Alginate Biosynthesis by *Pseudomonas mendocina*

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Exopolysaccharide-synthesizing variants of *Pseudomonas mendocina* NCIB 10541 were isolated on media containing carbenicillin. The exopolysaccharide was identified as alginic acid with a mannuronic acid : guluronic acid ratio of 1 : 2. These strains lost the ability to produce alginate at a high frequency, but more stable mutants which produced increased amounts of polysaccharide could be isolated by subsequent mutagenesis. High concentrations of polysaccharide (approximately 20 g l⁻¹) were obtained in nitrogen-limited continuous culture with a minimal glucose medium. In common with other bacterial alginates, the polymer is acetylated and has similar rheological properties to alginate from brown algae. An alginate lyase activity was present in cultures at sufficient specific activities to result in a low molecular weight, low viscosity polymer with rheology similar to printing grade alginate. This degradation was overcome by incorporation of a proteolytic enzyme into the growth medium without adverse effects on bacterial or polysaccharide yields. As an organism for the study of alginate biosynthesis, *P. mendocina* possesses advantages over *Azotobacter vinelandii* or *Pseudomonas aeruginosa* in terms of yield, strain stability, and absence of known pathogenicity.

INTRODUCTION

Alginic acid is a (1→4)-linked linear copolymer of β -D-mannuronic and β -L-guluronic acid synthesized by several species of marine algae (Booth, 1975) and produced as an exopolysaccharide by *Azotobacter vinelandii* (Gorin & Spencer, 1966) and *Pseudomonas aeruginosa* (Evans & Linker, 1973). *Azotobacter vinelandii* strains are usually mucoid when isolated and retain the ability to synthesize polysaccharide through repeated subculture (Jarman, 1979), although polysaccharide-negative mutants have been isolated and these remain viable under laboratory conditions (Sutherland, 1972). Alginate is also found in the cell walls of *Azotobacter* cysts (Sadoff, 1975).

Strains of *P. aeruginosa* do not in general produce alginate, but mucoid, alginate-producing variants are frequently isolated from the sputum of patients with cystic fibrosis (Doggett & Harrison, 1969). The environmental factors proposed to account for their gradual emergence in such patients include prolonged antibiotic therapy (Doggett & Harrison, 1969; Govan & Fyfe, 1978; Kulczcki *et al.*, 1978) and immunological selection through the protective effect of alginate on phagocytosis by alveolar macrophages (Schwarzmann & Boring, 1971) and reduced pulmonary clearance (Govan *et al.*, 1983). Alginate-producing mutants can be isolated *in vitro* by selection for resistance to phages (Martin, 1973; Govan, 1975) or antibiotics (Govan & Fyfe, 1978). Mucoid variants isolated *in vivo* or *in vitro* are often unstable; non-mucoid revertants are frequently found at high frequencies because of a growth rate advantage over the parent strain (Govan, 1975). Stability can be improved by growth in the presence of surfactants or with

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agitation (Govan, 1975). Large-scale microbial alginate synthesis is not feasible with *P. aeruginosa* because it is an opportunistic pathogen. Only poor carbon conversion efficiencies have been obtained with *A. vinelandii* (Deavin *et al.*, 1977; Jarman *et al.*, 1978).

More recently, alginate-synthesizing strains of *P. fluorescens*, *P. mendocina* and *P. putida* have been isolated by selection on growth media containing carbenicillin (Govan *et al.*, 1981). The present paper describes an investigation of alginate production by the saprophytic species *P. mendocina* (Palleroni *et al.*, 1970) for which no pathogenic role has been reported (Gilardi, 1972) and which has been very rarely isolated from clinical material (Farmer, 1976).

METHODS

Growth and maintenance of organisms. *Pseudomonas mendocina* (NCIB 10541) was routinely subcultured on nutrient agar slopes. *Pseudomonas aeruginosa* PsB (Mian *et al.*, 1978) was maintained on *Pseudomonas* isolation agar. Mucoid strains of *P. mendocina*, Muc 18, C7, U1 (NCIB 11687) and U3 (NCIB 11688) were maintained on agar (1.5%, w/v) containing a mineral salts medium (Jayasuria, 1955) plus glucose (2%, w/v) for maximum polysaccharide synthesis. Viability on this medium is poor because of acid production and subculturing must be carried out at monthly intervals. For growth of shake flask cultures using the same minimal medium, sodium gluconate (2%, w/v) as carbon source did not result in acid production. The medium used in continuous culture contained (g l⁻¹): glucose, 90; (NH₄)₂HPO₄, 1.25; K₂HPO₄, 1.25; Ca(OH)₂, 0.05; Mg(OH)₂, 0.07; MgSO₄·7H₂O, 0.14; citric acid, 0.99; yeast extract, 0.2; MnSO₄·4H₂O, 1.1 × 10⁻³; FeSO₄·7H₂O, 3.6 × 10⁻³; ZnSO₄·7H₂O, 0.7 × 10⁻³; CaSO₄·5H₂O, 0.25 × 10⁻³; CoSO₄·7H₂O, 0.28 × 10⁻³; H₃BO₄, 0.06 × 10⁻³, pH 7.0. Glucose was autoclaved separately to avoid charring.

