacterium.

Materials and methods

Bacterial sampling and isolation

Surface sediments were collected in plastic coring tubes from the Fraser River at New Westminster, British Columbia, Canada, in May of 1978. The cores were sealed with neoprene stoppers and transported to the laboratory on ice. Approximately 0.5 g amounts of mud were aseptically diluted in 10 mL of isolation medium, which consisted of the following: NaNO₃, 0.2 g; K₂HPO₄, 0.2 g; CaCl₂, 0.03 g; MgSO₄·7H₂O, 0.1 g; FeSO₄, 0.001 g; sodium citrate, 0.5 g; sodium succinate, 0.2 g; sucrose, 5.0 g; yeast extract, 0.5 g; 1 L distilled water, pH 7.9. The mud slurry was homogenized in a Waring blender for 1 min, then further diluted in isolation medium; portions of each dilution were spread on plates containing isolation medium plus 1.5% agar and incubated at 13°C. The dominant mucoid colonies which developed after 1 week were screened for capsule production using the India ink staining method of Duguid and Wilkinson (1953).

Electron microscopy

One dominant capsule-producing sediment isolate, which exhibited "Pseudomonas-like" characteristics (Mittelman and G. G. Geesey, to be published) but remains to be positively identified, was cultured in cell aggregation medium, which consisted of the following: NaCl, 0.2 g; K₂SO₄, 0.01 g; MgSO₄·7H₂O, 0.1 g; CaCl₂, 0.03 g; FeSO₄, 0.001 g; Na₂HPO₄, 9.2 g; K₂HPO₄, 0.8 g; sucrose, 5.0 g; (NH₄)₂SO₄, 2.36 g; 1 L distilled water, pH 7.9. Cell aggregates were collected with a Pasteur pipet and fixed in 0.067 M sodium cacodylate buffer, pH 7.0, containing 0.5% glutaraldehyde and 0.05% ruthenium red. Thin sections were prepared using the method of Patterson et al. (1975) and examined under a Phillips 300 transmission electron microscope.

Growth studies

Cells which grew as a collar on the inner surface of swirling culture flasks were used to inoculate a battery of flasks containing 50 mL of cell aggregation media. The flasks were incubated on a gyratory shaker at 13°C. At daily intervals the entire bacterial culture from three replicate flasks was collected by centrifugation and the cell pellet was assayed for protein (Lowry et al. 1951), carbohydrate (Dubois et al. 1956), and DNA (Burton 1955).

Exopolymer isolation

Five-day cultures were centrifuged at 8000 × g for 10 min and the cell pellet resuspended in holding medium (cell aggregation medium containing 10 mM EDTA, disodium salt, but without sucrose and (NH₄)₂SO₄) and homogenized in a Waring blender for 60 s. The cells were sedimented by centrifugation at 8000 × g for 10 min and portions of the supernatant liquid and cell pellet were assayed for protein, carbohydrate, DNA, and uronic acid (Gregory 1960; Blumenkrantz and Asboe-Hansen 1973). The supernatant and cell pellet were also assayed for glucose-6-phosphate dehydrogenase activity using the method of Lessie and Vander Wick (1972) to determine the extent of contamination of the exopolymer by intracellular material.

The remaining portion of the cell-free supernatant was treated with three volumes of isopropyl alcohol for 24 h at 4°C. The precipitate (crude exopolymer) was collected, dissolved in distilled water, dialyzed against distilled water, and centrifuged at 24 000 × g for 1 h to remove insoluble debris. Contribution of cell wall polymer (lipopolysaccharide) to exopolymer was determined by estimating the amount of 2-keto-3-deoxyoctonate (KDO) and heptose sugar using the methods of Osborn (1963).

The crude exopolymer suspension was subjected to the hexadecyltrimethyl ammonium bromide (cetavlon bromide) treatment of Scott (1965) as modified by Hungerer et al. (1967) to remove nucleic acids. The exopolymer solution was then extracted with an equal volume of ether to remove lipids and the aqueous phase was dialyzed against distilled water to remove traces of organic solvent.

