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Transcriptional Analysis of the *Pseudomonas aeruginosa* Genes *algR*, *algB*, and *algD* Reveals a Hierarchy of Alginate Gene Expression Which Is Modulated by *algT*

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Strains of *Pseudomonas aeruginosa* which colonize and infect the lungs of cystic fibrosis patients have a mucoid colony morphology due to the overproduction of the exopolysaccharide alginate. The response regulators AlgB and AlgR are required for the transcription of *algD*, a tightly regulated gene encoding GDP-mannose dehydrogenase, which is critical for *P. aeruginosa* alginate biosynthesis. Previous studies indicated that mutations in the *algT* gene of mucoid FRD1 *P. aeruginosa* result in nonmucoid derivatives. However, the specific role for *algT* in alginate gene regulation has not been elucidated. In this study, transcription of *algB*, *algD*, and *algR* was characterized by gene fusion and primer extension analysis. Expression of *algR* and *algD* was abolished in *P. aeruginosa* strains containing *algT*::Tn*501* insertions because of lack of transcription initiation at the *algR* and *algD* promoters. An *algR* mutation was constructed in FRD1, and this resulted in the loss of alginate production and a dramatic decrease in *algD* transcription. RNA and gene fusion analysis revealed that *algB* is not required for *algR* expression, nor is *algR* necessary for transcription of *algB*. Thus, with the exception of a requirement for AlgT, the AlgB and AlgR pathways appear to be independent of each other. In gel band mobility shift assays, a protein(s) present in extracts from mucoid and *algB* and *algR* mutant *P. aeruginosa* strains formed a specific complex with *algD* sequences located immediately upstream of the start of transcription. No binding to these sequences was observed when extracts from *algT* mutant strains were examined. A model proposed suggests that a hierarchy of alginate gene expression exists in which AlgT is required for transcription of the response regulators *algB* and *algR*, which in turn are necessary for *algD* expression. AlgT or a protein under *algT* control also binds to sequences located within the *algD* promoter.

During the last few years, great strides in understanding the basic genetic defect which causes cystic fibrosis (CF) have been made, including cloning of the CF gene and correction of the defect in cultured CF airway cells (27, 47, 48). Despite this, lung disease is still the cause of death in 95% of CF patients (52). Historically, individuals with CF are infected with *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and the nonmucoid form of *Pseudomonas aeruginosa* (45, 49). However, as the CF patient gets older, mucoid *P. aeruginosa* becomes the predominant pulmonary pathogen, present in up to 85% of cultures from patients with advanced disease (14). Mucoid strains of *P. aeruginosa* overproduce an *O*-acetylated polymer of β-1,4-linked D-mannuronic and L-guluronic acid called alginate (20), and these strains appear to be derived from the initially colonizing nonmucoid *P. aeruginosa* organisms (45). As the mucoid organisms emerge and persist, there is a distinct correlation with a poor clinical condition of the CF patient (14). Overproduction of alginate in the CF airway likely plays a selective role for *P. aeruginosa*, since mucoid organisms are more resistant to opsonization and phagocytosis (46).

The mucoid (Alg^+) phenotype of *P. aeruginosa* is unstable in the laboratory, and mucoid cells spontaneously convert to nonmucoid ones (Alg^-) at high frequency (16, 23). Most of the genes required for this conversion are clustered near the 68-min region of the chromosome (19, 32, 41). These genes have been referred to as *algT* (*algU*), *mucA*, and *algN* (*mucB*) (11, 17, 19, 21, 32–34). The genetic term *algS* (17) refers to spontaneous mutations in *algT* (13) (*algU*), a gene which encodes a *trans*-acting positive regulator required for activation of alginate genes. AlgT (AlgU) has been shown to have sequence similarity to the *Escherichia coli* alternative sigma factor (σ^E) involved in an environmental stress response system (12, 13). AlgT (AlgU) is controlled by the products of two closely linked genes, *mucA* and *algN* (*mucB*) (21, 33). In a number of *P. aeruginosa* CF isolates, including the strain FRD1 used in this study, mutations in *mucA* may inactivate the gene product so it cannot function as an inhibitor (anti-sigma factor) of AlgT (AlgU), which would lead to activation of alginate genes (12, 13).

In addition to the alginate conversion locus, a large operon located near *argF* at 34 min encodes most of the alginate biosynthetic enzymes (3). *algD*, the first gene in this operon, encodes GDP-mannose dehydrogenase. Overproduction of this enzyme is believed to commit the sugar intermediates to alginate production (50). For this reason, and the fact that *algD* is the first gene of the biosynthetic operon, many workers have studied the expression of *algD* (2, 8, 53, 55). The *algD* gene undergoes strong transcriptional activation in alginate-producing cells, yet no transcription of *algD* is detectable in nonmucoid cells (6).

Located near the 9-min region of the *P. aeruginosa* chromo-

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some are at least three regulatory genes which are required for maximal alginate production and *algD* transcription. The *algR* (also called *algR1*) gene product is an environmental response regulator (5) belonging to a class of two-component regulatory proteins commonly found in bacterial species (44). AlgR has been purified and shown to bind upstream from the start of *algD* transcription (24, 38, 39). AlgR also appears to be required for expression of an unlinked alginate biosynthetic gene, *algC* (18, 57).