Carbenicillin resistance. An exponential phase culture of *P. mendocina* in nutrient broth was serially diluted and spread on to nutrient agar plates containing carbenicillin, initially in the range 0 to 1000 µg ml⁻¹ in multiples of 100 µg ml⁻¹. This gave an approximate indication of the MIC which was defined further by a second set of determinations over a narrower range in multiples of 20 µg ml⁻¹.

Serial dilutions of an exponential phase culture of *P. mendocina* in nutrient broth were spread over nutrient agar plates containing 1.5 times the MIC of carbenicillin. The plates were examined after 36 h incubation at 30 °C. Resistant mucoid clones selected on the basis of their raised, glistening appearance were purified on minimal agar containing glucose (2%, w/v). The colony appearance of these isolates was recorded on deoxycholate/citrate agar, *Pseudomonas* isolation agar, minimal glucose agar (0.2, 1.0 and 2.0%, w/v), minimal gluconate agar (2.0%, w/v), nutrient agar and nutrient agar incorporating Tween 80 (1.0%, w/v) or Brij 35 (1.0%, w/v).

Mutagenesis. An exponential phase culture of *P. mendocina* in glucose (10 mM) mineral salts medium was harvested by centrifugation, washed in 0.5 M-Tris/maleate buffer, pH 6.0 and resuspended in 9 ml of this buffer to 0.5 × 10⁹ cells ml⁻¹. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) dissolved in the same buffer was added to a final concentration of 20 µg ml⁻¹. After incubation for 30 min at 30 °C, the cells were harvested by centrifugation, washed in mineral salts medium and resuspended in 100 ml salts medium plus glucose (2%, w/v). Measurements of viable cell concentration were made at all stages in this procedure. Cultures were grown for 24 h at 30 °C, the cells were collected by centrifugation, washed, resuspended in mineral salts medium, diluted and spread on to glucose (2%, w/v)/mineral salts agar plates to give 20 to 50 colonies per plate. Colony morphologies were examined after 4 to 6 d incubation at 30 °C.

Continuous culture. A laboratory fermentation vessel (L. H. Engineering Ltd, Stoke Poges, Buckinghamshire; type 1/1000) modified for continuous culture (working volume = 2.5 l) was used. The pH was maintained at 7.0 by the automatic addition of 2 M-NaOH. The temperature was 30 °C. Foaming was controlled by the addition of a silicone antifoaming agent. Air was supplied at 1 l min⁻¹ and the culture was mixed at impeller speeds of 300 to 750 r.p.m. so as to maintain the dissolved oxygen at about 20% of saturation.

For continuous culture, medium addition was started when the batch culture reached the stationary phase. The dilution rate was adjusted by alteration of the rate of flow of medium. Following a change in conditions, a steady state was considered to exist when cell dry weight and oxygen uptake had remained constant for three residence times. Glucose concentrations in culture broths were determined by the glucose oxidase method using a Model 23A glucose analyser (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.).

Cell dry weight. Bacterial cells and exopolysaccharide could only be separated in the presence of a chelating agent. Culture broth (40 ml) was mixed with 5 M-NaCl (0.8 ml) and 0.5 M-EDTA pH 7.0 (0.8 ml) and after standing for 10 min, was centrifuged at 24000 g for 40 min. The supernatant was removed, the precipitate was resuspended in distilled water and centrifuged as before. The final precipitate was dried at 105 °C to constant weight.

Polysaccharide determination. The first supernatant from the cell dry weight determination (25 ml) was added to propan-2-ol (75 ml). After mixing and standing for 10 min, the precipitate obtained was filtered on a pre-weighed Whatman GF/A filter disc which was dried *in vacuo* for 24 h and re-weighed.

