GDP-mannose dehydrogenase is the key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa*: evidence from metabolite studies

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The *Pseudomonas aeruginosa* enzyme GDP-mannose dehydrogenase (GMD) is encoded by the *algD* gene, and previous genetic studies have indicated that it is a key regulatory and committal step in the biosynthesis of the polysaccharide alginate. In the present study the *algD* gene has been cloned into the broad-host-range expression vector pMMB66EH and GMD overexpressed in mucoid and genetically-related non-mucoid strains of *P. aeruginosa*. The metabolic approach of P. J. Tatnell, N. J. Russell & P. Gacesa (1993), *J Gen Microbiol* 139, 119–127, has been used to investigate the subsequent effect of GMD overexpression on the intracellular concentrations of the key metabolites GDP-mannose and GDP-mannuronate, which have been related to GMD activity and total alginate production. The overexpression of *algD* in mucoid and non-mucoid strains resulted in elevated GMD activities compared to wild-type strains; there was a concomitant reduction in GDP-mannose concentrations and greatly increased GDP-mannuronate concentrations. However, significantly, alginate biosynthesis was detected only in mucoid strains and GMD overexpression resulted in only a marginal increase in exopolysaccharide production. The GDP-mannuronate concentrations in mucoid strains which overexpressed GMD were always significantly greater than those of GDP-mannose, indicating that GMD was no longer the major kinetic control point in the biosynthesis of alginate by these genetically-manipulated strains. The small but significant increase in alginate production by such strains together with the increased GDP-mannuronate concentrations is interpreted as meaning that a later enzyme of the alginate pathway has become the major kinetic control point and now determines the extent of alginate production. This study has provided direct metabolic evidence that GMD is the key regulatory enzyme in alginate biosynthesis in *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*, alginate biosynthesis, GDP-mannose dehydrogenase, cystic fibrosis, *algD* gene

INTRODUCTION

Pseudomonas aeruginosa is a major opportunistic pathogen causing severe lung infections in patients with cystic fibrosis (CF), which over the past 20 years, has emerged as the major cause of morbidity and mortality amongst this

cohort of patients (Govan & Glass, 1990). Typically, the *P. aeruginosa* strains isolated from the sputum of CF patients are mucoid and secrete copious quantities of the viscous extracellular polysaccharide alginate, which is considered to be a major virulence factor in the chronic colonization of the lungs of CF patients (Russell & Gacesa, 1988). Bacterial alginates are (1→4)-linked linear heteropolysaccharides consisting of β -D-mannuronate and its C5-epimer α -L-guluronate (Evans & Linker, 1973) in

Abbreviations: CF, cystic fibrosis; GMD, GDP-mannose dehydrogenase.

Table 1. Bacterial strains and plasmids

Strain	Genotype/phenotype	Reference or source
Bacteria		
<i>E. coli</i>		
HB101	<i>P</i> -A2 <i>L</i> -B6 <i>thi</i> -1 <i>lacY</i> 1 <i>hsdR</i> <i>recA</i> 13 <i>supE</i> 44 <i>rpsL</i> 20	Boyer & Roulland-Dussoix (1969)
S17-1	Chromosomally-encoded <i>tra</i> ⁺ genes	Simon <i>et al.</i> (1983)
<i>P. aeruginosa</i>		
FRD1	Prototrophic, <i>alg</i> ⁺ (mucoid), CF isolate	Ohman & Chakrabarty (1981)
FRD2	Spontaneous revertant of FRD1, <i>alg</i> (non-mucoid) <i>algS</i> (off)	Ohman & Chakrabarty (1982)
PAO1	Prototrophic, <i>alg</i> (non-mucoid)	Holloway <i>et al.</i> (1979)
PAO568	FP2 ⁺ <i>alg</i> ⁺ (mucoid) <i>muc</i> -2 <i>leu</i> -38	Fyfe & Govan (1980)
Plasmids		
pCC27	pCP19 with 23 kb <i>Bam</i> HI fragment of <i>P. aeruginosa</i> DNA containing the alginate biosynthetic gene cluster Tc ^r	Chitnis & Ohman (1990)
pMMB66EH	Ap ^r , <i>P</i> _{lac} , <i>mob</i> ⁺ , <i>lacI</i> ^q	Furste <i>et al.</i> (1986)
pPT1	pSP70 with 3.2 kb <i>Bgl</i> II/ <i>Cla</i> I fragment containing <i>algD</i> subcloned from pCC27	Present study
pPT2	pSP72 with 2.4 kb <i>Cla</i> I/ <i>Sma</i> I fragment containing <i>algD</i> subcloned from pPT1	Present study
pPT3	pMMB66EH with 2.4 kb <i>Bam</i> HI/ <i>Bgl</i> II fragment containing <i>algD</i> subcloned from pPT2. Forward clone with respect to <i>P</i> _{lac}	Present study
pPT4	pMMB66EH with 2.4 kb <i>Bam</i> HI/ <i>Bgl</i> II fragment containing <i>algD</i> subcloned from pPT2. Reverse clone with respect to <i>P</i> _{lac}	Present study
pSP70	Ap ^r , SP6 and T7 RNA polymerase promoters	Promega
pSP72	Ap ^r , SP6 and T7 RNA polymerase promoters	Promega

which the mannuronate residues may be mono- or di-O-acetylated (Sherbrock-Cox *et al.*, 1984).

