The *Pseudomonas aeruginosa* Ribbon-Helix-Helix DNA-Binding Protein AlgZ (AmrZ) Controls Twitching Motility and Biogenesis of Type IV Pili

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Pseudomonas aeruginosa is an opportunistic pathogen that is commonly found in water and soil. In order to colonize surfaces with low water content, *P. aeruginosa* utilizes a flagellum-independent form of locomotion called twitching motility, which is dependent upon the extension and retraction of type IV pili. This study demonstrates that AlgZ, previously identified as a DNA-binding protein absolutely required for transcription of the alginate biosynthetic operon, is required for twitching motility. AlgZ may be required for the biogenesis or function of type IV pili in twitching motility. Transmission electron microscopy analysis of an *algZ* deletion in nonmucoid PAO1 failed to detect surface pili. To examine expression and localization of PilA (the major pilin subunit), whole-cell extracts and cell surface pilin preparations were analyzed by Western blotting. While the PilA levels present in whole-cell extracts were similar for wild-type *P. aeruginosa* and *P. aeruginosa* with the *algZ* deletion, the amount of PilA on the surface of the cells was drastically reduced in the *algZ* mutant. Analysis of *algZ* and *algD* mutants indicates that the DNA-binding activity of AlgZ is essential for the regulation of twitching motility and that this is independent of the role of AlgZ in alginate expression. These data show that AlgZ DNA-binding activity is required for twitching motility independently of its role in alginate production and that this involves the surface localization of type IV pili. Given this new role in twitching motility, we propose that *algZ* (PA3385) be designated *amrZ* (alginate and motility regulator Z).

Pseudomonas aeruginosa is a formidable opportunistic pathogen causing deadly infections in individuals who are burn patients, undergoing cancer chemotherapy, or have the genetic disease cystic fibrosis (CF) (27). Initial colonizing strains in the CF lung have a nonmucoid phenotype, but gradually mucoid strains emerge (16). This mucoid phenotype is due to the overproduction of the exopolysaccharide alginate (14). This trait provides adherence properties and protects bacteria from the immune system, allowing *P. aeruginosa* to chronically infect the lung. For CF patients, chronic lung colonization by mucoid strains of *P. aeruginosa* leads to a potent inflammatory response and tissue destruction, with the eventual result in the vast majority of patients being respiratory failure and death (17).

Most of the biosynthetic genes required for alginate production are located in a large operon. Activation of *algD*, the first gene in this operon, is absolutely required for alginate production, and its expression is tightly controlled (10, 49). A number of genes have been shown to mediate control of *algD* transcription, including the alternative sigma factor AlgT (AlgU, σ^E) that is part of a family of sigma factors that regulate extracytoplasmic functions (13, 28). The *mucABCD* genes adjacent to *algT* negatively regulate the activity of AlgT. Mutations in these genes result in an active AlgT and subsequent transcription of genes, resulting in mucoidy (3, 15, 29). Regulators of *algD* that have been shown to be under AlgT control include

the response regulator proteins AlgB and AlgR and the ribbon-helix-helix (RHH) DNA-binding protein AlgZ (GenBank accession number AF139988 and *Pseudomonas aeruginosa* Genome Database [http://www.pseudomonas.com/current_annotation.asp] designation PA3385) (2, 49, 50). AlgR and AlgZ regulate *algD* by directly binding to sequences far upstream of the promoter. Each of these regulatory genes is required, but none appear to be sufficient for expression of the mucoid phenotype (1, 49).

In addition to alginate production, another important *P. aeruginosa* virulence factor is type IV pili (TFP). These polar appendages are responsible for attachment to epithelia, biofilm development (likely including DNA binding), and twitching motility (TM) (30, 38). TM allows *P. aeruginosa* to travel across solid surfaces via the extension and retraction of TFP (4). More than 40 genes have been identified as involved in TFP function or the regulation of TM. A loss of the TFP complex or the ability to extend and retract TFP results in cells that are avirulent in cytotoxicity with murine models of infection (9, 30).