Another key alginate regulatory gene called *algB* is essential for high-level synthesis of alginate in *P. aeruginosa* (22). Like AlgR, AlgB belongs to the response regulator superfamily of two-component signal transduction systems. In addition, *algB* is required for transcription of *algD*, the first gene in the alginate biosynthetic operon (55). Transcription of *algB* requires both the product of the *algT* gene and the DNA binding-bending protein integration host factor (IHF) (56).

In this study, we sought to determine the circuitry of alginate gene regulation. To accomplish this, we analyzed the transcription of *algD*, *algR*, and *algB* in mucoid, spontaneous nonmucoid, *algB*::Tn*501*, *algT*::Tn*501*, and *algR*-Ω *P. aeruginosa* FRD strains. We have determined that expression of the alginate biosynthetic operon requires both the response regulators, AlgB and AlgR, and AlgT. Expression of *algB* and *algR* is dependent on AlgT, suggesting a circuit in which AlgT activates *algB* and *algR*. Both *algB* and *algR* are necessary but not sufficient for *algD* transcription and alginate production. This study also provides genetic evidence that there are at least two independent signal transduction pathways required for alginate production by *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and plasmids, media, growth conditions, and enzyme assays. The *P. aeruginosa* strains utilized in this study are FRD1 (Alg^+, CF isolate) (41), FRD2 (Alg^-, spontaneous *algT18* derivative of FRD1) (13, 42), FRD440 (*algT*::Tn*501*) (16), FRD444 (*algB*::Tn*501*) (22), and FRD810 (*algR*-Ω) (see below). *E. coli* TB1, JM107, and HB101 used for routine cloning were described previously (55). The construction and use of the low-copy-number gene fusion vectors pKK61 (*algD-cat*) and pDJW161 (*algB-cat*) have been described elsewhere (55, 56). pRK2013 (15) was used to mobilize plasmids from *E. coli* HB101 to *P. aeruginosa* FRD strains by triparental mating. pUC18Ω was constructed by cloning a 2.0-kb *Sma*I restriction fragment, which contains the Ω gene cartridge (Sm^r Spc^r; Amersham), into *Sma*I-cleaved pUC18. pAL1 is pCP13 (4) containing a 20-kb *Hin*dIII insert with the *algR* gene (see below). pDJW100, pDJW235, pDJW236, pDJW243, and pDJW245 are derivatives of pAL1 which were used to construct an *algR* mutant (Fig. 1; see below). *P. aeruginosa* and *E. coli* strains were cultured as described elsewhere in LB medium with appropriate antibiotics (55). Streptomycin was used at concentrations of 100 μg/ml for *E. coli* and 500 μg/ml for *P. aeruginosa*. For gene fusion studies, *P. aeruginosa* strains containing pDJW161 (*algB-cat*) or pKK61 (*algD-cat*) were cultured to an A_{600} of 0.7 ($\sim 5 \times 10^8$ cells per ml), cell extracts were prepared, and chloramphenicol acetyltransferase (CAT) levels were determined as outlined elsewhere (55).

Nucleic acid manipulations. Most cloning manipulations were performed as previously described (1, 55, 56). *P. aeruginosa* genomic DNA was isolated by a technique described elsewhere (1). Plasmids were purified from *E. coli* with Qiagen columns (Qiagen Corp.). RNA was isolated from logarithmic-phase (A_{600} = 0.7) *P. aeruginosa* cells cultured in LB medium and precipitated in 5.7 M cesium chloride (56). Oligonucleotides were synthesized on an Applied Biosystems model 380B automated DNA synthesizer. Oligonucleotides for primer extension and DNA sequencing of *algD* and *algR* were synthesized from the DNA sequences (5, 7) and are 5′-AACAGGT TGAGTTTGTCCCT-3′ (located from +86 to +66 relative to the start of *algD* transcription) and 5′-GGTTCGTCATCGA CAATCAG-3′ (located from +101 to +81 relative to the *algR* transcription start site). These primers were end labeled by using polynucleotide kinase with [γ-^{32}P]ATP as described elsewhere (1). DNA sequences were determined from plasmid DNA as described previously, with modifications (55). The technique of Southern (51) was used to verify the construction of the *algR* mutant FRD810. The probe in the Southern blot shown in Fig. 1D was a 2.3-kb $algR^+$ fragment derived by *Eco*RI digestion of pDJW235 and was labeled by a random primer labeling kit (Promega Corp.) with [α-^{32}P]dCTP. Primer extension experiments were performed essentially as outlined previously (56), except that avian myeloblastosis virus reverse transcriptase (Promega Corp.) was substituted for Superscript during cDNA synthesis.