Protein extraction

Two methods were compared for the separation of protein from the polysaccharide exopolymers: phenol extraction and protease treatment. Phenol (45% final concentration) was heated with the exopolymer solution for 15 min at 69°C with periodic agitation. The mixture was cooled rapidly to 8°C and centrifuged to achieve phase separation according to the procedure of Westphal and Jann (1965) as modified by Osborn (1966). The aqueous phase was re-extracted two more times with phenol and then dialyzed at 4°C to remove residual phenol. Protease treatment followed the procedure of Spiro (1976). Pronase E (Sigma Chemical Co.) was mixed with exopolymer solution containing 1.5 mM calcium acetate and 0.15 M Tris-acetate buffer (pH 7.8). The enzyme concentration was adjusted to 1% of the total protein concentration. Additional portions of enzyme equal to one-half the initial amount were added after 24 and again after 48 h. After 72 h of digestion, the protein-depleted exopolymer was dialyzed against distilled water for 48 h at 4°C.

DEAE column chromatography

Phenol- or protease-treated exopolymer (containing approximately 1 mg carbohydrate) was applied to a DEAE Sephadex A-25 column (1 × 30 cm) equilibrated with 0.15 M NaCl in 0.05 M Tris buffer, pH 7.0, washed with 30 mL buffer, and eluted as 3-mL fractions with a linear salt gradient (0.15–0.45 M NaCl). Fractions containing the carbohydrate peak were pooled, the sodium chloride concentration was adjusted to 1 M, and five volumes of ethanol were added to precipitate the polysaccharide. The polysaccharide was dialyzed against distilled water and either frozen or lyophilized.

Determination of sugars in exopolysaccharide

Crude lyophilized exopolymer (approximately 0.3 g) was treated with "magic methanol" (methanol – chloroform – 12 M HCl (10:1:1)), reduced with sodium borodeuteride, and hydrolyzed according to the method of Fazio et al. (1982). The hydrolysate was adjusted to pH 5–7 with 1 M NaHCO₃ and the solution lyophilized. The salt was removed by three successive extractions of the monosaccharides in methanol for 10 min at 60°C and the salt-free sugar suspension evaporated to dryness under a stream of N₂ at 40°C. An internal standard of 100 µL of a 20-mM solution of inositol was added to each preparation; the samples were dried under a stream of N₂ and the sugars were derivatized with 1 mL of hydroxylamine-HCl – pyridine solution (15 mg/mL) for 1 h at 60°C. Peracetylated aldonitrile derivatives were formed by the addition of 1 mL acetic anhydride. Following 1 h incubation at 60°C, the reaction was terminated by the sequential addition of 3 mL chloroform and 3 mL 15% tartaric acid, followed by mixing with a Vortex mixer for 5 min. After three successive chloroform – tartaric acid extractions to remove the pyridine, the chloroform phase containing the derivatized sugars was evaporated to dryness under N₂ and the sugars were redissolved in 1 mL fresh chloroform. Authentic sugar standards were prepared by mixing 0.1 mL of each sugar (0.02 M) with 4 mL of a chloroform-methanol solution (3:1, v/v), drying under a stream of N₂, and derivatization with hydroxylamine HCl – pyridine and acetic anhydride as described above.

Gas-liquid chromatography (GLC)

Samples (1 µL) were injected onto a 25-m bonded phase fused silica capillary column (25QC2; SGE, Australia) in a Varian model 3700 gas chromatograph with a flame-ionization detector and a CDS-111 data system. The temperature was programmed to rise from 150 to 175°C at 3°C/min, remain at 175°C for 10 min, then increase at 2°C/min to a final temperature of 195°C, where it was held for 15 min.

The hydrogen carrier gas was introduced at 12–15 lb/in.² (1 lb/in.² = 6.895 KPa) and a flow rate of 1 mL/min. The injection port was held at 220°C and the detector at 280°C. The samples were analyzed using a splitless injection with a venting time of 0.5 min.

