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Cloning of *Pseudomonas aeruginosa algG*, Which Controls Alginate Structure

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The biochemical mechanism by which α-L-guluronate (G) residues are incorporated into alginate by *Pseudomonas aeruginosa* is not understood. *P. aeruginosa* first synthesizes GDP-mannuronate, which is used to incorporate β-D-mannuronate residues into the polymer. It is likely that the conversion of some β-D-mannuronate residues to G occurs by the action of a C-5 epimerase at either the monomer (e.g., sugar-nucleotide) or the polymer level. This study describes the results of a molecular genetic approach to identify a gene involved in the formation or incorporation of G residues into alginate by *P. aeruginosa*. Mucoid *P. aeruginosa* FRD1 was chemically mutagenized, and mutants FRD462 and FRD465, which were incapable of incorporating G residues into alginate, were independently isolated. Assays using a G-specific alginate lyase from *Klebsiella aerogenes* and ^{1}H-nuclear magnetic resonance analyses showed that G residues were absent in the alginates secreted by these mutants. ^{1}H-nuclear magnetic resonance analyses also showed that alginate from wild-type *P. aeruginosa* contained no detectable blocks of G. The mutations responsible for defective incorporation of G residues into alginate in the mutants FRD462 and FRD465 were designated *algG4* and *algG7*, respectively. Genetic mapping experiments revealed that *algG* was closely linked (>90%) to *argF*, which lies at 34 min on the *P. aeruginosa* chromosome and is adjacent to a cluster of genes required for alginate biosynthesis. The clone pALG2, which contained 35 kilobases of *P. aeruginosa* DNA that included the *algG* and *argF* wild-type alleles, was identified from a *P. aeruginosa* gene bank by a screening method that involved gene replacement. A DNA fragment carrying *algG* was shown to complement *algG4* and *algG7* in *trans*. The *algG* gene was physically mapped on the alginate gene cluster by subcloning and Tn*501* mutagenesis.

Patients with cystic fibrosis are at high risk for chronic pulmonary infection with the opportunistic pathogen *Pseudomonas aeruginosa* (39). Following colonization of the cystic fibrotic lungs by *P. aeruginosa*, the nonmucoid organisms are invariably replaced by mucoid variants, which are difficult to eradicate (8). This leads to a deteriorating respiratory condition and a reduced life expectancy for the patient (17). *P. aeruginosa* displays a mucoid phenotype because it secretes copious amounts of the exopolysaccharide alginate. Alginate is a nonrepeating copolymer of β-D-mannuronate (M) and its C-5 epimer, α-L-guluronate (G), which are linked by 1 → 4 glycosidic bonds (10). *P. aeruginosa* alginate is similar to the alginate produced by *Azotobacter vinelandii* (32) and certain species of brown seaweed (25) but differs from the alginate produced by the latter because it contains acetyl groups. Alginate forms highly viscous aqueous solutions that contribute to respiratory congestion, obstruction of small airways, and interference with mucociliary airway clearance in the cystic fibrotic lung. The polymer also provides the bacteria with an adherence mechanism (27, 34) and confers enhanced resistance to phagocytosis (1, 36).

The alginate-producing (Alg^+) trait is unstable, and mucoid clinical isolates of *P. aeruginosa* can rapidly switch back to the nonmucoid (Alg^-) phenotype in vitro. The clustered chromosomal loci, *algST*, are responsible for the spontaneous transformation between the Alg^+ and Alg^- phenotypes (13). These genes map to the 68-min region of the *P. aeruginosa* chromosome and have been cloned (12). *algS* acts in *cis* to control the expression of the closely linked gene, *algT*, which positively regulates alginate biosynthesis (12). Two other regulatory regions, *algRQ* (5, 7) and *algB* (16), have been cloned and map to the 9- and 13-min regions of the linkage map, respectively. *algB* is required for high levels of alginate biosynthesis (16). *algR* and *algQ* transcriptionally regulate *algD*, which encodes GDP-mannose dehydrogenase, one of the enzymes of the alginate pathway (6, 7). *algD* is the first gene in a cluster of genes involved in alginate biosynthesis located adjacent to *argF* at 34 min on the chromosome (6).

