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Alginate Production by Clinical Nonmucoid *Pseudomonas aeruginosa* Strains

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The slime material from a revertant nonmucoid variant, derived by serial passage of a heavily mucoid *Pseudomonas aeruginosa* strain isolated from a patient with bacteremia, was found to contain 16% uronic acids, 48.5% carbohydrates, 11% protein, and 2% lipids. Chromatographic analysis by ion exchange chromatography revealed that this extracellular material consisted of three fractions, one uronic acid fraction with properties similar to those of the alginate fraction of the parental strain and two other fractions consisting of neutral sugars and proteins in approximately a 5:1 ratio. In addition, the slime material from six other clinical macroscopic nonmucoid *P. aeruginosa* strains was found to contain alginate. These results demonstrate that alginate production in various amounts is a property shared by all *P. aeruginosa* strains.

Pseudomonas aeruginosa produces an extracellular slime layer characteristic of the species (17). The term slime has been used to denote extracellular substances of different chemical and physical characteristics such as neutral sugars (9), polysaccharide (7), hexosamine, and glucuronide (12) according to their extraction procedure. Bartell and coworkers have isolated a toxic glycolipoprotein (GLP) from the slime layers of all seven Fisher immunotypes with no qualitative and only certain minor quantitative differences from strain to strain (2, 10, 19, 30).

Mucoid strains of *P. aeruginosa* are isolated mainly from sputum cultures from patients with cystic fibrosis (CF) of the pancreas; such strains account for only 1 to 2% of clinical *P. aeruginosa* isolates in the general population (12). The extracellular polysaccharide produced by mucoid strains has been characterized as an O-acetylated polymer of mannuronic acid and guluronic acid that is similar to the alginic acid which is produced by brown seaweed (21) and the exopolysaccharide from the soil bacterium *Azotobacter vinelandii* (14). This exopolysaccharide has also been termed slime.

These mucoid strains seem to lose their ability to produce large amounts of extracellular polysaccharide with in vitro passage on nutrient agar plates, and this phenomenon is expressed by changes in colony morphology. This passage also enhances the simultaneous emergence of nonmucoid revertant colonies which usually belong to the same serotype and are considered to represent the more stable morphologic expression of the same strain (15, 24, 33).

Recently, we isolated a heavily mucoid *P. aeruginosa* strain from two blood cultures from a patient with drug-induced hepatitis. The major component of the extracellular material of this strain was characterized as alginate (1). The present study was undertaken to determine whether the revertant nonmucoid variant (RNMV) derived from our mucoid strain (MS) produced any extracellular material and, if so, to determine its chemical composition with respect to the extracellular material of the parental MS. In addition, the extracellular materials from six other clinical macroscopic nonmucoid *P. aeruginosa* strains were assayed specifically for the presence of alginate.

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MATERIALS AND METHODS

Bacterial strains. A clinical isolate of grossly mucoid *P. aeruginosa* was obtained from two blood cultures as previously described (1). RNMV were derived by serial passage of the parental MS on Trypticase soy agar plates (BBL Microbiology Systems, Cockeysville, Md.). Six other clinical macroscopic nonmucoid *P. aeruginosa* isolates (three from blood cultures and three from urine cultures) were also used in the present work.

Culture of bacteria. Mid-log-phase bacterial suspensions in Trypticase soy broth (BBL) were inoculated on cellophane sheets (12 cm in diameter) overlaying Trypticase soy agar plates and grown for 18 h in humidified chambers.

Extraction of extracellular products. Extracellular material was obtained by procedures which have been described previously for the extraction of GLP from the slime layer of *P. aeruginosa* (3, 29).

In brief, we harvested cells by washing the cellophane with 0.15 M NaCl and extracted the extracellular products by gentle shaking with glass beads. The extracts were precipitated with 95% ethanol in the presence of 10% sodium acetate and 1% glacial acetic acid. The precipitate was dissolved in distilled water, clarified by centrifugation at 27,000 × *g* for 30 min, and then dialyzed against distilled water for 3 days. The above procedure was repeated twice. The dialysates were centrifuged at 105,000 × *g* for 3 h, to remove any lipopolysaccharide, and the supernatants were lyophilized and stored at −20°C.

