

# *Pseudomonas aeruginosa* AlgR Regulates Type IV Pilus Biosynthesis by Activating Transcription of the *fimU-pilVWXYZIY2E* Operon<sup>∇</sup>

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**The response regulator AlgR is required for *Pseudomonas aeruginosa* type IV pilus-dependent twitching motility, a flagellum-independent mode of solid surface translocation. Prior work showed that AlgR is phosphorylated at aspartate 54, and cells expressing an AlgR variant that cannot undergo phosphorylation (AlgRD54N) lack twitching motility. However, the mechanism by which AlgR controls twitching motility is not completely understood. We hypothesized that AlgR functioned by activating genes within the prepilin *fimU-pilVWXYZIY2E* cluster that are necessary for type IV pilin biogenesis. Reverse transcriptase PCR analysis showed that the *fimU-pilVWXYZIY2E* genes are cotranscribed in an operon, which is under the control of AlgR. This supports prior transcriptional profiling studies of wild-type strains and *algR* mutants. Moreover, expression of the *fimU-pilVWXYZIY2E* operon was reduced in strains expressing AlgRD54N. DNase footprinting and electrophoretic mobility shift assays demonstrate that AlgR but not AlgRD54N bound with high affinity to two sites upstream of the *fimU-pilVWXYZIY2E* operon. Altogether, our findings indicate that AlgR is essential for proper pilin localization and that phosphorylation of AlgR results in direct activation of the *fimU-pilVWXYZIY2E* operon, which is required for the assembly and export of a functional type IV pilus.**

*Pseudomonas aeruginosa* is a prevalent opportunistic human pathogen and a major cause of acute pneumonia and septicemia in immunocompromised individuals (15). In addition, *P. aeruginosa* successfully establishes chronic infections in the airways of cystic fibrosis patients. Even rigorous treatment with antibiotics and anti-inflammatory agents cannot prevent lung damage and ultimately death due to respiratory failure in cystic fibrosis patients (15, 31). The first step in establishing an infection is the adherence and colonization of the epithelium, which is mediated, in part, by type IV pili (TFP) (8, 13, 18). TFP also mediate a flagellum-independent mode of surface translocation known as twitching motility (TM) (23, 24, 26). In vitro and in vivo studies show that mutants lacking functional TFP have a significant reduction in colonization, biofilm formation, and ability to spread (12, 17, 28, 38).

Control of TFP expression and TM is complex (9, 23, 37). One system that controls TM is a sensor kinase and a response regulator pair referred to as FimS/AlgR (35). FimS is also referred to as AlgZ (41). Mutations in either *algR* or its cognate sensor *fimS* result in a non-TM phenotype (35), and AlgR phosphorylation (AlgR~P) is required for TM (36). AlgR, which belongs to a novel family of response regulators with an unusual LyfTR DNA binding domain (27), plays an important role in the regulation of gene expression in *P. aeruginosa* (19). AlgR functions as an activator of *algD*, the first gene in the alginate biosynthetic operon (25, 26, 39), and *algC*, which is involved in alginate, lipopolysaccharide, and rhamnolipid production (19, 29). AlgR also controls hydrogen cyanide synthesis (20) and expression of type III secre-

tion genes (40). However, the role of AlgR~P in the control of such a diverse collection of genes is unknown.

TFP are polar organelles that are composed of a single protein subunit, PilA. PilA is exported out of the cell and polymerized to form the surface fimbrial strand. Assembly requires cleavage and methylation of a hydrophilic leader peptide by a type IV prepilin peptidase during pilin secretion (33). A striking feature of the TFP biogenesis and secretion pathway is its similarity to the type II (Xcp) secretion system, particularly the pseudopili or prepilin-like gene products shared by the two pathways in *P. aeruginosa* (21). Prepilin genes are located in one of the many TFP gene clusters and appear to play a role in TFP assembly, export, localization, and maturation and the general efficiency of the TFP machinery (14, 21–23, 32). To date, there are no reports that show the incorporation of these prepilin proteins in the TFP structure. A microarray study revealed that the *fimU-pilVWXYZIY2E* prepilin cluster is under the control of *algR* (20). In this study, AlgR-dependent expression of the *fimU-pilVWXYZIY2E* gene cluster was observed only during the stationary phase of growth, despite the fact that a plasmid containing the *fimTU-pilVWXYZIY2E* genes was able to restore TM to an *algR* mutant.

Our current report further defines the mechanism of AlgR-mediated control of TM. We hypothesized that the AlgR phosphorylation state is critical for its role as a transcriptional activator of the gene(s) involved in TFP biogenesis. We provide genetic and biochemical evidence that genes within the *fimU-pilVWXYZIY2E* cluster are indeed arranged in an operon and that AlgR but not AlgRD54N positively regulates the expression of the *fimU-pilVWXYZIY2E* operon. DNase I footprinting and electrophoretic mobility shift assays (EMSAs) indicate that this is mediated by AlgR binding specifically to two sites upstream of the *fimU-pilVWXYZIY2E* operon. The AlgR binding sites are atypical in that they overlap the *fimU-pilVWXYZIY2E* promoter region.

