Aust. J. Biol. Sci., 1987, **40**, 435–41

Composition of Alginate Synthesized during the Growth Cycle of *Pseudomonas aeruginosa*

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Abstract
A mucoid *P. aeruginosa* isolated from the sputum of a cystic fibrosis patient was grown in batch culture on a complex medium. During the growth cycle the amount of alginate produced was estimated and its composition was determined by proton magnetic resonance (^{1}H-n.m.r) spectroscopy. Exopolysaccharide production occurred mainly during the exponential phase of growth. The alginate samples isolated varied little in composition and were characterized by being highly acetylated, high mannuronate (0.83 - 0.93 mole fraction) polymers. Guluronate was present only within heteropolymeric regions of the polysaccharides which all displayed a complete absence of polyguluronate. Ca^{2+} ion supplementation of the medium was not observed to increase the levels of guluronate in the alginates produced.

Introduction

Alginic acid, a commercially important algal polysaccharide, is a co-polymer of (1–4)-linked α-*L*-guluronic acid and (1–4)-linked β-D-mannuronic acid. The residues are arranged in irregular blocks along the linear polymer chain (Grasdalen *et al.* 1981). Acetylated forms of alginic acid are also produced by *Azotobacter vinelandii* (Gorin and Spencer 1966) and *Pseudomonas aeruginosa* (Linker and Jones 1966).

The biosynthesis of alginate, which is originally synthesized as polymannuronic acid, in *A. vinelandii* has been described by Couperwhite and McCallum (1975); Pindar and Bucke (1975). The mannuronic acid residues are epimerized to guluronic acid on the polymer chain by an extracellular C-5-epimerase. *In vitro* studies have shown the action of the epimerase to be Ca^{2+} dependent (Haug and Larsen 1971; Couperwhite and McCallum 1974; Larsen 1981; Ofstad and Larsen 1981; Skjak-Braek and Larsen 1982) with changes in the level of the cation present affecting not only the amount of guluronic acid incorporated into the polymer but also its distribution. The level of Ca^{2+} also influences epimerase activity *in vivo* (Larsen and Haug 1971; Couperwhite and McCallum 1974; Annison and Couperwhite 1984) but more recent studies have demonstrated that the levels of polymer present also have an influence on the epimerase activity (Annison and Couperwhite 1986). Thus *A. vinelandii* alginates of highly varied composition have been isolated. Some have a preponderance of polymannuronic acid regions (M blocks) whilst in others polyguluronic acid regions (G blocks) dominate. The levels of heteropolymeric regions (MG blocks) may also vary.

The results of studies on the structure of *P. aeruginosa* alginate indicate that the polymer from this organism tends to consist mainly of M blocks with some MG blocks. No G blocks have been observed (Haug *et al.* 1974; Linker and Evans 1976; Piggott *et al.* 1982; Sherbrock-Cox *et al.* 1984). The findings of Piggot *et al.* (1981) demonstrated

0004-9417/87/040435$02.00

that guluronic acid is incorporated into the polymer by an extracellular, Ca^{2+}-dependent C-5-epimerase but it would seem that *P. aeruginosa* lacks the enzyme activity responsible for converting mannuronic acid to guluronic acid when guluronic acid is the neighbouring residue.

The absence of G blocks within the polysaccharide is of importance as in algal alginates the levels of these regions profoundly influence the physical properties of the polymer. This is of interest not only because *P. aeruginosa* is a potential commercial source of alginates but also because of the pathogenic nature of mucoid strains of the organism.

Chronic pulmonary infections due to *P. aeruginosa* are common in cystic fibrosis patients. It has been suggested that insoluble Ca^{2+}-polysaccharide complexes may contribute to chronic pulmonary obstructions (Dunne and Buckmire 1985) as elevated Ca^{2+} levels have been observed in the tracheobronchial secretions of cystic fibrosis patients (Gibson *et al.* 1971). The binding of Ca^{2+} by algal alginates has been shown to be almost entirely due to the chelation of the cation by G-block regions of the polymer (Smidsrød and Haug 1972; Grant *et al.* 1973; Morris *et al.* 1978). It is therefore important to establish whether *P. aeruginosa* can produce alginates with G blocks and if so under what conditions synthesis of this type of polymer is favoured.

