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Pseudomonas aeruginosa AlgG Is a Polymer Level Alginate C5-Mannuronan Epimerase

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Alginate is a viscous extracellular polymer produced by mucoid strains of *Pseudomonas aeruginosa* that cause chronic pulmonary infections in patients with cystic fibrosis. Alginate is polymerized from GDP-mannuronate to a linear polymer of β-1-4-linked residues of D-mannuronate and its C5-epimer, L-guluronate. We previously identified a gene called *algG* in the alginate biosynthetic operon that is required for incorporation of L-guluronate residues into alginate. In this study, we tested the hypothesis that the product of *algG* is a C5-epimerase that directly converts D-mannuronate to L-guluronate. The DNA sequence of *algG* was determined, and an open reading frame encoding a protein (AlgG) of approximately 60 kDa was identified. The inferred amino terminus of AlgG protein contained a putative signal sequence of 35 amino acids. Expression of *algG* in *Escherichia coli* demonstrated both 60-kDa pre-AlgG and 55-kDa mature AlgG proteins, the latter of which was localized to the periplasm. An N-terminal analysis of AlgG showed that the signal sequence was removed in the mature form. Pulse-chase experiments in both *E. coli* and *P. aeruginosa* provided evidence for conversion of the 60- to the 55-kDa size in vivo. Expression of *algG* from a plasmid in an *algG* (i.e., polymannuronate-producing) mutant of *P. aeruginosa* restored production of an alginate containing L-guluronate residues. The observation that AlgG is apparently processed and exported from the cytoplasm suggested that it may act as a polymer-level mannuronan C5-epimerase. An in vitro assay for mannuronan C5 epimerization was developed wherein extracts of *E. coli* expressing high levels of AlgG were incubated with polymannuronate. Epimerization of D-mannuronate to L-guluronate residues in the polymer was detected enzymatically, using a L-guluronate-specific alginate lyase of *Klebsiella aerogenes*. Epimerization was also detected in the in vitro reaction between recombinant AlgG and poly-D-mannuronate, using high-performance anion-exchange chromatography. The epimerization reaction was detected only when acetyl groups were removed from the poly-D-mannuronate substrate, suggesting that AlgG epimerization activity in vivo may be sensitive to acetylation of the D-mannuronan residues. These results demonstrate that AlgG has polymer-level mannuronan C5-epimerase activity.

Alginate is an unbranched polysaccharide produced by *Pseudomonas* species (10, 13), *Azotobacter vinelandii* (31), and several species of brown seaweed (22). Alginate is composed of D-mannuronate and its C5-epimer, L-guluronate, which are linked by β-1-4 glycosidic bonds (13). The L-guluronate is probably derived from D-mannuronate by the action of a C5-epimerase (Fig. 1). In bacteria, alginate is modified by the addition of *O*-acetyl groups on some D-mannuronate residues (8, 42). The sugar residues of alginate do not show repeating subunits characteristic of other bacterial exopolysaccharides (46). Mucoid strains of *Pseudomonas aeruginosa* produce alginate as a capsule-like exopolysaccharide and are responsible for chronic pulmonary infections in patients with cystic fibrosis. Alginate appears to be an important virulence factor of *P. aeruginosa* by inhibiting phagocytic engulfment by macrophage (2, 36, 40) and conferring an adherence mechanism (25, 33). The viscous nature of alginate in aqueous solution may also contribute to congestion in the cystic fibrosis-affected lung. Pulmonary infections with mucoid *P. aeruginosa* are almost impossible to eradicate despite intensive antibiotic therapy.

The proportions of the two residues in alginate, D-mannuronate (M) and L-guluronate (G), can vary widely, and this affects the physical properties of the polymer in aqueous solution. ^{1}H (4, 15, 16, 38) and ^{13}C (17, 27) nuclear magnetic resonance studies on the arrangement of the uronic acids in alginates have shown that *A. vinelandii* and seaweed produce alginates with G residues adjacent to other G residues (i.e., G blocks), in addition to M and MG blocks. However, *P. aeruginosa* produces alginate with only M and MG blocks. G blocks chelate calcium ions in an egg-box-like structure which results in stronger gels, whereas M-rich alginates bind calcium less strongly and thus form more flexible gels (13). In *A. vinelandii*, alginate appears to be initially synthesized as poly-D-mannuronate, and some M residues are subsequently epimerized to G by a polymer-level mannuronan C5-epimerase which can be found as a 122-kDa protein in culture supernatants (18, 21, 31, 43).

Most of the genes encoding enzymes for alginate biosynthesis in *P. aeruginosa* are found in a large cluster at 34 min on the chromosome (for a review, see reference 26). The alginate biosynthetic gene cluster appears to have an operonic structure (5) and encodes enzymes which include phosphomannose isomerase-GDP-D-mannose phosphorylase (*algA*) (39) and

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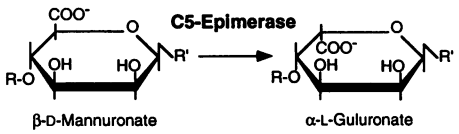


FIG. 1. Reaction catalyzed by an alginate-modifying, mannuronan C5-epimerase. R and R' indicate extensions of the unbranched polymer.

GDP-mannose dehydrogenase (*algD*) (9). Other genes identified in the alginate biosynthetic cluster include those for incorporation of L-guluronate residues into alginate (*algG*) (4), O acetylation of M residues (*algF*) (12), a D-mannuronate-specific alginate lyase (*algL*) (35), and a putative membrane-bound protein (*algE*) (6).

Mutants of *P. aeruginosa* which produce poly-M due to a defect in *algG* were isolated in this laboratory (4). The phenotype of *algG* mutants is consistent with a defect in an alginate C5-epimerase, if such an enzyme exists in mucoid *P. aeruginosa*. The location of *algG* within the alginate biosynthetic cluster was previously determined by transposon mutagenesis (4). Here, we present the DNA sequence of *algG* and inferred amino acid sequence of its protein product, AlgG. This information permitted the development of genetic constructions for the overexpression of *algG* in *Escherichia coli* and *P. aeruginosa*. Our observations suggest that AlgG has a signal sequence which is removed before it is exported from the cytoplasm, implying that AlgG acts upon alginate at the polymer level. By obtaining sufficient quantities of the recombinant protein in *E. coli*, we have developed assays which demonstrate that AlgG has polymer-level, mannuronan C5-epimerase activity.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are described in Table 1. *E. coli* and *P. aeruginosa* were routinely cultured in L broth (12). A minimal medium (48) was used to select for *P. aeruginosa* following triparental matings with *E. coli*. A defined medium (MAP) used to promote alginate production, similar to one previously described (29), contained 100 mM monosodium glutamate, 7.5 mM NaH_2PO_4, 16.8 mM K_2HPO_4, and 10 mM $MgSO_4$. M9 medium (per liter, 1 g of NH_4Cl, 3 g of KH_2PO_4, 1.6 g of Na_2HPO_4, and 1 ml of 1 M $MgSO_4$) or M63 medium [per liter, 13.6 g of KH_4PO_4, 2 g of $(NH_4)_2SO_4$, 0.5 g of $FeSO_4 \cdot 7H_2O$, and 1 ml of 1 M $MgSO_4$] with glucose (0.4%) was used in [^{35}S]methionine labeling studies. Antibiotics when used were at the following concentrations (per milliliter): ampicillin, 100 μg; carbenicillin, 300 μg; and tetracycline, 15 μg for *E. coli* and 100 μg for *P. aeruginosa*. Triparental matings were used to mobilize plasmids from *E. coli* to *P. aeruginosa*, using the conjugation helper plasmid pRK2013 (11).

