

## Alginate Biosynthetic Enzymes in Mucoïd and Nonmucoïd *Pseudomonas aeruginosa*: Overproduction of Phosphomannose Isomerase, Phosphomannomutase, and GDP-Mannose Pyrophosphorylase by Overexpression of the Phosphomannose Isomerase (*pmi*) Gene

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The specific activities of phosphomannose isomerase (PMI), phosphomannomutase (PMM), GDP-mannose pyrophosphorylase (GMP), and GDP-mannose dehydrogenase (GMD) were compared in a mucoïd cystic fibrosis isolate of *Pseudomonas aeruginosa* and in two spontaneous nonmucoïd revertants. In both revertants some or all of the alginate biosynthetic enzymes we examined appeared to be repressed, indicating that the loss of the mucoïd phenotype may be a result of decreased formation of sugar-nucleotide precursors. The introduction and overexpression of the cloned *P. aeruginosa* phosphomannose isomerase (*pmi*) gene in both mucoïd and nonmucoïd strains led not only to the appearance of PMI levels in cell extracts several times higher than those present in the wild-type mucoïd strain, but also in higher PMM and GMP specific activities. In extracts of both strains, however, the specific activity of GMD did not change as a result of *pmi* overexpression. In contrast, the introduction of the cloned *Escherichia coli* *manA* (*pmi*) gene in *P. aeruginosa* caused an increase in only PMI and PMM activities, having no effect on the level of GMP. This suggests that an increase in PMI activity alone does not induce high GMP activity in *P. aeruginosa*. The heterologous overexpression of the *P. aeruginosa* *pmi* gene in the *E. coli* *manA* mutant CD1 led to the appearance in cell extracts of not only PMI activity but also GMP activity, both of which are normally undetectable in extracts of CD1. We discuss the implications of these results and propose a mechanism by which overexpression of the *P. aeruginosa* *pmi* gene can cause an elevation in both PMM and GMP activities.

Commercial alginates have traditionally been isolated from brown seaweeds, but an exopolysaccharide resembling alginate is also produced by *Azotobacter vinelandii* (16) and mucoïd strains of *Pseudomonas aeruginosa* (14). Alginates are linear copolymers of  $\beta$ -1,4-linked residues of D-mannuronic acid and its C-5 epimer, L-guluronic acid, and their arrangement as well as their relative amounts vary greatly in alginates from different sources. The exopolysaccharide produced by *P. aeruginosa*, for example, contains mainly polymannuronic acid block structures and is highly acetylated (13, 14, 37). The epimerization of mannuronic acid units to guluronic acid is believed to occur in bacteria at the polymer level (32, 33, 38). Furthermore, the O-acetylation of some of the mannuronic acid residues was suggested to render them resistant to epimerization (10).

The biosynthesis of alginate has been studied in the alga *Fucus gardneri* (25, 26) and in *A. vinelandii* (33). The results of these studies demonstrated considerable similarity in the pathway leading to the synthesis of GDP-mannuronic acid in each organism. In the pathway for alginate biosynthesis (Fig. 1A), the glycolytic intermediate fructose 6-phosphate is first converted to mannose 6-phosphate by the action of phosphomannose isomerase (PMI). Mannose 1-phosphate is then formed by the action of phosphomannomutase (PMM) and further esterified with GTP by GDP-mannose pyrophos-

phorylase (GMP). Polymannuronic acid is then synthesized by the action of GDP-mannose dehydrogenase (GMD) and alginate polymerase. A similar biosynthetic pathway has been proposed to operate in mucoïd *P. aeruginosa* (32, 35).

Although *P. aeruginosa*, an opportunistic pathogen, in general does not produce alginate, mucoïd variants which produce copious quantities of extracellular alginate have been isolated from cystic fibrosis (CF) patients (12). Prolonged antibiotic therapy has been suggested as a causative factor in the emergence of mucoïd *P. aeruginosa* in the CF lung (22). The mucoïd variants are, however, unstable with respect to alginate synthesis and rapidly become nonmucoïd when cultured in vitro on artificial medium (17, 18, 28). Such a loss in alginate-producing ability has also been reported for *A. vinelandii* (20).

In the course of studying the genetics of alginate biosynthesis by *P. aeruginosa*, a 2.0-kilobase (kb) *Bam*HI-*Sst*I cloned fragment carrying the *P. aeruginosa* *pmi* gene (also designated *algA*) was mapped at 45 min on the PAO chromosome and shown to be part of a clustered set of genes involved in alginate biosynthesis (8, 9). This fragment has been subcloned into the broad-host-range expression vector pMMB24 under the *tac* promoter, and the PMI has been overproduced by induction with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) (9, 15). The 56-kilodalton (kDa) polypeptide seen in maxicell labeling experiments was consistent with a single major open reading frame in the *pmi* DNA sequence (7). A segment of the clustered alginate region located distal to the *pmi* gene had recently been shown to harbor the GMD gene (*gmd*, also designated *algD*) (Fig. 1B). *algD* was shown to be transcriptionally active in the mucoïd

