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Purification and Characterization of Phosphomannomutase/Phosphoglucomutase from *Pseudomonas aeruginosa* Involved in Biosynthesis of Both Alginate and Lipopolysaccharide

RICK W. YE, NICOLETTE A. ZIELINSKI, AND A. M. CHAKRABARTY*

Department of Microbiology and Immunology, University of Illinois at Chicago College of Medicine, Chicago, Illinois 60612

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The *algC* gene from *Pseudomonas aeruginosa* has been shown to encode phosphomannomutase (PMM), an essential enzyme for biosynthesis of alginate and lipopolysaccharide (LPS). This gene was overexpressed under control of the *tac* promoter, and the enzyme was purified and its substrate specificity and metal ion effects were characterized. The enzyme was determined to be a monomer with a molecular mass of 50 kDa. The enzyme catalyzed the interconversion of mannose 1-phosphate (M1P) and mannose 6-phosphate, as well as that of glucose 1-phosphate (G1P) and glucose 6-phosphate. The apparent K_m values for M1P and G1P were 17 and 22 μM, respectively. On the basis of K_{cat}/K_m ratio, the catalytic efficiency for G1P was about twofold higher than that for M1P. PMM also catalyzed the conversion of ribose 1-phosphate and 2-deoxyglucose 6-phosphate to their corresponding isomers, although activities were much lower. Purified PMM/phosphoglucomutase (PGM) required Mg^{2+} for maximum activity; Mn^{2+} was the only other divalent metal that showed some activation. The presence of other divalent metals in addition to Mg^{2+} in the reaction inhibited the enzymatic activity. PMM and PGM activities could not be detected in nonmucoid *algC* mutant strain 8858 and in LPS-rough *algC* mutant strain AK1012, while they were present in the wild-type strains as well as in *algC*-complemented mutant strains. This evidence suggests that AlgC functions as PMM and PGM in vivo, converting phosphomannose and phosphoglucose in the biosynthesis of both alginate and LPS.

Pseudomonas aeruginosa causes severe and debilitating pulmonary infections of children and young adults afflicted with cystic fibrosis (CF). *P. aeruginosa* isolated from the respiratory tracts of CF patients switches from a nonmucoid form to a mucoid, alginate-producing form upon progression of the disease (24). Alginate encapsulation is believed to protect the infecting bacterial cells from phagocytosis, as well as from antibiotic therapy. Alginate is a partially O-acetylated, linear copolymer of D-mannuronate and L-guluronate linked via β-1,4-glycosidic bonds (10). Fructose 6-phosphate was identified as an alginate precursor for the *P. aeruginosa* biosynthetic pathway and appears to be recruited from the carbohydrate pool via the Entner-Doudoroff pathway with the participation of fructose 1,6-bisphosphate aldolase (2). Fructose 6-phosphate is converted to mannose 6-phosphate (M6P), which is subsequently converted to mannose 1-phosphate (M1P), leading to the formation of GDP-mannose and GDP-manuronic acid. A bifunctional enzyme, phosphomannose isomerase (PMI)-guanosine diphosphomannose pyrophosphorylase (GMP), is responsible for the first and third steps of the reaction (34) while phosphomannomutase (PMM) carries out the second step of the reaction (25, 39). GDP-mannose dehydrogenase carries out the fourth step of the pathway (31). Very little is known about the polymerization of GDP-mannuronate to form the alginate polymer. A C_5 mannuronate epimerase has recently been isolated and shown to use polymannuronate as a substrate, converting some mannuronate residues to its C_5 epimer, L-guluronic acid (11). Modification of alginate by acetylation is under the control of the *algF* gene, which encodes an acetylase (12, 35). Substrate specificities, functional requirements, and critical structural domains for substrate binding, catalysis, and interaction with the cofactor NAD have been characterized in detail for the two initial enzymes, PMI-GMP and GDP-mannose dehydrogenase (23, 30), while very limited information on these aspects has been obtained for the enzyme PMM.

In addition to alginate, lipopolysaccharide (LPS) is another virulence factor of *P. aeruginosa*. Two distinct forms of LPS, the A and B bands, have been characterized. A-band LPS (D-rhamnan polysaccharide common antigen) consists mainly of a repeating trisaccharide of α-D-rhamnose, with smaller amounts of 3-*O*-methylrhamnose, ribose, mannose, glucose, and a 3-*O*-methylhexose (1). B-band LPS contains the O antigen (O side chain; 14, 17). A-band LPS is antigenically conserved, while B-band LPS is serologically variable. It has been shown that B-band LPS is a major virulence factor (3–5). Mutants devoid of O antigen were considerably more sensitive to serum components and phagocytic effects than their parent strains were. It has been recently demonstrated that the enzyme PMM is involved in LPS synthesis in *P. aeruginosa* PAO1 (13). Studies with LPS-rough mutant strain AK1012 showed a lack of glucose and rhamnose residues in the LPS and that the probable block in LPS synthesis was in the attachment of glucose or rhamnose residues to the galactosamine in the LPS core (15). In addition, strain AK1012 did not produce the O side chain of LPS. Upon introduction of the *algC* gene from the wild type into this mutant, the LPS-smooth phenotype was restored (13). It has also been shown that conversion of GDP–D-mannose to GDP-rhamnose is required for synthesis of A-band LPS (20). Mutant strain rd7513 isolated from *P. aeruginosa* had a deficiency in A-band LPS synthesis. Biochemical analysis indicated that the mutation blocked the conversion of GDP–D-mannose to GDP-rham-

* Corresponding author. Mailing address: Dept. of Microbiology & Immunology (M/C 790), University of Illinois at Chicago College of Medicine, 901 S. Wolcott Avenue, Chicago, IL 60612. Phone: (312) 996-4586. Fax: (312) 996-6415.

nose. This result suggested that there is a potential link between the alginate and LPS biosynthesis pathways with a common intermediate such as GDP-mannose (20).