Expression vectors. Plasmids containing the *trc* promoter, similar to those we previously described (12), were constructed for the expression of *algG* in *P. aeruginosa* based on the expression vector pKK233-2. To enable conjugal transfer of pKK233-2 to *P. aeruginosa*, a 1.3-kb *Eco*RI-*Bgl*II fragment containing a mobilization site (*oriT*) from pSF3 (37) was ligated into the *Eco*RI-*Bam*HI sites of pKK233-2, thus forming pMF15. To enable replication of this narrow-host-range plasmid in *P. aeruginosa*, a 1.8-kb *Eco*RI fragment from pMF17

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or phenotype[a]	Source or reference
E. coli		
JM101	*thi supE44* Δ*lac-proAB* (F' *traD36 proAB lacI*^{q}*ZΔM15*)	Promega
JM109	*endA1 recA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *relA1 supE44* Δ*lac-proAB* (F' *traD36 proAB lacI*^{q}*ZΔM15*)	Promega
P. aeruginosa		
FRD1	Prototrophic, mucoid, cystic fibrosis patient isolate	28
FRD462	*algG4* (poly-M-producing derivative of FRD1)	4
Plasmids		
pALTER-1	Tc^r Ap^s	Promega
pCC27	pCP19 (IncP1) Tc^r with 23-kb *P. aeruginosa* DNA containing all of the alginate biosynthetic operon except *algA*	4
pCC64	pT7-3 containing 3.7-kb DNA with *algG* oppositely oriented with respect to T7 promoter	This study
pCC65	pT7-3 containing 3.7-kb *Eco*RI fragment with *algG* correctly oriented with respect to T7 promoter	This study
pT7-3	T7 promoter, Ap^r	47
pGP1-2	p15A replicon, Km^r, λ *c*I857, λ p_L-T7 phage RNA polymerase gene (temperature induced)	47
pKK233-2	Inducible *trc* promoter expression vector, Ap^r	Pharmacia
pMF2	pALTER-1 containing 3.7-kb *Eco*RI fragment with *algG*, Tc^r Ap^s	This study
pMF3	pMF2 with *Nco*I site at start of *algG*, Tc^r Ap^r	This study
pMF4	pKK233-2 with *algG* on a 2.2-kb *Nco*I fragment	This study
pMF15	pMF4 with *oriT*	This study
pMF17	pUC4K with 1.8-kb *Eco*RI stabilized replication fragment, Ap^r	12
pMF18	pMF19 with 2.2-kb *algG* fragment in *Nco*I site	This study
pMF19	pKK233-2 with *oriT* and stabilized for replication in *P. aeruginosa*	This study
pMF54	pMF19 with *lacI*^{q}	This study
pMF55	pMF18 with *lacI*^{q}	This study
pMMB24	IncQ Sm^r Ap^r P_{tac} *lacI*^{q}	1
pRC5	Contains *aly* encoding a L-guluronate-specific lyase of *K. aerogenes*, Ap^r	3
pRK2013	ColE1-Tra(RK2)$^+$, Km^r	11
pSF3	Contains *oriT*(RK2), Tc^r	37

[a] Abbreviations for phenotypes: Tc^r, tetracycline resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cb^r, carbenicillin resistance; Tra$^+$, transfer by conjugation; poly-M, a variation of the alginate exopolymer which contains only D-mannuronate residues.

(12) which permits stable replication of ColE1 plasmids in *P. aeruginosa* (30) was ligated into the *Eco*RI site to obtain pMF19. The *lacI*^{q} repressor on a 1.2-kb *Eco*RI fragment from pMMB24 (1) was ligated into pMF19, partially digested with *Eco*RI, to allow controlled expression of the genes induced by the *trc* promoter, forming pMF54 (Fig. 2).

DNA manipulations. General DNA manipulations were performed as described previously (24). DNA sequences were determined by the chain termination technique using Sequenase (United States Biochemical Corp.), [5′-α-^{32}P]dCTP (>6,000 Ci/mmol, 10 mCi/ml; Amersham), and 7-deaza-dGTP (U.S. Biochemical) in procedures described by the manufacturers. Oligonucleotide primers for sequencing and site-directed mutagenesis were synthesized on an Applied Biosystems 380B DNA synthesizer. Site-directed mutagenesis of *algG* was performed with the Altered Sites system (Promega) in vector pALTER-1 (Promega) and a 30-base oligonucleotide (CGAGGAAACCGGCCATGGCCGACATTTCCC) which converted the initiating ATG codon of *algG* to an *Nco*I site (C′CATGG) by changing two base pairs (underlined).

^{35}S labeling of *algG* under the T7 and *trc* promoters. The T7 expression system described by Tabor and Richardson (47) was used to radiolabel preferentially *algG* products as well as plasmid-encoded β-lactamase in *E. coli*. A 3.7-kb *Eco*RI fragment containing *algG* (4) was cloned downstream of the T7 promoter in pT7-3 and transformed into *E. coli* JM101(pGP1-2). Cultures grew to mid-log phase at 30°C in L broth, and cells in 1 ml were collected by centrifugation, washed twice in M63 medium, and resuspended in 1 ml of M63 medium supplemented with glucose, thiamine, 18 amino acids (except methionine and cysteine), and appropriate antibiotics. Cells incubated for 1 h at 30°C and then were shifted to 42°C for 15 min to permit transcription from the T7 RNA polymerase gene. Rifampin (200 μg/ml) was added, and the incubation continued for 10 min at 42°C. The cells were shifted to 30°C for 20 min, then [^{35}S]methionine (10 μCi, 800 Ci/mmol; Amersham) was added, and the cells were incubated at 30°C for 5 min. The cells were washed twice in M63 medium.