The alginate biosynthetic pathway has been characterized in *Fucus gardneri* (Lin & Hassid, 1966) and *Azotobacter vinelandii* (Pindar & Bucke, 1975). However, full elucidation of the *P. aeruginosa* alginate pathway has proved difficult, partially due to low or barely detectable enzyme activities in even the most overtly mucoid strains (Narbad *et al.*, 1990). Evidence for the presence of crucial enzymes has been gained from molecular genetic studies, in which genes encoding the biosynthetic enzymes have been cloned and over-expressed (Sa'-Correia *et al.*, 1987). The application of molecular genetic techniques has also shown that the regulation of alginate biosynthesis is complex and involves an environmentally-responsive signal-transduction (or two-component) system and histone-like elements (see reviews by Deretic *et al.*, 1989, 1991; DeVault *et al.*, 1989; Ohman *et al.*, 1990; Russell *et al.*, 1992; Zielinski *et al.*, 1990).

The genes involved in alginate biosynthesis are located in three regions of the *P. aeruginosa* chromosome: the *muc* or switching region at 68 min (Fyfe & Govan, 1980; Flynn & Ohman, 1988); a regulatory cluster around 8 to 11 min (Deretic *et al.*, 1989; Deretic & Konyecsni, 1989); Goldberg & Ohman, 1987; Konyecsni & Deretic, 1990; Mohr *et al.*, 1991); and a biosynthetic gene operon at 34 min (Darzins *et al.*, 1985; Chitnis & Ohman, 1993). The *algD* gene is proximal to the promoter in the biosynthetic operon and encodes the enzyme GDP-mannose dehydrogenase (GMD), which catalyses the

essentially irreversible NAD⁺-dependent oxidation of GDP-mannose to GDP-mannuronate. Alginate production depends upon the transcriptional activation of the strong promoter immediately upstream of *algD* which is responsive, via *trans* activation, to environmental stimuli. The position of *algD* relative to the major promoter in the 34 min region of the *P. aeruginosa* chromosome, and the tightly controlled expression of this gene, indicate a key role for GMD in the biosynthesis of alginate.

The hexameric subunit structure of GMD (Roychoudhury *et al.*, 1989) is in accord with the proposed regulatory role of the enzyme in alginate biosynthesis. That GMD is the key regulatory enzyme and committal step in alginate biosynthesis is supported by recent metabolic studies by Tatnell *et al.* (1993), who showed that intracellular concentrations of GDP-mannose accumulate particularly during the exponential phase of growth in batch culture of both mucoid and non-mucoid strains. In contrast, the product of the GMD-catalysed reaction, GDP-mannuronate, was detectable only in mucoid strains at concentrations that were significantly lower than those of GDP-mannose.

One approach to clarifying the role of GMD in the regulation of alginate biosynthesis is to clone and overexpress *algD* in *P. aeruginosa*. Although this genetic manipulation has been done by some other research groups, no data have been published on how this affects the concentrations of the key metabolites GDP-mannose and GDP-mannuronate. In this paper we report the cloning and overexpression of GMD in *P. aeruginosa* and

Fig. 1. The construction of *P. aeruginosa* clones used in this study. (a) The recombinant plasmid pCC27 was used as the source of a 3.2 kb *Bgl*II/*Cla*I DNA fragment which contained the *P. aeruginosa algD* gene. Ligation of this fragment into pSP70 produced the construct pPT1. (b) the 2.4 kb *Cla*I/*Sma*I fragment from pPT1 was ligated into pSP72 to produce pPT2. (c) pPT3 and pPT4 were produced by insertion of a 2.4 kb *Bam*HI/*Bgl*II fragment from pPT2 into the *Bam*HI site of the expression vector pMMB66EH. Only restriction sites relevant to the subcloning of *algD* are shown (*tac* and *P* refer to the *tac* and *algD* promoters respectively and * refers to non-functional *Bam*HI/*Bgl*II sites).

the subsequent effects on the concentrations of these key nucleotide sugars and alginate production.

