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Comparison of the Outer Membrane Protein and Lipopolysaccharide Profiles of Mucoid and Nonmucoid *Pseudomonas aeruginosa*

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Laboratory-derived mucoid variants of *Pseudomonas aeruginosa* were selected by plating the standard PAO1 laboratory strain with bacteriophage. These mucoid variants formed two distinct groups of strains on the basis of phage typing. The first group had the same phage-typing pattern as the parent PAO1 strain, while the second group had a distinctly different phage-typing pattern. One strain from each group was assessed along with the parent PAO1 strain for its outer membrane protein (OMP) and lipopolysaccharide (LPS) profiles by sodium dodecyl sulfate-gel electrophoresis followed by appropriate staining. The mucoid derivatives were found to differ from the parent PAO1 nonmucoid strain in having lost a high-molecular-weight LPS species. Furthermore, the reversion of the mucoid strains to the nonmucoid phenotype was accompanied by a return of the missing high-molecular-weight LPS species. No observable difference between the mucoid derivatives and the parent nonmucoid strain was noted in the OMP profiles. The opposite was found in the case of four isolates of mucoid *P. aeruginosa* from patients with cystic fibrosis. Two OMP bands (of approximately 55 and 25 kilodaltons) were present in the mucoid isolates but missing in their sister nonmucoid strains. In the case of the cystic fibrosis isolates, no difference in the LPS profiles within mucoid-nonmucoid pairs was noted.

Mucoid variants of the standard PAO1 laboratory strain of *Pseudomonas aeruginosa* were isolated in our laboratory, having appeared as bacteriophage 7-resistant colonies in the midst of a sensitive PAO1 lawn. Despite being isolated by phage selection, upon further testing some of the mucoid strains were found to be sensitive to the lytic action of phage 7. The emergence of mucoid variants during routine phage typing of *P. aeruginosa* was first described by Martin (18) and has since been used as a method of selection for mucoid variants of *P. aeruginosa* (7, 19). An advantage of having mucoid strains derived from the standard PAO1 laboratory strain of *P. aeruginosa* is that the mucoid phenotype can be studied against a well-defined biochemical and genetic background, a point previously noted in the laboratories of Govan (5, 6) and Holloway (17). The present study was concerned with an examination of the outer membrane protein (OMP) and lipopolysaccharide (LPS) compositions of the mucoid variants in a comparison with the nonmucoid parent PAO1 strain. In addition, mucoid and nonmucoid *P. aeruginosa* pairs isolated from patients with cystic fibrosis (CF) were examined with regard to their OMP and LPS compositions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *P. aeruginosa* used in this study, along with their origins and phenotypes, are described in Table 1. The CF isolates were provided by David Speert of the Biomedical Research Centre of the Children's Hospital in Vancouver, British Columbia, Canada. These isolates were serotyped, showing that the nonmucoid variants, whether isolated from a patient with CF along with the mucoid strain or isolated in the laboratory, were of the same serotype as the mucoid strain (D. Speert, unpublished results). The laboratory-derived mucoid isolates arose and were isolated when phage 7 was plated with our standard laboratory strain of *P. aeruginosa* PAO1 (H103; see Results). Further phage sensitivity testing of the mucoid isolates was performed with a library of 22 phages by previously published methods (20). *P. aeruginosa* H103 and H330m were grown for 2 days in plastic chambers implanted in the peritonea of mice as previously described (13) for electron-microscopic visualization of their gross morphology. Scanning electron microscopy was performed on glutaraldehyde-fixed sections cut from the walls of the plastic chambers containing the *P. aeruginosa* cultures. The samples were washed in 0.05 M phosphate buffer and dehydrated in ethanol by standard procedures and visualized with a Cambridge Stereoscan 250 T scanning electron microscope operating at 20 kV.

OMP profile. OMs of mucoid and nonmucoid *P. aeruginosa* were prepared by sucrose gradient centrifugation of French-pressed cells in the absence of lysozyme treatment as previously described (9). The presence of the mucoid phenotype, after growth in PP2 broth culture for OM preparation, was confirmed by plating a sample onto PP2 agar. OMPs were separated on a sodium dodecyl sulfate (SDS)-14% polyacrylamide gel in the presence of 2-mercaptoethanol and stained with Coomassie blue (9).