In pulse-chase experiments, in which *algG* was expressed under the *trc* promoter in *E. coli* and *P. aeruginosa*, bacteria were grown for 18 h in L broth at 37°C. Cells from 0.75 ml of culture were collected by centrifugation (5,000 × *g*, 10 min), washed with M9 medium, resuspended in 3 ml of M9 medium supplemented with glucose (0.4%), and incubated at 37°C for 2 h. Isopropythiogalactopyranoside (IPTG) was added, with incubation for 0.5 h. [^{35}S]methionine (10 μCi, 800 Ci/mmol; Amersham) was added, and cells were incubated at 37°C for 2 min. Unlabeled L-methionine (0.1%) was added, and then samples were withdrawn at 0, 3, 5, 15, and 30 min after the chase began.

For visualization of proteins, cells were obtained by centrifugation and lysed in 60 μl of sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris-HCl, 2% [wt/vol] SDS, 10% [vol/vol] glycerol, 0.1 mg of bromophenol blue per ml, 5% [vol/vol] 2-mercaptoethanol [pH 6.8]). Proteins were denatured at 100°C for 5 min and separated on SDS–12% (wt/vol) polyacrylamide gels for polyacrylamide gel electrophoresis (PAGE) by the procedure of Laemmli (20). Gels were stained with Coomassie brilliant blue R250 (Bio-Rad). Following SDS-PAGE, radioactive proteins were electroblotted onto nitrocellulose by using a trans-blot apparatus (Bio-Rad) for 16 h at 14 V and 4°C, and the nitrocellulose membranes were exposed to X-ray film.

Cell fractionation. To obtain cytoplasmic and periplasmic proteins from *E. coli*, the procedure of Withlot et al. (49) was used to convert cells to spheroplasts. Briefly, following in vivo expression and labeling of AlgG from the T7 promoter, cells were collected by centrifugation, washed twice, and resuspended in 0.2 M Tris-HCl (pH 8.0). Spheroplasting buffer (1 M sucrose, 0.5 mM EDTA, 60 μg of lysozyme per ml, 0.2 M Tris-HCl [pH 8.0]) was added, and the suspension was rapidly diluted with cold distilled water. After 5 min, 3 mM $MgCl_2$ and pancreatic DNase (20 μg/ml) were added. The spheroplasts were recovered by centrifugation (4,000 × *g*, 15 min) and resuspended in sample buffer. Periplasmic proteins in the supernatant were precipitated with trichloroacetic acid and resuspended in sample buffer. Microscopic examination revealed that more than 95% of cells were converted to spheroplasts by this procedure.

Preparation of AlgG for enzymatic assays. To overproduce AlgG for biochemical studies, 1 liter of L broth was inoculated with 50 ml of an 18-h culture of *E. coli* JM109 containing pMF4 which had *algG* under the control of an IPTG-inducible *trc* promoter. The culture was incubated with aeration for 2 h at 28°C, IPTG (1 mM) was added, and the cells were incubated for 18 h. To visualize AlgG among the bacterial proteins, cells from 1 ml of culture were obtained by centrifugation (10,000 × *g*, 3 min) and resuspended in 0.5 ml of saline, and then 0.5 ml sample buffer was added. Proteins were denatured at 100°C for 5 min and separated by SDS-PAGE (12% gel). For assays of AlgG enzymatic activity, cells were obtained from the IPTG-induced cultures by centrifugation (10,000 × *g*, 15 min), resuspended in 10 ml of 20 mM Tris-HCl (pH 7.5), frozen at −70°C, thawed, and then disrupted at 0°C by sonication (Branson Sonifier model 450; three 5-s pulses with a microtip at 20% power). Unbroken cells were removed from the AlgG preparation by centrifugation (15,000 × *g*, 15 min), and the supernatant was filter sterilized and stored at −70°C.

Purification and deacetylation of alginate. Alginate and poly-D-mannuronate (i.e., alginate without G residues) were purified from cultures of *P. aeruginosa* FRD1 and FRD462, respectively, grown 24 h at 37°C with rapid aeration in 50 ml of MAP. Cultures were then mixed with an equal volume of 0.85% NaCl (saline) to reduce viscosity, and cells were removed by centrifugation (25,000 × *g*, 30 min). Polymers were precipitated by the addition of 50 ml of 2% (wt/vol) cetyl pyridinium chloride (Sigma Chemical Co.) and centrifuged (15,000 × *g*, 10 min). The precipitated polymers were resuspended in 50 ml of 1 M NaCl and precipitated again with 50 ml of isopropanol. The precipitated polymers were resuspended in saline and incubated with trypsin (0.5 mg/ml; Sigma) for 2 h at 37°C. NaCl was added to 1 M, and then the preparations were subjected to a cold isopropanol precipitation and dialysis against distilled water. The concentration of alginate in solution was determined by the carbazole method of Knutson and Jeanes (19), using *Macrocystis pyrifera* alginate (Sigma) as a standard. Prior to incubation with alginate lyase, samples (150 μl) of purified alginate were deacetylated by treatment with 50 μl of 1 M NaOH at 65°C for 15 min and then neutralized by the addition of 50 μl of 1 M HCl, 100 μl of 50 mM Tris-HCl (pH 8.0), 5 μl of 1 M $CaCl_2$, and 10 μl of 1 M $MgSO_4$.

Preparation of L-guluronate lyase. Clone pRC5 contains the *Klebsiella aerogenes aly* gene, which encodes an extracellular L-guluronate lyase that is specific for the glycosidic linkages with G residues in alginate (3). *E. coli* containing pRC5 was grown overnight in L broth at 37°C, and cells were removed by centrifugation (10,000 × *g*, 15 min). The L-guluronate lyase in supernatants was precipitated with $(NH_4)_2SO_4$ (80% saturation), resuspended in 10 ml of lyase buffer (19 mM NaH_2PO_4, 30 mM Na_2HPO_4, 50 mM NaCl [pH 7.0]), dialyzed extensively against lyase buffer, and stored at 5°C.

Enzymatic assay for mannuronan C5 epimerization. *P.*

aeruginosa AlgG, produced in *E. coli*, was tested for C5-epimerase activity in vitro, using purified poly-D-mannuronate from *P. aeruginosa* FRD462 as the substrate. Polymer (150 μl at 2 mg/ml) was mixed with 100 μl of E buffer (10 mM $CaCl_2$, 6 mM Tris-HCl [pH 6.8]) and 50 μl of *E. coli* extracts (as described above; ~60 μg of total protein) containing AlgG or vector control and then incubated at 30°C for 18 h. To detect L-guluronate in polymers, a preparation of L-guluronate lyase (10 μl) was added to the reaction mixture (or to purified, deacetylated alginates from *P. aeruginosa* supernatants) and incubated at 25°C for 1 h. The procedure described by Chitnis and Ohman (4) was used to measure the relative number of guluronates cleaved in the lyase-treated polymers. Briefly, the unsaturated residues formed in the cleavage of the glycosidic linkages by the G-specific lyase were oxidized with periodic acid and then detected by the formation of a chromogen (A_{535}) in a reaction with 2-thiobarbituric acid.