Chemical analyses. Chemical determinations were made on three independently prepared lots in triplicate, and average values were estimated. Uronic acids were determined by the method of Bitter and Muir with mannuronic acid standards (6), hexoses were determined by the anthrone method with glucose standards (31), protein was determined by the method of Lowry et al. with bovine serum albumin standards (22), total amino sugars were determined by the method of Belcher et al. (4), total lipids were determined by the gravimetric method of Salton (28) and the colorimetric method of Zöllner and Kirsch (kit was obtained from E.

TABLE 1. Chemical composition of MS and RNMV extracts

Component	MS extract (% dry wt)[a]	RNMV extract (% dry wt)[a]
Uronic acids	65.0	16.0
Neutral sugars	3.2	42.5
Amino sugars	2.2	5.5
Protein	1.0	11.0
Total lipids	0.9	2.0
KDO[b]	0.4	0.85
Phosphorus	—[c]	—

[a] The values represent the average estimates of three independently prepared lots in triplicate.
[b] KDO, 2-Keto-3-deoxyoctulosonic acid.
[c] —, Not significant.

Merck AG, Federal Republic of Germany), Darmstadt, 2-keto-3-deoxyoctulosonic acid was determined by the thiobarbituric acid assay (13, 26), and phosphorus was determined by the method of Chen et al. (8).

Thin-layer chromatography. Chromatographic separation of neutral sugars was performed as follows. Samples were hydrolyzed in 1 M HCl under nitrogen for 4 h at 100°C. After this period, the samples were cooled at room temperature and the HCl was evaporated in 40°C water bath under a nitrogen stream. The pellet was dissolved in distilled water to a final concentration of 2 mg/ml and centrifuged for 10 min in an Eppendorf centrifuge. Supernatants were spotted on silica gel plates (E. Merck AG) and analyzed in three different solvent systems: system I, *n*-butanol–acetone–water (40:50:10); system II, ethyl acetate-acetic acid-methanol-water (60:15:15:10), and system III, *n*-propanol–water (85:15). A diphenylamine-aniline-phosphoric acid-containing reagent (16) was used for developing the chromatograms. Rhamnose, ribose, arabinose, mannose, galactose, and glucose were used as standards.

Ion exchange chromatography. Ion exchange chromatography was carried out on DEAE-cellulose (DE 52; Whatman, Inc., Clifton, N.J.) columns at 4°C. Samples dialyzed in equilibration buffer (50 mM Tris hydrochloride [pH 8.2] 0.025 M EDTA) were applied on 15 ml bed volume columns. The bound fractions were eluted with a linear salt gradient (0 to 0.6 M NaCl) made in the same equilibration buffer. Fractions of 2.6 ml were collected and analyzed for uronic acids, neutral sugars, and protein as described above.

Infrared spectroscopy. Infrared spectra (KBr disk) were obtained with a spectrophotometer (model 137; The Perkin-Elmer Corp., Norwalk, Conn.).

RESULTS

Extracellular material was obtained from all strains as described in Materials and Methods. Harvested cells from 300 plates yielded approximately 12 g and 83 mg from the MS and RNMV, respectively. The extract from the RNMV was readily dissolved in distilled water, while the extract from the MS formed a very viscid solution in concentrations even less than 1 mg/ml. Gross chemical analyses of both extracts are presented in Table 1.

The chemical compositions of MS and RNMV extracts are qualitatively similar but show significant quantitative differences. Uronic acids are the major component of the MS extract, while neutral sugars predominate in the RNMV extract. Thin-layer chromatography revealed that the neutral-sugar fraction of the RNMV extract consists mainly of galactose, glucose, and ribose, with trace amounts of rhamnose and mannose.

The uronic acid fraction of the MS has been purified and characterized as alginate (1). To determine whether the uronic acid fraction of the RNMV is also alginate, we compared the elution profiles of both MS and RNMV extracts on DEAE-cellulose columns. The RNMV extract yielded one uronic acid fraction (Fig. 1B) between 0.2 and 0.4 M NaCl at the same position as the uronic acid fraction of the MS extract (Fig. 1A). The RNMV uronic acid fraction was precipitated with $CaCl_2$ but not with $MgSO_4$, a characteristic property of alginates, and yielded a similar infrared spectrum to that of the MS purified alginate fraction (Fig. 2).