Our laboratories have focused on the study of the proposed RHH DNA-binding protein AlgZ (1). Other members of this family, such as Mnt and Arc of *Salmonella enterica* serovar Typhimurium phage P22 (8, 39, 40) and NikR (8) of *Escherichia coli*, are repressors, while AlgZ was identified as a transcriptional activator (2). Recently, it has been found that AlgZ binds its own promoter and also represses transcription (31). Our search of the literature has not identified any other RHH protein that has been identified as both an activator and a repressor in vivo. All previous studies of AlgZ have been

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence	Source or reference
Strains		
<i>E. coli</i>		
JM109	e14 ⁻ <i>mcrA recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1 Δ(lac-proAB)</i> [F' <i>traD36 proAB lacI^qZΔM15</i>]	Promega
SM10	F ⁻ <i>supE44 thi-1 thr-1 leuB6 lacY1 tonA21 recA::RP4-2-Tc::Mu</i>	19
<i>P. aeruginosa</i>		
PAO1	Nonmucoid <i>P. aeruginosa</i> strain O1	D. J. Wozniak
WFPA1	<i>algD::tet</i> in PAO1	45
WFPA12	<i>algR::aacI</i> in PAO1	45
WFPA203	PAO1 <i>amrZΔ::tet attB::arabinose-inducible amrZ</i>	This study
WFPA205	PAO1 <i>amrZΔ::tet</i>	This study
AWO	<i>ΔpilA</i> in PAO1	C. Whitchurch
WFPA510	Wild-type <i>amrZ</i> replaced in WFPA205	This study
WFPA511	<i>amrZ</i> K18A in PAO1	This study
WFPA512	<i>amrZ</i> V20A in PAO1	This study
WFPA513	<i>amrZ</i> R22A in PAO1	This study
Plasmids		
pJF2	Mini-CTX with pBAD30-based sequence and wild-type <i>amrZ</i>	This study
pAB3	pEX18Ap <i>ΔamrZ::tet</i> with flanking sequence to construct deletion	31
pSW196	pBAD30-based vector; EcoRI, PstI, SmaI, NotI, and SacI cloning sites	This study
PDJW586	Wild-type <i>amrZ</i> in pEX18Ap for use in gene replacement	31
pPJ155	<i>amrZ</i> K18A in pEX18Ap for use in gene replacement	This study
pPJ156	<i>amrZ</i> V20A in pEX18Ap for use in gene replacement	This study
pPJ157	<i>amrZ</i> R22A in pEX18Ap for use in gene replacement	31
pEX18Ap	Shuttle vector based on pUC18	42
pEX100T	Gene replacement vector	33
Primers		
pET1	5'-CAATTCCTCTAGAAAGAAATTCTGTTTAACTTTAAGAAGG-3'	This study
pET2	5'-GGGCTTTGTTAGCAGCCGG-3'	This study
<i>amrZ</i> 14	5'-AACGACCGGTGGTCAGAAGG-3'	31
<i>amrZ</i> 22	5'-GTTACTCGCCATGGTTTGG-3'	This study
<i>amrZ</i> 19	5'-CAAATTCGTCGTTGCTCTGCCCCGAGGG-3'	31
<i>amrZ</i> 18	5'-CTGACAAATTCGCCGTTCTGCTG-3'	This study
<i>amrZ</i> 17	5'-AGCCGTACCGCTGACGCATTCGTCGTTCTGCTG-3'	This study

undertaken with mucoid *P. aeruginosa* strains. In the present work, we examined the potential role of AlgZ in the control of genes in the nonmucoid strain PAO1. We discovered that PAO1-derived *algZ* mutants were deficient in TM. The role of *algZ* in TM was shown to require its DNA-binding activity but was independent of its role in *algD* activation. Transmission electron microscopy and antipilin Western blot analysis of surface and whole-cell preparations suggest that *algZ* is required for proper assembly of surface-exposed TFP but not for expression of the major subunit *pilA*. Because of its dual role in controlling both alginate production and TFP-mediated TM, we propose that *algZ* (GenBank accession numbers AF139988 and PA3385) be renamed *amrZ* (alginate and motility regulator Z).

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides. All *P. aeruginosa* strains used in this study originated from PAO1 and are listed in Table 1. WFPA203 was constructed by inserting an arabinose-inducible *amrZ* at the neutral *attB* site with pJF2, a mini-CTX-based plasmid (19), whose construction is described below. Subsequently, pAB3 was used to replace the wild-type *amrZ* with an omega tetracycline cassette as described previously (31). Plasmid pJF2 was constructed by cloning the product of a PCR amplification with template pPJ145, which was previously described (50), and primers pET1 and pET2 into the EcoRI-SmaI site