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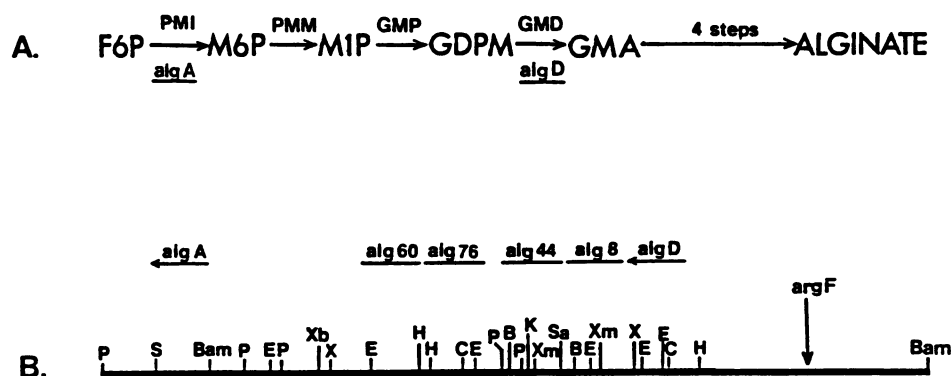


FIG. 1. (A) Alginic acid biosynthetic pathway. Abbreviations: F6P, fructose 6-phosphate; M6P, mannose 6-phosphate; M1P, mannose 1-phosphate; GDPM, GDP-mannose; GMA, GDP-mannuronic acid. The final four steps are proposed to be polymerization, epimerization, acetylation, and export, although these steps have not yet been demonstrated in *P. aeruginosa*. (B) Restriction endonuclease map of the *P. aeruginosa* clustered alginate region near 45 min on the PAO chromosome (7, 9). Abbreviations: B, *Bgl*II; Bam, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sst*I; Sa, *Sal*I; X, *Xho*I; Xb, *Xba*I; Xm, *Xma*I; *algA*, gene coding for PMI; *algD*, gene coding for GMD.

strain 8821 but inactive in the spontaneous nonmucoid revertant strain 8822 (11). The locations of the two remaining genes which participate in the formation of alginate precursors (i.e., *algB* and *algC*, encoding PMM and GMP, respectively), however, have not as yet been identified.

While Piggot et al. (32) provided evidence for the existence of enzymes required to synthesize GDP-mannuronic acid in *P. aeruginosa*, some reports have cited difficulties in detecting activities of the proposed alginate biosynthetic enzymes in crude extracts of *P. aeruginosa* by standard assay procedures. For instance, Banerjee et al. (2) were unable to detect PMI and PMM activities in mucoid *P. aeruginosa*, and GMD has been difficult to measure because of the presence of a nonspecific alcohol dehydrogenase in *P. aeruginosa* extracts (35).

In this communication we describe modified methods to assay very low levels of enzymes involved in alginate biosynthesis in *P. aeruginosa*. Using these methods, we compared the specific activities of PMI, PMM, GMP, and GMD in a mucoid and two spontaneous nonmucoid revertant strains of a mucoid CF isolate of *P. aeruginosa*. We demonstrate that the overexpression of the *pmi* gene in *P. aeruginosa* not only results in a very high specific activity for PMI but also in the appearance of high PMM and GMP activities. Studies dealing with the heterologous expression of the *P. aeruginosa pmi* gene in wild-type *Escherichia coli* and its *pmi* (*manA*) mutant were also undertaken.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and genetic procedures.** The bacterial strains and plasmids used in these experiments are listed in Table 1. Plasmid pAD4038 (*Ap*<sup>r</sup>) carries the *pmi* gene from *P. aeruginosa* on a 2.0-kb *Bam*HI-*Sst*I fragment which was subcloned from pAD4033 (7) as a single *Hind*III fragment and inserted into the broad-host-range expression vector PMMB24 (1) with the gene directly downstream of the *tac* promoter. Manipulation of DNA, agarose gel electrophoresis, and other genetic procedures have been described previously (6).

**Determination of enzymatic activities.** (i) **Growth conditions.** Cells were grown in L broth (10 g of peptone, 5 g of yeast extract, and 5 g of sodium chloride per liter). Carbenicillin (300  $\mu$ g/ml), ampicillin (50  $\mu$ g/ml), or tetracycline (50  $\mu$ g/ml) was used to maintain selection pressure on resistant

transformants or transconjugants. A 25-ml overnight liquid culture was used to inoculate 500 ml of L broth in 2-liter Erlenmeyer flasks, and the cultures were grown at 37°C with vigorous aeration for a total of 7 h. When carried out, induction with IPTG was performed after 5 h of growth, at a final IPTG concentration of 1 mM. The cultures were then grown for another 2 h before harvesting. For comparison, enzymes were also assayed in extracts prepared from cells of the mucoid strain 8821 grown for 9 h.