Hyperexpression of the *algA* gene, which encodes the PMI-guanosine diphosphomannose pyrophosphorylase bifunctional enzyme, results in an increase in levels of PMM activity (32). This led to the identification of nonmucoid strain 8858, which lacked PMM activity (39). The PMM-encoding gene *algC* was thus isolated by complementing strain 8858 to mucoidy. The amino acid sequence of PMM exhibits some similarities to that of rabbit muscle phosphoglucomutase (6), an enzyme that catalyzes the interconversion of glucose 1-phosphate (G1P) and glucose 6-phosphate (G6P). These similarities include the active site of phosphorylation, a metal ion-binding pocket, and an active-site flap (6). However, there is no detailed comparison between these two enzymes in terms of enzyme kinetics, substrate specificity, and cofactor requirements. Furthermore, many genes encoding PMM/phosphoglucomutase (PGM) activities from gram-negative bacteria have now been isolated and their gene products have been shown to have strong homology to the AlgC amino acid sequence (16, 18, 22, 33, 36). However, information regarding the characteristics of this group of enzymes is limited.

In this report, we describe the purification and characterization of PMM from *P. aeruginosa*. We found that this enzyme can efficiently use both phosphomannose and phosphoglucose as substrates and thus may participate in the biosynthesis of alginate as well as that of LPS. We determined its kinetic parameters, substrate specificities, and requirements for metal ions for activation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. $AlgC^-$ LPS rough strain AK1012, a derivative of *P. aeruginosa* PAO1, was kindly provided by Joanna Goldberg. All of the other bacterial strains and plasmids used in this study have been described previously (39). *P. aeruginosa* 8821 is a mucoid (alginate-producing) strain isolated from a CF patient, and strain 8830 is a stable alginate-producing mutant (7). Strain 8858 is an *algC* nonmucoid mutant isolated by chemical mutagenesis of strain 8830. Broad-host-range plasmid pMMB 66HE (9) was used for overexpression of the *algC* gene. Plasmid pMMB66HE contains an ampicillin resistance marker, the RSF1010 replicon, and the *tac* promoter upstream of the multiple cloning site. Thus, overexpression of the cloned gene can be achieved in *P. aeruginosa* upon induction by isopropyl-β-D-thiogalactopyranoside (IPTG). Luria broth was used for liquid culture. Solid media used were LB broth solidified with 1.5% agar for *E. coli* and *Pseudomonas* isolation agar (Difco) supplemented with 4 g of agar per liter for *P. aeruginosa*. Cultures were grown at 37°C and aerated with constant shaking (250 rpm) for liquid cultures. Antibiotic concentrations used for plasmid-containing strains were 350 μg of carbenicillin per ml for *P. aeruginosa* and 100 μg of ampicillin per ml for *E. coli*.

Crude extract preparation. The 1.8-kb DNA fragment containing the *algC* gene has previously been cloned into the *Hin*dIII-*Eco*RI sites of plasmid pMMB66HE, resulting in plasmid pNZ49 (39). The *algC* gene was under control of the *tac* promoter in this plasmid. To prepare a crude extract for PMM purification, a 10-ml overnight culture of *P. aeruginosa* 8858 containing pNZ49 was inoculated into 500 ml of LB broth in a 2-liter flask. Cells were induced to produce PMM by addition of IPTG at a 1 mM final concentration after 3 h of incubation. Cells were then harvested after an additional 5 h of growth. Cell pellets were washed twice with 100 mM MOPS buffer (pH 7.3) and suspended in the same buffer containing 1 mM dithiothreitol. Cells were disrupted by sonication, and the crude extract was obtained after centrifugation at 150,000 × *g* for 1 h. Crude extracts for enzymatic assays without IPTG induction were prepared by the same procedure, except that cells were harvested after overnight growth. As a standard procedure, crude extracts were treated with 1 mM EDTA before enzymatic assays to chelate trace amounts of metal ions bound to PMM or in solution.

Enzymatic assays. The activities of PMM and PGM were measured with either coupling assays or the direct assay for P_i. The coupling assay method provides a quick, accurate, and easy way to monitor the progress of the reaction by recording the increase in A_{340} due to NADPH formation. This is especially convenient for kinetic studies, since initial rates can be determined even at low substrate concentrations. The direct assay method is not reliable at low substrate concentrations. The coupling assays for PMM activities measured M6P formation from M1P by coupling the reaction to the reduction of NADP by G6P dehydrogenase via the activities of PMI and phosphoglucose isomerase (26, 27, 29, 32). For PGM activity, G6P formation from G1P was measured with G6P dehydrogenase as the only coupling enzyme (26). The standard reaction mixture in 1 ml of solution contained the following: 50 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.3), 5 mM $MgCl_2$, 10 μM glucose 1,6-diphosphate, 1.0 mM M1P or G1P, 1.0 mM NADP, and 1 U of each of the coupling enzymes. All coupling enzymes were purchased from Sigma and washed with Centricon concentrators before use. The reaction was monitored at 340 nm with a Gilford Response II spectrophotometer. One unit of enzymatic activity is defined as the amount required for the formation of 1.0 μmol of NADPH per min per mg of protein.

The direct assay method was based on the change in the concentration of the acid-labile phosphate at the carbon-1 position of phosphosugars. The amount of acid-labile phosphate was determined by the method of Fiske and SubbaRow (8) with a kit for P_i from Sigma. The standard direct assay mixture in a 1-ml volume contained 50 mM MOPS buffer (pH 7.0), 1 mM substrate, 10 μM glucose 1,6-diphosphate, and 5 mM $MgCl_2$. Aliquots of 150 or 200 μl were taken at different time points and added to equal amounts of 2 N HCl in 1.5-ml microcentrifuge tubes to stop the reaction. Samples were heated at 100°C for 10 min. After a brief centrifugation, the volume was adjusted to 1 ml. The A_{660} was measured after addition of 200 μl of an acid molybdate solution and 50 μl of Fiske and SubbaRow reducer. One unit of activity is defined as the amount required for the release of 1.0 μmol of phosphate per min per mg of protein. All of the results shown are averages of triplicate measurements.

Purification of PMM. (i) Q-Sepharose column. Crude extracts from 1 liter of cells were applied to a Q-Sepharose column (2 by 7 cm) which had been equilibrated with 100 mM MOPS buffer (pH 7.3) containing 0.5 mM dithiothreitol. The flow rate was 1 ml/min. After being washed with 30 ml of the same buffer, the column was eluted with a linear gradient of NaCl (0 to 300 mM) in a total volume of 80 ml. Fractions with PMM activity were pooled.

