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Role of an Alginate Lyase for Alginate Transport in Mucoid *Pseudomonas aeruginosa*

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The opportunistic pathogen *Pseudomonas aeruginosa* secretes a capsule-like polysaccharide called alginate that is important for evasion of host defenses, especially during chronic pulmonary disease of patients with cystic fibrosis (CF). Most proteins for alginate biosynthesis are encoded by the 12-gene *algD* operon. Interestingly, this operon also encodes AlgL, a lyase that degrades alginate. Mutants lacking AlgG, AlgK, or AlgX, also encoded by the operon, synthesize alginate polymers that are digested by the coregulated protein AlgL. We examined the phenotype of an Δ*algL* mutation in the highly mucoid CF isolate FRD1. Generating a true Δ*algL* mutant was possible only when the *algD* operon was under the control of a LacI^q-repressed *trc* promoter. Upon induction of alginate production with isopropyl-β-D-thiogalactopyranoside, the Δ*algL* mutant cells were lysed within a few hours. Electron micrographs of the Δ*algL* mutant showed that alginate polymers accumulated in the periplasm, which ultimately burst the bacterial cell wall. The requirement of AlgL in an alginate-overproducing strain led to a new model for alginate secretion in which a multiprotein secretion complex (or scaffold, that includes AlgG, AlgK, AlgX, and AlgL) guides new polymers through the periplasm for secretion across the outer membrane. In this model, AlgL is bifunctional with a structural role in the scaffold and a role in degrading free alginate polymers in the periplasm.

Pseudomonas aeruginosa is an opportunistic pathogen par excellence that causes severe and life-threatening infections in immunocompromised hosts such as patients with respiratory diseases, burns, cancers undergoing chemotherapy, and cystic fibrosis (CF). Virulence factors produced by *P. aeruginosa* include numerous extracellular toxins, proteases, hemolysins, and exopolysaccharides. The most striking feature of *P. aeruginosa* strains infecting the CF pulmonary tract is their highly mucoid phenotype, which is due to alginate overproduction (Alg$^+$) (10, 24). Mutations in *mucA*, encoding a transmembrane anti-sigma factor, are typically responsible for the mucoid conversion observed in clinical isolates of CF patients (25, 26).

The Alg$^+$ phenotype correlates with the ability of *P. aeruginosa* to persist in the lungs of CF patients and cause chronic bronchopulmonary infections (18). About 80% of the *P. aeruginosa* isolates from CF patients undergo mucoid conversion in vivo, whereas only about 1% of clinical *P. aeruginosa* isolates from other types of infections are Alg$^+$ (7, 8, 46). Thus, alginate appears to have an important role in the unique host-parasite relationship between the CF patient and *P. aeruginosa*. Alg$^+$ may confer several selective advantages on the bacterial invader, which have been reviewed (17) and include increased resistance to phagocytosis (a property typically associated with bacterial capsules) and reduced susceptibility to antibody-dependent bactericidal mechanisms (1, 36, 37, 43). Alginate also provides a polyanionic barrier that may exclude cationic peptide antibiotics (34). The biofilms of mucoid organisms in the CF lung show an unusual microcolony morphology (23), which is unique. Although alginate is not required for biofilm formation, an *algD* mutant (i.e., defective in alginate biosynthesis) shows a lag in initial biofilm development, suggesting its role in early attachment (30).

Structurally, alginate is a simple, unbranched polysaccharide of very high molecular weight that is composed of two uronic acids: β-D-mannuronic acid and its C5 epimer α-L-guluronic acid. In addition to *Pseudomonas* sp., members of the bacterial genus *Azotobacter* also synthesize alginate, where it forms part of a protective coat during cyst formation. Alginate is commonly found in brown algal seaweed as part of its gelatinous cell wall (19). The viscous nature of the algal alginate in aqueous solution makes it a commercially important product, especially in the food industry. In *P. aeruginosa*, alginate is produced as a capsule-like exopolysaccharide that loosely adheres to *P. aeruginosa* cells, and so most of it is found in the culture supernatant.

In *P. aeruginosa*, all but one of the known genes encoding the alginate biosynthetic machinery are clustered in an operon encoding 12 gene products: AlgD-8-44-K-E-G-X-L-I-J-F-A (5). The early pathway of biosynthesis utilizes AlgA, AlgD, and AlgC (unlinked to the operon) to form GDP-mannuronic acid, the primary precursor of alginate (28). The polymerase for alginate has not been positively identified, but the most likely candidate is Alg8, which shows structural homology to β-glycosyltransferases (41). The remainder of the proteins encoded by the operon bear little resemblance to known enzymes for

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capsule biosynthesis in other bacteria. AlgG was shown to be a periplasmic D-mannuronate C5 epimerase in *P. aeruginosa*, which is responsible for converting D-mannuronates to L-guluronates at the polymer level (4, 12). Other periplasmic proteins include AlgK (21) and AlgX (29), and mutants lacking these proteins are Alg$^-$. Secretion of polymer appears to occur through the outer membrane protein AlgE (38). AlgI, AlgJ, and AlgF are required to modify alginate with O-acetyl groups, but these proteins are not required for polymer formation (13–15). In addition, it is curious that the operon for alginate biosynthesis also includes a gene, *algL*, that encodes an alginate lyase, which can efficiently degrade alginate (42).