Analysis of exopolysaccharide. Infra-red spectral analysis of the sodium salt of the polysaccharide was carried out by the KBr disc method (Fillipov & Kohn, 1974) using a Perkin-Elmer 457 infrared spectrometer.

The acetate content of the polymer was determined by the hydroxamate method of McComb & McCready (1957).

Molecular weight profiles of polysaccharide were obtained by gel electrophoresis using 6% (w/v) polyacrylamide (Bucke, 1974).

Rheology. For a non-Newtonian fluid the apparent viscosity, η , is dependent upon the rate of shear and can be defined by: $\eta = K(d\gamma/dt)^n$ (Van Wazer *et al.*, 1963) where K is the consistency index (apparent viscosity at a shear rate of 1 s^{-1}), $d\gamma/dt$ is the rate of shear and n is the flow behaviour index. The higher the value of K , the more viscous the fluid. The flow behaviour index varies from 1 to 0 for pseudoplastic fluids; the lower its value the more pronounced are the non-Newtonian properties of the fluid.

Culture viscosity measurements were made using a model HAT cone-and-plate microviscometer (Wells-Brookfield Engineering Laboratories Inc., Stoughton, Mass., U.S.A.) at 25°C . For more viscous solutions a model HBT was used. The apparent viscosities were determined over a range of shear rates from 3.75 to 750 s^{-1} and K was obtained by extrapolation of a log-log plot of apparent viscosity against shear rate.

A more detailed rheological examination was made using a Rheomat-30 rotational viscometer with a cone-and-plate system (Contraves A. G., Zürich, Switzerland). A 1% (w/v) polysaccharide solution in (a) distilled water and (b) 100 mM-EDTA, pH 7.0, was prepared by continuous agitation for 1 h using a top-stirrer. Solutions were examined at 30 shear rates and values of K and n obtained from shear rate versus viscosity plots.

Preparation of cell-free extracts. Cells were harvested in the late-exponential phase of growth, washed in 0.1 M-phosphate buffer (pH 7.0) and suspended in four times their wet weight of the same buffer. In viscous cultures, 0.01 M-EDTA (sodium salt, pH 7.0) was added to the wash buffer. The cells were sonically disrupted by $4 \times 20 \text{ s}$ pulses (20 kHz) in a tube chilled to 0°C using an NaCl freezing mixture. The supernatant fluids were used for enzyme assays after centrifugation at $40000 g$ for 45 min at 0°C .

Assay of alginate lyase activity. This assay measures unsaturated uronic acids released from the polysaccharide using the periodate/thiobarbituric acid method (Weissbach & Hurwitz, 1959). Reaction mixtures (2.0 ml) contained 0.1% (w/v) Manugel in 0.1 M-potassium phosphate buffer (pH 7.0). Assays were started by the addition of bacterial extract (1–2 mg protein). Control mixtures lacking enzyme or alginate were included. Samples (0.2 ml) were withdrawn from reaction mixtures after 10, 20 and 30 min incubation at 30°C . The reaction was stopped by the addition of 62.5 mM- H_2SO_4 (0.2 ml) and the sample was mixed with 0.02 M-periodic acid in 62.5 mM- H_2SO_4 (0.2 ml). After 20 min incubation at room temperature, 2% (w/v) sodium arsenite in 0.5 M-HCl (0.5 ml) was added. The mixture was shaken and, after standing for 2 min, 0.3% (w/v) thiobarbituric acid (2 ml) was added followed by heating for 10 min at 100°C . After cooling, the absorbance at 549 nm was measured against a reagent blank. Enzyme activities are defined as nmol β -formyl pyruvic acid liberated $(\text{mg protein})^{-1} \text{ min}^{-1}$; 10 nmol β -formyl pyruvic acid produces an A_{549} of 0.29 under these conditions (Preiss & Ashwell, 1962).

Chemicals. Commercial algal alginates (Manugel and Manutex) were obtained from Alginate Industries, Girvan, Ayrshire, U.K. Deoxycholate/citrate agar was from Oxoid, *Pseudomonas* isolation agar from BBL, carbenicillin from Beecham Pharmaceuticals and Neutrase from Novo Industri, Bagsvaerd, Denmark.

