JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1982, p. 686–691
0095-1137/82/100686-06$02.00/0

Vol. 16, No. 4

Phenotypic Differences Among Clinically Isolated Mucoid *Pseudomonas aeruginosa* Strains

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Received 10 May 1982/Accepted 28 June 1982

Mucoid strains of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis or urinary tract infections displayed many phenotypic differences. The ratios of D-mannuronosyl to L-guluronosyl moieties of the extracellular alginate-like polysaccharides produced by the 19 strains examined varied from 99 to 0.8; the acetyl content of the polymers varied from 0.38 to 0.02 mol per mole of uronosyl residue. The strains also differed with regard to the stability of the mucoid phenotype. Of 15 isolates from patients with cystic fibrosis, 7 displayed stable mucoid phenotypes; 8 isolates were unstable and reverted to the nonmucoid phenotype at high frequency. The four strains isolated from patients with urinary tract infections were also unstable. Strains from urinary tract infections expressed the mucoid phenotype on six different media, both minimal and complex, whereas cystic fibrosis-associated strains varied widely with regard to medium-dependent expression of the mucoid phenotype. Of 15 cystic fibrosis strains, 5 were mucoid on each of six different media, 4 were mucoid on five media, 1 was mucoid on four media, 4 were mucoid on three media, and 1 yielded mucoid colonies on only one of the six media tested. There was no obvious correlation among polysaccharide structure, stability of the mucoid phenotype, and medium-dependent expression of the mucoid phenotype for any of the 19 strains investigated. These data suggest that although mucoid strains of *P. aeruginosa* must share some common property related to their ability to colonize their host, this property seems to be unrelated to polysaccharide composition, medium-dependent expression of the mucoid phenotype, or stability of the mucoid phenotype.

Mucoid strains of *Pseudomonas aeruginosa*, presently the most important bacterial pathogens in chronic pulmonary disease of cystic fibrosis (CF) patients, are responsible for much of the morbidity and mortality associated with CF (19, 31). These strains are rarely found in patients other than those with CF or urinary tract (UT) infections (4, 7–9, 11, 28, 32). The view that CF patients are colonized initially by a single nonmucoid strain of *P. aeruginosa* (31, 35, 37) which converts to a mucoid variant in vivo (10, 18) has been modified in the light of recent evidence (1, 6; B. K. Pugashetti, L. Vadas, H. M. Metzger, Jr., D. Edwards, and D. S. Feingold, unpublished data). It is now known that many CF patients are colonized by both mucoid and nonmucoid strains of *P. aeruginosa* (30, 32), and in some cases it has been possible to demonstrate the presence of multiple mucoid and nonmucoid strains of the organism in the respiratory tracts of individual patients. Multiple isolates from a single patient often differ with respect to polysaccharide composition, stability of the mucoid phenotype trait, medium-dependent expression of the mucoid phenotype, O-antigenicity, and antibiotic sensitivity (Pugashetti et al., unpublished data).

Polysaccharides produced by mucoid strains of *P. aeruginosa* are heteropolymers composed of D-mannuronosyl and L-guluronosyl moieties and invariably contain acetyl groups (3, 12, 22). Evans and Linker (12) compared algal alginate with the polysaccharides from mucoid strains of *P. aeruginosa* isolated from CF patients and from patients with UT infections. These workers found that the ratio of D-mannuronosyl to L-guluronosyl moieties (M/G ratio) and the acetyl content of the polymers differed, depending upon the strain of *P. aeruginosa*. In general, D-mannuronosyl residues predominated and in some cases represented as much as 90% but not less than 45% (M/G ratio, 9 to 0.8) of the polymer. Algal alginate, also composed solely of D-mannuronosyl and L-guluronosyl moieties (12,

22), is unacetylated. M/G ratios from 7 to 0.3, depending upon the polymer source, have been reported for algal alginate (12, 17). More recently, Lynn and Sokatch (A. R. Lynn and J. R. Sokatch, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, D55, p. 56) reported that mucoid strains from patients with UT infections yielded polysaccharides which contained only D-mannuronosyl moieties, whereas polysaccharides from CF-related strains of *P. aeruginosa* showed M/G ratios ranging from 20.6 to 2.5.