LPS profile. Proteinase K-digested whole-cell lysates were prepared from PP2 agar plate-grown cultures by the method of Hitchcock and Brown (12). The LPS profile was examined after electrophoretic separation on a 15% polyacrylamide gel containing 0.5% SDS by the method of Peterson and McGroarty (21), with the addition of 0.2 M EDTA to the same buffer to reduce LPS aggregation. The LPS bands were visualized by periodate treatment and silver staining (12).

Western immunoblotting. Whole-cell lysates were transferred from 15% polyacrylamide gels to nitrocellulose paper overnight at 10 mA. Western immunoblotting with monoclo-

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TABLE 1. Strains of *P. aeruginosa* used in this study

Strain	Phenotype	Source
H103	Nonmucoid	Laboratory strain PAO1
H325m	Mucoid	Derivative of H103
H326m	Mucoid	Derivative of H103
H327m	Mucoid	Derivative of H103
H328m	Mucoid	Derivative of H103
H329m	Mucoid	Derivative of H103
H330m	Mucoid	Derivative of H103
H328nm	Nonmucoid	Revertant of H328m
H330nm	Nonmucoid	Revertant of H330m
H246m	Mucoid	Isolated from CF patient
H247	Nonmucoid	Laboratory revertant of H246m
H411m	Mucoid	Isolated from CF patient
H412	Nonmucoid	Laboratory revertant of H411m
H658m	Mucoid	Isolated from CF patient
H659	Nonmucoid	Isolated from same patient as H658m
H666	Nonmucoid	Isolated from CF patient
H667m	Mucoid	Isolated from same patient as H666

nal antibody MA1-8, which is specific for the serotype antigen of *P. aeruginosa* H103 (11), was performed as previously described (10).

Enzyme-linked immunosorbent assay. Using both the monoclonal antibody and the specific enzyme-linked immunosorbent assay described by Pier et al. (22), we examined our laboratory-derived mucoid strains for the production of alginic acid.

RESULTS

Laboratory-derived mucoid *P. aeruginosa*. When our standard *P. aeruginosa* PAO1 laboratory strain (H103) was plated with phage 7, colonies of the mucoid phenotype were observed. Six independent mucoid colonies were isolated to pure culture and logged in our *P. aeruginosa* collection as strains H325m to H330m. Subsequent phage typing with a collection of 22 phages having different surface receptors located on the pilus, LPS, and OMPs of the pseudomonas cell (20) divided the mucoid isolates into two classes (Table 2). The first class of mucoid derivatives, represented by strains H325m and H328m, was similar to the parent H103 strain in being susceptible to all 22 phages tested, including phage 7. The second class, represented by strains H326m, H327m, H329m, and H330m, formed a distinct group of mucoid isolates having somewhat similar phage profiles to each other but having profiles different from that of the parent H103 strain (Table 2). One strain from each class, i.e., H328m and H330m, was selected for further characterization of its envelope properties. An enzyme-linked immunosorbent assay incorporating antibody specific to pseudomonas alginic acid (22) demonstrated that the laboratory-derived mucoid strains produced alginic acid (N. M. Kelly and R. E. W. Hancock, unpublished data). By using plastic chambers implanted in the peritonea of mice as a means of growing *P. aeruginosa* in vivo (13), the gross morphology of the mucoid derivative H330m was compared with that of the parent H103 strain. Upon electron-microscopic visualization (Fig. 1) of the in vivo-grown cultures, we found the mucoid bacteria to be intimately associated with an electron-dense material which was not evident in the nonmucoid parent H103 cultures.

As is seen with isolates from patients with CF (7, 24), the laboratory-derived mucoid isolates eventually yielded nonmucoid revertants upon multiple subculturing on nutrient agar. Two of these nonmucoid revertants, i.e., H328nm and H330nm, were also included in this study.

