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# Identification of *algF* in the Alginate Biosynthetic Gene Cluster of *Pseudomonas aeruginosa* Which Is Required for Alginate Acetylation

MICHAEL J. FRANKLIN AND DENNIS E. OHMAN*

*Department of Microbiology and Immunology, University of Tennessee, and Veterans Affairs Medical Center, Memphis, Tennessee 38163*

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**Mucoid strains of *Pseudomonas aeruginosa* produce a high-molecular-weight exopolysaccharide called alginate that is modified by the addition of O-acetyl groups. To better understand the acetylation process, a gene involved in alginate acetylation called *algF* was identified in this study. We hypothesized that a gene involved in alginate acetylation would be located within the alginate biosynthetic gene cluster at 34 min on the *P. aeruginosa* chromosome. To isolate *algF* mutants, a procedure for localized mutagenesis was developed to introduce random chemical mutations into the *P. aeruginosa* alginate biosynthetic operon on the chromosome. For this, a DNA fragment containing the alginate biosynthetic operon and adjacent *argF* gene in a gene replacement cosmid vector was utilized. The plasmid was packaged in vivo into lambda phage particles, mutagenized in vitro with hydroxylamine, transduced into *Escherichia coli*, and mobilized to an *argF* auxotroph of *P. aeruginosa* FRD. $Arg^+$ recombinants coinherited the mutagenized alginate gene cluster and were screened for defects in alginate acetylation by testing for increased sensitivity to an alginate lyase produced by *Klebsiella aerogenes*. Alginates from recombinants which showed increased sensitivity to alginate lyase were tested for acetylation by a colorimetric assay and infrared spectroscopy. Two *algF* mutants that produced alginates reduced more than sixfold in acetyl groups were obtained. The acetylation defect was complemented in *trans* by a 3.8-kb *Xba*I-*Bam*HI fragment from the alginate gene cluster when placed in the correct orientation under a *trc* promoter. By a merodiploid analysis, the *algF* gene was further mapped to a region directly upstream of *algA* by examining the polar effects of Tn*501* insertions. By gene replacement, DNA with a Tn*501* insertion directly upstream of *algA* was recombined with the chromosome of mucoid strain FRD1. The resulting strain, FRD1003, was nonmucoid because of the polar effect of the transposon on the downstream *algA* gene. By providing *algA* in *trans* under the *tac* promoter, FRD1003 produced nonacetylated alginate, indicating that the transposon was within or just upstream of *algF*. These results demonstrated that *algF*, a gene involved in alginate acetylation, is located directly upstream of *algA*.**

Chronic pulmonary disease in patients with cystic fibrosis is caused by mucoid strains of *Pseudomonas aeruginosa* that produce a viscous exopolysaccharide called alginate. Mucoid *P. aeruginosa* is almost impossible to eradicate in these infections and is responsible for much of the morbidity and mortality in this patient group (14). The persistence of mucoid *P. aeruginosa* in the lung may be related to the strain's increased resistance to phagocytosis compared with that of nonmucoid strains (1, 27, 31), the reduced rate of antibiotic diffusion through alginate (2), and the potential role of alginate as an adherence factor (21).

Alginate is a high-molecular-weight, linear, nonrepeating polymer of D-mannuronic acid and L-guluronic acid (8). Many of the genes involved in alginate biosynthesis from *P. aeruginosa* have been cloned and characterized for a better understanding of the pathway and regulation of alginate biosynthesis. Most of the alginate biosynthetic genes are at 34 min on the *P. aeruginosa* chromosome in a large cluster which has an operonic structure (4). These genes include *algA*, which encodes phosphomannose isomerase–GDP–D-mannose phosphorylase (30); *algD*, which encodes GDP-mannose dehydrogenase (7); and *algG*, which is required for C-5 epimerization (3). There are many genes in this cluster whose functions are not yet known. The final steps in the alginate biosynthetic pathway, which includes polymerization, export, epimerization, and acetylation, have not been elucidated.

Alginate is also produced by *Azotobacter vinelandii* and some algae, as well as several species of *Pseudomonas*. The bacterial alginate varies from the algal alginate in that the former is O acetylated. Acetylation of *A. vinelandii* alginate occurs primarily at the O-2 position of the mannuronic acid residues, but the O-3 position or both the O-2 and O-3 positions can be acetylated (32). Skjåk-Braek et al. (33) hypothesized that acetylation may affect the ratio of mannuronate to guluronate residues in *A. vinelandii* alginate. Because nonacetylated residues are more reactive with C-5 epimerase than acetylated residues, acetylation may influence the final structure of alginate by regulating the degree of epimerization. Acetylation also affects the physical and chemical properties of alginate, including viscosity and interaction with calcium ions, causing increased swelling of calcium alginate (34). Acetylated alginate is less sensitive to degradative alginate lyases than the nonacetylated form (33).

To better understand the mechanism of alginate acetylation in *P. aeruginosa*, we isolated mutants of the mucoid cystic fibrosis strain FRD that were defective in acetylation of this exopolysaccharide. We hypothesized that a gene for acetylation was located in the alginate biosynthetic gene cluster. To efficiently mutagenize this region of the *P. aeruginosa* chromosome, a technique for localized mutagen-