One of the difficulties which has hampered the study of the polysaccharides in question has been the lack of a convenient, precise analytical technique for determination of the M/G ratios of the polymers. The M/G ratios reported previously (3, 12, 22; Lynn and Sokatch, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, D55, p. 56) must be considered to be semiquantitative at best because of the imprecision of the analytical methods employed. Recent advances in methodology, with high-resolution nuclear magnetic resonance (16) or gas-liquid chromatography (33), now have made it possible to determine the M/G ratio with greater precision than was previously attainable, thus permitting differentiation of mucoid strains on the basis of polysaccharide composition. Fyfe and Govan (13) have proposed that mucoid strains of *P. aeruginosa* can be differentiated by their ability to yield mucoid colonies on certain types of media. Organisms which gave mucoid colony morphology on Pseudomonas Isolation Agar (PIA) (Difco Laboratories, Detroit, Mich.) and also on Vogel-Bonner minimal agar (34) were classed in group I, and strains which yielded mucoid colonies on PIA only were placed in group II. Yet another property considered to be characteristic of mucoid strains of *P. aeruginosa* is the in vitro instability of the mucoid phenotype, associated with a high reversion frequency (14, 15, 27, 36).

We now report the results of a comparison of 15 mucoid strains of *P. aeruginosa* isolated from the sputa of CF patients and 4 strains isolated from patients with UT infections. The results we have obtained confirm that pathogenic, mucoid strains of *P. aeruginosa* isolated from patients with CF or UT infections are phenotypically extremely heterogeneous, although they are similar in their ability to colonize their hosts.

MATERIALS AND METHODS

Isolation of *P. aeruginosa* strains. Sputum samples from CF patients were obtained through the courtesy of Joan Rodnan, Cystic Fibrosis Center at Children's Hospital, Pittsburgh, Pa. Mucoid strains of *P. aeruginosa* were isolated from sputum samples by plating on PIA and MacConkey agar base (Difco) with 10 g of glucose per liter (MCA). After 48 h at 41°C, mucoid colonies were purified by replating on the same media. Isolates which grew at 41°C but not at 4°C were confirmed as *Pseudomonas* species (20), maintained on PIA slopes at 4°C, and transferred monthly. Mucoid *P. aeruginosa* strains from patients with UT infections were obtained through the courtesy of Richard H. Michaels, Department of Pediatrics, Children's Hospital, Pittsburgh, Pa.

Expression of the mucoid phenotype on various agar media. All *P. aeruginosa* strains were grown on PIA from 24 to 48 h at 34°C. Strains which yielded mucoid colonies on PIA were further tested for their ability to produce mucoid colonies on several different agar media. These media included PIA, MCA, Vogel-Bonner minimal agar, L-agar (21), nutrient agar (Difco), and medium B agar (26) plates. Plates were examined for the presence of mucoid colonies after 24 and 48 h at 34°C. Strains which did not give mucoid colonies by 48 h were considered to be nonmucoid.

Stability of the mucoid phenotype. At monthly intervals, mucoid strains from agar maintenance slopes were plated on PIA and observed for nonmucoid revertants after 48 h at 34°C. One mucoid colony from each plate was suspended in water, and serial dilutions were plated and observed as above. Strains which did not yield detectable nonmucoid colonies were classified as stable; unstable mucoid strains were those which yielded nonmucoid revertants.

Isolation and purification of alginate-like polysaccharides from mucoid *P. aeruginosa* strains. A mucoid *P. aeruginosa* strain from a stock slant was streaked on an MCA plate and incubated for 24 h at 34°C. A single, well-separated mucoid colony was used to inoculate liquid medium B (26). After incubation on a rotary shaker (125 rpm) for 16 h at 34°C, 5 ml of the culture was transferred to 1 liter of medium B in a 2-liter flask, and the culture was incubated as above for 24 h. Two volumes of ice-cold ethanol were added to the chilled supernatant liquid obtained by centrifugation of the culture. After 12 h at 4°C, the polysaccharide precipitate was collected by centrifugation (12,000 × *g*, 20 min) and dissolved in 250 ml of water at 4°C (24 to 48 h). Sodium sulfate (1 M) was added to a final concentration of 0.02 M, followed by sufficient 3% cetyltrimethylammonium bromide to precipitate the polysaccharides. The precipitate was dissolved in 0.38 M sodium chloride, reprecipitated with ethanol, and dissolved in water as above. After treatment in 0.01 M magnesium chloride at 37°C for 30 min with 10 μg each of DNase and RNase per ml (Worthington Diagnostics, Freehold, N.J.), the solution was dialyzed exhaustively (24 to 48 h) against water at 4°C and finally treated with trifluorotrichloroethane to remove residual proteins (23). The purified polysaccharides were recovered by lyophilization of their aqueous solution.