We have shown that, in addition to its C-5 polymer level epimerase activity, AlgG is bifunctional, having another role in protecting alginate from degradation by AlgL during transport across the periplasm (16, 20). Similarly, a nonpolar Δ*algK* mutation, like an Δ*algG* mutation, results in the secretion of small alginate fragments due to AlgL-mediated polymer degradation (21), and so AlgK appears to share this second role. It was recently shown that an *algX* mutation also results in the secretion of AlgL-degraded alginate (40). Thus, AlgG, AlgK, and AlgX may be part of a periplasmic protein complex (scaffold) that guides alginate polymers to AlgE in the outer membrane. Furthermore, it appears that breakdown of this scaffold due to any one missing component protein leads to degradation of the freed polymer by AlgL depolymerase activity. In this study, we sought to evaluate the phenotype of a Δ*algL* mutation. We show evidence that despite the alginate-degrading activity of AlgL, it too is important for alginate secretion. This became evident through the discovery that alginate polymers are trapped in the periplasm in strains lacking AlgL, which ultimately leads to cell lysis. Our genetic analysis has led to a new model for alginate secretion whereby a protein scaffold that includes the alginate-degrading protein AlgL transports the growing alginate polymer chain through the periplasm.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* FRD1 is an Alg$^+$ CF isolate (31). Bacterial strains were routinely grown in L broth (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter). MAP defined medium (14) was used to reduce the background reaction when measuring the production of extracellular uronic acids. Isopropyl-β-D-thiogalactopyranoside (IPTG) was generally used with *P. aeruginosa* at a concentration of 5 mM to induce the P*trc* promoter. Antibiotics, when used, were at the following concentrations (per milliliter): ampicillin at 100 μg, carbenicillin at 300 μg, gentamicin at 15 μg for *Escherichia coli* and 300 μg for *P. aeruginosa*, and kanamycin at 40 μg. Sucrose (7.5%) was added to L agar to counterselect against the sucrose sensitivity caused by *sacB*. Plasmids were conjugated from *E. coli* to *P. aeruginosa* by triparental matings using the conjugative helper plasmid pRK2013 [ColE1-Tra(RK2) Km^r] (11) with selection on a 1:1 mixture of Pseudomonas Isolation Agar (Difco) and L Agar (Sigma).

Construction of P*trc-algD* mutant strain FRD1050. To control alginate biosynthesis, the *algD* promoter was replaced with an inducible P*trc* promoter as follows. A 600-bp fragment at the 5′ end of *algD* was obtained by PCR amplification using primers that placed the *algD* start ATG codon within an NcoI site, and the 3′ end contained an XbaI site. The fragment was cloned into the NcoI-XbaI sites and immediately downstream of the P*trc* promoter in expression vector pMF53 [*ori*(pBR322) *bla oriT*(RK2) *lacI*^{q} P*trc*], which was expression vector pMF54 (12) but without a stabilization fragment for replication in *Pseudomonas*. This plasmid, pJLS3, was conjugated into FRD1, and its *bla* marker for carbenicillin resistance permitted selection for exconjugants with single-crossover events by homologous recombination within *algD* on the chromosome. The carbenicillin-resistant merodiploids had the *algD-A* operon under P*trc* control, while the 0.6-kb *algD* fragment was under the control of the native P*algD* promoter. One such merodiploid, called FRD1050, was used in this study. The integration of pJLS3 at *algD* in the chromosome was confirmed by PCR amplification of genomic DNA with primers specific for vector sequences upstream of P*trc* and downstream to the terminator of the *algD* gene. DNA amplified by these primers with FRD1050 produced the expected 2,265-bp fragment, whereas no product was amplified with wild-type FRD1 or *E. coli*(pJLS3) (data not shown).

Construction of Δ*algL* Gm^r mutant FRD1300. pCC27 was pCP19 (IncP1, broad host range, Tc^r) with 23 kb of *P. aeruginosa* FRD1 DNA containing *algD-algF* (4). A 3.4-kb EcoRI fragment containing *′algX-algL-algI* from FRD1 was cloned into the SmaI site of the gene replacement vector pEX100T (*bla sacB oriT*) (44). Then, the 1,208-bp KpnI-XbaI fragment containing *algL* was replaced with a nonpolar gentamicin resistance cassette on a 785-bp KpnI-XbaI fragment from pSJ12 (21). The resulting plasmid, pSJ243, contained the Δ*algL*::Gm^r allele and was conjugated into FRD1050 with selection for gentamicin resistance. Colonies were screened for loss of sucrose sensitivity, which typically indicates a double-crossover event and thus gene replacement. In one isolate, called FRD1300, the Δ*algL*::Gm^r mutation was confirmed by PCR amplification of genomic DNA with primers specific for sequences upstream and downstream of the *algL* gene. DNA amplified by these primers with wild-type FRD1 produced the expected 1,485-bp fragment, and FRD1300 produced the predicted ~900-bp fragment (data not shown).

Alginate assay. To assay the amount of alginate secreted, cultures were grown for 18 h at 37°C in 10 ml of MAP medium or L broth. Cells were removed by centrifugation, and culture supernatants of L broth cultures were exhaustively dialyzed against saline. Samples were assayed for alginate concentration using a colorimetric test for uronic acids (22), with alginic acid from *Macrocystis pyrifera* (Sigma) used as the standard.

