A metabolic study of the activity of GDP-mannose dehydrogenase and concentrations of activated intermediates of alginate biosynthesis in *Pseudomonas aeruginosa*

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GDP-mannose dehydrogenase (GMD) is a key regulatory enzyme and the committal step in alginate biosynthesis. In this study, a metabolic approach has been used to investigate GMD activity in non-mucoid and isogenically related mucoid strains of *Pseudomonas aeruginosa*. Intracellular concentrations of GDP-mannose and GDP-mannuronate have been quantified using HPLC separation methods, and their concentrations have been related to GMD activity and total alginate production. In all strains of *P. aeruginosa* tested, GDP-mannose accumulated particularly during the exponential phase of growth in batch culture; the GDP-mannose concentrations in mucoid strains were significantly lower compared with isogenic non-mucoid strains. The product of GMD activity, GDP-mannuronate, was detectable only in mucoid strains, albeit at low but relatively constant levels irrespective of growth phase. The GDP-mannose concentrations in mucoid strains were always significantly greater than those of GDP-mannuronate, indicating that GMD is a rate-limiting enzyme in the biosynthesis of alginate. Significant GMD activity and extracellular alginate production were detected only in mucoid strains. The metabolic data reported here, together with previous genetic studies, provide strong evidence that GMD is the key regulatory enzyme controlling alginate biosynthesis in mucoid strains of *P. aeruginosa*.

Introduction

The sputum isolated from cystic fibrosis (CF) patients frequently contains mucoid strains of *Pseudomonas aeruginosa*. These strains secrete copious quantities of alginate (Govan & Harris, 1986), a viscous exopolysaccharide which plays an important role in the pathogenicity of this organism (Russell & Gacesa, 1988). During the past 20 years, *P. aeruginosa* has emerged as the major cause of morbidity and mortality amongst CF patients (Govan, 1988).

Bacterial alginate is a 1→4-linked linear heteropolymer consisting of β -D-mannuronate and its C5-epimer α -L-guluronate (Evans & Linker, 1973), in which the mannuronate residues can be mono- or di-O-acetylated (Sherbrock-Cox *et al.*, 1984). The alginate biosynthetic pathway has been studied in *Fucus gardneri* (Lin &

Hassid, 1966) and *Azotobacter vinelandii* (Pindar & Bucke, 1975), and a similar pathway has been proposed for *P. aeruginosa* (Piggott *et al.*, 1981). The complete elucidation of the pathway in *P. aeruginosa* has proved difficult, partially due to the low enzyme activities detected in even the most overtly mucoid strains (Narbad *et al.*, 1990). Recent molecular genetic approaches have greatly improved our understanding of both the biosynthesis of alginate and its regulation in response to environmental stimuli (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 biosynthetic enzymes at 34 min (Darzins *et al.*, 1985); a regulatory cluster at 8 min (Deretic *et al.*, 1989; Deretic & Konyecsni, 1989; Goldberg & Ohman, 1987; Konyecsni & Deretic, 1990a; Mohr *et al.*, 1991); and the *muc* or switching region at 68 min (Fyfe & Govan, 1980; Flynn & Ohman, 1988). The *algD* gene is present in the biosynthetic cluster at 34 min (Darzins *et al.*, 1985) and encodes the enzyme GDP-mannose dehydrogenase (GMD). This enzyme catalyses the essentially irreversible NAD⁺-dependent oxidation of GDP-mannose to GDP-

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Abbreviations: CF, cystic fibrosis; GMD, GDP-mannose dehydrogenase; PEI-cellulose; polyethyleneimine-cellulose; RP/IP, reverse-phase/ion-pairing; SAX, strong anion exchange.

mannuronate. Genetic studies have demonstrated that alginate production depends on transcriptional activation of the *algD* gene in mucoid strains, which occurs in response to environmental stimuli and leads to increased amounts of the enzyme being present (Deretic *et al.*, 1987a, b; 1990; Berry *et al.*, 1989). Several of the regulatory gene products are involved in the control of this environmentally responsive system (Konyecsni & Deretic, 1990a, b; Deretic & Konyecsni, 1989; Wozniak & Ohman, 1991; Kato & Chakrabarty, 1991).

