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Independent Regulation of MucD, an HtrA-Like Protease in *Pseudomonas aeruginosa*, and the Role of Its Proteolytic Motif in Alginate Gene Regulation

Lynn F. Wood and Dennis E. Ohman*

Department of Microbiology and Immunology, Virginia Commonwealth University, Medical College of Virginia Campus, Richmond, Virginia 23298-0678, and McGuire Veterans Affairs Medical Center, Richmond, Virginia 23249

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Expression of *mucD*, encoding a homologue of the HtrA(DegP) family of endoserine proteases, was investigated in *Pseudomonas aeruginosa*. Expressed from the *algT-mucABCD* operon, MucD was detected in mucoid (FRD1) and nonmucoid (PAO1) parental strains and also when polar insertions were placed upstream in *algT* or *mucB*. A transcriptional start site for a *mucD* promoter (P*mucD*) was mapped within *mucC*. Expression of single-copy *mucD217*, encoding MucD altered in the protease motif (S217A), was defective in temperature resistance and alginate gene regulation.

Pseudomonas aeruginosa strains causing chronic pulmonary tract infections in cystic fibrosis (CF) patients typically show a highly mucoid phenotype, indicating production of the exopolysaccharide alginate (14). The activation of alginate genes is complex (11, 26). Briefly, the *algT* gene (*algU*), which is essential for alginate production (8, 9), encodes the alternative sigma factor σ^{22}, which has homology to the extracytoplasmic function sigma E (σ^{E}) of *Escherichia coli* (6, 7, 15, 22). The *algT* operon consists of *algT-mucA-mucB-mucC-mucD*. MucA is an anti-sigma factor that sequesters σ^{22} (19, 27), and mutations in *mucA* are common in mucoid *P. aeruginosa* isolates from CF patients (17). MucB (AlgN) is a periplasmic, negative regulator (10, 16, 19). The role of MucC is unclear. MucD is a close homologue of the *E. coli* periplasmic serine protease HtrA (DegP) (3, 24), which is required in *E. coli* for resistance to high temperature and oxidative stress (25). HtrA degrades misfolded periplasmic proteins at elevated temperatures (12) and has a chaperone function at lower temperatures (33). Most organisms have genes for one or more HtrA-like proteases.

The *rpoE* and *algT* operons, where σ^{E} is under the control of anti-sigma RseA and periplasmic RseB, are similar (5, 21). The obvious difference in these operons is the location of the *htrA*/*mucD* gene. In *P. aeruginosa*, *mucD* is in the *algT* operon, and in *E. coli*, *htrA* is unlinked but is under σ^{E} transcriptional control. This interesting difference led us to examine *mucD* regulation and function in *P. aeruginosa*. To investigate this, rabbit polyclonal antibodies (Covance Research Products) were raised to purified His-tagged MucD (Novagen) in order to detect MucD protein by chemiluminescence in immunoblots of proteins from cell lysates separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Two strains were examined: wild-type (nonmucoid) PAO1 and a *mucA22* (mucoid) strain, FRD1, from a CF patient (23). The detection of MucD was not dependent upon alginate production in that both mucoid and nonmucoid strains contained MucD as a prominent band of ~48 kDa, with another less-intense band of lower molecular mass (Fig. 1A, lanes 1 and 5). In *E. coli*, mature HtrA (48 kDa) undergoes partial autocleavage under reducing conditions at the N terminus with the formation of two ~43-kDa truncated polypeptides (12, 31, 32). Mature MucD (48 kDa) in *P. aeruginosa*, with 57% similarity to HtrA, presumably undergoes a similar processing phenomenon. MucD was not observed in the *mucD* mutant, PDO350 (lane 4), showing that the antibodies were specific for MucD. Compared to PAO1 (lane 1), its mucoid *mucA22* derivative PDO300 (lane 3) contained more MucD, probably due to increased *algT* promoter activity (19). Interestingly, a polar insertion in *algT* in PAO1 (lane 2) or in FRD1 (lane 6) did not block production of MucD, although levels were reduced compared to parental strains. Since these polar insertions block transcription of *mucD* from the *algT* promoter (P*algT*), another promoter (P*mucD*) apparently allowed for *mucD* expression, which was not σ^{22} (i.e., *algT*) dependent.

In *E. coli*, disruption of *rpoE* results in compensatory mutation(s) (4). To avoid this possibility with *algT* mutations in *P. aeruginosa*, a PAO1 *mucB*Ω polar mutant (PDO353) was constructed using an *aacCI*Ω polar (gentamicin resistance) cassette (28) and allelic replacement to again examine the expression of *mucD* downstream. Semiquantitative Western analysis (Fig. 1B) showed that this polar insertion also permitted production of MucD at ~43% of the level in PAO1 as determined by densitometry. Thus, P*mucD* expression leads to about half of the amount of MucD in the cell during exponential growth. When primer extension analysis (1) was used, a primer complementary to DNA 165 bases upstream of the *mucD* start codon produced a product that began at nucleotide −245 relative to the start codon of the *mucD* open reading frame (Fig. 2). This placed the start of P*mucD* transcription in the middle of *mucC*.

To evaluate the P*mucD* promoter region, *mucD-lacZ* reporter fusions were constructed in *lacZ* vector pSS223 (34) and tested for β-galactosidase expression (measured in Miller

* Corresponding author. Mailing address: Dept. of Microbiology and Immunology, Virginia Commonwealth University, Medical College of Virginia Campus, 1101 E. Marshall St., 5-047 Sanger Hall, P.O. Box 980678, Richmond, VA 23298-0678. Phone: (804) 828-9728. Fax: (804) 828-9946. E-mail: deohman@vcu.edu.

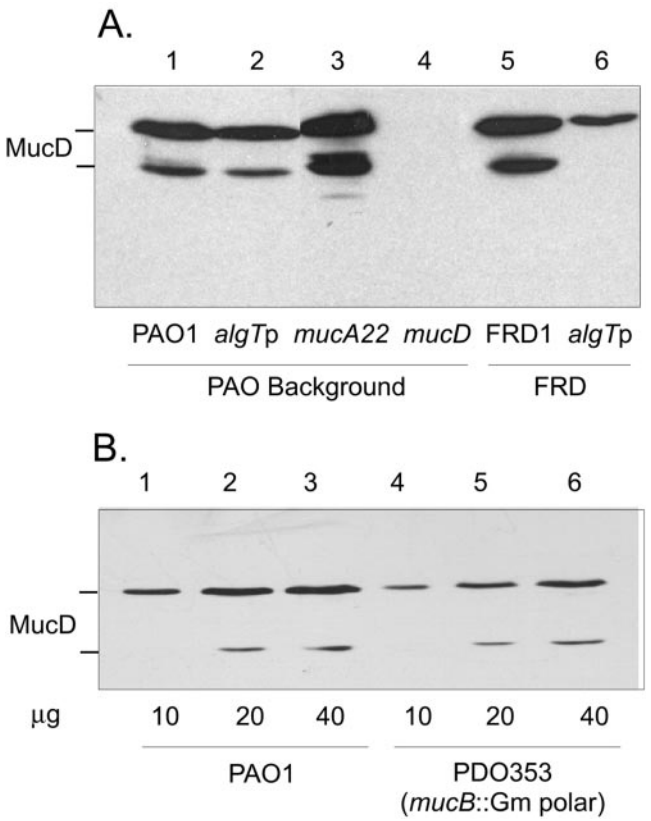

FIG. 1. Western blot analyses of MucD in *P. aeruginosa*. (A) