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Partial Chemical and Physical Characterization of Two Extracellular Polysaccharides Produced by Marine, Periphytic *Pseudomonas* sp. Strain NCMB 2021

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The marine bacterium *Pseudomonas* sp. strain NCMB 2021, which can attach to solid, and especially hydrophobic, surfaces, elaborates two different extracellular polysaccharides in batch cultures. One (polysaccharide A) was produced only during exponential growth and contained glucose, galactose, glucuronic acid, and galacturonic acid in a molar ratio of 1.00:0.81:0.42:0.32. It produced viscous solutions, formed gels at high concentrations, and precipitated with several multivalent cations. The other (polysaccharide B) was released at the end of the exponential phase and in the stationary phase. It contained equimolar amounts of *N*-acetylglucosamine, 2-keto-3-deoxyoctulosonic acid, an unidentified 6-deoxyhexose, and also O-acetyl groups. Despite its high molecular weight (10^5 to 10^6 as judged by gel filtration), the polysaccharide produced aqueous solutions with very low viscosities and was also soluble in 90% aqueous phenol, 80% methanol, and 80% ethanol.

Extracellular polysaccharides are thought to be involved in the adhesion of aquatic bacteria to solid surfaces (37). They may act directly as adhesives for the initial attachment (14) or form a complex slime layer surrounding the attached cells (5, 6, 13–15, 37).

A marine bacterium, *Pseudomonas* sp. strain NCMB 2021, originally isolated by Fletcher and Floodgate (14), has been used in several studies of attachment properties and mechanisms (12–16) and has become an important model in work of this kind. Electron microscopy (14, 15) demonstrated that the free-living bacteria are surrounded by a thin polymeric coat which can be stained by ruthenium red. This coat (primary polysaccharide) seems to bind the bacteria to solid surfaces. After attachment, a secondary polysaccharide, also stained by ruthenium red, is produced and builds up a gel-like layer between the cells.

Little is known about the nature of these polymers. Fletcher (13) isolated a nondialyzable fraction containing mannose, glucose, glucosamine, galactose, and ribose, but no uronic acids, from culture supernatants. Infrared spectroscopy suggested the presence of carboxyl groups, but no further characterization was attempted.

The extracellular polysaccharides from a few other surface-colonizing marine bacteria have been briefly described. Corpe (6) isolated an acidic polysaccharide containing mannose, glucose, galactose, glucuronic acid, and pyruvate from *Pseudomonas atlantica*. Sutherland (36) isolated extracellular polysaccharides with similar gross composition from several marine and fresh water strains. However, neither the physical properties nor the role of these polymers in the attachment process and biofilm formation was studied in detail.

This report deals with the isolation and characterization of two different extracellular polysaccharides produced by *Pseudomonas* sp. strain NCMB 2021. The aim was to see whether the chemical and physical properties of these polymers can give further information on their role in the initial attachment and biofilm buildup and also provide a basis for further studies in this field.

MATERIALS AND METHODS

Bacterial strain. The bacterium, a marine *Pseudomonas* sp. strain NCMB 2021 (National Collection of Marine Bacteria), was obtained from Madilyn Fletcher, Department of Environmental Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom. It was preserved in freeze-dried ampoules, and new stock cultures were established at regular intervals.

Media and growth conditions. The yeast extract used in the growth experiments contained significant amounts of high-molecular-weight carbohydrate. This material was removed before growth experiments by dialysis through tubing (Spectrapor 3787-F57) with a molecular weight cutoff of 8,000. Small-scale cultures were grown in 100 ml of medium in 500-ml flasks at 20 to 22°C on a rotary shaker (150 rpm; diameter, 14 cm). The medium contained peptone (0.3%, wt/vol; Difco Laboratories) or yeast extract (0.3%, wt/vol; Difco) in filtered seawater. Glucose (variable concentrations up to 5%, wt/vol) was added to this medium. Growth was measured turbidimetrically (660 nm). The cells were harvested by centrifugation (4,800 × *g* at 4°C for 20 min). The supernatants were filtered through a Whatman GF/F filter and dialyzed exhaustively against distilled water. The nondialyzable fractions were analyzed by colorimetric methods as described below.

Large-scale cultures for polysaccharide production were grown at 17 or 20°C in a 5-liter batch fermentor, aerated by bubbling. The medium contained yeast extract (Difco) (12 g) and glucose (8 g) in filtered (Whatman GF/F) seawater (4 liters). Samples (40 to 100 ml) were taken out at regular intervals for growth measurements and analysis after centrifugation, filtration, and dialysis as described above. Cells harvested in the late-stationary phase (after 48 to 50 h) were washed twice in filtered seawater, suspended in distilled water, and freeze-dried immediately. The filtered and dialyzed supernatant was designated the SL fraction.

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Solid cultures were grown on plates (18 cm) containing yeast extract (0.3%, wt/vol), glucose (0.2%, wt/vol), and Difco agar (1.5% wt/vol) in filtered seawater. The agar was overlaid by a circular piece of dialysis tubing to prevent contamination by agar polysaccharides. The plates were inoculated with 5 ml of a stationary-phase liquid culture. The cultures were incubated for 13 days at 10°C and harvested by carefully scraping off the lawn of bacteria from the upper side of the tubing. The cells were dispersed by shaking in 0.5 M NaCl (100 ml) for 60 min and then harvested by centrifugation at 19,000 × *g* for 30 min. The supernatant was filtered and dialyzed as described above and was designated the SA fraction.