(ii) **Preparation of cell extracts.** Cultures which were in the late exponential or early stationary phase of growth were harvested by centrifugation at 10,000  $\times$  *g* for 10 min at 4°C. The resulting cell pellet was then washed with 0.9% sodium chloride and recentrifuged. When enzyme assays were not to be performed immediately, cell pellets were frozen at -70°C until used. The cells were suspended in 3 ml of sonication buffer (10 mM MOPS [3-(*N*-morpholinopropanesulfonic acid)], 2 mM D,L-dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.0). Cell disruption was achieved by sonic vibration in an oscillator (Heat Systems-Ultrasonic Inc.). Cell debris was removed by centrifugation at 27,000  $\times$  *g* for 40 min at 4°C. The supernatants were then used directly for enzymatic analysis. No differ-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
<i>P. aeruginosa</i>		
8821	<i>his-1</i> (alginate producing)	6
8822	<i>his-1 alg-1</i> (spontaneous)	6
8823	<i>his-1 alg-2</i> (spontaneous)	This study
<i>E. coli</i>		
AC80	<i>thr leu met hsdR hsdM</i>	6
CD1	<i>thr leu met hsdR hsdM manA</i>	8
Plasmids		
pMMB24	IncQ <i>Ap</i> <sup>r</sup> <i>ptac-lacI</i> <sup>q</sup>	1
pAD4038	<i>ptac pmi</i> <sup>+</sup>	This study
pAD3 <sup>a</sup>	<i>manA</i> <sup>+</sup>	8
pAD451 <sup>b</sup>	<i>manA</i> <sup>+</sup>	8

<sup>a</sup> pAD3 contains a 6.2-kb *Hind*III chromosomal fragment of *E. coli* cloned in the broad-host-range cosmid vector pCP13.

<sup>b</sup> pAD451 contains a 1.5-kb subfragment from pAD3 cloned into the ColE1-based vector pBR325.

ence could be found between enzyme activities measured in these extracts and in extracts obtained after recentrifugation at  $100,000 \times g$  for 1 h to remove membranes. Enzyme assays were performed on freshly prepared extracts, and each value is an average for three experiments performed with different extracts.

**Enzyme assays.** Activities of PMI, PMM, GMP, and GMD were assayed in crude extracts by recording increases in optical density at 340 nm (due to the reduction of NAD or NADP) at 24°C in a Gilford model 2600 spectrophotometer-multiple sample absorbance recorder. Enzyme activities were calculated from the initial linear rates of cofactor reduction after subtraction of endogenous activities (which were measured in assays without substrate). Control assays lacking only the extracts were also carried out.

The protein concentration of extracts was estimated by the method of Bradford (3), with bovine serum albumin (BSA) as the protein standard. Conversion of the increase in optical density at 340 nm to micromoles of NADPH or NADH produced was based on their extinction coefficients. One unit of enzymatic activity was defined as that which reduced 1  $\mu\text{mol}$  of NADP or NAD per min under the specified assay conditions. Specific activities are given as milliunits per milligram of protein. Enzymes used in coupled reactions (units as defined by the manufacturer) were added in excess relative to the enzymatic activity under measurement.

**PMI.** PMI activity was measured by the method of Slein (39). The reaction mixture, in a total volume of 1 ml, contained 0.05 M Tris hydrochloride buffer, pH 7.5, 10  $\mu\text{mol}$  of  $\text{MgCl}_2$ , 0.5 U each of phosphoglucosyltransferase (PGI) and glucose 6-phosphate dehydrogenase (ZWF) and 1  $\mu\text{mol}$  of NADP. The reaction was initiated by the addition of 10 to 200  $\mu\text{l}$  of crude extract and 3  $\mu\text{mol}$  of D-mannose-6-phosphate.

**PMM.** PMM activity was assayed by the method described by Pindar and Bucke (33). The reaction mixture, in a total volume of 1 ml, contained 0.05 M Tris hydrochloride buffer, pH 7.5, 10  $\mu\text{mol}$  of  $\text{MgCl}_2$ , 0.25  $\mu\text{mol}$  of  $\alpha\text{-D-glucose-1,6-diphosphate}$ , 0.5 U each of PGI, ZWF, and PMI, and 1  $\mu\text{mol}$  of NADP. The reaction was started by the addition of 50 to 100  $\mu\text{l}$  of crude extract and 1  $\mu\text{mol}$  of  $\alpha\text{-D-(+)-mannose-1-phosphate}$ .

**GMP.** GMP activity was measured by the method of Munch-Petersen (29, 30). One milliliter of 0.05 M Tris hydrochloride buffer, pH 7.5, contained 10  $\mu\text{mol}$  of  $\text{MgCl}_2$ , 0.1  $\mu\text{mol}$  of ADP, 2  $\mu\text{mol}$  of pyrophosphate, 5  $\mu\text{mol}$  of sodium fluoride, and 0.5 U each of hexokinase, nucleoside-5'-diphosphate kinase, and ZWF, 0.8  $\mu\text{mol}$  of glucose, and 1  $\mu\text{mol}$  of NADP. The reaction was started by the addition of 50 to 100  $\mu\text{l}$  of crude extract and 0.4  $\mu\text{mol}$  of GDP-D-mannose.

**GMD.** GMD activity was measured by the method of Preiss (34). One milliliter of reaction mixture contained 0.05 M Tris hydrochloride, pH 7.5, 10  $\mu\text{mol}$  of  $\text{MgCl}_2$ , and 1  $\mu\text{mol}$  of NAD. The reaction was initiated by the addition of 50 to 100  $\mu\text{l}$  of crude extract and 1  $\mu\text{mol}$  of GDP-mannose.