of pSW196. This plasmid was then mobilized into PAO1 via *E. coli* strain SM10. The plasmid was integrated at the chromosomal *attB* site via the plasmid-encoded site-specific integrase (19). The cloning site in this vector is flanked by *Saccharomyces cerevisiae* Flp recombinase target sites. These sites facilitated in vivo excision of the plasmid, including the antibiotic resistance marker, via the Flp recombinase that was supplied on a different plasmid (pFLP2) via conjugation. This plasmid was cured via the plasmid-encoded *sacB* counterselectable marker (19). The pPJ145 template used contains the wild-type *amrZ* and has been previously described (50). pSW196 contains the promoter from pBAD30 (18) and a small multiple cloning site (EcoRI, PstI, SmaI, NotI, and SacI) cloned into mini-CTX (19). Briefly, pSW161, which has been previously described (46), was cut with EcoRI and HindIII, and a corresponding fragment containing *algT* was inserted (pSW186); this was then cleaved with EcoRI to remove the small polylinker with a KpnI site (pSW192). The KpnI-HindIII fragment containing *algT* with the polylinker and pBAD sequences was then cloned into the corresponding sites of mini-CTX (19) (pSW195), and the *algT* sequences were removed by cleavage with EcoRI and re-ligation (pSW196).

The PAO1 strains with mutations in the putative *amrZ* DNA-binding domain were constructed starting with a PAO1 *ΔamrZ::tet* strain (WFPA205). WFPA205 was constructed using pAB3 to replace the wild-type *amrZ* with an omega tetracycline cassette as described previously (31). Isogenic strains with the indicated mutations were constructed by allelic exchange of the omega tetracycline cassette with the designated *amrZ* allele. The alleles were constructed using site-directed mutagenesis (Promega Altered Sites) with the indicated oligonucleotides. Each mutant oligonucleotide was used with the plasmid pPJ136 (the 1.8-kb BamHI-HindIII fragment from PDJW585 containing *amrZ* in pAlter). A

chromosomal R22A mutation was introduced as described previously (31). Briefly, pPJ136 and mutagenic oligonucleotide *amrZ19* resulted in pPJ150 (*amrZ* R22A). The BamHI-HindIII fragment of this plasmid was cloned into pEX18Ap, resulting in pPJ157 (31). With the mutagenic oligonucleotide *amrZ18*, pPJ149 (*amrZ* V20A) was constructed and the BamHI-HindIII fragment was subcloned into pEX18Ap, resulting in pPJ156. With mutagenic oligonucleotide *amrZ17*, pPJ148 (*amrZ* K18A) was constructed and the BamHI-HindIII fragment was then cloned into pEX18Ap to produce pPJ155. The wild-type allele (pDJW585) and each construct (pPJ155, pPJ156, and pPJ157) were substituted for the Δ *amrZ::Uet* allele of WFA205. This resulted in strains WFA510 (complemented strain, *amrZ* Δ *amrZ*⁺), WFA511 (*amrZ* K18A), WFA512 (*amrZ* V20A), and WFA513 (*amrZ* R22A), respectively.

Site-directed mutagenesis of pPJ136 was used with mutant oligonucleotide *amrZ17* to generate the *amrZ17* allele (K18A, pPJ148). In the same manner, mutant oligonucleotide *amrZ18* was used to generate the *amrZ18* allele (V20A, pPJ149). The *amrZ19* allele (R22A, pPJ150) was constructed as previously described (31). To generate six-His-tagged alanine mutants of AmrZ, the mutant alleles were cloned into the BamHI-EcoRI sites of pTrcHisA (Invitrogen) by using primers *amrZ26* and *amrZ27* and template pPJ148, pPJ149, or pPJ150, resulting in pHL20 (AmrZ K18A), pHL21 (AmrZ V20A), and pHL22 (AmrZ R22A), respectively. Wild-type six-His-tagged AmrZ was constructed as previously described (31).

Cleared extracts derived from *E. coli* JM109/pDR2 and alanine substitution mutants were prepared as previously described (31). In brief, the cleared extract was added to a slurry containing Ni-nitrilotriacetic acid magnetic agarose beads (QIAGEN) and binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0), and the sample was allowed to mix at 4°C for 1 hour. Residual proteins were removed with two wash steps, and the pure protein was eluted from the beads by the addition of binding buffer containing 1 M imidazole and 0.005% Tween. Protein expression and purification steps were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue (Fisher).

DNA-binding assays and conditions are similar to those previously reported (2, 31). In brief, increasing amounts of purified wild-type N-terminal six-His AmrZ or alanine substitution mutants (AmrZ K18A, AmrZ V20A, and AmrZ R22A) were incubated in a DNA-binding assay with an end-labeled *algD* fragment amplified from template pDJW221 by using primers *algD5* and *algD7* (1, 48). All DNA-binding assays were performed at 25°C for 10 min in a reaction volume of 10 μ l and processed as previously described (2). Gels were dried at 80°C for 20 min and exposed to a phosphorimaging screen for variable amounts of time.

