Heterologous expression of an alginate lyase gene in mucoid and non-mucoid strains of *Pseudomonas aeruginosa*

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A 1.95 kb DNA fragment containing the *aly* gene from *Klebsiella pneumoniae*, which encodes an alginate lyase, has been ligated into the broad-host-range vector pLAFR3. Transfer of the resultant recombinant plasmid, pALY8, into mucoid and non-mucoid strains of *Pseudomonas aeruginosa* resulted in expression of the alginate lyase. The heterologously expressed alginate lyase, which had the same isoelectric point and substrate specificity as the native enzyme, altered the morphology of mucoid strains. Analysis of the extracellular material from mucoid strains revealed that lyase expression reduced the M_r and overall yield of alginate produced. The mature form of the recombinant enzyme was the same as that produced extracellularly by *Klebsiella pneumoniae*; however, most of the alginate lyase was retained intracellularly by *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes a variety of infections in compromised patients. In chronic infections a mucoid, exopolysaccharide-producing form of the organism tends to predominate and is invariably associated with a poorer prognosis for the patient. The exopolysaccharide, alginate, is an important virulence factor synthesized by mucoid strains of *Pseudomonas aeruginosa* (Gacesa & Russell, 1990). Approximately 80% of the isolates of *P. aeruginosa* from cystic fibrosis patients are mucoid, i.e. alginate-producing (Doggett *et al.*, 1966), and in other chronic clinical conditions a significant but lower incidence of mucoid isolates has also been observed (McAvoy *et al.*, 1989). Many mechanisms have been proposed for the role of alginate in pathogenesis and it appears that there is a complex interaction between the polysaccharide, the immune system and cell-surface components within the lungs of cystic fibrosis patients (Gacesa & Russell, 1990). Alginate is also synthesized by phytopathogenic pseudomonads, where it has been implicated as a virulence factor (Fett *et al.*, 1986; Osman *et al.*, 1986). Other pseudomonads (Govan *et al.*, 1981) and *Azotobacter vinelandii* (Anderson *et al.*, 1987) also synthesize alginate under appropriate environmental conditions.

Alginates are (1-4)-linked glycuronans comprised of residues of β -D-mannuronate and the C5-epimer α -L-guluronate. These monomers are arranged in block

structures which may be homopolymeric [poly β -D-mannuronate (poly M) or poly α -L-guluronate (poly G)] or heteropolymeric, occurring as random sequences (poly MG). The alginate produced by *P. aeruginosa* is devoid of poly G blocks and many of the D-mannuronate residues are O-acetylated at the 2 and/or 3 positions, unlike the alginate obtained from non-bacterial sources (Gacesa, 1988). The physical and chemical properties of alginates are dependent on the proportions of the various block structures within the polysaccharide chains and on the degree of O-acetylation. Alginates with a large proportion of poly G blocks form strong but brittle gels in the presence of divalent cations, especially Ca^{2+} , whereas those rich in the other block structures form weaker but more flexible gels. The types of alginate produced by clinical isolates of *P. aeruginosa*, i.e. no poly G blocks, O-acetylated and high M_r , result in the formation of relatively bulky gels with a high degree of water retention.

Alginates are degraded by a group of enzymes that catalyse the β -elimination of the 4-O-linked glycosidic bond with formation of unsaturated uronic-acid-containing oligosaccharides (Gacesa, 1987). These alginate lyases have been reported from a wide variety of bacterial and other sources and typically show preference for one or more of the block structures in alginate (Gacesa, 1992). A 'mannuronate-specific' alginate lyase has been detected in approximately 50% of isolates of mucoid *P. aeruginosa* (Dunne & Buckmire, 1985; Linker & Evans, 1984). The gene for a 'guluronate-specific' alginate lyase from *Klebsiella pneumoniae* has been cloned and the recombinant enzyme expressed in *Escherichia*

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coli (Caswell *et al.*, 1989). Although this enzyme preferentially degrades regions of alginate containing L-guluronate, it also degrades random block structures and alginate derived from isolates of mucoid *P. aeruginosa*.

The clinical management of mucoid *P. aeruginosa* infections in the lungs of cystic fibrosis patients has proved to be difficult. A complex regime of antibiotic therapy and physiotherapy is required to control mucoid *P. aeruginosa* and typically these infections become asymptomatic for varying periods before recurrence of the infection (Dinwiddie, 1990). One suggested approach to therapy has been the use of alginate lyases to degrade the alginate and aid dispersal of the bacterial infection by either physiotherapy or antibiotic treatment (Russell & Gacesa, 1988).