Gas chromatography – mass spectrometry (GC-MS)

GC-MS was performed with a DuPont DP-102 gas chromatograph – mass spectroscope using the column described above operated from 50 to 200°C at 25°C/min and from 200 to 230°C at 2°C/min with

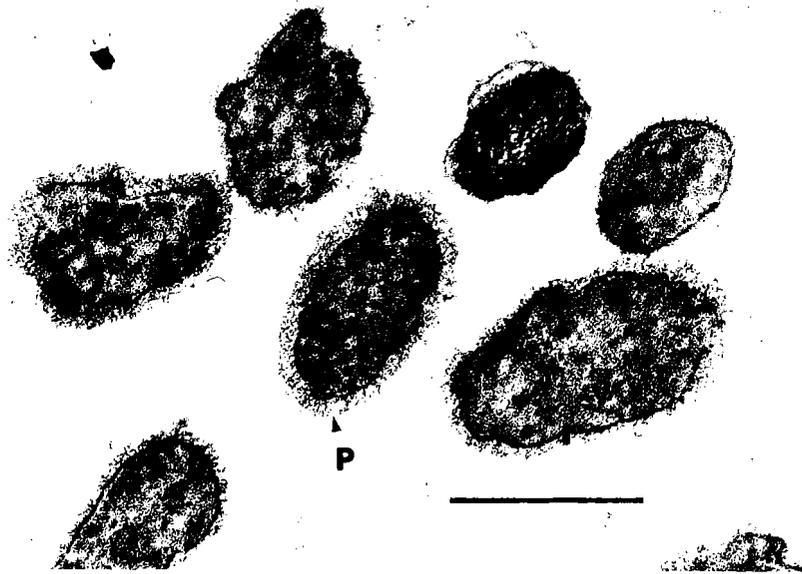


FIG. 1. Transmission electron micrograph of a thin-section preparation of the bacterium isolated from sediment which produced a firmly bound capsule (P). Bar = 1 μ m.

helium as the carrier gas. Samples (1 μ L) were injected onto the column using splitless injection and a venting time of 2.5 min with an additional 30-s delay before the temperature program was started. The mass spectrometer was calibrated and tuned with perfluorotributylamine at a scan speed of 200 atomic mass units (amu) per second.

Protein electrophoresis

Exopolymer proteins were separated by sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE). DNA- and lipid-extracted exopolymer material was dialyzed against 10 mM Tris buffer, pH 7.0, diluted 1:1 in sample buffer containing 2% SDS, 20% glycerol, 0.001% bromophenol blue, 0.1 M dithiothreitol, and 0.025 M Tris buffer, pH 6.8, and immersed in a boiling water bath for 1 min. Samples (100 μ L) were applied to a 10% polyacrylamide slab gel (0.8 mm thick) prepared according to the method of Reid and Bieleski (1968). Electrophoresis was carried out in an electrode buffer containing 0.5% SDS (w/v), 0.05 M Tris base, and 0.38 M glycine buffer until the tracking dye ran off the bottom of the gel (approximately 6 h) using a constant current of 15 mA. Gels were stained in Coomassie blue R, destained overnight, and dried on a slab drier.

Results

Growth characteristics

The bacterial cells grew in clumps when cultured in liquid broth in swirling flasks at 13°C. Clumping was so extensive that very little increase in culture turbidity occurred during cell replication. Under these conditions, increased culture biomass was detected visually by an increase in the number and size of bacterial clumps in suspension and by the formation of a collar of cells on the inner wall of the flask at the liquid-air boundary. Growth was also demonstrated by an increase in total protein, carbohydrate, and nucleic acid concentration over a 4-day period. Electron microscopic examination of thin-section preparations revealed the presence of a capsule around the cells (Fig. 1).

Exopolysaccharide isolation

The capsular material was selectively released from the cells of 5-day cultures by blending for 1 min in the presence of 10 mM EDTA. This treatment released up to 25% of the total cell

carbohydrate. The treatment did not, however, release significant amounts of intracellular polymeric material since only 3.3% of the total cellular glucose-6-phosphate dehydrogenase activity was recovered in the culture menstruum.