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Genotype and/or phenotype[a] | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* HB101 | *proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20* | Our laboratory |
| *P. aeruginosa* | | |
| FRD1 | Prototrophic, mucoid, cystic fibrosis isolate | 23 |
| PAO1 | Prototrophic, nonmucoid | D. Haas |
| FRD1152 | Mucoid, *algF2* | This study |
| FRD1153 | Mucoid, *algF3* | This study |
| FRD1003 | *algF*::Tn*501-3*, nonmucoid | This study |
| Plasmids | | |
| pEMR2 | pBR322 *cos oriT* Tn*5* $Ap^r$ $Km^r$ | 10 |
| pALG2 | pEMR2 with 35-kb *P. aeruginosa* DNA containing the alginate biosynthetic operon and *argF* | 24 |
| pCC29 | pEMR2 with 23-kb *P. aeruginosa* DNA containing *argF* and the alginate biosynthetic operon, except for *algA* | 24 |
| pCP19 | IncP1 *cos oriT* $Tc^r$ | 10 |
| pCC27 | pCP19 with 23-kb *P. aeruginosa* DNA containing *argF* and the alginate biosynthetic operon, except for *algA* | 3 |
| pHC79 | pBR322 *cos* $Ap^r$ $Tc^r$ | 16 |
| pMF27::Tn*501* | pHC79 with 23-kb *P. aeruginosa* DNA containing the alginate biosynthetic operon with a Tn*501* insertion | This study |
| pMF37 | pCC27 with 10-kb *Xho*I deletion | This study |
| pMF35 | pCC27 with 3.8-kb *Xba*I deletion | This study |
| pKK233-2 | Inducible *trc* promoter expression vector, $Ap^r$ | Pharmacia |
| pMF36 | pKK233-2 with 1.8-kb stabilization fragment and *oriT* | This study |
| pMF52 | pMF36 with 3.8-kb *Xba*I fragment, $algF^+$, from pCC27 | This study |
| pMF51 | pMF36 with 3.8-kb *Xba*I fragment from pCC27 in reverse orientation to *tac* promoter | This study |
| pCC75 | pMMB22 (IncQ) with *algA* under *tac* promoter | 4 |
| pRK2013 | ColE1-Tra(RK2)$^+$, $Km^r$ | 9 |

[a] Abbreviations: $Tc^r$, tetracycline resistance; $Hg^r$, mercury resistance; $Ap^r$, ampicillin resistance; $Km^r$, kanamycin resistance; $Cb^r$, carbenicillin resistance; $Tra^+$, transfer by conjugation.

esis that utilized in vitro mutagenesis and gene replacement strategies was developed. Screening methods were developed, and mutants that produced nonacetylated alginate were isolated. We took advantage of the operonic structure of the biosynthetic cluster to map the location of a gene required for acetylation, which was here termed *algF*. Further evidence for its role in acetylation was obtained by construction and characterization of mutants with chromosomal Tn*501* insertions that were defective in acetylation.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** Bacterial strains, phage, and plasmids used in this study are shown in Table 1. *Escherichia coli* and *P. aeruginosa* were routinely cultured in L broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, all per liter). *E. coli* strains used for λ phage propagation were grown in TM broth (10 g of tryptone, 5 g of NaCl, 2 g of maltose, all per liter). VB minimal medium (36) was used to select for *P. aeruginosa* following triparental matings with auxotrophic *E. coli*. AP defined medium (23) was used to promote alginate production by *P. aeruginosa*. A strain of *Klebsiella aerogenes* which can be induced to produce an α-L-guluronate-specific alginate lyase (3) was grown at 37°C in L broth containing 1% alginate (high-viscosity alginate from *Macrocystis pyrifera*; Sigma Chemical Co.). Lyase agar was used to screen nonmucoid mutants producing nonacetylated alginates that were especially sensitive to enzymatic degradation. Lyase agar was prepared by adding 100 ml of filter-sterilized supernatant from a 24-h culture of *K. aerogenes* to 900 ml of L agar. Antibiotics, when used, were at the following concentrations (per milliliter): ampicillin, 100 μg; carbenicillin, 300 μg; $HgCl_2$, 18 μg; and tetracycline, 15 μg for *E. coli* and 100 μg for *P. aeruginosa*.

**DNA manipulations.** General DNA manipulations were performed as described previously (20). Triparental matings were used to mobilize plasmids from *E. coli* to *P. aeruginosa* with the conjugation helper plasmid pRK2013 (9).

**Formation of merodiploids.** For the merodiploid analysis, pMF27::Tn*501-3*, *-26*, *-14*, and *-25* plasmids were constructed by ligation of the *Bam*HI fragments from pCC27::Tn*501-3*, *-26*, *-14*, and *-25* (3) into the *Bam*HI site of the cosmid vector pHC79 (16). To improve the cloning efficiencies of these large DNA fragments (23 plus 8.2 kb from Tn*501*), ligation mixtures were incubated with in vitro lambda packaging extracts (Promega, Inc.) and transduced into *E. coli* HB101 with selection on L agar containing ampicillin. The pMF27::Tn*501* derivatives were mobilized into *P. aeruginosa* with pRK2013 with selection on VB agar with carbenicillin. These narrow-host-range plasmids cannot replicate independently in *P. aeruginosa*, and carbenicillin-resistant colonies formed by homologous recombination with the chromosome, resulting in merodiploids.

**Construction of an expression vector for *P. aeruginosa*.** pMF36 was a broad-host-range *trc* expression vector constructed for these studies. New restriction sites were added to the *trc* vector pKK233-2 (Pharmacia) (Fig. 1) by hybrid-