The *algD* gene has been sequenced (Deretic *et al.*, 1987a) and the GMD enzyme purified and characterized. The active form exists as a hexamer with a subunit molecular mass of 48 kDa (Roychoudhury *et al.*, 1989), in common with a number of other four-electron transfer dehydrogenases (Feingold & Franzen, 1981). This oligomeric nature is characteristic of allosteric enzymes which are important in regulating the flow of metabolites through particular pathways (Fersht, 1985).

On the basis of such molecular genetic studies, it has been assumed that GMD is the key regulatory enzyme of alginate biosynthesis, and is the step whereby precursors are committed to the pathway (Deretic *et al.*, 1987b). In this paper we describe a metabolic study of the enzyme in isogenic mucoid and non-mucoid strains of *P. aeruginosa*. We demonstrate, for the first time, that the activity of GMD is regulated in a manner consistent with amounts of extracellular alginate production.

Methods

Bacterial strains. The strains of *Pseudomonas aeruginosa* used in this study are listed in Table 1. The organism was grown routinely at 37 °C on Pseudomonas Isolation Agar (PIA, Difco) or in L-broth supplemented with 0.25% glucose. Medium (1 litre) was inoculated with 5 ml of overnight culture. The conditions for growth of *Arthrobacter viscosus* were as described by Preiss & Wood (1964). The growth of cultures was monitored by turbidity measurements at 650 nm, and total bacterial numbers were estimated by epifluorescence microscopy using acridine orange (Hobbie *et al.*, 1977).

Preparation of nucleotide extracts. Cultures were harvested by centrifugation at 9600 g_{av} for 10 min at room temperature, washed with 15 mM-potassium phosphate buffer, pH 7.2, and resuspended in the same buffer. The nucleotide extract was prepared by utilizing a protein precipitation procedure in which the bacteria were homogenized in ice-cold 0.5 M-trifluoroacetic acid; the cell debris was removed by sedimentation in a microfuge at 12000 r.p.m. for 5 min at 4 °C. The supernatant was neutralized with freshly made, ice-cold 0.5 M-tri-*n*-octylamine in 1,1,2-trichloro-1,2,2-trifluoroethane. The upper nucleotide-containing aqueous layer was removed and stored at -20 °C (Dutta & O'Donovan, 1987). The extraction, which is optimized for the extraction of guanine-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 of nucleotide extract by HPLC. GDP-mannose was separated from other components by ion-pair HPLC fractionation on

a C18 Apex II ODS column (Jones Chromatography Ltd, Hengoed, Wales) having a particle-size of 5 µm and column dimensions of 250 mm × 4.6 mm (i.d.) using 100 mM-KH₂PO₄ buffer, pH 5.7, supplemented with 5 mM-tetrabutylammonium phosphate as the eluant at a flow rate of 1.5 ml min⁻¹.

GDP-mannuronate was separated from other components by anion-exchange HPLC fractionation on a Zorbax SAX column (Jones Chromatography) having a particle size of 5 µm and column dimension of 250 mm × 4.6 mm (i.d.). The column was eluted at a flow rate of 1 ml min⁻¹ with a buffer gradient system consisting of eluant A [15 mM-KH₂PO₄, pH 4.5, 10% (v/v) methanol] and eluant B [400 mM-KH₂PO₄, pH 4.1, 650 mM-KCl, 10% (v/v) methanol]; the percentage of eluant B in the buffer system was increased linearly from 0% to 25% during the initial 5 min, to 28% at 35 min, 100% at 40 min and this final percentage of eluant B maintained for 5 min. The column was re-equilibrated with 100% eluant A before application of the next sample.