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

| Strain, plasmid, phage, or primer       | Characteristic(s) or sequence  | Source or reference |
|---|--|---------------------|
| <b>Strains</b>                          |  |                     |
| PAO1                                    | Prototroph   | Lab                 |
| WFPA12                                  | <i>algR::aacC1</i>   | 36                  |
| WFPA6                                   | <i>algR::aacC1/algR<sup>+</sup></i>  | 36                  |
| WFPA8                                   | <i>algR7</i>   | 36                  |
| WFPA21                                  | <i>fimT::aacC1</i>   | This work           |
| WFPA22                                  | <i>fimU::aacC1</i>   | This work           |
| PAO AWO                                 | <i>pilA::tet</i>   | Lab                 |
| <b>Plasmids</b>                         |  |                     |
| pEX18Ap                                 | Cloning vector   | 16                  |
| <i>pfimT</i>                            | <i>fimT</i> clone in pEX18Ap   | This work           |
| <i>pfimU</i>                            | <i>fimU</i> clone in pEX18Ap   | This work           |
| pGMΩ1                                   | GEN cassette vector  | 16                  |
| pBB21                                   | <i>fimT</i> in pEX18Amp disrupted via GEN cassette; Amp <sup>r</sup> Gm <sup>r</sup> | This work           |
| pBB22                                   | <i>fimU</i> in pEX18Amp disrupted via GEN cassette; Amp <sup>r</sup> Gm <sup>r</sup> | This work           |
| pmini-CTX- <i>lacZ</i>                  | Integration vector; Tet <sup>r</sup>   | 16                  |
| pP <sub><i>fimU</i></sub> - <i>lacZ</i> | <i>fimT-fimU</i> intergenic region in mini-CTX- <i>lacZ</i> promoterless vector      | This work           |
| <b>Phage F116L</b>                      |  |                     |
| Phage F116L                             | Temperate, pilus-specific, generalized transducing phage                             | 7                   |
| <b>Primers used in assay</b>            |  |                     |
| <b>Cloning reaction</b>                 |  |                     |
| <i>fimUEFEcoR</i>                       | 5'-CCGGAATTCATGTCATATCGTTTC-3'   |                     |
| <i>fimUEREHind</i>                      | 5'-CCCAAGCTTGCATGACTGGGG-3'  |                     |
| <i>fimTEFEcoR</i>                       | 5'-CCGGAATTCATGGTCGAAAGG-3'  |                     |
| <i>fimTINRBam</i>                       | 5'-GAGGAGCCTCGGGAATCCGTTCCCTTCT-3'   |                     |
| <i>fimTINFBam</i>                       | 5'-AAGAAGGAACGGATTCCCAGGCTCCT-3'   |                     |
| <i>fimTERHind</i>                       | 5'-CCCAAGCTTTCATCCGGAAGTGCT-3'   |                     |
| minictxF                                | 5'-CGGAATTCGCTCTGCGGAAATAC-3'  |                     |
| minictxR                                | 5'-CGGGATCCGCTGCTTGAAGTTCGGAATGG-3'  |                     |
| <b>DNA binding and footprinting</b>     |  |                     |
| NSF                                     | 5'-CCCAAGCTTGAACCTCTTCCGCCGTCG-3'  |                     |
| NSR                                     | 5'-CCGGAATTCGTTGTTTGCTCTGCCGAT-3'  |                     |
| <i>algD33F</i>                          | 5'-AGCCCTTGTGGCGAATAGGC-3'   |                     |
| <i>algD7R</i>                           | 5'-AGGGAAGTTCGCCGCTTG-3'   |                     |
| F1F                                     | 5'-TTCTGCTCTGCGGAAGGCCGCATAC-3'  |                     |
| F1R                                     | 5'-GCTGCTTGAAGTTCGGAATGG-3'  |                     |
| F2F                                     | 5'-ACTTCCGGATGATCATGGAC-3'   |                     |
| F2R                                     | 5'-GGTCGAGTTGGAACGATATGA-3'  |                     |
| F3F                                     | 5'-GCTCTGCGGAAGGCATAC-3'   |                     |
| F3R                                     | 5'-GAAGTGCTGCATAGCTCACG-3'   |                     |
| <b>RT-PCR</b>                           |  |                     |
| <i>fimTF</i>                            | 5'-ATGGTCGAAAGGTCGCAG  |                     |
| <i>fimTR</i>                            | 5'-TCATCCGGAAGTGCTGCA  |                     |
| <i>fimUF</i>                            | 5'-ATGTCATATCGTTCCAACCTCGACC   |                     |
| <i>fimUR</i>                            | 5'-TCAATAGCATGACTGGGGCGC   |                     |
| <i>pilVF</i>                            | 5'-ATGCTATTGAAATCGCGACAC   |                     |
| <i>pilVR</i>                            | 5'-TCATGGCTCGACCTGAGG  |                     |
| <i>pilWF</i>                            | 5'-ATGAGCATGAACAACCGC  |                     |
| <i>pilWR</i>                            | 5'-TCATGGCAGAGATTCTGAG   |                     |
| <i>pilXF</i>                            | 5'-ATGAACAACCTCCCTGCACAAC  |                     |
| <i>pilXR</i>                            | 5'-TCAGTTGGTATAGAGACGGGC   |                     |
| <i>pilY1F</i>                           | 5'-ATGAAATCGGTAATCCACCAG   |                     |
| <i>pilY1R</i>                           | 5'-TCAGTTCTTTCCTTCGATGGG   |                     |
| <i>pilY2F</i>                           | 5'-ATGAAAGTGCTGCCTATGCTGCT   |                     |
| <i>pilY2R</i>                           | 5'-TCATCGGGCCTGCTCC  |                     |
| <i>pilEF</i>                            | 5'-ATGAGGACAAGACAGAAGGGC   |                     |
| <i>pilER</i>                            | 5'-TCAGCGCCAGCAGTCG  |                     |
| Primer A (411:430)                      | 5'-CGTTGCCTGG CAACTGATCC   |                     |
| Primer B (435:452)                      | 5'-CCGGAGGGCCGGCTCCGTACCG  |                     |
| Primer C (455:480)                      | 5'-CCGCGAGCGCCAGGGAGAACC GGAG  |                     |
| Primer D (499:518)                      | 5'-CTTCCGGATGATCATGGAC   |                     |

Continued on facing page

TABLE 1—Continued

| Strain, plasmid, phage, or primer | Characteristic(s) or sequence              | Source or reference |
|-----------------------------------|--|---------------------|
| Primer E (570:600)                | 5'-GCCCGTTTGGCATGCTTTCCAGGCGTAG            |                     |
| Primer F (593:618)                | 5'-CGTAGACCCCTGGAGCAACCGC                  |                     |
| Primer G (702:721)                | 5'-CATTCGAACTTCAAGCAGC                     |                     |
| <b>Real-time RT-PCR</b>           |  |                     |
| <i>fimU2F</i>                     | 5'-CGTCCTGCTGGCCATCA                       |                     |
| <i>fimU2R</i>                     | 5'-CTGCAGTTCGTTGCGTTCTG                    |                     |
| <i>fimU2P</i>                     | 5'-[DFAM]CGCCATTCCGAACCTTCAAGCAGGCT[DTAM]  |                     |
| <i>pilEF</i>                      | 5'-GCATCGCCATTCCCAGTT                      |                     |
| <i>pilER</i>                      | 5'-CCTCGGTGCGGGTTGA                        |                     |
| <i>pilEP</i>                      | 5'-[DFAM]CCAGAACTACGTGGATCC[DTAM]          |                     |
| <i>pilV2F</i>                     | 5'-TGCTATTGAAATCGCGACACA                   |                     |
| <i>pilV2R</i>                     | 5'-CGACCAGCACTTCGATCATG                    |                     |
| <i>pilV2P</i>                     | 5'-[DFAM]TCGCTGCACCAATCCGGCTTC[DTAM]       |                     |
| <i>rpoDF</i>                      | 5'-CCTGGCGGAGGATATTTC                      |                     |
| <i>rpoDR</i>                      | 5'-GATCCCCATGTCGTTGATCAT                   |                     |
| <i>rpoDP</i>                      | 5'-[DFAM]ATCCGGAACAGGTGGAAGACATCATCC[DTAM] |                     |

