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Mutant Analysis and Cellular Localization of the AlgI, AlgJ, and AlgF Proteins Required for O Acetylation of Alginate in *Pseudomonas aeruginosa*

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Alginate is an extracellular polysaccharide produced by mucoid strains of *Pseudomonas aeruginosa* that are typically isolated from the pulmonary tracts of chronically infected cystic fibrosis patients. Alginate is a linear polymer of D-mannuronate and L-guluronate with *O*-acetyl ester linkages on the O-2 and/or O-3 position of the mannuronate residues. The presence of *O*-acetyl groups plays an important role in the ability of the polymer to act as a virulence factor, and the *algF*, *algJ*, and *algI* genes are known to be essential for the addition of *O*-acetyl groups to alginate. To better understand the mechanism of O acetylation of alginate, the cellular locations of the AlgI, AlgJ, and AlgF proteins were determined. For these studies, defined nonpolar *algI*, *algJ*, and *algF* deletion mutants of *P. aeruginosa* strain FRD1 were constructed, and each mutant produced alginate lacking *O*-acetyl groups. Expression of *algI*, *algJ*, or *algF* in *trans* in the corresponding mutant complemented each O acetylation defect. Random *phoA* (alkaline phosphatase [AP] gene) fusions to *algF*, *algJ*, and *algI* were constructed. All in-frame fusions to *algF* and *algJ* had AP activity, indicating that both AlgF and AlgJ were exported to the periplasm. Immunoblot analysis of spheroplasts and periplasmic fractions showed that AlgF was released with the periplasmic contents but that AlgJ remained with the spheroplast fraction. An N-terminal sequence analysis of AlgJ showed that its putative AlgJ signal peptide was not cleaved, suggesting that AlgJ is anchored to the cytoplasmic membrane by its uncleaved signal peptide. AP gene fusions were also used to map the membrane topology of AlgI, and the results suggest that it is an integral membrane protein with seven transmembrane domains. These results suggest that AlgI-AlgJ-AlgF may form a complex in the membrane that is the reaction center for O acetylation of alginate.

Patients with cystic fibrosis (CF) are highly susceptible to bacterial pulmonary infections, especially chronic infections with *Pseudomonas aeruginosa*. Isolates of *P. aeruginosa* from CF patients with pulmonary infections typically display a highly mucoid phenotype due to the production of large amounts of the viscous extracellular polysaccharide, alginate (9). Alginate confers increased protection against immune- and nonimmune-mediated killing (2, 32, 36). The structure of alginate is variable and plays an important role in its function. Alginate is originally synthesized as polymannuronic acid linked by β-1,4-glycosidic bonds (9, 14). Some of the mannuronate residues in the polymer are then modified by C5-epimerase, which is encoded by *algG*, to L-guluronate. This results in alginate with random blocks of mannuronate residues and mannuronate blocks interspersed with L-guluronate residues (4, 11). The mannuronate residues are also modified by esterification with *O*-acetyl groups at the O-2 and/or O-3 position (7, 37), producing an O-acetylated polymer. These structural modifications of alginate affect its physical and chemical properties, including its viscosity and ability to bind to calcium ions (38). O acetylation of alginate is required for *P. aeruginosa* to form microcolonies in a biofilm, which may play a role in pathogenesis (24). O acetylation of alginate also maximizes the resistance of mucoid *P. aeruginosa* to antibody-independent opsonic killing and is the molecular basis for the resistance to normally nonopsonic but alginate-specific antibodies found in the sera of infected CF patients (26).

Most of the genes required for alginate biosynthesis are in an 18-kb operon of 12 genes on the *P. aeruginosa* chromosome (5). In vitro assays have been developed for three of the gene products: AlgD (GDP-mannose dehydrogenase [8]), AlgA (phosphomannose isomerase–GDP–D-mannose phosphorylase [34]), and AlgG (C5-mannuronan epimerase [11]). Another enzyme involved in alginate biosynthesis is AlgC (phosphomannomutase [40]), which is encoded by an unlinked gene and is involved in both alginate and lipopolysaccharide biosyntheses (15, 39). The functions of the remaining products of the open reading frames on the alginate biosynthetic operon have not been fully characterized. Of these, AlgK is required for alginate polymer formation (18) and AlgE may be involved in the export of alginate across the outer membrane (6). AlgL has alginate lyase activity in vitro, but its function in vivo is not known (3, 29). The functions of the *alg8*, *alg44*, and *algX* gene products have not been characterized.

The roles of *algI*, *algJ*, and *algF* have been characterized by mutation, complementation, and sequence analysis (12, 13, 35). All three of these genes are required for O acetylation of alginate. However, neither the mechanism nor the topology of

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TABLE 1. Bacterial strains, and plasmids, and primers