For each type of HPLC separation, the nucleotide extracts (100 µl) were filtered through a 0.45 µm ACRO LC13 filter (Gelman Sciences) just prior to injection. The nucleotides were detected by monitoring the absorbance of the column effluent at 254 nm using a Cecil CE212 spectrophotometer equipped with a 1 cm pathlength flow-through cell. The nucleotide-sugars were quantified by comparing their peak areas to those of known standards.

Acid phosphatase treatment of nucleotide extracts. Acid phosphatase (5 mg, Sigma) was added to each nucleotide extract (1 ml) and incubated for 5 h at 25 °C to remove terminal phosphate groups (phosphodiester linkages are unaffected by this treatment). The enzyme was removed by precipitation with trifluoroacetic acid (see above), and the extract re-analysed by HPLC using the same conditions as described above.

Enzyme assays. Cell-free extracts were prepared according to the procedure of Pugashetti *et al.* (1983) and GMD activity 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 of alginate. Alginate was isolated from liquid cultures and purified using the method of Narbad *et al.* (1988).

Thin-layer chromatography. A modification of the TLC system of Randerath & Randerath (1964), with PEI-cellulose containing fluorescent indicator as the adsorbent, was used to identify GDP-mannose and GDP-mannuronate. The nucleotide sugars were analysed directly, or in some instances, after acid hydrolysis (0.01 M-HCl for 15 min at 100 °C). Chromatograms were developed using the following procedure. Plates were placed in 0.2 M-LiCl for 1 min, transferred without drying to 1.0 M-LiCl and after 3.5 min transferred to 1.6 M-LiCl. The nucleotides and nucleotide sugars were visualized using UV light (254 nm). The sugar components derived from the acid-hydrolysed samples were visualized after spraying the chromatograms with alkaline silver nitrate reagent (Trevelyan *et al.*, 1950). The relative mobilities of samples were compared to those of appropriate standards. The mannuronate standard was prepared by treating commercially available mannurono-6,3-lactone with 1% (v/v) triethylamine (Miyamoto & Nagase, 1981).

Isolation of GDP-mannuronate from *Arthrobacter viscosus*. The nucleotide sugar GDP-mannuronate is not commercially available and therefore it was prepared by oxidation of GDP-mannose using GMD isolated from *Arthrobacter viscosus* according to the procedure of Preiss (1966). Separation of authentic GDP-mannuronate from assay components such as NAD⁺ and GDP-mannose was achieved using the Zorbax SAX HPLC system described above. The GDP-mannuronate peak fractions were collected and pooled; the solution was desalted by

Table 1. *Bacterial strains*

Strain	Genotype/phenotype	Reference
<i>P. aeruginosa</i>		
PAO 1	Prototrophic, Alg ⁻ (non-mucoid)	Holloway <i>et al.</i> (1979)
PAO 568	FP2 ⁺ Alg ⁺ <i>muc-2 leu-38</i> (mucoid)	Fyfe & Govan (1980)
FRD 1	Prototrophic, Alg ⁺ , CF isolate (mucoid)	Ohman & Chakrabarty (1981)
FRD 2	Spontaneous revertant of FRD 1, Alg ⁻ <i>algS</i> (off) (non-mucoid)	Ohman & Chakrabarty (1982)
<i>A. viscosus</i>		
NCIMB 9729	Wild-type strain equivalent to NRRL B1973 (ATCC 19584)	Preiss (1964)

adsorption on activated charcoal (NoritA) and eluted with 50% (v/v) aqueous ethanol containing 1% (v/v) NH₃. The GDP-mannuronate solution was lyophilized and re-dissolved in a minimum volume of distilled H₂O.

