The AlgT-Dependent Transcriptional Regulator AmrZ (AlgZ) Inhibits Flagellum Biosynthesis in Mucoid, Nonmotile Pseudomonas aeruginosa Cystic Fibrosis Isolates

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Pseudomonas aeruginosa is a microorganism associated with the disease cystic fibrosis. While environmental P. aeruginosa strains are generally nonmucoid and motile, isolates recovered from the cystic fibrosis lung frequently display a mucoid, nonmotile phenotype. This phenotypic conversion is mediated by the alternative sigma factor AlgT. Previous work has shown that repression of fleQ by AlgT accounts for the loss of flagellum biosynthesis in these strains. Here, we elucidate the mechanism involved in the AlgT-mediated control of fleQ. ELECTROPHORETIC mobility shift assays using purified AlgT and extracts derived from isogenic AlgT+ and AlgT− strains revealed that AlgT inhibits fleQ indirectly. We observed that the AlgT-dependent transcriptional regulator AmrZ interacts directly with the fleQ promoter. To determine whether AmrZ functions as a repressor of fleQ, we mutated amrZ in the mucoid, nonmotile P. aeruginosa strain FRD1. Unlike the parental strain, the amrZ mutant was nonmucoid and motile. Complementation of the mutant with amrZ restored the mucoid, nonmotile phenotype. Thus, our data show that AlgT inhibits flagellum biosynthesis in mucoid, nonmotile P. aeruginosa cystic fibrosis isolates by promoting expression of AmrZ, which subsequently represses fleQ. Since fleQ directly or indirectly controls the expression of almost all flagellar genes, its repression ultimately leads to the loss of flagellum biosynthesis.

Several gram-negative bacterial species show evidence of a reciprocal regulation of flagellum expression and exopolysaccharide synthesis. It has been suggested that this mechanism enables microbes to optimize their interaction with their prospective hosts or with particular niches in the environment. In Vibrio cholerae, for instance, epsD and epsE are involved in the coordinate control of flagellum and exopolysaccharide expression during the formation of biofilms (31). Disruption of the flagellar regulatory genes fltA and flrC in this microorganism results not only in the loss of flagellum expression but also in the induction of exopolysaccharide synthesis (1, 2). Recently, it has also been suggested that the sodium-driven flagellar motor may play a role in controlling the expression of exopolysaccharide in this microorganism (19). In Escherichia coli, an increase in the biosynthesis of colanic acid exopolysaccharide is evident upon repression of flagellum synthesis (24). In the symbiont Sinorhizobium meliloti, the ExoR protein and the ExoS/ChvI two-component system have been shown to control both succinoglycan and flagellum synthesis (35). The inverse regulation of flagellum biosynthesis and exopolysaccharide expression has also been observed in mucoid, nonmotile Pseudomonas aeruginosa cystic fibrosis (CF) isolates and is mediated by the alternative sigma factor AlgT (AlgU, σU) (11, 28).

The function of AlgT in the regulation of alginate synthesis has been well documented (12, 27). In nonmucoid P. aeruginosa isolates, the activity of AlgT is negligible due to the suppressive effect of the anti-sigma factor MucA (12, 34). However, in the majority of mucoid CF isolates, mutations in mucA result in a nonfunctional protein, which ultimately leads to a deregulation of AlgT (12, 27). Subsequently, AlgT positively controls several intermediate regulatory genes, including algB, algR, and amrZ. Their activities result in the expression of algD, which encodes a GDP-mannose dehydrogenase and ultimately commits the bacterium to the production of alginate (27). One of the mentioned AlgT-dependent intermediates, AmrZ, is a DNA-binding protein of the ribbon-helix-helix family (3) that is homologous to the repressors Mnt and Arc of Salmonella enterica serovar Typhimurium bacteriophage 22 (30). In each of these proteins, the amino terminus consists of a β-sheet involved in recognizing and binding to the DNA (18). Mutation of AmrZ residue K18 or R22, which reside within the proposed β-sheet, results in the loss of DNA binding (4, 26). In addition to its function in alginate production, AmrZ has also been shown to play a role in twitching motility and type IV pilus biosynthesis (4).