Electron microscopy. Samples of log-phase bacterial cultures were withdrawn periodically, and cells were collected by centrifugation in a Microfuge at a low speed (3,000 rpm). The pelleted cells were washed with saline and resuspended in glutaraldehyde. The samples were thin sectioned, stained, and examined using a Zeiss EM10CA transmission electron microscope in the Virginia Commonwealth University Core Electron Microscopy Facility.

RESULTS

Attempted construction of a Δ*algL* mutant of *P. aeruginosa* FRD1. Strain FRD1 was used because it is a CF clinical isolate of *P. aeruginosa* that displays the typical Alg$^+$ phenotype, and it is genetically manipulatable (20, 21). Previous studies have shown that mutants lacking AlgG, AlgK, or AlgX synthesize alginate polymers which are rapidly degraded by AlgL, an alginate lyase in the same operon as and thus coregulated with AlgG and AlgK (16, 20, 21, 40). Here we sought to evaluate the phenotype of a nonpolar Δ*algL* mutation in FRD1. The strategy used to construct an *algL* mutant was similar to one we have used previously (21). A clone was modified by replacing *algL* sequences with a nonpolar gentamicin resistance cassette, followed by allelic exchange using a suicide vector (pEX100T) that contains *sacB*, which confers sucrose sensitivity and thus provides counterselection on sucrose. However, when the resulting plasmid (pSJ243) was transferred to FRD1 with selection for Gm^r, an unusually low number of gene replacement candidates (i.e., sucrose-resistant colonies) was obtained. Among several *algL* mutants of FRD1 tested, all of which were nonmucoid, none could be complemented to the Alg$^+$ phenotype by a plasmid expressing *algL* in *trans*. This suggested that secondary suppressor mutations to block alginate biosynthesis are required for viability in a Δ*algL* mutant. Thus, this inability to construct a true Δ*algL* mutant suggested that AlgL might be required for cell viability in a highly mucoid strain like FRD1.

Placement of the alginate operon under IPTG control in *P. aeruginosa*. To test the hypothesis that a Δ*algL* mutation is lethal in a highly mucoid *P. aeruginosa* strain, a derivative of FRD1 was constructed for controlled expression of the Alg$^+$ phenotype. The 12-gene *algD* operon for alginate biosynthesis

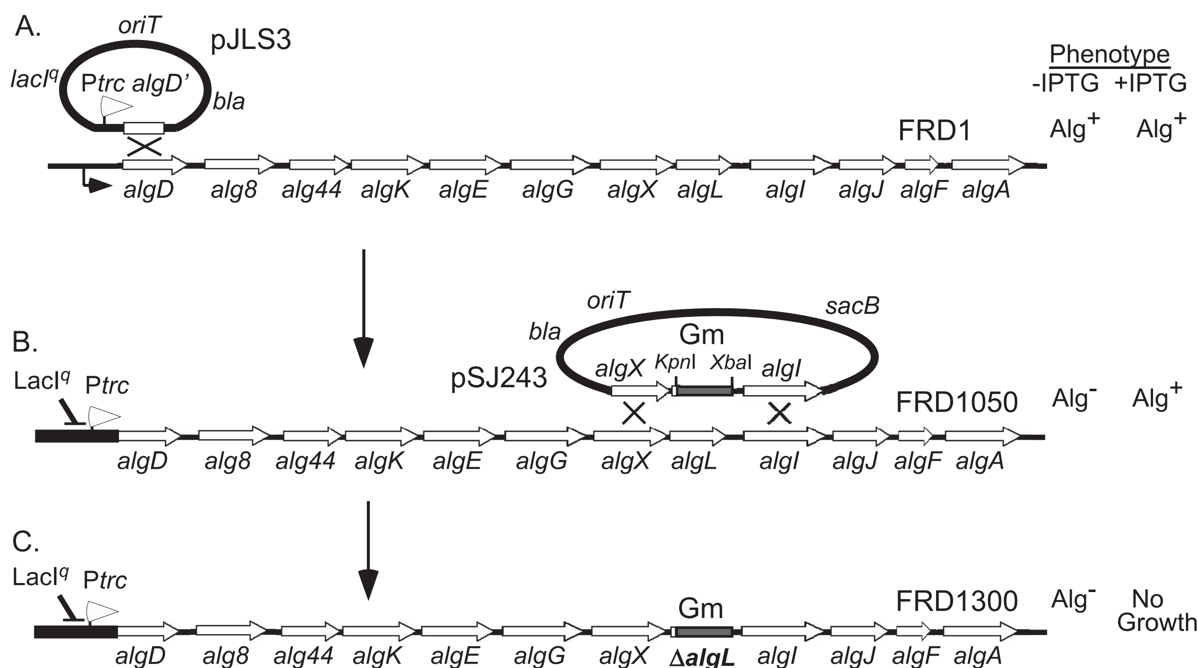

FIG. 1. Genetic manipulations of the *algD* operon encoding proteins for alginate biosynthesis in *P. aeruginosa* FRD1. (A). Suicide plasmid pJLS3 contained a P*trc-algD'* fragment and, when integrated into the chromosome of FRD1 at *algD*, formed strain FRD1050, which put the operon under LacI^q repression. (B) Suicide plasmid pSJ243, containing a fragment of the *algD* operon with a nonpolar Δ*algL*::Gm^r allele, was used to generate a Δ*algL* mutant of FRD1050 by allelic exchange. (C) Among the Gm^r transconjugants, FRD1300 was a Δ*algL* mutant that had lost the vector-encoded (Suc^s) phenotype. Induction of the *algD* operon in FRD1300 with IPTG prevented cell growth.