Furthermore, all extracts from the six clinical macroscopic nonmucoid *P. aeruginosa* isolates were found to contain 8 to 14% uronic acids (data not shown). These uronic acids were precipitated with $CaCl_2$ but not with $MgSO_4$ and

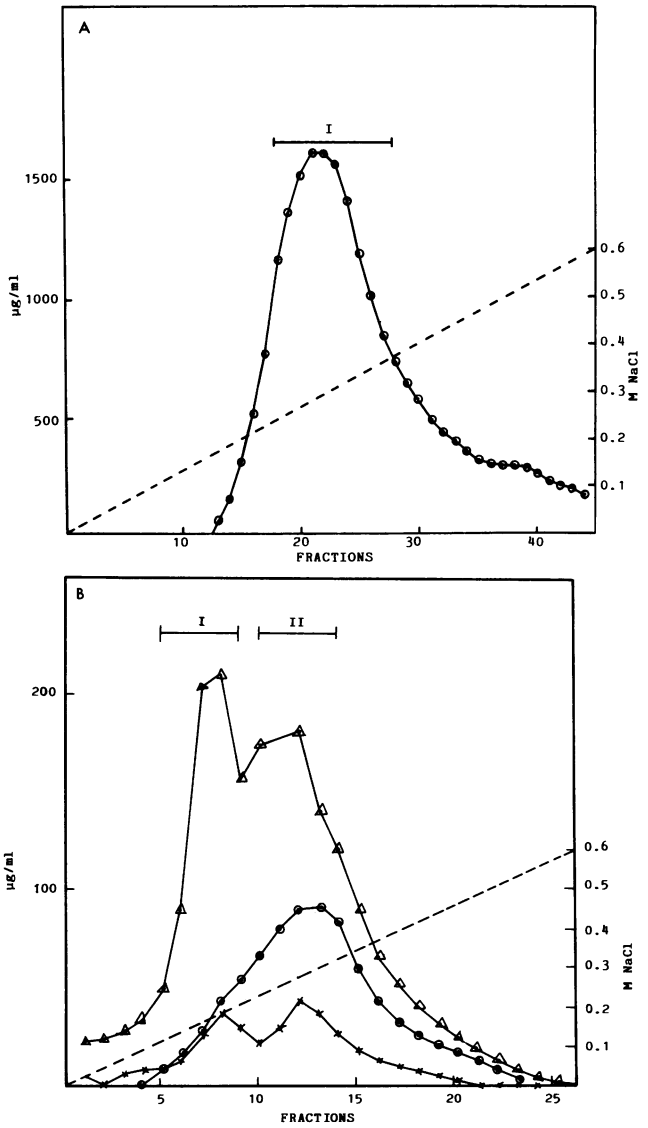

FIG. 1. Chromatographic analyses of extracts isolated from *P. aeruginosa* (A) MS and (B) RNMV. Samples, in 50 mM Tris hydrochloride (pH 8.2)–0.025 M EDTA, were applied in 15-ml bed volume columns, and the bound fractions were eluted with a linear salt gradient (0 to 0.6 M NaCl). Fractions of 2.6 ml were collected and analyzed for uronic acid (○) neutral sugar (△), and protein (×). Bars indicate the positions of the eluted peaks.