Separation of the capsular exopolymers from the cells by centrifugation or blending in the absence of EDTA resulted in the release of only 2 and 18% of the total carbohydrate, respectively. Efforts to release greater than 25% of the total cell carbohydrate using longer periods of blending resulted in the release of intracellular enzyme activity in amounts proportional to that of carbohydrate.

The menstruum of cell suspensions blended for 1 min in the presence of EDTA contained detectable amounts of protein, neutral hexose, and DNA (Table 1). The cell-free extract produced a positive carbazole reaction suggesting the presence of uronic acids; however, no uronic acids were detected when the extract was assayed by the method of Blumenkrantz and Asboe-Hansen (1973). The development of a yellow color in the latter reaction mixture indicated, instead, the presence of pentose sugar (Dische 1947).

The alcohol-precipitated, crude exopolymer contained the same components detected in the cell-free extract as well as KDO and heptose sugar (Table 1). Approximately 10% of the total cell KDO and 13% of the total cell heptose appeared with the crude exopolymer fraction. The heptose:KDO ratio of the crude exopolymer and whole cells was 15:1 and 20:1 (mole:mole), respectively. The hexose:heptose ratios in these fractions were also similar (Table 1). GLC of derivatized hydrolysis products of crude exopolymer indicated that the polysaccharide component contained glucose, arabinose, and rhamnose in a mole ratio of 25:1:1, respectively.

Crude exopolymer was analyzed by GC-MS to further investigate the possible presence of uronic acids. When samples of gum Arabic polysaccharide, which contains glucuronic acid, were reduced with sodium borodeuteride prior to polymer hydrolysis using the procedure of Fazio et al. (1982), analysis by GC-MS revealed the presence of deuterium-enriched fragments with an electronic mass (m/e) of 147 and 219 in the

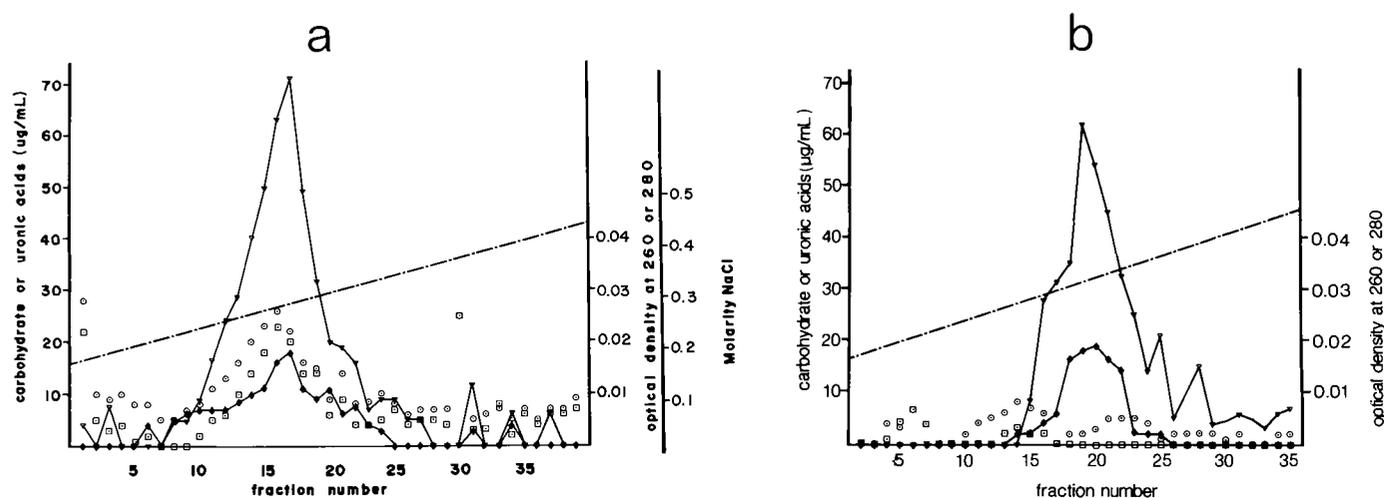


FIG. 2. Elution profiles of (a) phenol-extracted and (b) pronase-treated exopolymer from a DEAE Sephadex A-25 column. Carbohydrate (∇), carbazole-reactive material (\blacklozenge), relative absorbance at 280 (\odot) and 260 (\square) nm, and NaCl concentration (— — —) were monitored in each fraction.