Construction of a *P. aeruginosa* FRD *algR* mutant. To generate an *algR* mutant strain of *P. aeruginosa* FRD1 (Alg^+), a 2.3-kb *Eco*RI fragment containing the 5′ regulatory region and a portion of the *algR* coding sequence was subcloned into pUC18, resulting in pDJW235 (Fig. 1B). Two adjacent *Eco*RV sites are located 144 bp 3′ of the *algR* AUG initiation codon (5, 54). Therefore, pDJW235 was cleaved with *Eco*RV and ligated with a 2.0-kb Ω fragment derived from *Sma*I digestion of pUC18Ω, resulting in pDJW236 (Fig. 1B). This cloning places transcription and translation stop signals in the 5′ end of *algR* and also provides a convenient Sm^r marker. pDJW236 was then partially digested with *Eco*RI, and a 1.9-kb fragment containing adjacent 3′ *algR* sequences was cloned in the correct orientation downstream of the 4.3-kb *Eco*RI fragment containing *algR* Ω, resulting in pDJW243 (Fig. 1B). Interplasmid exchange (35) with pAL1 was performed to increase the amount of flanking DNA for homologous recombination in *P. aeruginosa*. To accomplish this, the rec^+ *E. coli* strain JM107 was transformed with both pDJW243 (pUC18 derivative, Ap^r Sm^r Spc^r) and pAL1 (IncP1, *cos* Tc^r) and colonies were selected on medium containing tetracycline and streptomycin. A portion of the cells was removed and cultured overnight with selection for both markers to permit recombination between the two plasmids. To package cosmid pAL1 derivatives, a λ cI857 lysate (31) was prepared on this culture. The cosmids were transduced into the *E. coli rec* mutant strain HB101, and colonies were selected again on medium containing tetracycline and streptomycin. The Sm^r Tc^r colonies were subsequently screened for the loss of the pUC18-derived Ap^r marker. By this technique, the 6.2-kb insert of pDJW243 was exchanged with the 4.3-kb homologous sequences present on pAL1 (Fig. 1B) to generate pDJW245. This plasmid is identical to pAL1 except that the 2.3-kb *Eco*RI fragment containing a portion of the wild-type *algR* is replaced with a 4.3-kb fragment containing the Ω gene cartridge present within the 5′ coding sequence of *algR*. To introduce the mutated *algR* gene into FRD1, F116L-mediated transduction (Fig. 1C) (43) was utilized. Colonies were initially selected on streptomycin plates and screened for the loss of Tc^r, which is encoded on the plasmid moiety of pDJW245. Gene replacements were verified by PCR and Southern blot comparisons of FRD1 and FRD810 genomic DNA.

Gel mobility shift assays. DNA-binding assays were performed essentially as described previously (53, 56) with *P. aeruginosa* extracts which were prepared identically to those