(ii) Phenyl Superose column. Solid ammonium sulfate (high-pressure liquid chromatography grade) was added to the pooled active fractions (1.2 g/10 ml) and mixed immediately. EDTA was added to a final concentration of 0.1 mM to chelate trace amounts of metals from the ammonium sulfate. The sample was then applied to a phenyl Superose column that had previously been equilibrated with 100 mM MOPS buffer

containing ammoniun sulfate (1.2 g/10 ml, pH 7.3). After the column was washed with 20 ml of equilibration buffer, the protein was eluted with a linear decreasing gradient of ammonium sulfate (1.2 g/ml to 0 g). The flow rate was 0.5 ml/min. Active fractions were pooled.

(iii) Superdex 75 gel filtration column. A Superdex 75 gel filtration column was equilibrated with 50 mM MOPS buffer (pH 7.3) containing 1 mM EDTA or 10 mM $MgCl_2$, depending on the experiment. The flow rate was 0.2 ml/min. The pooled fractions from the phenyl Superose step were concentrated with Centricon concentrators and then applied to the gel filtration column (200 μl for each run).

Molecular weight determination. The subunit molecular weight was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the molecular weight of the native protein was determined by fast protein liquid chromatography with a Superdex 75 gel filtration column. The protein standards for gel filtration were purchased from Pharmacia. They were RNase A (M_r 13,700), chymotrypsinogen A (M_r, 25,000), ovalbumin (M_r, 43,000), bovine serum albumin (M_r 67,000), and blue dextran 2000 (M_r, 2,000,000).

Kinetic studies. The K_m and V_{max} values for substrates were determined from a Lineweaver-Burke plot. K_{cat} values were calculated on the basis of a molecular mass of 50 kDa for the enzyme PMM/PGM. Thus, K_{cat} values (minute^{-1}) were obtained by dividing the V_{max} (micromoles minute^{-1} milligram^{-1}) by 0.02 to convert the protein concentration to molarity. The initial reaction rates for M1P and G1P were measured with the coupling assay system. The equilibrium constants were determined by the product/substrate concentration ratios when the reaction reached equilibrium in the direct assay method.

To determine the K_m value of $MgCl_2$, concentrations ranging from 0.2 to 2.0 mM were used. The K_m value was calculated by linear regression of the plot. To determine the K_i of $CaCl_2$ inhibition, the same range of concentrations of $MgCl_2$ was used in addition to 0.1 mM $CaCl_2$.

Site-directed mutagenesis. The 1.8-kb *Hin*dIII-*Eco*RI fragment containing *algC* was subcloned into pUC118 to generate single-stranded DNA. The primer used to change the putative active-site serine to alanine was 5′-GATGCTGACCGGCGC CCACAATCCG-3′. The in vitro mutagenesis system was purchased from Amersham Life Science. The mutation was confirmed by DNA sequencing.

Isolation of LPS and immunoblotting. The pellet from a 5-ml culture was lysed in 100 μl of 60 mM Tris-HCl (pH 6.8)–1% SDS by being heated at 100°C for 5 min and then diluted with the same buffer without SDS. Benzonase (400 U) was added and incubated at 37°C for 5 h to digest DNA and RNA. Proteinase K was added to a final concentration of 50 μg/ml, and the incubation was allowed to proceed overnight. Samples of 50 μl were taken and blotted onto a nitrocellulose filter. The immunoblot was developed with A-band-specific monoclonal antibody N1F10, provided kindly by J. S. Lam (19).

RESULTS

Purification of PMM/PGM. Upon induction of the *algC* gene under control of the *tac* promoter in pMMB66HE, PMM activity was greatly increased, facilitating PMM purification. A summary of the purification procedure is shown in Table 1. Purification of the enzyme was initiated with an ion-exchange column, which was followed by hydrophobic interaction and gel filtration chromatography. The enzyme was purified about 12-fold and was found to be capable of using both M1P and G1P as substrates. The purified enzyme migrated as a single polypeptide with a molecular mass of 52 kDa under denaturing conditions (Fig. 1). The native protein was estimated to have a molecular mass of 47 kDa as estimated by fast protein liquid chromatography with a Superdex 75 gel filtration column. The molecular mass calculated from the amino acid sequence was 50 kDa (39). Thus, the native enzyme is a monomer.

TABLE 1. Purification of PMM/PGM from *P. aeruginosa*[a]

Purification step	Protein (mg)	Total activity (U)		Sp act (U/mg)		Yield (%)	Purification (fold)
		G1P	M1P	G1P	M1P		
Crude extract	198	475	178	2.4	0.9	100	
Q-Sepharose	33	251	86	7.6	2.6	50	3
Phenyl Superose	5.7	123	41	21.6	7.2	25	9
Superdex 75	2.6	73	30	28	11.4	16	12

[a] Enzymatic activities were measured for both G1P and M1P with coupling assays. Yield and fold purification calculations were based on activities with both substrates.

Effect of EDTA. Rabbit muscle PGM has been shown to be activated by metal-chelating agents, such as EDTA, apparently by binding of inhibitory metal ions (28). We found that treatment with EDTA greatly increased the enzymatic activity of PMM for both M1P and G1P. This enhanced effect was found in crude extracts, as well as in collected fractions from different purification steps. Thus, EDTA treatment was routinely used for most of the enzymatic assays. Enzyme preparations frozen and stored at −70°C and then thawed also required addition of EDTA to restore activity.

