Overproduction and Assay of *Pseudomonas aeruginosa* Phosphomannose Isomerase

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Phosphomannose isomerase activity was undetectable in extracts of mucoid (alginate-producing) *Pseudomonas aeruginosa*. When a *P. aeruginosa* gene previously shown to complement an alginate-negative mutant was overexpressed under the control of the *tac* promoter in the broad-host-range controlled-expression vector pMMB22, phosphomannose isomerase activity could be measured in extracts of *P. aeruginosa* and in a *manA* (phosphomannose isomerase-negative) mutant of *Escherichia coli*. *P. aeruginosa* extracts containing induced levels of enzyme were shown to interconvert fructose 6-phosphate and mannose 6-phosphate. A 56,000-dalton polypeptide was visualized on sodium dodecyl sulfate-polyacrylamide gels after induction in both hosts. When RNA-DNA dot-blot hybridization analysis was used, transcription of *algA*, the gene coding for *P. aeruginosa* phosphomannose isomerase, was not measurable from the chromosomes of either mucoid or nonmucoid *P. aeruginosa*. However, a high level of *algA* transcription was detected after expression of *algA* under *tac* promoter control in pMMB22.

The alginate biosynthetic pathway of mucoid *Pseudomonas aeruginosa* has remained largely uncharacterized, although it has been proposed (17) to be similar to that of another alginate-producing organism, *Azotobacter vinelandii* (18). One problem in firmly establishing this pathway in *P. aeruginosa* has been the reported difficulties in detecting the activities of proposed biosynthetic enzymes in crude extracts. Using published assay procedures, Banerjee et al. (2) were unable to measure the activities of phosphomannose isomerase (PMI) and phosphomannomutase, the first two enzymes in the proposed pathway. The assay of another proposed enzyme, GDP-mannose dehydrogenase, has also been reported as unreliable owing to the presence of an interfering nonspecific alcohol dehydrogenase in *P. aeruginosa* extracts (19).

A stable mucoid strain which was recently obtained in our laboratory allowed the isolation by chemical mutagenesis of numerous alginate-negative mutants believed to be defective in genes controlling alginate production (5). Many of these mutations formed a cluster of at least six complementation groups which mapped at 45 min near the *argF* locus on the chromosome of *P. aeruginosa* PAO (7). Intergeneric complementation of one of these groups (I, see Fig. 1) by the *manA* gene isolated from *Escherichia coli* suggested that a cloned *P. aeruginosa* DNA fragment that also complemented group I mutants might harbor a gene coding for PMI (6). PMI activity, however, could not be measured in association with this *P. aeruginosa* DNA fragment cloned in the low-copy-number vector pCP13 (6). Thus, the functional identity of this gene as well as the involvement of PMI in the alginate biosynthetic pathway of mucoid *P. aeruginosa* remained unclear.

In this report, we present direct biochemical evidence for the functional identification of this *P. aeruginosa* gene, designated *algA*, coding for a polypeptide catalyzing the interconversion of fructose 6-phosphate (F6P) and mannose 6-phosphate (M6P). These results confirm the proposed role of PMI in the alginate biosynthetic pathway of mucoid *P. aeruginosa*. Overproduction of the *algA* gene product, al-

lowing its enzymatic characterization, demonstrates a potential approach for determining the functional identities of other alginate biosynthetic genes whose products may also be difficult to detect.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *P. aeruginosa* 8821 (mucoid), 8822 (spontaneous nonmucoid revertant of 8821), 8830 (stable mucoid), and PAO1 (nonmucoid) have been previously described (5, 14). *E. coli* CD5 is a PMI-negative mutant defective in colanic acid biosynthesis (6). CF1 is a mucoid *P. aeruginosa* strain isolated from the sputum of a cystic fibrosis patient at the University of Illinois Medical Center. CF1NM is a nonmucoid revertant of strain CF1. Plasmids are described throughout the text. All bacterial strains were grown and maintained as previously described (5). Triparental matings with *E. coli* HB101 harboring the mobilizing plasmid pRK2013 (12) were performed as described by Ruvkun and Ausubel (20).