## MATERIALS AND METHODS

**Strains and plasmids.** Bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* cultures were maintained at 37°C in Luria-Bertani (LB; 10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) broth, while *P. aeruginosa* was cultured in LB broth lacking NaCl (10 g/liter tryptone, 5 g/liter yeast extract). Solid media (LA) were prepared by adding 1.0 to 1.5% select agar (Gibco-BRL). Plasmids in *E. coli* were selected using media supplemented with the following antibiotics at the indicated concentrations: carbenicillin (CAR; 100 µg ml<sup>-1</sup>) and gentamicin (GEN; 10 µg ml<sup>-1</sup>). Plasmids in *P. aeruginosa* were selected on media containing CAR (300 µg ml<sup>-1</sup>), GEN (100 µg ml<sup>-1</sup>), and irgasan (Irg; 25 µg ml<sup>-1</sup>). *E. coli* strain JM109 was used for all cloning procedures, while *E. coli* SM10 was used to transfer plasmids into *P. aeruginosa* by biparental mating (34). The *P. aeruginosa* strains used were PAO1 and its derivatives WFPA8, WFPA12, WFPA20, WFPA21, and WFPA22. Vectors pGMΩ1, pEX18Gm, and pEX18Ap or derivatives were used for cloning and gene replacements (Table 1).

**Construction of *fimU* and *fimT* polar insertions.** A ~500-bp *fimU* fragment was amplified by PCR using primers *fimU*EFcoR and *fimU*ERHind (Table 1). The amplified gene was cloned into pEX18Ap to yield *pfimU*. *pfimU* was digested with BamHI, a unique restriction site present within *fimU* coding sequences. A 1.6-kb GEN resistance cassette (*aacC1*) was prepared by restriction digestion of pGMΩ1 with BamHI. This fragment was then gel purified and ligated with *pfimU*, resulting in pBB22. Plasmid pBB22 was transformed into SM10 for biparental mating into PAO1 following the protocol of Hoang et al. (16). Double recombinants that were sensitive to CAR but grew on LA lacking NaCl and GEN were selected as a positive *fimU::aacC1* integrant(s) and designated WFPA22 (Table 1). In the case of *fimT* insertions, we had to engineer a unique BamHI site in the *fimT* coding sequence via PCR. To do this, we amplified a 310-bp fragment using the primers *fimT*EFcoR and *fimT*INRBam and ligated this fragment to a 190-bp fragment created with primers *fimT*INFBam and *fimT*ERHind (Table 1). The ligated 510-bp fragment, containing the complete *fimT* sequence, was cloned into pEX18Ap to yield *pfimT*. The *aacC1* GEN cassette from pGMΩ1 was then inserted into *pfimT* to yield pBB21, which was transformed into SM10 for integration into the PAO1 chromosome via the procedure listed above (16). The *fimT::aacC1* strain was designated WFPA21 (Table 1). All gene replacements were verified by PCR.

**Phage sensitivity and subsurface TM assays.** Phage sensitivity was assayed by a plaque assay. Aliquots of serially diluted F116L phage lysate (1.1 × 10<sup>11</sup> PFU/ml) were mixed with 5 ml of top agar (0.3%) seeded with 0.1 ml of a stationary-phase culture of the strain being tested (10). PFU were counted for each strain tested after overnight incubation at 37°C and expressed relative to the phage sensitivity of PAO1. TM was assayed as described previously (36). Briefly, the *P. aeruginosa* strain to be tested was stab inoculated through a 1% agar plate and, after overnight growth at 37°C, the TM zone between the agar and petri dish interface was observed.

**DNA binding assays.** Conditions for the EMSA were similar to those previously reported (30, 36). DNA fragments were generated by PCR using forward (F) and reverse (R) primers listed in Table 1. DNA fragments that were used in Fig. 2B were labeled via PCR using [ $\alpha$ -<sup>32</sup>P]ATP (30). Unincorporated nucleo-

tides were removed by gel filtration through G-50 quick-spin columns (Amersham Biosciences). The two fragments used in the EMSA for Fig. 2B are a nonspecific DNA fragment (NS), which spans 500 bp of sequences encompassing *psl*, and the *algD* fragment, which spans -400 bp upstream of *algD*. The DNA fragments used in the EMSA in Fig. 2D (F1, F2, and F3) were also generated via PCR after end labeling the respective primers (Table 1) using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP. Unincorporated nucleotides were removed by gel filtration through G-50 quick-spin columns (Amersham Biosciences). Wild-type AlgR and AlgRD54N were overexpressed and purified as previously described (36). AlgR purity was estimated at 90% by Gelcode blue (Pierce) staining and densitometry. Protein concentrations for wild-type AlgR and AlgRD54N were determined by Bradford assay to be ~1,000 µM. In these experiments, we have used protein amounts ranging from 1.0 pmol to 5.0 pmol. All EMSAs were performed at 25°C for 10 min in a reaction volume of 10 µl. Gels were dried at 80°C for 20 min and exposed to a phosphorimaging screen for 24 h. The DNase I footprinting experiments utilized the F2 DNA fragment (Fig. 2C). Binding mixtures for DNase I footprinting were identical to those used for the EMSA in Fig. 2D. Partial digestion of protein-bound DNA was initiated by the addition of DNase I (100 U) (Promega Life Sciences) after dilution in 1× binding buffer containing 1 mM CaCl<sub>2</sub>. DNase I activity was terminated after 2 min by the addition of stop solution (0.4 M sodium acetate, 0.2% sodium dodecyl sulfate, 10 mM EDTA, 50 µg ml<sup>-1</sup> *Saccharomyces cerevisiae* tRNA). The protein-DNA mixtures were then extracted with phenol-chloroform, and DNA in the aqueous phase was ethanol precipitated and analyzed on 6% polyacrylamide-7 M urea gels. AlgR concentrations ranging from 0.33 to 8.33 pmol were used in these assays. Gels were run at 50 W at 25°C, dried for 50 min, and exposed to a PhosphorImager screen (Molecular Dynamics) and then developed with a Typhoon scanner.