In contrast to what is known regarding the function of AlgT in alginate regulation, the AlgT-mediated repression of flagellum biosynthesis remains to be further elucidated. In P. aeruginosa, a four-tiered transcriptional hierarchy tightly controls flagellum synthesis. In this cascade, proper expression of genes belonging to each particular tier/class requires the expression of genes of the previous tier (8). Previously published data indicate that AlgT inhibits flagellum synthesis by repressing the class I gene fleQ (28), which encodes an NtrC-like transcriptional activator (3). The FleQ protein has been referred to as the “master switch” of the flagellar regulatory circuit, as it is required for the expression of all other known flagellar genes with the exception of fliA (8).

The goal of this study was to elucidate the mechanism of the AlgT-mediated repression of fleQ in mucoid, nonmotile P. aeruginosa CF isolates. Biochemical approaches using the mu-
cid, nonmotile reference strain FRD1 (mucA22), as well as clinical mucoid, nonmotile CF isolates carrying mutations in mucA, revealed that AlgT inhibits fleQ by an indirect pathway. AmrZ, an AlgT-dependent regulator required for alginate production (3, 26, 33) and twitching motility/type IV pilus synthesis (4), was identified as the intermediate involved in the repression of fleQ. Electrophoretic mobility shift assays (EMSA) showed that AmrZ specifically binds the fleQ promoter and that this interaction is abolished if critical DNA-binding residues of the protein are mutated, which implied that AmrZ may function as a repressor of fleQ. This hypothesis was supported by results obtained from promoter fusion assays. Western blot analysis, and microscopy, which showed that mutation of amrZ in the mucoid, nonmotile P. aeruginosa CF isolate FRD1 results in increased fleQ promoter activity and restores flagellum expression as well as motility. Our data indicate that AlgT indirectly mediates the negative control of flagellum biosynthesis in mucoid, nonmotile P. aeruginosa CF isolates by increasing the expression of AmrZ. AmrZ subsequently represses the flagellar regulator fleQ, which ultimately results in loss of flagellum production.

**MATERIALS AND METHODS**

Strains, plasmids, oligonucleotides, and DNA manipulations. *Pseudomonas aeruginosa* PAO1, FRD1 (mucA22), FRD440 (mucA22 algT::Tn501), FRD831 (mucA22 algR::lacI), FRD840 (mucA22 algR::lacI), FRD1200 (mucA22 amrZ::zey£ae::lacI), FRD2234 (mucA22 amrZ::Z17; expresses AmrZ K18A) (4), and FRD2238 (mucA22 amrZ::Z19; expresses AmrZ R22A) (26) were used for this study. Other *P. aeruginosa* strains used included the mucoid, nonmotele CF isolates CF1 and CF2, and their isogenic algT mutants (28), as well as a collection of other CF-derived mucoid strains (5, 33). Cell density at 600 nm of 0.5. A 1.0-ml volume of cells was cultured per grid. After 1 min, excess liquid was wicked off without completely drying the grid to avoid flagellar shearing. Grids were washed twice by floating them on ultrapure water. Subsequently, a drop of 2% uranyl acetate was added and wicked off after 1 min. TEM was performed on a Philips TEM 400 operated at 80 kV. For phase-contrast microscopy, bacteria were grown overnight in LBNS. A drop of the suspension was added to a glass microscopy slide (Fisher) and covered with a glass coverslip (Fisher), and flagellar motility was examined at ×100 with a Nikon Eclipse E400 microscope.

**Western blot analysis.** Western blotting was performed with whole-cell lysates. The lysates were prepared from *P. aeruginosa* grown in LBNS to an optical density at 600 nm of 0.5. A 1.0-ml volume of cells was centrifuged for 3 min at 14,000 rpm, and the pellet was resuspended in 100 µl FB (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM MgCl2). A 10-µl volume of each suspension was examined by Western blotting. Western blotting was performed with rabbit anti-flagellin serotype B and anti-AmrZ antiserum as described previously (11, 33). Blots were developed with Kodak Image Station 2000RT.