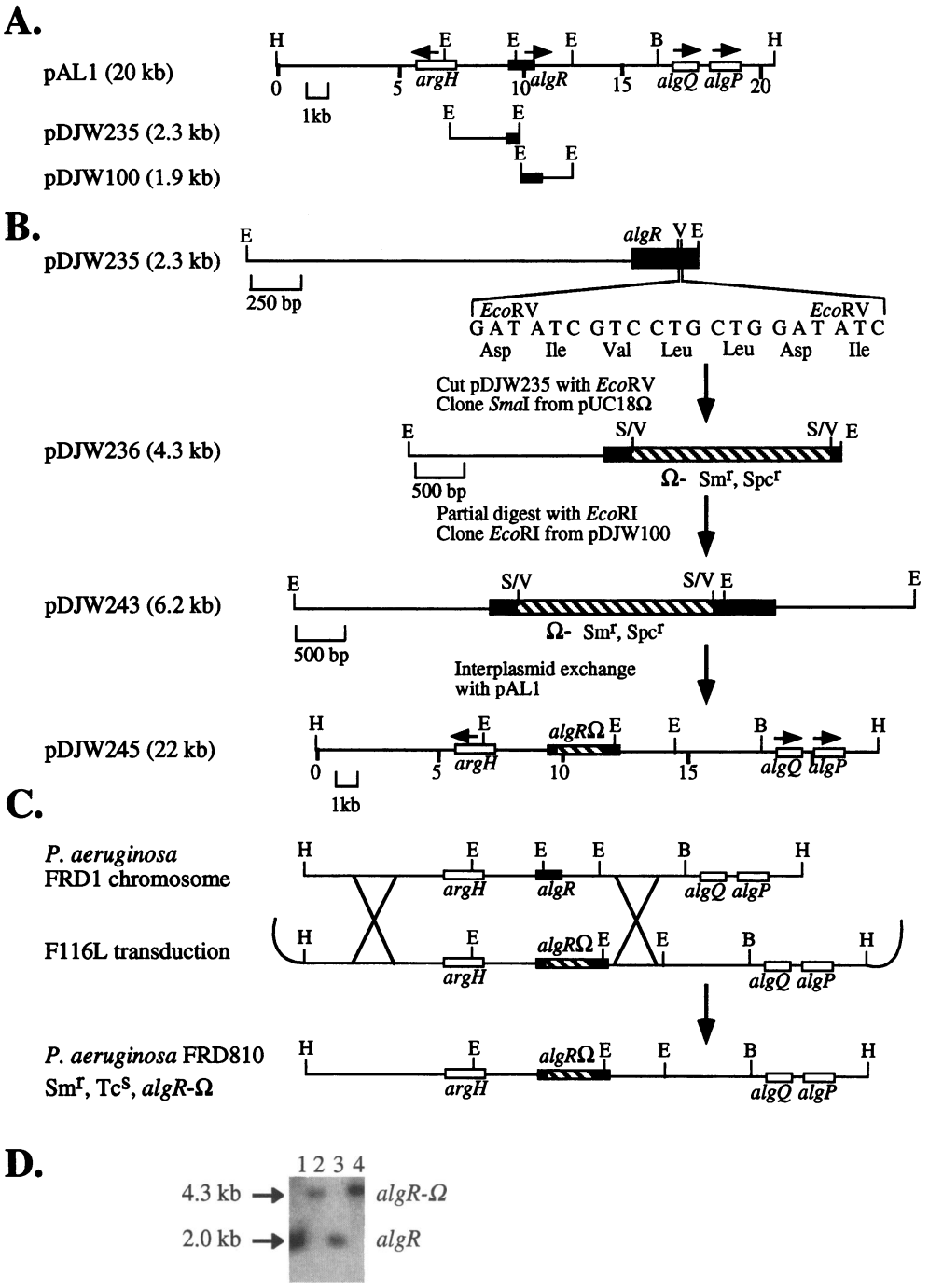

FIG. 1. Construction of an *algR* mutant of *P. aeruginosa* FRD. (A) Partial restriction map of pAL1 which contains the *algRPQ* and *argH* genes. The sizes to the right of the plasmid designations represent the size of the cloned insert DNA. Arrows indicate the direction of transcription. The filled box represents the *algR* gene. *algR* contains an *Eco*RI site (5), and two subclones of pAL1, pDJW100 and pDJW235, which contain a portion of the *algR* gene are depicted. (B) Cloning of the 2.0-kb Ω gene cartridge in *algR*. pDJW235 is a 2.3-kb *Eco*RI fragment containing the 5′ end of *algR*. Two adjacent *Eco*RV cloning sites with the intervening sequence are illustrated. The filled box represents *algR* sequences, while the hatched box depicts the 2.0-kb Ω gene cartridge. (C) Homologous recombination between the FRD1 chromosome (top) (*algR*$^+$) and a linear fragment of pDJW245 (middle) (*algR*-Ω). Selection for Sm^r and loss of Tc^r (pDJW245 derived) resulted in FRD810. (D) Southern blot illustrating insertion of the 2.0-kb Ω gene cassette in the *algR* gene of FRD810. The probe was a 2.3-kb *algR*$^+$ *Eco*RI fragment (from pDJW235) generated by random prime labeling. Lanes: 1, 5 ng of pAL1; 2, 5 ng of pDJW245; 3, 2 μg of genomic DNA from FRD1 (Alg$^+$); 4, 2 μg of genomic DNA from FRD810 (Alg$^-$ *algR*-Ω). Abbreviations: H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; S, *Sma*I; V, *Eco*RV; Sm^r, resistance to streptomycin; Spc^r, resistance to spectinomycin; Tc^s, sensitivity to tetracycline; Tc^r, resistance to tetracycline.

described above for the CAT assays. Oligonucleotides for PCR amplification of *algD* fragments for the experiment shown in Fig. 4 were as follows: fragment I, 5′-AAGGCGGAAATGC CATCTCC-3′ and 5′-AATGGCCACTAGTTGCAGAA-3′, which generated a 200-bp fragment located from −123 to −323 relative to the start of *algD* transcription; fragment II, 5′-AAGGCGGAAATGCCATCTCC-3′ and 5′-AGGGAAG TTCCGGCCGTTTG-3′, which generated a 300-bp fragment located from −23 to −323 relative to the start of *algD* transcription; and fragment III, 5′-AAGGCGGAAATGCCA TCTCC-3′ and 5′-TCAAGTTGCTCTGCCCATAA-3′, which generated a 360-bp fragment located from +36 to −323 relative to the start of *algD* transcription. PCR amplifications were performed by the GeneAmp process with *Taq* polymerase (Perkin-Elmer) as described elsewhere (53). Following electrophoresis, the gels were dried under a vacuum and subjected to autoradiography.

TABLE 1. Expression of *algD-cat* and *algB-cat* transcriptional fusions in *P. aeruginosa* strains

Strain[a]	Alginate phenotype	Genotype	*algD-cat* level[b]	*algB-cat* level[b]
FRD1	Alg^+	*algT*$^+$	1,431 ± 122	505 ± 45
FRD2	Alg^-	*algT18*	<10	166 ± 13
FRD440	Alg^-	*algT*::Tn*501*	<10	145 ± 13
FRD444	Alg^-	*algB*::Tn*501*	52 ± 6	554 ± 49
FRD810	Alg^-	*algR*-Ω	84 ± 10	523 ± 53

[a] Each strain contained a low-copy-number plasmid (IncP vector) with *algD-cat* (pKK61) (55) or *algB-cat* (pDJW161) (56).

[b] Cells were cultured to the same cell density (A_{595} = 0.8) in LB medium, and cell extracts were prepared and assayed for CAT by a sandwich enzyme-linked immunosorbent assay technique. CAT levels in dilutions of the cell extracts were determined by extrapolation from a standard curve and normalized for protein content. Values show picograms of CAT per microgram of protein in extracts.

RESULTS AND DISCUSSION

A defined *algR*-Ω mutation in the *P. aeruginosa* FRD background demonstrates an Alg^- phenotype. To our knowledge, only two *algR* mutants have been reported among *P. aeruginosa* strains: strain 8852 (*alg-22*), isolated by Darzins and Chakrabarty (4) using chemical mutagenesis, and CDM1/1, an *algR* mutant isolated by gene-scrambling mutagenesis in a PAO568 (*muc-2 leu-38*) genetic background (36). However, no defined *algR* mutation has been generated in *P. aeruginosa* CF isolates (e.g., strain FRD). Since different *P. aeruginosa* strains may utilize alternative mechanisms to become mucoid, it was important to test the phenotype of an *algR* mutant in the strain FRD1 genetic background. The basis for this strain diversity most certainly lies in the complex arrangement and expression of the *algT* (*algU*)-*mucA*-*algN* (*mucB*) loci located in the late region of the chromosome (13, 16, 21, 32, 34). Therefore, *algR* mutants would be valuable not only for examining the role of AlgR in alginate gene expression, but also for establishing the requirement for *algR* in the maintenance of mucoidy in FRD *P. aeruginosa* strains. As such, it was necessary to construct a *P. aeruginosa* FRD derivative which contained an *algR* mutation.