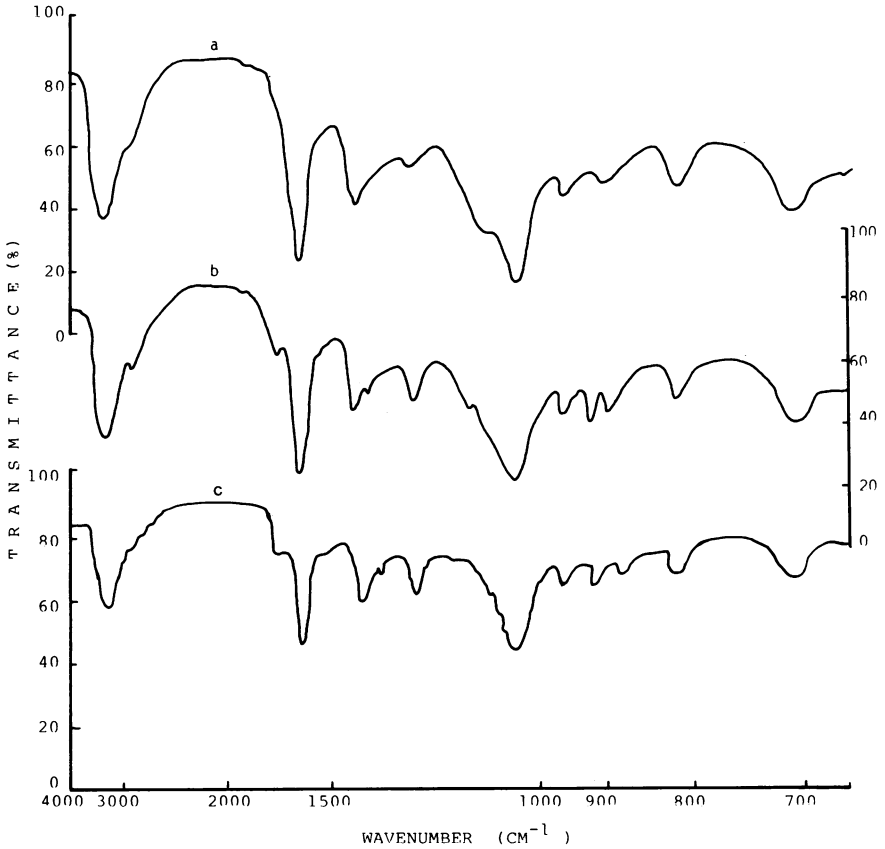

FIG. 2. Infrared spectra of commerical alginate (peak a), the MS alginate (peak b), and the RNMV alginate (peak c).

yielded similar infrared spectra to that of the MS purified alginate fraction, providing further evidence that the extracellular material of nonmucoid strains contains alginate as well.

The RNMV extract, in addition to uronic acid fraction, yielded two other major peaks, the first (I) between 0.1 and 0.2 NaCl and the second (II) between 0.2 and 0.4 M NaCl (Fig. 1B). Carbohydrate and protein analyses indicated that these two peaks contained neutral sugars and protein in approximately a 5:1 ratio. Similar neutral sugar and protein peaks could not be identified in the MS extract.

DISCUSSION

It is generally accepted that the slime of mucoid *P. aeruginosa* is an alginate (21). Ohman and Chakrabarty (25) identified chromosomal loci mediating alginate production by mucoid phenotypes (Alg^+), whereas nonmucoid variants (Alg^-) were characterized by their macroscopic appearance and by the absence of uronic acids in culture supernatants. Other workers, using the methodology of Bartell and coworkers (3, 29) for the extraction of the GLP from the slime layer of nonmucoid *P. aeruginosa*, have shown that uronic acid is a constant component of all seven Fisher immunotypes. However, this uronic acid constituent had been considered as making up part of the GLP macromolecule and had not been characterized (2, 10, 19). Antibody agglutination supports the existence of alginate in RNMV (27), while animal studies have shown that active immunization with purified alginate results in pulmonary clearance of the RNMV of a MS by 30 days after injection and clearance was correlated with antibody to alginate (32).

Our results clearly show that the RNMV produces extracellular material including alginate. Furthermore, the six clinical nonmucoid *P. aeruginosa* strains were very similar to our RNMV with respect to alginate. Therefore, gross appearance is insufficient for designating nonmucoid strains as lacking alginate.

In the environment, *P. aeruginosa* is isolated in the nonmucoid form. The similarity of our clinical isolates with the RNMV of our mucoid strain indicates that if the nonmucoid strains had the proper stimulating conditions (niche), they would ultimately revert to the mucoid phenotype. The hallmark of CF is the identification of mucoid *P. aeruginosa* strains in the sputum of such patients. However, colonization of the respiratory tracts of CF patients occurs first with nonmucoid *P. aeruginosa* strains which ultimately may revert to the mucoid strains; once removed from their niche (i.e., by serial passage) they re-revert to their nonmucoid environmental state (5, 11, 15). This finding is also supported by Lam et al. (20), who have shown that most nonmucoid *P. aeruginosa* isolates from CF patients with chronic lung infection are capable of mucoid growth if the correct media (niche) are used.

The GLP fraction of the slime layer of the seven Fisher immunotypes has been shown to possess many biological properties comparable to those of the viable cell, such as lethality and ability to cause leukopenia, while antibodies to GLP protect against infection by the homologous strain (3, 29, 30). On the other hand, alginic acid and substituted

alginates are nontoxic, have been approved by the Food and Agriculture Organization-World Health Organization Joint Expert Committee for human consumption, and are used for therapeutic purposes (18, 23).

Preliminary results indicate that the material of our RNMV has similar biological activities to those described for the GLP fraction, whereas the alginate obtained from the MS lacks such properties (unpublished results).

Although alginate production is energetically unfavorable for the microorganism (24), its overproduction most probably constitutes an adaptive mechanism for survival in the specific niche of CF patients and only indirectly may contribute to the impaired function of the lungs of CF patients.

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