JOURNAL OF BACTERIOLOGY, Dec. 1999, p. 7401–7404 Vol. 181, No. 23
0021-9193/99/$04.00+0

Negative Control of Flagellum Synthesis in *Pseudomonas aeruginosa* Is Modulated by the Alternative Sigma Factor AlgT (AlgU)

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Many respiratory isolates of *Pseudomonas aeruginosa* from cystic fibrosis patients are mucoid (alginate producing) yet lack flagella. It was hypothesized that an alginate regulator inhibits flagellar gene expression. Mutations in *algB*, *algR*, and *algT* resulted in nonmucoid derivatives, yet *algT* mutants expressed flagella. AlgT-dependent control of flagellum synthesis occurred through inhibition of *fliC* but not *rpoN* transcription.

Pseudomonas aeruginosa causes a variety of acute infections, but the organism is also responsible for most of the life-threatening chronic respiratory tract infections in people with cystic fibrosis (CF). Although lungs of CF patients are colonized by motile, nonmucoid *P. aeruginosa* strains, during the course of chronic infection there appears to be a selection for certain phenotypes (8, 16). Among these are rough lipopolysaccharide structure, mucoidy, and loss of motility. The mucoid phenotype is due to the overproduction of the exopolysaccharide alginate, a virulence factor which provides a selective advantage to the bacteria (references 8 and 16 and references therein). Nonmotility is also rare in *P. aeruginosa* except among CF isolates (11, 13). The occurrence of these two phenotypes (alginate and lack of flagella) in many CF isolates prompted us to examine whether alginate and flagellum synthesis were coordinately regulated.

Evidence for coordinate regulation between alginate synthesis and flagellum expression. A collection of mucoid and nonmucoid *P. aeruginosa* CF isolates (3) were cultured on L agar plates and scored for the mucoid phenotype (Luria broth [LB] contained the following [per liter]: 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl; L agar contained 1.5% agar in LB). Flagellum expression was examined by transmission electron microscopy (TEM) and Western blotting using antiserum against flagella which had been purified by published techniques (14) from strain PAK (serotype A) or PAO1 (serotype B). All nonmucoid *P. aeruginosa* strains examined synthesized flagella, whereas all mucoid isolates lacked flagella (data not shown). The results confirm those reported elsewhere (11, 13) and suggest a correlation between alginate synthesis and lack of flagellum expression.

It was unclear if these mucoid CF isolates acquired flagellar gene mutations during selection in the lungs of CF patients. This mechanism has been proposed as an explanation for the high frequency of nonmotile variants in *P. aeruginosa* CF isolates (6, 13). We propose an alternative explanation. Since most mucoid CF isolates acquire mutations in *mucA* resulting in elevated levels of the alternative sigma factor σ^{22} (8), we reasoned that the inhibition of flagellum expression was due to increased expression of σ^{22}. If this was true, inactivation of *algT*, encoding σ^{22}, in a mucoid, nonmotile strain should restore flagellum synthesis. However, if mucoid CF isolates were to acquire a mutation in a flagellar gene(s), *algT* mutants should not be able to express flagella. To distinguish these, isogenic strains FRD1 (*mucA22*) (22) and FRD440 (*mucA22 algT*::Tn*501*) (22) were examined for flagellum expression by Western blotting of whole-cell extracts derived from cells cultured in LB lacking NaCl. While the mucoid strain FRD1 lacked flagella, the *algT* mutant expressed flagella and was motile (Fig. 1, compare lanes 2 and 4). Analysis of the representative $AlgT^+$ strain *P. aeruginosa* FRD875 (*mucA22 algD*::*xylE-aacC1*) (21) by TEM (Fig. 2A), revealed that few, if any, bacteria were expressing a flagellum. However, the isogenic *algT* mutant clearly expressed a flagellum (Fig. 2B). Complementation studies with plasmid pJF15, which contains *algT* (7), revealed FRD440/pJF15 transconjugants were mucoid and lacked flagella (data not shown). This indicates the flagellum synthesis observed in FRD440 is due to the loss of the *algT* gene and not to polar effects on downstream genes.