Metal ion requirements and inhibition. The requirement for

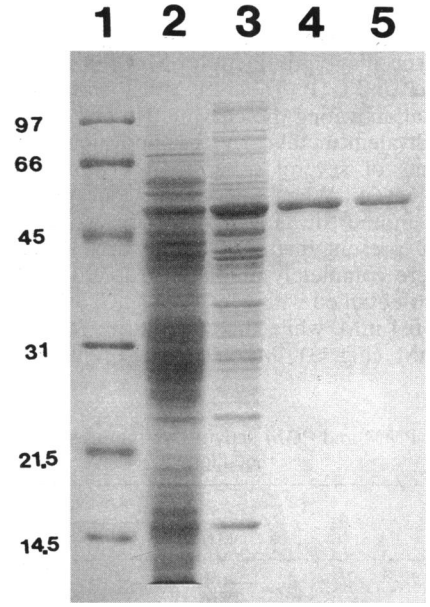

FIG. 1. Coomassie blue staining of proteins separated by SDS-polyacrylamide gel electrophoresis during the purification of PMM/PGM from *P. aeruginosa*. Samples were electrophoresed on a 10% polyacrylamide gel. Lanes: 1, molecular mass markers in kilodaltons; 2, crude extract; 3, pooled fractions from a Q-Sepharose column; 4, pooled fractions from a phenyl Superose column; 5, pooled fractions from a Superdex 75 column.

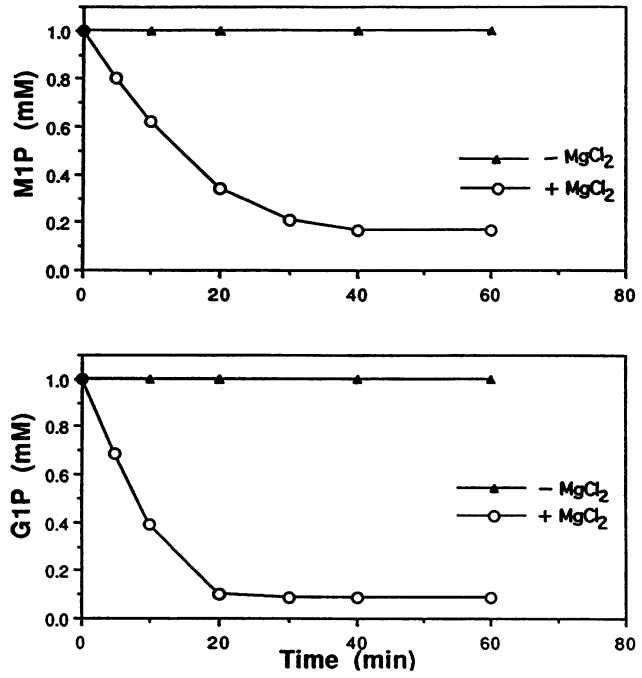

FIG. 2. Time course of the PMM/PGM enzymatic reaction with M1P or G1P as the substrate. The standard direct assay procedure described in Material and Methods was used for estimation of the M1P or G1P remaining in the reaction mixture. The amounts of the enzyme used were 2 and 3 μg, respectively, for M1P and G1P. The concentration of $MgCl_2$ was 5 mM.

metal ions for enzymatic activity was determined with the direct assay to avoid complications due to coupling enzymes. PMM from *P. aeruginosa* showed no activity when treated with EDTA without addition of 5 mM $MgCl_2$ (Fig. 2). The enzyme exhibited a specific requirement for $MgCl_2$ for optimal activity for both M1P and G1P (Table 2). $MnCl_2$ was only about 10% as effective at activating the enzyme. No activity was detected with other divalent metals or with monovalent Li^+.

The effects of several other metals were tested in the presence of $MgCl_2$. When equal molar concentrations of both $MgCl_2$ and another divalent metal, such as $NiCl_2$, $CoCl_2$, or $ZnCl_2$, were present in the assay mixture, PMM and PGM activities were completely inhibited (Table 3). Strong inhibition was also obtained with $MnCl_2$ or $CaCl_2$. The K_m value of $MgCl_2$ was 0.4 mM, while the competitive K_i value of $CaCl_2$ was 0.01 mM (Fig. 3). Monovalent Li^+ had only a slight

TABLE 2. PMM and PGM activities in the presence of different metal ions[a]

Metal	Activity (%)	
	M1P	G1P
Mg^{2+}	100	100
Mn^{2+}	10	8
Ca^{2+}	0	0
Co^{2+}	0	0
Ni^{2+}	0	0
Zn^{2+}	0	0
Li^+	0	0

[a] The metal ion concentration used was 5 mM. Disappearance of M1P or G1P in a direct assay system was measured.

TABLE 3. Inhibition PMM and PGM activities by different metal ions[a]

Metal(s)	Activity (%)	
	M1P	G1P
Mg^{2+} only	100	100
Mg^{2+}, Mn^{2+}	15	11
Mg^{2+}, Ca^{2+}	3	2
Mg^{2+}, Co^{2+}	0	0
Mg^{2+}, Ni^{2+}	0	0
Mg^{2+}, Zn^{2+}	0	0
Mg^{2+}, Li^+	66	74

[a] The metal ion concentration used was 5 mM. Disapparence of M1P or G1P in the direct assay system was measured.

inhibitory effect. These results suggest a strong inhibitory effect of divalent metals on PMM/PGM activity and explain the enhanced activity after EDTA treatment under standard assay conditions in the presence of $MgCl_2$.

Kinetic properties and substrate specificities. The purified enzyme catalyzed the interconversion of M1P and M6P, as well as that of G1P and G6P. To compare the reactivity of this enzyme towards these two substrates, kinetic paramaters were measured. Similar apparent K_m values for M1P and G1P were found (Table 4). Low K_m values were also observed with PMI-GMP and GDP-mannose dehydrogenase (31, 34), which are involved in the initial steps of alginate biosynthesis, suggesting that these enzymes can function at low substrate concentrations. The K_{cat} value of PMM/PGM for G1P was higher than it was for M1P. Thus, the catalytic efficiency based on the K_{cat}/K_m ratio was about twofold higher for G1P than it was for M1P. The equilibrium constants were 10 for G1P and 5 for M1P when they were used as the starting substrates, indicating that the reaction conditions used favored the formation of G6P or M6P.