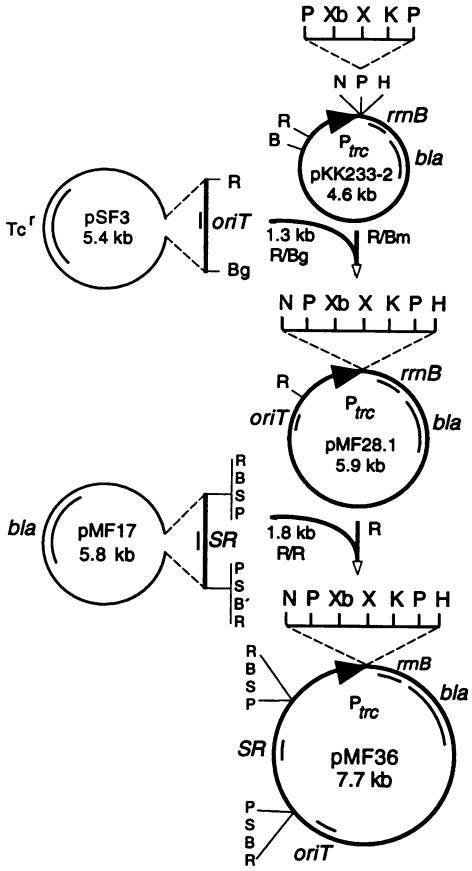


FIG. 1. Construction of *P. aeruginosa trc* expression vector. Restriction sites were added by ligating a synthetic polylinker into the *Pst*I site of pKK233-2. A 1.3-kb *Eco*RI-*Bgl*II fragment from pSF3 (28) containing *oriT* was ligated into the *Eco*RI-*Bam*HI sites, resulting in pMF28.1. A 1.8-kb *Pst*I fragment, which enables stable replication (*SR*) of ColE1 plasmids in *P. aeruginosa* (26), was ligated into the *Pst*I site of pUC4K to obtain flanking *Eco*RI sites, resulting in pMF17. This *Eco*RI fragment from pMF17 was ligated into the *Eco*RI site of pMF28.1, resulting in pMF36, which is mobilizable and replicates in both *E. coli* and *P. aeruginosa*. $P_{trc}$, strong *trc* promoter; *rrnB*, strong transcriptional terminator; *bla*, β-lactam resistance; Tc$^r$, tetracycline resistance; *oriT*, origin of transfer; P, *Pst*I; Xb, *Xba*I; X, *Xho*I; K, *Kpn*I; N, *Nco*I; H, *Hin*dIII; R, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; S, *Sal*I.

izing two synthetic oligonucleotides containing *Xba*I, *Xho*I, and *Kpn*I sites and ligating the DNA into the *Pst*I site of pKK233-2. A 1.3-kb *Eco*RI-*Bgl*II fragment from pSF3 (28), containing the *oriT* site for conjugal transfer, was ligated into the *Eco*RI–*Bam*HI-digested plasmid, resulting in pMF28.1. A 1.8-kb *Pst*I fragment from pPZ101 (gift from P. Phibbs), which permits stable replication of ColE1-based plasmids in *P. aeruginosa* (26), was ligated into the *Pst*I site of pUC4K (Pharmacia), resulting in pMF17. The *Eco*RI fragment from pMF17 was ligated into the *Eco*RI site of pMF28.1, resulting in pMF36.

**Localized mutagenesis.** Localized chemical mutagenesis was used to induce mutations in the chromosomal alginate biosynthetic operon of *P. aeruginosa*. pALG2 has been described previously (3) and contains the entire alginate biosynthetic gene cluster and *argF*$^+$ on 35 kb of *P. aeruginosa* FRD1 DNA in the gene replacement vector pEMR2 (10). λ*c*I857 lysates were prepared as described previously (20) on *E. coli* HB101(pALG2) to package the cosmid pALG2 into λ particles. These lysates were mutagenized by treatment in vitro with hydroxylamine according to Tessman (35) by incubating ~$10^{11}$ phage particles with phosphate-buffered hydroxylamine (500 mM $NH_2OH$, 100 mM potassium phosphate, 1 mM EDTA, 1 mM $MgSO_4$ [pH 6.0]) for 24 h at 37°C. The mutagenized plasmids in the treated phage were transduced into HB101 and then selected on L agar with ampicillin. Colonies containing pALG2 with random chemical mutations were pooled (10 pools of ~$10^3$ colonies per pool).

The *P. aeruginosa* DNA in pALG2, mutagenized as described above, was mobilized to *P. aeruginosa* by pRK2013. pALG2 cannot replicate in *P. aeruginosa* because of its narrow host range, and recombination occurs with the chromosome in the region homologous to the inserted DNA. The vector contains Tn*5*, which shows reduced stability in *P. aeruginosa* (13) and facilitates excision of the clone from the chromosome by homologous recombination, and this often results in gene replacement (10, 12). Here, the *argF* gene adjacent to the *alg* cluster served as the selectable marker in *P. aeruginosa*. Mutagenized pALG2 plasmids were conjugated en masse into the Arg$^-$ strain *P. aeruginosa* FRD492, and recombinants were obtained by selection for Arg$^+$ on minimal agar medium. Under these conditions, the mutagenized alginate biosynthetic operon would be coinherited at a high frequency with the adjacent *argF*$^+$ marker. Prototrophic colonies were pooled (10 pools with ~$10^3$ colonies per pool) and grown for 18 h in VB liquid medium to allow gene replacement to occur.

**Construction of *alg*::Tn*501* mutants.** Construction of mutants with Tn*501* insertions in the alginate gene cluster was done by gene replacement by a transduction method previously described (25). Derivatives of pCC27 containing Tn*501* insertions in the *alg* cluster (3) were mobilized into *P. aeruginosa* PAO1 and then selected for tetracycline resistance. Lysates of the generalized transducing phage F116L were prepared on PAO1(pCC27::Tn*501*) strains and used to transduce plasmid fragments into FRD1. Recombinants were selected on L agar containing $HgCl_2$ and screened for loss of the vector-encoded tetracycline resistance marker, which indicated gene replacement.

**Isolation of alginates.** Alginates were collected from culture supernatants of strains grown for 24 to 48 h in AP medium as follows. Samples (5 ml) of cultures were mixed with 5 ml of saline to reduce viscosity, and the cells were removed by centrifugation (25,000 × *g* for 15 min). The culture supernatant was mixed with 5 ml of 2% cetylpyridinium chloride (Sigma), and the precipitated alginate was collected by centrifugation (25,000 × *g* for 15 min). The pellet was dissolved in 5 ml of 1 M NaCl, precipitated again with 5 ml of cold (−20°C) isopropanol, and dissolved in 2 ml of saline. The isopropanol precipitation procedure was repeated for samples examined by infrared (IR) spectroscopy. Where indicated, *P. aeruginosa* alginate was deacetylated by incubating 500 μl of purified alginate with 300 μl of 1 M NaOH at 65°C for 30 min, and the reaction mixture was neutralized with 300 μl of 1 M HCl.