TABLE 1. Exopolymer composition after various stages of purification of polysaccharide component

Preparation	Carbohydrate ^a	Protein	DNA	Uronic acid			
				Carbazole	Metahydroxydiphenyl	KDO	Heptose
Whole cells (222 mg dry weight)	13.8 mg	85 mg	6 mg	3.9 mg	ND ^b	80 μ g	1 mg
Cell-free extract (25 mL)	2759 μ g	2350 μ g	474 μ g	350 μ g	ND	ND	ND
Crude exopolymer precipitated by alcohol (21 mg dry weight)	1975 μ g	1247 μ g	342 μ g	340 μ g	ND	8 μ g	128 μ g
Partially purified exopolymer after: Cetavlon bromide treatment	1266 μ g	1212 μ g	ND	279 μ g	ND	ND	114 μ g
Phenol treatment	570 μ g	189 μ g					
Pronase treatment	1101 μ g	346 μ g					
Exopolymer after DEAE chromatography							
Phenol treated	750 μ g	150 μ g		285 μ g			
Pronase treated	751 μ g	75 μ g		150 μ g			

^aTotal neutral hexose sugar as glucose.

^bND, not detected.

component with a retention time of glucose. Quantitative determinations indicated that glucuronic acid was recovered from gum arabic at 65% efficiency by this technique. Samples of crude exopolymer prepared along with the gum arabic sample, however, showed no deuterium enrichment in any fragments obtained from the separated sugars. These results provide additional evidence that uronic acids were not present in the exopolymer of this sediment bacterium.

Partial purification of exopolysaccharide

Treatment of the cell-free exopolymer material with cetavlon bromide removed >98% of the DNA, 36% of the neutral hexose, 18% of the carbazole-reactive material, and 11% of the heptose sugar. No significant loss of these components was observed following lipid extraction.

PAGE of the partially purified exopolymer yielded a variety of bands representing peptides ranging in size from <15 000 to >150 000 daltons. Separation of protein from the polysaccharide components proved difficult. Removal of 85% of the protein through repeated aqueous phenol extraction resulted

in a 55% loss of polysaccharide (Table 1). Digestion of the exopolymer with pronase E removed 73% of the protein with a 13% loss of polysaccharide.

DEAE column chromatography of the phenol-extracted and pronase-treated exopolymer produced similar profiles for the respective neutral hexose and carbazole-reactive components when eluted with a sodium chloride gradient (Figs. 2a and 2b). A major, broad neutral hexose peak, representing polysaccharide, eluted from both preparations at a 0.25–0.30 M salt concentration. Protein elution profiles based on absorbance at 280 nm differed slightly between the two treatments. Total protein concentration was relatively low in each case, however. Final 16- and 11-fold purifications of polysaccharide relative to protein were achieved after the eluants from peak tubes were pooled from pronase-treated and phenol-extracted exopolymer material, respectively.

Discussion

Capsular polymers were consistently produced by cells of

this sediment bacterium when the population adhering to the wall of the culture vessel was used as the inoculum for subcultures. These polymers, which at the ultrastructural level resembled those elaborated by natural sediment bacterial communities (Moriarty and Hayward 1982), also promoted adhesion under laboratory conditions as demonstrated by the collar that formed on the inner wall of culture flasks. It is likely, therefore, that the selective pressures used to encourage exopolymer production in this study mimicked, to some extent, those that exist in natural sediments.

Determination of glucose-6-phosphate dehydrogenase, KDO, and total carbohydrate provided a means of evaluation and selection of an exopolymer isolation procedure that produced a sufficient amount of capsular material for characterization yet was contaminated with only small quantities of cell wall and intracellular polymers. Unlike the capsular material of some bacteria (Duguid and Wilkinson 1953; Farrah and Unz 1976; Williams and Wimpenny 1978; Sutherland 1979; Brown and Lester 1980), the exopolymers of the sediment bacterium described above could not be efficiently separated from the cells by centrifugation or blending alone. The presence of EDTA was required during the blending and centrifugation treatments to achieve the release of sufficient amounts of exopolymer for subsequent characterization.