The chemical identity of the prepared standard of GDP-mannuronate was confirmed using several independent techniques. First, the concentrations of substrates and products of the enzymic reaction were estimated at various time intervals to ascertain the stoichiometry of the reaction. This involved anion-exchange HPLC fractionation of the intermediates, as described above. From a quantification of the time-dependent change in concentrations of the reactants it was determined that 2 moles of NAD⁺ were reduced stoichiometrically to 2 moles of NADH + H⁺ while 1 mole of GDP-mannose was converted to 1 mole of a more negatively-charged compound which was presumed to be GDP-mannuronate. Second, the presence of uronic acid in the putative GDP-mannuronate was confirmed using the assay of Blumenkrantz & Asboe-Hansen (1973). The nucleotide component was also confirmed as GDP by its characteristic UV absorption spectra at pH values of 1, 7 and 12, which were compared with those of the GDP in authentic GDP-mannose. Third, the component parts of the nucleotide sugar, i.e. GDP and mannuronate, were identified by thin-layer chromatography of the sample following acid hydrolysis (see above) in which they co-migrated with authentic GDP and mannuronate standards.

Statistical analysis of data. Results are presented as means $\pm$ standard deviations where *n* equals the number of experimental determinations. When appropriate, probability values have been calculated using Student's paired *t*-test with statistical significance set at the 90% confidence limit.

Results

Detection of GDP-mannose and GDP-mannuronate in nucleotide extracts of P. aeruginosa

A variety of HPLC separation systems utilizing a wide range of buffer concentrations and pH gradients were tested for their ability to separate the components of nucleotide extracts from *P. aeruginosa*, in particular to facilitate the quantification of GDP-mannose and GDP-mannuronate. No one system proved capable of giving good enough separations to quantify both components on a single chromatogram. Instead, two chromatographic systems having different bases for the separation were used, namely a reverse-phase (RP) column in conjunction with the ion-pairing (IP) agent tetrabutylammonium phosphate for separation and quantification

of GDP-mannose and a strong-anion-exchange (SAX) system for GDP-mannuronate. These gave good baseline separations of the key nucleotide sugars (Figs 1 and 2), although there was some peak broadening of GDP-mannuronate. A comparison of *P. aeruginosa* strains showed that GDP-mannuronate could be detected only in mucoid strains.

The retention times of GDP-mannose and GDP-mannuronate corresponded with those of authentic standards and were confirmed by co-chromatography. In the RP/IP system GDP-mannose eluted from the column at approximately 20 min, while in the SAX system GDP-mannuronate eluted at approximately 36 min. These times varied marginally depending on the overall nucleotide composition of the extract, which changed with the growth phase at which cultures were harvested. However, the addition of small quantities of standards to the extracts confirmed the identity of the peaks. Pre-treatment of nucleotide extracts with acid phosphatase also altered their overall composition, which in turn changed marginally the retention times of GDP-mannose and GDP-mannuronate in the extracts and of exogenously added authentic standards.

Identification of GDP-mannose and GDP-mannuronate in nucleotide extracts of P. aeruginosa

Once the retention times for each nucleotide-sugar had been determined, peaks were collected and pooled for further structural identification. The identity of GDP-mannose was confirmed firstly by its UV absorption spectrum, which was identical to that of a standard solution of GDP-mannose in the same RP/IP buffer system as used for the HPLC separation, and secondly by the fact that it co-migrated with an authentic GDP-mannose standard (dissolved in either water or the RP/IP buffer system) after thin-layer chromatography on PEI-cellulose. The presence of the hexose moiety, i.e. mannose, was confirmed by a positive colour reaction in the phenol/H₂SO₄ test (Dubois *et al.*, 1956).

GDP-mannuronate was identified on the basis of the presence of a uronic acid moiety as determined by the

Fig. 1. Fractionation of a *P. aeruginosa* FRD1 nucleotide extract using reverse-phase ion-pair HPLC. The nucleotide extract was obtained from cultures harvested at the late exponential phase of growth and analysed directly without prior alkaline phosphatase treatment. The peak corresponding to GDP-mannose is indicated by an arrow.