**Alginate and acetylation assays.** The concentration of alginate in culture supernatants was determined by the carbazole method of Knutson and Jeanes (19). A solution of purified alginate (30 μl) was mixed with 1.0 ml of borate-sulfuric acid reagent (10 mM $H_3BO_3$ in concentrated $H_2SO_4$), and 30 μl of carbazole reagent (0.1% in ethanol) was added. The mixture was heated to 55°C for 30 min, and the

alginate concentration was determined spectrophotometrically at 500 nm with *M. pyrifera* alginate (Sigma) as a standard. The method described by Hestrin (15) was used to measure alginate acetylation. Briefly, 500 μl of an alginate solution was incubated with 500 μl of alkaline hydroxylamine (0.35 M $NH_2OH$, 0.75 M NaOH) for 10 min at 25°C. The reaction mixture was acidified with 500 μl of 1.0 M perchloric acid, and 500 μl of 70 mM ferric perchlorate in 0.5 M perchloric acid was added. The concentration of acetyl groups was determined spectrophotometrically at 500 nm on the basis of a standard curve with ethyl acetate as the substrate.

**IR spectroscopy.** Alginate acetylation was examined by Fourier transform (FT) IR spectroscopy (FT-IR). Others have shown that absorbances at 1,732 and 1,250 $cm^{-1}$ are associated with alginate O-acetyl ester bonds (29). Purified alginates were spotted onto IR cards (3M Co.) and air dried in a laminar flow hood. Spectra were collected with a Mattson Polaris spectrometer as single-beam interferograms in the transmittance mode. Fourier-processed interferograms were divided by a background spectrum obtained with an IR card containing no alginate.

## RESULTS

**Isolation of *P. aeruginosa algF* mutants defective in alginate acetylation.** We predicted that a gene (here termed *algF*) for acetylation of alginate would probably be located in the alginate biosynthetic gene cluster at 34 min on the *P. aeruginosa* chromosome. A method for localized mutagenesis was developed to mutagenize this region of the chromosome at a high frequency in an effort to isolate mutants defective in alginate acetylation. The technique developed bears a resemblance to one used by Hong and Ames (17), who obtained temperature-sensitive mutants in *Salmonella typhimurium* by in vitro mutagenesis of a transducing phage lysate prior to transduction. For localized mutagenesis in *P. aeruginosa*, we utilized pALG2 (3), which contains 35 kb of *P. aeruginosa* DNA encoding the alginate biosynthetic operon and *argF*$^+$ in the cosmid gene replacement vector, pEMR2 (Fig. 2A). Lambda lysates were prepared on *E. coli* HB101(pALG2) to package these cosmids into phage particles. The packaged cosmids were mutagenized in vitro by treatment with hydroxylamine and then transduced into *E. coli* (Fig. 2B). Mutagenized pALG2 plasmids were conjugated en masse into an Arg$^-$ strain of *P. aeruginosa*, FRD492, and recombinants were obtained by selection for Arg$^+$ on minimal agar medium (Fig. 2C). Under these conditions, the mutagenized alginate biosynthetic operon with potential mutations in *algF* would be coinherited at a high frequency along with the adjacent *argF*$^+$ marker (Fig. 2D).

An L-guluronate lyase produced by a strain of a *Klebsiella* species has been previously shown to efficiently depolymerize deacetylated alginate, but acetylated alginate is more resistant (3, 33). This property of the enzyme was used to screen for mutants producing nonacetylated alginates. The pooled recombinants were plated on L agar containing L-guluronate lyase at the highest concentration that did not change the mucoid phenotype of colonies producing wild-type, acetylated alginate. Nonmucoid colonies observed on the lyase agar, which showed a mucoid phenotype on L agar in the absence of the enzyme, were examined further. The alginate of the parent strain FRD492 was highly resistant to this L-guluronate lyase, not only because of acetylation, but also because of an *algG* mutation which results in the production of alginate without L-guluronate residues (3). Thus, high sensitivity to this guluronate-specific lyase indicated coinheritance of *algG*$^+$ from pALG2 as well as a potential *algF* mutation. From approximately 10,000 colonies screened on the lyase medium, alginates were collected from about 300 potential *algF* mutants and tested for acetyl

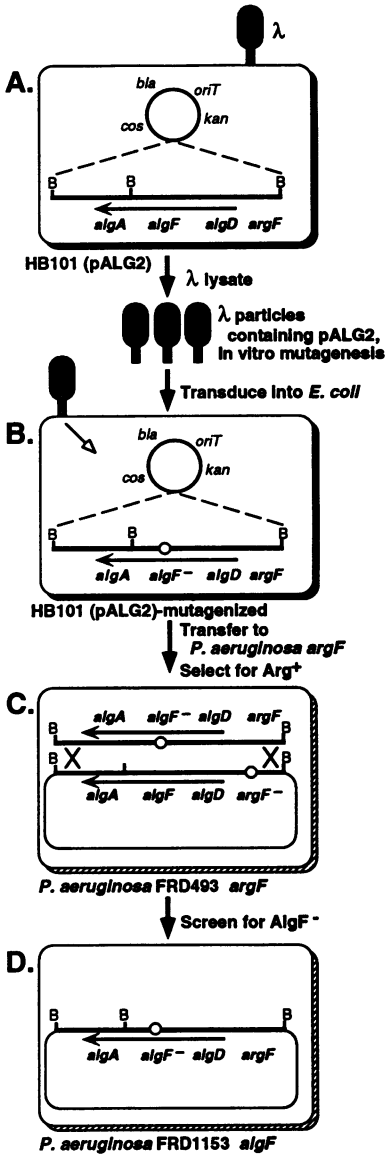


FIG. 2. Method for region-specific chemical mutagenesis of alginate biosynthetic operon in chromosome of *P. aeruginosa* FRD. (A) pALG2 contains the alginate biosynthetic operon and the adjacent *argF* gene in the cosmid vector pEMR2. *E. coli*(pALG2) was infected with λ*c*I857 to package the plasmid into phage particles in vivo, and the lysates containing pALG2 were mutagenized in vitro by treatment with hydroxylamine. (B) Plasmids with potential mutations in *algF* were transduced into *E. coli* HB101. (C) Mutagenized pALG2 plasmids were mobilized en masse into FRD493 with selection for Arg$^+$. (D) Recombinants were pooled and screened for the AlgF$^-$ phenotype by plating on L agar containing guluronate lyase, which preferentially degrades deacetylated alginate such that *algF* mutants appear almost nonmucoid. Abbreviations: *cos*, λ packaging site; *oriT*, origin of transfer by conjugation; *bla*, β-lactam resistance; *kan*, kanamycin resistance; B, *Bam*HI.