**RNA isolation and reverse transcriptase PCR (RT-PCR) analysis.** *P. aeruginosa* strains (PAO1, WFPA8, WFPA12, WFPA21, and WFPA22) were grown on LANS plates for 16 to 18 h and resuspended in phosphate-buffered saline at ~1.0 × 10<sup>7</sup> CFU/ml. Total RNA was isolated using the Ribopure minikit (Ambion) per the manufacturer's instructions. RNA samples were subjected to RNase-free DNase treatment (Ambion) to remove any residual contaminating chromosomal DNA, and the purity of the RNA was verified by agarose gel electrophoresis. RNA concentrations were determined by measuring absorbance on an Eppendorf Biophotometer. Primer and probe sets used in these experiments are listed in Table 1.

For the RT-PCR assays (Fig. 1 and 4), cDNA derived from PAO1 was prepared as outlined by the manufacturer (Invitrogen) and used for PCR analysis of mRNA of the prepilin cluster. Briefly, RNA, 50 ng random primers, 10 mM deoxynucleoside triphosphates, and sterile distilled water were heated at 65°C for 5 min and then chilled on ice for 1 min. First-strand buffer (5×), RNase Out, and 0.1 M dithiothreitol were added, and the mixtures were incubated at 25°C for 10 min. The reverse transcription reaction was carried out at 50°C using Superscript III (Gibco) and *Taq* polymerase (Gibco) according to the manufacturer's instructions. Each PCR experiment was accompanied by controls including a reaction of cDNA with no primers, a reaction with genomic DNA, and a reaction with RNA and no RT. Primer sets that amplify the open reading frames of genes

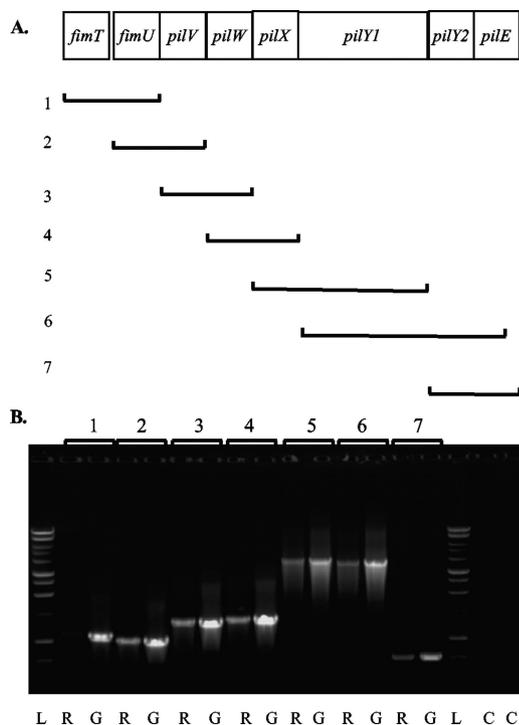


FIG. 1. The *fimU-pilVWXYZIY2E* gene cluster is an operon. (A) Map of the *P. aeruginosa fimTU-pilVWXYZIY2E* prepilin gene cluster. Numbers 1 to 7 represent the respective primer pairs spanning two adjacent genes used in RT-PCR analysis. Table 1 shows sequences for primers. (B) Agarose gel analyses of RT-PCRs. Lanes 1 to 7 are primer pairs as denoted in Fig. 1A. Lanes designated "R" represent the reactions where cDNA derived from PAO1 was amplified by the primer pairs 1 to 7, lanes G are controls with genomic DNA, lanes C are negative controls (RNA without RT or cDNA without primers, respectively), and lanes L are DNA ladders.

in the prepilin cluster (Fig. 1A) include *fimTF-fimUR* (set 1), *fimUF-pilVR* (set 2), *pilVF-pilWR* (set 3), *pilWF-pilXR* (set 4), *pilXF-pilY1R* (set 5), *pilY1F-pilY2R* (set 6), and *pilY2F-pilE2* (set 7). The sequences of the primer sets used to localize the *fimU* promoter by RT-PCR (Fig. 4A) are depicted in Table 1. Each individual primer of primers A to F was used in combination with primer G for RT-PCR analyses (Fig. 4A).

The TaqMan real-time RT assays were performed with an ABI 7700 instrument (Applied Biosystems, Forest City, CA) and the TaqMan One-Step RT-PCR Master Mix reagent kit (Roche). The amplification profile used was as follows: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. The standard curve method was applied, and serially diluted genomic DNA was used as a template to obtain the relative standard curve. The quantity of transcript for each experimental gene was normalized to the quantity of the constitutively transcribed housekeeping gene (*rpoD*).

**Statistical analysis.** Results were analyzed using the SigmaSTAT statistical program. Individual means from three or more experiments were compared using an unpaired Student *t* test. *P* values considered significant are denoted in the respective figures.

## RESULTS

**Evidence that the *fimU-pilVWXYZIY2E* gene cluster is an operon.** To date, no report exists regarding the potential operon structure of the *fimTU-pilVWXYZIY2E* prepilin cluster. We used RT-PCR and polar insertion mutagenesis strategies to investigate this. RT-PCR was conducted using total RNA isolated from surface-grown PAO1 (wild type). The RNA was

converted to cDNA as outlined by the manufacturer (Invitrogen) and amplified using primer pairs spanning two consecutive adjacent genes within the *fimTU-pilVWXYZIY2E* cluster (labeled 1 to 7, Fig. 1A; Table 1). Each primer pair was used to amplify the cDNA (R lanes, Fig. 1B) as well as the genomic DNA (G lanes, Fig. 1B), which served as a positive control. The RT-PCR analysis indicates that each gene in the cluster downstream of *fimU* was cotranscribed with the adjacent gene (Fig. 1B, lanes 2 to 7). The results also indicate that *fimT* does not appear to be cotranscribed with *fimU* (Fig. 1B, lane 1). There were no amplicons in control lanes (C), which are mock reactions (RNA with no RT) and cDNA without primers, respectively. There were no transcriptional terminators apparent within the prepilin operon, except one positioned ~50 bp downstream of *pilE* (data not shown). Thus, we propose that the *fimU-pilVWXYZIY2E* genes form an operon consisting of seven cistrons.