The pathway for alginate biosynthesis has been elucidated in *A. vinelandii* (32), and a similar pathway appears to operate in *P. aeruginosa*. In the proposed alginate biosynthetic pathway of *P. aeruginosa*, fructose 6-phosphate is first converted to the nucleotide-linked intermediate GDP-mannuronate in four steps by the enzymes phosphomannose isomerase, phosphomannomutase, GDP-mannose pyrophosphorylase, and GDP-mannose dehydrogenase (6).

The steps of the pathway that follow the synthesis of GDP-mannuronate in *P. aeruginosa* are not understood. In *A. vinelandii*, GDP-mannuronate residues are linked by 1 → 4 glycosidic bonds to make polymannuronate, which is exported from the cell. An extracellular, Ca^{2+}-dependent C-5 epimerase then epimerizes some of the M residues to G at the polymer level to give the mature mixed-sequence polymer (20, 23). The incorporation of G residues markedly affects the structure of alginate. Because of the different chair conformations of M ($^4C_1$) and G ($^1C_4$) residues (Fig. 1), blocks of M residues have a physical structure different from those of blocks of G residues or mixed blocks of alternating M and G residues (14). The relative number of M and G residues (referred to as the M/G ratio) and the way in which they are distributed in the different blocks determine the

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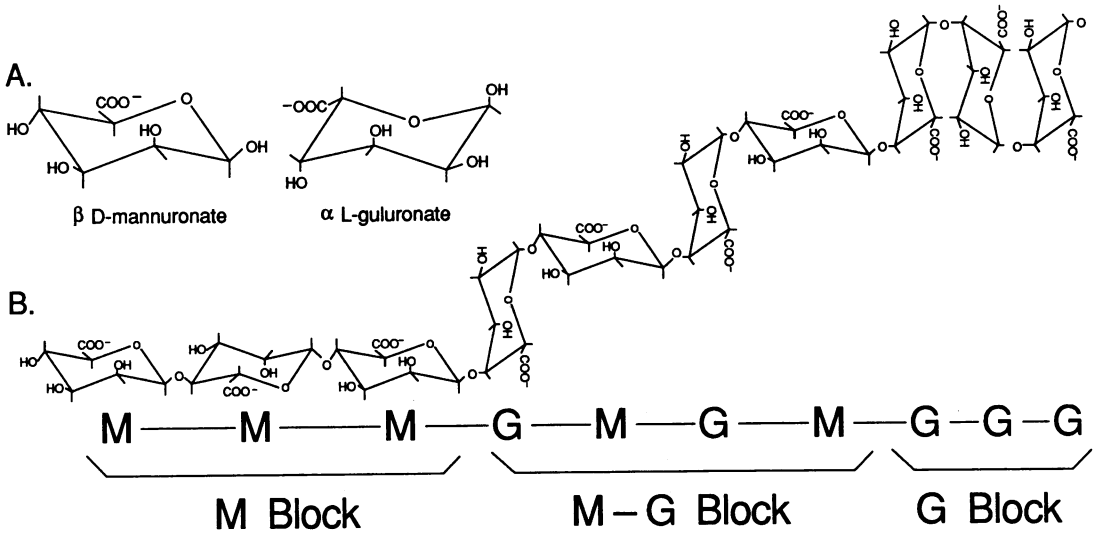

FIG. 1. (A) Monomers of alginate, M and G, in their most favored chair conformations, $^4C_1$ and $^1C_4$, respectively. (B) Monomers within the alginate molecule, arranged as a series of block structures. Shown are the alginate chain conformations of poly-M (M blocks), heteropolymeric conformations with alternating M and G (M-G blocks), and poly-G (G blocks). The physical properties of alginate depend upon both the ratio of D-mannuronate to L-guluronate (M/G ratio) and block distribution.

physical properties of alginate, such as viscosity and relative ability to form gels in the presence of cations (14).

The mechanism by which G residues are incorporated into *P. aeruginosa* alginate is not known, but a C-5 epimerization step similar to that used by *A. vinelandii* may play a role. Others have attempted to identify such a C-5 epimerase enzyme in mucoid *P. aeruginosa* using a biochemical approach (37). This paper describes our molecular genetic approach, which identified a gene required for the formation or incorporation of G residues into alginate.