This paper details experiments in which the fine structure of the alginate produced by *P. aeruginosa* was examined.

Methods

Bacterial Strain

The *P. aeruginosa* strain studied was isolated from the sputum of a cystic fibrosis patient. The culture was provided by the Prince of Wales Hospital, Sydney. The isolate (termed isolate M) was identified by the use of the analytical profile index (API) 20 NE system (Analytab Products Inc., Plainview, New York U.S.A.).

Nutrient Media

P. aeruginosa isolate M was maintained on brain heart infusion agar (BHI) (Oxoid, Basingstoke, England) slopes and routinely subcultured every 2–3 months. For cultivation in liquid medium, tryptone soya broth (TSB) (Oxoid) was used.

Alginate Determinations

Two samples of culture broth (40 ml) were adjusted to 0.1 M NaCl (0.8 ml 5 M NaCl) and 0.01 M ethylene diaminetetra-acetate (0.8 ml, 0.5 M Na_4-EDTA). After vortexing, the samples were centrifuged (20 000 *g*, 20 min) and the supernatant was poured into two volumes of 2-propanol. The precipitate (alginate) was recovered by centrifugation (bench centrifuge), washed twice with fresh 2-propanol (10 ml) and once with ether. The alginate was dried to constant weight *in vacuo* over P_2O_5.

Alginate Analysis

The uronic acid composition of alginates was determined by proton magnetic resonance (^{1}H-n.m.r.) spectroscopy. The dried alginate from duplicate samples was dissolved in distilled water (25 ml) and dialysed against 1% (w/v) NaCl and 0.05% (w/v) EDTA for 5 days and then distilled water for 2 days. The alginate was reprecipitated in two volumes of 2-propanol and dried. A portion (10 mg) was dissolved in 0.1 M NaOH and agitated for 1 h at 30°C to cause *O*-deacetylation. The mixture was neutralized (0.1 M HCl) and the *O*-deacetylated polymer was recovered by precipitation in absolute ethanol and dried *in vacuo* over P_2O_5. Samples of the *O*-deacetylated polymer (2–3 mg) were dissolved in D_2O (0.4 ml). The solutions were adjusted to pD 7 with NaOD. EDTA (1–2 mg) was added to complex any divalent cations. Each sample was subjected to ultrasonification for 5 min (Branson Model B-30 Sonifier, Consolidated Ultrasonics, N.S.W.) to cause partial depolymerization to reduce solution viscosities. Before ^{1}H-n.m.r. analysis the samples were freeze-dried and resuspended twice in D_2O to remove excess H_2O.

The acetate content of the dialysed, native polymer was determined by the method of McComb and McCready (1957).

^{1}H-n.m.r. Analysis

^{1}H-n.m.r. spectra were obtained with a Bruker CXP300 nuclear magnetic resonance spectrometer operating at 300 mHz. A sweep width of 3000 Hz was used with 8 K data points and line broadening of 0.5 Hz. The spectra were recorded at 80°C. The ^{1}H-n.m.r. method was checked by using alginate fractions of known M/G ratio, kindly provided by Dr B. Larsen, Institute of Marine Biochemistry, Trondheim, Norway. The mole fractions of mannuronic acid, guluronic acid, polymannuronic acid, polyguluronic acid blocks and mixed blocks were determined from ^{1}H-n.m.r. spectra using the method of Grasdalen *et al.* (1979).

^{13}C-n.m.r. Analysis

^{13}C-n.m.r. spectra of alginate samples were obtained using procedures based on those of Grasdalen *et al.* (1981). Dialysed alginate samples (25 mg) were dissolved in distilled water (3.5 ml) with EDTA (25 mg) and D_2O (0.5 ml). The sample solutions were adjusted to pH 7 with 1 M NaOH. ^{13}C-n.m.r. spectra were recorded with a Baker CPX 300 spectrometer at 80°C using 8 K data points, 15 000 Hz spectral width, 75° pulse and 0.7 s pulse repetition time. The number of scans was 30 000–40 000. All spectra were broadband decoupled. The deuterium resonance was used as a field lock. Chemical shifts in some samples were checked using acetone as an internal standard with a chemical shift of 31 p.p.m. relative to trimethylsilane.