**Ion-exchange chromatography.** Crude extracts containing up to 200 mg of protein were fractionated by high-pressure liquid chromatography (HPLC) with a Bio-Rad Bio-gel TSK SP-5-PW column (150 by 21.5 mm). The elution buffer was 10 mM MOPS (pH 7.0) containing 1 mM DTT. Following sample injection, the column was washed with 60 to 80 ml of elution buffer, and bound material was eluted with a linear 0 to 0.6 M NaCl gradient which was prepared in elution buffer and run over a 45-min time span. The flow rate was 4.0

TABLE 2. Specific activities of alginate biosynthetic enzymes in mucooid *P. aeruginosa* 8821 and in spontaneous nonmucooid revertants 8822 and 8823

Strain	Growth <sup>a</sup> (h)	Sp act (mU/mg)			
		PMI	PMM	GMP	GMD
8821	7	+ <sup>b</sup>	2.0	1.2	8.0
8821	9	1.0	14.0	1.6	25.0
8822	7	+	0.4	3.4	2.8
8823	7	ND <sup>c</sup>	ND	ND	0.7 <sup>d</sup>

<sup>a</sup> A 25-ml overnight culture was used to inoculate 500 ml of fresh L broth. Cultures were grown at 37°C for the indicated times before harvesting.

<sup>b</sup> +, Barely detectable activity (about 0.5 mU/mg).

<sup>c</sup> ND, Not detected.

<sup>d</sup> *P. aeruginosa* possesses an NAD-linked alcohol dehydrogenase that is slightly reactive with GDP-mannose, but catalyzes the formation of a product other than GDP-mannuronic acid (35). Thus, the low GMD activity observed, based on measurement of NAD reduction, may be nonspecific activity.

ml/min, and 4-ml fractions were collected. Fractions were maintained on ice prior to assay.

**Biochemicals.** Disodium carbenicillin was obtained from Roerig (Pfizer, Inc., New York, N. Y.). Ampicillin (sodium salt) and tetracycline (hydrochloride) were obtained from Sigma Chemical Co., St. Louis, Mo. IPTG, DTT, PMSF, phosphorylated sugars, nucleotides, sugar nucleotides, NAD, NADP, BSA, and all enzymes used in coupling reactions were also from Sigma. MOPS was obtained from Research Organics, Cleveland, Ohio, and the protein assay reagent was from Bio-Rad Laboratories, Richmond, Calif. *Pseudomonas* isolation agar, peptone, and yeast extract were supplied by Difco Laboratories, Detroit, Mich. All other chemicals and reagents were purchased from commercial sources and were standard reagent grade or better.

## RESULTS

**Comparison of the activities of alginate biosynthetic enzymes in mucooid and nonmucooid *P. aeruginosa*.** The normally low or undetectable activity levels of enzymes involved in alginate biosynthesis previously reported for *P. aeruginosa* (2) prompted us to develop assay conditions (described in Materials and Methods) that allowed detection of such low activity, allowing reliable comparisons to be made between enzyme levels of mucooid and nonmucooid strains. Under these conditions, activities of PMI, PMM, GMP, and GMD were detected in mucooid and nonmucooid *P. aeruginosa* strain 8821, 8822, and 8823 (Table 2). While the activity levels were still relatively low (note PMI and PMM levels in *E. coli* and *P. aeruginosa*, Table 3), especially in the nonmucooid strains, they were of the same order of magnitude as those reported for *A. vinelandii* (19, 20, 33) and for GMD from *P. aeruginosa* (35) and were reproducible. The successful detection of PMI, PMM, GMP, and GMD in *P. aeruginosa* reported here coupled with the genetic characterization of the *algA* and *algD* genes (7, 11) further support the hypothesis that the alginate biosynthetic pathway in *P. aeruginosa* is indeed similar to the proposed pathway for *A. vinelandii* (33), *F. gardneri* (25, 26), and other strains of *P. aeruginosa* (32, 35).

The phase of growth in which cells were harvested was an important factor in detecting enzymatic activities. If the mucooid strain 8821 was harvested in the late exponential phase of growth (7 h of growth), PMM, GMP, and GMD activities were detected, but PMI activity was very low or undetectable (Table 2). In contrast, when strain 8821 cells

were harvested in the stationary phase of growth (9 h of growth), the activities of PMM and GMD were elevated substantially, and PMI activity was easily detectable. These results are consistent with the findings of Pugashetti et al. (35), that GMD activity in *P. aeruginosa* follows the growth curve, and the findings of Horan et al. (19), that PMI, GMP, and GMD activities in *A. vinelandii* are undetectable in exponentially growing cells but reach maximum levels in stationary-phase cells. It should be noted that the nonmucoid strains 8822 and 8823 exhibited a faster growth rate than the mucoid strain 8821 (data not shown). Thus, for direct comparison of enzyme activities (Table 2), the nonmucoid and mucoid strains were grown for 7 and 9 h, respectively, so that all cells were harvested in the early stationary phase of growth.

**Effect of overexpression of the *P. aeruginosa pmi* gene on specific activities of alginate biosynthetic enzymes.** The introduction of plasmid pAD4038, which harbors the *P. aeruginosa pmi* gene as a 2.0-kb *Bam*HI-*Sst*I fragment (7), into both mucoid and nonmucoid *P. aeruginosa* strains and subsequent overexpression of the *pmi* gene by IPTG induction of the *tac* promoter resulted in the appearance in cell extracts of high PMI specific activities (15). This 2.0-kb segment has been completely sequenced and contains a single open reading frame corresponding to a 53-kDa polypeptide (7). When overproduced under the *tac* promoter, the high PMI specific activity correlated very well with the increasing intensity of a 56-kDa polypeptide band on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (15).