The enhanced recovery of polysaccharide in the presence of EDTA probably resulted from the capacity of the chelator to complex divalent metal ions forming coordination complexes among the polymers. However, EDTA is known to extract lipopolysaccharide (LPS) from Gram-negative bacterial cell envelopes (Gray and Wilkinson 1965; Leive 1965; Rogers et al. 1969). Electron microscopic examination of thin-section preparations indicated that the sediment isolate possessed a cell envelope architecture consistent with that of Gram-negative bacteria. The recovery of KDO and heptose sugar in ratios similar to those reported for core polysaccharide of LPS isolated from other bacteria (Osborn 1963) indicates that LPS was extracted with the exopolymer of the sediment isolate.

The ratio of glucose to heptose or KDO in the crude exopolymer, however, was considerably different from those reported for LPS in other bacteria (Osborn 1963; Prehm et al. 1975). On the basis of GLC results which demonstrated that glucose contributed 93% of the total hexose sugars detected, crude exopolymer possessed a glucose:heptose (mole:mole) ratio of 17:1. In contrast, LPS from a galactose-4-epimerase mutant of *Salmonella typhimurium* exhibited a glucose:heptose ratio which ranged from 1:2 to 1:1 (Osborn 1963) and that of a wild-type *Escherichia coli* was found to have a ratio of 2:3 (Prehm et al. 1975). If the recoveries of heptose and KDO were similar to that of glucose, and the LPS of the sediment isolate contained a glucose:heptose ratio of 2:3, then only 2.4% of the total exopolymer glucose appeared to be in the form of LPS. The remaining 97.6% is therefore likely to be associated with capsular polysaccharide. However, the possibility exists that the exopolymer consists of a portion of the LPS, such as a glucose-rich O-antigen segment of the molecule, which was sheared from the cell surface by the blending and centrifugation treatment.

Although all exopolymer fractions produced a positive carbazole reaction, the presence of uronic acids could not be confirmed by the more specific assay of Blumenkrantz and Asboe-Hansen (1973) or by GC-MS using the technique of Fazio et al. (1982). Furthermore, subsequent studies showed that quantities of glucose (hexose) and arabinose (pentose)

comparable with those present in the exopolymer samples contributed the bulk of the carbazole-positive material initially attributed to uronic acids (data not shown).

Uronic acids are often difficult to recover quantitatively from polysaccharides (Blake and Richards 1968; Lindberg et al. 1975). Guluronic acid and mannuronic acid were not recovered quantitatively from alginic acid and xanthan gum, respectively, when evaluated by the method of Fazio et al. (1982) in the present study (data not shown). Thus, the possibility of uronic acids being present in the exopolymer of this sediment bacterium cannot be completely ruled out at this time.

The detection of a large number of Coomassie blue stained bands following PAGE suggests that a complex assemblage of proteins was associated with the exopolymers of this sediment bacterium. Protein has been found to be associated with the capsules of numerous bacteria (Povoni et al. 1972; Usui et al. 1981; Orr et al. 1982). Linker and Jones (1966) reported problems in removing protein from slime produced by a pseudomonad without considerable loss of polysaccharide. Carbohydrate chains of bacterial capsules have been shown to trap and bind small and large molecular weight substances (Rees 1976). The difficulty encountered during attempts to separate protein from polysaccharide in the present study indicates that these exopolymer components are firmly associated with each other. Some of the proteins may be extracellular enzymes and other proteins released from intact cells.

The development of a procedure for the isolation of firmly bound exopolymers from sessile sediment bacteria provides the opportunity to determine specific interactions between these ubiquitous cell surface components and substances in the environment without substantial interference from other cellular constituents. Such an approach should lead to a better understanding of the importance of exopolymers to the microorganism and the environment.

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