One potential explanation for the loss of flagellum expression in mucoid strains was that expression of alginate blocked the secretion of flagellin or the assembly of a functional flagellum. This was apparently not the case as a nonmucoid FRD1 derivative with an insertion in the *algD* gene (FRD875) remained nonmotile and lacked a flagellum (Fig. 1, lane 3; Fig. 2A). Taken together, these data suggest an inverse coordinate regulation between alginate synthesis and flagellum production.

Mutations in *algB* or *algR* do not affect flagellum expression. σ^{22} directs the expression of several alginate transcriptional regulators (8, 22). These include *algB* and *algR*, encoding response regulators (8, 12). To determine if the σ^{22}-dependent inhibition of flagellum synthesis was mediated through *algB* or *algR*, whole-cell extracts of isogenic *algB* and *algR* mutants were analyzed for flagellum expression and motility (Fig. 1). The *algB* mutant FRD444 (22) did not express flagella (Fig. 1, lane 5) and was nonmotile. Likewise, two *algR* mutants, FRD810 (22) and FRD831 (12), lacked motility and failed to synthesize flagella (Fig. 1, lanes 6 and 7, respectively). This suggests that the σ^{22}-mediated inhibition of flagellum synthesis does not require the σ^{22}-dependent *algB* or *algR* gene products.

Overexpression of σ^{22} in a motile *P. aeruginosa* isolate inhibits flagellum expression and motility. A prediction from the results above is that overexpression of σ^{22} in a motile *P. aeruginosa* strain should inhibit flagellum synthesis. This was shown to be the case in the experiment whose results are depicted in Fig. 3. The full-length *algT* gene was cloned by PCR amplification of *P. aeruginosa* FRD1 genomic DNA into the expres-

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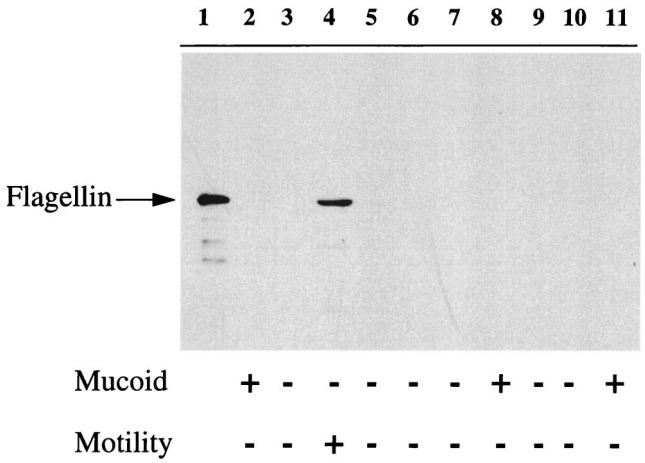

FIG. 1. Western blot analysis of flagellum expression in *P. aeruginosa* strains. Whole-cell extracts were prepared from *P. aeruginosa* strains by culturing cells in 10 ml of LB lacking NaCl at 37°C to an A_{580} of 0.4. The culture was centrifuged (5,000 × *g* for 10 min), and pellets were suspended in 2% of the original culture volume in fractionation buffer (10 mM Tris HCl [pH 8.0], 100 mM NaCl, 1 mM $MgCl_2$). A 10-μl sample of this preparation was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting with anti-flagellum B antibodies. Polyclonal antiserum against flagella was elicited in New Zealand White rabbits (Covance) using flagella (0.75 mg) purified from sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Anti-flagellum antibodies were used in Western blots at a dilution of 1:25,000 with chemiluminescent reagents by procedures outlined by the manufacturer (Amersham), and film was exposed for 30 s prior to development. Lane 1, flagella B (250 ng) purified from PAO1. Lanes 2 through 11 contain extracts derived from strains FRD1 (*mucA22*), FRD875 (*mucA22 algD*::*xylE aacC1*), FRD440 (*mucA22 algT*::Tn*501*), FRD444 (*mucA22 algB*::Tn*501*), FRD810 (*mucA22 algR*::Ω*str*), FRD831 (*mucA22* Δ*algR*::Ω*aacC1*), FRD1230 (*mucA22 fliC*::*xylE aacC1*), FRD1234 (*mucA22 algT*::Tn*501 fliC*::*xylE aacC1*), FRD1240 (*mucA22 algT*::Tn*501 rpoN*::*xylE aacC1*), and FRD1242 (*mucA22 rpoN*::*xylE aacC1*), respectively. The mucoid and motility phenotypes of the strains analyzed in each lane are depicted along the bottom. Motility assays were performed by inoculating a single colony into 0.3% L agar lacking sodium chloride. Following overnight growth at 37°C, motility was assessed qualitatively by examining colonies which spread beyond the point of inoculation (2).