is under the control of a complex hierarchy of gene regulators (6, 32). In order to turn alginate on or off at will, a genetic technique was employed that placed the chromosomal *algD* operon under P*trc* control, which could then be repressed by the LacI^q repressor but derepressed in the presence of IPTG. To accomplish this, a 5′-terminal fragment of *algD′* was placed under the control of a P*trc* promoter in a suicide plasmid (pJLS3) and integrated into the FRD1 chromosome by homologous recombination at *algD* (Fig. 1). The merodiploids thus formed had the *algD* operon under P*trc* control, and the 0.6-kb *algD′* fragment was then under the control of the native P*algD* promoter. The merodiploids obtained were nonmucoid on L agar but produced the Alg$^+$ phenotype when plated on L agar plus IPTG (Fig. 2). One of these FRD1 derivatives, showing alginate production only in the presence of IPTG, was called FRD1050 and used as described below. Under typical conditions in L broth, cultures of FRD1 accumulated about 1,000 ± 50 μg of alginate per ml. Similar cultures of FRD1050 accumulated 700 ± 20 μg of alginate per ml with IPTG and 80 ± 10 μg of alginate per ml without IPTG. Thus, P*tac* was nearly as strong a promoter as P*algD* in *P. aeruginosa*.

Construction of a conditionally lethal Δ*algL* mutant of *P. aeruginosa*. FRD1050 was grown under conditions in which alginate was not produced (L broth without IPTG), and the Δ*algL*::Gm^r gene replacement plasmid described above, pSJ243, was introduced to replace the chromosomal *algL* gene (Fig. 1B). Unlike the experience with Alg$^+$ FRD1, using FRD1050 as a host produced numerous Δ*algL*::Gm^r mutants. One potential Δ*algL*::Gm^r mutant, called FRD1300, was selected, and its mutation was confirmed at the DNA level by

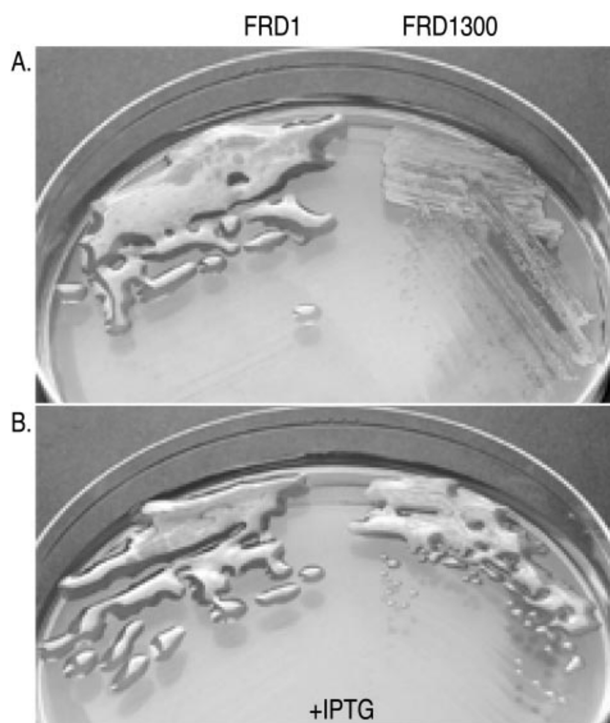

FIG. 2. Alginate phenotypes of FRD1 derivatives. (A) The mucoid (Alg$^+$) phenotype on L agar was observed with parent strain FRD1 but not with FRD1050 (P*trc-algD-A*), which has the *algD* operon repressed by LacI^q. (B) The mucoid phenotype on L agar plus IPTG (1 mM) was observed with both FRD1 and FRD1050 (P*trc-algD-A*), which has the *algD* operon induced.

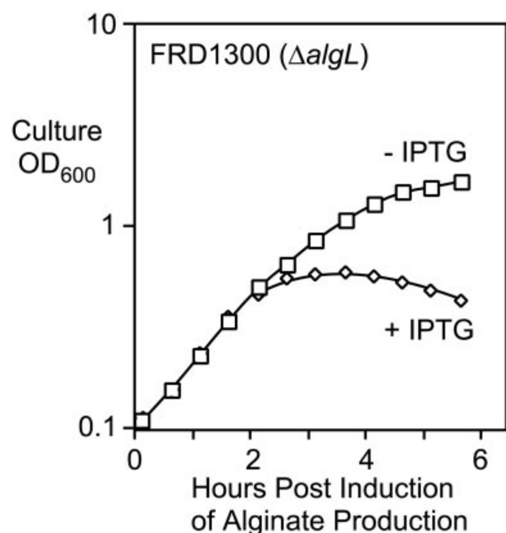

FIG. 3. Effect of the induction of alginate production on the growth of FRD1300 (P*trc-algD-A* Δ*algL*::Gm^r). A 0.3-ml sample of an FRD1300 overnight culture was inoculated into 25 ml L broth, which was incubated with aeration at 37°C. After 1 h, the culture was split and 5 mM IPTG was added to one flask. A plot of culture density (OD_{600}) versus minutes postinduction is shown.