Strain, plasmid, or primer	Genotype, phenotype[a] and/or description	Source or reference
P. aeruginosa strains		
FRD1	CF patient isolate; Alg^{+}	25
FRD1154	Δ*algJ5*::Gm^{r} Alg^{-}	13
FRD1155	Δ*algI6*::Gm^{r} Alg^{-}	13
FRD1156	Δ*algF1*::Gm^{r} Alg^{-}	13
FRD1175	Δ*algF2* Alg^{+}	This study
FRD1176	Δ*algJ6* Alg^{+}	This study
FRD1177	Δ*algI7* Alg^{+}	This study
Plasmids		
pRK2013	ColE1-Tra(RK2)$^{+}$ Km^{r}	10
pEX100T	ColE1-*sacB* gene replacement vector; Ap^{r}	33
pMF174	pEX100T with *algIJ*-Δ*algF2*-*algA*	This study
pMF180	pEX100T with *algI*-Δ*algJ*-*algF*	This study
pMF181	pEX100T with *algI′*-*algJ*-*algF*	This study
pMF54	Ptrc expression vector pKK233–2 with *oriV*$_{SF}$ *oriT lacI*^{q} Ap^{r}	11
pPHO7	pTZ18R containing a 2.6-kb *phoA* fragment lacking a signal peptide	16
pMF145	pMF54 with *algF* on a 0.8-kb *Nco*I-*Hin*dIII fragment	This study
pMF153	pMF145 with *phoA* on a 2.6-kb *Xba*I-*Xho*I fragment	This study
pMF150	pMF54 with *algJ* on a 1.1-kb *Nco*I-*Hin*dIII fragment	This study
pMF155	pMF150 with *phoA* on a 2.6-kb *Xba*I-*Xho*I fragment	This study
pMF149	pMF54 with *algI* on a 1.6-kb *Nco*I-*Hin*dIII fragment	This study
pMF154	pMF149 with *phoA* on a 2.6-kb *Xba*I-*Xho*I fragment	This study
pET28b	Polyhistidine fusion vector; Km^{r}	Novagen
pMF148	pET28b with a *Sac*I-*Xho*I *algF* fragment	This study
pMF142	pET28b with an *Eco*RI-*Not*I *algJ* fragment	This study
Primers[b]		
5′ algF	GCCGCAACGATTGAACGAAC	
3′ algF	GGCGGGATATCTCGTTACCG	
5′ algJ	CGGCCGATACGCCACTGCTC	
3′ algJ	CGTTCAATCGTTGCGGCTGG	
5′ algI	GCGATCTTCCCGCACCTGAT	
3′ algI	CTGGTTGTAGAACTGGCGCA	
algF-5′*Nco*I	ATACCCATGGGACCCGATGACCCGCC	
algF-3′*Hin*dIII	ATCAAGCTTCTCGAGTCTCTAGAGCCCCGGGTGACCTCTCCAGTG	
algJ-5′*Nco*I	CACGCCATGGCACAGAGCATTTCCC	
algJ-3′*Hin*dIII	GCGAAGCTTCTCGAGGTTCTAGACCTTGGCTGTGTTCGTTCAATC	
algI-5′*Nco*I	GACGCCATGGTCTTTTCTTCAAACG	
algI-3′*Hin*dIII	TAGAAGCTTCTCGAGGGTCTAGAAATGCTCTGTGTCATGGCGTGG	

[a] Abbreviations for phenotypes: Alg^{+}, alginate overproduction; Alg^{-}, nonmucoid; Ap^{r}, ampicillin resistance; Km^{r}, kanamycin resistance; Gm^{r}, gentamicin resistance cartridge; Tra^{+}, transfer by conjugation.

[b] All primer sequences are 5′ to 3′, reading from left to right, and the underlined sequences are those altered from the wild type to introduce restriction sites during PCR.

this O acetylation reaction is known. To better understand the mechanism of O acetylation of alginate, we determined the cellular locations of the AlgI, AlgJ, and AlgF proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains, plasmids, and oligonucleotides 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, each per liter). The following antibiotics were used at the indicated concentrations: ampicillin at 100 μg/ml; carbenicillin at 300 μg/ml; gentamicin at 15 and 250 μg/ml for *E. coli* and *P. aeruginosa*, respectively; and tetracycline at 15 and 100 μg/ml for *E. coli* and *P. aeruginosa*, respectively.

Construction of nonpolar chromosomal deletions of *algF*, *algJ*, and *algI*. General DNA manipulations were performed as described previously (22). Triparental matings with pRK2013 were used to mobilize plasmids from *E. coli* to *P. aeruginosa* (10). Nonpolar deletions of *algI*, *algJ*, or *algF* were generated by replacing a gentamicin-resistant (Gm^{r}) insertion allele with a deletion allele in the chromosomes of *P. aeruginosa* strains FRD1154, FRD1155, and FRD1156, previously described (13). A 5.8-kb *algI-algJ-algF-algA Xba*I fragment or a 3.8-kb *algI-algJ-algF Xba*I-*Bam*HI fragment was blunt ended and ligated into the *Sma*I site of pEX100T (33). To generate a Δ*algF* mutant, the 0.8-kb *Sma*I fragment was deleted to form pMF174, which was introduced into strain FRD1156 (Δ*algF1*::Gm^{r}) with selection for carbenicillin resistance (Cb^{r}) to produce a single crossover by homologous recombination with the chromosome. A second recombination event was obtained by plating strain FRD1156::pMF174 on L agar with 7% sucrose to eliminate the vector, which contained *sacB* for sucrose sensitivity, and the mucoid Gm^{s} Δ*algF2* strain obtained was called FRD1175. To construct a Δ*algJ* mutant, the *Sap*I-*Not*I DNA fragment containing *algJ* was deleted from the 3.8-kb *algI-algJ-algF* DNA (pMF180) and introduced into FRD1154 (Δ*algJ5*::Gm^{r}), and selection for sucrose resistance resulted in a mucoid Gm^{s} Δ*algJ6* strain that was called FRD1176. To construct a Δ*algI* strain, the 0.8-kb *Sex*AI internal fragment of *algI* was deleted from the 3.8-kb *algI-algJ-algF* DNA (pMF181) and introduced into FRD1155 (Δ*algI6*::Gm^{r}) and the sucrose-resistant mucoid Gm^{s} Δ*algI7* strain obtained was called FRD1177. DNA amplification by PCR using oligonucleotide primers flanking the *alg* genes was performed to verify the *alg* deletions in *P. aeruginosa* chromosomal DNA.