Additional genetic evidence for *fimU-pilVWXYZIY2E* operon structure comes from our analyses of *fimT* and *fimU* polar insertion mutants. We generated *fimT* and *fimU* mutants (strains WFA21 and WFA22, respectively) by insertion of a polar GEN resistance cassette within the coding sequences of these genes (see Materials and Methods). RNA was isolated from surface-grown PAO1, WFA21, and WFA22 and converted to cDNA. The mRNA levels of three representative genes of the *fimU-pilVWXYZIY2E* operon, *fimU*, *pilV*, and *pilE*, were measured by real-time RT-PCR (Table 2). In strain WFA21, there was no significant reduction in expression of *fimU*, *pilV*, and *pilE* compared to the wild-type strain. This indicates that disruption of *fimT* does not affect expression of the downstream *fimU*, *pilV*, and *pilE* genes. However, the mutation in *fimU* (strain WFA22) resulted in severe polar effects on *fimU*, *pilV*, and *pilE* expression (Table 2).

Subsurface TM assays performed as previously described (2, 35, 36) showed that WFA22 but not WFA21 was defective in TM (Table 3). Phage sensitivity assays also indicate that WFA21 is as sensitive as PAO1 to pilus-specific phage F116L (Table 3). Collectively, these data provide additional genetic evidence that the *fimU-pilVWXYZIY2E* gene cluster is indeed an operon. *fimT* does not appear to be cotranscribed with the *fimU-pilVWXYZIY2E* operon, and we confirm that *fimT* is not required for TM (3).

TABLE 2. Expression of prepilin genes in *P. aeruginosa* strains<sup>a</sup>

| Strain                       | Normalized value of transcript of prepilin gene (% of wild-type value) |             |             |
|------------------------------|--|-------------|-------------|
|                              | <i>fimU</i>  | <i>pilV</i> | <i>pilE</i> |
| PAO1 (wild type)             | 100  | 100         | 100         |
| WFA8 ( <i>algR7</i> )        | 4.0 ± 0.4  | 25.0 ± 2.0  | 21.0 ± 4.0  |
| WFA12 ( <i>algR::aacC1</i> ) | 2.0 ± 1.4  | 9.2 ± 0.7   | 19.0 ± 10.1 |
| WFA21 ( <i>fimT::aacC1</i> ) | 97.0 ± 2.8   | 99.0 ± 2.1  | 88.0 ± 8.1  |
| WFA22 ( <i>fimU::aacC1</i> ) | 5.0 ± 0.8  | 6.0 ± 2.9   | 3.0 ± 2.5   |

<sup>a</sup> Total RNA was isolated from plate-grown *P. aeruginosa* strains, and *fimU*, *pilV*, and *pilE* transcription levels were determined by real-time RT-PCR (TaqMan). The standard curve method was applied, and serially diluted genomic DNA was used as a template to obtain the relative standard curve. Average threshold cycle values for each prepilin gene tested were normalized to the average *rpoD* values. These values were then set as percentages of the PAO1 values. The data obtained represent the means and standard errors obtained from three independently isolated RNA samples.

TABLE 3. TM and phage F116L sensitivity of *P. aeruginosa* strains

| Strain (genotype)             | TM <sup>a,b</sup> (mm) | Phage sensitivity <sup>b,c</sup> (% of wild-type strain) |
|-------------------------------|------------------------|--|
| PAO1 (wild type)              | 23 ± 0.8               | 100  |
| WFPA8 ( <i>algR7</i> )        | 0                      | 0.117  |
| WFPA12 ( <i>algR::aacC1</i> ) | 0                      | 0.0012   |
| WFPA21 ( <i>fimT::aacC1</i> ) | 23 ± 0.8               | 123  |
| WFPA22 ( <i>fimU::aacC1</i> ) | 0                      | 0.0019   |
| AWO ( <i>pilA::tet</i> )      | 0                      | 0  |

<sup>a</sup> Quantification of TM was performed via measurements of the averages of respective halo sizes in mm (diameter of the colony – diameter of the TM zone). Assays were performed in triplicate, and means and standard errors were determined. Data analysis was performed via unpaired *t* test.

<sup>b</sup> All values are statistically significant ( $P < 0.001$ ).

<sup>c</sup> Phage (F116L) sensitivity assays were performed in triplicate, and values are expressed as percentages of wild-type (PAO1) sensitivity.

**AlgR but not AlgRD54N regulates the prepilin operon by binding to sequences upstream of *fimU*.** Based on the hypothesis that AlgR controls TM by activating the *fimU-pilVWXYZIY2E* operon, we analyzed the expression of *fimU*, *pilV*, and *pilE* in the wild type and two *algR* mutants (Table 2). RNA was isolated from surface-grown PAO1, WFPA8 (*algR7*; encoding AlgRD54N), and WFPA12 (*algR::aacC1*; *algR* null) converted to cDNA, and *fimU*, *pilV*, and *pilE* levels were measured by real-time RT-PCR. Compared with the wild type, *fimU*, *pilV*, and *pilE* mRNA levels were significantly reduced in both *algR* mutants (Table 2).

The above results (Tables 2 and 3) and published data (1–3, 36) indicate that *P. aeruginosa* strains expressing wild-type AlgR but not AlgRD54N are TM proficient and express the *fimU-pilVWXYZIY2E* genes. Although there are several potential explanations for this, we hypothesized that AlgR activates the *fimU-pilVWXYZIY2E* operon by binding directly to sequences in the *fimU* promoter region. Additionally, we hypothesized a difference in the DNA binding affinity between wild-type AlgR and AlgRD54N for *fimU* sequences. To address this, AlgR and AlgRD54N were purified as previously described (36). Purity was estimated at 90% by Gelcode blue staining and densitometry (Fig. 2A). AlgR and AlgRD54N were tested for binding to an *algD* fragment with a known AlgR binding site as well as a nonspecific (NS) DNA target. Both AlgR and AlgRD54N bound to *algD* sequences, albeit with different affinities (Fig. 2B, lanes 2 to 4 versus lanes 5 to 7). There was no protein-DNA complex formed when AlgR was incubated with the NS DNA target (Fig. 2B, lanes 9 and 10).