PCR analysis (see Materials and Methods). When this mutant was grown on agar medium lacking IPTG, it displayed a nonmucoid phenotype like its parent FRD1050. However, when incubated on L agar containing IPTG, it was unable to grow. When a self-replicating plasmid expressing *algL* in *trans* was introduced into FRD1300, it restored both viability on L agar with IPTG and the Alg$^+$ phenotype. Thus, AlgL (i.e., an alginate lyase) was required for viability in Alg$^+$ strain FRD1.

Kinetics of cell death due to Δ*algL* mutation in Alg$^+$ *P. aeruginosa*. To better understand the nature of the growth defect due to the Δ*algL* mutation, cultures were examined to determine the effect of alginate induction on growth over time. FRD1300 (P*trc-algD-A* Δ*algL*) was grown for 1 h in L broth with aeration, and then IPTG was added to half of the divided culture. At 3 h postinduction, the growth of the alginate-induced culture was clearly slower than that of the uninduced culture (Fig. 3). By 4 h postinduction, the density of the induced culture actually began to decline, suggesting rapidly dying cells (Fig. 3). Thus, a Δ*algL* mutation resulted in a lethal phenotype in mucoid *P. aeruginosa* FRD1. In that FRD1300 (an AlgL$^-$ mutant) was viable as long as it was nonmucoid (i.e., IPTG was not included), then apparently AlgL is not required for viability as long as alginate is not being overproduced.

Loss of AlgL results in accumulation of periplasmic alginate. Transmission electron microscopy was used to investigate the cause of cell lysis upon induction of alginate biosynthesis in the Δ*algL* mutant. Samples were withdrawn over time from a culture of FRD1300 (P*trc-algD-A* Δ*algL*) following IPTG induction, and thin sections were prepared for transmission electron microscopy. Prior to IPTG induction, these gram-negative bacterial cells showed a normal cell structure and morphology (Fig. 4A). However, by 2 h of IPTG induction to induce alginate production, most cells of the Δ*algL* mutant showed obvious zones of separation between the inner and outer membranes (Fig. 4B). By 4 h of IPTG induction, the periplasm of the Δ*algL* mutant was dramatically swollen (Fig. 4C). Increasing the magnification (Fig. 4D) showed more clearly that the periplasm had accumulated a material (i.e., alginate) that caused large separations between the inner and outer membranes. By 6 h of induction, nearly all of the Δ*algL* mutant cells had been lysed and few showed remnants of their original cell morphology (Fig. 4E and F). These observations suggested that the loss of AlgL in Alg$^+$ FRD1 prevented newly formed alginate polymers in the periplasm from being transported through the outer membrane, which ultimately led to periplasmic accumulation of alginate and caused the cell walls to burst from the pressure.

Reduced P*tac*. *algD* expression in the Δ*algL* mutant causes lysis. We took advantage of the P*trc-algD* inducible system and reduced the amount of IPTG to reduce alginate production to see if this would allow the Δ*algL* mutant to survive. Nearly identical cultures of FRD1050 (*algL*$^+$) and FRD1300 (Δ*algL*) were grown in the presence of 4.0 mM (high expression) or 0.4 mM (lower expression) IPTG and then compared for cell growth (optical density at 600 nm [OD_{600}]) and amounts of alginate produced over time. This 10-fold difference in IPTG resulted in approximately a twofold difference in the amount of alginate made by the Alg$^+$ strain during the log phase of growth: 4.0 mM and 0.4 mM IPTG induction for 10 h in the *algL*$^+$ strain resulted in 725 ± 75 μg/ml and 410 ± 35 μg/ml alginate, respectively (Fig. 5A and B). However, alginate accumulation was approximately the same with either IPTG concentration, ~900 ± 75 μg/ml, after 22 h. Lower concentrations of IPTG were also tested, but they did not induce detectable alginate production in the *algL*$^+$ strain (data not shown). With FRD1300 (Δ*algL*), 4.0 mM and 0.40 mM IPTG both induced approximately 95 ± 25 μg/ml alginate at 10 h postinduction and 150 ± 20 μg/ml alginate at 22 h postinduction (Fig. 5). The log-phase cells of FRD1300 were able to grow to a higher OD with the lower IPTG level but were still lysed at approximately the same time. Thus, reducing the level of alginate production in FRD1300 did not appear to abrogate the lethal phenotype, suggesting that AlgL still plays a role in strains producing lower levels of alginate.

DISCUSSION

A wide range of organisms is known to produce alginate lyases (alginases), which are enzymes that catalyze the degradation of alginate polymers into unsaturated oligosaccharides. Many marine bacteria produce alginate lyases in order to use the alginate of brown algae (seaweed) as a carbon source (49). Alginate-producing bacteria like *Azotobacter* and *Pseudomonas* spp. have AlgL, a periplasmic alginate lyase, but they do not use alginate as a carbon source (39, 42). The AlgL protein (40 kDa) has a signal peptide, which is processed during periplasmic localization (42). *Pseudomonas* AlgL has been shown to preferentially cleave bonds between pairs containing at least one D-mannuronate residue, which thus classifies it as a D-mannuronate-specific lyase (9).