METHODS

Bacterial strains. The strains of *Escherichia coli* and *Pseudomonas aeruginosa* used in this study are listed in Table 1. Cultures of transformed *E. coli* strains were grown at 37 °C on L-agar or in L-broth supplemented with either ampicillin (100 µg ml⁻¹) or tetracycline (15 µg ml⁻¹) as appropriate. *P. aeruginosa* trans-conjugants were grown routinely at 37 °C on Pseudomonas Isolation Agar (PIA, Difco) or in L-broth supplemented with 0.25% (w/v) glucose; both solid and liquid media contained 500 µg carbenicillin ml⁻¹. The growth of *E. coli* and *P. aeruginosa* cultures was monitored by turbidity measurements at 600 nm and 650 nm respectively and, where appropriate, total *P. aeruginosa* numbers were estimated by epifluorescence microscopy using acridine orange (Hobbie *et al.*, 1977).

Construction and manipulation of recombinant plasmids. Routine DNA manipulations were performed as described by Sambrook *et al.* (1989). The strategy adopted to facilitate the sub-cloning of *algD* is outlined in Fig. 1. The recombinant plasmid pCC27 contains a 23 kb fragment of *P. aeruginosa* DNA (which includes *algD*) and was used to facilitate the sub-cloning of *algD* into the cloning vectors pSP70 and pSP72, and into the broad host range expression vector pMMB66EH. Plasmids pSP70 and pSP72 were utilized as a means of introducing new restriction sites flanking *algD*, thereby facilitating the final

subcloning of *algD* into pMMB66EH. The presence of the *algD* gene in pMMB66EH was confirmed by the amplification of an internal DNA fragment using the polymerase chain reaction (PCR). The orientation of the cloned fragment with respect to the pMMB66EH *tac* promoter was established by restriction site mapping.

The subsequent constructs pPT3 and pPT4 (forward and reverse clone respectively), and pMMB66EH were transformed into *E. coli* HB101 or S17-1. Recombinant DNA was mobilized into isogenic mucoid and non-mucoid strains of *P. aeruginosa* by filter-mating experiments, and transconjugants of *P. aeruginosa* were selected by growth on M9 minimal medium (Sambrook *et al.*, 1989) containing citrate as the sole carbon source and carbenicillin (both at 500 µg ml⁻¹).

DNA amplification and sequencing. The oligonucleotide primers PA1 and PA3 (McIntosh *et al.*, 1992) were used to amplify a 292 bp internal fragment of *algD* to confirm the presence of this gene in the various recombinant constructs. Amplification was achieved by using a Techne PHC-2 programmable heating block. After an initial denaturation step of 94 °C for 5 min the following thermocycle was performed: 94 °C for 20 s, 60 °C for 45 s and 74 °C for 2 min. The heating rate was 48 °C min⁻¹ and after 30 cycles the heating block was returned to room temperature. The amplified DNA was either digested with appropriate restriction enzymes to confirm the position of internal restriction sites or sequenced directly using the Promega fmol DNA sequencing system using the thermocycle described above.

Overexpression of GDP-mannose dehydrogenase. Transformants of *E. coli* S17-1 and transconjugants of *P. aeruginosa* were grown in the appropriate media at 37 °C. At the mid-exponential growth phase 1 mM IPTG was added and the temperature was reduced to 30 °C. Bacteria were harvested at the appropriate phase and cell-free extracts were prepared according to the procedure of Pugashetti *et al.* (1983). Total cellular protein extracts were produced at the early stationary phases and analysed by SDS-PAGE according to the method of Laemmli (1970) using Gibco BRL pre-stained protein molecular mass standards.

Preparation of nucleotide extracts. Nucleotide extracts were prepared by utilizing a protein precipitation procedure and extraction with ice-cold 0.5 M tri-*n*-octylamine in 1,1,2-trichloro-1,2,2-trifluoroethane (Tatnell *et al.*, 1993). This procedure, which is optimized for the extraction of guanosine-containing compounds (Dutta & O'Donovan, 1987), is quantitative (Woodland & Pestell, 1972) and the extracts may be stored for up to one month at -20 °C without measurable degradation of the GDP-sugars.

Fractionation and quantification of nucleotide-sugars by HPLC. GDP-mannose was separated from other components using reverse-phase ion-pair HPLC (C₁₈ Apex II ODS column, retention time 20 min) whereas GDP-mannuronate was fractionated by anion-exchange HPLC (Zorbax SAX column, retention time 36 min) as described previously (Tatnell *et al.*, 1993). In both separations, the identity of the nucleotide sugars was confirmed by co-chromatography with authentic standards and by separate analysis of the eluted material according to procedures utilized by Tatnell *et al.* (1993). GDP-mannose was identified by TLC on PEI cellulose, the characteristic UV absorption spectrum of guanosine-containing compounds and a positive colour reaction with the phenol/H₂SO₄ test. Similar analytical techniques were used to confirm the identity of GDP-mannuronate and its acid-degradation products, except that the presence of uronic acid was confirmed using the assay of Blumenkrantz & Asboe-Hansen (1973). The nucleotide-sugars were quantified by comparing their peak areas to those of authentic standards of known concentration.