Sequence analyses of the *fimT-fimU* intergenic region located a potential AlgR binding site with the sequence 5'-CC GTTTGGC-3' that closely matches the high-affinity AlgR binding sites upstream of *algD* and *algC* (25, 29, 42). Therefore, we generated end-labeled DNA fragments that span the putative AlgR binding site in the *fimT-fimU* intergenic region. F1 spans sequences from –251 to +80, relative to the *fimU* translational start site. The F1 fragment was further divided into two additional fragments, F2 and F3 (Fig. 2C). After incubations with AlgR or AlgRD54N, the DNA fragments were analyzed by EMSA. Two AlgR-*fimU* complexes were observed with fragments F1 and F2 (Fig. 2D, lanes 2 to 4 and lanes 14 to 16, respectively). AlgR binding to F1 and F2 was concomitant with the loss of free *fimU* DNA (Fig. 2D). No binding to F1 or

F2 was evident with AlgRD54N (Fig. 2D, lanes 5 and 6 and lanes 17 and 18). There was little detectable binding of AlgR or AlgRD54N to fragment F3 (Fig. 2D, lanes 8 to 13). The limited DNA binding activity seen with AlgRD54N provides a clear explanation for the reduced *fimU-pilVWXYZIY2E* operon expression observed in WFPA8 (Table 2), as well as the TM and TFP phenotypes associated with this strain (Table 3) (36). From these assays, we conclude that the AlgR binding sites are positioned within the F2 fragment, which spans positions –117 to +23 relative to the *fimU* translational start site.

**Localization of the AlgR binding sites upstream of the prepilin operon.** To precisely define the AlgR binding sites upstream of *fimU*, we performed DNase I footprinting with AlgR or AlgRD54N on the end-labeled F2 DNA fragment illustrated in Fig. 2C. AlgR protected sequences encompassing sites –60 to –50 (ABS2) and –30 to –10 (ABS1) on the F2 fragment (Fig. 3, lanes 4 to 6). This region contained the sequences 5'-CCGTTTGGC-3' (ABS2) and 5'-CCCTCGGG C-3' (ABS1), which resemble AlgR binding sites upstream of *algD* (25). In contrast, AlgRD54N failed to provide protection from DNase cleavage of the F2 fragment (Fig. 3, lanes 8 to 12).

Since the transcriptional start site of *fimU* is not known, the F1 fragment (Fig. 2C) was directionally cloned into the promoterless vector (mini-CTX-*lacZ*), yielding mini-CTX-pP<sub>*fimU*</sub>-*lacZ* (Table 1). This plasmid was used to generate chromosomal *fimU-lacZ* fusions at the neutral *attB* site (16) in *P. aeruginosa* strains PAO1, WFPA8, and WFPA12. Compared with the wild-type strain, which produced 220 Miller units of β-galactosidase, strains WFPA8 and WFPA12 produced 20 and 53 Miller units, respectively (data not shown). This finding suggests that the AlgR-regulated *fimU* promoter is located within the F1 fragment.

Despite numerous attempts, we were unable to map the *fimU* transcriptional start site by the method of primer extension or 5' rapid amplification of cDNA ends. To localize the *fimU* promoter, we used RT-PCR. RNA derived from surface-grown PAO1 was converted to cDNA as described above (Materials and Methods) and amplified using primer pairs (labeled A to G in Fig. 4A) spanning the region between *fimT* and *fimU*. Each individual primer of primers A to F was used in combination with primer G for RT-PCR to amplify the cDNA (R lanes, Fig. 4B), the genomic DNA (G lanes, Fig. 4B), and mock reactions (RNA with no RT; C lanes, Fig. 4B). The results reconfirmed that *fimT* is not cotranscribed with *fimU* (Fig. 4B, fragments A-G, B-G, C-G, and D-G). There were RT-PCR amplicons using primer sets E-G and F-G (Fig. 4B), which localized the *fimU* promoter from –45 to –97 bp upstream of the *fimU* start codon (italicized region, Fig. 4C). A sequence resembling the σ-70 consensus –35 element (5'-TTGACA-3') was observed (positions 522 to 527, Fig. 4C), but a –10 element was not apparent. Surprisingly, this region also contains both of the AlgR binding sites (ABS1 and ABS2, Fig. 4C), indicating that these *cis* elements overlap the *fimU* promoter.

## DISCUSSION

While many of our findings are consistent with the work of others (4, 19, 20, 36), several novel aspects of this study should be emphasized. Notably, all our experiments compared effects of surface-grown wild-type *P. aeruginosa* and two classes of