(A preliminary account of some of these data was reported at the International Symposium on Basic Research and Clinical Aspects of *Pseudomonas aeruginosa* Infection, Copenhagen, Denmark, 1–4 September 1988 [29].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are shown in Table 1. L medium was 1% tryptone (Difco Laboratories), 0.5% yeast extract (Difco), and 0.5% NaCl. The minimal medium used has been described (38). Antibiotics were used in selection media at the following concentrations: tetracycline, 10 μg/ml for *Escherichia coli* and 100 μg/ml for *P. aeruginosa*; kanamycin, 25 μg/ml for *E. coli*; ampicillin, 50 μg/ml for *E. coli*; carbenicillin, 300 μg/ml for *P. aeruginosa*. An alginate lyase that is specific for glycosidic linkages with α-L-guluronate residues (G'ase) was obtained from the culture supernatants of *Klebsiella aerogenes* type 25 (2), grown for 18 h at 37°C with aeration in L broth with 1% alginate (high-viscosity alginate from *Macrocystis pyrifera*; Sigma Chemical Co.). G'ase plates were made by mixing 1 volume of 2× L agar with 1 volume of supernatant (sterilized by filtration) from the *K. aerogenes* type 25 culture.

Assay for G in alginate using *K. aerogenes* G'ase. To isolate alginate for this assay, overnight cultures of *P. aeruginosa* on L agar (containing appropriate antibiotics) were harvested with saline and cells were removed by centrifugation and filtration (0.45-μm-pore-size filters). NaCl was added to 1%, and alginate was precipitated with 2 volumes of ethanol, collected by centrifugation, and then dissolved in water. To improve the reaction with the *K. aerogenes* G'ase, bacterial alginate was deacetylated by treatment with 0.5 N NaOH for 1 h at room temperature and dialyzed against water. The alginate was lyophilized and dissolved in water (to approximately 5 mg/ml), and the concentration was determined by the method of Knutson and Jeanes (22), with seaweed alginate (Sigma) as the standard. Samples at the same alginate concentration (i.e., 3 to 5 mg/ml) were treated for 1 h at room temperature with a G'ase preparation obtained by concentrating a *K. aerogenes* culture supernatant 25-fold by ammonium sulfate (80% saturation) precipitation (2, 3). When the G'ase cleaved the glycosidic linkages at G, it created unsaturated residues at the nonreducing end. These residues were oxidized by periodate to form β-formyl pyruvate which produced a chromogen (A_{535}) when reacted with 2-thiobarbituric acid, by the procedure of Preiss and Ashwell (33).

NMR spectroscopy of alginates from seaweed and *P. aeruginosa*. *P. aeruginosa* alginate was deacetylated as described above. Seaweed (*M. pyrifera*) alginate does not have acetyl groups and was used directly. Alginate was prepared for nuclear magnetic resonance (NMR) analysis as described by Grasdalen et al. (18) for seaweed alginate. Briefly, the alginate samples were partially depolymerized to reduce viscosity by mild sulfuric acid hydrolysis (pH 2.8 to 3.0, 100°C for 90 min), returned to neutral pH, and lyophilized overnight. Samples (10 mg) were dissolved in 0.5 ml D_2O (Sigma), and 0.5 mg of triethylenetetramine hexacetic acid (Sigma) was added to chelate trace divalent cations. NMR spectra were recorded at 180 MHz in the Fourier transform mode at 90°C by using a 180°-t-90° pulse sequence to reduce the solvent (HDO) peak. Chemical shifts were expressed in parts per million downfield from internal sodium 3-(trimethylsilyl)1-propane sulfonate.

Isolation of mutants producing G'ase resistant alginate. Cultures of *P. aeruginosa* FRD1 in late logarithmic growth were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (60 μg/ml) for 10 min at 37°C, washed with saline, and grown overnight in L broth. Appropriate dilutions of mutagenized cultures were plated onto G'ase plates, which were then

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype[a]	Source or reference
E. coli HB101	*proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20*	This laboratory
P. aeruginosa		
PAO1	Prototrophic, Alg^-	D. Haas
FRD1	Prototrophic, Alg^+ cystic fibrosis isolate	28
FRD40	*pro-3*	28
FRD462	*algG4*	This study
FRD465	*algG7*	This study
FRD493	*algG4 argF1*	This study
K. aerogenes type 25	Secretes G'ase	J. Turvey
pCP19	IncP1 *cos oriT* Tc^r	12
pEMR2	pBR322 *cos oriT* Ap^r Km^r	12
R68.45	IncP1 Tc^r Cb^r Km^r Tra^+ Cma^+	D. Haas
RSF1010::Tn*501*	IncQ Hg^r	30
pRK2013	ColE1-Tra(RK2)$^+$ Km^r	11
pALG2	pEMR2 with 35-kb *P. aeruginosa* DNA containing $argF^+$ $algG^+$	29
pCC27	pCP19 with 23-kb *P. aeruginosa* DNA from pALG2 containing $argF^+$ $algG^+$	This study
pCC28	pCP19 with 12-kb *P. aeruginosa* DNA from pALG2	This study