TABLE 2. Phage sensitivity profiles of *P. aeruginosa* H103 and its mucoid derivatives

Phage	Sensitivity[a] of strain:						
	H103	H325m	H326m	H327m	H328m	H329m	H330m
119x	+	+	−	−	+	+	−
M6	+	+	−	−	+	+	+
B39	+	+	−	−	+	−	−
44	+	+	+	+	+	+	+
109	+	+	−	−	+	−	−
F8	+	+	−	−	+	−	−
352	+	+	−	+	+	−	+
PB1	+	+	+	−	+	+	−
S1	+	+	+	+	+	+	+
C3A	+	+	+	+	+	+	+
E79	+	+	+	+	+	+	+
7	+	+	−	−	+	−	−
C21	+	+	−	−	+	−	−
A8A	+	+	+	+	+	+	+
B6B	+	+	−	−	+	−	−
B6C	+	+	±	−	+	−	−
B9E	+	+	−	−	+	−	−
2	+	+	−	−	+	−	−
D3C^{-}1^{+}	+	+	+	+	+	−	−
B5A	+	+	±	−	+	−	−
C7B	+	+	+	+	+	+	+
B7A	+	+	−	+	+	+	+

[a] +, Phage sensitive; −, phage resistant; ±, phage sensitive in at least one of three individual phage-typing experiments.

OMP profiles of laboratory-derived and CF isolates of mucoid and nonmucoid *P. aeruginosa*. OM preparations of the standard laboratory H103 strain, the mucoid derivatives H328m and H330m, and their nonmucoid revertants H328nm and H330nm and of four mucoid-nonmucoid pairs of *P. aeruginosa* isolated from patients with CF (Table 1) were prepared by a standard laboratory technique (9). The proteins were separated on SDS-14% polyacrylamide gels in the presence of 2-mercaptoethanol and visualized by Coomassie blue staining (Fig. 2 and 3). The mucoid isolates from patients with CF displayed a prominent protein band of approximately 55 kilodaltons and a second band of approximately 25 kilodaltons which were absent or underexpressed in the concomitantly isolated or laboratory-reverted nonmucoid strains (Fig. 2, lanes 1 to 8). Other differences in protein profiles were noted within individual mucoid-nonmucoid pairs. The most notable of these was the overproduction of a protein of approximately 44 kilodaltons in the laboratory-derived nonmucoid revertants of mucoid CF isolates (Fig. 2; compare lanes 5 and 7 with lanes 6 and 8).

In the case of the laboratory strains of *P. aeruginosa*, no major difference was noted between the OMP profile of the parent H103 strain and those of the two mucoid strains which were derived from it (Fig. 3, lanes 1 to 3). Similarly, no protein bands were gained or lost in the reversion of the mucoid strains to the nonmucoid phenotype (Fig. 3; compare lanes 2 and 3 with lanes 4 and 5).

LPS profiles of laboratory-derived and CF isolates of mucoid and nonmucoid *P. aeruginosa*. Whole-cell lysates of mucoid and nonmucoid laboratory strains and CF isolates grown on PP2 agar medium were prepared by the method of Hitchcock and Brown (12). The cell lysates were electrophoresed through SDS-15% polyacrylamide gels and stained