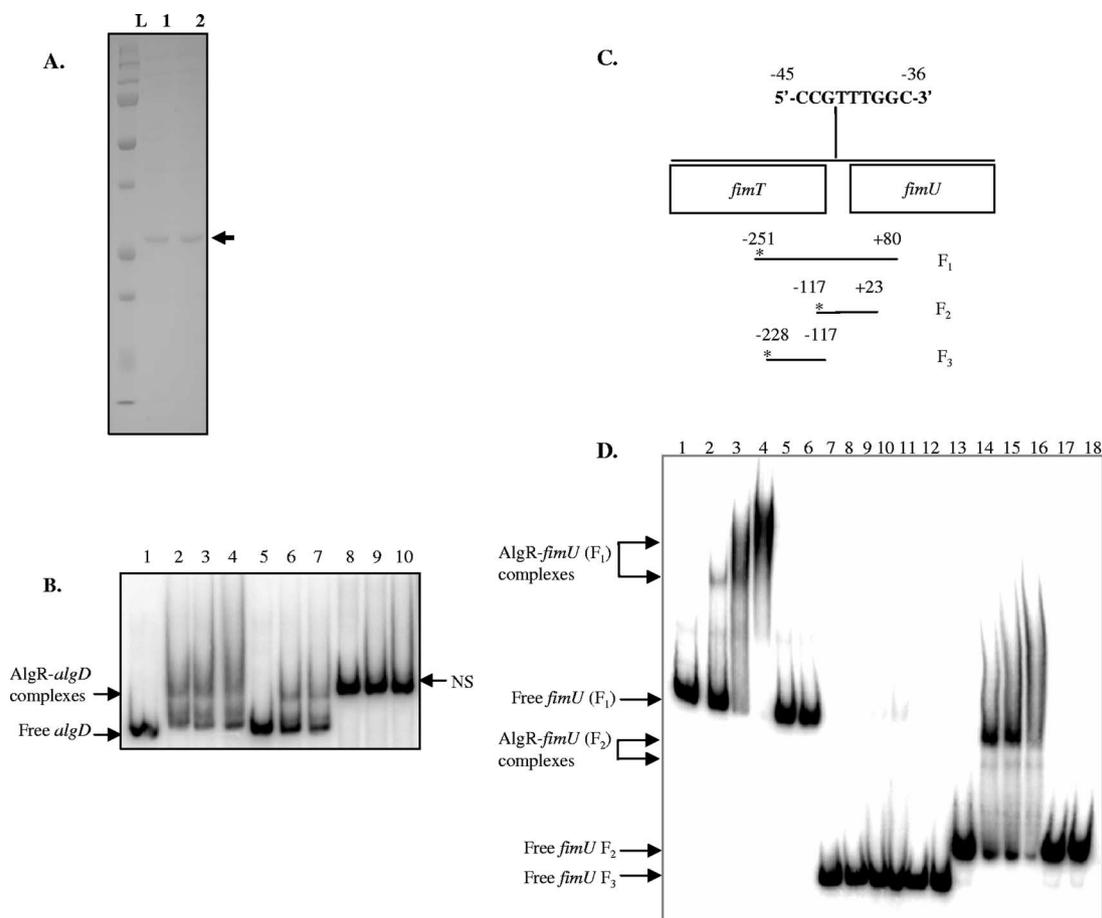


FIG. 2. AlgR but not AlgRD54N binds directly to sequences upstream of *fimU*. (A) Gelcode blue stain of a sodium dodecyl sulfate-polyacrylamide gel with purified AlgR (lane 1) or AlgRD54N (lane 2). The arrow indicates the positions of these proteins (~30 kDa). Lane L, protein ladder. (B) EMSA of DNA-protein complexes formed in the presence of AlgR and AlgRD54N and *algD* or NS DNA. Lanes 1 (*algD*) and 8 (NS DNA) represent free DNA (2.27 pM and 1.5 pM, respectively). Lanes 2 to 4 are incubated with 2.0, 4.27, or 6.0 pmol of AlgR, respectively; Lanes 5 to 7 are incubated with 2.0, 4.27, or 6.0 pmol of AlgRD54N, respectively. Lanes 9 and 10 have 2.0 pmol and 4.27 pmol of AlgR, respectively. (C) AlgR binding site (–45 CCGTTTGGC –36) localized with respect to *fimU* ATG. F1, F2, and F3 DNA fragments (–251 to +80, –117 to +23, and –228 to –117, respectively, with respect to *fimU* ATG). The asterisk denotes the  $\gamma$ - $^{32}$ P label. (D) DNA-protein complexes formed in the presence of AlgR and AlgRD54N using DNA fragments illustrated in panel C. Lanes 1, 7, and 13 represent free DNA for F1 (1.5 pM), F3 (1.46 pM), and F2 (1.5 pM), respectively. Lanes 2 to 4, 8 to 10, and 14 to 16 are incubated with 1.0, 2.0, and 4.27 pmol of wild-type AlgR, respectively. Lanes 5 and 6, 11 and 12, and 17 and 18 are incubated with 2.0 and 4.27 pmol of AlgRD54N, respectively.

*algR* mutants (WFA12 and WFA8), the latter expressing a variant of AlgR (AlgRD54N), which does not undergo *in vitro* phosphorylation (36). Collectively, our results reveal that the *fimU-pilVWXYIY2E* cluster is indeed an operon, that *fimT* is not cotranscribed with *fimU*, and that *fimU* is the first gene of this operon. Real-time RT analysis shows that insertions in *fimT* did not exert any polar effects because *fimU*, *pilV*, and *pilE* mRNA levels were similar to wild-type levels, which provides additional evidence that *fimT* and *fimU* are in separate transcriptional units. However, insertions in *fimU* did result in polar effects on *pilV* and *pilE*. Surprisingly, *fimT* mutations do not affect TM (Table 3), despite the finding that overexpression of *fimT* can bypass mutations in *fimU*, indicating that *fimU* and *fimT* may have similar functions (3). Conversely, strains WFA12 and WFA8 and the *fimU* mutant WFA22 are each defective in TM. Phage sensitivity assays show that WFA12, WFA8, and WFA22 exhibit some detectable resistance to infection by phage F116L (Table 3), despite the fact that they

fail to express polar pili (3, 36). This suggests that these strains still extrude some F116L-accessible components in their respective incompletely assembled pili. However, the *fimT* mutant WFA21 is as sensitive to F116L infection as the wild-type strain (Table 3).

Several lines of evidence reveal a link between AlgR and regulation of the *fimU-pilVWXYIY2E* operon. First, mutations in most of the *fimU-pilVWXYIY2E* genes result in strains incapable of TM (1, 32) (Table 3). Second, the TFP phenotype observed with *algR* mutants (improper assembly of surface TFP) resembles that observed in *fimU* and *pilV* mutants (2, 3). Finally, prior transcriptional profiling analyses indicate that the *fimU-pilVWXYIY2E* locus is under the control of AlgR. In that study (20), AlgR-dependent *fimU-pilVWXYIY2E* expression was observed only during stationary phase, in liquid growth conditions. Our real-time RT-PCR analysis using surface-grown *P. aeruginosa* cells, the DNA binding studies, and prior published work (20) suggest that expression of the *fimU*-