Gene expression under the *Ptrc* promoter and *phoA* fusions. The *algI*, *algJ*, and *algF* genes were each PCR amplified with primers that produced an *Nco*I site at the 5′ translation initiation codon of each gene and an *Xba*I (in *algI*), *Xho*I (in *algJ*), or *Hin*dIII (in *algF*) site at the 3′ end. Each fragment was ligated into the *Ptrc* expression vector pMF54 (11) to create pMF149, pMF150, and pMF145 (Table 1). The *phoA Xba*I-*Xho*I fragment from pPHO7, which encodes alkaline phosphatase (AP) without its signal peptide, was ligated downstream of *algF*,

algJ, and *algI*, resulting in pMF153, pMF155, and pMF154, respectively; these were then digested with *Xba*I and *Kpn*I and exposed to exonuclease by use of the Erase-a-Base system (Promega). The *alg* gene was progressively digested with exonuclease due to the 5′ overhang of an *Xba*I site. The plasmids were blunt-end ligated and transformed into *E. coli* CC118 (*phoA*). Fusions expressing active AP were screened as blue colonies on 5-bromo-4-chloro-3-indolylphosphate (XP). In-frame but inactive *algI-phoA* fusions were identified by sequence analysis. To determine AP activity, 0.5 ml of an 18-h culture was placed in 5 ml of L broth plus ampicillin at 100 μg/ml, incubated with aeration for 1.5 h, and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. The cells from 1 ml of the solution were pelleted in a microcentrifuge for 5 min, washed with saline, and resuspended in 1 ml of saline. A 25-μl sample was transferred to a microtiter dish well containing 200 ml of AP buffer (30 mM Tris, pH 8.5), and the A_{600} was determined. Then, 100 μl of AP substrate (0.4% [wt/wt] *p*-nitrophenyl-phosphate in AP buffer; Sigma) was added, and the change in A_{415} was determined, with AP activity reported as the increase in A_{415} per min/A_{600}.

Elicitation of antibodies. PCR-amplified *algJ* and *algF* were ligated into pET28b (Novagen) to produce polyhistidine-tagged proteins. Plasmid pMF148 encoded *algF* without a signal peptide and an N-terminal polyhistidine fusion, whereas pMF142 encoded a similar polyhistidine-*algJ* fusion. Cultures of *E. coli* BL21::DE3 carrying these plasmids were induced with IPTG (1 mM) for 6 h, and the cells were resuspended in binding buffer (Novagen) and lysed by sonication. The proteins were purified with nickel affinity columns (Novagen). Antisera were raised in New Zealand White rabbits (Immunodynamics, Inc.), diluted in phosphate-buffered saline (PBS), and applied to affinity columns prepared by linking purified polyhistidine-AlgF or polyhistidine-AlgJ to cyanogen bromide-activated Sepharose (Pharmacia). After being washed with PBS, the antibodies were eluted with 5 mM glycine–500 mM NaCl (pH 2.3), neutralized with solid Na_2PO_4–$7H_2O$, and then dialyzed against PBS.

Periplasm extraction and analysis. *P. aeruginosa* strains FRD1 (wild type), FRD1175 (Δ*algF2*), and FRD1176 (Δ*algJ6*) in 0.5 ml of an 18-h culture were inoculated into 5 ml of L broth and incubated for 8 h at 37°C with aeration. The cells were centrifuged for 10 min at 5,000 × *g* and washed with saline. The pellets were washed with 5 ml of periplasm extraction buffer (PEB; 20% sucrose–30 mM Tris [pH 8.0]), centrifuged at 5,000 × *g* for 10 min, resuspended in 500 μl of PEB, and transferred to a 1.5-ml microcentrifuge tube. Twenty-five microliters of lysozyme solution (10 mg of lysozyme/ml of PEB) was added, and the samples were incubated at room temperature for 5 min. Ten microliters of EDTA solution (100 mM EDTA in PEB) was added, and the samples were incubated for 15 min. Spheroplast formation was examined microscopically. The samples were centrifuged for 5 min, and the pellets containing the spheroplasts were separated from the supernatants enriched for periplasmic contents. The proteins in whole cells, periplasmic extracts, and spheroplasts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by use of 4% stacking gels with 12% resolving gels (21) and electroblotted onto nitrocellulose membranes (1), which were probed with affinity-purified AlgF or AlgJ antibodies with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase as the secondary antibody for detection by chemiluminescence analysis (1).

Amino-terminal sequence analysis of AlgJ. Anti-AlgJ was used to immunoprecipitate AlgJ from FRD1. The immunoprecipitation pellet was subjected to SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (1). The proteins were visualized by Coomassie blue staining, and AlgJ (43 kDa) was excised from the membrane for amino-terminal sequence analysis (performed at St. Jude Research Hospital, Memphis, Tenn.).