[a] Abbreviations for phenotypes: Tc^r, tetracycline resistance; Hg^r, mercury resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cb^r, carbenicillin resistance; Tra^+, transfer by conjugation; Cma^+, chromosome mobilization ability.

incubated at 37°C. Potential polymannuronate-producing mutants were identified by their mucoid phenotypes, unlike the wild-type mucoid strain FRD1, which appeared nonmucoid on G'ase medium.

Genetic mapping. Isolation of auxotrophic derivatives of *P. aeruginosa* and plasmid R68.45-mediated matings were performed as previously described (28). Recombinants were tested for coinheritance of the unselected *algG* marker by testing the alginate for the presence of guluronate residues using the *K. aerogenes* G'ase.

Construction and manipulation of recombinant plasmids. General DNA manipulations were performed as described by Maniatis et al. (26). A gene bank of *P. aeruginosa* FRD1 genomic DNA was constructed in the gene replacement cosmid vector pEMR2 (13), as previously described (12). Subclones were constructed in the broad-host-range vector pCP19 (13). Triparental matings were used to mobilize recombinant plasmids from *E. coli* to *P. aeruginosa* as previously described (15) by using the conjugative helper plasmid pRK2013 (11). Transconjugants of *P. aeruginosa* were isolated on minimal medium containing appropriate antibiotics and amino acid supplements. Complementation of *algG4* and *algG7* was scored by testing the alginate for the presence of α-L-guluronate residues by the G'ase spectrophotometric assay described above. Recombinant plasmids with Tn*501* insertions were obtained as previously described (30), by using plasmid RSF1010::Tn*501* as the transposon donor. The sites of Tn*501* insertion were mapped by restriction fragment analysis.

RESULTS

Isolation of mutants defective in incorporation of guluronate into alginate. The expected phenotype of a mutant defective in the incorporation of G residues into alginate was the production of poly-M alginate. Cultures of the wild-type alginate-producing strain FRD1 were mutagenized with nitrosoguanidine, and survivors were plated on L agar containing the *K. aerogenes* G'ase, which cleaves alginate at G residues (see Materials and Methods). FRD1 colonies appeared nonmucoid on this medium because the wild-type alginate was depolymerized (Fig. 2). However, among the survivors of mutagenesis, mucoid colonies were observed on the G'ase medium at a frequency of about 10^{-4}. Two independently isolated mutants, FRD462 and FRD465, which produced G'ase-resistant alginates were characterized further. The mutant and wild-type alginates were tested in a quantitative assay in which a chromogen (maximum A_{535}) was produced if the alginate was cleaved by G'ase. The reaction of *P. aeruginosa* alginate with the lyase was improved if alginate was first treated with NaOH to remove acetyl groups, whereas this had little effect on seaweed alginate, which was not acetylated (Table 2). The alginates from FRD462 and FRD465 produced almost no detectable reaction in this assay, which suggests that they were not cleaved by the G'ase (Table 2).

^{1}H-NMR spectroscopy of wild-type and mutant alginates. The structure and composition of alginates produced by seaweed have been studied extensively by using ^{1}H-NMR spectroscopy (18). Using this technique, we compared the wild-type alginate of FRD1 with the alginate of seaweed and the G'ase-resistant alginates of FRD462 and FRD465. Figure 3A shows the 180 MHz ^{1}H-NMR spectra for seaweed and *P. aeruginosa* FRD1 alginates. The seaweed alginate spectrum has four characteristic peaks arising from the anomeric protons on G (G-1) and M (M-1) residues, from the protons on C-5 of G residues with M neighbors (*G*M-5), and from the protons on C-5 of G residues with G neighbors (*G*G-5) (18). The FRD1 alginate spectrum has peaks from both M and G residues, but the *G*G-5 peak is missing, indicating that blocks of contiguous G residues are rare or absent in FRD1 alginate. We observed that G blocks were also absent in the alginates

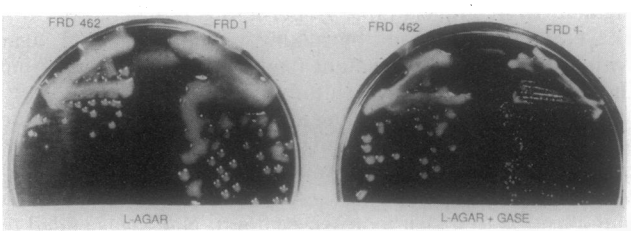

FIG. 2. Mucoid phenotypes of wild-type FRD1 and *algG4* mutant FRD462 on L agar (left) and L agar containing G'ase (right).