To clone *algR*, we took advantage of the fact that *algR* and the prototrophic marker *argH* are located on the same 20-kb *Hin*dIII restriction fragment (Fig. 1) (4). A clone (pAL1) was obtained by transferring a gene bank containing *Hin*dIII fragments of strain FRD1 to an *argH* auxotroph of *P. aeruginosa* and selecting for prototrophy (Fig. 1A) (40a). Two subclones were generated from pAL1: pDJW100 and pDJW235 (Fig. 1A). Restriction mapping, partial DNA sequence analysis of pDJW100 and pDJW235, and comparison with previously published *algR* data from other laboratories (4, 5) verified that pAL1 indeed contained the *P. aeruginosa* FRD *algR* gene (data not shown).

To generate an *algR* mutant strain of FRD1, a derivative of pAL1 was constructed which contained the omega (Ω) gene cassette cloned within the amino-terminal coding sequence of *algR* (Fig. 1B) (see Materials and Methods). F116L-mediated transduction (Fig. 1C) (43) was utilized to introduce the *algR*-Ω allele into FRD1 (Fig. 1C). Colonies were initially selected on streptomycin plates and screened for the loss of Tc^r, which is encoded on the plasmid moiety of pDJW245 (Fig. 1C). As an initial screen, genomic DNA from several Sm^r Tc^s colonies was isolated and tested by PCR (using *algR*- and Ω-specific primers). Genomic DNAs from clones which by PCR analysis appeared to contain the Ω fragment in *algR* were tested by Southern hybridization. Of these, most contained a unique 4.3-kb *Eco*RI fragment which hybridized to an *Eco*RI *algR*$^+$ probe, indicating insertion of the 2.0-kb Ω cassette in *algR*. One derivative (FRD810) which had undergone gene replacement at the *algR* locus with sequences present on pDJW245 was chosen for further study. When probed with a 2.3-kb *Eco*RI *algR* fragment derived from pDJW235, an *Eco*RI digest of FRD810 chromosomal DNA demonstrated a 4.3-kb band (Fig. 1D, lane 4) which is identical to that observed with an *Eco*RI digest of pDJW245 (the plasmid used for allele replacement) (lane 2). These *Eco*RI fragments are 2.0 kb larger than wild-type sequences contained on pAL1 (Fig. 1D, lane 1) or FRD1 (lane 3), indicating insertion of the Ω fragment in *algR*. Finally, restriction analysis with other enzymes and Southern blot hybridization tests confirmed these observations (data not shown).

When plated on L agar medium, the *algR*-Ω mutant FRD810 had a nonmucoid phenotype and did not produce detectable alginate as determined by the carbazole assay (29). Moreover, the loss of alginate in FRD810 was not due to spontaneous conversion to the nonmucoid phenotype, since *algR* supplied in *trans* (pAL1) restored alginate production to wild-type levels. Therefore, *algR* is required for alginate overproduction in *P. aeruginosa* FRD.

Transcription of *algD* in *P. aeruginosa* FRD strains is under *algT*, *algB*, and *algR* control. A committed step in the overproduction of alginate is transcriptional activation of *algD* (6, 50), the first gene in an operon encoding most of the enzymes required for alginate biosynthesis (3). *algD* is tightly regulated; no transcription is apparent in nonmucoid *P. aeruginosa* strains, but *algD* undergoes strong transcriptional activation in mucoid cells (6). Therefore, we chose to examine *algD* transcription in various *P. aeruginosa* strains with a low-copy-number plasmid-borne *algD-cat* operon fusion (Table 1) and by primer extension analysis (Fig. 2). In *P. aeruginosa* FRD strains, transcription of *algD* is clearly regulated by the mucoid status of the cell; *algD* is expressed at high levels in the mucoid strain FRD1 (Table 1; Fig. 2, lane 1), whereas no *algD* transcription is detectable in the spontaneous nonmucoid derivative, FRD2 (Table 1; Fig. 2, lane 2). In FRD1, *algD* transcription initiates at a G residue, 366 nucleotides upstream of the AUG translation codon, and this is identical to the start site identified by others (7) with another *P. aeruginosa* strain. In addition, no transcription of *algD* is detectable in a strain containing a Tn*501* mutation in the regulatory gene *algT* (Table 1; Fig. 2, lane 3). In the FRD1-derived *algB* mutant FRD444 (Table 1; Fig. 2, lane 4) and the *algR* mutant FRD810 (Table 1; Fig. 2, lane 5), *algD* expression is reduced 28- and 17-fold, respectively. Taken together, these results indicate that the regulatory genes *algT*, *algR*, and *algB* are all required