General fractionation methods. (i) Gel filtration. Small samples were chromatographed on a column (2.6 by 95 cm) of Sepharose CL-2B or a column (1.6 by 80 cm) of Sepharose CL-6B, both equilibrated and eluted with 50 mM Na_2HPO_4-NaH_2PO_4 (pH 7.0) containing 0.5 M NaCl. The flow rate was kept constant (3 to 5 cm/h) with a peristaltic pump. The elution was monitored by continously measuring the UV absorbance at 206 nm (ISCO model 1840 detector) or 280 nm (Pharmacia UV-1 detector) and by chemical analysis of the fractions. Large samples were chromatographed on a column (6.0 by 110 cm) of Sepharose CL-2B with the same buffer and flow rate.

(ii) Anion-exchange chromatography. The samples were dissolved in 10 to 20 ml of 50 mM Tris hydrochloride (pH 7.0) and applied to a column (2.6 by 40 cm) of DEAE-Sephadex A-25 equilibrated with the same buffer. The column was eluted with a linear gradient (0 to 2.0 M) of NaCl in the buffer. The flow rate was kept at 5 cm/h with a peristaltic pump, and the elution was monitored as described above.

Viscosity measurement. Measurements of viscosity were performed at 20°C in an Ubbelohde capillary viscometer. The polysaccharide solutions were diluted directly in the viscometer by the addition of isoionic NaCl solution. The intrinsic viscosity was determined as a function of ionic strength with NaCl concentrations of 0.01, 0.1, and 1.0 M.

Charge density measurements. Solutions of polysaccharides were dissolved in distilled water and dialyzed thoroughly against 0.1 M $MgCl_2$ and then distilled water. Bound magnesium was released by dialyzing several times against 0.2 M HCl. The dialyzable fractions were combined, and the magnesium content was analyzed by atomic absorption spectroscopy (Perkin-Elmer 560 atomic absorption spectrometer).

Analytical methods. (i) Colorimetric analyses. Uronic acids were measured by the carbazole-sulfuric acid method (9) with D-glucuronic acid or purified polysaccharide as the standard. 6-Deoxyhexoses were analyzed by the cysteine-sulfuric acid method (10) with L-rhamnose or purified polysaccharide as the standard. Total carbohydrates were analyzed by the phenol-sulfuric acid method (11) with D-glucose as the standard. Total protein was analyzed by the method of Lowry et al. (24) with bovine serum albumin as the standard.

(ii) Acid hydrolysis before chromatography. Acid-labile components were liberated by hydrolysis in 0.05 M H_2SO_4 at 80°C for 60 min. The hydrolysates were neutralized by the addition of calcium carbonate and filtered. Other sugars were liberated by hydrolysis in 0.5 M H_2SO_4 at 100°C for 5 to 24 h. Hydrolysates prepared with 2 M trifluoroacetic acid (100°C for 5 to 24 h) were neutralized by evaporation to dryness under reduced pressure. Complete hydrolysis of uronic acids was performed by suspending freeze-dried polysaccharide (10 mg) in 0.2 ml of ice-cold 80% (vol/vol) H_2SO_4. The suspension was left overnight on a slowly melting ice bath. Distilled water (2.8 ml) was added, and the solution was heated at 100° C for 5 h.

Neutral and acidic sugars in the hydrolysates were separated by passing the neutralized solution (adjusted to pH 8.5 to 9 to saponify uronic acid lactones) through a column (1.0 by 5.0 cm) of Dowex 1 × 8 (200-400 mesh, acetate form). Neutral components were eluted with 15 ml of distilled water, and acidic components were eluted with 15 ml of 2 M acetic acid. Acetic acid was removed from the eluates by several evaporations with water under reduced pressure.

(iii) Paper chromatography and electrophoresis. Ascending paper chromatogaphy (Selecta no. 2043 paper, 20- by 31-cm Schleicher & Schuell) was performed with the following solvents: (A) 1-butanol–pyridine–0.1 M HCl (5:3:2, vol/vol); (B) 2-propanol–acetic acid–water (58:8:18, vol/vol); (C) pyridine–ethyl acetate–acetic acid–water (5:5:1:3, vol/vol); (D) benzene–1-butanol–pyridine–water (1:5:3:3, vol/vol); (E) 1-butanol–pyridine–water (6:4:3, vol/vol). Neutral sugars were chromatographed in solvents C and D, amino sugars where chromatographed in solvent E, acid-labile sugars were chromatographed in solvents A and B, and uronic acids were chromatographed in solvent C. Paper electrophoresis of acidic components was performed as described by Haug and Larsen (18).

Neutral sugars and uronic acids were detected by spraying the chromatograms with *p*-anisidine hydrochloride and heating at 110°C for 3 to 10 min (20). Amino sugars were detected by spraying with ninhydrin (E. Merck AG) and heating at 105°C for 5 min. 2-Keto-3-deoxy sugars were detected with the periodate-thiobarbituric acid method (2).

(iv) Gas-liquid chromatography of alditol acetates. The samples were hydrolyzed in 2 M trifluoroacetic acid as described above. De-N-acetylated sugars were re-N-acetylated by the method of Levvy and McAllan (22). Alditol acetates were prepared as described by Sawardeker et al. (31). Gas-liquid chromatography was performed on a Perkin-Elmer Sigma 2 gas chromatograph equipped with a flame ionization detector. The carrier gas was nitrogen. A stainless steel column (2 m by 2.2 mm) of 1.5% XE-60 plus 1.5% HI-EFF-2BP on Chromosorb W.HP (100-120 mesh) was operated isothermally at 190°C or with a linear gradient (155 to 190°C, 1°C/min). N-acetylated hexoses were eluted by increasing the temperature to 200°C.