Mucoid *P. aeruginosa* strains which did not produce alginate-like polysaccharides in medium B were grown to confluence on PIA plates at 34°C for 24 h. The viscous mucoid growth was scraped from the agar, suspended in water, and treated as described above to isolate the polysaccharides.

Uronic acid composition of alginate-like polysaccharides. The method described by Vadas et al., (33) was used to determine the M/G ratios of the purified polysaccharides. This technique permits determination of the ratios with an accuracy of ±5%.

Acetyl content of alginate-like polysaccharides. The

acetyl content of the polysaccharides was estimated according to the method of McComb and McCready (25).

Determination of *P. aeruginosa* serotypes. Serotypes (O-antigen) of *P. aeruginosa* isolates were determined with *P. aeruginosa* antisera (Difco) and *P. aeruginosa* antigens (Difco) as positive controls. *P. aeruginosa* strains were grown on Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.). Slide agglutination assays were done with an autoclaved (30 min at 121°C), washed bacterial suspension as the antigen (2).

RESULTS

Polysaccharide production and composition. In liquid medium B at 34°C, maximal yields of polysaccharides were obtained at 24 h. Depending upon the strain, yields varied from 20 mg to more than 1 g per liter of medium (data not shown). Since strains DE-5, DE-7, DE-8, DE-10, and DE-24 did not produce polysaccharides on medium B agar, polysaccharides were isolated from cultures of these strains on PIA plates at 34°C.

The relative proportions of D-mannuronosyl and L-guluronosyl moieties in the isolated polysaccharides from different strains varied widely (Table 1). Any particular strain of *P. aeruginosa* always yielded polysaccharides with the same M/G ratios, confirming the observation by Evans and Linker (12) that polysaccharide composition is a stable, strain-related character. Only trace amounts of L-guluronosyl moieties were present in polymers from CF strains DE-8 and DE-22, whereas the polymers produced by strains DE-27 and DE-36 contained more than 50% L-guluronosyl moieties. A similar variation in the content of L-guluronosyl moieties existed among the polysaccharides from the *P. aeruginosa* strains isolated from UT infections. Acetyl content, however, did not vary widely, with the exception of strain DE-36. There is no correlation between M/G ratio and acetyl group content.

O-antigen types of mucoid *P. aeruginosa* strains. The 19 strains examined belonged to seven O-antigen groups; group 1 was predominant (5 of 15) among the CF strains, and group 6 (3 of 4) was predominant among the UT strains (Table 1).

Stability of the mucoid phenotype. A tendency to revert to the nonmucoid phenotype is considered to be characteristic of mucoid strains of *P. aeruginosa* (15, 24, 36). Of the 15 mucoid strains isolated from CF patients, 7 were stable and

TABLE 1. Comparison of mucoid strains of *P. aeruginosa* isolated from clinical sources

Strain[a]	Stability of mucoid phenotype[b]	O-antigen type	Polysaccharide[c]		Colony morphology on[e]:						Proposed group
			% D-Mannuronosyl moieties	Acetyl groups[d]	PIA	MCA	VBMA	LA	NA	MBA	
UT-3799	U	6	≥97	0.33	+	+	+	+	+	+	VI
DE-8	U	3	≥96	0.27	+	+	−	−	+	−	III-A
DE-22	U	1	≥95	0.34	+	+	−	+	+	+	V
UT-4174	U	5	93	0.38	+	+	+	+	+	+	VI
DE-7	U	3	92	0.27	+	+	−	+	−	−	III-B
DE-24	S	10	84	0.28	+	+	−	+	−	−	III-B
UT-3969	U	6	77	0.31	+	+	+	+	+	+	VI
DE-10	S	3	76	0.16	+	+	−	+	−	−	III-B
DE-1	S	10	75	0.30	+	+	+	+	−	+	V
DE-33	S	1	73	0.28	+	+	+	−	−	+	IV
DE-12	S	10	72	0.25	+	+	+	+	+	+	VI
DE-23	U	10	70	0.25	+	+	+	+	+	+	VI
DE-30	U	1	67	0.24	+	+	+	+	+	+	VI
DE-25	U	9	64	0.19	+	+	+	+	+	+	VI
DE-5	S	NT[f]	62	0.34	+	−	−	−	−	−	I
DE-32	U	1	57	0.31	+	+	+	+	+	+	VI
UT-4176	U	6	57	0.38	+	+	+	+	+	+	VI
DE-36	U	9	45	0.02	+	+	−[g]	+	+	+	V
DE-27	S	1	44	0.26	+	+	−	+	+	+	V