RESULTS

Isolation of mucoid strains

The MIC of carbenicillin for the wild-type strain of *P. mendocina* in the single cell dilution technique on nutrient agar was $80 \mu\text{g ml}^{-1}$. When suspensions of this strain were plated on to nutrient agar containing carbenicillin ($120 \mu\text{g ml}^{-1}$), resistant colonies grew after 3 to 4 d incubation at 30°C as described by Govan *et al.* (1981). Mucoid colonies (e.g. Muc 18; C7; Table 1) were identified by their raised glistening appearance on glucose (2%, w/v) minimal agar. Resistant colonies could not be obtained by plating directly on to glucose minimal agar containing carbenicillin even at concentrations as low as $50 \mu\text{g ml}^{-1}$. The capsular polysaccharide was provisionally identified as alginate by the formation of a white flocculent precipitate in 0.05 M- H_2SO_4 and by gelation in 0.01 M- CaCl_2 . Confirmation was later obtained by IR analysis and rheology.

Of the media tested, capsule production (as judged by colony morphology) in the mucoid strains was only apparent in mineral salts containing more than 1% (w/v) glucose, or on deoxycholate/citrate agar. The active components of deoxycholate/citrate medium appeared to be sodium deoxycholate and sodium thiosulphate, although neither alone was as effective in eliciting polysaccharide synthesis as the complete formulation. Incorporation of other

Table 1. *Alginate production by various strains of P. mendocina in shake flask cultures*

All strains were grown in gluconate (2%, w/v)/minimal salts medium. Propan-2-ol precipitation was carried out after 4 d incubation at 30 °C.

Strain	Alginate produced (g propan-2-ol precipitate l ⁻¹)
Wild-type (NCIB 10541)	0.45
Muc18 (First stage carbenicillin-resistant isolate)	2.0
C7 (First stage carbenicillin-resistant isolate)	1.6
U1 (NCIB 11687) (Nitrosoguanidine mutagenesis of Muc18)	2.8
U3 (NCIB 11688) (Nitrosoguanidine mutagenesis of Muc18)	3.2

Table 2. *Alginate synthesis by mutant U1 growing in continuous culture under nitrogen limitation at various dilution rates*

Culture conditions and medium were as described in Methods. Glucose was supplied at 90 g l⁻¹ and the dissolved oxygen tension was maintained at approximately 20% of saturation. The yield of polysaccharide is defined as the ratio of the propan-2-ol precipitate to the glucose consumed by the culture.

Dilution rate (h ⁻¹)	Cell dry weight (g l ⁻¹)	Propan-2-ol precipitate (g l ⁻¹)	Cell: poly- saccharide ratio	Residual glucose (g l ⁻¹)	Glucose used by culture (g l ⁻¹)	Yield of poly- saccharide
0.03	2.7	17.5	6.5	33.5	56.5	0.31
0.04	2.5	18.0	7.5	32.0	58	0.31
0.06	2.5	23.5	9.2	25.5	64.5	0.36

surfactants, for example Tween 80 (polyoxyethylene sorbitan mono-oleate) or Brij 35 (polyoxyethylene-23-lauryl ether), into growth media was not effective. Colonies did not, however, appear mucoid on gluconate (2%, w/v) minimal agar, but alginate could be recovered from liquid cultures using this medium. An alginate-synthesizing strain of *P. aeruginosa* PsB appeared mucoid on all media tested.

When *P. mendocina* cultures grown on a non-inducing medium were transferred back to 2% glucose or deoxycholate/citrate agar the mucoid appearance was regained, but all strains tested were unstable. Constant segregation to small non-mucoid colonies occurred but instability was particularly apparent in liquid culture. In some cases less than 20% of the population retained polysaccharide synthesis after 60 mass doublings.

In an attempt to improve stability and polysaccharide yield, nitrosoguanidine-induced mutagenesis was carried out on one carbenicillin-resistant isolate (Muc 18). Distinct large spreading mucoid colonies could be observed amongst survivors of the mutation. A total of 35 such isolates was obtained by screening 10⁵ clones on glucose (2%, w/v)/mineral salts agar without selective enrichment. Consistently greater propan-2-ol precipitates were obtained in shake flask cultures of these strains than in strain Muc18 or any of the first stage carbenicillin-resistant isolates (Table 1). Shake flask culture using glucose as sole carbon source was difficult to reproduce because of high glucose dehydrogenase activity and release into the medium of gluconic acid which inhibited growth by lowering the pH. Increasing the buffering capacity of the medium inhibited polysaccharide synthesis, so replacement of glucose by sodium gluconate was adopted as a standard procedure, but polysaccharide concentrations were never as high as using glucose in a pH-controlled fermenter. Approximately 50% of the large mucoid clones were unstable in shake flask culture as judged by the rapid appearance of small colonies, but the remainder (e.g. U1, U3; Table 1) were successfully subcultured for 70 mass doublings.

