Isolation of mucoid strains of *Pseudomonas aeruginosa* from non-cystic-fibrosis patients and characterisation of the structure of their secreted alginate

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Summary. When the incubation period of primary isolation plates was extended to 48 h, mucoid strains of *Pseudomonas aeruginosa* were found in specimens from various infected sites in patients who did not have cystic fibrosis. The 17 mucoid isolates were characterised in terms of mucoid type, pyocin type, and their sensitivity or resistance to seven β -lactam and two aminoglycoside antibiotics. The carbohydrate, uronic acid (alginate) and protein content of the water-soluble extracellular material of 15 strains was determined. This material was fractionated by ion-exchange chromatography, and the presence of alginate confirmed by the chemical assay of uronic acids and their quantitation by gas-liquid chromatography. Uronic acids were absent from a non-mucoid revertant of one strain. The strains produced alginate with a high content of mannuronic acid and substituted with O-acetyl groups. By proton nuclear magnetic resonance (^1H -nmr) analysis the alginate from three strains was shown to lack poly-guluronate blocks in its structure. These properties are also found in the alginate of mucoid *P. aeruginosa* strains from patients with cystic fibrosis.

Introduction

Pseudomonas aeruginosa can produce the polysaccharide alginate as a large-volume, viscous gel around the cell (Carlson and Mathews, 1966; Linker and Jones, 1966). This mucoid phenotype is observed most frequently in strains isolated from sputum of cystic fibrosis (CF) patients. Most CF patients become colonised, probably by the non-mucoid form of *P. aeruginosa* which later becomes mucoid (Govan and Harris, 1986). It is not known what stimulates the bacteria to synthesise alginate (Russell and Gacesa, 1988). Assumptions have been made that some factor specific to the CF condition is responsible and that the presence of mucoid *P. aeruginosa* can be used as a diagnostic indicator of cystic fibrosis. These assumptions are probably invalid, because mucoid *P. aeruginosa* strains are isolated occasionally from patients without CF (Evans and Linker, 1973) but comprehensive studies of these strains have not been made. It has not been shown whether they also secrete alginate or another extracellular polysaccharide and, if alginate is produced, whether it has the same

structure as that from mucoid isolates from CF patients. If there is a difference between mucoid strains from CF and non-CF patients, this might provide a valuable clue to the control of alginate synthesis which, like the pathway, is poorly understood (Russell and Gacesa, 1988).

We have previously purified alginate from mucoid isolates of *P. aeruginosa* from patients with CF, studied its composition and structure, and used proton nuclear magnetic resonance (^1H -nmr) to elucidate the arrangement in the polymer of the two uronic acid monomers, β -D-mannuronic acid and α -L-guluronic acid (Sherbrock-Cox *et al.*, 1984). We now report some of the properties of mucoid isolates of *P. aeruginosa* from patients without CF and show that the alginate produced by them has essentially the same structure as that from isolates from patients with CF.

Materials and methods

Isolation and maintenance of bacteria

Over a 16-month period (Oct. 1983–Feb. 1985), specimens from patients with neither symptoms nor

medical history of cystic fibrosis were plated out on MacConkey Agar (Difco) and grown at 37°C for 48 h. Overtly mucoid colonies thought to be pseudomonads were picked, purified on Pseudomonas Isolation Agar (PIA; Difco 0927-01) and maintained on deoxycholate citrate agar slopes at 4°C. For longer term storage they were kept in skimmed milk 10% w/v at -70°C.

Identification of bacteria

Isolates were identified as *P. aeruginosa* by colonial morphology, a positive oxidase reaction (Kovacs, 1956), oxidative utilisation of glucose (Hugh and Leifson, 1953), growth in peptone water after incubation for 48 h at 37°C and 42°C, and production of pigment on PIA (King *et al.*, 1954). Some isolates were also identified with API 20NE kits (API Laboratory Products Ltd, Basingstoke).

The isolates were also examined for their ability to grow on minimal medium in the presence or absence of leucine. This is the basis of a simple typing method to determine the characteristics of mucoid growth (Fyfe and Govan, 1980).