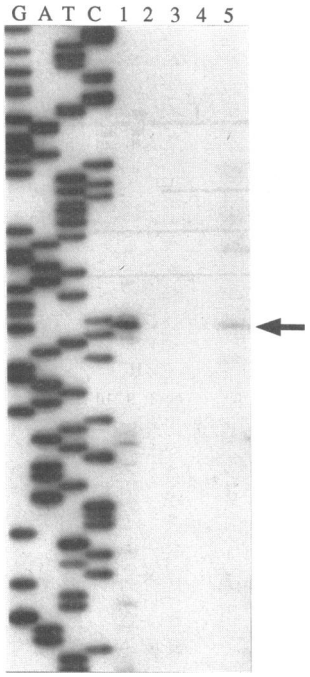

FIG. 2. Primer extension analysis of *algD*. RNA was prepared as described in Materials and Methods. The oligonucleotide 5′-AACAG GTTGAGTTTGTCCCT-3′ was end labeled and used in a primer extension experiment with 50 μg of total cellular RNA from the following strains: FRD1 (Alg^+) (lane 1), FRD2 (Alg^- *algT18*) (lane 2), FRD440 (*algT*::Tn*501*) (lane 3), FRD444 (*algB*::Tn*501*) (lane 4), and FRD810 (*algR*-Ω) (lane 5). The arrow represents the start site of *algD* transcription. The *algD* sequencing ladder (GATC) was produced by the oligonucleotide used for synthesis of the probe in the primer extension experiment.

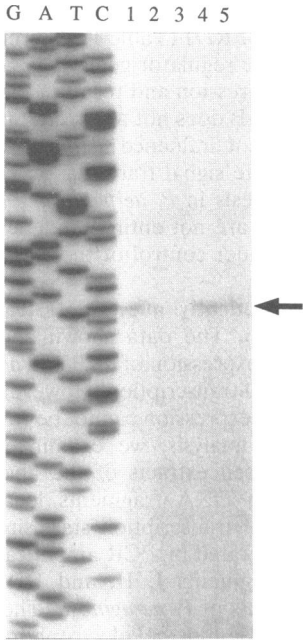

FIG. 3. Primer extension analysis of *algR*. RNA was prepared as described in Materials and Methods. The oligonucleotide 5′-GGT TCGTCATCGACAATCAG-3′ was end labeled and used in a primer extension experiment with 50 μg of total cellular RNA from the following strains: FRD1 (Alg^+) (lane 1), FRD2 (Alg^- *algT18*) (lane 2), FRD440 (*algT*::Tn*501*) (lane 3), FRD444 (*algB*::Tn*501*) (lane 4), and FRD810 (*algR*-Ω) (lane 5). The arrow represents the start site of *algR* transcription. The *algR* sequencing ladder (GATC) was produced by the oligonucleotide used for synthesis of the probe in the primer extension experiment.

for *algD* transcription and that mutations in any one of these genes block *algD* transcription. In addition, mutations in these genes affect transcription initiation at the *algD* promoter and not at any other promoters which might be involved in *algD* expression. The reduction or elimination of *algD* transcription in these mutants is not due to spontaneous Alg^+-to-Alg^- conversion (e.g., *algT* mutations), since each mutant can be complemented to Alg^+ in *trans* with plasmids containing the respective wild-type gene (54).

***algR* transcription in *P. aeruginosa* FRD is under *algT* control but *algB* and *algR* independent.** The above results indicate that transcription of *algD* is reduced or absent in *algT18*, *algT*::Tn*501*, *algB*::Tn*501*, and *algR*-Ω *P. aeruginosa* FRD strains. Among these, only the *algR* gene product has been shown to be directly involved in binding *algD* sequences (24, 39). It is possible that each of the gene products affects *algD* transcription directly by *algD* promoter activation. However, it is more likely that these genes are part of a cascade or hierarchy that leads to *algD* expression. To explore this possibility, we chose to examine whether the loss of *algD* transcription in the above-mentioned strains was mediated through expression of the *algR* gene. RNA from the wild type and the various mutants used in the experiment described above was characterized for *algR* transcription by primer extension analysis (Fig. 3). Like that of *algD*, *algR* transcription is absent in *algT18* and *algT*::Tn*501 P. aeruginosa* strains (Fig. 3, lanes 2 and 3, respectively). However, expression of *algR* is apparent in the mucoid strain FRD1 (Fig. 3, lane 1), in *algB*::Tn*501* strains (lane 4), and in the *algR*-Ω strain FRD810 (lane 5). In these strains, *algR* transcription initiates at a G residue located 73 bp upstream of the presumed translation initiation codon. This start site is identical to that determined for *algR* in other *P. aeruginosa* CF isolates (9, 28). In contrast to results observed elsewhere (40), we did not observe *algR* transcription arising from an upstream constitutive promoter (54). In these FRD strains, *algR* expression does not appear to be autoregulated, since wild-type levels of *algR* are present in the FRD810 *algR*-Ω strain (Fig. 3, lane 5). The involvement of AlgR in its own synthesis is controversial because of conflicting reports (28, 36). Our results regarding this issue appear to support those of Mohr and Deretic (36). In addition, AlgB is not required for *algR* transcription as levels of *algR* expression in FRD1 (Fig. 3, lane 1) and FRD444 (*algB*::Tn*501*) (lane 4) are identical.