(v) Methanolysis and gas-liquid chromatography of trimethylsilylated or trifluoroacetylated methyl glycosides. Freeze-dried samples (0.2 to 0.5 mg) were dried in vacuo over P_2O_5. Methanolysis was performed by adding 1.0 ml of 1.0 M HCl in dry methanol and heating in screw-capped vials with Teflon-lined septa at 80°C for 24 h. The samples were dried under a stream of nitrogen. Dry methanol was added, and the evaporation was repeated twice. The samples were dried overnight in vacuo over KOH pellets. In some cases, the methanolysis was repeated once or twice to increase the recovery of uronic acids. The methyl glycosides were converted into the corresponding trimethylsilyl derivatives by adding 0.2 ml of Sylon HTP (Supelco). Gas chromatography was performed on a Carlo Erba 4200 gas chromatograph equipped with flame-ionization detector. The carrier gas was hydrogen. A fused silica capillary column (15 m) of DP5 was operated with a temperature gradient (140 to 170°C, 1°C/min). Methanolysis and gas-liquid chromatography of trifluoroacetylated methyl glycosides were performed as described by Bryn and Jantzen (4).

Reduction of carboxyl groups. Carboxyl groups in the hydrolysates were esterified by refluxing overnight in 1.0 M methanolic HCl. The sample was neutralized by several

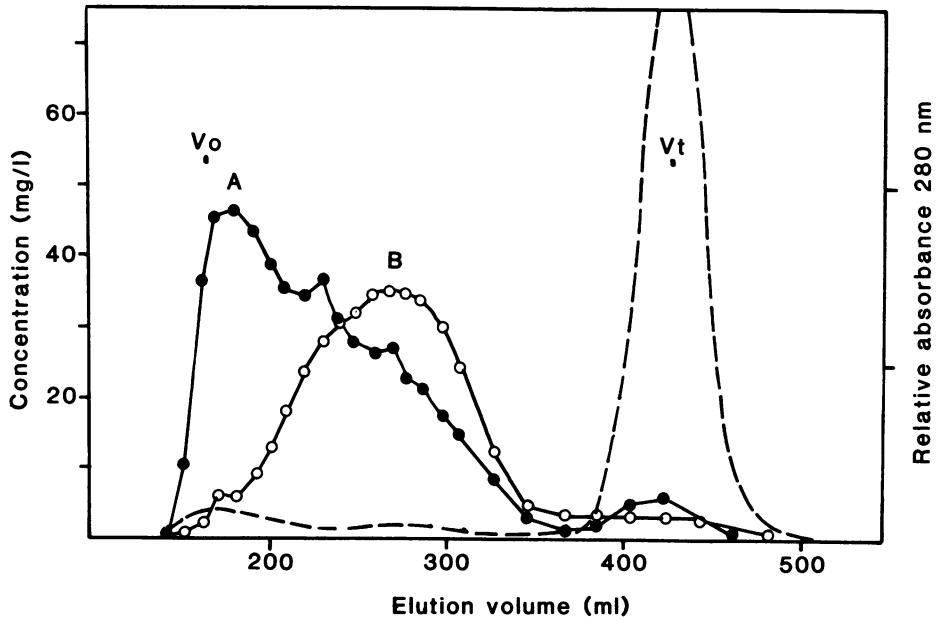

FIG. 1. Gel filtration of the crude SL fraction on a column (2.6 by 94 cm) of Sepharose CL-2B. The eluent was 50 mM sodium phosphate (pH 7.0) containing 0.5 M NaCl. The flow rate was 14.6 ml/h. Fractions (4.9 ml) were analyzed for uronic acids (●) (9) and 6-deoxyhexoses (○) (10). Proteins were detected by continously measuring the UV absorbance at 280 nm (——). V_0 and V_t represent the void and the total volumes of the column, respectively.

evaporations with methanol under reduced pressure. Reduction was performed by the addition of methanolic $NaBH_4$. After 20 min, the solution was neutralized by the addition of Dowex 50W × 8 resin (H^+ form). The sample was filtered, and the filtrate was evaporated to dryness three times with methanol. The methyl glycosides were hydrolyzed in 2 M trifluoroacetic acid and analyzed by gas-liquid chromatography as described above. Esterification of carboxyl groups by carbodiimide and subsequent reduction without degradation of the polymer were performed as described by Taylor et al. (39).

De-O-acetylation. The sample (15 mg) was dissolved in 2 ml of 0.1 M NaOH and heated at 37°C for 2 h. The solution was neutralized by adding 0.2 ml of 1.0 M HCl, desalted on a PD-10 column (Pharmacia), and freeze-dried.

RESULTS

Growth and polysaccharide production. When the bacterium was grown in batch culture in a medium containing yeast extract (0.3%) and glucose (0.2%) in seawater, approximately 240 mg of nondialyzable, soluble extracellular polysaccharide (SL fraction) per liter was recovered from the culture supernatant in the late-stationary phase (after 48 to 50 h). The medium itself (i.e., the yeast extract) contained significant amounts of high-molecular-weight carbohydrate, but this was easily removed by dialyzing the yeast extract before use and using only the diffusible fraction. Neither growth nor polysaccharide production was affected by dialyzing the yeast extract. Further details on polysaccharide production in batch cultures are given at the end of this section.

When grown on agar plates, the bacterium produced a slimy layer on the agar. The slime was extracted with 0.5 M NaCl (SA fraction) after 13 days of growth at 10°C. It contained slightly less nondialyzable carbohydrate (72 mg/g [dry weight] of cells) than the SL fraction (87 mg/g). The ratio of 6-deoxyhexoses to uronic acids was lower in the SA fraction (0.47 compared with 0.78), and it had a higher protein content (33% compared with 26%). The SA fraction was not studied further.