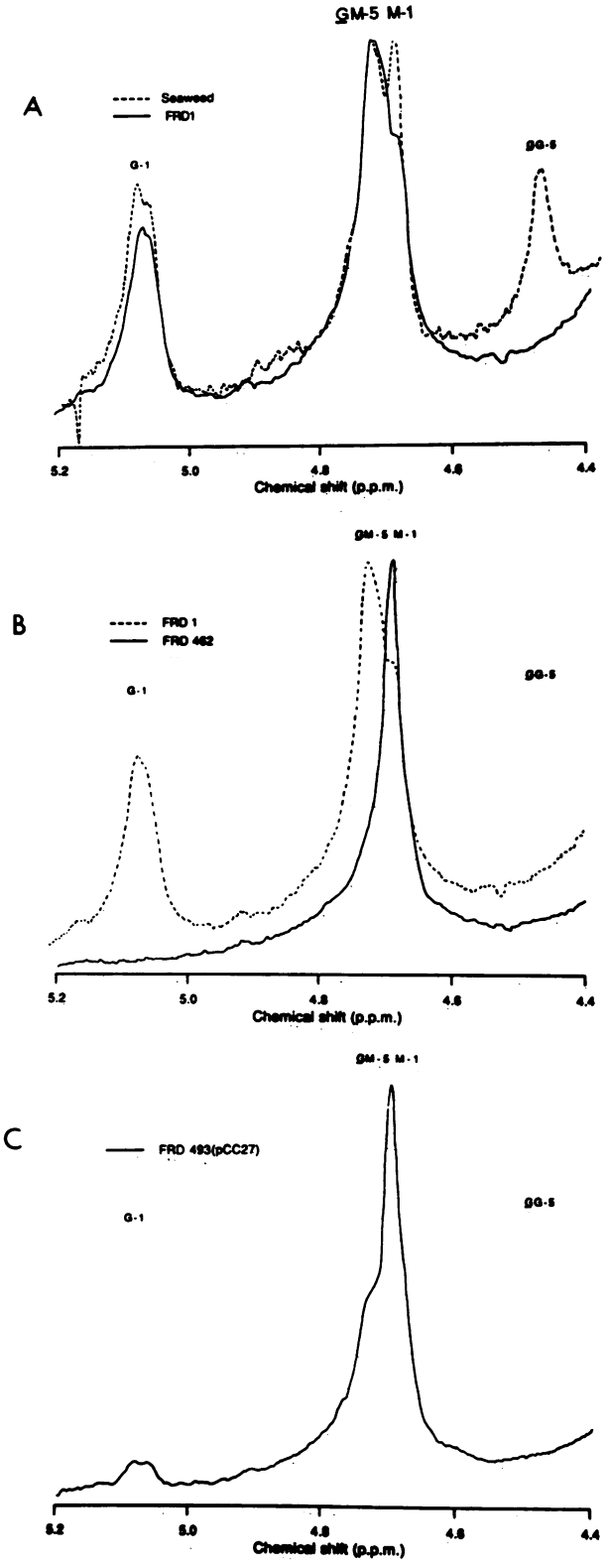

TABLE 2. Quantitative reaction of *P. aeruginosa* alginates with G'ase from *K. aerogenes* type 25

Alginate source	NaOH treatment[a]	G'ase reaction (A_{535})[b]
Seaweed	−	2.15
Seaweed	+	2.50
FRD1	−	1.11
FRD1	+	3.21
FRD462 *algG4*	+	0.14
FRD465 *algG7*	+	0.04
FRD493 *algG4*(pCC28)	+	0.18
FRD465 *algG7*(pCC28)	+	0.04
FRD493 *algG4*(pCC27)	+	1.23
FRD465 *algG7*(pCC27)	+	1.39

[a] Alginate (5 mg/ml) samples (150 μl) were pretreated with 50 μl of 1 N NaOH (20 min at 22°C) to remove acetyl groups from bacterial alginates. Samples were neutralized by the addition of 50 μl of 1.0 N HCl. +, NaOH treated; −, not treated.