**RESULTS**

AlgT controls expression of fleQ indirectly. Recent work in our laboratory revealed that AlgT controls *P. aeruginosa* flagellum synthesis by inhibiting expression of the flagellar master regulator fleQ (28). However, the underlying mechanism of the repression of fleQ remains unknown. To test whether fleQ expression is inhibited directly or indirectly, we first examined the fleQ promoter region for the AlgT promoter recognition sequence, GAAC-16/TGCA-TGCA (12), but were unable to detect this consensus. Subsequently, we incubated radiolabeled fleQ promoter DNA either with purified AlgT-His6 or with protein extracts derived from the mucoid, nonmotile AlgT+ CF isolate FRD1 or its isogenic algT mutant and analyzed these binding reactions by EMSA. There were no DNA-protein complexes in lanes containing purified AlgT-His6 or with protein extracts derived from the mucoid, nonmotile AlgT+ CF isolate FRD1 or its isogenic algT mutant and analyzed these binding reactions by EMSA. There were no DNA-protein complexes in lanes containing purified AlgT-His6 (Fig. 1A, lanes 4 to 9), which suggested that AlgT does not directly repress fleQ. The lack of binding observed for purified AlgT is not likely due to the loss of activity upon purification, as the same preparation of AlgT-His6 was used in an in vitro transcription assay and found to be active (unpublished data).

However, a protein-DNA complex was evident for the AlgT+-derived protein extract (Fig. 1A, lane 2). A gradual loss of free DNA was observed as increasing quantities of the AlgT+-derived protein extract were added to fleQ (Fig. 1B, lanes 2 to 9). In contrast, the protein-DNA complex was missing in the lane containing extracts derived from the isogenic
algT mutant (Fig. 1A, lane 3). Thus, the EMSA results suggest that under the conditions employed here, AlgT does not directly interact with fleQ; rather, a gene product under the control of AlgT binds to the fleQ promoter DNA.

To determine whether the observed AlgT-mediated binding activity is conserved among mucoid, nonflagellated \textit{P. aeruginosa} cells, we analyzed extracts from two additional clinical mucoid, nonmotile \textit{CF} isolates and their isogenic \textit{algT} mutants by EMSA. As with the AlgT\textsuperscript{+} reference strain FRD1 (Fig. 1C, lane 2), a protein-DNA complex was observed for each extract derived from AlgT\textsuperscript{+} \textit{P. aeruginosa} (Fig. 1C, lanes 4 and 6) but was absent when extracts from the isogenic \textit{algT} mutants were used (Fig. 1C, lanes 3, 5, and 7).

**AmrZ interacts directly with the fleQ promoter.** Previously published data show that there is an AlgT-mediated inverse regulation of alginate and flagellum expression in mucoid, nonmotile \textit{P. aeruginosa} CF isolates (11, 28). In the alginate regulatory pathway, \textit{algB}, \textit{algR}, and \textit{amrZ} are known AlgT-dependent genes, all of which encode regulators of alginate synthesis. We hypothesized that one or more of these gene products might also be involved in the repression of fleQ and, ultimately, flagellum biosynthesis. To test this hypothesis, radiolabeled
fleQ promoter DNA was incubated with protein extracts isolated from isogenic FRD1-derived AlgT+ strains carrying null mutations either in algB, algR, or amrZ. The binding reactions were subsequently analyzed by EMSA. DNA-protein complexes, which migrated to a position identical to that seen with the extract derived from the parental strain, were observed in both the algB and the algR mutants (Fig. 2A, lanes 4 and 5). This indicated that neither AlgB nor AlgR directly interacts with fleQ. On the other hand, no DNA-protein complex was observed for the amrZ mutant (Fig. 2A, lane 6). To be certain that the observed loss of binding to the fleQ promoter DNA was indeed due to amrZ, another EMSA was performed with protein extract derived from an amrZ mutant which had been complemented with a functional copy of amrZ (Fig. 2B). While no DNA-protein complex was evident for the amrZ mutant (Fig. 2B, lane 3), a DNA-protein complex was observed when extract of the complemented amrZ mutant was used (Fig. 2B, lane 4). This suggested that either AmrZ or an AmrZ-dependent gene product binds to the fleQ promoter.