Antibiotic sensitivity testing

All strains were tested for their sensitivity to a variety of antibiotics. Diagnostic Sensitivity Test Agar (DST, Oxoid CM 261) plates were inoculated with a swab charged with bacteria from a suspension in peptone water (2 ml) at a density giving rise to semi-confluent growth. Antibiotic-containing disks were then applied to the agar surface and the plates were incubated for 24 h at 37°C. The disks contained gentamicin 10 µg, tobramycin 10 µg, amoxycillin 10 µg, carbenicillin 100 µg, mezlocillin 75 µg, azlocillin 75 µg, cephadrine 30 µg, cefuroxime 30 µg or ceftriaxone 30 µg. A known sensitive strain of *P. aeruginosa* (NCTC 10662) was tested in a similar manner. Isolates were classified as sensitive, intermediate or resistant to antibiotics by comparison with the control strain according to the criteria specified in the WHO Technical Report Series No. 210 (1966).

Pyocin typing

Both the active technique of Gillies and Govan (1966) and the liquid-culture method of Williams and Govan (1973) were used for the production and detection of pyocins. Indicator strains 1-8 and sub-typing indicator strains A-E (Govan, 1978), obtained from Dr T. Pitt, Division of Hospital Infection, Central Public Health Laboratory, Colindale, were used to prepare bacterial lawns for testing the activity of pyocins from the isolates. In addition, control strains of known pyocin type were also tested within each set of experiments. The isolates were classified according to the criteria of Govan (1978).

Extraction and purification of polysaccharide

For each isolate, the growth from three PIA plates incubated at 37°C for 72 h was removed with a glass rod and crude water-soluble polysaccharide was separated from the cells by centrifugation (Sherbrock-Cox *et al.*, 1984).

Samples (50 mg) of extract were fractionated on Dowex 1-X2 by step-wise elution with NaCl as described before (Sherbrock-Cox *et al.*, 1984), desalted on a Biogel P-6DG column (3.2 × 36.0 cm), dialysed and freeze-dried before analysis for protein, carbohydrate and uronic-acid content.

Analytical methods

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Total carbohydrate was assayed with phenol-sulphuric acid reagent (Dubois *et al.*, 1956) and uronic acids with carbazole-borate reagent (Knutson and Jeanes, 1968) with either D-mannurono 6,3-lactone or D-galacturonic acid as standard. The O-acetyl content of the alginate samples was assayed by the method of Buscher *et al.* (1974) with α-D-glucose penta-acetate as standard.

Proton nuclear magnetic resonance (¹H-Nmr) spectroscopy. Samples (5 mg) of freeze-dried alginate the viscosity of which had been reduced by minimal hydrolysis (30 min, 100°C, pH 2.9) in HCl followed by neutralisation with NaOH, were added to EDTA (3 mg) and dissolved in D₂O (0.5 ml) at pD 7. Spectra were obtained at 90°C with a Bruker WM-360 nmr spectrometer operating in the Fourier transform mode. A 180°-τ-90° pulse sequence (τ ~ 3 s) with a recycle time of 5 s was used to eliminate the solvent peak (HDO). ¹H-Chemical shifts were expressed in ppm downfield from the internal standard of sodium 4,4-dimethyl-4-silapentane sulphonate. The assignment of peaks was based on previous work (Grasdalen *et al.*, 1979) and confirmed by the use of algal alginate block structures of known composition (Haug and Larsen, 1962).

Gas-liquid chromatography. Samples of polysaccharide were methanolysed and the resulting methyl glycosides analysed as the trimethylsilyl ether derivatives by a modification of the method of Clamp *et al.* (1967). Thus, for samples in which the amino-sugar content was low, smaller than recommended amounts of acetic anhydride were used in the re-N-acetylation step. These amounts were determined empirically as the maximum concentration which did not cause O-acetylation, as judged by the appearance of mono- and di-acetylated derivatives of the internal standard D-mannitol. The trimethylsilyl ether derivatives were analysed on a Pye 104 gas-liquid chromatograph on a column of SE30 3% w/w on Gas Chrom Q (Phase Separation Ltd, Queensferry, Clwyd), temperature programmed from 140 to 250°C at 2°C/min with a N₂ carrier gas flow rate of 80 ml/min and injector and detector temperatures of 255°C. Methyl glycosides were identified on the basis of their retention times relative to the internal standard, D-mannitol, and by comparison with those of known standards.

Results

Isolation and characterisation of mucoid strains

Seventeen mucoid strains of *P. aeruginosa* were isolated from a large number of routine specimens taken over a 16-month period from patients without cystic fibrosis. The strains came from various clinical sites (table I).