The role of an alginate-degrading enzyme within the alginate biosynthesis-secretion pathway has not been intuitive, and so the function of AlgL has been somewhat controversial. AlgL may control the length of the polymer produced (27). In sup-

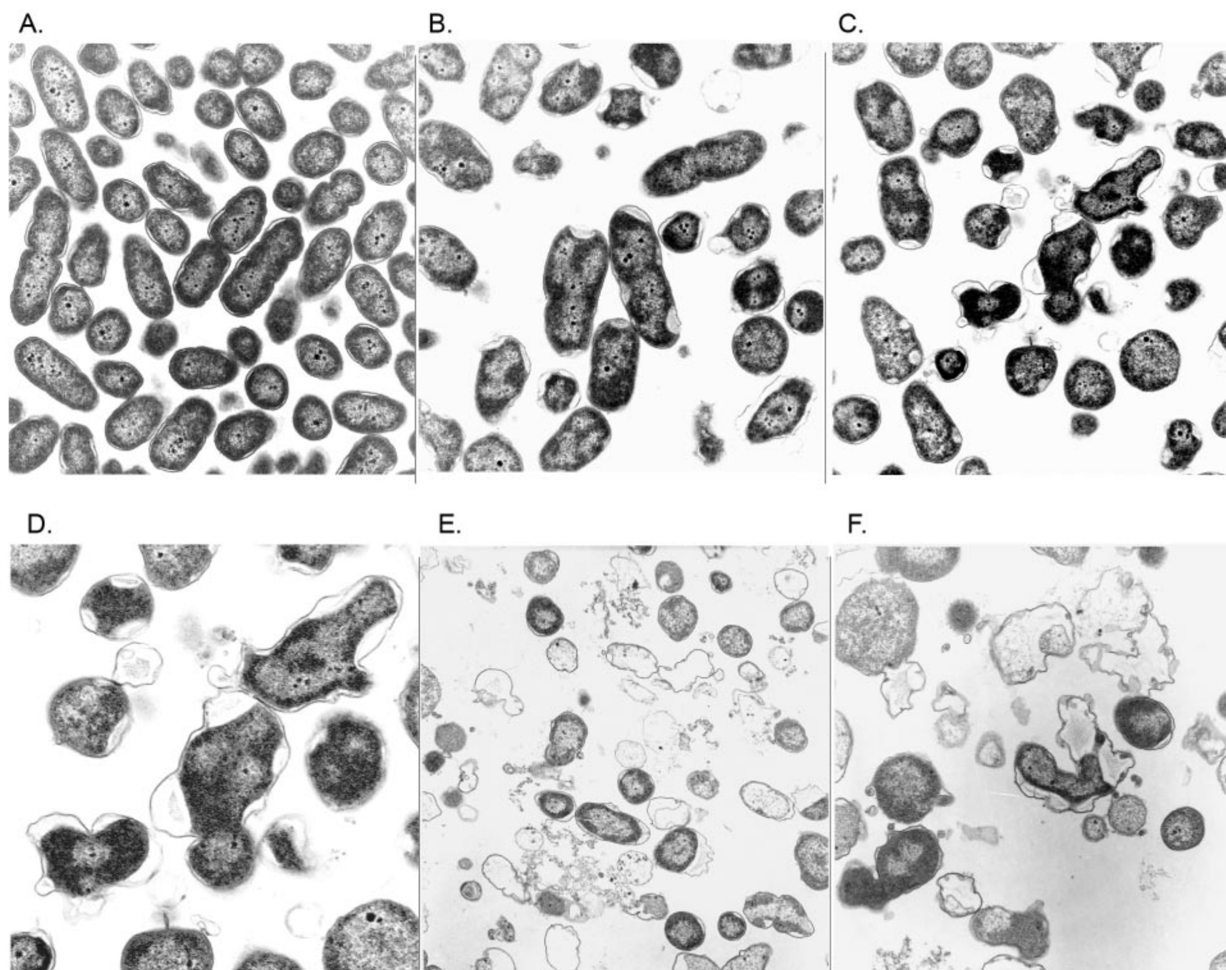

FIG. 4. Electron micrographs of thin-sectioned FRD1300 (P*trc-algD-A* Δ*algL*::Gm^r) following incubation in L broth and comparing cells at 0, 2, 4, and 6 h post IPTG induction of the *algD* operon. (A) Uninduced, normal-appearing cells (×40,000). *P. aeruginosa* rods are approximately 1 by 2 μm in size. (B) Induction for 2 h shows zones of separation between membranes. (C) Induction for 4 h shows larger zones of separation. (D) Induction for 4 h; this enlargement of panel C shows periplasmic polymer accumulation. (E) Induction for 6 h showed general lysis of the cells. (F) Enlarged image of FRD1300 cells after 6 h of induction with IPTG.