Baynham et al. (5) showed that recombinant AmrZ derived from overexpression in E. coli specifically binds at algD. Thus, to examine whether AmrZ directly interacts with the fleQ promoter, radiolabeled fleQ promoter DNA was incubated with increasing amounts of recombinant AmrZ (Fig. 2C). A gradual loss of free DNA was observed as increasing quantities of AmrZ were added to fleQ (Fig. 2C, lanes 4 to 9). The position of the AmrZ-fleQ complexes was identical to that observed for the extract derived from the AlgT+ strain (Fig. 2C; compare lane 2 with lanes 7 to 9).

Previous work showed that alanine substitutions of AmrZ residues K18 and R22, which reside in the proposed β-sheet DNA-binding domain, resulted in a loss of DNA binding at algD and amrZ (4, 26). Therefore, we wanted to determine whether these residues are also essential for DNA binding at fleQ. Radiolabeled fleQ promoter DNA was incubated with increasing amounts of recombinant wild-type, K18A, or R22A mutant AmrZ proteins (Fig. 2D). Mutation of either K18 or R22 resulted in a loss of interaction with fleQ DNA (Fig. 2D, lanes 5 to 7 and 8 to 10, respectively). Thus, AmrZ recognizes fleQ DNA in a fashion similar to that seen at algD and amrZ. Together, these data led to the hypothesis that AmrZ is the AlgT-dependent repressor responsible for the inhibition of fleQ expression and, ultimately, flagellum synthesis in the mucoid, nonmotile P. aeruginosa CF isolate FRD1.

AmrZ controls flagellum biosynthesis. To explore whether AmrZ represses fleQ, the fleQ promoter was fused with a promoterless lacZ and the fusion was integrated into the chromosome of the mucoid, nonflagellated AlgT+ P. aeruginosa CF isolate FRD1 and its isogenic amrZ mutant. The data revealed that fleQ promoter activity was approximately fourfold higher in the amrZ mutant than in the parental AlgT+ strain (Fig. 3A). To examine whether the binding of AmrZ to the fleQ promoter (Fig. 2) and the observed reduction in fleQ promoter activity translate into a flagellum phenotype, we analyzed the mucoid, nonmotile AlgT+ CF isolate FRD1 and its isogenic amrZ mutant for flagellin and AmrZ expression. The results showed that the AlgT+ strain expressed AmrZ and lacked flagellin (Fig. 3B, lane 1). In contrast, the amrZ mutant lacked AmrZ but expressed flagellin (Fig. 3B, lane 3). Upon complementation with amrZ, flagellin expression was inhibited and

![Figure 2: AmrZ binds to the fleQ promoter.](image-url)

(A) Mutation of amrZ in AlgT+ P. aeruginosa abolishes fleQ binding activity. Lane 1, free fleQ promoter DNA; lane 2, AlgT+ extract (3 μg); lane 3, AlgT+ extract (3 μg); lane 4, AlgT+ AlgB extract (3 μg); lane 5, AlgT+ AlgR extract (3 μg); lane 6, AlgT+ AmrZ extract (3 μg). (B) Complementation of the amrZ mutant restores binding to fleQ promoter DNA. Lane 1, free fleQ promoter DNA; lane 2, AlgT+ extract (3 μg); lane 3, AlgT+ AmrZ extract (3 μg); lane 4, extract derived from an AlgT+ AmrZ+ strain complemented with amrZ (3 μg). (C) AmrZ binds to the fleQ promoter. Lane 1, free fleQ promoter DNA; lane 2, AlgT+ extract (3 μg); lane 3, AlgT+ AmrZ extract (3 μg); lane 4, extract derived from an AlgT+ AmrZ+ strain complemented with amrZ (3 μg). (D) Mutations of critical residues abolishes the ability of AmrZ to bind to fleQ. Lane 1, free fleQ promoter DNA; lane 2 to 4, increasing amounts of recombinant AmrZ (5, 10, 25, 50, and 100 ng, respectively); lanes 5 to 7, increasing amounts of recombinant AmrZ K18A (20, 40, and 60 ng, respectively); lanes 8 to 10, increasing amounts of recombinant AmrZ R22A (20, 40, and 60 ng, respectively).
AmrZ expression was restored (Fig. 3B, lane 4), which was accompanied by the synthesis of alginate (data not shown). Moreover, TEM revealed that ~95% of the amrZ mutant cells expressed a characteristic single polar flagellum (Fig. 3C, panel II), which was absent in the parental strain (Fig. 3C, panel I). Examination of swimming behavior by phase-contrast microscopy showed that the mucoid, nonmotile AlgT\(^{+}\) strain was nonmotile whereas its isogenic amrZ mutant was motile. Upon complementation of the mutant with amrZ, motility was lost (data not shown). These data provide further evidence that the AlgT-dependent regulatory protein AmrZ plays a role in the direct repression of fleQ in mucoid, nonmotile P. aeruginosa CF isolates and thus in flagellum biosynthesis.