Purification of the SL fraction. Gel filtration of the SL fraction on a column of Sepharose CL-2B separated it into a polysaccharide and a protein fraction. The protein eluted in the total volume (V_t) (Fig. 1). The carbohydrate fraction was composed of two populations with different sugar compositions and elution profiles. The first component (polysaccharide A) was detected with the carbazole-sulfuric acid reaction, but did not respond in the cysteine-sulfuric acid reaction, indicating the presence of uronic acids but no 6-deoxyhexoses. Most of polysaccharide A eluted in the void volume (V_0). The second component (polysaccharide B) was detected with the cysteine-sulfuric acid reaction, indicating the presence of a 6-deoxyhexose. It did not react significantly in the uronic acid analysis and had a typical bell-shaped elution profile with a peak maximum at a K_{av} value of 0.40. [$K_{av} = (V - V_0)/(V_t - V_0)$, where V is the elution volume, V_0 is the void volume, and V_t is the total volume of the column.] Only small amounts of the polysaccharides were detected in the protein fraction.

The presence of the two different polysaccharides in the SL fraction was also demonstrated by anion-exchange chromatography (Fig. 2). Both polysaccharides bound to the anion-exchange resin (DEAE-Sephadex A-25) at pH 7.0 and low ionic strength. By increasing the concentration of NaCl in the buffer, both polysaccharides were eluted. Polysaccharide B eluted at a somewhat lower ionic strength than polysaccharide A (peak maxima at 0.30 and 0.40 M NaCl, respectively). Both polysaccharides gave distorted elution profiles with a sharply rising front and considerable tailing. The components were not very well separated in this experiment. The resolution was not improved by using a phos-

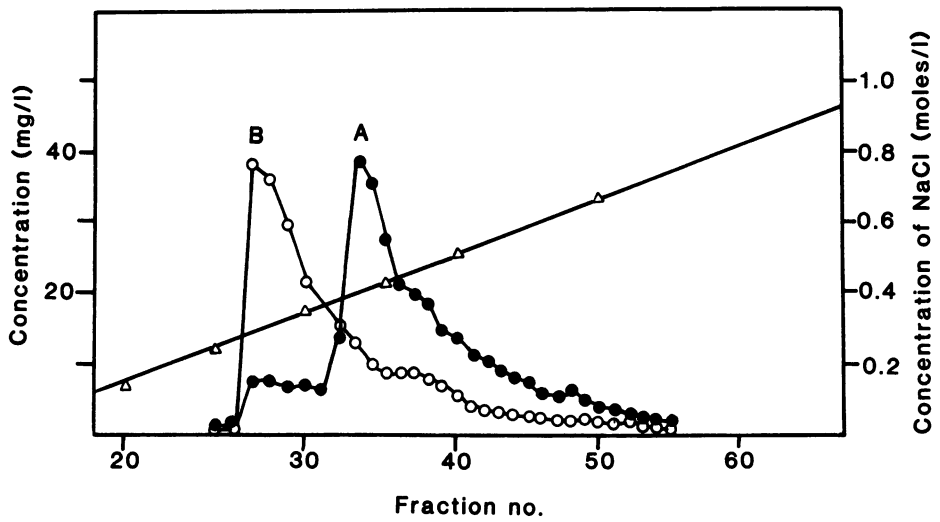

FIG. 2. Chromatography of the crude SL fraction on a column (2.6 by 40 cm) of DEAE-Sephadex A-25 equilibrated with 50 mM Tris hydrochloride (pH 7.0). The column was eluted with a linear gradient of NaCl (0 to 2 M) in the Tris hydrochloride buffer. The flow rate was 25 ml/h. The fractions (12.5 ml) were analyzed for uronic acids (●) (9) and 6-deoxyhexoses (○) (10). The content of NaCl (△) was analyzed by titration with silver nitrate.

phate gradient or eluting with an NaCl gradient in sodium acetate buffer at pH 5.0.

Partial acid hydrolysis and separation of the polysaccharides. The two polysaccharides had very different stabilities in dilute acid. Figure 3 shows that B was rapidly degraded at pH 3.0 and 90°C, leaving A almost undegraded. Thus it should be possible to separate A and B by gel filtration after partial hydrolysis. Figure 4 shows that they separated on Sepharose CL-2B after hydrolysis at pH 3.0 for 30 min at 90°C. B eluted as a narrow peak in the total volume, whereas the elution profile of A was little affected by the acid treatment. There was some cross-contamination between the two components, but this was probably due in part to mutual interference in the colorimetric analyses. Gas-liquid chromatographic analyses showed that the A fraction was almost completely devoid of contamination by B, whereas there were only traces of A in the B fraction.