Chromatographic assay for mannuronan C5 epimerization. M and G monomers were separated by high-performance anion-exchange chromatography (HPAEC), using a procedure similar to that described by Sonesson and Jantzen (45). A sample (1.5 ml at 2 mg/ml) of purified deacetylated alginate was mixed with 1.5 ml of 4 M trifluoroacetic acid (Fluka) and hydrolyzed at 100°C for 2 h. The mixture was evaporated to dryness in a Speed-Vac concentrator (model AS290; Savant). The residue was dissolved in 2 ml of purified (>18 MΩ/cm; Millipore) water, passed through a disposable C_{18} column (Waters Corp.), vacuum evaporated, redissolved in 3 ml of purified water, and filtered through a 0.22-μm-pore-size filter. HPAEC separations of 25-μl samples were performed with a pellicular CarboPac PA1 anion-exchange column (4 by 250 mm) and a PA1 guard column on a Dionex series 4500 high-pressure liquid chromatography system. The mobile phase used was 100 mM NaOH with a linear gradient of sodium acetate from 0 to 100 mM during 20 min starting from injection. The flow rate was 1 ml/min. Monomers were detected with a Dionex pulsed electrochemical detector (PED) operated in the integrated amperometric mode, with electrode potentials set at E_1 0.1 V, E_2 0.6 V, and E_3 −0.8 V, with 300-, 120-, and 300-ms applied duration times, respectively. Peaks were identified by their retention times compared with commercial sources of algal alginate (*M. pyrifera*; Sigma) and D-mannuronate (Sigma).

Nucleotide sequence accession number. The nucleotide sequence data reported here for *algG* have been deposited in the GenBank data base under accession number UO6720.

RESULTS

DNA sequence analysis of *algG*. The *algG* gene, which encodes a protein (AlgG) required for the incorporation of G residues into alginate, was previously (4) mapped by transposon mutagenesis to a region within a 3.7-kb *Eco*RI fragment in the alginate biosynthetic gene cluster of *P. aeruginosa* (Fig. 2). Approximately 3 kb of this region was sequenced, and an open reading frame (ORF) preceded by a likely ribosome binding site (AGGA) 8 bp from the ATG initiation codon was observed (Fig. 3). The predicted amino acid sequence of the AlgG ORF gave a protein with a molecular mass of 59,807 Da. Codon usage analysis showed high G+C content in the third position, characteristic of *P. aeruginosa* DNA, which is high (67%) in G+C. The inferred amino acid sequence of AlgG predicted a protein with a charge of +6.4 at pH 7.0 and an isoelectric point of 8.9, suggesting that *algG* encodes a basic protein. A hydropathy plot of the predicted amino acid sequence of AlgG indicated a generally hydrophilic protein (data not shown). The AlgG amino acid sequence was used to perform a homology search using the GenBank data base, but no significant similarities were identified. However, DNA sequences immediately upstream of *algG* matched those previously described (6) for *algE* (Fig. 3).

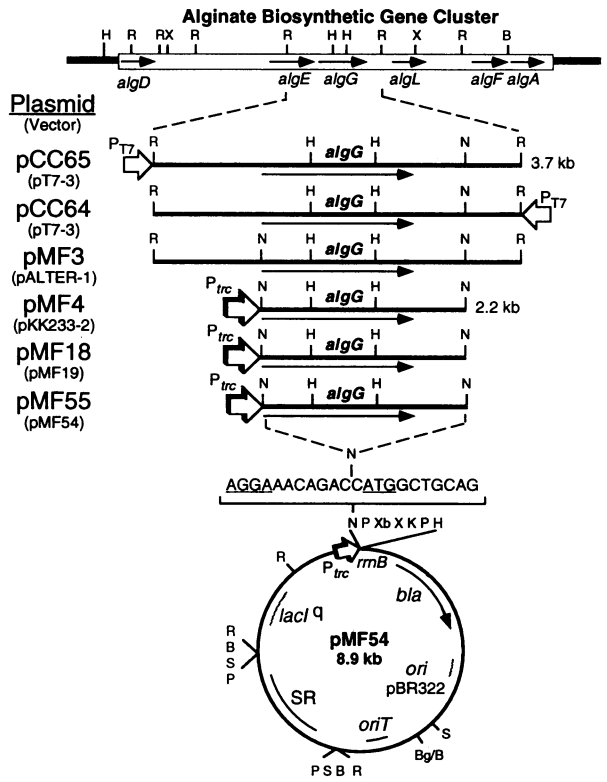


FIG. 2. Restriction maps of *P. aeruginosa* FRD1 DNA showing the alginate biosynthetic gene cluster and fragments used in this study containing *algG*. P_{T7}, T7 promoter; P_{trc}, *trc* (*trp-lac*) promoter; *lacI*^{q}, repressor of P_{trc}; *bla*, β-lactamase gene encoding ampicillin and carbenicillin resistances; SR, stabilized replication in *P. aeruginosa*; *oriT*, origin of transfer; *ori*, origin of replication; H, *Hin*dIII; R, *Eco*RI; X, *Xho*I; Xb, *Xba*I; K, *Kpn*I; B, *Bam*HI; N, *Nco*I; P, *Pst*I; S, *Sal*I; Bg, *Bgl*II.

We also observed that the sequence of AlgG contained a putative signal sequence at the N terminus (shown in italics in Fig. 3). This sequence contained a positively charged N terminus of 17 amino acids, followed by an 11-amino-acid hydrophobic region and then by polar residues (for a review, see reference 32). There was also a putative signal peptidase recognition sequence (Ala-Trp-Ala ↓ Ala) at the 3′ end of the signal sequence. This suggests that AlgG may be initially synthesized as a precursor (preAlgG) of about 60 kDa and then processed to the 55-kDa mature protein.