In this paper we report the cloning of an alginate lyase gene into mucoid and non-mucoid strains of *P. aeruginosa* and the effects of heterologous expression on the properties of the bacterial alginate. This provides a useful model system for studying the effects of alginate lyase on the exopolysaccharide produced by mucoid strains of *P. aeruginosa*.

Methods

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this work are listed in Table 1. Strains of *E. coli* and *P. aeruginosa* were grown with aeration in L broth (Tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g l⁻¹) at 37 °C. Antibiotics were used in selective media at the following concentrations: ampicillin (50 µg ml⁻¹ for *E. coli*), tetracycline (10 µg ml⁻¹ for *E. coli*; 100 µg ml⁻¹ for *P. aeruginosa*). Media were solidified by the addition of 15 g agar l⁻¹.

Expression of alginate lyase activity was detected by growth on L-broth, containing appropriate antibiotics, solidified with 1% (w/v) agarose and containing 0.1% sodium alginate (high-viscosity alginate from *Macrocystis pyrifera*; Sigma). Clearing zones were detected following addition of 10% (w/v) cetyl pyridinium chloride (Gacesa & Wusteman, 1990).

A minimal medium was used to grow bacteria for the subcellular localization of alginate lyase. *P. aeruginosa* was grown overnight at

37 °C on M9-salts with D-gluconate as the carbon source and supplemented with amino acids and antibiotics as appropriate.

Construction and manipulation of recombinant plasmids. DNA manipulations were performed as described by Sambrook *et al.* (1989). The recombinant plasmid pRC5 (Caswell *et al.*, 1989) was used as the source of a 1.95 kb *Hind*III fragment which contained the alginate lyase gene from *Klebsiella pneumoniae*. The 1.95 kb fragment was inserted into the *Hind*III site of the broad-host-range vector pLAFR3 and transformed into *E. coli* JM107. Clones expressing alginate lyase, as detected using alginate/agarose plates, were used as donors in triparental mating experiments. Recombinant DNA was mobilized into mucoid and non-mucoid strains of *P. aeruginosa* by triparental mating using the conjugative helper plasmid pRK2013 (Goldberg & Ohman, 1984). Recipient strains of *P. aeruginosa* were selected by growth on Vogel and Bonner minimal medium containing tetracycline (Goldberg & Ohman, 1984). The donor strain of *E. coli* is unable to grow in the absence of thiamin and the helper strain is sensitive to tetracycline. Transconjugants capable of expressing alginate lyase were detected by zones of clearing on alginate agarose plates.

Purification and analysis of polysaccharides. *P. aeruginosa* was grown on L-agar plates at 37 °C for 72 h. The growth from three plates was gently removed with a glass rod, centrifuged and the supernatant, containing water-soluble polysaccharide, was dialysed and freeze-dried (Sherbrock-Cox *et al.*, 1984). This material was analysed for total carbohydrate, uronic acid and protein prior to further purification. The crude freeze-dried extract was redissolved in a minimum of distilled water and cetyl pyridinium chloride (CPC) added to a final concentration of 2% (w/v) to precipitate the alginate. The alginate/CPC complex was collected by centrifugation (5000 g for 10 min at 4 °C) and the alginate redissolved by the slow addition of solid NaCl (final concentration 1 M) (Narbad *et al.*, 1988). Further purification was achieved by the addition of an equal volume of propan-2-ol to precipitate the alginate. The precipitate was redissolved in water, the propan-2-ol precipitation procedure repeated once and samples freeze-dried.

Protein was measured by the method of Bradford (1976) with bovine serum albumin as standard. Total carbohydrate was assayed with phenol/sulphuric acid reagent (Dubois *et al.*, 1956) and uronic acids with 3-phenylphenol/borate reagent (Blumenkrantz & Asboe-Hansen, 1973) with D-mannurono 6,3-lactone as standard. The O-acetyl content of the alginate sample was assayed by the method of Buscher *et al.* (1974) with β-D-glucose penta-acetate as standard.