Mucoid strains grew more slowly than non-mucoid strains and other organisms on primary isolation plates and incubation had to be extended to 48 h to permit the recognition of the mucoid phenotype. Six strains produced mucoid growth on minimal medium in the absence of leucine (type 1) and five required the addition of leucine to give mucoid growth (type 2). The remaining six isolates did not grow on minimal media and were non-typable (table I).

As in previous studies, several mucoid strains were non-typable by the pyocin typing method of Gillies and Govan (1966). Nearly all, however, were typable when liquid pyocin preparations were used (see table I).

The results of antibiotic sensitivity tests are summarised in table II. Most strains showed multiple resistances; all were resistant to cephradine, all but four to cefuroxime and all but five to amoxycillin. Two strains (N9 and N10) showed

intermediate resistance to ceftriaxone and they were the only isolates that were resistant to carbenicillin and mezlocillin. Except for strain N10, all were sensitive to azlocillin, and all but strains N8 and N9 were sensitive to the aminoglycosides.

Analysis of mucoid material

All strains readily produced mucoid material when grown on PIA plates. Analysis of water-soluble extracts of the material invariably showed the presence of carbohydrate, uronic acid and smaller amounts of protein (table III). Although the proportions of these three constituents differed among isolates, for most, carbohydrate comprised at least 60% of the total material and protein less than 20% of the total material. There was considerable variation in the uronic acid content, e.g., from 18 to 77% of the total carbohydrate extracted from these isolates (table III). With two strains (N5 and N10), the crude material was insoluble after lyophilisation and, therefore, analysis was not possible.

Fractionation of the water-soluble mucoid material on Dowex 1-X2 (Cl⁻ form) showed that most of the uronic-acid-containing material was eluted with 0.6, 0.8 and 1.0 M NaCl. Samples of algal alginate also eluted from the Dowex 1-X2 column with 0.6 and 0.8 M NaCl. Both non-uronic-acid-containing carbohydrate (i.e., total carbohydrate less uronic acid) and protein were eluted over a wide range of salt concentrations with the latter, in particular, being eluted at the lower salt concentrations (figure).

All fractions of each sample from ion-exchange chromatography were analysed qualitatively by GLC to determine their sugar composition. In all fractions containing sufficient material for analysis (i.e., 105 of 119 samples) glucose, galactose, mannose, xylose and rhamnose were detected. D-Mannuronate was produced by all isolates and was found principally in the fractions eluting at 0.6, 0.8 and 1.0 M NaCl. L-Gulonate was also found in these fractions from all isolates except strains N2 and N9, in which this sugar could not be detected. Whether the absence of L-gulonate in these two isolates is because of insufficient material for analysis or the production of alginate composed entirely of polymannuronate is unclear. A non-mucoid revertant of strain N11 also had all of the sugars present in the water-soluble extract of the mucoid strains, but neither of the two uronic acids was detected.

Whenever sufficient material for analysis was

Table I. The source, mucoid type and pyocin type of 17 mucoid isolates of *P. aeruginosa* from patients without CF

Strain designation	Source	Mucoid type*	Pyocin type†
N1	Throat swab	NT	5NT
N2	Urine	NT	3e
N3	Sputum	2	1e
N4	Sputum	1	1e
N5	Urine	1	3e
N6, N7	Ear swab	NT	3h
N8	Ear swab	NT	3NT
N9	Urine	2	NT
N10	Urine	1	3e
N11	Endotracheal tube	2	5y
N12	Urine	1	3e
N13	Sputum	1	1e
N14	Sputum	2	NT
N15	Sputum	NT	NT
N16	Sputum	1	1b
N17	Lung abscess	2	1NT

* Typed according to the system of Fyfe and Govan (1980).

† Typed by the liquid-culture method for the production of pyocins (Williams and Govan, 1973). The number refers to the indicator strain and the letter to the sub-typing indicator strain. NT = non-typable.