We found that induction of the *pmi* gene not only promoted high PMI activity, but also resulted in the simultaneous appearance of PMM and GMP activities that were several times higher than those present in strain 8821 or 8822 without plasmids or harboring pAD4038 without IPTG induction (Table 3). In contrast, in extracts of 8821 or 8822 (even after IPTG induction of pAD4038), the specific activity of GMD was similar to that observed in mucoid and nonmucoid strains harboring the expression vector pMMB24. Introduction of the cloned *E. coli pmi* gene (pAD3) (7) into *P. aeruginosa* 8821 and 8822 resulted in PMI activities that were about one-half of those attained following IPTG induction of pAD4038 in the same cells (Table 3). However, in contrast to pAD4038 (induced), which promoted high levels of PMM and GMP in addition to PMI, pAD3 promoted elevated levels of PMM (but not GMP) in addition to PMI.

**Effect of overexpressing the *P. aeruginosa pmi* gene on PMI and GMP levels in *E. coli*.** We could not detect GMP activity in cell extracts of the non-colanic acid-producing *E. coli* strain AC80 by the same spectrophotometric method used to assay this activity in mucoid *P. aeruginosa*. It should be noted that our assay method was not suitable for detecting low GMP activity in crude extracts of *E. coli* because of relatively high background rates without added substrate. However, after introduction and IPTG induction of pAD4038 in *E. coli*, we observed a high level of GMP activity (Table 3) that could easily be quantitated by subtracting background rates and presumably resulted only from expression of the *P. aeruginosa pmi* gene in *E. coli*. In contrast, the overexpression of the *E. coli pmi* gene (pAD451) in *E. coli* had no effect on the level of GMP (Table 3). Because of high endogenous activity of PMI and PMM in *E. coli* (Table 3), it was not possible to determine whether any additional PMI and PMM activity was being expressed in *E. coli* specifically from the induction of the *P. aeruginosa pmi* gene in pAD4038. Therefore, plasmid pAD4038 was

introduced into the *E. coli pmi* mutant CD1 and overexpressed by IPTG induction. Crude extracts of CD1 alone or CD1 containing the expression vector pMMB24 contained no measurable PMI or GMP activity. However, after the introduction and induction of pAD4038 in CD1, crude extracts possessed high PMI and GMP levels (Table 3).

**Fractionation of PMI, PMM, and GMP activities present in cell extracts.** To investigate whether the high GMP and PMM activities present in extracts of *P. aeruginosa* overexpressing the *pmi* gene corresponded to different proteins or to a single multifunctional protein, HPLC fractionation of a crude extract of the nonmucoid strain 8822 harboring pAD4038 and induced with IPTG was carried out as described in Materials and Methods. Figure 2A shows the chromatographic profile obtained for *P. aeruginosa* carrying pAD4038 and induced with IPTG. The PMI and GMP activities eluted together in the same fractions in the 0 to 0.6 M NaCl gradient, while PMM eluted earlier in the gradient, separate from the other two activities. This result suggested that the gene product of the 2.0-kb *Bam*HI-*Sst*I fragment of pAD4038 may be a protein with at least two enzymatic activities. To test this possibility further, pAD4038 was induced with IPTG in *E. coli* AC80, and a crude extract was fractionated in the same manner as the *P. aeruginosa* extract. As a control, *E. coli* AC80 harboring only the expression vector pMMB24 was also examined. Figure 2B shows the elution profile for PMI and PMM from *E. coli* AC80 containing pMMB24. No GMP activity was detected in this strain. An identical HPLC fractionation of the *E. coli* crude extract obtained from pAD4038-harboring cells (induced with IPTG) demonstrated that, besides the PMI and PMM of *E. coli*, an additional peak of PMI activity also possessing GMP activity appeared in the same fractions in which it eluted when the *P. aeruginosa pmi* gene was

TABLE 3. Specific activities of alginate biosynthetic enzymes in *P. aeruginosa* and *E. coli* strains harboring various plasmids

Strain	Sp act (mU/mg) <sup>a</sup>			
	PMI	PMM	GMP	GMD
8821	≤0.5	2.0	1.2	8.0
8821(pMMB24)	≤0.5	2.3	1.8	15.0
8821(pAD4038)	1.3	2.1	6.9	13.5
8821(pAD4038) (+ IPTG)	113	23	43	14.0
8821(pAD3) <sup>b</sup>	60.2	5.9	1.6	12.0
8822	≤0.5	0.6	3.6	3.0
8822(pMMB24)	≤0.5	0.5	3.4	2.8
8822(pAD4038)	1.2	≤0.5	7.1	2.6
8822(pAD4038) (+ IPTG)	100	12.0	49	3.2
8822(pAD3) <sup>b</sup>	51	4.6	4.0	2.0
AC80	306	113	ND <sup>c</sup>	— <sup>d</sup>
AC80(pMMB24)	445	80	ND	—
AC80(pAD4038)	348	146	6.0	—
AC80(pAD4038) (+ IPTG)	390	179	34	—
AC80(pAD3)	1,031	124	ND	—
AC80(pAD451) <sup>b</sup>	1,380	73	ND	—
CD1	ND	92	ND	—
CD1(pAD4038) (+ IPTG)	82	108	37	—

<sup>a</sup> A value of ≤0.5 indicates that activity was detectable, but just within the limit of detection.