[b] Deacetylated samples were treated with 10 μl of G'ase for 1 h 22°C, 250 μl of 0.025 N periodic acid in 0.125 N H_2SO_4 for 40 min at 22°C, 100 μl of 2% sodium arsenate in 0.5 N HCl for 1 min at 22°C, and then 1 ml of 0.6% 2-thiobarbituric acid in 7 mM NaOH for 30 min at 65°C. Samples were cooled and immediately read at A_{535}.

of other strains of mucoid *P. aeruginosa* (data not shown), and others have reported similar findings (35). The NMR spectrum of the mutant FRD462 alginate shows only one peak from the anomeric protons of M residues, and no evidence of G residues was detected (Fig. 3B). FRD465 alginate has a spectrum identical to that of FRD462 (data not shown), indicating that both of these mutant strains secrete polymannuronate.

Chromosomal mapping of *algG4*. The mutations responsible for defective incorporation of G residues into alginate in the mutants FRD462 and FRD465 were designated *algG4* and *algG7*, respectively. To determine the chromosomal location of *algG4*, random auxotrophic mutants of FRD462 were isolated to obtain selectable markers that were potentially adjacent to *algG4* for genetic mapping experiments. One auxotroph, FRD493, grew on minimal medium only if it was provided with arginine or citrulline and was unable to grow with ornithine, indicating that it had a mutation in *argF* (19), which is located at 34 min on the chromosome (31). In matings between recipient FRD493 *algG4 argF1* and the chromosomal donor FRD40 *pro-3* (R68.45), the $argF^+$ recombinants coinherited the $algG^+$ allele at high frequency (>90%). This indicated that the *algG4* mutation was located at approximately 34 min. This also indicates that *algG4* was within or near the cluster of alginate genes previously shown to be adjacent to the *argF* gene (4).

Cloning of *algG* using a gene replacement vector. The closely linked *argF* mutation in FRD493 was used to clone the wild-type *algG* gene. A gene bank of the *P. aeruginosa* FRD1 chromosomal DNA (partially digested *Bam*HI fragments) was prepared in the cosmid pEMR2. This vector permits cloning of large DNA fragments, mobilization by conjugation, and gene replacement in *P. aeruginosa* as

Fig. 3. NMR spectra of alginates from seaweed and *P. aeruginosa*. Alginates were prepared for ^{1}H-NMR analysis as described by Grasdalen et al. (18). Partially depolymerized samples were dissolved in deuterium, and NMR spectra were recorded at 180 MHz at 90°C. (A) Spectra of alginates from seaweed *M. pyrifera* (---------) and wild-type *P. aeruginosa* FRD1 (———); (B) spectra of alginates from wild-type *P. aeruginosa* FRD1 (---------) and *algG4* mutant FRD462 (———); (C) spectrum of alginate from FRD493(pCC27) (———).