Table II. Antibiotic sensitivity profiles of mucoid isolates of *P. aeruginosa* from patients without CF

Strain designation	Disk antibiotic sensitivities								
	MEZ	CXM	AZL	AML	CE	CN	TOB	PY	ROC
N1	S	R	S	R	R	S	S	S	S
N2	S	R	S	S	R	S	S	S	S
N3	S	S	S	S	R	S	S	S	S
N4	S	S	S	S	R	S	S	S	S
N5	S	R	S	R	R	S	S	S	S
N6, N7	S	R	S	R	R	S	S	S	S
N8	S	R	S	R	R	R	I	S	S
N9	I	R	S	R	R	R	R	I	I
N10	R	R	I	R	R	S	S	R	I
N11	S	R	S	R	R	S	S	S	S
N12	S	R	S	R	R	S	S	S	S
N13	S	R	S	R	R	S	S	S	S
N14	S	I	S	I	R	S	S	S	S
N15	S	S	S	S	R	S	S	S	S
N16	S	R	S	R	R	S	S	S	S
N17	S	S	S	S	R	S	S	S	S

MEZ, mezlocillin; CXM, cefuroxime; AZL, azlocillin; AML, amoxycillin; CE, cephadrine; CN, gentamicin; TOB, tobramycin; PY, carbenicillin; ROC, ceftriaxone; S, sensitive (zone of inhibition similar to or greater than control); I, intermediate (zone diameter 4–10 mm smaller than control); R, resistant (zone diameter > 10 mm smaller than control).

available, a semi-quantitative estimate was made of the proportions of the uronic acids in the samples based on GLC analysis (table IV). All isolates produced alginate containing a much higher proportion of mannuronate than guluronate, with 7 of 9 samples having >80% mannuronate. Seven samples were also analysed for the presence of O-acetyl groups. These were found in all but in differing amounts (table IV). The alginate fractions isolated from three strains were analysed quantitatively by ¹H-nmr to obtain information about the nearest neighbour frequency of mannuronate and guluronate residues within the polysaccharide (table V). There was good agreement between the fractional compositions obtained separately by ¹H-nmr and GLC analyses of these samples (see tables IV and V). The proportions of the various doublet frequencies (i.e., nearest neighbour analysis) showed that the alginate contained both polymannuronate and poly-mannuronate/guluronate (i.e., mixed) blocks, but that there was a complete absence of poly-guluronate blocks (table V).

Discussion

We have found that mucoid strains of *P. aeruginosa* can be isolated from patients who do not have CF, although the frequency of isolation is very

much less than that with CF patients. Mucoid strains of *P. aeruginosa* can be isolated from the sputum samples of c.50% of patients with CF. In a 16-month period we obtained 17 mucoid isolates from non-CF patients without resort to special screening or selection procedures other than extending the incubation period. Because the mucoid phenotype is often slowly expressed, if primary isolation plates are only incubated for the conventional period of 18–24 h, mucoid strains may not be recognised. We suspect, therefore, that mucoid *P. aeruginosa* strains occur more frequently than hitherto believed. We recommend that plates be incubated for 48 h whenever chronic *P. aeruginosa* infection is suspected, because the presence of mucoid strains has considerable bearing on the decision as to the best antibiotic or other form of therapy.

Like mucoid strains of *P. aeruginosa* from CF patients, the 17 mucoid isolates from patients without CF showed typical resistance to cephradine whilst some were sensitive to amoxycillin and cefuroxime. This contrasts with non-mucoid strains from patients with or without CF which are always resistant to these antibiotics. Only two strains showed multiple resistance to β -lactam antibiotics and one of them was also resistant to the aminoglycosides. The phenomenon of increased sensitiv-

Table III. Composition of the extracellular material isolated from mucoid strains of *P. aeruginosa* from patients without CF

Strain designation	Percentage composition* of extracellular material			uronic acid as percentage of carbo-hydrate†
	protein	carbo-hydrate	uronic acid†	
N1	8	75	26	35
N2	25	49	13	26
N3	27	61	21	34
N4	24	84	22	26
N5	N.D.	N.D.	N.D.	N.D.
N6, N7	16	40	31	77
N8	14	89	42	47
N9	12	72	13	18
N10	N.D.	N.D.	N.D.	N.D.
N11	10	63	22	35
N12	15	59	15	25
N13	11	69	28	41
N14	9	84	21	25
N15	8	63	40	63
N16	15	69	18	26
N17	11	57	30	53

* Values are expressed as percentages of the total weight on the basis of chemical assays of individual components and gravimetric determination of total weight. The presence of variable amounts of water in different samples probably accounts for the fact that the sum of the individual components is not always 100%.

† Uronic acids give a positive reaction in both the total carbohydrate and the uronic-acid assays.

N.D. = not determined.