[a] Strains UT-3799, UT-4174, UT-3969, and UT-4176 were from patients with UT infections; all others were from CF patients.

[b] S, Stable; U, unstable.

[c] From growth in liquid medium B, except for strains DE-8, DE-7, DE-24, DE-10, and DE-5, which were grown on PIA plates.

[d] Moles of acetyl per mole of uronic acid residue.

[e] VBMA, Vogel-Bonner minimal agar; LA, L-agar; NA, nutrient agar; MBA, medium B agar; +, mucoid; −, nonmucoid.

[f] NT, Nontypable.

[g] Did not grow.

have not reverted to the nonmucoid phenotype over a period of 6 months. All four of the UT isolates, on the other hand, were unstable and yielded nonmucoid revertants at high frequency (Table 1).

Medium-dependent expression of the mucoid phenotype. The strains examined were not identical in their ability to produce mucoid colony morphology on different media, although with one exception they grew well on all test media used. As shown in Table 1, 5 of 15 CF strains were mucoid on each of six media tested, 4 were mucoid on five media, 1 was mucoid on four media, 4 were mucoid on three media, and 1 was mucoid on PIA only. CF strain DE-36 also differed in that it did not grow on one of the test media, the minimal agar of Vogel and Bonner (34). All four UT strains gave mucoid colony morphology on each of the six media tested.

DISCUSSION

Alginate-like polysaccharides produced by strains of *P. aeruginosa* isolated from patients with CF and UT infections varied widely with regard to M/G ratios (Table 1). These results, obtained with the sensitive technique of Vadas et al. (33), confirm those of earlier workers, who employed less precise analytical methods (3, 12, 22; Lynn and Sokatch, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, D55, p. 56).

Since the patients from whom the strains were isolated scored (29) in the severe to moderate range (score, 35 to 55) with one exception, there seems to be no relationship between the severity of infection and the M/G ratios or acetyl content of the polysaccharides. It is also of interest that the polysaccharides produced by UT organisms are not noticeably different from those obtained from CF isolates. Most of the polysaccharides that we studied have an M/G ratio of greater than 1, which seems to be characteristic of bacterial alginate-like polysaccharides (3, 12, 22; Lynn and Sokatch, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, D55, p. 56). Nonetheless, two strains of *P. aeruginosa* (DE-27 and DE-36) from CF patients yielded polysaccharides with M/G ratios of 0.8.

Despite the wide variability of the M/G ratios, the acetyl content of the polysaccharides differed at most by a factor of 2.5 (with the exception of strain DE-36). Strains DE-8 and DE-27 had essentially identical acetyl contents, but their M/G ratios (>30 and 0.8, respectively) were significantly different. A similar comparison can be made between strains DE-22 and DE-5. These data are not in accord with those of Evans and Linker (12), who considered that the O-acetyl content of the polymers was proportional to the content of D-mannuronosyl moieties. It remains to be shown whether the acetyl groups are involved in the protection of D-mannuronosyl moieties from 5-epimerization (conversion of the D-mannuronosyl moiety to the L-guluronosyl moiety) during biosynthesis of the polymers, as suggested by Davidson et al. (5). The M/G ratios are strain dependent and reproducible; however, it is not known whether these ratios are medium dependent. Were this the case, polysaccharides produced in vitro might not be identical to polymers produced by the same strain of *P. aeruginosa* in vivo, since the respiratory tract can be considered to be a "medium" of unknown composition.