Expression of *algG* and periplasmic localization of mature product in *E. coli*. The *algG* gene was expressed in *E. coli* to confirm that the ORF observed from the sequence analysis encoded a protein of the predicted size. The 3.7-kb *Eco*RI fragment containing *algG* was cloned into the inducible T7 promoter expression vector pT7-3, forming pCC65 (Fig. 2). Examination of ^{35}S-labeled proteins from JM101(pGP1-2, pCC65) revealed two unique proteins encoded by the *P. aeruginosa* DNA of approximately 60 and 55 kDa (Fig. 4A, lane 2) that were not seen in the vector control strain, JM101(pGP1-2, pT7-3) (Fig. 4A, lane 1) or in pCC64, in which

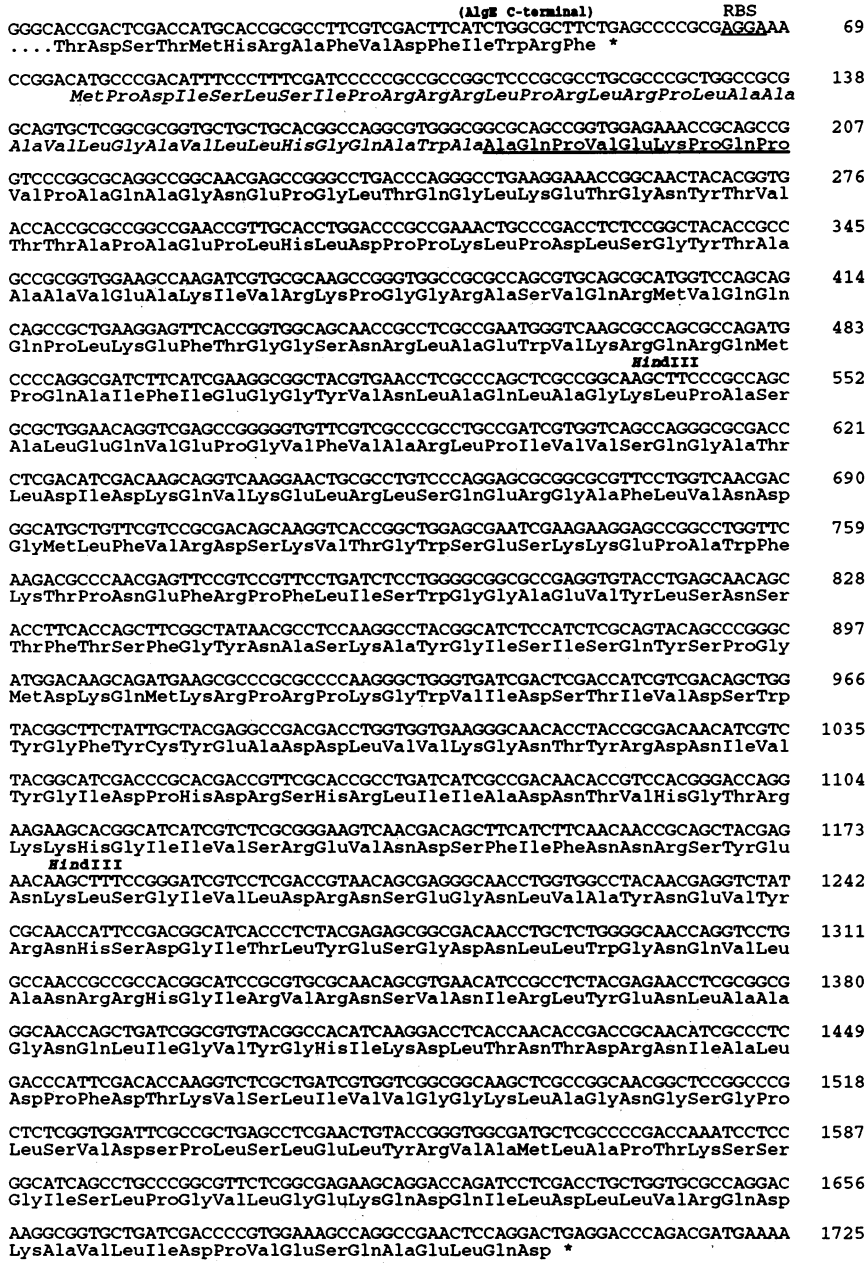


FIG. 3. Sequence from the region of an *Eco*RI fragment containing the *algG* ORF and its inferred protein sequence. Indicated in the sequence are the ribosome binding site (RBS), signal sequence (in italics), and results of an N-terminal analysis of AlgG (underlined). The sequence encoding the C terminus of *algE* (6) is shown immediately upstream of *algG*.

the fragment was in the opposite orientation (data not shown). The sizes of these proteins matched the inferred sequences of pre-AlgG and AlgG.

The processing of the *algG* product to 55 kDa suggested that it may be localized to the periplasm of *E. coli*. Spheroplasts were prepared from JM101(pGP1-2, pCC65) in which proteins were labeled with [^{35}S]methionine following induction of the T7 promoter. The disrupted spheroplasts (Fig. 4A, lane 3) included both the 60- and 55-kDa proteins, with the 60-kDa species being predominant. The periplasmic fraction revealed only the 55-kDa protein (Fig. 4A, lane 4). These results suggest that the preAlgG protein was processed and that AlgG protein was exported to the periplasm.

High-level expression of *algG* in *E. coli*. In pCC65, the *algG* ORF translational start codon was 1.1 kb downstream of the inducible vector T7 promoter, and so the amount of protein obtained with pCC65 was low. To improve expression of *algG*, and thus obtain sufficient quantities of the protein to perform functional assays in vitro, the 1.1 kb of DNA upstream of *algG* was deleted as follows. The 3.7-kb *Eco*RI fragment containing *algG* was cloned into pALTER-1 to form pMF2. A mutagenic oligonucleotide was then used to change the sequences con-

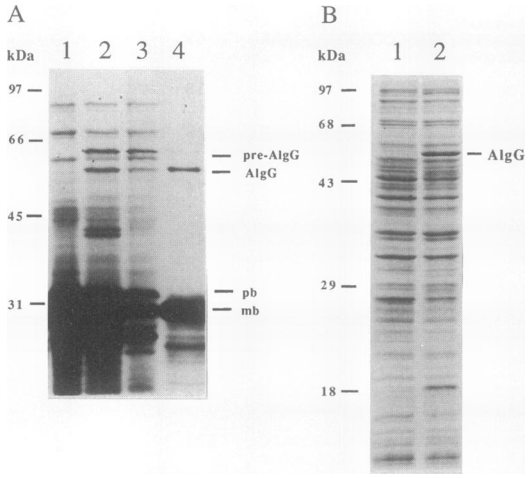


FIG. 4. Expression of *algG* and periplasmic localization of the mature gene product in *E. coli*. (A) Overexposed autoradiogram of proteins expressed from the T7 promoter, labeled with [^{35}S]methionine for 5 min, denatured, and separated by SDS-PAGE from strains JM101(pGP1-2, pCC65) (lane 1) and JM101(pGP1-2, pCC65) whole cells (lane 2), spheroplasts (lane 3), and a periplasm-enriched fraction (lane 4). Migration of pre-AlgG (60 kDa), mature AlgG (55 kDa), pre-β-lactamase (pb), and mature β-lactamase (mb) are indicated on the right. Positions of protein size markers are also indicated. (B) SDS-PAGE analysis of the expression of AlgG of pMF4 in *E. coli* cells. JM109 cells carrying pKK233-2 (lane 1) or pMF4 (lane 2) were treated with IPTG to induce expression from the *trc* promoter. Proteins from lysed cells were denatured, separated by SDS-PAGE, and stained with Coomassie brilliant blue R250. Numbers on the left indicate molecular size markers: the position of the protein expressed from *algG* is shown on the right.