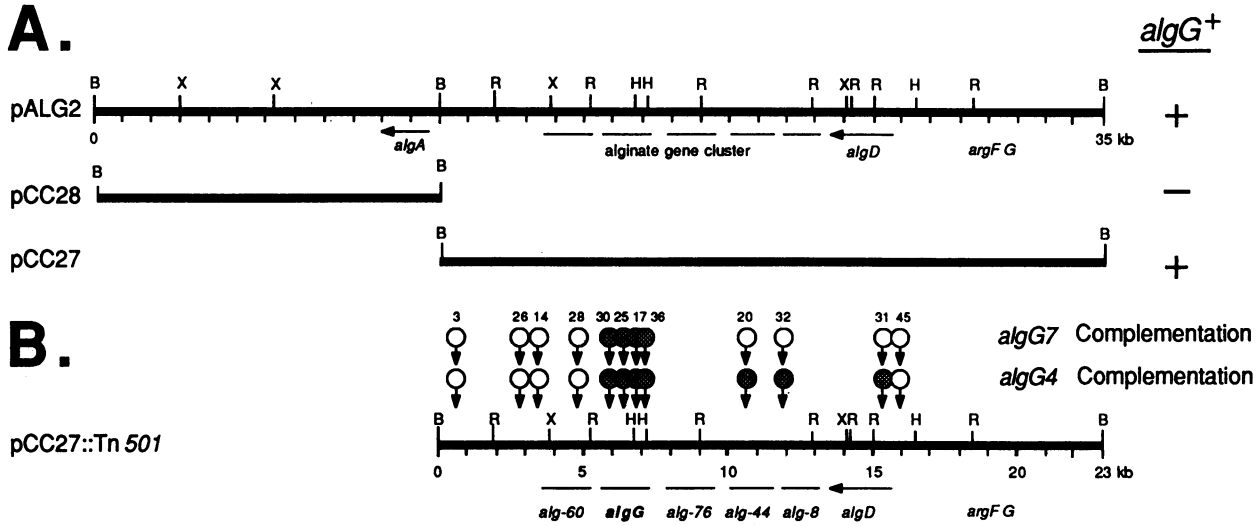

FIG. 4. (A) Restriction maps of *P. aeruginosa* FRD1 DNA fragments and their ability (+ or −) to complement *algG4* and *algG7* mutations in *trans* in *P. aeruginosa* strains. (B) Physical mapping of *algG* by Tn*501* mutagenesis of pCC27. Shown are the sites of Tn*501* insertions that do (●) and do not (○) block complementation of the defect in incorporation of guluronate residues into alginate by pCC27::Tn*501* derivatives in FRD465 *algG7* and FRD493 *algG4*. The locations of genes other than *algG* were determined previously (4, 6). B, *Bam*HI; X, *Xho*I; H, *Hin*dIII; R, *Eco*RI.

previously described (12). Briefly, pEMR2 is a derivative of pBR322 and cannot autonomously replicate in *P. aeruginosa*. It has a *cos* site, *oriT* sequence, *bla* (β-lactamase) gene, and a Tn*5* insertion with an internal *Bam*HI cloning site. When plasmids from this gene bank were mobilized from *E. coli* to FRD493 with selection for Arg^+, the $argF^+$ recombinants frequently (>10%) coinherited $algG^+$, which was determined by reaction with G'ase. This indicated that the gene bank included a clone containing both $argF^+$ and $algG^+$. To isolate this clone, the bank was divided into pools of 50 *E. coli* colonies, and each pool was tested for the ability to convert FRD493 to $argF^+$ and $algG^+$. The individual members of a positive pool were then tested to identify a clone containing $argF^+$ and $algG^+$. One positive clone, called pALG2, was transferred to FRD493 with selection for Arg^+. Approximately 50% of the transconjugants obtained had undergone gene replacement, as indicated by the loss of the vector markers for carbenicillin and kanamycin resistance. Among these Arg^+ recombinants with gene replacements, approximately 20% had coinherited the pALG2-borne $algG^+$ allele and secreted mixed sequence alginate, indicated by the production of an alginate susceptible to G'ase.

Physical mapping of *algG* by subcloning and transposon mutagenesis. Restriction fragment analysis of the 35 kilobases (kb) of *P. aeruginosa* DNA in pALG2 (Fig. 4A) revealed that the locations of *Bam*HI, *Eco*RI, *Hin*dIII, and *Xho*I sites were identical to those described for the alginate gene cluster adjacent to *argF* in *P. aeruginosa* 8821 (4). The 12- and 23-kb *Bam*HI fragments of pALG2 were subcloned in the broad-host-range vector pCP19 for *trans* complementation studies. The resulting subclones, pCC27 and pCC28 (Fig. 4A), were both transferred to FRD493 and FRD465 by conjugation, and complementation in *trans* of *algG4* and *algG7* was examined by using the G'ase assay and ^{1}H-NMR. The *algG* gene was localized to the 23-kb *Bam*HI fragment of *P. aeruginosa* DNA from pALG2, since only pCC27 could complement the defects in guluronate incorporation in FRD493 and FRD462 (Table 2). Although pCC27 could complement the *algG4* mutation in FRD493, the NMR spectrum revealed that the ability to incorporate guluronate residues conferred by pCC27 was only partially restored compared with that of the wild-type alginate (Fig. 3C, G-1 peak). Comparable results were obtained in the NMR spectrum of FRD465(pCC27) (data not shown).