Mucoid strains of *P. aeruginosa* isolated from CF patients have been reported to revert to the nonmucoid phenotype with high frequency (14, 24, 36). Surprisingly, 7 of 15 strains that we isolated were stable and have not yielded nonmucoid variants over a period of 6 months. The origin of such stable mucoid strains is obscure. We have not observed in vitro conversion of unstable mucoid strains of *P. aeruginosa* to the stable mucoid phenotype. Stable strains could arise in vivo as the result of a series of successive interrelated mutational events, e.g., loss of repressor function or integration of a control plasmid, coupled with an in vivo selective advantage afforded by the presence of the mucoid trait. An alternative possibility is that stable mucoid strains of *P. aeruginosa* normally exist as saprophytes in the environment, although none have as yet been detected, and that colonization of CF patients is a result of primary infection by organisms in the environment. Another possibility is cross-infection from another CF patient.

From the limited data available, there seems to be little correlation between the stable or unstable mucoid phenotype of the infecting strain and the severity of disease, as shown by the patient score. However, it should be emphasized that the role of mucoid strains in the morbidity and mortality in CF can only be ascertained by a long-term longitudinal study of respiratory colonization of CF patients, rather than by the kaleidoscopic view available from our present results. Our failure to detect stable mucoid strains among the UT isolates probably is not significant because of the small sample studied.

The use of six different plating media permitted further differentiation of mucoid strains; a proposed expanded classification is presented (Table 1). The medium-dependent expression of the mucoid phenotype is indicated by Roman numerals, which designate the number of media which yielded mucoid colonies for a given strain. Thus, isolate DE-5, which was mucoid only on PIA, is in group I, DE-22, which was mucoid on five media, is in group V, and so on. In cases in which mucoid colonies were produced on the same number of media but in which

the strains differed with regard to which media, the Roman numeral is followed by a letter; e.g., strain DE-8 is in group III-A, and strain DE-10 is in group III-B. In this way, the 19 strains under investigation could be arranged into five distinct groups ranging from single strains (DE-5 and DE-33) in groups I and IV to 9 strains in group VI. No group II strains (mucoid on two different media) have been detected to date. Except for DE-12, strains in group VI, which includes the four UT strains and four CF strains, were unstable. Thus, there seems to be some correlation between unstable strains and the ability to produce polysaccharides on a variety of media. The high degree of phenotypic similarity of the UT and CF strains suggests that the environmental pressures which select the mucoid phenotype in vivo are similar in the respiratory tracts of CF patients and the UTs of non-CF patients.

Group I strains, which were mucoid on only one of six different media, would seem less likely to be mucoid in vivo than group VI strains. It is possible that not all strains of *P. aeruginosa* designated as mucoid in laboratories express the mucoid phenotype in vivo. Whether this is actually the case can only be answered by direct comparison on the in vitro product with polysaccharides isolated from the respiratory tract of the patient, an aim which has not yet been accomplished.

According to present views, mucoid strains of *P. aeruginosa* rarely occur except in the respiratory tracts of patients with CF and much less frequently in UT infections. Given the unique habitat of such strains, they would be expected to be phenotypically similar, especially in features associated with the mucoid phenotype. However, the strains we have examined are heterogeneous with regard to M/G ratios and acetyl content of their polysaccharides, the stability of the mucoid phenotype, and the medium-dependent expression of the mucoid phenotype. These strains are also heterogeneous with regard to characters not related to the mucoid phenotype, such as O-antigen groups. The only obvious common property of the 19 strains that we have been able to demonstrate is their ability to yield mucoid colony morphology on PIA and (with the exception of strain DE-5) on MCA. Whether the pathogenicity of mucoid strains of *P. aeruginosa* is indeed related to their production of alginate-like polysaccharides or some other as yet unappreciated property of the organism remains to be demonstrated.

ACKNOWLEDGMENTS

We thank Anna Marie Bowser, Michelle Baker, and Mercedes Kozameh for excellent technical assistance.

This investigation was supported in part by Public Health Service grant AI 16993 from the National Institutes of Health (to D.S.F.), fellowship T32 GM07349-04 from the National Institutes of Health (to B.K.P.), and a fellowship from the Swiss National Fund for Scientific Research (to L.V.).

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