Media and chemicals. All *P. aeruginosa* strains were cultured in LBNS (10 g liter⁻¹ tryptone and 5 g liter⁻¹ yeast extract) or on LANS (LBNS with 15 g liter⁻¹ agar). *E. coli* strains were grown in LB (10 g liter⁻¹ tryptone, 5 g liter⁻¹ yeast extract, 5 g liter⁻¹ sodium chloride) or on LA (LB with 15 g liter⁻¹ agar). Incubations were carried out at 37°C. Antibiotic concentrations (when necessary) for culture of *E. coli* were as follows: for tetracycline, 15 μ g/ml; for ampicillin, 100 μ g/ml; and for gentamicin, 15 μ g/ml. For *P. aeruginosa*, antibiotics were used at the following concentrations: tetracycline, 100 μ g/ml; carbenicillin, 300 μ g/ml; and gentamicin, 100 μ g/ml. The medium for culturing of *P. aeruginosa* strains after mating was LANS supplemented with Irgasan (25 μ g/ml). Sucrose counterselection via *sacB* was performed at 30°C with 5% sucrose in LANS. All chemicals were obtained from Sigma unless otherwise stated.

Twitching motility assays. Twitching motility was assayed by stab inoculating strains through a thin 1% LANS plate containing 0.1% tetrazolium red (with 2% arabinose supplementation where indicated) with 24 to 48 h of incubation at 37°C under humidified conditions. Twitching motility zones were visualized at the agar plate interface (43).

Transmission electron microscopy (TEM). Bacterial strains were inoculated onto LANS plates and placed at 37°C overnight. After 12 h, the plates were flooded with 3 ml of LBNS and the colonies were gently suspended. From each plate, 1.0 ml was collected and placed into microfuge tubes. Copper grids were treated with 100% ethyl alcohol for 1 minute. A fresh suspension of each strain was placed on the corresponding grid, barely covering the darker side of the grid. After 1 minute, the edge of the grid was wicked with filter paper. The treated surface of the grid was washed with cold double-distilled water three times. Upon washing, the excess of moisture was wicked from the grid. A drop of 2% uracyl acetate was placed on the grid for 1 minute to negatively stain the sample. The grid was dried with filter paper from the edge. Visualization up to $\times 25,000$ was made via a Philips TEM 400 (Micromed, Wake Forest University School of Medicine). The images are of a representative bacterium that corresponded to 90 to 95% of those observed for each strain analyzed.

Western blot assays. Whole-cell PilA preparations for Western blotting were prepared by resuspending bacterial cultures from plates in LBNS to an optical density of 1.0 at 600 nm. Samples (1 ml) were centrifuged, and the pellets were resuspended in 50 μ l of 1 $\times$ loading buffer (0.25 M Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 0.0125% bromophenol blue, 5% 2-mercaptoethanol). Chromosomal DNA was sheared by drawing samples through a 27.5-G needle approximately 10 to 20 times or until the solution was no longer viscous. The samples were boiled for 4 min, resolved in a 4 to 20% precast gel (Bio-Rad Laboratories) via sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred electrophoretically using a semidry transfer apparatus (Bio-Rad) to Immobilon-P polyvinylidene difluoride membranes (Millipore). PilA was detected with anti-PilA antibody (a gift of Randall Irving) used at a concentration of 1:5,000 in Tris-buffered saline (20 mM Tris base, 137 mM NaCl, pH 7.6) with 0.1% Tween 20, followed by donkey anti-rabbit immunoglobulin G, horseradish peroxidase-linked antibody (Amersham Biosciences). Chemiluminescent detection reagents (Amersham Biosciences) were used according to the manufacturer's procedure, and CL-XPosure Clear-Blue X-ray film (Pierce Biotechnology) was exposed to the blots and developed. Fimbrial-surface preparations for Western blotting were prepared as done previously (45) by scraping whole plates of bacteria into 1.5 ml phosphate-buffered saline (8 g liter⁻¹ NaCl, 1.16 g liter⁻¹ Na₂HPO₄, 0.2 g liter⁻¹ KH₂PO₄, 0.2 g liter⁻¹ KCl, pH 7.0). Samples were vortexed to resuspend bacteria and to shear the fimbriae from the cell surface. The cells were centrifuged twice, and the supernatants were transferred to fresh 1.5-ml microfuge tubes. MgCl₂ (1 M, 150 μ l) was added to precipitate the fimbriae, and the tubes were vortexed briefly and placed at 4°C overnight. The TFP were pelleted by centrifugation and resuspended in 100 μ l of 1 $\times$ loading buffer. Samples were resolved and detected as described above. When included, flagellin-specific antibody was provided at a 1:20,000 dilution. Loading control gels were stained with Gelcode Blue (Pierce) as instructed by the manufacturer.