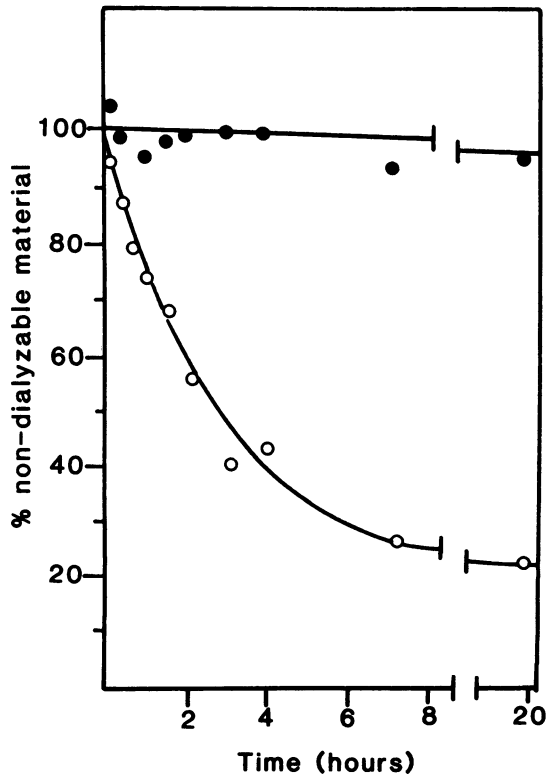

FIG. 3. Degradation of the SL fraction at pH 3.00 at 90°C. Samples were taken at regular intervals, neutralized with dilute NaOH, and dialyzed against distilled water. The remaining uronic acids (●) and 6-deoxyhexoses (○) were analyzed as in Fig. 1.

A smaller portion of the A peak (shaded area in Fig. 4) was selected for further chemical and physical studies of polysaccharide A.

To minimize the degradation of polysaccharide B, the partial hydrolysis was also performed at pH 5.0 (30 min at 90°C). The size distribution of B after partial hydrolysis at both pH 3 and pH 5 was investigated by gel filtration on Sepharose CL-6B. When degraded at pH 5.0, it had a bell-shaped elution profile with peak maximum at a K_{av} value of 0.53, indicating a molecular weight of 10^5 to 10^6 (28). The elution profile was not changed when a detergent (0.2% Triton X-100) replaced the sodium chloride in the eluent. After degradation at pH 3.0, the polysaccharide eluted as a narrow peak near the total volume (K_{av} = 0.87), suggesting a much lower molecular weight.

Chemical composition of polysaccharide A. Paper chromatography, paper electrophoresis, and gas-liquid chromatography indicated that purified polysaccharide A contained glucose, galactose, glucuronic acid, and galacturonic acid. The nature of the uronic acids was confirmed by reduction of the carboxyl groups followed by hydrolysis or methanolysis and gas-liquid chromatographic analysis. Extended methanolysis was necessary to liberate the uronic acids, especially glucuronic acid. The monomer composition of polysaccharide A obtained by different methods is shown in Table 1. ^1H nuclear magnetic resonance analysis (B. E. Christensen, unpublished results) after degradation by sonication indicated that the polysaccharide contained substituents. A peak

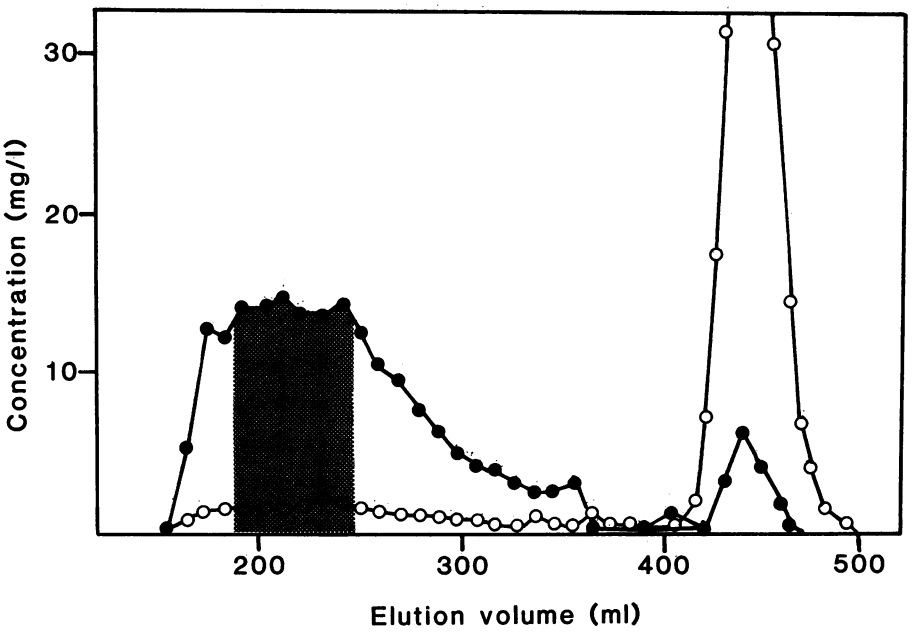

FIG. 4. Gel filtration of the protein-free SL fraction (obtained as shown in Fig. 1) on Sepharose CL-2B after partial hydrolysis (pH 3.00 at 90°C for 30 min). Column, elution, analyses of fractions, and symbols are as in Fig. 1. The material in the shaded area was selected for chemical and physical studies of polysaccharide A.

at 2.1 ppm indicated the presence of O-acetyl groups. The spectrum also contained a broad peak at 2.7 ppm, but its origin is not known. These peaks disappeared when the sample was degraded by partial hydrolysis. No sulfate or phosphate groups were detected in the polysaccharide.

Physical properties of polysaccharide A. Polysaccharide A produced very viscous solutions in water. At concentrations above approximately 1% (wt/vol), it produced a transparent gel rather than a true solution. The gel dissolved reversibly upon heating at 70 to 80°C. The intrinsic viscosity [η; defined as $|\lim c\rightarrow 0|(\eta_{sp}/c)$, where η_{sp} is the specific viscosity] at 20°C was 19.2 dl/g in 10 mM NaCl, decreasing to 11.8 dl/g in 100 mM NaCl and 8.9 dl/g in 1.0 M NaCl. When plotted against the resiprocal of the square root of the ionic strength, the intrinsic viscosity yielded a straight line with a slope (S) of 1.13. The stiffness parameter B, which is defined by Smidsrød and Haug (34) as S divided by the intrinsic viscosity at an ionic strength of 0.1 M to the power of 1.3, could then be calculated and was found to be 0.047. The polysaccharide was precipitated by Zn^{2+}, Cu^{2+}, Al^{3+}, and Fe^{3+}. It was also precipitated by Cetrimide (E. Merck AG), a quarternary ammonium salt.