TABLE 2. Assay of alginate acetylation and effects of complementing plasmids

| Strain (genotype) | Plasmid (genotype) | Molar ratio of acetyl groups to sugar residues[a] |
|---|---|---|
| FRD1 (*algF*$^+$) | | 0.54 |
| FRD1152 (*algF2*) | | 0.08 |
| FRD1152 (*algF2*) | pCC27 (*algF*$^+$) | 0.24 |
| FRD1152 (*algF2*) | pMF37 (*algF*$^+$) | 0.20 |
| FRD1152 (*algF2*) | pMF35 (*algF*$^-$) | 0.10 |
| FRD1152 (*algF2*) | pMF51 (*algF*) | 0.13 |
| FRD1152 (*algF2*) | pMF52 (*algF*$^+$) | 0.74 |
| FRD1153 (*algF3*) | | 0.03 |
| FRD1153 (*algF3*) | pCC27 (*algF*$^+$) | 0.12 |
| FRD1153 (*algF3*) | pMF37 (*algF*$^+$) | 0.13 |
| FRD1153 (*algF3*) | pMF35 (*algF*$^-$) | 0.01 |
| FRD1153 (*algF3*) | pMF51 (*algF*$^+$) | 0.10 |
| FRD1153 (*algF3*) | pMF52 (*algF*$^+$) | 0.68 |
| FRD1153 (*algF3*) | pCC29 | 0.63 |
| FRD1153 (*algF3*) | pMF27::Tn*501-3* | 0.01 |
| FRD1153 (*algF3*) | pMF27::Tn*501-26* | 0.02 |
| FRD1153 (*algF3*) | pMF27::Tn*501-14* | 0.02 |
| FRD1153 (*algF3*) | pMF27::Tn*501-25* | 0.01 |
| FRD1003 (*algF*::Tn*501-3*) | pCC75 (*algA*$^+$) | 0.01 |

[a] Values shown represent the average of duplicates in one of three comparable experiments.

groups by the colorimetric assay. Of the 300 potential *algF* mutants, two independent isolates, FRD1152 and FRD1153, produced alginates that demonstrated weak reactions in the assay for acetylation. Compared with the wild-type exopolysaccharide of FRD1, which contained 0.54 mmol of acetyl groups per mmol of alginate, the exopolysaccharides from the *algF* mutants FRD1152 and FRD1153 contained approximately 15 and 7%, respectively, of wild-type levels of acetylation (Table 2).

**FT-IR of *algF* mutant alginates.** Figure 3A shows the FT-IR spectrum of FRD1 alginate. Figure 3B shows the spectrum of FRD1 alginate that was chemically deacetylated by alkaline treatment. The results show the loss of two distinct peaks (at 1,732 and 1,250 $cm^{-1}$) associated with alginate O-acetyl ester bonds. Figure 3C shows the spectrum of purified alginate from the *algF* mutant FRD1153, which also had reduced absorbances at 1,732 and 1,250 $cm^{-1}$ compared with those of wild-type alginate. The spectrum of alginate from mutant FRD1152 was similar to that of FRD1153 (data not shown). These results verified that the mutant alginate was deficient in O acetylation (AlgF$^-$).

**Genetic complementation of *algF* mutations.** The acetylation defects in FRD1152 and FRD1153 were complemented in *trans* by pCC27 (Table 2), a low-copy-number plasmid that contains a 23-kb *Bam*HI fragment from pALG2 encoding all of the alginate biosynthetic gene cluster except for *algA* in a broad-host-range plasmid vector (Fig. 4A). Although the molar ratio of acetyl groups to sugar residues did not reach wild-type levels in alginates FRD1152(pCC27) and FRD1153(pCC27), the increase in acetylation was readily apparent. Thus, *algF* was located in this gene cluster, as expected from the localized mutagenesis procedure used to isolate these mutants. Deletion derivatives of pCC27, which still maintained the operon under expression from the promoter upstream of *algD*, were used to localize *algF*. Plasmid pMF37 was constructed by deletion of a 10-kb internal *Xho*I fragment from pCC27 (Fig. 4A), and this plasmid complemented both *algF* mutants, as determined by the acetylation