Experimental

Production and Composition of P. aeruginosa *Alginate during Growth Cycle*

P. aeruginosa isolate M was plated onto BHI agar and incubated for 24 h (30°C). Material from a single mucoid colony was used to inoculate TSB broth (50 ml). Following incubation (to mid log phase) aliquots (0.5 ml) were used to inoculate 40 replicate flasks containing TSB (50 ml) supplemented with $CaCl_2$ (0.1 g l^{-1}). The cultures were incubated on an orbital shaker (30°C, 140 rpm) and periodically two flasks were removed for analysis. The optical density at 600 nm of the culture was read and the amount of extracellular polysaccharide and its composition was determined. One polysaccharide sample (26 h) was analysed by ^{13}C-n.m.r. in its native and *O*-deacetylated form.

Influence of Ca^{2+} on the Composition of P. aeruginosa *Alginate*

An inoculum (0.5 ml) of *P. aeruginosa* strain M (prepared as described previously) was added to replicate flasks containing TSB (50 ml) with and without added $CaCl_2.2H_20$ (3.4 mM). After incubation on an orbital shaker (30°C, 140 rpm) for 24 and 48 h duplicate flasks were removed, alginate was recovered from the cultures and its composition determined.

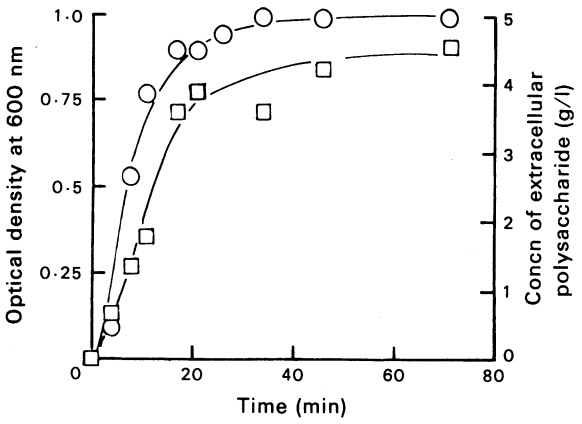

Fig. 1. Optical density at 600 nm of culture (○) and the level of extracellular polysaccharide (□) produced during the growth of *P. aeruginosa* isolate M.

Results

The data presented in Fig. 1 show that rapid growth of *P. aeruginosa* isolate M began with inoculation (no lag period being recorded) and the stationary phase was reached in 20 min. Growth was accompanied by production of the polysaccharide

which reached high levels in the culture (4.6 g/l) and production almost ceased with the onset of stationary phase.

The mole fraction data (Table 1) indicate that the composition of the polyuronate isolated from the cultures did not alter greatly during the incubation period. The polysaccharides were predominantly mannuronate with galuronate mole fraction values ranging from 0.07–0.17. The polysaccharides were found to be highly acetylated (degree of acetylation 0.40–0.70) and possessed no G-block regions.

Table 1. Composition of the extracellular polysaccharides produced by *P. aeruginosa* isolate M during growth in batch culture

The mole fraction data were determined by ^{1}H-n.m.r. analysis and the degree of acetylation was determined by chemical analysis

Time (h)	Mannuronic acid	Guluronic acid	Mole fraction M blocks	G blocks	MG blocks	Degree of acetylation
11	0.83	0.17	0.67	0	0.33	0.57
21	0.90	0.10	0.79	0	0.21	0.63
26	0.90	0.10	0.79	0	0.21	0.70
46	0.85	0.15	0.71	0	0.29	0.52
71	0.88	0.12	0.75	0	0.25	0.46
124	0.93	0.07	0.86	0	0.14	0.40

The addition of 3.4 mM Ca^{2+} to the growth medium did not greatly influence the composition of the polysaccharide produced by *P. aeruginosa* isolate M (Table 2). The polysaccharides isolated after 24 h had higher levels of guluronate (0.3 mole fraction) and a lower degree of acetylation than those isolated after 48 h ($\simeq$ 0.1 mole fraction). No G blocks were detected in the samples either by ^{1}H-n.m.r. analysis (see Tables 1 and 2; Fig. 3) or by ^{13}C-n.m.r. analysis (Fig. 2).