port of this, an *algL* mutant of *Azotobacter vinelandii* produced an alginate with a higher mean molecular weight than the parent strain, although neither encystment nor cyst germination was affected (47). AlgL may also be important in facilitating dissemination of bacteria; overexpression of *algL* in mucoid *P. aeruginosa* leads to a decrease in alginate polymer length and an increase in bacterial detachment from an adherent surface (2). With *algL* in the operon for alginate biosynthesis, and thus coexpressed with alginate biosynthetic enzymes, it seemed reasonable that a periplasmic AlgL protein might be part of a polymerization or transport complex in the periplasm. In *Pseudomonas syringae* pv. syringae, the absence of lyase activity reduces alginate production by about 50% (35). In *P. aeruginosa*, one study reports that AlgL is not required for alginate production by *P. aeruginosa* (3), yet in another a lyase-negative *P. aeruginosa* isolate appeared nonmucoid and produced only small amounts of alginate (29).

In this study, we examined the effect of a nonpolar Δ*algL* mutation in strain FRD1, a highly mucoid (Alg$^+$) CF clinical isolate of *P. aeruginosa*. This construction was problematic in that we found such mutants to be very difficult to construct using a well-established gene replacement technology for this organism. This was circumvented by artificially replacing the promoter of the *algD* operon with a LacI^q-repressed *tac* promoter, which then permitted IPTG induction of the operon. In this background, Δ*algL* mutants could readily be constructed (e.g., FRD1300), as long as expression of the operon was low. However, when the *algD* operon was reactivated with IPTG in the Δ*algL* mutant, the cells were lysed within a few hours. Electron microscopy showed over time a dramatic separation of the inner and outer membranes, with accumulation of alginate in the periplasm that ultimately led to cell rupture. Alginate was detected in the supernatant of lysed FRD1300 cultures, indicating that AlgL was not required for alginate production. These microscopic observations are similar to those seen with mutations that block ABC transporter-dependent secretion of K1 polysaccharide out of the cell in *E. coli*, which leads to cytoplasmic accumulation of polymer (33, 45). In contrast, our observation of periplasmic accumulation of polymer in *P. aeruginosa* suggests that the AlgL$^-$ block in

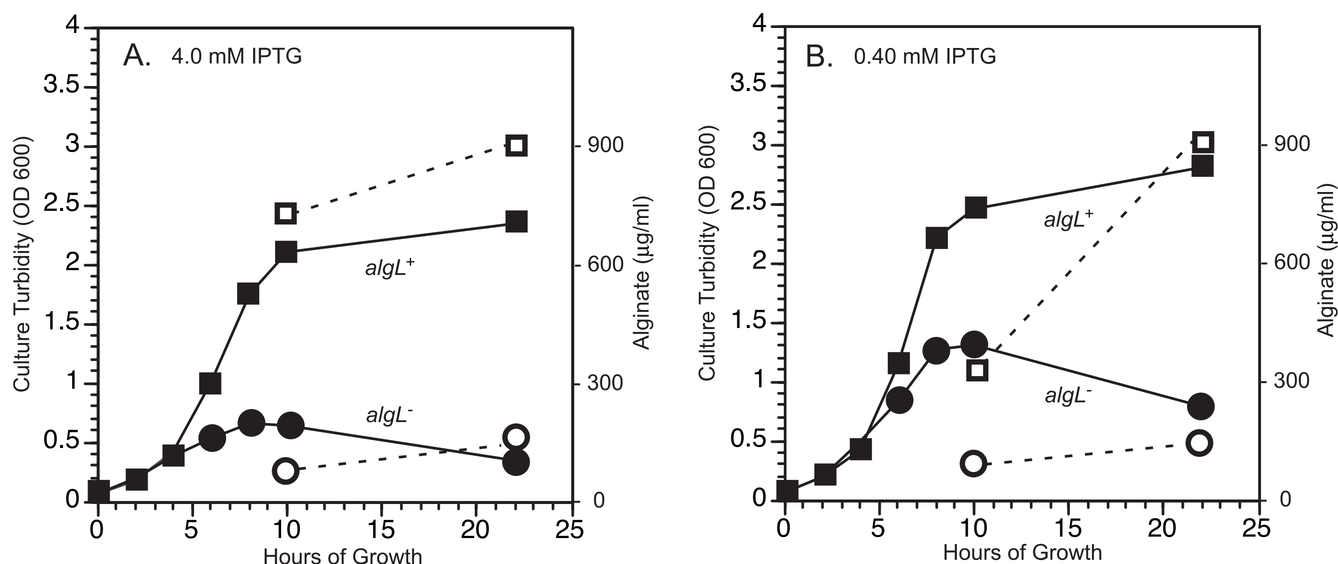

FIG. 5. Effect of IPTG concentrations on growth and alginate production in FRD1050 and FRD1300. Overnight cultures were used to inoculate MAP medium and incubated with aeration at 37°C to an OD_{600} of 0.20. Twenty-five-milliliter volumes of log-phase cultures were incubated with 4.0 mM IPTG (A) or 0.4 mM IPTG (B) to induce alginate production. Samples were taken periodically for measurements of growth as OD_{600} (closed symbols) and alginate accumulation in culture supernatants (open symbols). FRD1050 *algL*$^+$, squares; FRD1300 Δ*algL*, circles. A repeat of this experiment produced similar results.

alginate transport is by a distinctly different mechanism. We have isolated IPTG-resistant mutants of FRD1300 which were Alg$^-$, and they presumably have secondary mutations that prevent alginate production, which is currently under study.