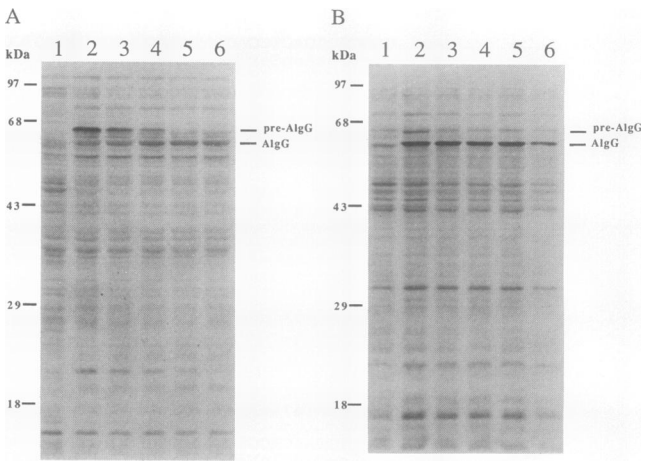


FIG. 5. Autoradiograms of pulse-labeled proteins in cells expressing *algG*. Cells were labeled with [^{35}S]methionine for 2 min and chased with nonlabeled methionine for 0 (lane 2), 3 (lane 3), 5 (lane 4), 15 (lane 5), and 30 (lane 6) min. Cells were lysed, and samples were analyzed by SDS-PAGE. (A) Lane 1, *E. coli* JM109(pKK233-2); lanes 2 to 6, *E. coli* JM109(pMF4). (B) Lane 1, *P. aeruginosa* FRD462(pMF54); lanes 2 to 6, *P. aeruginosa* FRD462(pMF55). Numbers on the left indicate molecular size markers. Pre-Alg and AlgG indicate the precursor (60-kDa) and mature (55-kDa) proteins expressed by *algG*, respectively.

taining the *algG* initiation codon (ACATGC) to an *Nco*I site (C′CATGG) and form pMF3 (Fig. 2). This also changed the second proline-encoding codon (CCC) to encode alanine (GCC). The resulting 2.2-kb *Nco*I fragment containing *algG* from pMF3 was then cloned into the expression vector pKK233-2, forming pMF4 (Fig. 2). This placed *algG* under the inducible *trc* (i.e., *trp-lac* hybrid) promoter and in optimal position relative to a vector-encoded ribosome binding site. Protein extracts from JM109(pMF4), separated by SDS-PAGE and stained with Coomassie blue (Fig. 4B), showed a 55-kDa AlgG protein which was not found in the vector-containing control strain. The 55-kDa protein corresponded in size to AlgG, as predicted from the DNA sequence analysis. To verify that this 55-kDa protein was AlgG, an N-terminal analysis was performed. The sequence obtained was A-Q-P-X-E-X-P-Q-P-X-P-A-Q-A (where X residues were not interpretable), and this corresponded to the predicted N-terminal sequence of mature AlgG following cleavage of the first 35 amino acids by signal peptidase (Fig. 3).

Pulse-chase analysis shows conversion of pre-AlgG to AlgG. A pulse-chase analysis was performed to confirm the sequencing and gene expression studies described above which suggested that AlgG has a signal peptide that is probably removed during export from the cytoplasm. Proteins in JM109(pMF4) were labeled with [^{35}S]methionine and then incubated in excess nonradioactive L-methionine to block further incorporation of labeled amino acid. Samples were withdrawn at various intervals, and proteins in extracts were separated by SDS-PAGE and autoradiographed (Fig. 5A). The 60-kDa pre-AlgG band was observed in *E. coli* at 2 min (lane 2), and it gradually faded in intensity during the chase along with a concomitant increase in intensity of the 55-kDa protein (lanes 3 to 6). This suggested that pre-AlgG was processed by signal peptidase in *E. coli*.

A similar pulse-chase experiment was performed in mucoid *P. aeruginosa*. The 2.2-kb *Nco*I fragment from pMF3 was ligated into the broad-host-range expression vector pMF54, forming pMF55 (Fig. 2). Induction of labeled AlgG production in a logarithmic-phase culture of *P. aeruginosa* FRD462(pMF55) was obtained by the addition of IPTG and [^{35}S]methionine. Excess nonradioactive L-methionine was then added, samples were withdrawn over time, and proteins were examined by SDS-PAGE and autoradiography (Fig. 5B). The 60-kDa pre-AlgG band was seen in FRD462(pMF55) at 2 min (lane 2), but it quickly faded by the 3 min of chase (lane 3). These results showed that the change made by site-directed mutagenesis in the second amino acid of pre-AlgG (Pro-2 to Ala) did not block processing by signal peptidases in *E. coli* or *P. aeruginosa*. These data also suggest that the synthesis of AlgG in *P. aeruginosa* involves the removal of a signal sequence and export from the cytoplasm.

Genetic complementation of *algG4* in *P. aeruginosa*. To ensure that the 2.2-kb *Nco*I fragment containing *algG* constructed above still encoded a functional AlgG protein, its ability to complement an *algG* mutation in mucoid *P. aeruginosa* was examined. The fragment was cloned into pMF19 (which is similar to pMF54 except for the omission of *lacI*^{q}) to form pMF18 and was mobilized by triparental mating into FRD462. This strain is an *algG4* mutant of FRD1 and so produces an alginate devoid of G residues (i.e., poly-M). Alginates were collected and purified from FRD1, FRD462, FRD462(pMF18), and FRD462(pMF19) after 24 h of growth at 37°C in MAP medium (in which carbenicillin was added to cultures of plasmid-bearing strains). To assay the relative amount of G residues in alginates, samples were treated with an L-guluronate-specific lyase which forms unsaturated residues at cleavage sites that can then be measured in a colori-

TABLE 2. Enzymatic assay for in vivo epimerization of D-mannuronate to L-guluronate residues by complementation of *algG* mutation in *P. aeruginosa*

Alginate source	Phenotype	Reaction with G-specific lyase (U)[a]
FRD1	$AlgG^+$	1.38
FRD462	$AlgG^-$	0.28
FRD462(pMF19)	$AlgG^-$	0.26
FRD462(pMF18)	$AlgG^+$	1.46

[a] Units represent the A_{530} following treatment of 0.3 mg of deacetylated alginate with a G-specific alginate lyase followed by assay for the formation of unsaturated uronic acids. Data shown represent averages of three samples which gave comparable values.

metric assay. FRD1 (wild-type) alginate contains G residues and gave a strong reaction (1.38 U). FRD462 and FRD462 (pMF19) produce poly-M and showed only weak reactions (0.28 and 0.26 U, respectively) with the L-guluronate lyase (Table 2). In contrast, alginate from FRD462(pMF18), which contains the plasmid-encoded *algG* gene, produced a strong reaction (1.46 U) in this assay for G residues which was similar to values obtained with alginate from FRD1 (Table 2). Thus, pMF18, containing *algG* under the *trc* promoter, was able to complement the *algG* defect in *P. aeruginosa* FRD462.