Chemical composition of polysaccharide B. Nuclear magnetic resonance studies (B. E. Christensen, unpublished results) indicated the presence of three different monosaccharides in equimolar amounts, in addition to O-acetyl groups (0.95 per trisaccharide unit). They also indicated the presence of an N-acetyl group. The same results were obtained for both undegraded (before separation from polysaccharide A) and degraded and purified polysaccharide B. Paper chromatography after acid hydrolysis showed one component which cochromatographed with glucosamine and stained with ninhydrin. Gas-liquid chromatographic analysis confirmed the presence of *N*-acetylglucosamine.

The response in the cysteine-sulfuric acid analysis suggested the presence of a 6-deoxyhexose. The chromatographic analyses showed one component with mobilities close to, but clearly different from, those of L-rhamnose, L-fucose, and 6-deoxy-D-glucose (quinovose), indicating another unidentified 6-deoxyhexose.

The presence of a 2-keto-3-deoxy acid was suggested by characteristic peaks in the nuclear magnetic resonance spectra as well as the great lability toward acid and the polyanionic character of polysaccharide B. Mild acid hydro-

TABLE 1. Monosaccharide compositon of polysaccharide A

Method	Monsaccharide composition (mmol/g [dry wt])						Total negative charges	Recovery (% of dry wt)[a]
	Glucose	Galactose	Total hexoses	Glucuronic acid	Galacturonic acid	Total uronic acids		
Gas-liquid chromatography[b]	1.45	1.18	2.63	0.64	0.43	1.07		70.5
Colorimetric analyses[c]			2.77			0.99		71.3
Charge density measurements[d]							1.37	

[a] Uronic acids were in the sodium form.
[b] Obtained after methanolysis in 1.0 M HCl of 80°C for 24 h. The methanolysis was repeated 3 times.
[c] Obtained by a combination of the phenol-sulfuric acid (11) and the carbazole-sulfuric acid (9) analyses.
[d] Obtained by measuring the amount of bound magnesium after having transferred the polysaccharide to the magnesium form.

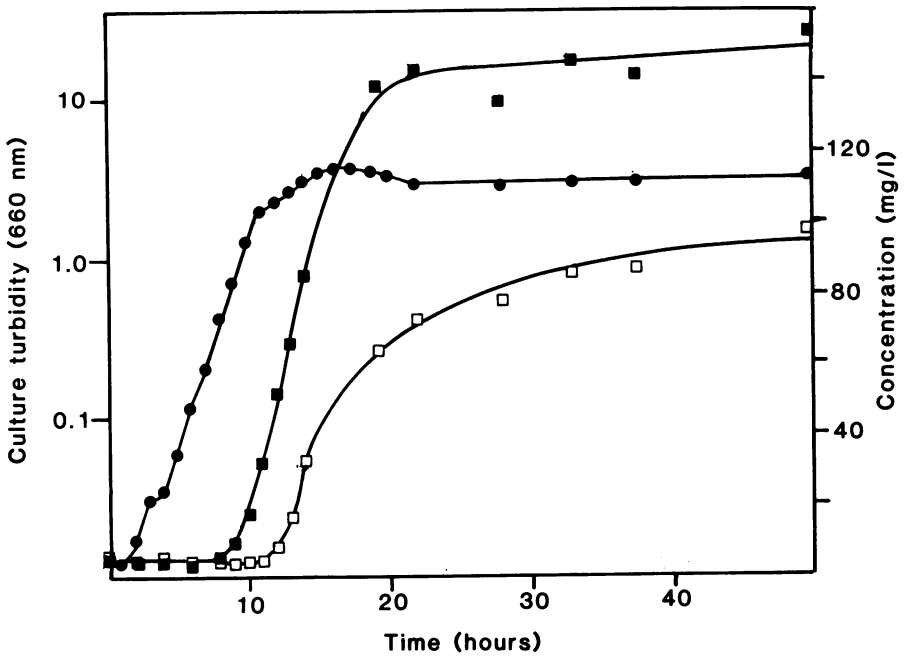

FIG. 5. Growth curve (culture turbidity at 660 nm) (●) and the production profiles of polysaccharide A (■) and polysaccharide B (□) in a batch culture of *Pseudomonas* sp. strain NCMB 2021 grown at 17°C. Samples (30 to 50 ml) were taken at regular intervals, centrifuged, filtered, and dialyzed. The content of polysaccharide A was measured by the carbazole-sulfuric acid method (9), and the content of polysaccharide B was analyzed by the cysteine-sulfuric acid method (10).

lysis (0.05 M H_2SO_4, 80°C for 60 min) and subsequent paper chromatography gave three spots reacting in the periodate-thiobarbiturate test. The central spot cochromatographed with 2-keto-3-deoxyoctulosonic acid. The presence of 2-keto-3-deoxyoctulosonic acid was confirmed by the gas-liquid chromatographic method of Bryn and Jantzen (4). The origin of the other spots was not investigated.