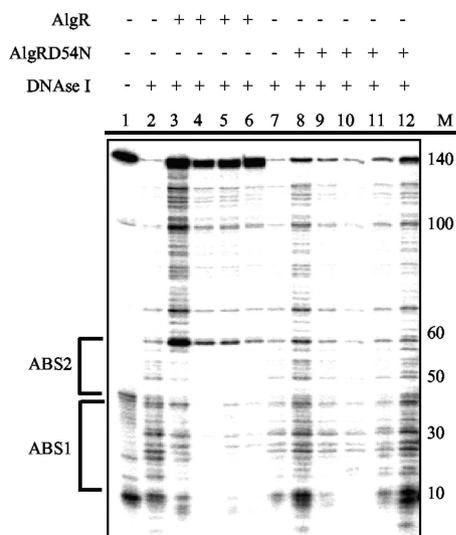


FIG. 3. DNase I protection assays of the *fimU* promoter with AlgR and AlgRD54N. The binding reaction mixture contained the end-labeled DNA template strand of the F2 fragment (1.5 pM) illustrated in Fig. 2C. Lanes 3 to 6 contained 0.33, 0.83, 1.67, and 3.33 pmol of AlgR, respectively. Lanes 8 to 12 contained 0.33, 0.83, 1.67, 3.33, and 8.33 pmol of AlgRD54N, respectively. Lane 1 contains control reaction mixtures with free DNA lacking AlgR and DNase I; lanes 2 and 7 are DNase I-treated control reaction mixtures without protein. Molecular size markers (M) are indicated on the right in nucleotides. The brackets mark regions of DNA protected from DNase cleavage by AlgR and also containing the consensus AlgR binding sites: ABS2 (5'-CCGTTTGGC-3') and ABS1 (5'-CCCGTTTGGC-3').

*pilVWXYIY2E* operon fully accounts for the effect of AlgR on TFP expression and biogenesis (Table 2). The TFP defect observed in *algR* mutants also correlates with a decrease in attachment to 16HBE14 human epithelial cells (data not shown). This is likely due to the failure of AlgR to directly activate the *fimU-pilVWXYIY2E* operon in these strains.

DNA binding assays show that AlgR but not AlgRD54N specifically binds to sites located upstream of the *fimU* translational start site (Fig. 2 to 4). These binding sites closely resemble the AlgR consensus core sequence at *algD* and *algC* (11, 25). The arrangement of these AlgR binding sites, which appear to overlap the *fimU* promoter region (italicized area, Fig. 4C), is quite different from the arrangement of the AlgR binding sites at *algD* or *algC* (11, 25). It is not unprecedented that the binding of a transcriptional factor could overlap and/or extend into -35 elements and still function as an activator. For example, the binding site of the *Bordetella pertussis* response regulator BvgA overlaps the -35 region of the *fha* promoter (6). Additional analysis revealed that three BvgA dimers bind one face of the DNA helix at the *fha* promoter, allowing for recruitment of RNA polymerase, which binds to the other face of the DNA helix (5). Similarly, the arrangement of ABS1 and ABS2 with respect to the *fimU* promoter would appear atypical, and the mode of AlgR-mediated activation of the *fimU* operon may be novel. We also cannot exclude the possibility that this region contains more than one promoter, each of which responds differentially to AlgR bound at ABS1 or ABS2.

A body of work on TFP biogenesis in *Neisseria* species and

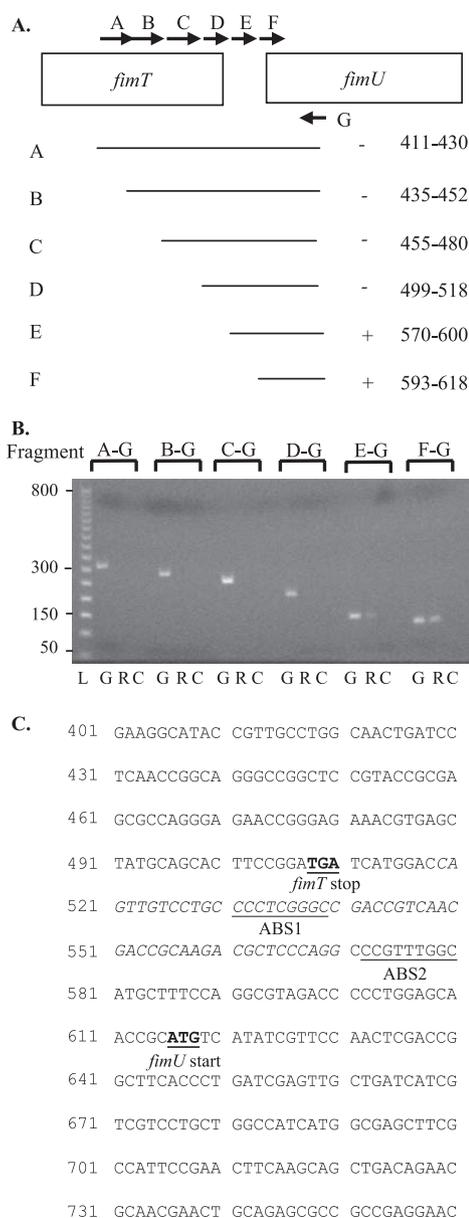


FIG. 4. Localization of the *fimU-pilVWXYIY2E* promoter. (A) Positions of primers (A to G) spanning *fimT* and *fimU* used in RT-PCR analysis. The positions of these primers are relative to the *fimT* start codon. Table 1 shows the primer sequences. The minus and plus signs denote the absence or presence of RT-PCR product, respectively, using each primer indicated in combination with primer G. (B) Agarose gel analyses of RT-PCRs. Lanes denote primer pairs as indicated in panel A. Lanes designated "R" represent the cDNA reactions with the primer pairs indicated, lanes G are controls with genomic DNA, lanes C are negative controls (RNA without RT), and lane L is a DNA ladder. Sizes (bp) are indicated on the left. (C) Sequences of the 3' end of *fimT* and the 5' region of *fimU* (*fimT* stop codon and *fimU* start codons are indicated). The *fimU* promoter region, as identified by RT-PCR, is shaded. AlgR binding sites (ABS1 and ABS2) are underlined (5'-CCCTCGGGC-3' and 5'-CCGTTTGGC-3', respectively).

other pseudomonads implicates the prepilin genes in export, maturation, and presentation of the major fimbrial subunit, when these genes are expressed in the correct stoichiometric ratios (2, 3). We postulate a model in which phosphorylated

AlgR controls the *fimU-pilVWXYIY2E* prepilin operon and consequently TM by binding to ABS1 and/or ABS2 in the promoter. This model involves assumptions that require further analysis such as phosphorylation-induced conformational changes of AlgR that allow high-affinity binding to the *fimU-pilVWXYIY2E* promoter region. Work is in progress to delineate the contribution of AlgR phosphorylation to the transcriptional machinery of the *fimU-pilVWXYIY2E* operon.

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