Enzyme assays. GDP-mannose dehydrogenase activity was assayed according to the method of Preiss (1964). Specific enzyme activities were corrected for endogenous dehydrogenase activity (< 80 pmol min⁻¹ mg⁻¹). Protein concentration was determined using the procedure of Bradford (1976).

Isolation and quantification of alginate. Alginate was isolated from liquid cultures and purified using the method of Narbad *et al.* (1988). Alginate was quantified by estimation of the uronic acid content, using the assay of Blumenkrantz & Asboe-Hansen (1973).

Statistical analysis of data. Results are presented as means ± SD. When appropriate, probability values have been calculated using Student's paired *t*-test with statistical significance set at the 90% confidence limit.

RESULTS

Sub-cloning of *algD* into the expression vector pMMB66EH

The strategy used to subclone *algD* is outlined in Fig. 1. The recombinant plasmid pCC27 was used as the source of a 3.2 kb *Bgl*II/*Cla*I fragment which contained the *algD* gene from *P. aeruginosa*. Ligation of this fragment into pSP70 produced the construct pPT1. A 2.4 kb *Cla*I/*Sma*I

fragment from pPT1 was ligated into pSP72 to produce pPT2. Plasmids pPT3 and pPT4 were constructed by insertion of a 2.4 kb *Bam*HI/*Bgl*II fragment from pPT2 into the *Bam*HI site of pMMB66EH. Restriction digests with *Sma*I or *Bam*HI/*Pst*I were used to establish the orientation of the 2.4 kb *Bam*HI/*Bgl*II insert in the plasmids pPT3 and pPT4. Plasmid pPT3 is the forward clone and contains the insert in the correct orientation for the transcription of *algD* from the vector *tac* promoter, whereas in the reverse clone pPT4 the cloned fragment is in the opposite orientation. The presence of the *algD* gene in each vector was confirmed by PCR, restriction mapping of the PCR product, and the sequencing of approximately 200 bases of one strand of the amplified DNA using the oligonucleotide primer PA1.

GMD activity in recombinant *P. aeruginosa*

The GMD activity in transconjugants of isogenic mucoid and non-mucoid strains of *P. aeruginosa* is given in Table 2. Transconjugants containing the plasmid pMMB66EH had specific activities, irrespective of the addition of IPTG, that were similar to those reported previously for the same strains lacking the plasmid (Tatnell *et al.*, 1993). The GMD specific activities in these transconjugants were higher in mucoid compared with isogenic non-mucoid strains, and increased in the former by approximately 2–3-fold as the cultures progressed from mid-exponential to early-stationary phase. The addition of 1 mM IPTG to cultures had no significant effect on the specific activity of GMD.

The introduction of pPT4 into mucoid strains of *P. aeruginosa* did not result in an increased specific activity of GMD. However, non-mucoid transconjugants showed a marked increase in GMD specific activity with values approaching 0.3–0.5-fold those for the corresponding isogenic mucoid organisms. The addition of IPTG did not affect *algD* expression (as measured by GMD activity) in either mucoid or non-mucoid *P. aeruginosa* strains containing pPT4.

Transconjugants containing pPT3 and grown in the absence of IPTG exhibited specific activities that were comparable to those strains containing pPT4. However, the addition of IPTG to the culture medium resulted in a 6–22-fold increase in the specific activity of GMD in isogenic mucoid and non-mucoid strains (Table 2).

Total cellular protein extracts from *E. coli* S17-1 and isogenic mucoid and non-mucoid strains of *P. aeruginosa* were analysed by SDS-PAGE. Those bacteria containing pPT3 and grown in the presence of IPTG produced a visible protein band of approximately 48 kDa (Fig. 2). However, this band was not evident in extracts from bacteria containing either pMMB66EH or pPT4 and grown in the presence of IPTG. The determined molecular mass for the GMD monomer compares favourably with the predicted mass of 47.6 kDa (Deretic *et al.*, 1987b) and to 48 kDa determined by SDS-PAGE (Deretic *et al.*, 1987a). The intensity of the protein band corresponding to the GMD monomer paralleled the detected GMD activity.

Table 2. GMD activity in mucoid and non-mucoid strains of *P. aeruginosa*

The specific activity of GMD was assayed in cell-free extracts of *P. aeruginosa* grown in the presence or absence of 1 mM IPTG. Results, which have been corrected for endogenous activity, are expressed as means $\pm$ SD of a minimum of three separate cultures.