Preparation of cell-free extracts. For induction experiments, 5-ml cultures were grown overnight and used to inoculate 200 ml of fresh medium (1:100 dilution). Cultures were grown for 1 to 2 h to the early log phase, supplemented with isopropyl β -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and allowed to grow for an additional 3 h. Unless otherwise stated, all subsequent operations were performed at 0 to 4°C. Induced cells were harvested by low-speed centrifugation ($5000 \times g$, 10 min), washed once with 0.9% sodium chloride, and frozen overnight for subsequent use. Thawed cells were suspended in 4 ml of extraction buffer, which contained 50 mM Tris hydrochloride (pH 7.6), 1 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol. Suspended cells were broken by sonication, and extracts were centrifuged at $40,000 \times g$ for 30 min. Pellets were discarded, and supernatant liquids were centrifuged at $105,000 \times g$ for 60 min to remove the remaining cell debris and membranes. The high-speed supernatant liquid was retained for subsequent analyses.

Analyses of PMI activity. (i) **Assay 1.** PMI activity was measured in a spectrophotometric assay system by following M6P conversion to F6P coupled to exogenous phosphoglu-

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cose isomerase and glucose-6-phosphate dehydrogenase by the method of Kang and Markovitz (15) with some modifications. A typical assay mixture contained 25 mM Tris hydrochloride (pH 8.5), 5 mM MgCl₂, 1 mM NADP⁺, 3 mM M6P, 0.5 U of phosphoglucose isomerase, 1.0 U of glucose-6-phosphate dehydrogenase, and 10 to 100 µl of soluble extract to a final volume of 1 ml. Reduction of NADP⁺ by glucose-6-phosphate dehydrogenase was monitored at 340 nm in a Gilford double-beam spectrophotometer at room temperature. Absorbance changes occurring in a reference cell lacking M6P were subtracted during the assay to yield the M6P-dependent activity.

(ii) **Assay 2.** PMI activity was also determined qualitatively by monitoring the direct interconversion of M6P and F6P. Equivalent amounts (25 to 50 µg of protein) of soluble extracts were incubated at room temperature in the presence of 25 mM Tris hydrochloride (pH 8.5)–5 mM MgCl₂—either 25 mM F6P or 25 mM M6P in a final volume of 30 µl. After 20 min, 5 U of alkaline phosphatase was added, and the mixtures were incubated at 37°C for an additional 10 min. Samples (2 to 4 µl) were then spotted on Whatman no. 1 paper and electrophoresed for 45 min at 400 V by using the borate buffering system of Ryder et al. (21) with some modifications. Electrophoresis buffer contained 0.1% ammonium carbonate and 0.05% boric acid. The pH was adjusted to 9.2 with concentrated ammonium hydroxide. Sugars were identified after staining with silver nitrate (23).

Polyacrylamide gel electrophoresis. Denaturing polyacrylamide gel electrophoresis (16) was carried out with a 12.5% acrylamide running gel and a 4.5% acrylamide stacking gel. Samples were denatured by being boiled for 2 min in solubilization buffer containing 10 mM Tris hydrochloride (pH 6.8), 6% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue. Gels were stained with Coomassie blue by the method of Fairbanks et al. (10). Protein was determined by the method of Bradford (3) with bovine serum albumin as the standard.

RNA-DNA dot blot analysis. Total cellular RNA was isolated by a modification of the cesium chloride method of Glisin et al. (13). RNA dot blots were prepared by the method of Thomas (22) with Gene Screen Plus membranes. DNA probes were labeled with [α -³²P]dCTP (3,000 Ci/mmol) by either primer extension of the 2.0-kilobase (kb) *Bam*HI-*Sst*I fragment of plasmid pAD4033 cloned in M13mp19 as template by the method of Burke (4) or random primer extension of the double-stranded 8.0-kb *Eco*RI A fragment of plasmid pVD12 (9) by the method of Feinberg and Vogelstein (11).

Chemicals. Materials from commercial sources included NADP⁺, F6P, M6P, alkaline phosphatase, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, IPTG, and phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.); Gene Screen Plus membranes (Dupont NEN Research Products, Boston, Mass.); *E. coli* rRNA (Boehringer GmbH, Mannheim, Federal Republic of Germany); and [α -³²P]dCTP (Amersham Corp., Arlington Heights, Ill.). The M13 sequencing primer (15mer) and the Klenow fragment of *E. coli* DNA polymerase I were parts of the sequencing kit from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis molecular weight standards were also purchased from Bethesda Research Laboratories.