Assays for alginate. Mucoid *P. aeruginosa* strains were grown for 48 h at 37°C in L broth with 1 mM IPTG and carbenicillin. Alginates in the culture supernatants were precipitated once with 2% cetyl pyridinium chloride and twice with isopropanol as described previously (13) and measured according to the method of Knutson and Jeanes (20). Briefly, a 30-μl sample 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) and incubated at 55°C for 30 min, and then the A_{500} was measured. *Macrocystis pyrifera* alginate (Sigma) was used as a standard.

Assays for O acetylation of alginate. O acetylation of alginate was measured as described by Hestrin (17). Briefly, a 500-μl sample was incubated with 500 μl of alkaline hydroxylamine (0.35 M NH_2OH, 0.75 M NaOH) for 10 min at 25°C, and then 500 μl of 1.0 M perchloric acid was added, followed by the addition of 500 μl of 70 mM ferric perchlorate in 0.5 M perchloric acid. The A_{500} was measured with ethyl acetate as a standard. Alginate acetylation was also examined by Fourier transform infrared (IR) spectroscopy as described previously (13). Purified alginates (250 μl) were spotted onto IR cards (3M Co.), air dried in a laminar-flow hood, and stored in a desiccator. Spectra were collected with a Mattson Galaxy series 2020 spectrometer.

RESULTS AND DISCUSSION

Construction and characterization of nonpolar *algI*, *algJ*, and *algF* deletion mutants. To study the roles of the AlgI, AlgJ, and AlgF proteins in the O acetylation of alginate, we constructed nonpolar deletion mutants of mucoid *P. aeruginosa* that still expressed the downstream *algA* gene (encoding phosphomannose isomerase–GDP–D-mannose phosphorylase), which is essential for alginate biosynthesis. The strategy used to generate these mutants is illustrated in Fig. 1A, where a polar *algF*::Gm insertion mutation was replaced with a clean *algF* deletion. A Δ*algF*-containing DNA fragment in pEX100T (containing *sacB* for sucrose sensitivity) was introduced into *P. aeruginosa* FRD1156 (Δ*algF1*::Gm^r), and the carbenicillin-resistant colonies obtained were merodiploids. A second crossover, forced by exposure to sucrose, resulted in replacement of the *algF*::Gm^r allele with the Δ*algF2* allele and restoration of the mucoid, Gm^s, and sucrose-resistant phenotypes following loss of the vector. This strategy for replacing a chromosomal polar Gm^r marker with a deletion allele was used to construct the Δ*algF*, Δ*algJ*, and Δ*algI* mutants named FRD1175, FRD1176, and FRD1177, respectively (see Materials and Methods). The mucoid colony phenotype of these strains on L agar was indistinguishable from that of the parent strain, FRD1, verifying that *algI*, *algJ*, and *algF* were not involved in alginate polymerization or its export as previously reported (13). Since the mutant strains did not retain an antibiotic resistance marker, this technique could permit the construction of strains with multiple genomic deletions without adding foreign DNA that could have downstream effects.

Parent strain FRD1 contained approximately 0.45 mol of *O*-acetyl group per mol of alginate, indicating that approximately half of the uronic acid residues in wild-type alginate were acetylated. FRD1175, FRD1176, and FRD1177 contained no detectable *O*-acetyl groups, as determined by the spectrophotometric assay. A Fourier transform IR spectroscopy analysis of alginates from culture supernatants also demonstrated the lack of alginate O acetylation in the deletion strains (Fig. 2). Thus, *algI*, *algJ*, and *algF* were required for the O acetylation of alginate as previously reported (13). The ability of cloned genes to complement these mutants was also tested. The *algI*, *algJ*, and *algF* genes were each amplified by PCR and cloned downstream of an inducible promoter, *Ptrc*, in the *P. aeruginosa* expression vector pMF54. Assays for O acetylation demonstrated that each clone would complement its respective mutant if induced with IPTG, and O acetylation levels ranging from 0.33 to 0.39 mol of *O*-acetyl group per mol of alginate were observed (data not shown).

Periplasmic localization of AlgF. The *algF* gene encodes a 23-kDa pre-AlgF protein with a typical signal peptide that is known to be removed during localization to the periplasm (13, 35). AlgF localization was confirmed by use of *phoA* fusions and a periplasmic analysis. In-frame gene fusions to *phoA*, which encode AP without a signal peptide, are active only when *phoA* is exported to the periplasm (23). Two clones containing *algF-phoA* fusions were constructed at D99 and V193, which were confirmed to be in frame by sequence analysis, and both expressed AP activity. Purified AlgF antibodies used in an immunoblot analysis of total proteins demonstrated that a ~23-kDa AlgF was present in extracts of FRD1 and