Fig. 2. Fractionation of a *P. aeruginosa* FRD1 nucleotide extract using anion-exchange HPLC. The lower trace represents nucleotide extract obtained from cultures harvested at the late exponential phase of growth and analysed directly without prior alkaline phosphatase treatment. The upper trace represents the same sample containing added GDP-mannuronate (0.2 µg). For the sake of clarity, only the region of the trace around the GDP-mannuronate peak is shown. The peak corresponding to GDP-mannuronate is indicated by an arrow.

Blumenkrantz and Asboe-Hansen assay (see Methods) and its UV absorption spectrum compared with other guanosine-containing compounds, including authentic GDP-mannuronate prepared from GDP-mannose using the isolated GMD from *A. viscosus*. The standards were dissolved in the effluent collected from the HPLC column at a retention time corresponding to that of GDP-mannuronate; this column effluent was used as the blank. Further confirmation of identity was provided by thin-layer chromatography on PEI-cellulose, when the

GDP-mannuronate from *P. aeruginosa* co-migrated with the prepared standard sample of GDP-mannuronate.

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

Quantification of both nucleotide sugars was achieved by comparison of peak areas with standards of known concentrations (see Tables 2 and 3). Treatment of the nucleotide extracts with acid phosphatase reduced the

Table 2. *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 terms of nmol GDP-mannose per 10^{12} cells, are the means $\pm$ SD of a minimum of three observations. Extracts were analysed either directly (untreated) or following incubation with acid phosphatase (AP-treated).

Growth phase*	PAO 1 (non-mucoid)		PAO 568 (mucoid)		FRD 2 (non-mucoid)		FRD 1 (mucoid)	
	Untreated	AP-treated	Untreated	AP-treated	Untreated	AP-treated	Untreated	AP-treated
Early exponential	33.40 $\pm$ 1.19	30.44 $\pm$ 3.47	30.73 $\pm$ 1.39	23.05 $\pm$ 1.90	33.47 $\pm$ 1.37	32.14 $\pm$ 1.57	27.08 $\pm$ 2.44	25.07 $\pm$ 1.54
Mid-exponential	35.53 $\pm$ 4.63	27.51 $\pm$ 1.24	24.99 $\pm$ 1.08	22.70 $\pm$ 1.75	31.11 $\pm$ 0.54	28.66 $\pm$ 3.04	21.17 $\pm$ 2.83	22.14 $\pm$ 2.35
Late exponential	32.02 $\pm$ 3.66	30.03 $\pm$ 2.32	23.24 $\pm$ 0.93	21.55 $\pm$ 1.40	30.80 $\pm$ 1.92	28.86 $\pm$ 1.63	19.24 $\pm$ 1.68	18.62 $\pm$ 1.48
Early stationary	17.13 $\pm$ 0.88	16.38 $\pm$ 0.50	12.87 $\pm$ 1.41	12.85 $\pm$ 1.52	9.13 $\pm$ 0.38	8.00 $\pm$ 0.69	6.24 $\pm$ 0.34	6.29 $\pm$ 0.23
Mid-stationary	6.96 $\pm$ 0.19	6.16 $\pm$ 0.19	6.09 $\pm$ 0.36	5.53 $\pm$ 0.41	3.33 $\pm$ 0.26	3.54 $\pm$ 0.10	2.81 $\pm$ 0.22	3.01 $\pm$ 0.18
Late stationary	2.58 $\pm$ 0.12	2.79 $\pm$ 0.23	2.25 $\pm$ 0.20	1.99 $\pm$ 0.29	2.00 $\pm$ 0.35	1.76 $\pm$ 0.22	0.84 $\pm$ 0.11	1.19 $\pm$ 0.06

* Early exponential $OD_{650} = 0.10$; mid-exponential, $OD_{650} = 0.35$; late exponential $OD_{650} = 0.60$; early stationary, $OD_{650} = 1.30$; mid-stationary, $OD_{650} = 1.50$; late stationary, $OD_{650} = 1.70$.