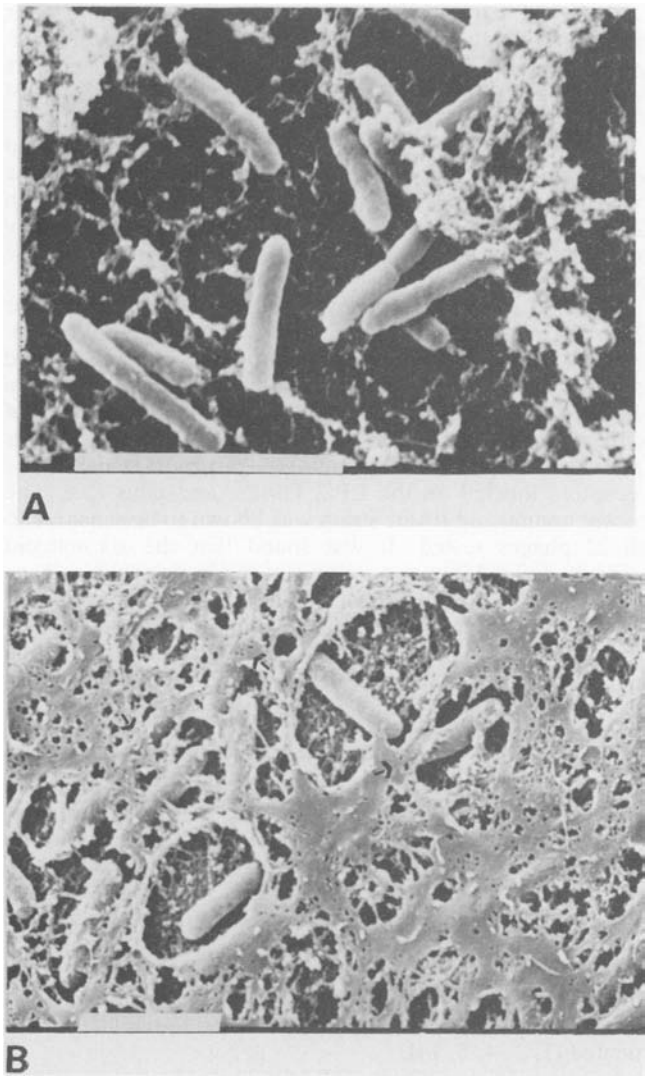

FIG. 1. Scanning electron micrographs of the walls of chambers containing cultures of strain H103 (A) or H330m (B) removed from the peritoneal cavities of mice 44 h after implantation. In contrast to H103, the bacteria of strain H330m appeared to be intimately associated with an electron-dense material (indicated by arrows). (A) Bar, 4 μm; (B) bar, 2 μm.

with silver for visualization of the LPS (Fig. 4 and 5). The four mucoid-nonmucoid pairs of *P. aeruginosa* isolated from patients with CF showed no difference in LPS profile when the mucoid and nonmucoid members of the pair were compared (Fig. 4).

In contrast, the laboratory-derived mucoid strains H328m and H330m appeared to have lost a high-molecular-weight LPS species present in the parent nonmucoid H103 strain (Fig. 5). This pattern was further elucidated when cell lysates of strains H103, H328m, and H330m were reacted with a monoclonal antibody specific for the O antigen of H103 by a Western blotting technique (data not shown). Furthermore, the reversion of these mucoid strains to the nonmucoid phenotype was accompanied by the return of the higher-molecular-weight LPS species (Fig. 5).

DISCUSSION

Mucoid variants of *P. aeruginosa* were isolated in our laboratory by plating phage 7 together with our standard

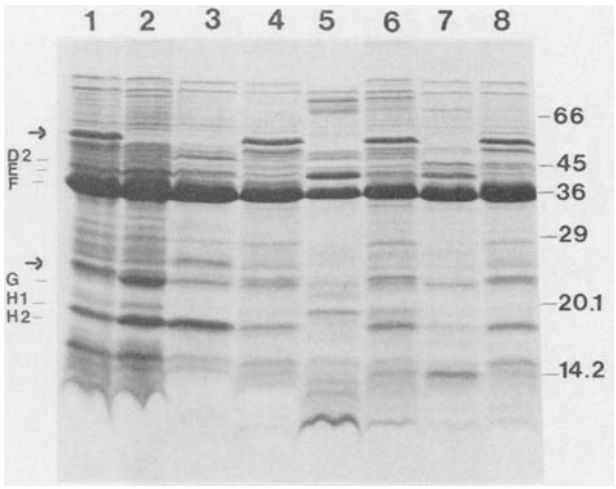

FIG. 2. OMP profiles of mucoid and nonmucoid *P. aeruginosa* isolated from patients with CF. The CF isolates represented paired mucoid (designations ending in "m") and nonmucoid strains isolated at the same time from the same patient, as in the cases of H667m and H666 (lanes 1 and 2, respectively), and H659 and H658m (lanes 3 and 4, respectively), and nonmucoid laboratory revertants of mucoid isolates paired with those mucoid isolates, as in the cases of H247 and H246m (lanes 5 and 6, respectively) and H412 and H411m (lanes 7 and 8, respectively). The proteins were loaded in amounts of approximately 35 mg and separated on an SDS-14% polyacrylamide gel in the presence of 2-mercaptoethanol. The running positions of molecular mass standards (in kilodaltons) are indicated on the right side of the figure. The running positions of the major OMPs are indicated on the left side of the figure, and arrows point to the proteins present in mucoid CF isolates (lanes 1, 4, 6, and 8) which are missing in their nonmucoid counterparts.