To explain these results, we propose a model for alginate biosynthesis in which AlgL has both an enzymatic function as an alginate lyase and a structural role as a component of a periplasmic transport apparatus, or scaffold, in mucoid *P. aeruginosa* (Fig. 6, left). The term scaffold has also been applied to the biosynthesis-transport complex for K5 capsule production in *E. coli* (48). Our previous studies indicate that the periplasmic AlgG protein is also bifunctional, having an enzyme activity (i.e., polymer level C-5 mannuronan epimerase) (12) and a structural role in protecting nascent alginate polymers from the enzymatic attack of the AlgL lyase (20). The AlgK protein is a putative lipoprotein in the periplasm, which likewise is required to protect the polymer from the enzymatic attack of the AlgL lyase (21). AlgX mutants have a similar phenotype (40). Thus, mutants lacking AlgG, AlgK, or AlgX produce polymers that fail to be secreted, but they are rapidly degraded by AlgL. High levels of low-molecular-weight uronic acids, the products of alginate degradation, are found in the culture supernatant of these mutants. Thus, a model for the alginate scaffold includes at least AlgG, AlgK, AlgX, and probably AlgL. It is conceivable that AlgL plays its major role in degrading alginate that accumulates in the periplasm under highly mucoid conditions, and that it has a minor role in transporting polymers across the outer membrane. However, previous studies did not reveal the release of AlgL-degraded alginate in the highly mucoid CF strain FRD1 (21). The attempt here to reduce alginate production levels by FRD1300 by lowering the IPTG induction still resulted in the lysis of cells. Most striking are the electron micrographs here that show periplas-

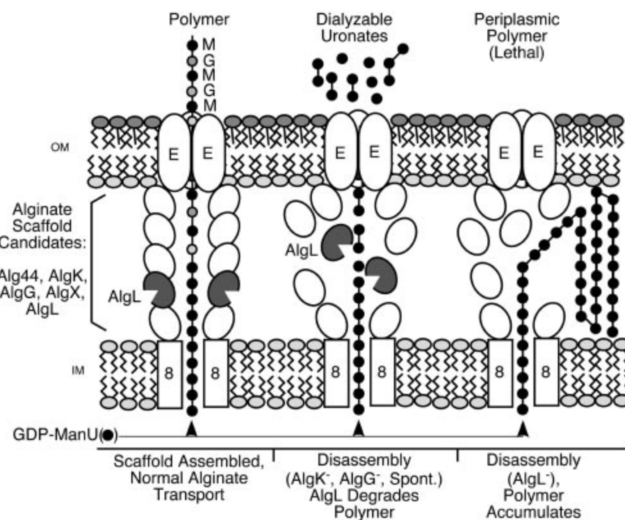

FIG. 6. Model for alginate secretion. (Left) Polymerization of mannuronates (●) from GDP-mannuronate probably occurs via Alg8, which appears to be an inner membrane (IM) glycosyltransferase. A multicomponent protein scaffold is proposed to transport polymer across the periplasm to AlgE in the outer membrane (OM). AlgG, a periplasmic C-5 mannuronan epimerase, converts some D-mannuronate residues to L-guluronate (grey circles). Likely candidates as components of the scaffold complex include the periplasmic proteins Alg44, AlgK, AlgG, AlgX, and AlgL. (Middle) The scaffold fails to assemble correctly when AlgK or AlgG protein is absent. AlgL's alginate lyase activity is then free to digest any newly formed polymer in the periplasm, thus releasing dialyzable uronates into the extracellular environment. Occasionally, the scaffold may disassemble spontaneously in the wild type, allowing AlgL to digest periplasmic polymer. (Right) When AlgL is absent, the scaffold again does not assemble correctly but the absence of periplasmic lyase activity causes polymer to accumulate in the periplasmic space, ultimately bursting the cells.

mic blebs of alginate after just 2 h of induction of the alginate biosynthetic operon.

In concurrence with the mutant analysis, our model for alginate biosynthesis in *P. aeruginosa* suggests that the periplasmic scaffold does not form correctly when any single component is missing and results in polymer being trapped in the periplasm. We also propose that the depolymerase activity of AlgL is at least partially masked while it is part of the transport scaffold. If the missing component is AlgK, AlgG, or AlgX, the trapped alginate is subject to attack by active AlgL, and this results in the release of small uronic acids (Fig. 6, middle). However, when AlgL is not present due to mutation, then polymer rapidly builds up in the cell's periplasmic space (Fig. 6, right), with lethal effects. Other periplasmic components of the scaffold may include Alg44, which is periplasmic and also required for polymer formation. A putative role for AlgL lyase activity, under normal conditions, is to digest any alginate that remains in the periplasm after spontaneous disassembly of the scaffold. This would indirectly affect polymer size as well. Overall, these data are consistent with the idea that AlgL is a vital part of the alginate transport scaffold, as well as having a role in degrading alginate as a lyase. Future studies will examine the enzymatic role of AlgL in polymer formation and identify the interacting components of the putative alginate transport scaffold.

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