RESULTS

PMI, the product of the *P. aeruginosa* *algA* gene. Despite efforts to optimize conditions with a coupled spectrophotometric

TABLE 1. Measurement of *P. aeruginosa* PMI activity

Strain (plasmid)	IPTG (mM)	PMI sp act ^a (nmol/min per mg) ^b
<i>P. aeruginosa</i>		
8821 (Mucoid)	0 or 1	<0.1
8822 (Nonmucoid)	0 or 1	<0.1
8830 (Mucoid)	0 or 1	<0.1
PAO1 (Nonmucoid)	0 or 1	<0.1
8821 (pAD4033)	0	0.4
8821 (pAD4033)	1	23.1
8821 (pMMB22)	1	<0.1
<i>E. coli</i>		
CD5 (pAD4033)	0	0.5
CD5 (pAD4033)	1	42.6

^a Average of duplicate determinations.

^b Nanomoles of NADP⁺ reduced by glucose-6-phosphate dehydrogenase per minute per milligram of soluble protein in the coupled spectrophotometric PMI assay (15).

metric assay (15), we were unable to detect PMI activity in *P. aeruginosa* strains (Table 1). To establish the involvement of PMI activity in alginate biosynthesis by *P. aeruginosa*, our approach was to overexpress the cloned gene coding for the putative PMI (6) to overproduce the corresponding gene product.

The recent availability of broad-host-range controlled-expression vectors has made it possible to control the expression of *Pseudomonas* genes in various hosts. The vector pMMB22 contains the strong *tac* promoter as well as the *lacI^q* gene, which codes for a repressor of the *lac* operon (1). Any gene cloned under *tac* promoter control can be overexpressed with the addition of the inducer IPTG to the culture medium. In this study, we overexpressed the gene residing on the 2.0-kb *Bam*HI-*Sst*I fragment cloned into vector pMMB22 (Fig. 1 and Table 1). This construct, pAD4033, was previously used to demonstrate intergeneric complementation of an *E. coli* *manA* (PMI-deficient) mutant (CD5) by restoring the ability of this mutant to produce colanic acid from glucose after IPTG induction (8). When mucoid *P. aeruginosa* 8821 (pAD4033) was grown in the presence of IPTG, high levels of PMI activity were measured in crude cell extracts (Table 1). Similar induction was also observed in the nonmucoid revertant strain 8822 without restoration of the mucoid phenotype (data not shown). The addition of IPTG to cultures of various mucoid and nonmucoid strains or to a culture of mucoid strain 8821 (pMMB22) had no effect on PMI activity.

To rule out the possibility that the *Bam*HI-*Sst*I fragment coded for a product which might indirectly activate the true PMI-coding gene located at another chromosomal site in *P. aeruginosa*, we also induced plasmid pAD4033 in CD5, the *E. coli* *manA* mutant. High levels of PMI activity were also detected in this mutant only after induction with IPTG (Table 1). IPTG induction of plasmid pAD4033 in either *E. coli* or *P. aeruginosa* produced a 56,000-dalton polypeptide, as seen on a sodium dodecyl sulfate-polyacrylamide gel stained with Coomassie blue (Fig. 2). Recent nucleotide sequence analysis of the 2.0-kb *Bam*HI-*Sst*I fragment revealed a single open reading frame translating a protein sequence of approximately 53,000 daltons (8). Thus, the *Bam*HI-*Sst*I fragment (Fig. 1) codes for a single polypeptide with associated PMI activity. We designate this gene *algA*

FIG. 1. Physical map of the alginate gene cluster. Shown are the approximate locations of six alginate complementation groups (I to VI) known to be clustered near the *argF* locus at 45 min on the chromosome of *P. aeruginosa* PAO (7). Plasmid pAD4033 contains the 2.0-kb *Bam*HI-*Sst*I fragment, complementing alginate-negative group I, ligated by using a 100-base-pair (bp) polylinker derived from pCP13 into the *Eco*RI and *Sst*I sites of the *tac-lacI^q* expression vector pMMB22 (8). Abbreviations of restriction endonuclease sites are as follows: B, *Bam*HI; Bg, *Bgl*II; C, *Cl*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I; St, *Sst*I; X, *Xba*I.