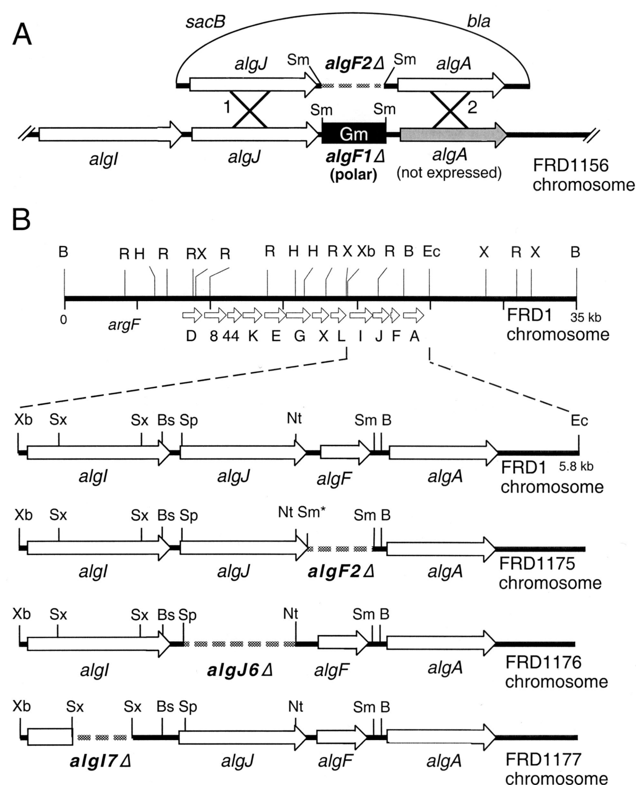

FIG. 1. Construction and genotypes of *algF*, *algJ*, and *algI* deletion mutants in *P. aeruginosa* FRD1. (A) The strategy used to generate mucoid mutants through the construction of a nonpolar deletion of *algF* is illustrated. *algF* was deleted from a DNA fragment in vector pEX100T (33) and introduced into *P. aeruginosa* FRD1156, a strain that is nonmucoid due to a Δ*algF1*::Gm^r mutation that is polar on *algA* (13). The vector's narrow host range prevents autonomous replication, but Cb^r colonies are merodiploids formed by chromosomal integration of the plasmid via a single-crossover homologous recombination (labeled 1). A second crossover on the other side of the deletion (labeled 2) results in the replacement of the *algF*::Gm^r allele by the nonpolar Δ*algF2* allele, restoration of the mucoid and Gm^s phenotypes, and loss of the vector DNA. Sm, *Sma*I restriction site. (B) The restriction map of the alginate biosynthetic operon with an expanded view of the *algI-algJ-algF-algA* region in wild-type FRD1 is shown. The genotypes of mucoid strains FRD1175, FRD1176, and FRD1177 with nonpolar deletions of the *algI*, *algJ*, and *algF* genes are illustrated. The *alg* genes are represented by open arrows above their designations. Restriction site abbreviations: Xb, *Xba*I; Sx, *Sex*AI; Bs, *Bst*XI; Sp, *Sap*I; Nt, *Not*I; Ec, *Ecl136*II; B, *Bam*HI; R, *Eco*RI; X, *Xho*I; H, *Hin*dIII; Sm, *Sma*I. The asterisk indicates the Sm site introduced by oligonucleotide mutagenesis.

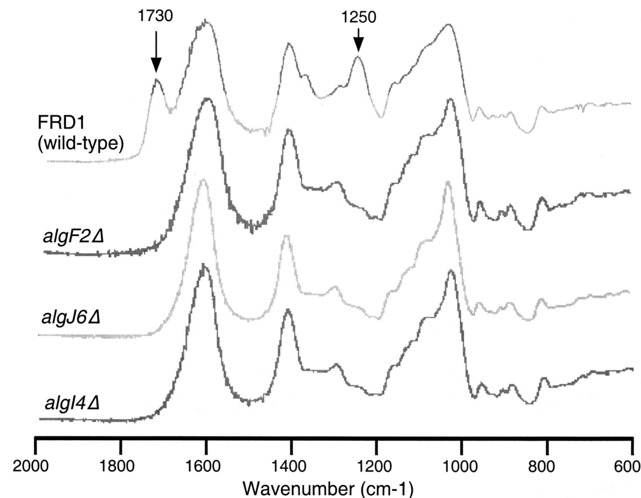

FIG. 2. Fourier transform IR spectroscopy of alginates purified from mucoid FRD strains. The IR spectrum of alginate produced by FRD1 (wild type) has absorbance peaks at 1,730 and 1,250 cm^{-1} that indicate the presence of the acetyl ester bond associated with O acetylation. In contrast, spectra from acetylation-deficient alginates from FRD1175 (Δ*algF2*), FRD1176 (Δ*algJ6*), and FRD1177 (Δ*algI7*) lack these peaks.

FRD1175(pMF145) but was missing in the Δ*algF2* mutant, FRD1175 (Fig. 3A). It was curious that AlgF migrated slightly more slowly than the predicted 20-kDa protein for processed AlgF. Varying the amounts of DNA upstream and downstream of *algF* in expression clones had no effect on the protein's size (data not shown), so AlgF presumably has a slightly anomalous migration in SDS-PAGE gels. Proteins enriched from the periplasm of FRD1 showed a strong band corresponding to AlgF and a weaker 20-kDa band that may represent a degradation product (Fig. 3B). In contrast, the remaining spheroplasts representing the membrane fraction showed only a faint AlgF band (Fig. 3B). Overall, these tests clearly confirm that AlgF is a periplasmic protein.