<sup>b</sup> See Table 1, footnotes a and b. The *E. coli pmi* gene is known to be inefficiently expressed in *P. aeruginosa* (8).

<sup>c</sup> ND, Not detected.

<sup>d</sup> —, Not tested, but such activities are known to be undetectable in *E. coli* (<0.1 mU/mg; see reference 11).

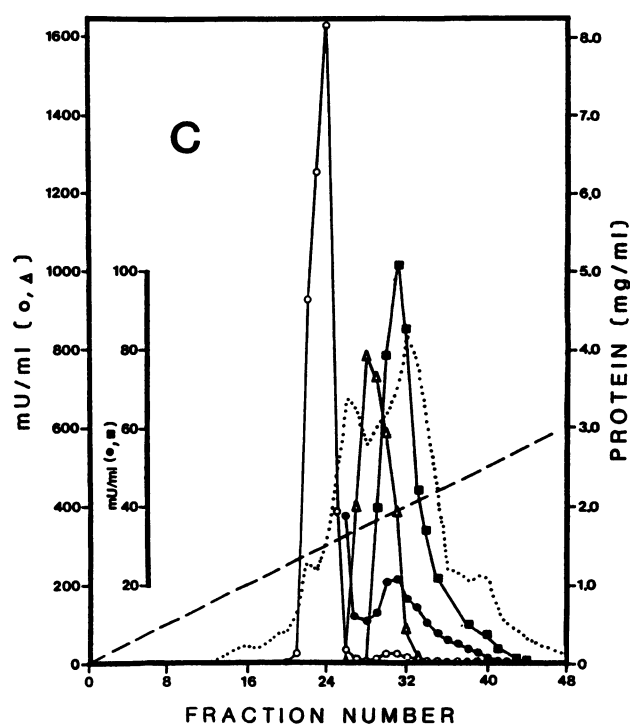
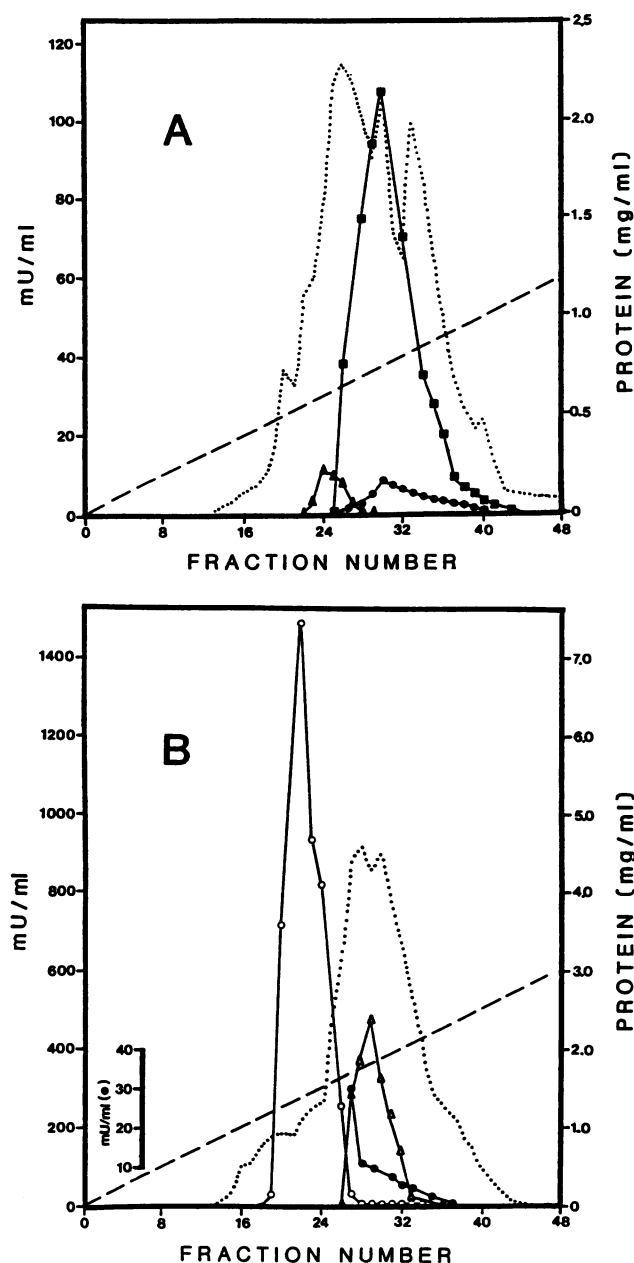