(Fig. 1), since PMI has been proposed to catalyze the first step in the alginate biosynthetic pathway.

Catalysis of a reversible reaction by *P. aeruginosa* PMI. The PMI activity measured in the experiments in Table 1 involved the conversion of M6P to F6P in the coupled spectrophotometric assay (15). An alternative system was employed to measure directly the interconversion of F6P and M6P. After incubation of extracts with either M6P or F6P, the addition of alkaline phosphatase to the assay mixture permitted quantitative dephosphorylation of the sugar phos-

phates to their corresponding free sugars. Mannose and fructose were then separated by high-voltage paper electrophoresis (21). After incubation of either F6P or M6P with extracts containing induced levels of the *algA* gene product (Fig. 3, lanes 4 and 8) there was an accumulation of mannose and fructose, respectively. Low-level accumulation of these products was also observed in extracts from uninduced cells (Fig. 3, lanes 3 and 7). The accumulation of mannose or fructose products was not observed in extracts from mucoid strain 8821 (Fig. 3, lanes 1 and 5) or nonmucoid revertant strain 8822 (Fig. 3, lanes 2 and 6). Incubation of *P. aeruginosa* extracts with mannose or fructose alone resulted in no direct interconversion of fructose and mannose. These results are consistent with our observations in the coupled spectrophotometric assay (Table 1) and show that the *algA* gene product catalyzes the interconversion of F6P and M6P.

Transcription of the *P. aeruginosa* *algA* gene. RNA-DNA dot blot analysis of *algA* transcripts from both mucoid and nonmucoid strains revealed no significant increase in the hybridization signal as compared with the background level obtained with rRNA from *E. coli* (Fig. 4A, dots 1 to 5). By contrast, a high level of *algA*-specific mRNA was observed when *algA* was expressed in *P. aeruginosa* 8822 from the *tac* promoter after induction with IPTG (Fig. 4A, dot 7). A lower level of *algA* transcript formation was detectable even without IPTG induction (Fig. 4, dot 6). This may account for

FIG. 2. Overproduction of *P. aeruginosa* PMI. Equivalent amounts (50 μ g) of soluble protein added to each lane were electrophoresed through a sodium dodecyl sulfate-polyacrylamide gel. Lanes: (1), 8821(pAD4033), no IPTG; (2), 8821(pAD4033), 1 mM IPTG; (3), CD5(pAD4033), no IPTG; (4), CD5(pAD4033), 1 mM IPTG. Molecular mass markers (in kilodaltons [kD]) are as follows: 68kD, bovine serum albumin; 45kD, ovalbumin; 26kD, chymotrypsinogen A.

FIG. 3. Interconversion of M6P and F6P by *P. aeruginosa* PMI. Extracts were incubated in the presence of either F6P (lanes 1 to 4) or M6P (lanes 5 to 8). Lanes: 1 and 5, mucoid strain 8821; 2 and 6, nonmucoid strain 8822; 3 and 7, 8821(pAD4033), no IPTG; 4 and 8, 8821(pAD4033), 1 mM IPTG. Shown at right are the relative migration distances of the standards mannose and fructose.

the low level of PMI activity measurable in *P. aeruginosa* extracts without IPTG induction of pAD4033 (Table 1). To rule out the possibility that failure to detect a positive hybridization signal from chromosomal transcripts of *algA* might have been due to technical problems, we hybridized the same RNA preparations with another alginate-specific DNA, the 8.0-kb *EcoRI* A fragment of a chromosomal region located at 19 min on the PAO chromosome and complementing the *alg-22* mutation (5). A portion of this region was previously shown to undergo chromosomal gene amplification in *rec-2 P. aeruginosa*, resulting in a stable mucoid phenotype (9). Results obtained with this DNA as a hybridization probe (Fig. 4B) revealed an apparent constitutive expression of a gene(s) in this region at comparable levels in both mucoid and nonmucoid backgrounds. Thus, although it was possible to detect mRNA transcripts from a chromosomal region harboring at least one other alginate-specific

FIG. 4. RNA-DNA dot blot analysis of *P. aeruginosa* *algA* gene transcription. RNA samples (10 μ g) were *E. coli* rRNA (spots 1) and total cellular RNAs from nonmucoid strain 8822 (spots 2); mucoid strain 8821 (spots 3); mucoid strain CF1 (spots 4); nonmucoid strain CF1NM (spots 5); 8822(pAD4033), no IPTG (spots 6); and 8822(pAD4033), 5 mM IPTG (spots 7). The filter was hybridized with either the 2.0-kb *Bam*HI-*Sst*I fragment of pAD4033 (A) or the 8.0-kb *Eco*RI A fragment of pVD12 (B).

gene and possibly other non-alginate-specific genes, we were unable to detect measurable chromosomal transcription of *algA*.