Periplasmic, membrane-associated localization of AlgJ in FRD1. The predicted amino acid sequence of AlgJ (13) includes an amino terminus that resembles a signal peptide (i.e., 12 hydrophilic residues followed by 18 hydrophobic residues), but it is missing a typical signal peptidase cleavage site (e.g., Ala-X-Ala [28]). To determine the potential periplasmic localization of AlgJ, random *phoA* fusions to *algJ* were constructed by use of progressive exonuclease digestion and blunt-end ligation. *E. coli* transformants were screened on XP medium, and AP-active blue colonies were observed, suggesting that AlgJ is exported to the periplasm. Five random *algJ-phoA* fusions with AP activity were sequenced, showing that AP had fused to AlgJ in frame at amino acids E257, E265, D280, Y321, and G340. The open reading frame of *algJ* is predicted to encode a 43-kDa protein (13), and based on immunoblots of total protein extracts, AlgJ antibodies detected a ~43-kDa band in FRD1 and FRD1176(pMF150) that was absent in the Δ*algJ6* mutant, FRD1176 (Fig. 4A).

The AlgJ antibodies were used to immunoprecipitate AlgJ from strain FRD1. The results of an amino-terminal analysis produced the sequence GQSISRPVQYA, which was in close agreement with AlgJ's predicted unprocessed N-terminal sequence of MTQSISRPLQYA. Thus, AlgJ apparently has a signal peptide that is not removed during localization to the periplasm. To directly examine the cellular location of AlgJ, FRD1 cells were spheroplasted to release the contents of the periplasmic space. When examined by immunoblot analysis, AlgJ (43 kDa) was not observed in the periplasmic fractions (Fig. 4B), even though the *algJ-phoA* fusions described above were indicative of a periplasmic localization. Instead, AlgJ was readily found in the spheroplast membrane sample (Fig 4B). These data (i.e., from the N-terminal sequence analysis, *phoA*

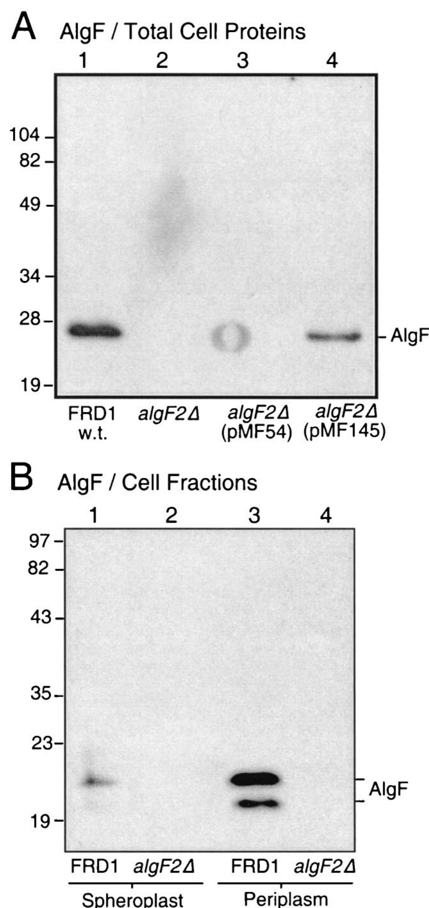

FIG. 3. Immunoblot analysis of AlgF in *P. aeruginosa* strains. (A) Proteins in whole-cell lysates were separated by SDS-PAGE, blotted, and probed with AlgF antibodies. Lanes: 1, FRD1 (w.t., wild type); 2, FRD1175 (Δ*algF2*); 3, FRD1175(pMF54); 4, FRD1175(pMF145) with *algF* expressed in *trans*. Molecular weight markers (10^3) are shown on the left, and AlgF is labeled on the right. (B) Proteins in fractions enriched for the periplasm and spheroplast membranes were separated by SDS-PAGE, blotted, and probed with AlgF antibodies. Lanes: 1, FRD1 spheroplasts; 2, FRD1175 (Δ*algF2*) spheroplasts; 3, FRD1 periplasm; 4, FRD1175 periplasm. Note that AlgF is mostly associated with the periplasmic fraction.