In vitro assay of C5 epimerization of poly-M. The phenotype of *algG* mutants suggested that AlgG may be a C5-epimerase and is directly responsible for the conversion of M to G during alginate biosynthesis (Fig. 1). As AlgG appears to be an exported protein, and epimerization during alginate biosynthesis in *A. vinelandii* appears to occur at the polymer level, we examined whether AlgG could epimerize residues in poly-M to form an alginate with mixed M and G sequences. An in vitro assay using a poly-M substrate purified from culture supernatants of the *algG* mutant, FRD462, was developed. Poly-M (0.3 mg) was incubated with cell sonic extracts (60 μg of protein) of JM109(pMF4) induced to express AlgG. However, there was only a small increase in the reactivity with the G-specific lyase compared with poly-M treated with control sonic extracts from JM109(pKK233-2) (Table 3).

Because polymer-level epimerization may be inhibited by the *O*-acetyl groups, which are substituted on the two and/or three positions of D-mannuronate on poly-M from FRD462, the substrate was deacetylated by alkaline treatment prior to testing for epimerization. When AlgG-containing cell sonic extracts of JM109(pMF4) were incubated with deacetylated poly-M, an increase of nearly 10-fold in reactivity with the G-specific lyase was seen over the control reactions with JM109(pKK233-2) sonic extracts (Table 3). This reaction was not due to any M-specific lyase activity in AlgG because incubation of deacetylated poly-M with sonic extracts of JM109(pKK233-2) or JM109(pMF4) gave only a weak reaction (A_{530} of ~0.18) with thiobarbituric acid, which indicates that unsaturated uronic acids were not formed unless the G-specific lyase was added. Therefore, these studies showed that AlgG encodes a mannuronan C5-epimerase that is inhibited by the presence of acetyl groups on the substrate.

TABLE 3. Enzymatic assay for in vitro epimerization by recombinant AlgG of D-mannuronate to L-guluronate residues in poly-D-mannuronate

Plasmid expressed in *E. coli* before sonication	Phenotype	Reaction with G-specific lyase (U)[a]	
		Acetylated poly-M	Deacetylated poly-M
pKK233-2	$AlgG^-$	0.13	0.15
pMF4	$AlgG^+$	0.15	1.43

[a] Poly-M from FRD462 was acetylated or deacetylated prior to the assay by treatment with KOH and dialysis. Units represent the A_{530} following treatment of 0.3 mg of alginate with a G-specific alginate lyase followed by assay for the formation of unsaturated uronic acids, and the background reaction obtained without the G-specific lyase (A_{530} of 0.18) was subtracted. The reaction between the G-specific lyase and deacetylated poly-M (without extracts of *E. coli*) was an A_{530} of 0.23. Data shown represent averages of three samples which gave comparable values.

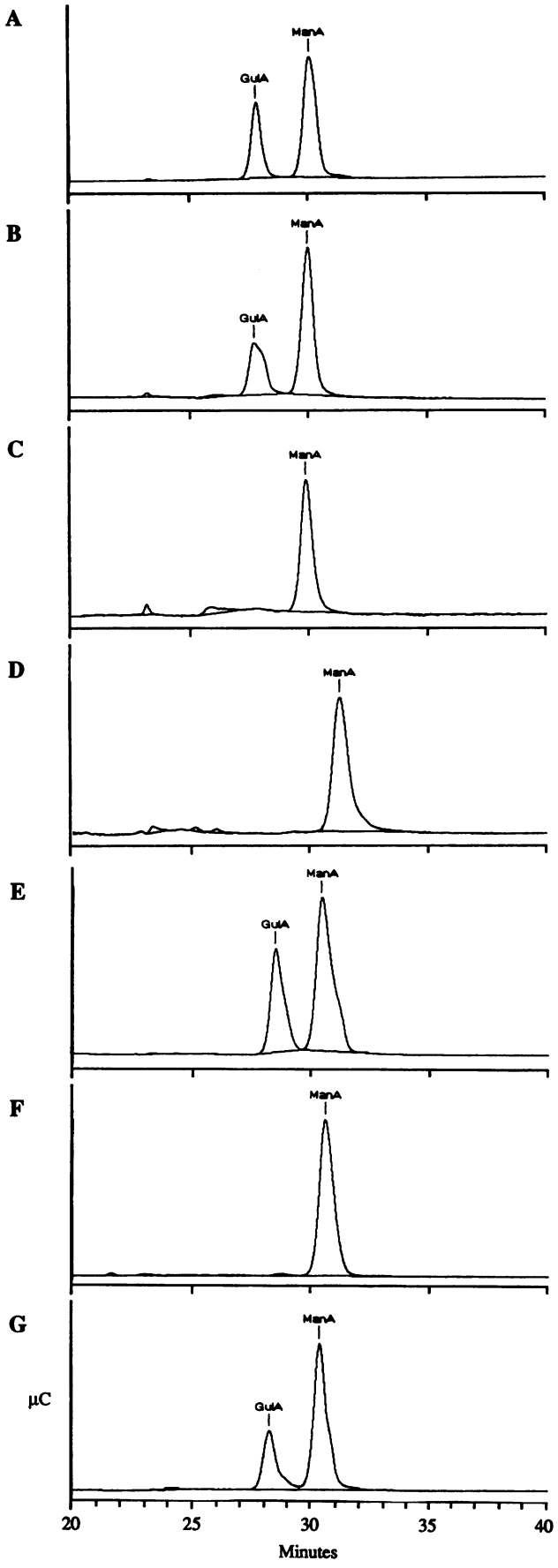
HPAEC-PED analyses of alginates produced following in vivo genetic complementation and in vitro, polymer-level epimerization. HPAEC with a PED was also used to analyze alginates from the foregoing strains. These analyses showed that alginate from seaweed (Fig. 6A) and wild-type FRD1 (Fig. 6B) produced two peaks, with retention times corresponding to G (approximately 27.7 min) and M (approximately 30.0 min). The poly-M of *algG* mutant FRD462 (Fig. 6C) and FRD462(pMF19) (Fig. 6D) showed only the one peak associated with M residues. However, alginate from FRD462 (pMF18) (Fig. 6E) with *algG* expressed in *trans* produced chromatograms with two peaks that were similar to those observed for FRD1 (Fig. 6B). These results verified that the 2.2-kb *Nco*I fragment contained a functional *algG* gene which enables incorporation of G residues into alginate.