sion vector pMMB503EH (M. Bagdasarian), resulting in pWG21. The primers algT2 (5′-CGGGATCCTCAGGCTTCT CGCAACAAAGG-3′) and algT3 (5′-GGAATTCGAAGAG GAGCTTTCATG-3′) were used for PCR amplification with *Taq* polymerase by previously outlined conditions (3). The *algT* gene in pWG21 was sequenced, and the sequence was found to be identical to that published previously (4). Plasmid pWG21 or pMMB503EH was introduced into the motile strain *P. aeruginosa* WFPA14. WFPA14 was generated by allelic exchange of PAO1 wild-type *algD* with an *algD*::*xylE aacC1* cassette from pDJW530 (21) by previously described techniques (12, 21). WFPA14 was chosen because the *algD*::*xylE* fusion provides a convenient screen for σ^{22} activity. A Western blot of whole-cell extracts demonstrated that expression of σ^{22} in WFPA14 inhibited flagellum expression (Fig. 3, compare lanes 3 and 4). σ^{22} expressed from pWG21 was active, since an increase in *algD*::*xylE* levels was observed (Fig. 3) (XylE assays were determined as described previously [21]). σ^{22}-mediated inhibition of flagellum synthesis was also observed in the parental, motile strain PAO1 as well as in the serotype A strain PAK (data not shown).

The σ^{22}-mediated inhibition of flagellum expression occurs through *fliC* transcription. To determine if σ^{22}-mediated inhibition of flagellum expression occurs through transcription of one or more flagellar genes, strains with *fliC*::*xylE aacC1* or *rpoN*::*xylE aacC1* operon fusions were constructed in *P. aeruginosa* FRD1 (*mucA22*) and FRD440 (*mucA22 algT*::Tn*501*). We chose *rpoN* and *fliC* since these genes represent early and late markers of flagellar gene expression, respectively (2, 20). Both the *rpoN* gene, which encodes the alternative sigma factor, σ^{54}, as well as *fliC*, encoding the flagellar subunit protein flagellin, are essential for flagellum synthesis and motility (6, 20). A portion of *fliC* was obtained by PCR amplification of PAO1 genomic DNA with the primers fliC1 (5′-GCCTGCAG ATCTCCAAC-3′) and fliC2 (5′-GCAGCTGGTTGGCCTG G-3′). The PCR fragment was cloned into pUC18 (23), resulting in pDJW567. The presence of *fliC* in pDJW567 was verified by DNA sequencing. The *fliC* gene of pDJW567 was subcloned into the gene replacement vector pEX100T (9) to generate pWG26. This plasmid was digested with *Age*I, which cleaves within the *fliC* coding sequence, the ends were treated with Klenow fragment, and a 2.2-kb *Sma*I *xylE aacC1* fragment from pX1918 (18) was inserted. The resulting plasmid, pDJW600, was used for generating chromosomal *fliC*::*xylE aacC1* insertions by techniques outlined previously (12, 21). To create *rpoN*::*xylE aacC1* operon fusions, a 10-kb fragment containing *rpoN* was obtained by *Bam*HI digestion of pKI10 (10) and cloned into the gene replacement vector pDJW525 (12). The resulting plasmid, pWG23, was digested with *Nsi*I, which cleaves within *rpoN*, and the 2.4-kb *xylE aacC1 Pst*I cassette from pX1918GT (18) was inserted. The resulting plasmid, pWG24, was used for generating chromosomal *rpoN*::*xylE aacC1* insertions.

Transcription levels of *rpoN* were similar in strains harboring either the wild-type or the *algT*::Tn*501* allele, decreasing by only about 20% from 8.1 to 6.4 U of XylE in the *algT* mutant. By contrast, expression of the *fliC*::*xylE* operon fusion was increased by about 100-fold in the *algT* mutant from 0.24 to 22.4 U of XylE. This suggests that the σ^{22}-dependent control of flagellum synthesis and motility occurs through inhibition of *fliC* transcription. As expected, stains with chromosomal *fliC*::*xylE aacC1* or *rpoN*::*xylE aacC1* insertions did not express any flagella detectable by Western blotting and were nonmotile (Fig. 1, lanes 8 through 11).