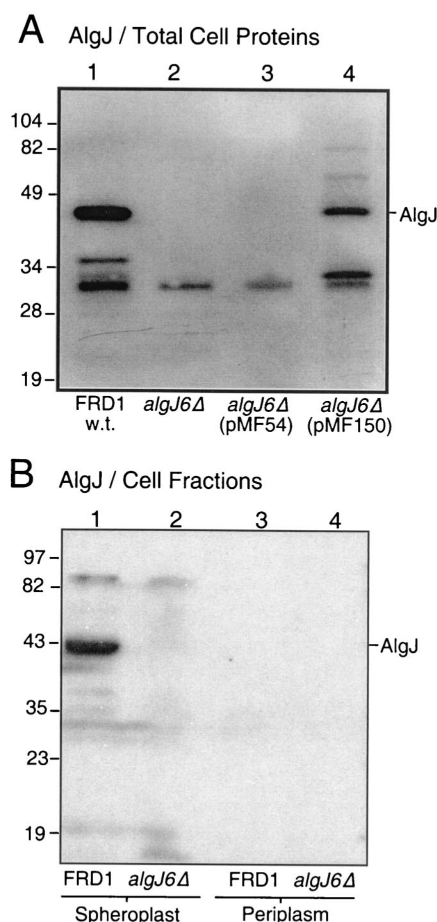

FIG. 4. Immunoblot analysis of AlgJ in *P. aeruginosa* strains. (A) Proteins in whole-cell strain lysates were separated by SDS-PAGE, blotted, and probed with AlgJ antibodies. Lanes: 1, FRD1 (w.t., wild type); 2, FRD1176 (Δ*algJ6*); 3, FRD1176(pMF54); 4, FRD1176 (pMF150) with *algJ* expressed in *trans*. Molecular weight markers (10^3) are shown on the left, and AlgJ is labeled on the right. (B) Proteins in various fractions enriched for the periplasm and spheroplast membranes were separated by SDS-PAGE, blotted, and probed with AlgJ antibodies. Lanes: 1, FRD1 spheroplasts; 2, FRD1176 (Δ*algJ6*) spheroplasts; 3, FRD1 periplasm; 4, FRD1176 periplasm. Note that AlgJ is mostly associated with the spheroplast (i.e., membrane) fraction even though PhoA fusions indicate that it is periplasmic.

fusions, and periplasmic studies) suggest that AlgJ is a periplasmic protein that remains tethered to the inner membrane, presumably by the hydrophobic portion of its uncleaved signal peptide. Thus, AlgJ is characteristic of the type IA membrane proteins described by Pugsley (28).

Topology of AlgI in the membrane. A sequence analysis of AlgI suggests that this component of the O acetylation complex may be a membrane protein (13). To obtain experimental data regarding the membrane topology of AlgI, random *algI-phoA* translational fusions were constructed. Some colonies expressed active *algI-phoA* fusions on XP, suggesting that domains were exposed to the periplasm, and these were sequenced. To identify the regions of AlgI localized to the cytoplasm, in-frame but enzymatically inactive *algI-phoA* fusions were identified with AP antibodies and confirmed by sequence analysis. The AP activities of 33 well-distributed, in-frame active and inactive *algI-phoA* fusions expressed in *P. aeruginosa* were measured. These results, together with those of the hydrophobicity plot, were used to construct a model for the membrane topology of AlgI (Fig. 5A). An AlgI-PhoA fusion exhibiting high AP activity suggested a periplasmic localization of the insertion domain, and a low AP activity (i.e., comparable to that of the vector control) indicated a cytoplasmic domain in AlgI. Some fusions had intermediate AP activities, but these appeared to be primarily in periplasmic domains. Our model predicted that AlgI has seven transmembrane (TM)-spanning helices, with four located near the N terminus of the protein and three located near the C terminus (Fig. 5A).

We compared our experimental model of AlgI topology to a computer model produced by the Simple Modular Architecture Research Tool (SMART) (30, 31). SMART predicted five TM regions near the N terminus and four in the C terminal