Several other phosphosugars were tested as substrates with the direct assay system (Table 5). Activities were detected with D-ribose 1-phosphate and 2-deoxyglucose 6-phosphate, but

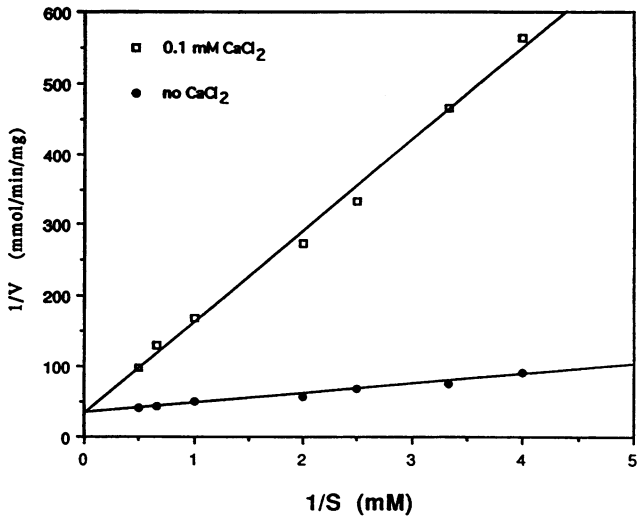

FIG. 3. Double-reciprocal plot of enzyme activity as a function of $MgCl_2$ concentration with and without $CaCl_2$. The best-fit equation for data obtained with $MgCl_2$ alone was $1/V = 34 + [13.4(1/S)]$, and it was $1/V = 33 + [129(1/S)]$ with the presence of 0.1 mM $CaCl_2$.

TABLE 4. Kinetic parameters of PMM/PGM

Substrate	K_m (μM)	K_{cat} (min^{-1})[a]	K_{cat}/K_m (min^{-1} μM^{-1})	K_{eq}[b]
M1P	17	1,350	79	5
G1P	22	3,000	136	10

[a] Substrate concentrations ranging from 5 to 75 μM were used to determine K_m and V_{max}. K_{cat} values were calculated on the basis of PMM/PGM as a 50-kDa monomer, and its V_{max} of M1P and G1P were 27 and 60 μmol min^{-1} mg^{-1}, respectively.

[b] K_{eq} is based on the C-6 phosphate/C-1 phosphate ratio when the reaction reached equilibrium as shown in Fig. 2.

these activities were much lower than those found with M1P or G1P. No activities were detected with the rest of the phosphosugars tested.

PMM and PGM activities in different mutants. It has been demonstrated that LPS-rough mutant AK1012 has a glucose and rhamnose residue deficiency in its LPS and lacks the O side chain (B band; 13, 15). This deficiency is caused by a mutation in the *algC* gene (13). To test the possibility that the enzyme PMM/PGM is responsible for converting phosphomannose as well as phosphoglucose in vivo, the activities of both PMM and PGM were measured in *algC* mutants. As shown in Table 6, neither PMM nor PGM activity was detected in the crude extract prepared from mutant strain AK1012. However, activities were present in wild-type strain PAO1. Activities were also found in *algC*-complemented strain AK1012. Similarly, no PMM and PGM activities were detected in nonmucoid *algC* mutant strain 8858. The activities were found in wild-type strain 8830 (Table 6). PMM and PGM activities were simultaneously restored upon introduction of the *algC* gene into mutant strain 8858. These results indicate that AlgC functions as PMM and PGM in vivo.

To further study the structure and function of PMM/PGM, the conserved active-site amino acid residue, serine-107, was changed to alanine by site-directed mutagenesis. The mutant *algC* gene was then subcloned into pMMB66HE, resulting in clone pSA107. The mutant protein lost both PMM and PGM activities (Table 6), suggesting similar reaction mechanism modes for both substrates.

In an immunodot blot developed with A-band-specific monoclonal antibody N1F10, no A-band LPS was detected in LPS isolated from mutant strain AK1012. In contrast, A-band LPS was detected in LPS isolated from both wild-type strain PAO1 and strain AK1012 complemented with *algC* in pNZ49 (Fig. 4), suggesting the involvement of *algC* in the biosynthesis of A-band LPS.

TABLE 5. Substrate specificity of purified PMM/PGM from *P. aeruginosa*[a]

Substrate	Sp act (U/mg)
G1P	33
M1P	13
D-ribose 1-phosphate	0.38
2-Deoxyglucose 6-phosphate	0.25
Fructose 1-phosphate	BD[b]
Galactose 1-phosphate	BD
D-arabinose 5-phosphate	BD
Glucosamine 6-phosphate	BD
N-Acetyl-α-D-glucosamine 1-phosphate	BD

[a] The substrate concentration used was 1.0 mM, and the direct assay method was used to measure enzymatic activity.

[b] BD, below detection limit (0.01 U/min).

TABLE 6. PGM and PMM activities in crude extracts from mutant and wild-type strains of *P. aeruginosa*[a]

Strain	Sp act (mU/mg)	
	PGM	PMM
PAO1[b]	24 ± 2	10 ± 1
AK1012[b]	BD[c]	BD
AK1012(pNZ49)	792 ± 62	334 ± 18
8830[b]	258 ± 22	100 ± 11
8858[b]	BD	BD
8858(pNZ49)	1,060 ± 50	371 ± 17
8858(pSA107)[d]	BD	BD

[a] Cells were inoculated into 100 ml of LB medium and grown overnight at 37°C for 16 h. Crude extracts were prepared as described in Materials and Methods. The amount of crude extract used in the coupling assay was 10 to 50 μl, depending on the activity.

[b] Strain AK1012 is a rough-LPS $AlgC^-$ mutant of wild-type *P. aeruginosa* PAO1 (13, 15). Strain 8830 is a mucoid *P. aeruginosa* isolated from the lungs of a CF patient (7). Strain 8858 is a nonmucoid $AlgC^-$ mutant derived from strain 8830 (39).

[c] BD, below detection limit (1.0 mU/mg).

[d] Plasmid pSA107 harbors the mutated *algC* gene with a change of residue 107 from serine to alanine. This serine residue is believed to be the active site on the basis of homology comparison to the rabbit muscle PGM sequence (Fig. 5).