Table 3. *GDP-mannuronate concentration in 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 terms of nmol GDP-mannuronate per 10^{12} cells, are the means $\pm$ SD of a minimum of three observations. Extracts were analysed either directly (untreated) or following incubation with acid phosphatase (AP-treated). ND, not detected.

Growth phase	PAO 568 (mucoid)		FRD 1 (mucoid)	
	Untreated	AP-treated	Untreated	AP-treated
Early exponential	ND	ND	ND	ND
Mid-exponential	0.88 $\pm$ 0.30	0.78 $\pm$ 0.23	1.25 $\pm$ 0.27	0.93 $\pm$ 0.48
Late exponential	1.37 $\pm$ 0.66	0.48 $\pm$ 0.14	1.02 $\pm$ 0.28	1.03 $\pm$ 0.12
Early stationary	0.72 $\pm$ 0.12	0.59 $\pm$ 0.16	0.96 $\pm$ 0.16	0.93 $\pm$ 0.16
Mid-stationary	0.48 $\pm$ 0.23	0.68 $\pm$ 0.10	0.63 $\pm$ 0.26	0.41 $\pm$ 0.09
Late stationary	1.25 $\pm$ 0.23	0.56 $\pm$ 0.19	0.85 $\pm$ 0.09	0.75 $\pm$ 0.23

Table 4. *GMD activity in mucoid strains of P. aeruginosa*

The specific activity of GMD was assayed in cell-free extracts of *P. aeruginosa* harvested at various stages of growth. Results, which have been corrected for endogenous activity, are expressed as means $\pm$ SD (number of estimates = 6). The specific activity of non-mucoid strains was < 0.1 nmol min⁻¹ mg⁻¹.

Growth phase	Specific activity (nmol min ⁻¹ mg ⁻¹)	
	PAO 568	FRD 1
Early exponential	7.0 $\pm$ 1.9	4.3 $\pm$ 0.3
Mid-exponential	7.0 $\pm$ 2.9	6.8 $\pm$ 1.4
Late exponential	11.5 $\pm$ 1.3	8.6 $\pm$ 1.4
Early stationary	15.2 $\pm$ 0.3	21.3 $\pm$ 2.6
Mid-stationary	16.1 $\pm$ 1.3	28.9 $\pm$ 0.9
Late stationary	15.1 $\pm$ 0.6	22.7 $\pm$ 4.8

total number of peaks in the chromatogram but did not alter significantly the quantitative estimation of GDP-mannose. The lower limit of detection of GDP-mannose using the RP/IP HPLC system was 50 pmol per ml of extract chromatographed. This limiting value is 20-fold less than the lowest amount of GDP-mannose detected in any of the samples from mucoid or non-mucoid strains.

The concentrations of GDP-mannose in nucleotide extracts of PAO and FRD strains of *P. aeruginosa* depended on the time of harvesting during batch culture: the amounts did not alter during the exponential phase of growth but decreased 10–20-fold during the stationary phase (Table 2). There was a small, but statistically significant ($P < 0.12$), difference between the amounts of GDP-mannose in pairs of mucoid/non-mucoid isogenic strains except at late stationary phase.

GDP-mannuronate could be detected only in the mucoid strains PAO 568 and FRD 1 and there was no statistically significant variation ($P > 0.20$) in the concentration of this metabolite during growth in batch culture (Table 3). The lower limit of detection of GDP-

mannuronate using the Zorbax SAX HPLC system (see Methods) was 0.1 nmol per ml of extract chromatographed. This limiting value is threefold less than the lowest amount of GDP-mannuronate detected in mucoid strains.