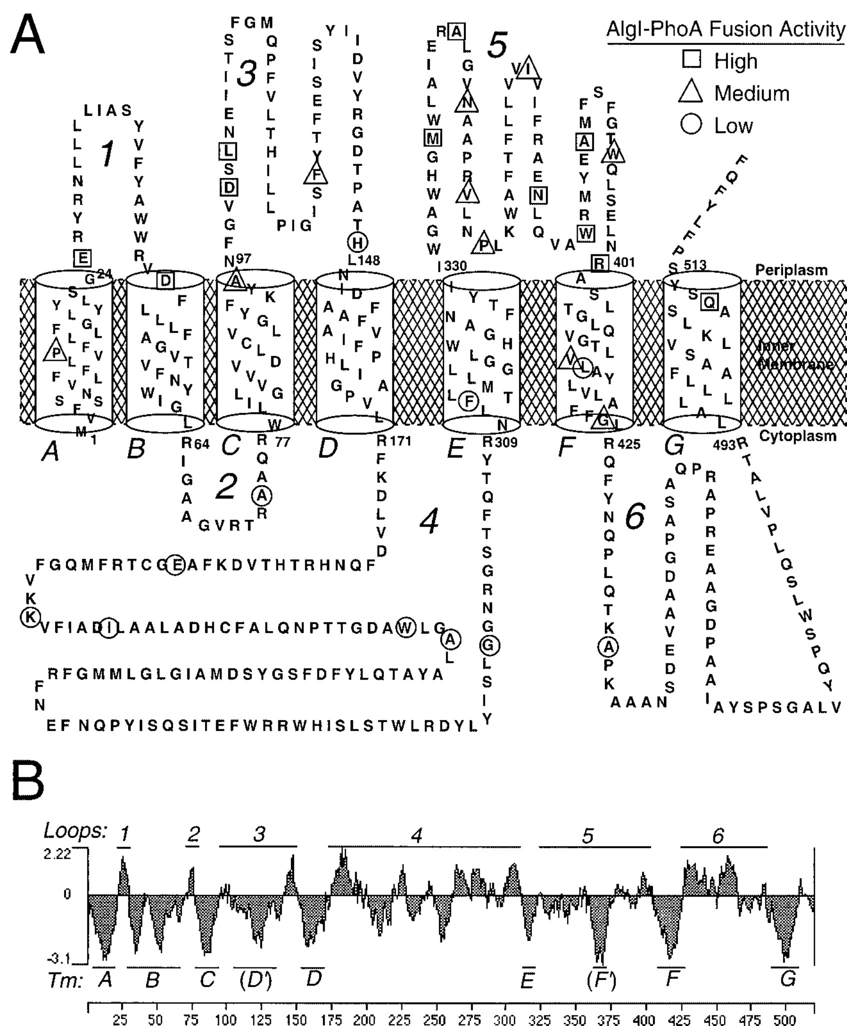

FIG. 5. Model for the topology of AlgI in the inner membrane of *P. aeruginosa*. (A) The amino acid sequence of AlgI, the TM domains based upon the AP activities of 33 random AlgI-PhoA fusions, and the results of a hydrophobicity analysis are shown. AP activity was measured as $\Delta A_{420} \cdot \text{min}^{-1} \cdot A_{600}{}^{-1}$. The open boxes indicate fusions with AP activities of greater than 100 U, the triangles indicate fusions with activities of 10 to 100 U, and the circles indicate fusions with activities equal to that of the vector control strain. The loops are labeled *1* to *6*, with loops located in the periplasm represented by odd numbers and loops located in the cytoplasm represented by even numbers. The TM domains are labeled *A* to *G*. (B) A hydrophilicity plot (Kyte-Doolittle) of the AlgI amino acid sequence, with the TM domains labeled *A* to *G* and the corresponding loops labeled *1* to *6* as in panel A. Computer modeling predicted this structure plus two more TM domains, labeled D′ and F′, which do not fit the experimental data (see Results and Discussion).

half. Four of the TM domains near the N terminus predicted by SMART matched those from our predictions (Fig. 5A, domains A to D) and encompassed residues M1 to L22 (domain A), V45 to I65 (domain B), Q76 to A96 (domain C), and N150 to L170 (domain D). SMART also predicted a TM domain at G111 to F131 (Fig. 5B, domain D′); however, this seems unlikely because an added TM domain here would force loop 4 to be oriented toward the periplasm despite the six AlgI-PhoA fusions in this region that had no AP activity. From the information at hand, it is possible that domain D′ and not domain D is the actual TM domain, but domain D is more hydrophobic and so was chosen here as the actual TM domain. The SMART analysis predicted three TM domains in the C-terminal half of AlgI encompassing residues L311 to W331 (domain E), L404 to L424 (domain F), and S485 to L505 (domain G) that are also in our experimentally based model (Fig. 5A). SMART predicted that residues N355 to A375 (Fig. 5A, domain F′) constituted a TM domain, but this seems unlikely in that an added TM domain would orient several active PhoA fusions in loop 5 to the cytoplasmic side. Therefore, domain F′ is likely to be a hydrophobic region in periplasmic loop 5 of AlgI. It is possible that this hydrophobic region may fold into the membrane on the periplasmic side, similar to what has been observed for the ABC transport protein KpsM (27).

Model for the alginate O acetylation protein complex. The O acetylation of alginate plays an important role in human pathogenesis by protecting infecting organisms from the antibodies typically found in the sera of infected CF patients (26), and it is required for *P. aeruginosa* to form microcolonies in a

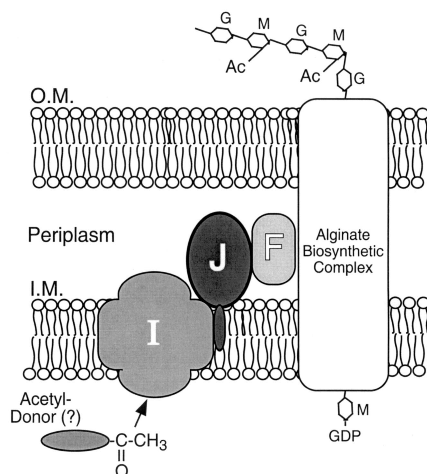

FIG. 6. Model for the organization of an AlgI-AlgJ-AlgF complex for alginate O acetylation in *P. aeruginosa*. The data presented here suggest that AlgI is an integral membrane protein, that AlgJ is periplasmic but associated with the membrane, and that AlgF is predominantly associated with the periplasmic fraction. These proteins may form a complex (yet to be demonstrated) that is responsible for the O acetylation of D-mannuronates (M), but not of L-guluronates (G), in alginate. At this time, the cytoplasmic acetyl donor is not known, but it may be acetyl coenzyme A and/or an acetyl carrier protein. Also, the mechanism, specific substrate, and topology of this O acetylation reaction are not yet understood. AlgI-AlgJ-AlgF may interact with the alginate biosynthetic complex of proteins and with alginate (at the monomer or polymer level) to bring about efficient modification of the polymer by O acetylation. O.M., outer membrane; I.M., inner membrane.

biofilm (24), which is seen with mucoid *P. aeruginosa* in the lungs of CF patients. Little is currently known about the mechanism of O acetylation of alginate except that it requires at least three gene products, AlgI, AlgJ, and AlgF. This study allows us to begin drawing a molecular model of the O acetylation machinery (Fig. 6) as an inner membrane complex of proteins, which presumably interacts with the alginate polymerization-transport complex. Next, we would like to examine the individual roles of these proteins in alginate O acetylation. AlgI has some sequence similarity to DltB, a membrane protein involved in the O alanylation of lipoteichoic acid in gram-positive bacteria, and a polymer-level esterification model has been proposed for O alanylation (19). Thus, polymer-level O acetylation of alginate in *P. aeruginosa* is a possibility that needs to be tested.

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