Tn*501* mutagenesis was used to localize the *algG* gene on pCC27. Figure 4B shows the sites of Tn*501* insertions and their effect on the ability of pCC27 to complement *algG4* and *algG7* in *trans*. The sites of transposon insertions that could not complement the *algG7* mutation were clustered and revealed the location of the *algG* gene. The *algG* gene appeared to be located between two genes of unknown function required for alginate biosynthesis previously called *alg-60* and *alg-76* (4). Complementation of the *algG4* mutation with pCC27::Tn*501* derivatives showed a pattern similar to that seen in FRD465, except that the Tn*501* insertions that were upstream of *algG* and in the cluster of alginate genes also blocked the complementation of *algG4*. This suggested a polar effect of upstream genes on *algG* in this strain. Tn*501* insertions upstream of the alginate cluster or downstream of *algG* had no effect on complementation of *algG4* or *algG7*.

DISCUSSION

Unlike most polysaccharides, alginates do not have a repeating subunit structure and can contain blocks of M residues, blocks of G residues, and mixed blocks of alternating M and G residues, all of which are linked in a random manner (14, 21). The M and G monomers adopt the $^4C_1$ and $^1C_4$ chair conformations, respectively, so that the bulky carboxyl group is in the energetically favorable equatorial orientation (Fig. 1). As a result, the glycosidic bonds between M residues are equatorial and M blocks form an extended ribbonlike structure, whereas the glycosidic bonds between G residues are axial and G blocks resemble a buckled chain. A mixed block of alternating M and G residues introduces a bend in the linear chain (Fig. 1). Thus, the physical structure and properties of alginate are determined by both the M/G ratio and the way in which the epimers are distributed in the different blocks. For example,

G-rich alginates form strong but brittle gels in the presence of divalent cations like Ca^{2+}, whereas M-rich alginates form gels that are weak but flexible (14).

The biochemical steps by which the alginate pathway intermediate GDP-mannuronate is synthesized in *P. aeruginosa* are now understood. However, the mechanism by which G residues are formed and incorporated into the polymer has not been characterized. In *A. vinelandii*, a C-5 epimerase converts M residues to G at the polymer level (20, 23). Our ability to isolate mutants of *P. aeruginosa* that secrete polymannuronate is consistent with the possibility of a similar epimerization step for incorporation of G residues into alginate. Also, the epimerization reaction is not strictly required for the synthesis of the polymer. In *A. vinelandii*, the M/G ratios of alginate depend on the Ca^{2+} ion concentration in the growth medium because the C-5 epimerase is extracellular and Ca^{2+} dependent (23). However, the M/G ratio of *P. aeruginosa* alginate is independent of external conditions such as Ca^{2+} ion concentration and is tightly regulated in each strain (9; unpublished data). While *P. aeruginosa* alginate does not have any G blocks, as seen from the NMR data, *A. vinelandii* alginate has all three kinds of blocks (20, 23, 24). These differences suggest that the mechanisms of incorporation of G residues into alginate may be different in *P. aeruginosa* and *A. vinelandii*.

Both *algG4* and *algG7* mutations map to the same gene within the cluster of alginate genes that are located at 34 min on the revised (31) chromosomal linkage map. *algG* appears to lie between *alg-60* and *alg-76*, two alginate genes whose roles in the pathway are not yet known. The *algG* mutants isolated here still have a mucoid phenotype, whereas *alg-60* and *alg-76* mutants are nonmucoid, suggesting that *algG* is distinct from these two genes. The cluster has several genes involved in the alginate pathway, and more genes may be found between *alg-60* and *algA*. It has been shown that *algD*, the first gene in the alginate cluster, is transcriptionally activated in Alg^+ strains (6). Previous studies on the expression of this gene cluster using Northern (RNA) blot hybridization did not provide any evidence for coordinate regulation of downstream genes (6). This is supported by the fact that our Tn*501* insertions on pCC27 located upstream (i.e., on the *algD* side) of *algG* showed no obvious polar effect on the complementation of *algG7*. However, Tn*501* insertions between *algD* and *algG* appeared to have a polar effect on the complementation of *algG4*. This suggests that the alginate gene cluster may have some operonic structure, and this possibility is under investigation.

The *algG* gene product is clearly necessary for the formation or incorporation of G residues into alginate and may encode a C-5 epimerase. In an effort to understand the biochemical role of the *algG* product, constructions which allow for its overexpression in *E. coli* are being made and its ability to epimerize M residues to G at the polymer and/or monomer levels is being tested. These studies should improve our understanding of the late steps in the pathway of alginate biosynthesis and control of alginate structure in *P. aeruginosa*.

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