Growth phase*	Specific activity (nmol min ⁻¹ mg ⁻¹)							
	PAO1 (non-mucoid)		PAO568 (mucoid)		FRD2 (non-mucoid)		FRD1 (mucoid)	
	-IPTG	+IPTG	-IPTG	+IPTG	-IPTG	+IPTG	-IPTG	+IPTG
pMMB66EH-transconjugants								
Mid-exponential	0.40 $\pm$ 0.08	0.91 $\pm$ 0.42	9.92 $\pm$ 1.05	12.32 $\pm$ 3.97	0.89 $\pm$ 0.20	0.76 $\pm$ 0.16	9.68 $\pm$ 1.55	6.85 $\pm$ 2.28
Early-stationary	1.06 $\pm$ 0.62	0.56 $\pm$ 0.24	18.86 $\pm$ 5.26	23.22 $\pm$ 4.32	1.30 $\pm$ 0.04	0.94 $\pm$ 0.52	24.18 $\pm$ 6.28	25.74 $\pm$ 5.81
pPT3-transconjugants								
Mid-exponential	7.49 $\pm$ 1.87	125.85 $\pm$ 28.78	18.91 $\pm$ 0.10	162.72 $\pm$ 31.88	13.12 $\pm$ 3.10	136.37 $\pm$ 10.55	14.36 $\pm$ 3.03	175.78 $\pm$ 39.71
Early-stationary	9.36 $\pm$ 0.63	203.73 $\pm$ 69.98	29.94 $\pm$ 6.60	191.87 $\pm$ 2.68	12.83 $\pm$ 3.48	219.57 $\pm$ 55.17	32.08 $\pm$ 6.29	209.68 $\pm$ 32.74
pPT4-transconjugants								
Early-stationary	14.72 $\pm$ 1.35	14.01 $\pm$ 1.47	25.14 $\pm$ 2.74	25.42 $\pm$ 1.39	12.49 $\pm$ 1.13	10.97 $\pm$ 1.95	29.05 $\pm$ 4.27	26.71 $\pm$ 4.59

* Mid-exponential, OD₆₅₀ = 0.40; early-stationary, OD₆₅₀ = 1.30.

Fig. 2. SDS-PAGE of the total protein extract derived from the non-mucoid *P. aeruginosa* strain PAO1 containing the plasmid pPT3 induced with 1 mM IPTG (lane 1) and non-induced (lane 2). The protein band at 48 kDa in lane 1 represents the GMD monomeric subunit. SDS-PAGE gels were run under reducing conditions and stained with Coomassie blue.

Quantification of GDP-mannose and GDP-mannuronate in nucleotide extracts from *P. aeruginosa* transconjugants

GDP-mannose accumulated in all of the *P. aeruginosa* transconjugants irrespective of the phenotype of the host strain or the plasmid, although the concentration de-

creased by between 20% and 80% as cells entered stationary phase (Table 3).

The addition of IPTG to the pMMB66EH-containing strains had no significant effect on the intracellular GDP-mannose concentration irrespective of the phenotype. The mucoid pMMB66EH-containing strains generally contained lower levels of GDP-mannose compared to the equivalent isogenic non-mucoid strain which is in accord with Tatnell *et al.* (1993), although this trend was not always statistically significant.

When grown in the absence of IPTG, pPT3-transconjugants irrespective of strain phenotype generally contained a GDP-mannose concentration that was marginally smaller than that determined in the equivalent pMMB66EH-containing strains, though this difference was not always statistically significant. Indeed, the GDP-mannose concentration exhibited by non-mucoid pPT3-containing strains was more characteristic of that exhibited by the equivalent mucoid strain. Significantly, the addition of IPTG to cultures of pPT3-containing transconjugants caused a 30–50% reduction in GDP-mannose concentration relative to that of the corresponding uninduced cultures (Table 3).

GDP-mannuronate was detectable (irrespective of the addition of IPTG) only in mucoid *P. aeruginosa* pMMB66EH-containing transconjugants, albeit at low but relatively constant levels irrespective of growth phase (Table 4). The lower limit of detection of GDP-mannuronate was 0.1 nmol (ml extract)⁻¹, which is approximately fivefold less than the lowest amount of GDP-mannuronate detected.

Relatively high concentrations of GDP-mannuronate occurred in all pPT3-transconjugants irrespective of strain phenotype or addition of IPTG. However, the nucleotide-sugar concentration decreased as cultures entered the stationary phase. The GDP-mannuronate intracellular concentration in the mucoid pPT3-containing strains was 7–104-fold greater than in the equivalent mucoid

Table 3. GDP-mannose concentrations in mucoid and non-mucoid strains of *P. aeruginosa*

GDP-mannose concentrations were determined in nucleotide extracts obtained from cultures of *P. aeruginosa* harvested at various stages of growth. Results, which are expressed in nmol GDP-mannose per 10^{12} cells, are the means $\pm$ SD of a minimum of three separate cultures. Extracts were analysed from cultures grown in the presence or absence of 1 mM IPTG.