Table 2. Composition of alginate produced by *P. aeruginosa* isolate M in TSB both with and without added Ca^{2+} after 24 h and 48 h incubation

Mole fraction data were determined by ^{1}H-n.m.r. analysis and degree of acetylation was determined by chemical analysis

Culture medium	Mole fraction Mannuronic acid	Guluronic acid	M blocks	G blocks	MG blocks	Degree of acetylation
TSB						
24	0.71	0.29	0.42	0	0.58	0.50
48	0.92	0.08	0.84	0	0.16	0.65
TSB + 3.4 mM Ca^{2+}						
24	0.69	0.31	0.38	0	0.02	0.49
48	0.89	0.11	0.78	0	0.22	0.63

Discussion

The alginates produced by *P. aeruginosa* isolate M during the investigations reported here are similar to those described earlier (Linker and Evans 1976; Sherbrock-Cox *et al.* 1984), being highly 0-acetylated polymers with high content of mannuronic acid. The polysaccharide was produced mainly during the exponential phase of growth (Fig. 1) which has been observed previously in *P. aeruginosa* alginate production (Mian *et al.* 1978), although Piggott *et al.* (1982) observed polysaccharide synthesis to commence with the onset of stationary phase.

In spite of the fact that the pattern of alginate production by *P. aeruginosa* isolate M is similar to that reported for *A. vinelandii*, which also synthesizes alginate almost exclusively in the exponential phase of growth (Deavin *et al.* 1977; Annison and Couperwhite 1984), the factors influencing the composition of the polysaccharide produced

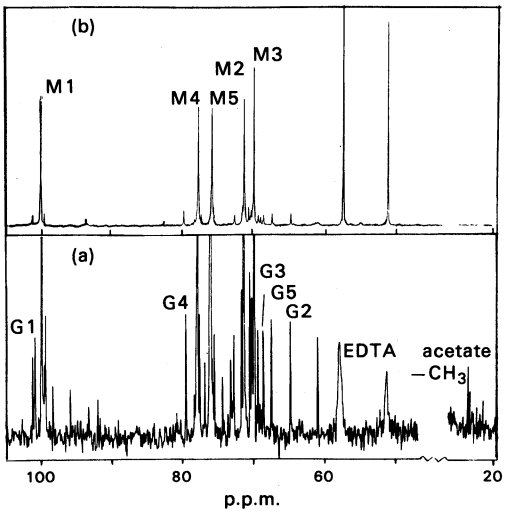

Fig. 2. ^{13}C-n.m.r. spectra of alginate from *P. aeruginosa* isolate M: (*a*) native polysaccharide; (*b*) *O*-deacetylated polysaccharide.

by the two organisms are quite different. The structure of the alginate samples isolated during the growth cycle did not vary greatly whereas, when *A. vinelandii* is cultivated under similar conditions, high guluronic acid alginates, with elevated G-block content, are synthesized early in the growth cycle and later the polymer has a preponderance of mannuronic acid and M blocks (Larsen and Haug 1971; Annison and Couperwhite 1984). The total absence of G blocks in all the samples analysed and the apparent independence of composition and Ca^{2+} levels (Table 2) indicates that the epimerase of *P. aeruginosa* isolate M is not the same as that of *A. vinelandii*.