PAO1 strain, H103, in an experiment analogous to those previously described by Martin (18), Govan (7), and Miller and Rubero (19). Although these mucoid variants arose as resistant to phage 7, subsequent phage typing showed that

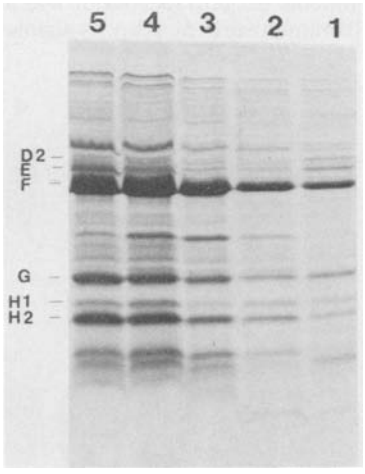

FIG. 3. OMP profiles of laboratory-derived mucoid strains H328m (lane 2) and H330m (lane 3) and their nonmucoid revertants H328nm (lane 4) and H330nm (lane 5) compared with the parent laboratory strain, H103 (lane 1). The proteins were loaded in amounts of approximately 35 mg and separated on an SDS-14% polyacrylamide gel in the presence of 2-mercaptoethanol. The running positions of the major OMPs are indicated on the left side of the figure.

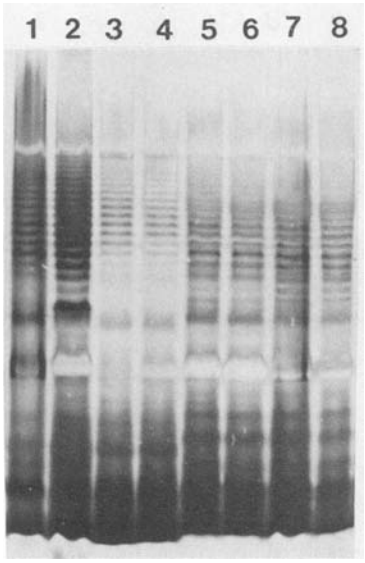

FIG. 4. SDS-15% polyacrylamide gel electrophoretogram of whole-cell lysates of CF isolates of *P. aeruginosa* stained for LPS. Lanes 1 to 4 represent the mucoid CF isolates H246m and H411m (lanes 1 and 3) alongside their respective nonmucoid laboratory revertants H247 and H412 (lanes 2 and 4). Lanes 5 to 8 represent the simultaneously isolated mucoid-nonmucoid pairs H658m and H659 (lanes 5 and 6, respectively) and H667m and H666 (lanes 7 and 8, respectively).

one subset of mutants was in fact sensitive to the lytic action of phage 7 (Table 2). Martin (18) described the same phenomenon in her isolation of mucoid variants during routine typing of *P. aeruginosa* clinical isolates and described it as pseudolysogeny. By further characterizing our laboratory-derived PAO1 mucoid variants, it was determined that they resembled mucoid variants isolated from patients with CF in the following ways: (i) they produced copious amounts of mucoid covering if left growing on nutrient agar at room temperature for 1 week (N. M. Kelly, unpublished observations), (ii) the mucoid exopolysaccharide was identified in an enzyme-linked immunosorbent assay as alginic acid, and (iii)

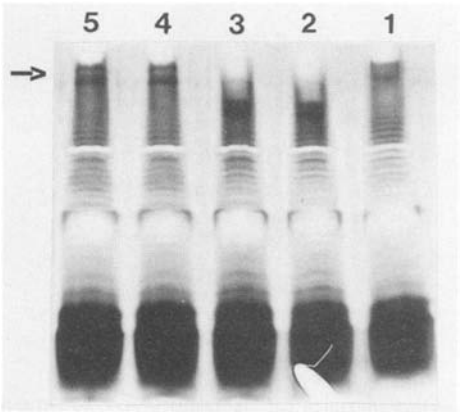

FIG. 5. SDS-15% polyacrylamide gel electrophoretogram of whole-cell lysates of laboratory-derived mucoid strains of *P. aeruginosa* and their nonmucoid revertants stained for LPS. Lane 1, H103; lane 2, H328m; lane 3, H330m; lane 4, H328nm; lane 5, H330nm. The arrow points to the high-molecular-weight LPS species present in the nonmucoid strains but missing in the mucoid strains.

the mucoid variants yielded nonmucoid revertants after multiple subculturing on agar medium. These results suggested that the laboratory-derived PAO1 mucoid variants might be a valid model for study of the mucoid phenotype of *P. aeruginosa* that arises in the lungs of patients with CF (14). An electron-microscopic study of one of the mucoid variants, H330m, did not reveal microcolonies such as have been described for *P. aeruginosa* isolated from a patient with CF (16) but did show that the mucoid variant was covered by an electron-dense material which was missing from the nonmucoid parent strain (Fig. 1).