Growth phase*	PAO1 (non-mucoid)		PAO568 (mucoid)		FRD2 (non-mucoid)		FRD1 (mucoid)	
	-IPTG	+IPTG	-IPTG	+IPTG	-IPTG	+IPTG	-IPTG	+IPTG
pMMB66EH-transconjugants								
Mid-exponential	33.62 $\pm$ 5.26	32.90 $\pm$ 4.46	27.03 $\pm$ 2.71	25.89 $\pm$ 5.18	30.85 $\pm$ 5.32	31.31 $\pm$ 4.34	26.04 $\pm$ 5.60	23.45 $\pm$ 1.72
Early-stationary	22.91 $\pm$ 2.10	20.00 $\pm$ 3.91	17.34 $\pm$ 4.13	20.70 $\pm$ 2.84	14.13 $\pm$ 0.92	12.26 $\pm$ 2.16	9.48 $\pm$ 0.68	7.58 $\pm$ 0.68
pPT3-transconjugants								
Mid-exponential	27.42 $\pm$ 1.38	19.27 $\pm$ 1.32	27.03 $\pm$ 7.17	17.88 $\pm$ 0.52	27.08 $\pm$ 3.08	16.40 $\pm$ 2.99	23.50 $\pm$ 2.13	15.64 $\pm$ 1.78
Early-stationary	15.69 $\pm$ 2.92	9.31 $\pm$ 2.30	13.38 $\pm$ 0.94	8.97 $\pm$ 1.54	9.28 $\pm$ 1.31	3.52 $\pm$ 0.83	7.36 $\pm$ 0.54	3.67 $\pm$ 0.67

* Mid-exponential, OD₆₅₀ = 0.40; early-stationary, OD₆₅₀ = 1.30.

Table 4. GDP-mannuronate concentrations in mucoid and non-mucoid strains of *P. aeruginosa*

GDP-mannuronate concentrations were determined in nucleotide extracts obtained from cultures of *P. aeruginosa* harvested at various stages of growth. Results, which are expressed in nmol GDP-mannuronate per 10^{12} cells, are the means $\pm$ SD of a minimum of three separate cultures. Extracts were analysed from cultures grown in the presence or absence of 1 mM IPTG.

Growth phase*	PAO1 (non-mucoid)		PAO568 (mucoid)		FRD2 (non-mucoid)		FRD1 (mucoid)	
	-IPTG	+IPTG	-IPTG	+IPTG	-IPTG	+IPTG	-IPTG	+IPTG
pMMB66EH-transconjugants								
Mid-exponential	ND	ND	1.90 $\pm$ 0.55	1.84 $\pm$ 0.40	ND	ND	1.72 $\pm$ 0.40	1.41 $\pm$ 0.19
Early-stationary	ND	ND	1.29 $\pm$ 0.18	0.97 $\pm$ 0.13	ND	ND	1.20 $\pm$ 0.20	1.87 $\pm$ 0.17
pPT3-transconjugants								
Mid-exponential	107.07 $\pm$ 10.63	264.53 $\pm$ 43.00	76.03 $\pm$ 8.10	191.24 $\pm$ 27.77	86.64 $\pm$ 11.55	278.10 $\pm$ 29.62	54.42 $\pm$ 10.19	193.41 $\pm$ 13.93
Early-stationary	21.50 $\pm$ 3.05	121.89 $\pm$ 14.67	20.36 $\pm$ 4.08	75.12 $\pm$ 3.97	8.58 $\pm$ 0.67	110.92 $\pm$ 15.74	7.95 $\pm$ 1.48	75.99 $\pm$ 7.32

ND, Not detected.

* Mid-exponential, OD₆₅₀ = 0.40; early-stationary, OD₆₅₀ = 1.30.

Table 5. Quantification of extracellular alginate

The alginate concentration in the extracellular material, which was derived from cultures of *P. aeruginosa* grown in the presence and absence of 1 mM IPTG, was determined as described in Methods. Results are expressed as means $\pm$ SD of a minimum of three separate cultures.

Growth phase*	Alginate produced (mg per 10^{12} cells)							
	PAO1 (non-mucoid)		PAO568 (mucoid)		FRD2 (non-mucoid)		FRD1 (mucoid)	
	-IPTG	+IPTG	-IPTG	+IPTG	-IPTG	+IPTG	-IPTG	+IPTG
pMMB66EH-transconjugants								
Mid-exponential	1.90 $\pm$ 0.18	2.70 $\pm$ 0.59	5.80 $\pm$ 1.29	7.30 $\pm$ 0.71	2.89 $\pm$ 0.21	2.70 $\pm$ 1.04	2.90 $\pm$ 0.78	3.66 $\pm$ 0.50
Early-stationary	0.66 $\pm$ 0.31	0.81 $\pm$ 0.22	26.99 $\pm$ 8.11	27.06 $\pm$ 3.77	1.69 $\pm$ 0.50	1.09 $\pm$ 1.17	30.48 $\pm$ 5.69	36.28 $\pm$ 2.04
pPT3-transconjugants								
Mid-exponential	1.76 $\pm$ 0.30	3.33 $\pm$ 0.88	10.57 $\pm$ 1.01	13.26 $\pm$ 1.05	2.80 $\pm$ 0.44	3.09 $\pm$ 0.33	11.64 $\pm$ 1.30	13.45 $\pm$ 1.59
Early-stationary	2.07 $\pm$ 0.53	1.04 $\pm$ 0.32	34.42 $\pm$ 2.72	45.66 $\pm$ 6.93	1.92 $\pm$ 0.23	1.31 $\pm$ 0.33	38.89 $\pm$ 7.16	46.46 $\pm$ 3.24