RESULTS

AmrZ (AlgZ) is required for twitching motility, and this role does not involve alginate production. While AmrZ (previously referred to as AlgZ, encoded by *algZ* [PA3385]) was identified as a DNA-binding protein required for the production of alginate in mucoid *P. aeruginosa* isolates (2), it was unclear what role this protein may play in nonmucoid strains, which are the predominant phenotype found in the environment and in acute-infection isolates. An *amrZ* deletion was constructed in the nonmucoid strain PAO1, and this resulted in a domed-colony morphology lacking a rough "ground glass" edge. This phenotype was suggestive of a defect in TM (35). Complementation was accomplished by placing an arabinose-inducible *amrZ* at the neutral *attB* site. This resulted in the wild-type spreading, rough-edged colony type of the parental PAO1 strain upon arabinose induction (data not shown).

TM assays were performed in order to more precisely analyze the TM phenotype of these strains (Fig. 1A). This involved stab inoculation of a 1% agar plate and visualization of TFP-mediated growth at the agar plate interface by utilizing tetrazolium red. In the absence of arabinose, only PAO1 (Fig. 1A) demonstrates a halo of bacterial motility at the agar plate interface. Both the *amrZ* deletion strain (WFA205) and the deletion strain containing an inducible *amrZ* (WFA203) are TM deficient. However, in the presence of arabinose, both PAO1 and WFA203 demonstrate a TM⁺ phenotype (Fig. 1B), as WFA203 is complemented by the arabinose-inducible *amrZ*. These data indicate a previously unrecognized role for *amrZ* in the control of TM. This was not due to a growth defect, since the *amrZ* deletion and parental PAO1 strain have similar growth curves under rich-medium growth conditions (data not shown). Due to its function as a regulator of at least two distinct surface

FIG. 1. TM analysis of *amrZ* and *algD* strains. A thin 1% agar plate containing LA with no sodium chloride and tetrazolium red without (A) or with (B) 2% arabinose supplementation was stab inoculated to the bottom of the plate and incubated for 24 to 48 h at 37°C. The central growth of the colony on top of the agar is seen in all cases, while TM is shown as a lighter circle surrounding the colony. The following strains were analyzed: strain 1, WFP205 (*amrZ* deletion); strain 2, PAO1 (wild type, TM⁺); strain 3, WFP203 (*amrZ* deletion with an arabinose-inducible *amrZ* at *attB*). (C) TM analysis of *algD* and *amrZ* strains performed as described above. The following strains were analyzed: strain 1, WFP1 (*algD* deletion); strain 2, WFP205 (*amrZ* deletion).

components in *P. aeruginosa*, the *algZ* gene is now given the designation *amrZ* (alginate and motility regulator Z).

Since *amrZ* is required both for the production of alginate and for TM, the simplest explanation would be that alginate may be required for TM. Perhaps this exopolysaccharide aids in TM by decreasing friction and allowing the bacteria to move more easily. In order to address this possibility, an isogenic strain (WFP1) in which *algD* was deleted was constructed and the TM phenotypes of the *amrZ* and *algD* deletion strains were examined (Fig. 1C). WFP1 displays a TM⁺ phenotype, compared with WFP205 (*amrZ* deletion) (Fig. 1C), which is TM⁻. This indicates that alginate is not required for TM and that the role AmrZ plays in TM is independent of *algD* activation.

TEM reveals that the *amrZ* deletion strain produces no detectable surface pili. Previously, Bradley found that many TM mutants lacked functional TFP and were either apiliated or hyperpiliated (4). In order to determine the phenotype of the *amrZ* deletion strain with regard to production of pili, the surfaces of the various bacterial strains were analyzed via TEM. Upon examination of numerous negatively stained cells, no TFP were visualized on the surface of bacteria harboring a

FIG. 2. TEM analysis of *P. aeruginosa* strains. Bacteria were harvested from plates cultured overnight, negatively stained using 2% uracyl acetate, and visualized using a Philips TEM 400. Strains analyzed were (A) PAO1 (wild type), (B) AWO (*pilA* deletion), and (C) WFP205 (*amrZ* deletion).

deletion in *pilA* or *amrZ*, while the wild-type PAO1 strain clearly possessed these appendages (Fig. 2). These data suggest that *amrZ* is required for *pilA* expression, TFP assembly, or TFP export.