DISCUSSION

The PMM/PGM enzyme isolated from *P. aeruginosa* is very similar to the PGM enzyme from rabbit muscle (28). Although the overall homology between the amino acid sequences of these two enzymes is marginal, critical functional domains are conserved (6, 39). These include the active-site serine residue, the metal-binding pocket, and the active-site flap (Fig. 5). These structural similarities are consistent with the biochemical characteristics of both enzymes. Both require glucose 1,6-diphosphate as the cofactor and Mg^{2+} for maximum activity. They can use phosphoglucose, phosphomannose, and phosphoribose as substrates. The reaction equilibria for both enzymes are most favorable for conversion of C_1 phosphosugars to C_6 phosphosugars. However, the enzymes differ in their activities towards G1P and M1P. A high K_{cat}/K_m ratio usually reflects high catalytic efficiency of an enzyme towards a particular substrate. PGM from rabbit muscle has a much higher K_{cat}/K_m value for G1P (2,550 min^{-1} μM^{-1}) than for M1P (5 min^{-1} μM^{-1}), suggesting that this enzyme is very specific for G1P (21). The K_{cat}/K_m values of PMM/PGM encoded by *algC*

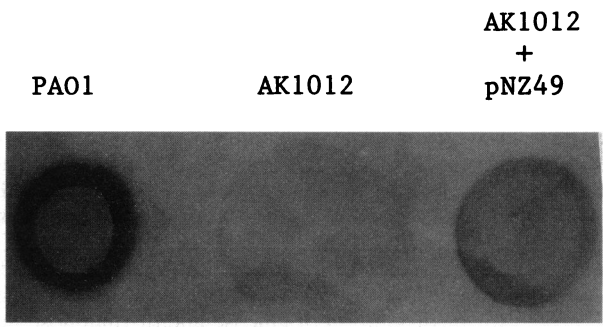

FIG. 4. Immunodot blot of LPS from wild-type *P. aeruginosa* PAO1, mutant strain AK1012, and strain AK1012 complemented with *algC*-containing plasmid pNZ49. The blot was developed with A-band-specific monoclonal antibody N1F10 and a goat anti-mouse antibody conjugated with alkaline phosphatase.

	Function	Active site	Mg binding	Sugar binding
PGM (Rm)	glycogen	GIILTASHNP	DFGAAFDGDGDR	GEESFG
PMM (Pa)	Alg/LPS	GVMLTGSHNP	DLGLAFDGDGDR	GEMSG
XanA(Xa)	Xanthan	GVMVTASHNP	DFGIAWDGDFDR	GEMSA
CpsG(Se)	M antigen	AIMVTGSHIP	DAIFSTDGDGDR	GEMSA
RfbK(St)	O antigen	GIEVTASHNP	DMGIAFDGDFDR	GEMSA
RfbK(Ec)	O antigen	GIEVTASHNP	DMGIAFDGDFDR	GEMSA
PGM (Ng)	LPS	GVMITGSHNP	EIGLAFDGDADR	GEMSG
RfbB(Vc)	LPS	GIEVTASHNP	DMGIAFDGDFDR	GEMSA

FIG. 5. Comparison of conserved motifs among phosphomutases. Abbreviations: Rm, rabbit muscle; Pa, *P. aeruginosa*; Xa, *X. campestris*; Se, *S. enterica*; St, *S. typhimurium*; Ec, *E. coli*; Ng, *N. gonorrhoeae*; Vc, *V. cholerae*. Definitions of various domain structures are given in reference 6.

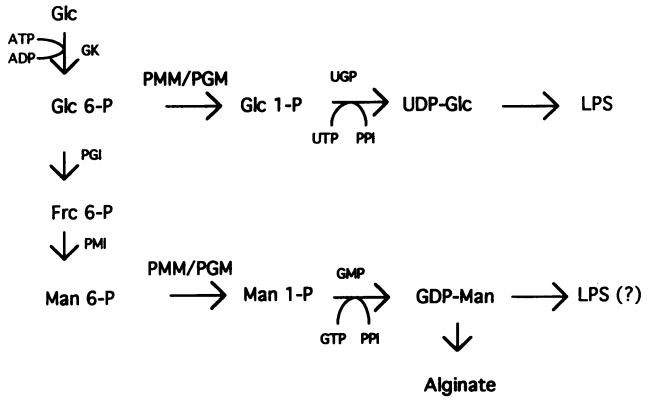

FIG. 6. Proposed roles of PMM/PGM in the biosynthesis of alginate and LPS. Abbreviations: Glc, glucose; GK, glucose kinase; Glc 6-P, G6P; Glc 1-P G1P; UDP-Glc, UDP-glucose; UGP, UDP-glucose pyrophosphorylase; PGI, phosphoglucose isomerase; Frc 6-P, fructose 6-phosphate; Man 6-P, M6P; Man 1-P, M1P; GDP-man, GDP-mannose; GMP, GDP-mannose pyrophosphorylase.

were 79 min^{-1} μM^{-1} for M1P and 136 min^{-1} μM^{-1} for G1P. There is only a 2-fold difference instead of the 500-fold difference found with PGM. Thus, PMM/PGM from *P. aeruginosa* can efficiently catalyze the isomerization of both phosphoglucose and phosphomannose, although the activity is higher for phosphoglucose. The other difference between these two enzymes is that rabbit muscle PGM can be activated by Ni^{2+} and Co^{2+} but PMM/PGM from *P. aeruginosa* cannot. Reasons for this difference in divalent metal requirements are unknown.

Besides AlgC, both PMM and PGM activities have been demonstrated in XanA from *Xanthomonas campestris* (18) and in PGM from *Neisseria gonorrhoeae* (33). The amino acid sequences of these proteins are similar. Similarities were also found in other genes encoding PGM and/or PMM from *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio cholerae* (16, 22, 36, 37). These proteins may form a subclass of phosphohexomutases. The PGM enzymes from *Agrobacterium tumefaciens* (ExoC; 38) and *Acetobacter xylinum* are very similar to the PGM enzymes from rabbit muscle and humans and may form another subclass. This subclass may be very specific for phosphoglucose. These two subclasses share active sites in domains I and II, as mentioned above, but there is little similarity in domains III and IV, which may control substrate specificity. The loop interacting with the sugar ring in rabbit muscle PGM is GEESFG in domain III. The corresponding loop in the PMM/PGM subclass of phosphohexose could be GEMSG (Fig. 5). It is interesting that Arg-420, the site mutated in *P. aeruginosa* 8858 (39), is conserved in the PMM/PGM subclass and may be responsible for interaction with the phosphate group of the substrate, similar to the functions of the arginine residues in domain IV of rabbit muscle PGM. Very little information is available regarding the potential third subclass of phosphohexomutases from bacteria that are only specific for phosphomannose.