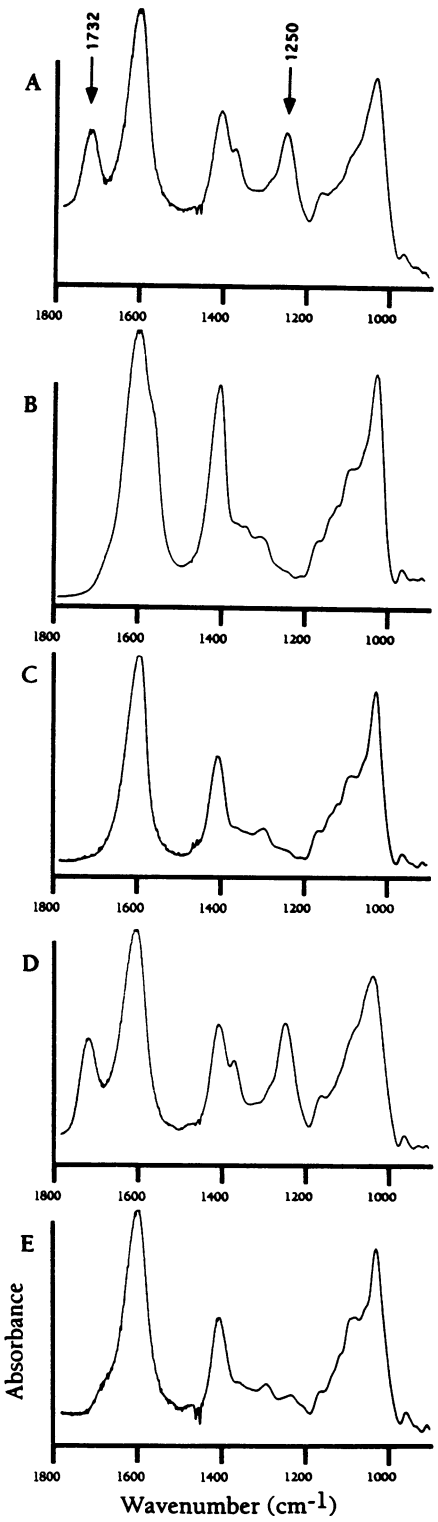


FIG. 3. FT-IR spectra of purified alginates from *P. aeruginosa* FRD strains. Absorbance peaks at 1,732 and 1,250 $cm^{-1}$ indicate the presence of the acetyl ester bond. (A) FRD1 wild-type alginate; (B) FRD1 alginate which was chemically deacetylated with NaOH; (C) FRD1153 *algF3* alginate; (D) FRD1153 *algF3* (pMF52 *algF*$^+$) alginate; (E) FRD1003 *algF*::Tn*501-3* (pCC75 *algA*$^+$) alginate.

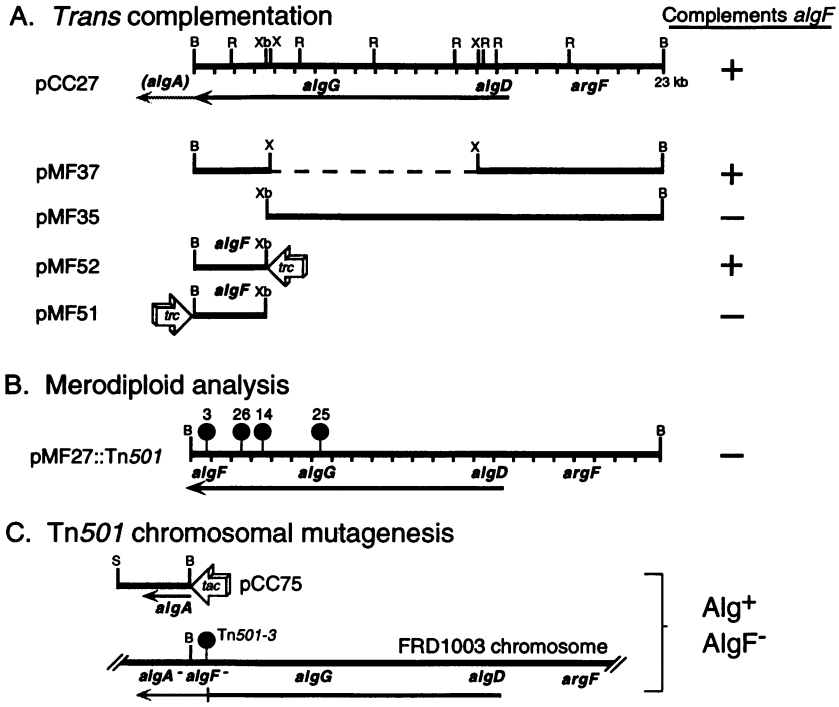


FIG. 4. *P. aeruginosa* DNA in plasmid constructions used in *algF* complementation analyses. (A) Plasmids shown were used to examine complementation of *algF* mutations in FRD1152 and FRD1153 in *trans*. The upstream 23-kb region of the alginate biosynthetic operon (pCC27) complemented the *algF* mutations. The shaded arrow indicates a portion of the operon not expressed. Complementation also occurred when the 10-kb internal *Xho*I fragment was deleted (pMF37) but not when the 3.8-kb *Xba*I-*Bam*HI fragment was deleted (pMF35). Complementation occurred when the 3.8-kb fragment was under the control of the *trc* promoter (pMF52) but was reduced when the *trc* promoter was in the reverse orientation (pMF51). (B) Shown are Tn*501* insertion derivatives used in merodiploid complementation tests to map the physical location of *algF*. Circles indicate the locations of Tn*501* insertions originally in pCC27 (3). The *Bam*HI fragment with Tn*501* (which has no *Bam*HI sites) was cloned into the narrow-host-range vector pHC79, which can then be mobilized to *P. aeruginosa* and integrated into the chromosome by homologous recombination. None of these merodiploids containing Tn*501* complemented *algF* mutations, indicating that the farthest downstream transposon, Tn*501-3*, was within or just upstream of *algF*. (C) FRD1003 with a chromosomal *algF*::Tn*501-3* insertion in the chromosome was nonmucoid because of the polar effect on *algA* expression. When *algA* was expressed in *trans* under the *tac* promoter on pCC75, FRD1003(pCC75) was mucoid ($Alg^+$) but produced a nonacetylated alginate ($AlgF^-$). For abbreviations, see the legend to Fig. 1.

assay (Table 2) and FT-IR analysis of the alginate produced (data not shown). Plasmid pMF35 was constructed by deletion of the end 3.8-kb *Xba*I-*Bam*HI fragment from pCC27 (Fig. 4A). This plasmid failed to complement either *algF* mutant (Table 2), indicating that *algF* was located on this terminal 3.8-kb fragment. The 3.8-kb *Xba*I-*Bam*HI fragment containing *algF* was cloned into the high-copy-number expression vector, pMF36 (Fig. 1); pMF52 and pMF51 contained this 3.8-kb fragment in the correct and opposite orientations, respectively, for transcription under the control of the *trc* promoter (Fig. 4A). pMF52 (*trc→algF*) complemented the $AlgF^-$ phenotype of both FRD1153 and FRD1152 to wild-type levels, whereas pMF51 (*←trc algY*) showed relatively little complementation activity (Table 2). The FT-IR spectrum of alginate from FRD1153(pMF52) demonstrated absorbance peaks at 1,732 and 1,250 $cm^{-1}$, thus verifying the presence of the O-acetyl ester bond (Fig. 3D).