The in vitro epimerization of deacetylated poly-M by AlgG, detected enzymatically (see above), was verified by HPAEC-PED to detect the presence of G residues. Deacetylated poly-M showed one peak with retention time equivalent to M residues following incubation with control sonicates of JM109(pKK233-2) (Fig. 6F). In contrast, incubation with AlgG-containing extracts of JM109(pMF4) showed an additional peak with a retention time equivalent to G residues, indicating the presence of a mixed species (i.e., M and G) alginate (Fig. 6G). These results confirmed the results of the assay with the L-guluronate-specific alginate lyase above to show that AlgG acts as a polymer-level mannuronan C5-epimerase.

DISCUSSION

In this study, a gene (*algG*) which is involved in epimerization of M to G residues in *P. aeruginosa* was characterized to better understand this late step in pathway of alginate biosynthesis. Earlier efforts to detect a polymer-level C5-epimerase in mucoid *P. aeruginosa* were unsuccessful, suggesting that such an enzyme was not part of the pathway of alginate biosynthesis (41). However, failure to detect epimerase activity in *P. aeruginosa* may be due to low levels of epimerase in the cell. The other enzymes encoded by the alginate biosynthetic gene cluster are also difficult to detect (7, 34). Using a genetic approach to identify the putative C5-epimerase, we recently described *algG* mutants of *P. aeruginosa* which produce alginate without G residues (i.e., poly-M), the expected phenotype of a putative C5-epimerase mutant (4). The inferred sequence of AlgG from a DNA sequence analysis did not provide any direct evidence for its function because it lacked homology to any protein in the current data base.

Oligonucleotide-directed mutagenesis permitted the construction of a plasmid with *algG* in optimal position relative to both a strong promoter and a ribosome binding site. This led to the production of ample quantities of AlgG protein in *E. coli* for use in assays of its enzymatic function. The *algG* mutants of *P. aeruginosa* provided a convenient source of poly-M which could be purified and used as a substrate to test for polymer-level epimerization. The effect of incubating deacetylated poly-M with crude AlgG preparations showed a dramatic



increase in the G content of the substrate, as determined by reactions with a G-specific lyase. Epimerization of the substrate was confirmed by HPAEC-PED analysis. Thus, these studies established that AlgG acts as a polymer-level mannuronan C5-epimerase. Future studies will examine the kinetics of this reaction with purified AlgG and the possible cofactors or conditions that may be necessary for optimal reaction.

Our data showed that AlgG's C5-epimerase activity in vitro was markedly inhibited by the presence of *O*-acetyl groups on the M residues. Chemical removal of the *O*-acetyl groups from the poly-M substrate was necessary to demonstrate epimerization by AlgG in vitro. Most bacterial alginates are acetylated, and this modification protects the polymer from degradative alginate lyases. The *algG* mutants of *P. aeruginosa* produced a poly-M that was still acetylated, indicating that epimerization was not a prerequisite for acetylation in alginate biosynthesis. The finding that acetylated poly-M is a poor substrate for AlgG might suggest that epimerization precedes acetylation in the pathway of biosynthesis. However, it was curious that the overexpression of *algG* in *P. aeruginosa* did not appear to significantly increase the G content in alginate over that of the wild-type strain, suggesting that the inherent specificity of the enzyme limits the number of G residues that can be incorporated into alginate, although a cofactor in the reaction may be limiting. Alternatively, epimerization activity in alginate-producing bacteria could be partially under the control of O-acetylation activity, a theory that has been previously suggested (44). We are currently examining this hypothesis by using our genetic constructs for the overproduction of AlgG in *P. aeruginosa* mutants that are alginate O acetylation defective (*algF*) (12) and characterizing their alginates for incorporation of G residues.

An interesting feature of AlgG, revealed by the sequence analysis and expression studies, is that this protein is synthesized as a 60-kDa protein with an N-terminal (signal) sequence that is removed to produce a 55-kDa mature protein. This suggests that AlgG is exported from the cytoplasm to another compartment where it acts upon nonacetylated M residues in the polymer. The mechanism for polymerizing GDP-D-mannuronate into alginate and the cellular compartment where this occurs in *P. aeruginosa* are currently not known. AlgG may epimerize M residues in the periplasm following polymerization, and this possibility is under investigation.

Studies of alginate biosynthesis in *A. vinelandii* (18, 21, 31) and the alga *Pelvetia canaliculata* (23) have shown that these organisms also contain extracellular, polymer-level mannuronan epimerases. However, the current data suggest that the mannuronan C5-epimerases of *P. aeruginosa* and *A. vinelandii* are quite different. The purified *A. vinelandii* epimerase had a molecular mass of 122 kDa, as determined by electrophoresis on denaturing SDS-polyacrylamide gels (43), whereas the *P. aeruginosa* enzyme was approximately 55 kDa. The pI of the *A.*

FIG. 6. Chromatographic separation of acidic monosaccharides D-mannuronate (ManA) and L-guluronate (GulA) from alginates by HPAEC-PED. Separations were performed on a CarboPac1 PA1 column (4 by 250 mm) and PA1 guard column, and detected with a PED operated in the integrated amperometric mode. Note that small variations in the retention times of the M and G peaks were common among the samples, although their positions relative to each other remained constant. Profiles of retention times for alginates are from seaweed (commercial) (A) and *P. aeruginosa* FRD1 (B), FRD462 (C), FRD462(pMF19) (D), FRD462(pMF18) (E), FRD462 deacetylated and incubated with proteins from JM109(pKK233-2) (F), and FRD462 deacetylated and then incubated with proteins from JM109(pMF4) (G).

vinelandii enzyme was 4, whereas the predicted pI of the *P. aeruginosa* epimerase (with the signal sequence removed) was 8.6. These structural differences in the two enzymes may account for their apparent functional differences. First, the *A. vinelandii* epimerase was isolated from culture supernatant, suggesting that the *A. vinelandii* epimerase is secreted (18), whereas epimerase activity is not readily detected in culture supernatants from *P. aeruginosa* (41). Second, the activity of the *A. vinelandii* epimerase is dependent on calcium ion concentration, resulting in different M-to-G ratios depending on the calcium ion concentration of the environment (43). In contrast, various calcium concentrations in the culture medium of *P. aeruginosa* did not appear to affect the ratio of G to M residues (28). Also, the *A. vinelandii* epimerase can convert up to 90% of the M residues to G residues with extensive G blocks (42), whereas G blocks are not observed in *P. aeruginosa* alginate. Future studies will include studies on the biochemical mechanism of epimerization by *P. aeruginosa* AlgG.

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