Polysaccharide production in continuous culture

High concentrations of polysaccharide were achieved by growth of one of the large colony isolates (strain U1) in continuous culture (Table 2). Optimal rates of polysaccharide synthesis

Table 3. *Stability of exopolysaccharide synthesis by mutant U1 in continuous culture*

Culture conditions were as described in Methods. The dilution rate was maintained at an average of 0.05 h^{-1} and dissolved oxygen tension at 20% of saturation.

Time after inoculation (h)	Propan-2-ol precipitate (g l^{-1})	Percentage of small colonies
0	0	0
25.6	15.6	0
52	19.0	5
122	15.1	5
197	18.5	17
298	20.3	21
381	18.4	35

Table 4. *Effect of incorporation of various amounts of a proteolytic enzyme on culture viscosity*

Cultures were maintained at a dilution rate of 0.05 h^{-1} and dissolved oxygen tension at approximately 20% of saturation. Other culture conditions and medium were as described in Methods.

Neutrase (Anson units l^{-1})	Propan-2-ol precipitate (g l^{-1})	Cell dry weight (g l^{-1})	Culture consistency index, K (mPa s^n)
0	18.5	3.4	32
0.1	21.0	3.6	4700
0.25	19.6	3.0	5100

were obtained by maintaining the dissolved oxygen tension between 18 and 25% of saturation. Below this range polysaccharide concentration declined while above it concentrations were maintained but carbon conversion efficiencies from glucose fell, presumably because of increased substrate oxidation. Polysaccharide concentration increased with dilution rate to 0.06 h^{-1} but then fell sharply to less than 10 g l^{-1} at 0.08 h^{-1} , although cell dry weight remained constant. Nitrogen was established as the limiting nutrient in the medium used by adding $1 \text{ g NH}_4\text{Cl l}^{-1}$ to a culture at a steady state. Cell density rose from 2.7 g l^{-1} to 4.1 g l^{-1} after 12 h but then remained constant due to the establishment of a new limit.

When the mutant U1 was maintained at a constant dilution rate of 0.5 h^{-1} , strain instability as estimated by the appearance of small colonies was apparent (Table 3). This was not accompanied, however, by a corresponding drop in polysaccharide concentration, although when polysaccharide synthesis by these small colony types was measured in shake flask culture it was less than 50% of the starter culture of mutant U1.

Degradation of polysaccharide

All strains of *P. mendocina* used in this work were found to possess an alginate lyase activity which was present at specific activities of between 0.2 and $0.35 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ in crude extracts independent of polysaccharide synthesis or growth substrate. The specific activities also remained unaltered throughout the course of continuous fermentations. Despite high concentrations of alginate, culture viscosities (as estimated by the culture consistency index) remained low unless a proteolytic enzyme was incorporated into the medium (Table 4). Addition of 'Neutrase' increased culture viscosities substantially. This enzyme preparation did not have an adverse effect on cell growth or polysaccharide synthesis.

Evidence was also obtained that the alginate lyase activity was normally intracellular and was only released by cell lysis. PAGE of polysaccharide indicated only high molecular weight material in exponential phase batch cultures. Lower molecular weight bands only appeared after 12 to 24 h in the stationary phase. Addition of sonicated cell extracts to high viscosity cultures resulted in a rapid drop in viscosity and faster migrating bands on electrophoresis. Similar results were obtained when polysaccharide breakdown was estimated by the concentration of free uronic acid groups using the thiobarbituric acid assay.

Fig. 1. Infra-red spectra of exopolysaccharide from *P. mendocina* grown in continuous culture under nitrogen limitation (—) and commercial algal alginate (----).

Table 5. *Rheology of alginate samples*

All samples were prepared as 1% (w/v) solutions and rheology measurements made as described in Methods.