FIG. 2. Elution profiles of PMI, PMM, and GMP following fractionation of crude extracts of *P. aeruginosa* and *E. coli* via HPLC ion-exchange chromatography (see text). In all three panels, the dotted line represents the distribution of protein, and the dashed line represents the NaCl gradient (0 to 0.6 M). (A) Elution profiles of PMI (●), PMM (▲), and GMP (■) from *P. aeruginosa* 8822 harboring pAD4038 and induced with IPTG. (B) Elution profiles of PMI (○) and PMM (△) from *E. coli* AC80 harboring the expression vector pMMB24. The tail of the PMI activity peak (fractions 27 to 37) was also plotted on an increased scale (see inset ordinate, ●) to demonstrate the absence of an additional peak of PMI activity in these fractions (see panel C). (C) Elution profiles of PMI (○; also plotted on an increased scale for fractions 26 to 42 (●)), PMM (△), and GMP (■; also plotted on an increased scale) from *E. coli* AC80 harboring pAD4038 and induced with IPTG. Open symbols, Activity peaks endogenous to *E. coli*; solid symbols, activity peaks of *P. aeruginosa* origin, being a result of the *P. aeruginosa pmi* gene carried on pAD4038.

overexpressed in *P. aeruginosa* (Fig. 2C). In extracts of *E. coli* harboring pAD4038 (induced with IPTG), no additional peak of PMM activity was found.

If the *P. aeruginosa pmi* gene does in fact encode a bifunctional PMI-GMP, a proportional increase in the two activities following induction of pAD4038 would be expected. However, after induction of pAD4038 (in *P. aeruginosa*), PMI activity increased 100-fold but GMP activity increased only 7-fold (Table 3). A considerable amount of additional data obtained with these and other strains of *P. aeruginosa* show that unlike PMI activity, which consistently underwent a ca. 100-fold increase in response to induction of pAD4038, the level of GMP activity varied in different crude preparations of induced cells (unpublished observations). However, following initial fractionation of crude extracts, both the level of GMP and the GMP-PMI ratio were consistent with a proportional induction of both activities. For example, the GMP-PMI ratio in uninduced

cells was about 7 (Table 3). After induction, the PMI activity increased about 100-fold, while GMP activity apparently only increased 7-fold. However, after fractionation of crude extracts (Fig. 2A and C), the GMP-PMI ratio was 6 to 10. This GMP-PMI ratio was maintained throughout subsequent purification steps, which also failed to separate the two activities (A. Berry, unpublished data). Thus, the apparent disproportional increase in PMI and GMP activities in response to induction of pAD4038 appears to be a result of some component in crude cell-free preparations that affects GMP activity. This component is apparently removed during the initial purification step.

## DISCUSSION

The four alginate biosynthetic enzymes PMI, PMM, GMP, and GMD reported to be present in extracts of alginate-producing strains of *A. vinelandii* (33) and *F. gardneri* (25, 26) were detected and quantitated in the mucoid *P. aeruginosa* strain 8821. We previously described the cloning and nucleotide sequence determination of two genes from *P.*

*aeruginosa*, *algA* and *algD* (encoding PMI and GMD, respectively), and their involvement in alginate synthesis (7, 11). Together, these results support the contention that a similar alginate biosynthetic pathway operates in *P. aeruginosa*, *A. vinelandii*, and *F. gardneri*.

In *A. vinelandii* the loss of the mucoid phenotype has been reported to be accompanied by undetectable levels of PMI, GMP, and GMD, which were all present in extracts of the alginate-producing wild type (20). A similar pattern was also reported for the enzymes involved in the biosynthesis of the exopolysaccharide colanic acid by *E. coli* K-12 (23, 24, 27). Other observations, however, indicate that the loss of the ability to produce different exopolysaccharides cannot always be associated with a decrease in the specific activity of the enzymes involved in the formation of its nucleotide-sugar precursors. For example, several mutants of *Klebsiella aerogenes* which had lost the ability to produce an exopolysaccharide after mutagenesis contained wild-type levels of the biosynthetic enzymes involved in the formation of precursors (31). It has been further demonstrated that in *P. aeruginosa*, the level of alginate precursors does not necessarily reflect the amount of polysaccharide produced, which is dependent on the medium composition, environmental factors, and rate and stage of growth (40). While the differences in enzyme levels we observed in mucoid and nonmucoid strains were small (Table 2), they were nevertheless reproducible, and in addition were comparable to the difference in enzyme levels reported previously for mucoid and nonmucoid *A. vinelandii* (19, 20) and *P. aeruginosa* (35). Thus, our results indicate that, at least for the few nonmucoid strains we examined, the spontaneous reversion phenomenon may be due to the repression or loss of activation (or both) of the synthesis of the key alginate biosynthetic enzymes. Our results also suggest the possibility that different levels of repression or activation exist (e.g., compare enzyme levels in the two nonmucoid strains 8822 and 8823). Mutations in two different regulatory genes (*capR* and *capS*) were reported to cause increased synthesis of the capsular polysaccharide colanic acid in *E. coli*, but some of the enzymes involved in the polysaccharide biosynthesis which were derepressed in *capR* mucoid strains were found not to be derepressed in the *capS* mucoid strains (23). In the future, additional attention will have to be focused on the role that the enzymes involved in polysaccharide polymerization and excretion play in the spontaneous reversion phenomenon.