Samples of purified alginates were analysed by ¹H-NMR as described previously (McAvoy *et al.*, 1989). The *M_r* distributions of alginate preparations were determined by gel permeation chromato-

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Genotype or phenotype	Reference
Bacteria		
<i>E. coli</i>		
JM107	Δ(lac-pro) thi gyrA96 endA1 hsdR17 relA1 supE44 mcrA (F' traD36 proAB lacZΔM15)	Yanisch-Perron <i>et al.</i> (1985)
<i>P. aeruginosa</i>		
AK957	PAO1 equivalent, prototrophic, Alg ⁻	A. Kropinski, Kingston, Ontario
FRD 1	Prototrophic, Alg ⁺ cystic fibrosis isolate	Ohman & Chakrabarty (1981)
FRD 130	met-1, arg-1, trp-2, Nal ^r , Alg ⁺ stable	Goldberg & Ohman (1984)
Vectors		
pALY8	pLAFR3 with 1.95 kb <i>K. pneumoniae</i> DNA containing <i>aly</i>	This paper
pLAFR3	IncP Tc ^r λ cos ⁺	Staskawicz <i>et al.</i> (1987)
pRC5	pHG327 with 1.95 kb <i>K. pneumoniae</i> DNA containing <i>aly</i>	Caswell <i>et al.</i> (1989)
pRK2013	ColEI-Tra(RK2) ⁺ Km ^r	Figurski & Helinski (1979)

graphy on Sepharose CL-4B. Alginate samples (approximately 1 mg) were eluted from the column at a flow rate of 0.83 ml min^{-1} and fractions (2 ml) were analysed for uronic acid using the 3-phenylphenol/borate reagent.

Properties of the recombinant gene products. Crude preparations of intracellular and extracellular enzyme were prepared and assayed as described previously (Caswell *et al.*, 1986). Enzyme degradation of sodium alginate was monitored by measuring either the release of reducing sugar equivalents or the increase in absorbance at 232 nm (Boyd & Turvey, 1977). One unit of enzyme activity is defined as the release of $1 \mu\text{mol}$ reducing sugar min^{-1} under the conditions of assay. The substrate specificity of the recombinant enzyme was determined using samples of characterized algal and bacterial alginate, and specific block structures which were prepared as described previously (Gacesa & Wusteman, 1990).

The isoelectric point of alginate lyase was determined by isoelectric focusing and detection of enzyme activity by a substrate-overlay technique (Caswell *et al.*, 1986).

Sub-cellular localization of the alginate lyase. Strains of *P. aeruginosa* were grown overnight at 37°C on M9-salts containing D-gluconate as carbon source and supplemented with amino acids and antibiotics as appropriate. Extracellular, periplasmic and cytoplasmic fractions were obtained as described previously (Cheng *et al.*, 1970).

Results

Isolation of clones containing the *aly* gene

The cloned alginate lyase gene, *aly*, on a 1.95 kb *Hind*III fragment was ligated into the single *Hind*III site in the broad-host-range plasmid pLAFR3 as described in Methods. Plasmids were transformed into *E. coli* JM107 selected for the plasmid-encoded tetracycline resistance and screened for alginate lyase activity, as detected by clearing zones on alginate/agarose plates. Plasmids were isolated from *Aly*⁺ *E. coli* transformants and restriction endonuclease digestion of plasmid DNA with *Hind*III was used to confirm the acquisition of the 1.95 kb *Hind*III fragment into pLAFR3. The recombinant plasmid was then transferred to *P. aeruginosa* by triparental mating and all transconjugants produced alginate lyase as detected by clearing zones on alginate/agarose plates. The absence of detectable endogenous alginate lyase was confirmed as control strains containing pLAFR3 alone did not produce clearing zones under these experimental conditions. Analysis of plasmid DNA by restriction digestion and electrophoresis on agarose gels confirmed that all *Aly*⁺ transconjugants tested contained a 1.95 kb insert in pLAFR3. The recombinant plasmid containing the 1.95 kb insert was designated pALY8.

The phenotypes of the transconjugants grown on L-agar were compared to control strains containing vector alone. Non-mucoid transconjugants containing pALY8 showed no alteration in morphology. However, mucoid transconjugants containing pALY8 had a less mucoid

Fig. 1. Colonial morphology on L-agar of the 'mucoid' strain FRD 1 containing vector (pLAFR3; left) or the recombinant plasmid (pALY8) which contains the 1.95 kb fragment encoding alginate lyase (right).

appearance at low cell density and were essentially non-mucoid at high cell density (Fig. 1).