FIG. 3. Analysis of PilA production and localization via Western blotting. Whole-cell extracts (A) with identical samples in a stained-gel control (C) and sheared-surface TFP (B) were visualized via Western blotting with enhanced chemiluminescence (ECL) detection. The primary antipilin antibody was a gift from Randall Irvin and was used at a 1:5,000 dilution. Antiflagellin antibody at 1:20,000 was used as a loading control for the surface samples (D) with detection via ECL reagents. The following strains were analyzed: strain 1, PAO1 (wild type); strain 2, AWO (*pilA* deletion); strain 3, WFP205 (*amrZ* deletion); strain 4, WFP203 (*amrZ* deletion with an arabinose-inducible *amrZ* at *attB*) without arabinose supplementation; strain 5, WFP203 with 3% arabinose supplementation.

AmrZ is required for proper surface localization of TFP but not for *pilA* expression. In order to determine whether *amrZ* plays a role in *pilA* expression, we prepared whole-cell extracts of PAO1, AWO (*pilA* deletion), WFP205 (*amrZ* deletion), and WFP203 (*amrZ* deletion with an arabinose-inducible *amrZ* at *attB*) both with and without arabinose supplementation and analyzed these via Western blotting using antipilin antibodies (Fig. 3A). Bacteria were harvested from plates, and the numbers of cells used were normalized by dilution of the samples to the same optical density at 600 nm. A stained gel of the samples indicates that similar amounts of total protein were in each lane (Fig. 3C). The amounts and masses of PilA present in the wild-type PAO1 strain and the *amrZ* deletion strains are similar, while PilA is not detectable in the *pilA* deletion strain AWO (Fig. 3A, compare lanes 1, 3, and 4 to lane 2). This indicates that *amrZ* is not required for *pilA* expression or PilA maturation. Since the amount of PilA in the *amrZ* deletions was similar to that of the wild type, we wanted to determine if the TM defect was due to PilA export or TFP assembly. To address this, we made fimbrial-surface preparations of the above strains and subjected these to Western blot analysis (Fig. 3B). In order to confirm that similar amounts of the preparations were loaded into each lane, antiflagellin antibody was used in addition to anti-PilA antibody in probing the blots of the surface samples. The amount of flagellin detected in each lane is similar, indicating that comparable numbers of cells were analyzed (Fig. 3D). Compared to the wild-type strain, which possesses copious amounts of surface PilA, the *amrZ* deletions show extremely low levels (Fig. 3B, compare lane 1 with lanes 3 and 4). When arabinose is supplied in

order to induce *amrZ* in the WFP203 strain (*amrZ* deletion with an arabinose-inducible *amrZ* at *attB*), the result is wild-type amounts of surface PilA (Fig. 3B, lane 5). This confirms the TEM data and demonstrates that *amrZ* is required for proper localization of wild-type amounts of TFP.

AmrZ DNA binding is required for TM. Since AmrZ-mediated autoregulation and *algD* expression require the DNA-binding activity of AmrZ (2, 31), we examined if this is the case with TM. The amino termini of the RHH DNA-binding proteins Arc and Mnt are responsible for operator recognition and binding, forming a unique DNA-binding domain (22). The DNA-binding domains of the dimeric protein Arc (53 residues, monomer) and the tetrameric protein Mnt (82 residues, monomer) are similar in structure and assume the shape of an orthogonal bundle (37). The orthogonal bundle is comprised of a beta-strand, which contacts DNA, followed by two alpha helices that form a coiled coil and comprise a dimerization domain (6). Previous studies have shown that residues Arg2, His6, Asp8, and Arg10 of Mnt are responsible for operator recognition and binding (21). Of the first 26 residues of the N terminus of AmrZ, residues 13 to 26 share amino acid identity with the beta strands of Arc and Mnt (Fig. 4A) (1).

We first addressed whether these residues would be required for DNA-binding activity to a published AmrZ-binding target. To test this, we engineered amino acid substitutions in place of AmrZ residues K18, V20, and R22 by using site-directed mutagenesis and tested purified wild-type and mutant proteins for binding to an *algD* DNA fragment containing an AmrZ-binding site (Fig. 4B to E).