The dual functions of AlgC as PMM and PGM may have important physiological roles in the synthesis of alginate and LPS. This is supported by the fact that *algC* mutant strain AK1012 showed a lack of glucose and rhamnose residues in its LPS (15). Furthermore, this mutant exhibits a rough phenotype due to a deficiency of the O side chain. The core LPS usually contains 2-keto-3-deoxyoctonate, heptose, glucose, rhamnose, *N*-acetylgalactosamine, and alanine (29). $AlgC^-$ mutant strains have been shown to be blocked in the attachment of glucose residues to the core (13). Thus, the gene *algC* is required for incorporation of glucose and/or rhamnose into the growing core of LPS. It is possible that the PGM activity of AlgC provides phosphoglucose for this incorporation and for other part of LPS. It has been demonstrated that the major component of A-band LPS is D-rhamnose (1). Studies with a monoclonal antibody specific for A-band LPS further support the role of AlgC in the biosynthesis of A-band LPS (Fig. 4). Conversion of GDP–D-mannose to GDP-rhamnose has been shown to be one of the steps leading to the biosynthesis of A-band LPS (20). Figure 6 summarizes the roles of PMM/PGM (AlgC) in the synthesis of alginate and the synthesis of LPS in *P. aeruginosa*. It is worth noting that PMM/PGM can also use phosphoribose (Table 5) and this conversion could have a role in LPS synthesis since a small amount of ribose is also present.

Alginate production is triggered by unique environmental signals in CF-afflicted lungs, such as nutritional starvation, dehydration, and high osmolarity. In this environment, the O antigen is often no longer expressed in mucoid strains (19). It is unknown whether other enzymes besides PMM involved in the formation of GDP-mannuronic acid during alginate production participate in the synthesis of LPS. Similarly, whether the regulatory components for alginate biosynthesis also control the expression of LPS remains to be determined. Thus, it is worthwhile to further study the potential links between alginate biosynthesis and LPS biosynthesis.

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REFERENCES

1. **Arsenault, T. L., D. W. Hughes, D. B. MacLean, W. A. Szarek, A. M. B. Kropinski, and J. S. Lam.** 1991. Structural studies on the polysaccharide portion of "A-band" lipopolysaccharide from a mutant (AK1410) of *Pseudomonas aeruginosa* strain PAO1. Can. J. Chem. **69:**1273–1280.
2. **Banerjee, P. C., R. I. Vanags, A. M. Chakrabarty, and P. K. Maitra.** 1983. Alginic acid synthesis in *Pseudomonas aeruginosa* mutants defective in carbohydrate metabolism. J. Bacteriol. **155:** 238–245.
3. **Cryz, S. J., Jr., E. Furer, and R. Germanier.** 1983. Passive protection against *Pseudomonas aeruginosa* infection in an experimental leukopenic mouse model. Infect. Immun. **40:**659–664.