Sample	Solvent	Consistency index, K (mPa s ⁿ)	Flow behaviour index (n)
Nitrogen-limited culture without protease	H ₂ O	9.6	0.86
	EDTA (100 mM)	8.4	0.86
Nitrogen-limited culture plus protease	H ₂ O	8043	0.27
	EDTA (100 mM)	557	0.43
Low molecular weight algal alginate (Manutex F)	H ₂ O	31.4	0.91
	EDTA (100 mM)	12.8	0.98

Polysaccharide characterization

The IR spectrum of exopolysaccharide from mutant U1 grown in continuous culture differs from algal alginate by having additional absorption peaks at 1250 cm⁻¹ and 1720 cm⁻¹ which correspond to *O*-acetyl ester (Fig. 1). It is similar to the spectra of alginate from *P. aeruginosa* and *A. vinelandii* (Evans & Linker, 1973; Mian *et al.*, 1978). The acetate content of the polymer was determined to be 12% (w/v) and an approximate mannuronic:guluronic acid ratio of 1:2 was estimated from the absorbances at 1125 cm⁻¹ and 1030 cm⁻¹.

The rheological data (Table 5) indicate that without the addition of a proteolytic enzyme the *P. mendocina* polysaccharide has similar properties, including low pseudoplasticity, to the low molecular weight commercial alginate obtained from brown algae. With addition of protease a high viscosity product with similar properties to the high molecular weight algal product is obtained. Addition of EDTA lowers viscosity by chelation of divalent metal ions which form cross-links between chains.

DISCUSSION

Alginic acid can be synthesized by variants of *P. mendocina* selected in the presence of normally inhibitory concentrations of carbenicillin (Govan *et al.*, 1981). A similar response has been observed in some strains of *P. aeruginosa* (Govan & Fyfe, 1978). The mucoid variants of *P.*

mendocina are unstable. Stability and yields of polysaccharide can be improved by selection of large mucoid clones after mutagenesis of carbenicillin-selected isolates. The reasons for this response to carbenicillin are unclear. Continued selection on carbenicillin attempted in this study resulted in the eventual dominance of non-mucoid resistant strains. Similarly, attempts to stabilize polysaccharide synthesis using surfactants were unsuccessful although this technique was used with *P. aeruginosa* (Govan, 1975). Polysaccharide synthesis by *P. mendocina* was also dependent upon growth medium, high concentrations of glucose giving the best results, whereas a mucoid strain of *P. aeruginosa* synthesized exopolysaccharide on all growth media tested.

The polymer from *P. mendocina* is partially acetylated, in common with that from *A. vinelandii* (Larsen & Haug, 1971) and *P. aeruginosa* (Evans & Linker, 1973) but unlike that from the brown algae. This does not, however, seem to result in gross differences in rheology. The similarity in structure of a polysaccharide from such a range of organisms is interesting in view of the synthesis of very different exopolysaccharides by different wild-type strains of *P. aeruginosa* (Brown *et al.*, 1969; Bartell *et al.*, 1970).

The presence of an alginate lyase, albeit at very low intracellular activities, dramatically reduces the viscosity of culture broths. This suggests an endo-enzyme making possibly only a few breaks in a long chain. A similar effect has been observed in *A. vinelandii* cultures (Jarman, 1979). An inducible alginate lyase has been studied in *Beneckeia pelagia* which is able to utilize alginate as a carbon source (Pitt & Raisbeck, 1978), but *P. mendocina* in common with *A. vinelandii* (Davidson, 1975) possesses low specific activities of this enzyme and is unable to grow on alginate. In *A. vinelandii*, however, a role for the enzyme in breaking down alginate during encystment or cyst germination can be postulated. Lyase activity has been reported in *P. putida* and *P. maltophila* (Sutherland & Keen, 1981) but not in *P. aeruginosa*. In this work intracellular activities similar to those in *P. mendocina* were detected in the alginate-synthesizing *P. aeruginosa* PsB although this organism does not degrade polymer in fermentations (Mian *et al.*, 1978). The difference may lie in a lower rate of cell lysis or in a poor activity of the enzyme towards an acetylated polymer.

Alginate lyase activity can be overcome in continuous culture of *P. mendocina* by incorporation of a protease in the growth medium and concentrations of alginate of 20 g l^{-1} were maintained for 400 h at a dilution rate of 0.05 h^{-1} . The stability of this strain of *P. mendocina* was considerably better than that of *P. aeruginosa* PsB where 45% non-mucoid colonies were apparent after 112 h (Mian *et al.*, 1978). Higher polysaccharide:cell ratios were also obtained in this work. Alginate synthesis by *A. vinelandii* suffers from poor carbon conversion efficiencies because the organism synthesizes poly- β -hydroxybutyrate (Jarman *et al.*, 1978), and at higher oxygen tensions has very high respiration rates which result in low polysaccharide synthesis (Deavin *et al.*, 1977). At present, therefore, *P. mendocina* appears to be the most suitable organism to study bacterial alginate synthesis on a larger scale.

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