Transcription of *algB* in *P. aeruginosa* FRD is under *algT* control but *algR* and *algB* independent. Our previous studies revealed that *algB* transcription requires AlgT and a *P. aeruginosa* IHF homolog (56). In *P. aeruginosa algT18* and *algT*::Tn*501* strains, *algB* expression is reduced threefold (Table 1), indicating that the mucoid status of the cell and AlgT affect *algB* expression. Similar to the results presented above for *algR* expression, AlgB is not required for its own expression. To determine if *algR* affects *algB* expression, *algB-cat* fusions were transferred to FRD810 (*algR*-Ω) and expression of *algB* in this strain was compared with expression in the parental Alg^+ strain FRD1. The results indicate that AlgR does not affect *algB* transcription since the levels of CAT in

FRD810 (*algR* mutant) are identical to those observed in the wild-type Alg^+ strain FRD1 (Table 1). Therefore, mutations in either of the response regulator genes *algR* and *algB* decrease or eliminate *algD* expression and result in the loss of mucoidy. In addition, since AlgB does not affect *algR* transcription (Fig. 3) and AlgR does not influence *algB* expression (Table 1), there are two separate signal transduction pathways required for alginate biosynthesis in *P. aeruginosa* FRD strains. However, these pathways are not entirely independent, since both *algR* and *algB* are under control of the *algT* gene (Fig. 3 and Table 1).

AlgT may independently affect *algD* transcription via a DNA-binding protein. The data shown above indicate that AlgT is required for expression of *algB* and *algR*, which in turn are necessary for *algD* transcription. However, an independent role for AlgT in *algD* expression cannot be ruled out. Using gel band mobility shift analysis, we examined the binding of proteins present in cell extracts of the above-mentioned *P. aeruginosa* strains to DNA fragments located immediately upstream of the *algD* transcription start site (Fig. 4). Three DNA fragments generated by PCR amplification of a plasmid containing *algD* (fragments I, II, and III) (Fig. 4A) were incubated with extracts of *P. aeruginosa*. The complexes were separated on polyacrylamide gel electrophoresis gels and then analyzed by autoradiography (Fig. 4B). A protein(s) present in FRD1 (mucoid), FRD444 (*algB*::Tn*501*), and FRD810 (*algR*-Ω) bound specifically to *algD* DNA fragments II and III (Fig. 4B, lanes 8, 11, and 12 for fragment II located from −323 to −23 and lanes 14, 17, and 18 for fragment III located from −323 to +36 relative to the start of *algD* transcription). However, no DNA binding to these fragments was observed with extracts from the *algT18* or the *algT*::Tn*501 P. aeruginosa* strains FRD2 and FRD440, respectively (Fig. 4B, lanes 9 and 10 for fragment II and lanes 15 and 16 for fragment III). When extracts from each of these strains were tested with fragment I (−323 to −123 relative to the start of *algD* transcription), little specific binding was detected (Fig. 4B, lanes 1 through 6). Thus, AlgT or a protein under AlgT control is binding to a region of *algD* between −123 and −23. Since AlgT is probably a sigma factor (13, 32) and most full-length sigma factors, in the absence of the core subunits of RNA polymerase, do not bind DNA (58), the mobility shift observed in the experiment shown in Fig. 4B is likely not due to direct AlgT-*algD* interactions. Thus, we favor the hypothesis that a protein under AlgT control is binding to this region. This protein is not AlgB or AlgR, since the binding still occurs when extracts from *algB* and *algR* mutants are used (Fig. 4B, lanes 11, 12, 17, and 18).

We also considered a role for IHF in the above-described gel band mobility shift analyses. IHF from *E. coli* has been shown to bind with low affinity to a region located approximately 80 bp upstream from the start of *algD* transcription (Fig. 4A, boxed region) (37, 53). However, the following data strongly imply that the binding observed in this region is not due to IHF. (i) The mobility of the complexes formed with proteins in *P. aeruginosa* extracts is different from that of those formed with purified IHF (53). (ii) IHF is present in mucoid and nonmucoid *P. aeruginosa* strains (53, 56), yet no binding to these fragments is observed when extracts from the nonmucoid strains FRD2 and FRD440 are tested (Fig. 4B, lanes 9, 10, 15, and 16). (iii) *algD* DNA fragments with mutations in this low-affinity IHF-binding site were recently generated (53, 54). Although these fragments no longer bind purified IHF in vitro, they still retain the ability to form complexes with a protein(s) present in extracts from *P. aeruginosa* FRD1, FRD444, and FRD810 that are identical to the complexes observed in the experiment shown in Fig. 4B with wild-type *algD* sequences (54).