Localization of the alginate lyase activity

The bulk of the alginate lyase was retained in the cytoplasm of the non-mucoid strain, with small but detectable activity in the periplasmic fraction. Sub-cellular fractionation of mucoid strain FRD 1(pALY 8) showed that almost one-third of the enzyme was present in the medium (Table 2).

Effects of alginate lyase on the properties of alginate

The crude water-soluble extracellular material produced by transconjugants of *P. aeruginosa* contained carbohydrate, uronic acid and small amounts of protein ($<3\%$, w/w). Analysis of the purified material from transconjugants containing pALY8 revealed that only 14–19% of the carbohydrate was uronic acid. In contrast, transconjugants containing vector alone produced polysaccharide which was 87–92% uronic acid (Table 3). ¹H-NMR analysis of the material from the control bacteria gave a spectrum typical of alginate, whereas spectra of polysaccharide from transconjugants containing pALY8 lacked the peaks at 4.65 and 5.05 p.p.m. which are characteristic of alginates. The alginates derived from FRD 1 and FRD 130 were identical, with a guluronate content of 34% and a nearest-neighbour fractional composition of $F_{GG} = 0$, $F_{MM} = 0.32$ and $F_{MG} + F_{GM} = 0.68$.

The M_r profiles of alginate from *P. aeruginosa* on gel permeation chromatography showed significant differ-

Table 2. Cellular distribution of alginate lyase activity

<i>P. aeruginosa</i> strain	Plasmid	Enzyme*	Percentage distribution of enzyme activity		
			Medium	Periplasm	Cytosol
AK957 (non-mucoid)	pLAFR3	AP	44.8	35.3	19.8
	pALY8	AP	43.0	40.3	16.7
	pLAFR3	Lyase	ND	ND	ND
	pALY8	Lyase	ND	8.9	91.1
FRD1 (mucoid)	pLAFR3	AP	7.3	90.6	2.1
	pALY8	AP	5.9	92.1	2.0
	pLAFR3	Lyase	ND	ND	ND
	pALY8	Lyase	30.0	10.0	60.0

ND, Not detected.

* AP, alkaline phosphatase (periplasmic marker enzyme).

Table 3. Analysis of extracellular material from cultures of *P. aeruginosa*

Strain	Plasmid	Crude material	Cetyl trimethylammonium bromide-purified material		
		Percentage uronic acid	Percentage uronic acid	O-Acetyl/uronate ratio	O-Acetyl/mannuronate ratio
FRD 1	pLAFR3	55.7	86.8	0.75	1.12
FRD 1	pALY8	14.1	19.0	0.74	ND*
FRD 130	pLAFR3	59.9	91.9	0.76	1.15
FRD 130	pALY8	12.5	14.5	0.73	ND*
Manucol DH†		87.5	91.1	NA‡	NA‡

* Insufficient material available to determine the mannuronate to guluronate ratio.

† A sample of algal alginate.

‡ Not applicable as algal alginate is devoid of O-acetyl groups.

Fig. 2. M_r profile of alginate derived from mucoid strains of *P. aeruginosa*. Samples of alginate from *P. aeruginosa* containing the recombinant plasmid, pALY8 (dashed line) or vector, pLAFR3 (solid line) were chromatographed on a column of Sepharose 4B-CL. Experimental details are described in Methods. V_0 indicates the void volume of the column.

ences between strains. Alginate of lower M_r was produced by those bacteria which contained pALY8 and expressed the alginate lyase (Fig. 2).

Table 4. Substrate specificity of native and recombinant alginate lyase

Substrate	Relative activity	
	Native enzyme	Recombinant enzyme
Oligomeric		
Random block	1.00*	1.00†
Poly M block	0.41	0.36
Poly G block	0.86	0.88
Polymeric		
Manucol DH (algal alginate)	0.77	0.85
CF492a (<i>Pseudomonas</i> alginate)	0.25	0.27

* Measured activity was 28 nmol product produced min^{-1} .† Measured activity was 85 nmol product produced min^{-1} .

Properties of the alginate lyase

The recombinant enzyme expressed by *P. aeruginosa* had the same substrate specificity as the alginate lyase expressed by the *K. pneumoniae* strain from which the original *aly* gene had been obtained. The recombinant

enzyme degraded polymeric algal alginate (Manuol DH), bacterial alginate and various block structures, and had the same ratio of activities towards the substrates as the native enzyme. Poly G and poly MG blocks were degraded preferentially, with less degradation of poly M (Table 4).