Published data (6, 8, 15, 16, 22) as well as data discussed in this paper can be summarized by a working model for explaining the coordinate control of flagellum synthesis with alginate. CF patients are colonized by motile *P. aeruginosa* strains, and there is evidence that flagella play a critical role in the early events of colonization of the lungs of CF patients and abiotic surfaces (2, 6, 15). Following or coincidental with this initial attachment, type IV fimbriae and twitching motility are utilized to form tighter association with the epithelium and to initiate microcolony formation. In the lungs of CF patients, impaired activity of mucociliary clearance, decreased defensin activity, decreased bacterial uptake and desquamation, and increased expression of ganglioside surface receptors for *P. aeruginosa* favor progression of the infection. Concurrently with these processes airway inflammation occurs. Because of this, and the fact that they are convenient ligands for phagocytic cells, flagella become detrimental to *P. aeruginosa*. In CF, a strong selection is imposed on the organisms and *mucA* mutants predominate. Loss of the anti-sigma factor MucA function results in increased expression of the AlgT (σ^{22}) regulon. The *algT* gene modulates a hierarchy of gene expression, leading to expression of the alginate operon. Induction of the σ^{22} regulon also appears to inhibit flagellum synthesis and motility.

The negative control of flagellum expression by σ^{22} could theoretically occur at any point in the flagellar hierarchy which includes the alternative sigma factors *rpoN* and *fliA* as well as several positive transcriptional regulators (1, 17, 19, 20). The σ^{22}-mediated inhibition occurs through transcriptional control of *fliC* but is independent of *rpoN*. Since most sigma factors lack the ability to bind DNA without being complexed with