DISCUSSION

The objective of this work was to determine the functional identity of a gene known to complement an alginate-negative mutation in *P. aeruginosa*. Two factors necessitated this characterization. First, we wished to clarify the discrepancy between previous genetic data suggesting that this gene coded for PMI and the inability to detect PMI activity in association with the cloned gene (6). Second, the alginate biosynthetic pathway in mucoid *P. aeruginosa* has remained largely uncharacterized, and the proposed role of PMI in the alginate biosynthetic pathway was unclear.

The availability of vector pMMB22 allowed the overexpression of the *algA* gene. Measurement of PMI activity in extracts of either *E. coli* or *P. aeruginosa* by two independent assay systems was dependent on the IPTG-mediated expression of *algA* under *tac* promoter control (Table 1 and Fig. 3). These results provide direct biochemical evidence for the identification of this alginate-specific gene (*algA*) as coding for PMI and confirm the proposed role of PMI as an essential enzyme in the alginate biosynthetic pathway of mucoid *P. aeruginosa*.

Previous genetic evidence from our laboratory (6) suggested that the putative *P. aeruginosa* PMI might catalyze a unique unidirectional reaction (i.e., only conversion of F6P to M6P). However, as we report here, the reverse reaction (M6P to F6P) was readily measured by two independent assay systems after overexpression of *algA* (Table 1 and Fig. 3). When the high-voltage paper electrophoresis assay was used, both products (mannose and fructose) appeared to accumulate in comparable amounts in crude extracts incubated with either substrate (M6P or F6P). There was no apparent accumulation of any additional products, suggesting that both M6P and F6P were not significantly metabolized under the conditions of incubation. Based on this result, it appears that *P. aeruginosa* PMI may catalyze a freely reversible reaction. Before this suggestion can be firmly established, however, a thorough kinetic characterization of the purified enzyme must be performed. As demonstrated in this report, our ability to overproduce *P. aeruginosa* PMI to high levels (Fig. 2) and to assay PMI activity in extracts of *P. aeruginosa* (Table 1 and Fig. 3) should allow the rapid isolation of the purified enzyme.

The observed difficulty in measuring PMI activity in *P. aeruginosa* appears to be due to the low-level expression of *algA* (Fig. 4). Thus, *algA* may be expressed in mucoid *P. aeruginosa* at a low level which provides a sufficient amount of PMI activity for alginate production but not for detection by RNA-DNA dot blot or enzyme assay. The *algA* gene is not the only point of potential transcriptional regulation in the alginate biosynthetic pathway in *P. aeruginosa*. This conclusion is underscored by the observation that when plasmid pAD4033 was mobilized into nonmucoid strain 8822, overproduction of PMI was not sufficient to render the cells mucoid. Recent evidence from our laboratory, however, suggests that another alginate biosynthetic gene (*algD*), located approximately 15 kb from *algA* (Fig. 1) and coding for GDP-mannose dehydrogenase, is highly transcribed from the chromosome of mucoid *P. aeruginosa* but remains unexpressed in nonmucoid revertants (V. Deretic, J. F. Gill, and A. M. Chakrabarty, submitted for publication). Thus, transcriptional control of alginate biosynthesis in mucoid *P. aeruginosa* appears to be exerted at a point beyond *algA*.

We have focused here on the overproduction and assay of *P. aeruginosa* PMI. Using the same approach as that described above, we have been able to overexpress and identify the product of the GDP-mannose dehydrogenase (*algD*) gene (Deretic et al., in preparation). Work is under way to isolate other DNA fragments complementing mutations near *algA* and *algD* in the alginate gene cluster (Fig. 1) to overexpress the genes present on these fragments and determine their functional identities. This approach will provide the foundation for elucidating the precise mechanism(s) by which alginate biosynthesis is controlled in mucoid strains of *P. aeruginosa*.

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