Physical properties of polysaccharide B. The preparations obtained by gel filtration after partial hydrolysis at pH 5.0 were used for studying the physical properties of polysaccharide B. It was freely soluble in water. At a concentration of 80 mg/ml, the solution was only slightly viscous. No viscosity measurements were performed. The de-O-acetylated polysaccharide was equally soluble in water, but curiously it produced turbid solutions at elevated temperatures (50 to 90°C). The turbidity disappeared immediately upon cooling. The polysaccharide was not precipitated by any of the multivalent cations tested (Ca^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Al^{3+}, Fe^{3+}). However, when mixed with polysaccharide A, both precipitated with several cations.

When suspended in 90% (wt/wt) aqueous phenol (41), 60% of the material dissolved within 30 min. When subjected to gel filtration on a column of Sepharose CL-6B, the phenol-soluble fraction had the same elution profile as samples degraded at pH 5.0, suggesting that the solubility in phenol did not result from degradation.

Aqueous solutions of B did not precipitate upon the addition of ethanol or methanol unless the final concentration of alcohol exceeded 80% (vol/vol).

Production profiles of polysaccharides A and B and the effects of different medium composition on polysaccharide production. The specific colorimetric analyses for uronic acids and 6-deoxyhexoses made it possible to analyze the content of both polysaccharides A and B in the culture supernatant without prior separation. A batch culture was grown at 17°C in a medium containing yeast extract (dialyzed, 0.3%) and glucose (0.2%) in seawater. The production profiles of the two polysaccharides are shown together with the growth curve in Fig. 5.

Production of polysaccharide A was mainly observed in the late-exponential growth phase. There was only a small increase in the polysaccharide content in the stationary phase. Polysaccharide B had a quite different production profile. Its release into the medium started in the late growth phase, and none was produced during exponential growth. The production continued well into the stationary phase, but at a lower rate.

The production of both polysaccharides depended upon the composition of the medium (Table 2). By adding glucose (up to 1%) to the medium base of either peptone (0.3%) or yeast extract (0.3%), both denser cultures and higher polysaccharide production (relative to the maximum culture

TABLE 2. Amounts of polysaccharides A and B recovered from the culture supernatant (SL fraction) after growth in media of different compositions.

Medium composition (%)			Culture turbidity (660 nm)	Polysaccharide concn (mg/liter)	
Peptone	Yeast extract	Glucose		A	B
0.3			0.44	30.8 (69.9)[a]	49.1 (111.5)
0.3		0.2	0.47	63.7 (135.6)	70.8 (150.7)
0.3		1.0	0.71	118.9 (167.4)	79.8 (112.4)
		5.0	0.65	130.2 (200.4)	49.1 (75.5)
	0.3		0.61	26.6 (43.6)	21.1 (34.6)
	0.3	0.2	0.75	97.5 (130.1)	99.5 (132.7)
	0.3	1.0	0.92	103.9 (112.9)	142.7 (155.1)
	0.3	5.0	0.61	82.9 (135.8)	74.3 (121.8)

[a] The values within parentheses represent the concentration of the polysaccharides relative to the concentration of cells. The latter is expressed as the turbidity of the culture in the stationary phase.

densities) were obtained. In general, the ratio of polysaccharide A to polysaccharide B increased with increasing concentration of glucose. With 5% glucose the production of the cells and the polysaccharides was lower than with 1% glucose.

DISCUSSION

The fractionation experiments clearly indicated that *Pseudomonas* sp. strain NCMB 2021 produced two fundamentally different extracellular, soluble polysaccharides. They were separated and purified in a two-step procedure based upon gel filtration and controlled, partial hydrolysis. Other common methods for purifying the polysaccharides, for instance, precipitation with Cetavlon, gave less satisfactory results.

Polysaccharide A, containing glucose, galactose, glucuronic acid, and galacturonic acid, resembles many slime or capsular polysaccharides from other bacteria, including nonmarine organisms (6, 35, 36). Different methods for analyzing the sugar composition of polysaccharide A gave somewhat different results (Table 1). The analysis of charge density suggested a higher content of uronic acids than that found in the other analyses. One major reason for these differences is the general problem of hydrolyzing glycosidic bonds when uronic acids are involved (33). Mild hydrolysis conditions give incomplete release of uronic acids, whereas stronger conditions may lead to further degradation of the liberated sugars. Some undetected substituents were probably also present in the polysaccharide, as indicated by unidentified peaks in the nuclear magnetic resonance spectra and the fact that only 71% of the dry weight could be accounted for in the sugar analysis. These substituents might possibly contribute to the observed charge density. Further work on the chemical composition of polysaccharide A is in progress.

Aqueous solutions of polysaccharide A were quite viscous, and the intrinsic viscosity was dependent on the ionic strength. The stiffness parameter B was 0.047. Such values are typical for polyelectrolytes with relatively stiff chains, for instance, β-1,4-linked glucans (34).

Polysaccharide A was only produced by growing cells. The production in the stationary phase was almost negligible. Most exopolysaccharides are produced both in the exponential and stationary phases (35, 40); an exception has been noted by Williams and Wimpenny (42) for another nonmarine pseudomonad, which produces exopolysaccharide only in the stationary phase.

Since polysaccharide A was able to form gels it may possibly function as the secondary polysaccharide described by Fletcher and Floodgate (14), i.e., in maintaining a hydrated and mechanically stable matrix between the attached cells and consequently determine many of the physical properties of established biofilms (5). The ability to bind several multivalent cations may be a mechanism for protecting the cells from toxic heavy metal ions (7).