Table IV. Fractional uronic acid content and extent of O-acetylation of the alginate in samples isolated from some mucoid strains of *P. aeruginosa*

Strain designation	Fractional content of residues		Molar ratio of acetate:uronate
	mannuronate	guluronate	
N1	0.82	0.18	0.48
N7	0.82	0.18	N.D.
N8	0.83	0.17	0.26
N11	0.62	0.38	0.08
N12	0.78	0.22	0.23
N14	0.88	0.12	0.60
N15	0.90	0.10	N.D.
N16	0.84	0.16	0.52
N17	0.85	0.15	0.06

N.D. = not determined.

Figure. Ion-exchange chromatography elution profile of crude extracellular material isolated from *P. aeruginosa* strain N1. The sample (436 mg) was fractionated on Dowex 1-X2 with stepwise elution with NaCl as described by Sherbrock-Cox *et al.* (1984). Fractions were analysed for uronic acid (top), total carbohydrate (centre) and protein (bottom).

ity to antibiotics (when measured in laboratory tests) of mucoid strains of *P. aeruginosa* from patients with CF compared with other non-mucoid strains has been termed “hypersensitivity” (Govan *et al.*, 1983). In this paper we report the first demonstration of “hypersensitivity” in strains of *P. aeruginosa* from patients without CF.

Table V. ^1H -nmr analysis of the alginate from three mucoid strains of *P. aeruginosa*

Strain designation	Fractional composition					
	guluronate	mannuronate	F _{GG}	F _{MM}	F _{MG}	F _{GM}
N1	0.23	0.77	0	0.54	0.23	0.23
N11	0.27	0.73	0	0.45	0.28	0.28
N12	0.30	0.70	0	0.41	0.29	0.29

GG = guluronate adjacent to guluronate.

MM = mannuronate adjacent to mannuronate.

MG = mannuronate adjacent to guluronate.

GM = guluronate adjacent to mannuronate.

Although it had been shown previously (Evans and Linker, 1973) that mucoid isolates from patients without CF occurred rarely and could produce alginate, there has been no comprehensive study of the extracellular material from these organisms. We have demonstrated that, as with mucoid strains from patients with CF, this material contained a mixture of alginate and other polysaccharides, including lipopolysaccharide (LPS), and a smaller amount of protein. On fractionation by ion-exchange chromatography, the fractions containing most alginate eluted in 0.6 and 0.8 M NaCl, as did those containing alginate from isolates from patients with CF (see, however, Sherbrock-Cox *et al.*, 1984). The identity of alginate was confirmed both by chemical analysis of uronic acids and by quantitation of their relative amounts by GLC analysis. The latter technique also showed that the extracellular material contained LPS and neutral sugars. Since the latter are known to be components of LPS in *P. aeruginosa* (Chester *et al.*, 1972) and were present in the water-soluble extract of the non-mucoid revertant of strain N11, it would appear that both neutral sugars and LPS are

contaminants rather than components of the extracellular material and that they were removed from the bacterial outer membrane during isolation of the mucoid material.

It was of particular interest to analyse the structure of the alginate to determine whether any differences existed between that of strains from patients with or without CF, because this might give clues as to the regulation of biosynthesis of this polymer. The isolates from patients without CF all produced alginate that was rich in mannuronate, although the proportion of alginate in the extracellular material varied quite widely among isolates. Moreover, ^1H -nmr analysis demonstrated that the alginate in all three strains investigated comprised blocks of poly-mannuronate and poly-mannuronate/guluronate whilst blocks of poly-guluronate were completely absent. The same block structure was found previously for the alginate from isolates from patients with CF (Sherbrock-Cox *et al.*, 1984). Furthermore, all of the samples examined contained O-acetyl groups and although the molar ratio of acetate:uronate varied from sample to sample, the values obtained were similar to those reported for isolates from patients with CF (Evans and Linker, 1973; Sherbrock-Cox *et al.*, 1984).

In conclusion, mucoid strains of *P. aeruginosa* from patients without CF may be more common than hitherto realised. This is because the development of the mucoid phenotype requires longer than the usual incubation times used in routine bacteriology laboratories. The extracellular material produced by these strains is composed mainly of alginate and has a similar composition to that produced by isolates of *P. aeruginosa* from patients with CF.

We are grateful to the Welsh Scheme for the Development of Health and Social Research for financial support.

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