**DISCUSSION**

P. aeruginosa has a host of virulence factors at its disposal to successfully establish and maintain infections; these factors include endotoxin, elastase, alkaline protease, exotoxin A, cytotoxin, hemolysin, type III secretion proteins, type IV pili, flagella, and the exopolysaccharide alginate (12, 29). Previous work in our laboratory revealed that in mucoid, nonmotile P. aeruginosa CF isolates, alginate expression and flagellum biosynthesis are inversely controlled by the alternative sigma factor AlgT (11, 28). AlgT modulates the increased expression of several intermediates, including algB, algR, and amrZ, which result in the expression of the algD operon and thus in alginate production (27). Our lab discovered that in mucoid, nonmotile P. aeruginosa strains, inactivation of algT restores flagellum expression and motility (11, 28). Recent work suggested that AlgT mediates the loss of flagellum synthesis in mucoid, nonmotile CF isolates by repressing the flagellar regulator fleQ (28), which is often referred to as the “master switch” of the flagellar regulon, as fleQ is vital for the expression of almost all other known flagellar genes (2, 8, 17). In this study, we propose a model (Fig. 4) in which AlgT represses fleQ by an indirect mechanism. Under physiological conditions, AlgT activity is controlled by the anti-sigma factor MucA. In the CF lung, mutations in mucA result in a nonfunctional MucA and, subsequently, the derepression of AlgT. AlgT then promotes expression of amrZ, which functions as both a repressor of fleQ and an activator of algD, thus resulting in the loss of flagellum biosynthesis and alginate production, respectively.

Baynham et al. (5) first discovered AmrZ as a DNA-binding activity upstream of the algD promoter. Here, AmrZ binds an A/T-rich sequence centered 282 bp upstream of the transcriptional start site. Mutation of this site abolishes AmrZ binding in vitro and results in an almost complete loss of algD promoter activity (5). Expression of AmrZ is conserved among mucoid P. aeruginosa CF isolates and is absolutely required for both activation of algD transcription and alginate expression (3). Ramsey et al. (26) discovered that AmrZ binds two sites at the algT promoter and functions as an autorepressor. While ribbon-helix-helix proteins usually function as repressors, AmrZ plays a role in both activation and repression of particular target genes. More recent data revealed that AmrZ is also required for twitching motility (4). However, while the data convincingly showed that the DNA-binding activity of AmrZ is required in this process, the AmrZ-dependent genes involved in twitching motility remain to be identified. In the present work, AmrZ is ascribed yet another novel function as a repressor of flagellum synthesis in mucoid, nonmotile P. aeruginosa CF isolates.

Each of the diverse functions of the AmrZ protein described
above requires its ability to bind DNA. AmrZ residues K18 and R22, which reside within the proposed β sheet, are required for DNA binding to sites at algD, amrZ (4, 26), and fleQ (Fig. 2D). Replacement of either of these residues with an alanine results in a complete loss of DNA binding in each case, suggesting that the requirement for particular AmrZ residues in DNA binding is conserved independently of the target DNA. When AmrZ binds to sites at algD (3, 5) or amrZ (26), several protein-DNA complexes are evident, which are likely due to AmrZ oligomerization. Interestingly, only a single protein-DNA complex is present when AmrZ binds to fleQ.