After IPTG induction of *P. aeruginosa* strains harboring pAD4038, PMI, PMM, and GMP were simultaneously overproduced, initially suggesting to us that the increased activity of PMI was inducing the next two enzymes in the alginate biosynthetic pathway. However, the increase in PMI activity alone could not explain the higher values of GMP, because the introduction and expression of the cloned *E. coli pmi* gene (pAD3) in *P. aeruginosa* had no effect on the GMP activity (Table 3). The heterologous overexpression of the *P. aeruginosa pmi* gene from pAD4038 in the *E. coli pmi* (*manA*) mutant CD1 led to the appearance of high levels of PMI and GMP, activities which were both undetectable in this mutant in the absence of pAD4038. Because of the large PMM activity already present in extracts of *E. coli*, it was not possible to determine whether any additional PMM activity was present as the result of the induction of pAD4038. However, this possibility is unlikely since (i) fractionation studies (Fig. 2) revealed that PMM is clearly a separate gene product from PMI-GMP in *P. aeruginosa* and (ii) high PMI levels alone (caused by overexpression of the

*P. aeruginosa pmi* gene) in *E. coli* would not cause overproduction of PMM, since hyperproduction of the *E. coli* PMI (from pAD451) in *E. coli* did not lead to elevated levels of PMM (Table 3). The latter finding suggests that the mechanism by which overproduction of PMI causes elevation of PMM in *P. aeruginosa* does not exist in *E. coli*. Since PMM in *P. aeruginosa* was elevated in response to overexpression of either the *P. aeruginosa* or the *E. coli pmi* gene, the most likely explanation is that the product of the PMI reaction, mannose 6-phosphate, induces PMM in *P. aeruginosa*. This situation would be similar to the induction of tryptophan synthase by indoleglycerol 3-phosphate in *P. aeruginosa* and *Pseudomonas putida* (5).

At present we are left with a few possibilities concerning the function of the *P. aeruginosa pmi* gene. One hypothesis is that the product of the *pmi* gene induces GMP activity in both *P. aeruginosa* and *E. coli* by increasing the levels of intermediates of alginate synthesis. This possibility is unlikely, however, since the presence of the *E. coli pmi* gene in *P. aeruginosa* did not cause an elevation in GMP activity (Table 3). Likewise, the overproduction of the *E. coli* PMI (pAD451) in *E. coli* did not cause an elevation in GMP activity. Another hypothesis is that the *P. aeruginosa pmi* gene codes for a single protein with two separate enzymatic activities, namely PMI and GMP. The possibility that the 2.0-kb *Bam*HI-*Sst*I fragment which harbors the *P. aeruginosa pmi* gene also contains a second gene specifying GMP activity is untenable for the following reasons. It has previously been shown that the 2.0-kb fragment contains a 1,440-base-pair (bp) open reading frame in the orientation from *Bam*HI toward *Sst*I, initiating 40 bp from the *Bam*HI site and terminating at bp 1480 (7). In addition, no alternative significant open reading frames were found on either DNA strand. *E. coli* maxicell studies also indicate the presence of a single polypeptide on the SDS-polyacrylamide gels (7). Furthermore, subcloning of sequences 3' of the *pmi* gene has revealed no effect on GMP levels when the various constructs were introduced into either 8821 or 8822 (I. Sá-Correia and A. Darzins, unpublished results). In light of these results, it is unlikely that a separate gene encoding GMP is also present within the 2.0-kb fragment.

Results of fractionation of the alginate biosynthetic enzymes present in cell extracts of *P. aeruginosa* and *E. coli*, both harboring pAD4038 and induced with IPTG (Fig. 2), are consistent with the possibility that the *P. aeruginosa pmi* gene encodes a bifunctional protein. PMI and GMP activities present in extracts prepared from *P. aeruginosa* harboring pAD4038 and induced with IPTG eluted in exactly the same fractions. Furthermore, fractionation of crude extracts of *E. coli* overexpressing pAD4038 revealed that the peak of *E. coli* PMI activity was followed by another PMI activity peak also possessing GMP activity that eluted in the same position as the *P. aeruginosa* PMI-GMP activity peak. This additional PMI-GMP activity was not detected in extracts prepared from *E. coli* harboring only the expression vector pMMB24 (i.e., lacking the inserted *P. aeruginosa pmi* gene). Work is presently under way to purify what could possibly be a bifunctional protein.

Many of the bi- and multifunctional enzymes that have been identified (see reference 36 for an overview) catalyze contiguous reactions in a given pathway. The best examples of such enzymes are those of the tryptophan pathway (5, 41). Fewer examples of bifunctional enzymes that catalyze noncontiguous reactions are known, the two best studied being the aspartokinase-homoserine dehydrogenase of *E. coli* (4) and the 3-deoxy-D-arabino-heptulosonate 7-

phosphate synthase-chorismate mutase of *Bacillus subtilis* (21). If the PMI-GMP reported here proves to be a single polypeptide, it will represent yet another example of a bifunctional enzyme catalyzing noncontiguous reactions. It is tempting to speculate that PMM is associated with PMI-GMP in a protein-protein interaction in *P. aeruginosa*, especially since PMM levels increase in response to elevated PMI levels. This would indeed provide an efficient means for channeling the initial substrate of alginate synthesis, fructose 6-phosphate, to GDP-mannose, thus avoiding loss of diffusible intermediates to competing reactions. Examination of this possibility awaits the acquisition of purified PMI-GMP and PMM.

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