As the concentration of wild-type six-His AmrZ increases

FIG. 4. Amino acid residues K18 and R22 are absolutely required for binding of AmrZ to the *algD* promoter. (A) Amino acid alignment between Arc (residues 1 to 53) and AmrZ (residues 10 to 62). Amino acid residues that correspond to the beta-strand (Arc residues 9 to 13), alpha helix A (Arc residues 16 to 28), or alpha helix B (Arc residues 33 to 47) are indicated in bold. Amino acids conserved between the two proteins are underlined. Numerical references for a portion of the amino acids are listed above their corresponding residues. (B to E) Electrophoretic mobility shift assays with the following amounts of the wild-type (B), K18A (C), V20A (D), or R22A (E) N-terminal domain (NTD) six-His-tagged AmrZ incubated with end-labeled DNA containing the AmrZ-binding site at *algD*: lane 1, 1.22 pmol; lane 2, 2.44 pmol; lane 3, 3.66 pmol; lane 4, 4.88 pmol; lane 5, 6.10 pmol; lane 6, 12.2 pmol; lane 7, 24.4 pmol; lane 8, 36.6 pmol; lane 9, 48.8 pmol.

(Fig. 4B), the AmrZ-*algD* complexes were observed and eventually shifted away 100% of the free DNA (Fig. 4B, lanes 2 to 10). Distinct complex formation was observed at higher concentrations of six-His AmrZ only (Fig. 4B, compare lanes 5 to 7 with lanes 8 to 10). Similar to wild-type AmrZ, AmrZ V20A bound to *algD* DNA (Fig. 4D), and this mutant protein was capable of shifting 100% of the free DNA at high protein concentrations (Fig. 4D, lanes 7 to 10). The migration patterns of the complexes formed by AmrZ V20A differ from wild-type AmrZ in that the most distinct complex appears to migrate much higher in

the gel than that observed for the wild type (compare Fig. 4B and D, lanes 2 to 7). In contrast to wild-type AmrZ, AmrZ K18A and AmrZ R22A demonstrated a complete loss of DNA-binding activity (Fig. 4C and E, respectively). This suggests that residues K18 and R22 are absolutely required for AmrZ-mediated DNA-binding activity at *algD*.

To test the effect of these mutations in vivo, we utilized gene replacement strategies to construct isogenic strains expressing these AmrZ mutant proteins in place of wild-type AmrZ (see Material and Methods). The *amrZ* deletion WFP205 shows

FIG. 5. TM analysis of *amrZ* alleles with a mutation in the DNA-binding domain. Strains analyzed were PAO1 (wild type), WFA510 (*amrZ* deletion complemented with WT *amrZ*), WFA511 (*amrZ* K18A), WFA512 (*amrZ* V20A), WFA513 (*amrZ* R22A), and WFA205 (*amrZ* deletion).

no TM (Fig. 5). However, complementation of the *amrZ* deletion with wild-type *amrZ* (WFA510) restored the TM⁺ phenotype (note that strain WFA510 demonstrates a twitching zone identical to that of PAO1 [Fig. 5]). The strains with mutations in residues required for AmrZ DNA binding and/or activation at *algD* are shown at the bottom of the plate (Fig. 5). In each situation, there is no detectable TM, demonstrating that these amino acids are required for TM. Since AmrZ V20A is able to bind the *algD* DNA in a manner similar to that of the wild-type AmrZ protein, this may indicate either a function for this residue other than DNA binding or that AmrZ V20A is defective in steps after DNA binding. Transmission electron microscopy was used to analyze the surface of the strains. The complemented strain (Fig. 6A) demonstrates wild-type TFP (compare with Fig. 2A). In contrast, bacteria harboring AmrZ K18A, AmrZ V20A, or AmrZ R22A do not express detectable surface TFP (Fig. 6B to D). This demonstrates that a mutation of any of these residues results in the apilicate appearance of an *amrZ* null strain and suggests that the DNA-binding activity of AmrZ is essential for TM. Unfortunately, the amount of AmrZ produced by wild-type PAO1 and PAO1-derived strains with the above AmrZ variants are below the level of detection by Western blotting. However, when these constructs were placed in a mucoid strain background, AmrZ was readily detectable (P. J. Baynham and D. J. Wozniak, unpublished data).