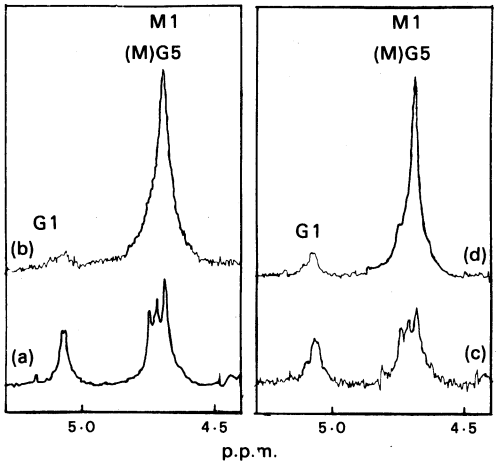

Fig. 3. ^{1}H-n.m.r. spectra of alginate of *P. aeruginosa* isolate M produced in batch culture in TSB: (*a*) without added Ca^{2+}, 24 h; (*b*) without added Ca^{2+}, 48 h; (*c*) with added Ca^{2+} (3.4 mM), 24 h; (*d*) with added Ca^{2+} (3.4 mM), 48 h.

The absence of G blocks was established using a standard ^{1}H-n.m.r. procedure (Grasdalen *et al.* 1979) developed for the analysis of algal alginates. Quantitation of each uronic acid and block type was calculated from the integrals of the resonances

in the downfield region of the spectra (Figs 3*a*–3*d*). The ^{13}C-n.m.r. spectrum (Fig. 2*a*) of the sample of *O*-deacetylated *P. aeruginosa* isolate M alginate, however, confirms the relatively simple structure of the polymer. In comparing the anomeric resonances (at $\triangleq$ 100 p.p.m.) with ^{13}C-n.m.r. data reported by Grasdalen *et al.* (1981) the signal assigned to guluronic acid residues present as G blocks is absent. The spectrum of the native polysaccharide (Fig. 2*b*) shows considerable splitting of resonances but this is due to the high content of *O*-acetyl groups present on the polymer. *O*-Acetyl substitution is known to cause both upfield and downfield shifts of resonances (depending on whether the carbon is α or β to the position of substitution). As the native polysaccharide forms much more viscous solutions than the de-*O*-acetylated polymer, less sample was used for the ^{13}C-n.m.r. analysis (ca 25 mg and 75 mg respectively). This accounts for the large difference in signal to noise in the two spectra obtained (Figs 2*a*, 2*b*). The acetates of bacterial alginates have been localized solely on the mannuronic acid residues (Davidson *et al.* 1977) but the exact site (oxygen 2 or 3 by default) is not known. Sherbrock-Cox *et al.* (1984) found levels of *O*-acetylation in some *P. aeruginosa* alginates which indicated that either some mannuronic acid residues carried two *O*-acetyl groups or alternatively that guluronic acid also carried the substituent. The alginates analysed in the investigation reported here were not so highly acetylated.

The variability in the level of acetate groups in *P. aeruginosa* alginates and the absence of G blocks is likely to affect the physical properties of the polymer and this may ultimately have ramifications regarding its commercial usefulness.

More importantly, the variability in fine structure of the polysaccharide may influence its antigenic nature. *O*-Acetyl groups have been shown to be the immunodominant region of bacterial polysaccharide antigens. Furthermore, Larsen *et al.* (1985) reported that an antibody raised against alginate had a great specificity for the G-block regions of the polymer but showed little reactivity with M- and MG-block regions. Woods and Bryan (1985) have demonstrated that immunization of rats with alginate induces high antibody levels, which may, in some cases, lead to clearance of *P. aeruginosa* in the lungs of infected animals. They concluded, however, that alginate is not a good protective immunogen due to the deposition of immuno-complexes in lung tissue.

Clearly further work is required to establish the composition of *P. aeruginosa* alginate when it is produced during infection and determine the factors which influence it. Investigations into the use of alginate as a protective immunogen should then be pursued using polysaccharide samples of defined structure both in terms of uronic acid composition and distribution and also acetate content. It may well be that to provide an effective protective immunogen the polysaccharide should be produced in carefully controlled growth conditions so that the structure is very similar to that of the antigen produced during infection.

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Manuscript received 16 March 1987, accepted 30 June 1987