* Mid-exponential, OD₆₅₀ = 0.40; early-stationary, OD₆₅₀ = 1.30.

pMMB66EH-containing strains. The non-induced GDP-mannuronate concentration in pPT3-containing non-mucoid strains was generally in excess of that exhibited by the equivalent non-induced isogenic mucoid strains.

Induction of the cloned *algD* gene with IPTG resulted in a 2.5–3.5-fold increase in the intracellular GDP-mannuronate concentration at mid-exponential growth phase with a more marked effect (4–13-fold increase) by

early-stationary phase. The concentration of GDP-mannuronate in IPTG-induced pPT3-transconjugants was always greater in the non-mucoid strain compared with the equivalent isogenic mucoid strain (Table 4).

Production of extracellular alginate

The amounts of alginate produced by the non-mucoid strains containing either pMMB66EH or pPT3 were small compared to mucoid strains and were unaffected by the induction of the cloned *algD* gene with IPTG (Table 5). Alginate production in mucoid pMMB66EH-containing strains was independent of IPTG addition and increased by 4–10-fold as the cultures progressed from mid-exponential to early-stationary phase. The alginate concentration for the pMMB66EH-transconjugants (irrespective of phenotype) and for non-mucoid pPT3-transconjugants were comparable to those reported previously for the same strains lacking either plasmid (Tatnell *et al.*, 1993).

Mucoid strains transconjugated with pPT3 and grown in the absence of IPTG generally produced greater quantities of alginate compared to equivalent pMMB66EH-containing strains. The IPTG-mediated induction of the cloned *algD* gene within mucoid pPT3-transconjugants resulted in a modest but significant increase (approximately 23%) in alginate production at both mid-exponential and early-stationary phases of growth (Table 5).

DISCUSSION

Genetic studies have established that GMD is the key regulatory enzyme and committal step in the biosynthesis of alginate in *P. aeruginosa*, with transcriptional activation of the *algD* promoter occurring only in mucoid strains (Deretic *et al.*, 1987a). Complementary metabolic studies in which the concentrations of GDP-mannose and GDP-mannuronate have been measured in wild-type mucoid and non-mucoid isogenic pairs of strains have demonstrated that GMD is the major kinetic control point in alginate biosynthesis and regulates exopolysaccharide production (Tatnell *et al.*, 1993).

Several groups have over-expressed the *algD* gene in *P. aeruginosa* under the control of the *tac* promoter. Deretic *et al.* (1987a) subcloned the *P. aeruginosa* 3.2 kb *Bgl*II/*Cla*I *algD*-containing fragment (equivalent to that subcloned to form pPT1) into the *tac*-promoter-controlled expression vector pMMB24 so as to produce the forward and reverse constructs, pVD211 and pVD210 respectively. Reported IPTG-induced GMD activities from pVD211-containing strains ranged from approximately 25 to 100 nmol min⁻¹ mg⁻¹ (Deretic *et al.*, 1987a; Roychoudhury *et al.*, 1989; Martins & Sa'-Correia, 1991), values which are approximately 50% lower than those obtained in the present work. This difference is unlikely to be a gene-dosage effect as the broad-host-range expression vectors used in both laboratories are based on the same RSF1010 replicon (Bagdasarian *et al.*, 1983; Furste *et al.*, 1986) which is maintained at approximately 13 copies per

cell (Morales *et al.*, 1990). However, in the present study both the forward and reverse clones (pPT3 and pPT4 respectively) differed from those of Deretic *et al.* (1987a) in that they lacked approximately 800 bp of DNA sequence immediately downstream of *algD*. Deletion of this DNA appears to have increased the efficiency of expression as judged by our experiments.