**Merodiploid analysis with Tn*501* insertions to map *algF*.** The results above show that *algF* was located in the terminal 3.8-kb *Xba*I-*Bam*HI fragment of pCC27 between *algG* and *algA*. The *algF* gene was further mapped with Tn*501* insertions in complementing clones and by testing for the $AlgF^+$ phenotype in *algF* mutants. For these studies, a merodiploid analysis was used. pCC29 (24) contained the same 23-kb *Bam*HI fragment as that in pCC27 (Fig. 4A), which encodes the alginate gene cluster except for *algA*, but in a narrow-host-range plasmid. When pCC29 integrated into the chromosomes of FRD1152 and FRD1153 by homologous recombination (selecting for the vector-encoded carbenicillin resistance marker), all merodiploids were $AlgF^+$ (Table 2) because one of the operons formed can still transcribe $algF^+$ (Fig. 5A). A series of narrow-host-range plasmids (pMF27::Tn*501*) containing the 23-kb *Bam*HI fragment with Tn*501* insertions near the end of the alginate biosynthetic operon were constructed (Fig. 4B). These pMF27::Tn*501* derivatives were used to construct mucoid merodiploids of the *algF* mutants. Nonmucoid merodiploids were predicted to form when crossovers were downstream of Tn*501* (because of the polar effect of the transposon on *algA* transcription), and these colonies were not evaluated here. Mucoid merodiploids resulted when the crossover occurred upstream of Tn*501*, as shown in Fig. 5. If the transposon insertion were downstream of $algF^+$ in pMF27::Tn*501*, then $AlgF^+$ merodiploids would be obtained (Fig. 5B), since one functional operon containing the *algF* mutant allele and one truncated operon containing $algF^+$ would be transcribed. However, if the transposon insertion were upstream of $algF^+$ in pMF27::Tn*501*, then $AlgF^-$ merodiploids would be obtained (Fig. 5C). When merodiploids were formed and

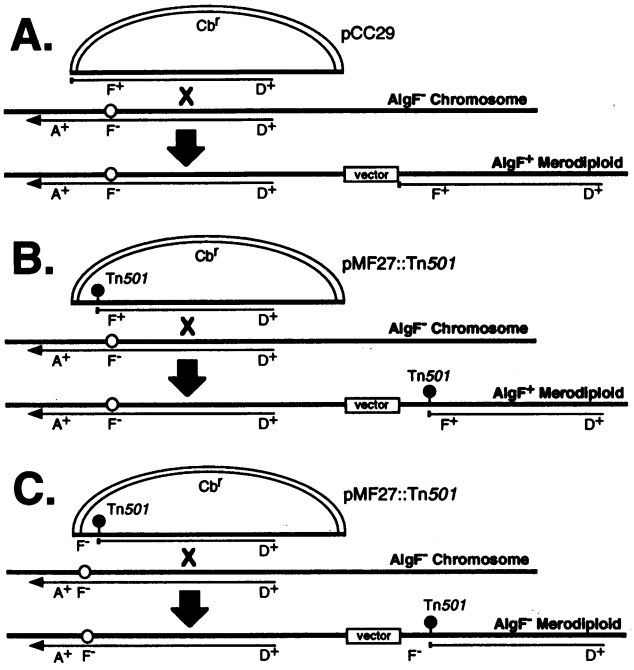


FIG. 5. Analysis of mucoid merodiploids formed in *algF* mutants with upstream 23-kb fragment of alginate biosynthetic operon. (A) Integration of pCC29 results in expression of an $algF^+$ allele and a complete transcript containing the downstream gene $algA^+$, resulting in an $AlgF^+$ (acetylated alginate) phenotype. (B) The $AlgF^+$ phenotype would be predicted if Tn*501* were downstream of *algF*, since one complete transcript containing $algA^+$ and one truncated transcript containing $algF^+$ would be produced. (C) The $AlgF^-$ phenotype (nonacetylated alginate) would be predicted if Tn*501* were upstream of *algF*, since $algF^+$ would not be expressed from either transcript. Arrows indicate *alg* operon. X indicates single crossover by homologous recombination. $Cb^r$, carbenicillin resistance; $F^+$, $algF^+$; $D^+$, $algD^+$; $A^+$, $algA^+$; $F^-$, $algF^-$.

analyzed with pMF27::Tn*501-3*, *-26*, *-14*, and *-25* (Fig. 4B) in both *algF* mutants, all formed only $AlgF^-$ merodiploids (Table 2; Fig. 5C). Considering that Tn*501-3* is at nearly the end of the alginate gene cluster in pMF27 (Fig. 4B), these results indicated that Tn*501-3* must be within or just upstream of *algF*.

**Phenotype of *alg*::Tn*501-3* mutants.** A plasmid-borne *algF*::Tn*501-3* insertion was transferred from pCC27::Tn*501-3* to the chromosome of FRD1 by a transduction method for gene replacement. The resulting strain, FRD1003 (Fig. 4C), did not produce alginate because of (i) the polar effect of the transposon on *algA* expression and (ii) the possibility that Tn*501-3* could be in a gene essential for alginate production. The latter possibility was tested by construction of FRD1003 containing pCC75, a plasmid with *algA* under the control of the *tac* promoter (Fig. 4C). With expressed *algA* provided in *trans*, FRD1003(pCC75) displayed a mucoid ($Alg^+$) phenotype, indicating that Tn*501-3* was not located in a gene essential for alginate production. However, the alginate produced by FRD1003(pCC75) was not acetylated, as determined by the colorimetric assay (Table 2). The FT-IR spectra of alginate from FRD1003 (pCC75) showed little absorbance at 1,250 and 1,732 $cm^{-1}$, indicative of the lack of acetyl ester bonds (Fig. 3E). These results confirmed that the Tn*501-3* was probably within (or just upstream of) *algF*.