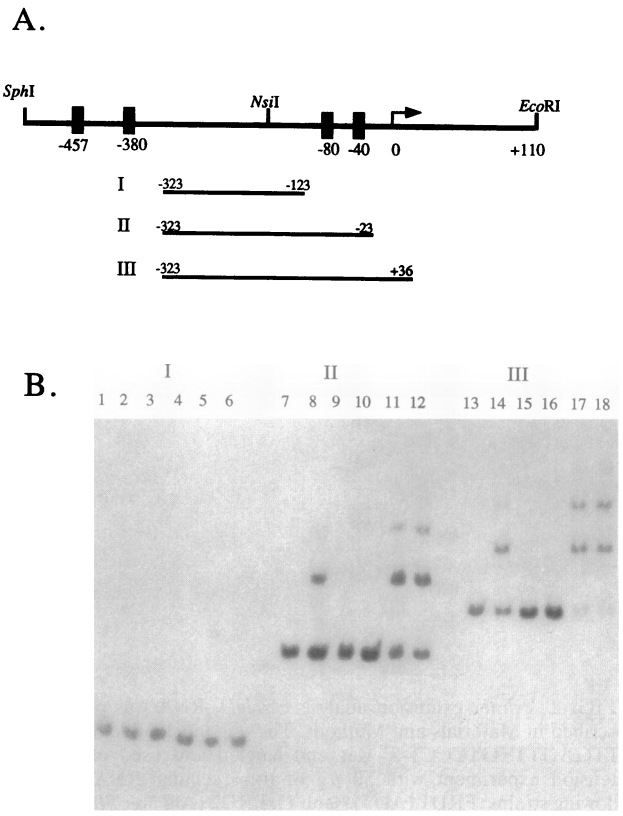

FIG. 4. Analysis of AlgT or a protein under AlgT control binding to *algD* sequences by the gel band mobility shift assay. (A) Partial map of the region upstream of *algD* where PCR fragments I, II, and III (shown below map) were generated. Stippled boxes (−457, −380, and −40) denote AlgR-binding sites (39), and the filled box (−80) represents an IHF-binding site (37, 53). (B) Approximately 1 pmol of PCR-generated fragment I (lanes 1 through 6), fragment II (lanes 7 through 12), or fragment III (lanes 13 through 18) was incubated with 1 μg of cell extracts from the following strains of *P. aeruginosa*: FRD1 (lanes 2, 8, and 14), FRD2 (lanes 3, 9, and 15), FRD440 (lanes 4, 10, and 16), FRD444 (lanes 5, 11, and 17), and FRD810 (lanes 6, 12, and 18). Lanes 1, 7, and 13 contain fragments I, II, and III, respectively, with no extracts added. Complexes were separated from free DNA by electrophoresis in nondenaturing polyacrylamide gels followed by autoradiography as described in Materials and Methods.

Model for a hierarchy of alginate gene expression in *P. aeruginosa*. The biosynthesis of alginate is regulated in a complex manner involving a hierarchy of several genes. As such, it was essential to determine where the regulators fall in this hierarchy. This study employed RNA and gene fusion analysis with a variety of *P. aeruginosa* strains to contribute to the model shown in Fig. 5. Genes *algT* (*algU*), *mucA*, and *algN* (*mucB*) clustered near the 68-min region of the chromosome (13, 16, 19, 21, 30, 32, 41) appear at the top of the hierarchy and are required for spontaneous alginate conversion ($Alg^+ \leftrightarrow Alg^-$). *mucA* and *algN* (*mucB*) appear to have a negative effect on alginate production, since mutations in these genes result in the conversion to mucoidy in both laboratory and clinical *P. aeruginosa* strains (13, 21, 33, 34). The *trans*-acting positive regulatory protein AlgT (AlgU) has homology to σ^E, a global stress response sigma factor (12, 13, 32). AlgT is required for expression of *algB* and *algR*, two genes that

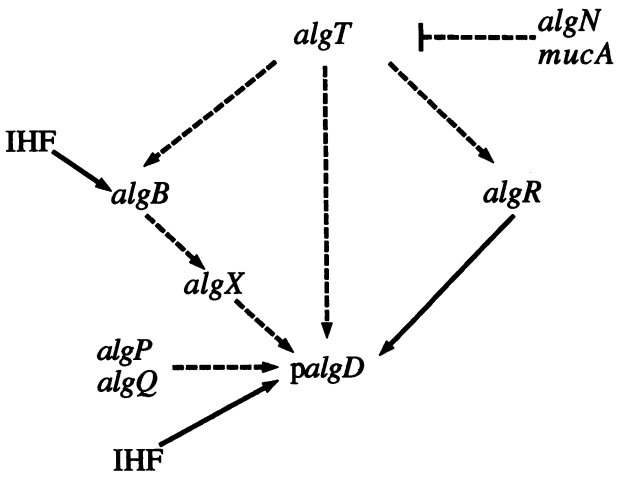

FIG. 5. Proposed model for the hierarchy of alginate gene expression. Dashed lines indicate that the association exists but a direct effect has not been demonstrated. Solid lines demonstrate that a direct association has been shown. Alternative gene designations in use are *algT* (*algU*), *algN* (*mucB*), *algR* (*algR1*), *algP* (*algR3*), and *algQ* (*algR2*).

encode response regulators, and both these genes are necessary for transcription of the *algD* promoter. *algD* is the first gene in a large (~18-kb) operon encoding most of the enzymes necessary for the production and modification of alginate. The histone-like proteins IHF (37, 53) and AlgP (10, 26), as well as AlgQ (9, 25), are also necessary for *algD* transcription. DNA-binding studies indicate that AlgR is directly involved in activating *algD* (24, 38). Although AlgB is necessary for *algD* transcription, there is no evidence for its binding in the *algD* promoter region (54). Thus, there may be another gene (*algX*) in the circuit between *algB* and *algD* (Fig. 5). In addition, *algB* does not appear to be required for *algR* expression, nor is *algR* necessary for transcription of *algB*. Moreover, with the exception of a requirement for AlgT, the AlgB and AlgR pathways appear to be operating independently of each other.

Alginate overproduction apparently provides a selective advantage for *P. aeruginosa* in the CF lung. Our studies provide a closer, but as yet incomplete, understanding of the genetic mechanisms involved in alginate overproduction by *P. aeruginosa* CF isolates. In the future, it will be necessary to determine whether direct interactions occur with each of the components of the hierarchy. Additionally, an understanding of the conditions in nature which trigger alginate biosynthesis is essential.

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