Both in chemical composition and physical properties, polysaccharide B was very different from polysaccharide A. Two of the monosaccharides, *N*-acetylglucosamine and the 6-deoxyhexose, are typical constituents of bacterial exopolysaccharides (35). 2-Keto-3-deoxyoctulosonic acid is usually associated with the core region of bacterial lipopolysaccharides (33, 35), but has also been found in exopolysaccharides (3, 21). The presence of 2-keto-3-deoxyoctulosonic acid explains the great lability of the polysaccharide toward hydrolysis in dilute acids (33). Nuclear magnetic resonance studies (unpublished results) showed that the molar ratio between the monosaccharides was 1:1:1, suggesting a trisaccharide repeating unit, and with 0.95 O-acetyl groups per repeating unit.

The physical properties of polysaccharide B were quite unusual. Despite its high molecular weight as demonstrated by gel filtration, it produced solutions with low viscosity, suggesting that the molecule has a very flexible chain (random coil). It was also quite soluble in 90% aqueous phenol and in very high concentrations of methanol and ethanol, thus exhibiting a more protein-like behavior. This phenomenon is probably due to the hydrophobic methyl and acetyl groups, and the molecule would therefore be expected to participate in hydrophobic interactions (38). However, self-association of polysaccharide chains into larger aggregates or formation of micelle-like structures probably did not occur in aqueous solutions, since no change in the elution properties was obtained by adding Triton X-100 (32).

Polysaccharide B was also different from polysaccharide A in that it was released only in the very late growth phase and in the stationary phase, similar to the exopolysaccharide of the pseudomonad described by Williams and Wimpenny (42).

A production profile similar to that of polysaccharide B has been described for the polysaccharide emulsan, which is produced by *Acinetobacter calcoaceticus* RAG-1 (17, 43). In the early-exponential phase, emulsan exists as a thin capsule on the cell surface and is gradually released into the medium when the cells approach the stationary phase (17). Preliminary investigations have shown that polysaccharide B can be extracted from washed cells, even in the late-stationary phase, suggesting that some kind of capsule may be present on the bacterial surface, also in this case.

Compared with many exopolysaccharide-producing bacteria, low yields of the two polysaccharides were obtained. Higher exopolysaccharide production is often obtained by increasing the carbon/nitrogen ratio of the medium, for instance, by adding more glucose (35). This effect was indeed observed for both polysaccharide A and B, but to a rather limited extent. For further studies of polysaccharide production, defined media should be used instead of the complex media used in this work.

The production of two different polysaccharides in pure cultures has been observed for other bacteria, for instance, *Serratia marcescens* (1), *Streptococcus faecalis* (27), and *Rhodopseudomonas capsulata* Sp11 (26). Variations in the sugar compositon of the polysaccharide fraction produced under different growth conditions or in different growth phases have been described for several bacterial strains (1, 40). It is not unlikely, in view of the findings mentioned above, that these organisms produce two or more polysaccharides and that variations in the sugar composition reflect changes in the production rates of the different polysaccharides rather than structural changes in one of them.

It has been assumed by several authors that cell surface polysaccharides are involved in the initial adhesion of bacteria to solid surfaces (6, 8, 13, 14). The polysaccharides described in this study may be important in such interactions, but no direct evidence for such a function has yet been obtained. The chemical structure and the solution properties of polysaccharide B correlate to some degree with the tendency of the pseudomonad to attach preferentially to hydrophobic surfaces (16). However, the polysaccharides studied were obviously not associated with the cell surface, but were excreted into the medium as soluble molecules. Since washed cells are still able to adhere (12, 16), the adhesion-enhancing molecules would be expected to be

present on the cell surface after washing. Preliminary investigations suggested that the two polysaccharides were still present on the surfaces of washed cells.

Several adhesion-related phenomena which have been explained as hydrophobic interactions have been described for many bacterial strains (for review, see reference 25). These phenomena include aggregation of the cells above certain critical salt concentrations, binding to oil-water or air-water interfaces, binding to hydrophobic gels (hydrophobic interaction chromatography) or fatty acids, and partitioning in a two-phase liquid system. In most of these cases it has been assumed that the molecular basis for the hydrophobic properties lies in cell surface proteins (19, 23, 25). Polysaccharides, usually in the form of a loose slime or discrete capsules, have been reported to enhance or reduce binding to hydrophobic surfaces. Hogt et al. (19) found that several encapsulated staphylococci adhered better to hydrocarbons than did many noncapsulated strains, whereas slimes usually reduced the binding. On the other hand, the presence of emulsan (a polysaccharide containing 17% fatty acid esters [43]) as a capsule on *A. calcoaceticus* RAG-1 greatly reduced the ability of the cells to adhere to hydrocarbons compared with noncapsulated bacteria (30), although purified emulsan itself is able to bind to and emulsify hydrocarbons in water. Pringle et al. (29) isolated two spontaneous mutants from continous cultures of *Pseudomonas fluorescens*. The wild type, which produced little exopolysaccharide, was foam fractionated, i.e., it adhered to the air bubbles. A mucoid mutant producing an alginate-like, water-soluble exopolymer accumulated in the liquid phase. The other mutant adhered to the glass walls of the fermentor. It had a crenated colony morphology and produced little exopolymer.

These data from the literature thus give no clear indications as to whether the two polysaccharides described in this study are involved in the attachment of the *Pseudomonas* sp. strain NCMB 2021 to solid surfaces, as suggested by earlier electron microscopic investigations. As in the case of emulsan, the chemical composition and the solution properties of the polysaccharides are probably not the only factors which determine the effects of these substances on the adhesion of bacterial cells to surfaces. The orientation of the polymers relative to the cell surface and the surface of the substratum may be equally important (38).

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