GMD activity in P. aeruginosa

The activities of GMD in the mucoid strains PAO 568 and FRD 1 are given in Table 4. The enzyme activity in corresponding isogenic non-mucoid strains was barely detectable [0.08 nmol min⁻¹ (mg protein)⁻¹] above endogenous dehydrogenase activity. As batch cultures progressed from exponential into stationary phase, the enzyme activity increased approximately twofold in *P. aeruginosa* PAO 568 and fivefold in strain FRD 1 (Table 4). The specific activity of GMD in the clinical strain FRD 1 was similar to that in the laboratory strain PAO 568 during exponential phase, but significantly more ($P < 0.1$) during stationary phase.

Production of extracellular alginate

The production of extracellular alginate was measured by quantification of the uronic acid content in extracellular material secreted into the culture medium by mucoid and non-mucoid strains (Table 5). The amounts produced by the non-mucoid strains PAO 1 and FRD 2 were very small compared with the mucoid strains. The quantity of alginate produced by the mucoid strains increased by 9–10-fold as cultures progressed from late-exponential to early-stationary phase, after which there was no further increase.

Discussion

Mucoid and non-mucoid strains of *P. aeruginosa* are genetically competent to synthesize alginate as they all possess the alginate biosynthetic and regulatory genes (Fialho *et al.*, 1990). The term 'mucoid' is used to

Table 5. *Quantification of extracellular alginate*

The alginate concentration in the extracellular material was determined as described in Methods. Results are expressed as means $\pm$ SD of a minimum of three experiments.

Growth phase	Alginate produced (mg per 10 ¹² cells)			
	PAO 1	PAO 568	FRD 2	FRD 1
Early exponential	0.85 $\pm$ 0.05	2.75 $\pm$ 0.69	0.14 $\pm$ 0.01	2.95 $\pm$ 0.02
Mid-exponential	0.52 $\pm$ 0.05	2.96 $\pm$ 0.44	0.72 $\pm$ 0.01	4.15 $\pm$ 0.27
Late exponential	0.14 $\pm$ 0.03	8.31 $\pm$ 0.52	0.37 $\pm$ 0.02	11.64 $\pm$ 0.95
Early stationary	0.23 $\pm$ 0.04	28.95 $\pm$ 4.00	0.28 $\pm$ 0.03	38.43 $\pm$ 3.63
Mid-stationary	0.16 $\pm$ 0.04	30.70 $\pm$ 5.19	0.27 $\pm$ 0.09	38.89 $\pm$ 3.54
Late stationary	0.23 $\pm$ 0.11	25.45 $\pm$ 4.10	0.47 $\pm$ 0.08	30.25 $\pm$ 3.12

describe those strains which produce copious amounts of alginate and, according to the classification scheme of Phillips (1969), give the type 5 watery colonial morphology within 24 h on agar-based media (Govan, 1990). There has been one report (Anastassiou *et al.*, 1987) that non-mucoid strains also produce alginate, albeit in very small quantities. In the present study, the isogenic non-mucoid strains were found to produce from 3- to 180-fold less alginate than the isogenic mucoid strain, depending on the stage of growth in batch liquid culture (Table 5). Indeed, it is possible that the small amount of uronic-acid-positive extracellular material made by non-mucoid strains was derived from a polymer other than alginate, particularly since the amount did not alter during the growth cycle. The lipopolysaccharide of some strains of *P. aeruginosa* contains small quantities of L-gulonate and D-mannuronate (Kelly *et al.*, 1990), which could be the source of this uronic-acid-positive material.

On the basis of genetic evidence, GMD appears to be the key regulatory enzyme and the committal step for alginate biosynthesis in mucoid *P. aeruginosa*. Genetic experiments have demonstrated that the *algD* gene, which encodes GMD, is transcriptionally activated in mucoid but not non-mucoid strains (Deretic *et al.*, 1987a). The postulated stringent control over the *algD* promoter leads to tight regulation of alginate biosynthesis, which is believed to be necessary as so much of the carbon and energy sources available to mucoid strains is committed to extracellular polysaccharide production (Ohman *et al.*, 1990). Transcriptional regulation of GMD, however stringently controlled, can only influence the total quantity of enzyme which is synthesized and thereby the capacity to consume substrate. However, this type of control may not be sufficiently precise to regulate the flux through the alginate pathway in response to more immediate changes in such metabolic conditions as adenylate charge. The multi-subunit structure of the enzyme and its inhibition by various guanosine derivatives (Roychoudhury *et al.*, 1989) support the concept that GMD could be a metabolically regulated enzyme.