## DISCUSSION

A major difference between the alginate of bacteria and that of algae is that the former contains O-acetyl groups on the mannuronate residues (6). Previous studies have shown that acetylation affects the physical and chemical properties of alginate (34). However, the mechanism of alginate acetylation in bacteria has not been investigated. In this study, a molecular genetic approach was used to isolate mutants of *P. aeruginosa* that were defective in alginate acetylation in order to identify a gene (here called *algF*) responsible for acetylation.

The gene replacement technique called excision marker rescue has been previously used to alter the *P. aeruginosa* chromosome by the introduction of site-specific transposon insertions (12, 18), introduction of specific deletion mutations (12), allelic exchange with adjacent selectable markers (10), and introduction of specific base pair substitutions constructed in vitro (22). Here we used a combination of region-specific chemical mutagenesis (17) and this gene replacement technique to introduce random mutations into the alginate biosynthetic operon of *P. aeruginosa*. We took advantage of the λ*cos* site on the gene replacement vector, pEMR2, to package a clone containing the alginate gene cluster into phage particles; this allowed efficient chemical mutagenesis in vitro and simple manipulation of the plasmid. Region-specific chemical mutagenesis of the alginate biosynthetic operon should result in a high concentration of mutations in the region of interest, while leaving the remainder of the chromosome unaffected.

The method available to us for recognizing *algF* mutants on alginate lyase medium led to numerous false positives, and ~300 colonies had to be tested for acetylation in a more labor-intensive assay before two *algF* mutants were eventually obtained. However, localized chemical mutagenesis probably enabled us to screen far fewer colonies than if the entire chromosome had been mutagenized by standard procedures. We took advantage of an adjacent prototrophic marker (*argF*) on the clone being mutagenized, and this provided a convenient selectable marker for selecting recombinants. Alternatively, a transposon insertion (e.g., Tn*501*) could have been used as an adjacent selectable marker to obtain the same results. Thus, this method for localized mutagenesis may have other applications when one expects a clone to contain a cluster of related genes.

Localized mutagenesis allowed us to rapidly identify the location of *algF*, since the clone used for mutagenesis already contained the wild-type gene. Also, localized chemical mutagenesis was advantageous over transposon mutagenesis in the initial phases of this study, since *algF* is located in a large operon and transposon insertions cause polar mutations on the downstream alginate biosynthetic genes (4). The disadvantage of this technique would be if a gene of interest were not located within the region being mutagenized. Only one known alginate biosynthetic gene (*algC*) is not located within the alginate biosynthetic operon, and so it seemed likely to us that a gene involved in alginate acetylation would also be in the alginate biosynthetic operon; this was confirmed by our results. However, we cannot discount the possibility that there is another gene elsewhere in the chromosome that is required for alginate acetylation but that would not have been discovered by our methods.

We also hypothesized that mannuronate acetylation would not be required for alginate biosynthesis, and our mutant-screening procedure was restricted to mucoid colonies on the basis of this assumption. Since the *algF* mutants found

were indeed able to synthesize nonacetylated alginate, then acetylation is apparently not required for polymer biosynthesis. Preliminary studies indicate that the *algF* mutations had no effect on the amount of alginate synthesized compared with results from the wild-type strain (11).

Chitnis and Ohman (4) recently used genetic analyses and Northern (RNA) hybridizations to demonstrate that the alginate biosynthetic gene cluster has an operonic structure, with expression of the gene cluster under the primary control of the promoter upstream of *algD*. Although other studies have suggested that the alginate biosynthetic gene cluster also contains weaker internal promoters (e.g., reference 5), this study showed that *algF* did not have an alternate, internal promoter that was strong enough to complement the *algF* mutations. If *algF* contained its own promoter, then merodiploids with Tn*501* far upstream of the *algF* mutation would be expected to complement the mutation, but this was not the case. Even *algD*::Tn*501* insertions examined in the merodiploid analysis did not complement *algF* mutations (11). These results suggest that *algF* is transcribed from the promoter upstream of *algD* and further support the operon theory recently proposed for the alginate biosynthetic gene cluster (4).

We took advantage of the operonic nature of the gene cluster to locate *algF* within the 3.8-kb *Xba*I-*Bam*HI region upstream of *algA*. This was accomplished by constructing merodiploids with a variety of Tn*501* insertions in the chromosome. A merodiploid formed by chromosomal integration of a plasmid containing the entire gene cluster except for *algA* reconstructed a single operon which resulted in the $AlgF^+$ phenotype. However, none of the merodiploids containing Tn*501* insertions complemented the *algF* mutations. This indicated that the Tn*501* insertion farthest downstream (Tn*501-3*) was probably within *algF* or just upstream of this gene. Considering that Tn*501-3* was ~1 kb upstream of *algA*, this suggests that *algF* may be the next alginate gene adjacent to *algA*. Defined Tn*501-3* mutants were constructed, and these displayed the expected nonmucoid phenotype due to the polar effect of the transposon on *algA*. This was confirmed when the downstream gene, *algA*, was provided in *trans* under the *tac* promoter and the ability of the mutants to synthesize alginate was restored. Consistent with the merodiploid analysis, the alginate from the Tn*501-3* mutant with *algA* in *trans* was not acetylated. These results verified that *algF* was located directly upstream of *algA*.

The results of this study provide the molecular basis for studies on alginate acetylation. The *algF* gene, which may encode an acetyltransferase, is presently being sequenced and expressed in *E. coli* to study its role in alginate acetylation. It has been proposed previously that mannuronate acetylation in *A. vinelandii* may occur intracellularly at the sugar or sugar-nucleotide level (33). If acetylation occurs intracellularly in *P. aeruginosa*, then the enzymes involved in alginate polymerization are equally able to incorporate nonacetylated or acetylated mannuronate residues into the final alginate structure. We are presently investigating the mechanism of alginate acetylation, which may occur at the sugar, sugar-nucleotide, or polymer level in *P. aeruginosa*.

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