The first question which we decided to ask about our laboratory-derived mucoid variants was whether the phenotypically observed switch from the nonmucoid to the mucoid colony type had been accompanied by any other changes in the envelope composition. For a preliminary method of screening for changes at the cell surface, we used our laboratory collection of *P. aeruginosa* phages which have receptors located on the LPS, OMPs, and pilus (20). The parent nonmucoid PAO1 strain was known to be sensitive to all 22 phages tested. It was found that the six mucoid variants divided into two groups on the basis of their phage sensitivity. Two of the six displayed the global phage sensitivity of the parent strain. The remaining four had become resistant to approximately half of the 22 phages, representing loss of susceptibility to phages having LPS, OMPs, and pilus receptors. It seems unlikely that the transition from a nonmucoid to a mucoid-producing variant would have been accompanied by 12 or 13 independent changes in at least three different surface structures in the latter case but accompanied by none of these changes in the former case. An alternative explanation is that minor differences in the composition of the alginic acid produced by these two groups of mucoid variants, resulting in different gelling properties, might have been responsible for the covering of phage receptors in the case of the latter group of four mucoid variants. That the mucoid phenotype represents a heterogeneous group in terms of the physical and chemical organization of the exopolysaccharide has been previously documented (1, 2, 4, 5, 23).

One mucoid strain from each of the two phage groups was chosen for further analysis of its envelope composition in terms of its OMP and LPS profiles. No differences between these two mucoid strains were observed; however, both mucoid strains differed from the parent nonmucoid strain in having lost a high-molecular-weight LPS band. Furthermore, the reversion of the mucoid phenotype to the nonmucoid phenotype was accompanied by the return of this high-molecular-weight LPS species (Fig. 5). Knirel et al. (15) have shown that guluronic acid and mannuronic acid, the constituents of the alginic acid in the mucoid covering, are part of the O-specific polysaccharide portion of the LPS in *P. aeruginosa* serogroup O:3 in the Lanyi classification. This Lanyi serogroup encompasses the Habs serogroup 5 of our PAO1 strain (11). Given this information, we suggest that the loss of a high-molecular-weight LPS species in our mucoid strains may represent competition between the assembly of LPS and alginic acid for precursors or for processing enzymes.

Mucoid *P. aeruginosa* isolates from patients with CF were examined along with concomitantly isolated or laboratory-reverted nonmucoid strains for their OMP and LPS profiles. While no changes in the LPS profiles between the CF isolates of mucoid and nonmucoid strains were observed, there was a difference in the OMP profiles. Two protein species, of approximately 55 and 25 kilodaltons, were

present in all four mucoid strains but absent in the equivalent nonmucoid strains (Fig. 2). Dunne and Buckmire (3) described an exopolysaccharide depolymerase activity associated with mucoid strains of CF origin which was missing from their nonmucoid revertants. As seen by polyacrylamide gel electrophoresis, this depolymerase activity was contained in two protein species, namely, a major species of 53 kilodaltons and a minor species of 23 kilodaltons. Depolymerase was released from the cell after EDTA treatment, suggesting a periplasmic or cell wall location. On the basis of molecular masses, it seems plausible that the OMP species detected in our CF isolates of mucoid *P. aeruginosa* and missing in their nonmucoid counterparts may represent this exopolysaccharide depolymerase. Isolation of these proteins accompanied by assaying for their depolymerase activity should resolve this hypothesis.

In conclusion, the findings from this study demonstrating differences in envelope properties between mucoid *P. aeruginosa* of laboratory origin and that of clinical origin adds to the growing body of knowledge which suggests that the mucoid variant of *P. aeruginosa* cannot be represented by one distinct phenotype but rather represents a heterogeneous group of strains having in common the production of alginic acid (1, 2, 4, 5, 23). The environmental pressure resulting in the induction of, and continued selection for, exopolysaccharide synthesis may play a pivotal role in determining the resulting phenotype.

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ADDENDUM

Just recently, while the manuscript was being reviewed, Grabert et al. (8) reported on a 54-kilodalton OMP present in mucoid strains of *P. aeruginosa* and absent in their nonmucoid counterparts. Whether their 54-kilodalton protein and our 55-kilodalton protein are one and the same protein is at this point unclear.

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