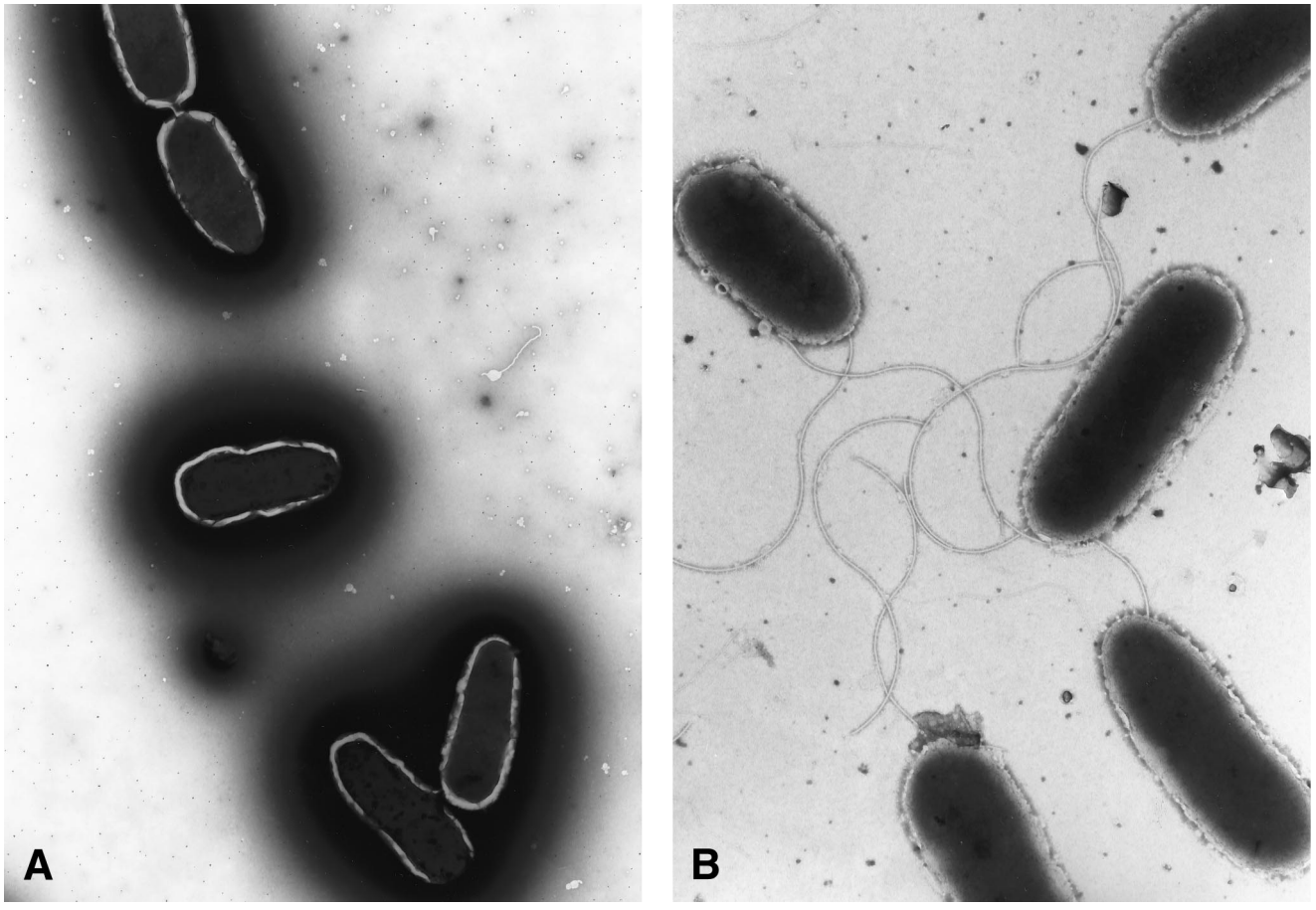

FIG. 2. TEM of *P. aeruginosa* strains. Magnification, ×18,090. (A) FRD875 (AlgT$^+$*mucA22 algD*::*xylE aacC1*); (B) FRD440 (*mucA22 algT*::Tn*501*). TEM was performed by scraping individual colonies from plates cultured overnight and resuspending the samples in 15 μl of LB containing alpha lactalbumin carrier protein (500 μg/ml). Glow-discharged, Formvar-coated copper mesh grids were floated on this suspension for 2 min. The excess suspension was wicked off, and the grid was floated on a drop of 2% phosphotungstic acid for 1 min and wicked dry. TEM was performed on a Philips TEM 400 operated at 80 kV.

core RNA polymerase (5), it is unlikely that σ^{22} directly represses flagellar gene expression. Instead, σ^{22} probably controls the expression of a negative effector of flagellum synthesis. Understanding the specific point in the flagellar genetic pathway in which σ^{22}-dependent inhibition occurs is a focus of future studies. These experiments will provide insights into the pathogenesis of *P. aeruginosa* in chronic lung infections of CF patients and yield information regarding the natural role of the σ^{22} regulon in *P. aeruginosa*.

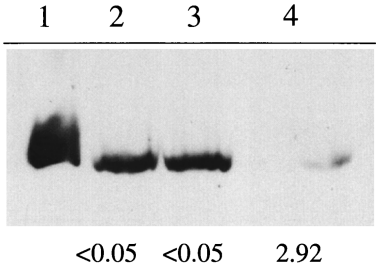

FIG. 3. Expression of σ^{22} in the nonmucoid, motile isolate PAO1 results in the inhibition of flagellum expression. Whole-cell extracts of PAO1-derived cells were analyzed on a Western blot probed with anti-flagellum B antibodies. Lane 1, a sample of purified flagella B (250 ng). Lanes 2 through 4 contain whole-cell extracts derived from strains WFPA14 (*algD*::*xylE aacC1*), WFPA14/pMMB503EH (vector), and WFPA14/pWG21 (*algT*), respectively. The *algD*::*xylE* activities (nanomoles of 2-hydroxymuconic semialdehyde/min/A_{540} of culture) of strains analyzed are indicated at the bottom of each lane.

This work was supported by Public Health Service grant 5R01 HL58334 (D.J.W.) from the National Heart, Lung, and Blood Institute.

We are grateful to S. Lory and N. Baker for advice and for providing antiserum and strains. Assistance in TEM was provided by K. Grant and G. Jerome of the Micromed electron microscopy facility in the Department of Pathology at WFUBMC. We are also indebted to G. O'Toole, C. Whitchurch, and J. Mattick for helpful discussions. The DNA Synthesis Core Laboratory of the Cancer Center of Wake Forest University provided oligonucleotides. E. Jung of the DNA Sequencing Core Lab performed DNA sequencing. Both facilities are supported in part by NIH grant CA-12197.

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