Our data, based on the quantification of key metabolites, show that GMD is indeed the controlling step of the alginate biosynthetic pathway. In mucoid strains, the specific activity of GMD increases progressively with growth of the culture and alginate production. This is paralleled by a decrease in the steady-state quantity of the substrate GDP-mannose. However, the immediate product of the GMD reaction, i.e. GDP-mannuronate, remains at a low and fairly constant level regardless of the specific activity of GMD or the rate of alginate production. These observations are in accord with the concept that the rate of alginate biosynthesis is governed by the activity of GMD.

Calculations based on our data provide estimates for the GDP-mannose concentration within *P. aeruginosa* in batch culture of between 60 μM in early exponential phase to 6 μM in late stationary phase. Reported kinetic data for GMD provide an estimate of 15 μM for the K_m for GDP-mannose (Roychoudhury *et al.*, 1989). Therefore, the calculated concentrations of GDP-mannose in *P. aeruginosa* lie within the range of 0.4 to 4 times the K_m value for GMD. In effect, this means that the rate of flux through this step of the pathway is dependent on available GMD activity.

Using HPLC we have determined the free intracellular concentrations of GDP-mannose and GDP-mannuronate in isogenic pairs of mucoid and non-mucoid strains of *P. aeruginosa*, and have related these substrate/product levels both to the GMD activity in cell-free extracts and to the quantity of extracellular alginate produced. The fact that we could detect GMD activity in mucoid strains is in accord with the genetic observations of others that *algD* transcription is switched on in such strains. Moreover, the specific activity of the enzyme increased as cultures entered stationary phase in concert with an increase in the amount of alginate produced (compare Tables 4 and 5). The higher GMD activity in FRD 1 compared with PAO 568 also correlates with the greater production of alginate by the mucoid FRD strain. These metabolic data support the genetic evidence that the amount of alginate produced is governed by the activity of GMD. The rate of chemical flux through a metabolic pathway is often regulated by controlling not only the expression but also the activity of a key, rate-limiting enzyme in the pathway (Fersht, 1985). Our metabolic data on GMD activity and substrate and product levels are consistent with metabolic regulation of enzyme activity being a possibility.

The substrate of the GMD reaction, GDP-mannose, was found in comparable amounts in mucoid and non-mucoid isogenic pairs of strains, despite the large extra demand for GDP-mannose in mucoid strains making great quantities of extracellular alginate. Although the mucoid strains contained statistically significantly lower levels of GDP-mannose compared with the respective isogenic non-mucoid strains, the actual differences were small. This indicates that the flux of GDP-mannose through the enzyme is tightly regulated, even in non-mucoid strains, which probably reflects the relatively constant demand for GDP-mannose in Gram-negative bacteria for lipopolysaccharide biosynthesis (Sutherland, 1985). Despite the large metabolic demand for GDP-mannose, the amounts present in all strains were small. This observation supports the idea of a tightly regulated flux of GDP-mannose through the GMD enzyme, probably by allosteric effectors, which is consistent with the observation that GDP-mannose concentrations are

constant during exponential growth but fall during stationary phase when metabolic activity and therefore metabolite pool sizes are likely to decline.

In conclusion, our study has provided firm metabolic evidence that the activity of GMD regulates the flux of metabolites through the alginate pathway. The total available activity of GMD is certainly regulated by transcriptional control and may also be modulated by allosteric effectors. The activity of the enzyme is tightly regulated and this emphasizes the role which GMD plays as the key committal step for alginate biosynthesis in mucoid *P. aeruginosa*.

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