4. **Cryz, S. J., Jr., T. L. Pitt, E. Furer, and R. Germanier.** 1984. Role of lipopolysaccharide in virulence of *Pseudomonas aeruginosa*. Infect. Immun. **44:**508–513.
5. **Cryz, S. J., Jr., J. C. Sadoff, D. Ohman, and E. Furer.** 1988. Characterization of the human immune response to a *Pseudomonas aeruginosa* O-polysaccharide–toxin A conjugate vaccine. J. Lab. Clin. Med. **111:**701–707.
6. **Dai, J.-B., Y. Liu, W. J. Ray, Jr., and M. Konno.** 1992. The crystal structure of muscle phosphoglucomutase refined at 2.7-Angstrom resolution. J. Biol. Chem. **267:**6322–6337.
7. **Darzins, A., and A. M. Chakrabarty.** 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. J. Bacteriol. **159:**9–18.
8. **Fiske, C. H., and Y. SubbaRow.** 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66:**375–400.
9. **Fürste, J. P., W. Pansegau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48:**119–131.
10. **Evans, L. R., and A. Linker.** 1973. Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. J. Bacteriol. **116:**915–924.
11. **Franklin, M. J., C. E. Chitnis, P. Gacesa, A. Sonesson, D. C. White, and D. E. Ohman.** 1994. *Pseudomonas aeruginosa* AlgG is a polymer level alginate C5-mannuronan epimerase. J. Bacteriol. **176:**1821–1830.
12. **Franklin, M. J., and D. E. Ohman.** 1993. Identification of *algF* in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. J. Bacteriol. **175:**5057–5065.
13. **Goldberg, J., K. Hatano, and G. B. Pier.** 1993. Synthesis of lipopolysaccharide O side chains by *Pseudomonas aeruginosa* PAO1 requires the enzyme phosphomannomutase. J. Bacteriol. **175:**1605–1611.
14. **Hatano, K., J. B. Goldberg, and G. B. Pier.** 1993. *Pseudomonas aeruginosa* lipopolysaccharide: evidence that the O side chains and common antigens are on the same molecule. J. Bacteriol. **175:**5117–5128.
15. **Jarrell, K. F., and A. M. Kropinski.** 1981. *Pseudomonas aeruginosa* bacteriophage ϕPLS27-lipopolysaccharide interactions. J. Virol. **40:**411–420.
16. **Jiang, X.-M., B. Neal, F. Santiago, S. J. Lee, L. K. Romana, and P. R. Reeves.** 1991. Structure and sequence of the rfb (O antigen) gene cluster of *Salmonella* serovar *typhimurium* (strain LT2). Mol. Microbiol. **5:**695–713.
17. **Knirel, Y. A., E. V. Vinogradov, N. A. Kocharova, N. A. Paramonov, N. K. Kochetkov, B. A. Dmitriev, E. S. Stanislavsky, and B. Lanyi.** 1988. The structure of O-specific polysaccharides and serological classification of *Pseudomonas aeruginosa*. Acta Microbiol. Hung. **35:**3–24.
18. **Köplin, R., W. Arnold, B. Hötte, R. Simon, G. Wang, and A. Pühler.** 1992. Genetics of xanthan production in *Xanthomonas campestris*: the *xanA* and *xanB* genes are involved in UDP-glucose and GDP-mannose biosynthesis. J. Bacteriol. **174:**191–199.
19. **Lam, M. Y. C., E. J. McGroarty, A. M. Kropinski, L. A. MacDonald, S. S. Pedersen, N. Hoiby, and J. S. Lam.** 1989. Occurrence of a common lipopolysaccharide antigen in standard and clinical strains of *Pseudomonas aeruginosa*. J. Clin. Microbiol. **27:**962–967.
20. **Lightfoot, J., and J. S. Lam.** 1993. Chromosomal mapping, expression and synthesis of lipopolysaccharide in *Pseudomonas aeruginosa*: a role for guanosine diphospho (GDP)-D-mannose. Mol. Microbiol. **8:**771–782.
21. **Lowry, O. H., and J. V. Passonneau.** 1969. Phosphoglucomutase kinetics with the phosphates of fructose, glucose, mannose, ribose, and galactose. J. Biol. Chem. **244:**910–916.
22. **Marolda, D. L., and M. A. Valvano.** 1993. Identification, expression, and DNA sequence of the GDP-mannose biosynthesis genes encoded by the O7 *rfb* gene cluster of strain VW187 (*Escherichia coli* O7:K1). J. Bacteriol. **175:**148–158.
23. **May, T. B., D. Shinabarger, A. Boyd, and A. M. Chakrabarty.** 1994. Identification of amino acid residues involved in the activity of phosphomannose isomerase-guanosine 5′-diphospho-D-mannose pyrophosphorylase. J. Biol. Chem. **269:**4872–4877.
24. **May, T. B., D. Shinabarger, R. Maharaj, J. Kato, L. Chu, J. D. DeVault, S. Roychoudhury, N. A. Zielinski, A. Berry, R. K. Rothmel, T. K. Misra, and A. M. Chakrabarty.** 1991. Alginate synthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. Clin. Microbiol. Rev. **4:**191–206.
25. **Padgett, P. J., and P. V. Phibbs, Jr.** 1986. Phosphomannomutase activity in wild-type and alginate-producing strains of *Pseudomonas aeruginosa*. Curr. Microbiol. **14:**187–192.
26. **Phillips, A. T.** 1994. Enzymatic activity, p. 555–586. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
27. **Pindar, D. F., and C. Bucke.** 1975. The biosynthesis of alginic acid by *Azotobacter vinelandii*. Biochem. J. **152:**617–622.
28. **Ray, W. J., Jr., and E. J. Peck, Jr.** 1972. Phosphomutase, p. 407–477. *In* P. D. Boyer (ed.), The enzymes, vol. 6. Academic Press, Inc., New York.
29. **Rowe, P. S., and P. M. Meadow.** 1983. Structure of the core oligosaccharide from the lipopolysaccharide of *Pseudomonas aeruginosa* PACR1 and its defective mutants. Eur. J. Biochem. **132:**329–337.
30. **Roychoudhury, S., K. Chakrabarty, Y.-K. Ho, and A. M. Chakrabarty.** 1992. Characterization of guanosine diphospho-D-mannose dehydrogenase from *Pseudomonas aeruginosa*: structural analysis by limited proteolysis. J. Biol. Chem. **267:**990–996.
31. **Roychoudhury, S., T. B. May, J. F. Gill, S. K. Singh, D. S. Feingold, and A. M. Chakrabarty.** 1989. Purification and characterization of guanosine diphospho-D-mannose dehydrogenase. J. Biol. Chem. **264:**9380–9385.
32. **Sá-Correia, I., A. Darzins, S.-K. Wang, A. Berry, and A. M. Chakrabarty.** 1987. Alginate biosynthetic enzymes in mucoid and nonmucoid *Pseudomonas aeruginosa*: overproduction of phosphomannose isomerase, phosphomannomutase, and GDP-mannose pyrophosphorylase by overexpression of the phosphomannose isomerase (*pmi*) gene. J. Bacteriol. **169:**3224–3231.
33. **Sandlin, R. C., and D. C. Stein.** 1994. Role of phosphoglucomutase in lipopolysaccharide biosynthesis in *Neisseria gonorrhoeae*. J. Bacteriol. **176:**2930–2937.
34. **Shinabarger, D., A. Berry, T. B. May, R. Rothmel, A. Fialho, and A. M. Chakrabarty.** 1991. Purification and characterization of phosphomannose isomerase-guanosine diphospho-D-mannose pyrophosphorylase. J. Biol. Chem. **266:**2080–2088.
35. **Shinabarger, D., T. B. May, A. Boyd, M. Ghosh, and A. M. Chakrabarty.** 1993. Nucleotide sequence and expression of the *Pseudomonas aeruginosa algF* gene controlling the acetylation of alginate. Mol. Microbiol. **9:**1027–1035.
36. **Stevenson, G., S. J. Lee, L. K. Romana, and P. R. Reeves.** 1991. The *cps* gene cluster of *Salmonella* strain LT2 includes a second mannose pathway: sequence of two genes and relationship to genes in the *rfb* gene cluster. Mol. Gen. Genet. **227:**173–180.
37. **Stroeher, U. H., L. E. Karaggeorgos, R. Morona, and P. A. Manning.** 1992. Serotype conversion in *Vibrio cholerae* O1. Proc. Natl. Acad. Sci. USA **89:**2566–2570.
38. **Uttaro, A. D., G. A. Cangelosi, R. A. Geremia, E. W. Nester, and R. A. Ugalde.** 1990. Biochemical characterization of avirulent *exoC* mutants of *Agrobacterium tumefaciens*. J. Bacteriol. **172:**1640–1646.
39. **Zielinski, N., A. M. Chakrabarty, and A. Berry.** 1991. Characterization and regulation of the *Pseudomonas aeruginosa algC* gene encoding phosphomannomutase. J. Biol. Chem. **266:**9754–9763.