Some expression of *algD* was observed in mucoid and non-mucoid strains of *P. aeruginosa* containing pPT3 or pPT4, even in the absence of IPTG, whereas this was not the case with transformants of *E. coli* (data not shown); similar results have been reported elsewhere (Roychoudhury *et al.*, 1989; Deretic *et al.*, 1987a; Martins & Sa'-Correia, 1991). It is unlikely that basal expression of plasmid-encoded *algD* in *P. aeruginosa* could have resulted from incomplete repression of the vector *tac* promoter (Bagdasarian *et al.*, 1983) as the vector pMMB66EH is apparently well regulated in Pseudomonads (Furste *et al.*, 1986). Also, the residual expression in *P. aeruginosa* (but not *E. coli*) of GMD from pPT4, in which *algD* is in the opposite orientation to the vector *tac* promoter, indicates that some form of vector-independent expression is occurring in this construct. The numerous inverted-repeat sequences and the presence of a putative ribosomal binding site (Deretic *et al.*, 1987b) in the 367 bp of DNA upstream of *algD*, which is present in all of the constructs (pVD211, pPT3 and pPT4), has a positive effect on transcription and is the likely cause of residual expression of GMD.

The effect of over-expression of *algD* in *P. aeruginosa* mucoid strains containing pPT3 is to cause a small but significant increase in alginate production (see Tables 2 and 5), whereas in non-mucoid strains the amounts of uronic-acid-positive material were close to the lower limits of detection irrespective of the addition of IPTG. This uronic-acid-containing material from non-mucoid strains is most likely to be LPS, since ¹H-NMR spectra (results not shown) did not indicate peaks characteristic of alginate. Several *P. aeruginosa* O-specific polysaccharide serogroups contain uronic acid moieties (Knieral *et al.*, 1988) including L-gulonate and D-mannuronate (Kelly *et al.*, 1990).

The production of alginate was proportional to GMD activity in non-induced cultures, which is consistent with data for non-recombinant *P. aeruginosa* (Tatnell *et al.*, 1993). Although induction of mucoid cultures containing pPT3 led to a large increase in GMD activity, alginate production increased only marginally compared to non-induced levels. In contrast, Martins & Sa'-Correia (1991) found that addition of IPTG to pVD211-containing mucoid *P. aeruginosa* strains reduced levels of alginate production compared to non-induced levels. These workers proposed that the IPTG-induction of GMD interfered with the biosynthesis of outer membrane components and thereby affected the export of alginate from the bacterial cell. However, our data do not support this theory.

Quantitative analysis of the data from Tables 2 and 5 shows that the flux control coefficient for the GMD-

catalysed step of the pathway approaches 1.0 for strains expressing normal amounts of GMD. The sum of the coefficients for each step in the pathway should equal 1.0, therefore such a high value for the flux control coefficient for GMD demonstrates that the biosynthesis of alginate is effectively controlled by the rate of conversion of GDP-mannose to GDP-mannuronate under these conditions. Over-expression of GMD results in a large reduction in the flux control coefficient to values of approximately 0.19 for mucoid strains and approximately 0.03 for non-mucoid strains.

IPTG induction of GMD resulted in relatively small changes in the pool sizes of GDP-mannose compared to GDP-mannuronate (see Tables 3 and 4). This is because certain nucleotide-sugars, e.g. GDP-mannose, can inhibit biosynthetic enzymes such as GDP-mannose pyrophosphorylase (GDMPP), thus effectively self-limiting metabolite pool sizes (Kornfeld & Ginsburg, 1966). Tight regulation of the GDP-mannose intracellular pool size probably ensures that this important metabolite is available for LPS biosynthesis and other essential pathways. Lightfoot & Lam (1993) have demonstrated that conversion of GDP-mannose into GDP-rhamnose is essential for A-band biosynthesis. Therefore, even though LPS biosynthesis is apparently down-regulated in mucoid strains (Lam *et al.*, 1989) the metabolic demand for GDP-mannose remains high.

Accumulation of GDP-mannuronate in both induced and non-induced pPT3-transconjugated mucoid strains of *P. aeruginosa* indicates that the later enzymes of alginate biosynthesis are unable to utilize the increased quantity of this nucleotide sugar. The alginate biosynthetic gene cluster is strictly and co-ordinately regulated as a single operon (Chitnis & Ohman, 1993), so elevated expression *in trans* of GMD activity causing greatly increased GDP-mannuronate concentrations results in only a marginal increase in alginate production. Speculation as to the rate-limiting step in alginate biosynthesis in these recombinants must centre on the polymerase or some aspect of the export mechanism. It is unlikely that the polymer-level epimerase or the O-acetyltransferase (encoded by *algG* and *algF* respectively) are rate limiting, because both these genes affect alginate structure and not alginate production levels (Chitnis & Ohman, 1990; Franklin & Ohman, 1993).

In conclusion, our studies have shown that over-expression of *algD* causes significant changes in the pool sizes of GDP-mannose and GDP-mannuronate. This has only a minimal effect on alginate production as other enzymes in the pathway become rate-limiting. It is clear that in wild-type *P. aeruginosa* the expression of GMD is tightly regulated and that the enzyme is the rate-determining and committal step for alginate biosynthesis.

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