Isoelectric focusing

Both the native and recombinant alginate lyases produced identical patterns on isoelectric focusing gels, with activity detected at a pI value of 8.9.

Discussion

The expression of the *Klebsiella* alginate lyase in mucoid *P. aeruginosa* affects the properties of the alginate produced by this organism. Evidence for enzyme activity is based on the observable clearing zones on alginate/agarose plates, the altered morphology and the fractionation and quantitative estimate of extracellular material. It is unlikely that the observed effects are due to reversion of the mucoid strains rather than the effect of the lyase. No evidence of abnormal rates of reversion was noted in mucoid *P. aeruginosa* transconjugants containing the vector alone. Also, the same alteration of morphology was noted in both the wild-type organism, FRD 1, and the stable mucoid derivative FRD 130.

The expressed alginate lyase in the recombinant isolates of *P. aeruginosa* had the same isoelectric point and substrate specificity as the native enzyme from *K. pneumoniae*. Therefore, the observed enzyme activity is due to expression of the *K. pneumoniae* *aly* gene rather than to induced endogenous activity. Although alginate lyase activity has been observed in up to 50% of clinical mucoid isolates of *P. aeruginosa* (Dunne & Buckmire, 1985; Linker & Evans, 1984), no endogenous alginate lyase was observed in our strains under the culture conditions used. It is conceivable that all strains of *P. aeruginosa* possess a gene(s) for an alginate lyase but this is likely to be tightly regulated, as is the case for genes involved in alginate biosynthesis (Mohr *et al.*, 1990).

The alginate lyase was located predominantly in the cytoplasm or periplasmic space in non-mucoid *P. aeruginosa*. However, a significant proportion of the enzyme activity was located extracellularly in the mucoid strain, although this may have been partly due to cell lysis. The enzyme in *K. pneumoniae* is located extracellularly (Caswell *et al.*, 1989) although there have been reports that substantial quantities of lyase remain in the bacterial cell (Lange *et al.*, 1989). Expression of the *aly* gene in *E. coli* results in production of a pro-enzyme (Caswell & Gacesa, 1990b) which is processed to the

mature form of the lyase and exported into the periplasm or medium (Caswell & Gacesa, 1990a). The fact that expression of the *aly* gene in *P. aeruginosa* results in a protein with a pI of 8.9, i.e. identical to the mature protein in *K. pneumoniae*, implies that *P. aeruginosa* is able to process the pro-protein although export is less effective and may be deficient. However, it is conceivable that growth rate or the age of the culture may influence the amount of enzyme that is exported by *P. aeruginosa*. This is undoubtedly the case with *K. pneumoniae* (Caswell & Gacesa, 1990b). Some other periplasmic enzymes of *K. pneumoniae*, e.g. pullulanase, are exported from the bacterial cell as lipoprotein complexes (D'Enfert *et al.*, 1987); however, there is no evidence that this is the case with alginate lyase.

The secretion of a greater proportion of the alginate lyase by the mucoid compared to the non-mucoid strain may be the result of any one of a number of effects. For example, the measurable endogenous exolipase activity of *P. aeruginosa* is greater in alginate-producing strains (Wingender & Winkler, 1984) and it has been proposed that alginate promotes the release of exolipase from the lipopolysaccharides of the outer membrane (Wingender, 1990). In contrast, it has been reported that mucoid strains of *P. aeruginosa* secrete less elastase than their non-mucoid counterparts (Ohman & Chakrabarty, 1982; Goldberg & Ohman, 1987). However, this probably reflects transcriptional control in the latter example rather than the release of latent enzyme from the surface of the outer membrane (Goldberg & Ohman, 1987; Mohr *et al.*, 1990).

It will be interesting to study the virulence of the recombinant strains. Alginate has been reported to inhibit phagocytosis of *P. aeruginosa* by acting as a free-radical scavenger (Smith & Simpson, 1990). This is supported by other studies which indicate that alginate lyase treatment of mucoid *P. aeruginosa* enhances phagocytosis of the bacteria by human monocyte-derived macrophages (Eftekhari & Speert, 1988). The availability of recombinant strains containing the *aly* gene is a useful model system. Such a model system will allow the problems outlined above to be investigated more thoroughly than has been possible hitherto, as the alginate lyase may be expressed in the recombinant organism in a controlled manner.

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