DISCUSSION

Previously, we identified AmrZ as a proposed RHH DNA-binding protein absolutely required for *algD* transcription in mucoid *P. aeruginosa* strain FRD1 (1). Since these strains are mucoid due to mutations that lead to alginate overproduction, we wanted to examine if *amrZ* had any role in nonmucoid strains of *P. aeruginosa*. In these studies, we constructed an *amrZ* deletion in nonmucoid PAO1 that resulted in a TM⁻ phenotype. Since an *algD* deletion remains TM⁺, this provides strong evidence that AmrZ enhances TM through some mechanism other than *algD* activation. Furthermore, mutagenesis of three amino acids crucial for AmrZ DNA binding reveals that the DNA-binding activity is required for its function in pro-

moting TM. We also examined whether *amrZ* is involved in *pilA* expression or PilA maturation. To address this, we performed TEM and Western blot analysis of surface and whole-cell preparations. A deletion in *amrZ* resulted in no detectable pili on the surface of the cells via TEM. The Western blot analysis showed that PilA present in the whole-cell preparation of the deletion strains was of the same size and amount as that present in the wild type. However, there were only trace amounts of surface-localized PilA in the *amrZ* mutants. This reveals a defect in TFP surface localization in the *amrZ* deletion strain. The involvement of *amrZ* in TM is confirmed by previous studies in which an elaborate transposon library was constructed and then screened for mutations that result in a loss of TM. Although these studies were not pursued, Jacobs et al. also discovered that a mutation in *amrZ* (PA3385) exhibited a TM⁻ phenotype (20).

It is not unusual for regulators of *algD* to have roles in the regulation of other genes. Since alginate may afford protection from environmental stresses encountered during infection (12, 36, 47), having regulators coordinately controlling other genes necessary for successful infection would be an advantage. Integration host factor is required for full *algD* activation and also functions in the regulation of enzymes involved in heme biosynthesis, which occurs largely under anaerobic conditions (23, 48). In addition to alginate production, AlgR has been shown to regulate TM and cyanide production (7, 43). AmrZ also regulates multiple genes, functioning as an activator of *algD* and a repressor of its own synthesis (1, 2, 31), and data from this study demonstrate an additional role for AmrZ in TM.

Both AmrZ and AlgR bind the *algD* promoter and are required for *algD* activation (1, 11), although the exact mechanism of activation is not known. It is not clear whether a precise three-dimensional structure is required for RNA polymerase binding or if there may be specific protein-protein interactions in which one or both of these regulators participate. Since AlgR and AmrZ both participate in *algD* activation and regulate TM (43), it is interesting to speculate that they may both be required for expression of the gene(s) involved in TFP assembly and localization. Consistent with this hypothesis is the finding that both *amrZ* and *algR* mutants exhibit similar TM phenotypes (45). Recently, it has been found that the TM genes under AlgR control are in the *fimTU-pilVWXYIY2E* operon (26). A DNA-binding consensus for AmrZ has recently been proposed: 5'-gGCCAttACCagcc-3' (31). Upon examination of the *fimTU-pilVWXYIY2E* operon, there does not appear to be any obvious AmrZ DNA-binding site (P. J. Baynham, unpublished data). AlgR binding to this promoter has not been demonstrated, and so both AmrZ and AlgR may regulate this promoter indirectly. An attempt to complement the *amrZ* deletion with a plasmid (pVDtacPIL, a gift of M. J. Schurr) containing the *fimTU-pilVWXYIY2E* operon was unsuccessful, while an *algR* deletion strain was complemented in tandem, supporting the hypothesis that AmrZ regulates TM in some other manner (data not shown). Since AmrZ has both activator and repressor functions (1, 31), it is unclear how AmrZ may control TM in *P. aeruginosa*. Genomics experiments in progress should elucidate the AmrZ-dependent genes involved in TM and TFP biogenesis.

P. aeruginosa has a number of virulence factors, which include an array of extracellular proteases, toxins, the ability to form biofilms, and type III secretion. Additionally, there are a

FIG. 6. TEM analysis of *P. aeruginosa* strains containing DNA-binding-domain mutations. Bacteria were harvested from plates cultured overnight, negatively stained using 2% uracyl acetate, and visualized using a Philips TEM 400. Strains analyzed were all PAO1 derivatives: (A) WFP510 (complemented strain, *amrZ*Δ *amrZ*⁺), (B) WFP511 (*amrZ* K18A), (C) WFP512 (*amrZ* V20A), and (D) WFP513 (*amrZ* R22A).

number of regulators that control more than one *P. aeruginosa* virulence factor. For example, *vfr* is necessary for exotoxin A and protease production (41). The quorum sensing systems control a plethora of virulence traits, including elastases, alkaline protease, exotoxin A, and pyocyanin (5, 25, 32, 34). Likewise, a three-component regulatory network controls both bio-film development and the type III secretion system (24).

As our knowledge of bacterial gene regulation advances, the coordinate regulation of virulence factors is elucidated (7, 24, 43, 44). An understanding of how this regulation occurs is central to determining how to counter these traits. The targeting of important regulators may be an effective strategy after the determination of how they function. The examination of the role of AmrZ in TM is a crucial step in this process.

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