Comparison of the three known AmrZ-binding sites at algD and amrZ resulted in a proposed consensus motif, 5'-gGCCA ttaACCagcc-3', where uppercase letters indicate nucleotides conserved among all three known AmrZ-binding sites and lowercase letters represent nucleotides that are found at only two binding sites (26). We searched the 250-bp fleQ promoter fragment utilized in the DNA binding assays for evidence of the proposed AmrZ consensus motif but found only a minimal match. Therefore, footprinting assays will be necessary to identify the specific AmrZ-binding site(s) at fleQ. It should also be pointed out that currently only three genes are known to be under the direct control of AmrZ. Therefore, identification of additional AmrZ-dependent genes and corresponding binding sites is vital to eventually derive a more concise AmrZ consensus motif.

There are many other gram-negative bacterial species that coordinate regulation of flagellum synthesis and expression of exopolysaccharides. It has been proposed that this mechanism enables microbes to optimize their interaction with prospective hosts or with particular niches in the environment. For example, Cano et al. (6) reported that in Salmonella enterica, the igaA gene encodes a pleiotropic regulator that positively controls the flagellar master operon flhDC and inhibits expression of the colanic acid gene cluster wca. Mutation of igaA activates the two-component system RscB-RscC, which in turn results in the repression of flhDC and a derepression of the wca genes, leading to nonmotile, mucoid S. enterica variants. This is functionally similar to the mechanism involved in the reciprocal control of flagellum synthesis and alginate expression in mucoid, nonmotile P. aeruginosa, as described in this study. Here, AlgT controls expression of AmrZ, which in turn represses the flagellar master regulator fleQ and promotes production of alginate. Therefore, AlgT is the functional equivalent of IgaA, and AmrZ plays a role functionally similar to that of the two-component system RscB-RscC in S. enterica.

It has been suggested that both the mucoid phenotype and the lack of flagella provide P. aeruginosa with a selective advantage in the CF lung (7, 21). The copious amounts of alginate form a barrier that shields the bacteria from some antimicrobials. For instance, Learnt et al. (20) showed that alginate is able to scavenge hypochlorite produced by phagocytic cells, and Pedersen et al. (23) reported that alginate reduces the chemotaxis of polymorphonuclear leukocytes into the CF lung and inhibits activation of the complement system. Moreover, Cobb et al. (7) presented evidence that infection of Calu-3 cells with mucoid P. aeruginosa results in increased expression of genes with antiapoptotic effects in the infected cells. Thus, the presence of alginate not only attenuates host responses but also aids in bacterial circumvention of host defenses. In contrast, flagellin, the major structural subunit of the bacterial flagellum, induces a potent proinflammatory response (7, 9, 15, 16, 25). In fact, flagellin appears to be the major proinflammatory signal of P. aeruginosa (9, 16). Therefore, the ability to shut off flagellum expression may provide P. aeruginosa with yet another way to successfully evade host immune defenses and facilitate its persistence in the CF lung.

While the AlgT-mediated inverse control of flagellum ex-

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**FIG. 4.** Proposed model for AlgT-mediated inverse regulation of flagellum synthesis and alginate production in mucoid, nonmotile P. aeruginosa CF isolates. Under most physiological conditions, the activity of the alternative sigma factor AlgT is inhibited by the anti-sigma factor MucA. Unique environmental conditions in CF airways result in mutations of mucA and, subsequently, deregulation of AlgT. AlgT is now free to up-regulate expression of the ribbon-helix-helix-protein AmrZ, which has dual functions as a repressor of fleQ and an activator of algD.
pression and alginate production seems particularly beneficial to the bacterium in the CF lung, it also provides an interesting target for future therapeutic strategies aimed at controlling chronic P. aeruginosa infections. Today, early aggressive antibiotic treatment is used to delay the onset of chronic P. aeruginosa infection and the appearance of mucoid, nonmotile variants (14). However, once these variants arise, they are generally a poor prognostic indicator for CF patients, as it is impossible to eradicate them (12). Thus, the possibility of being able to reverse the mucoid, nonmotile phenotype to a nonmucoid, motile one would be an appealing therapeutic strategy for successfully managing chronic P. aeruginosa infections in the CF lung. Together with traditional therapeutic approaches, this strategy may therefore yield an improved prognosis for patients suffering from CF.

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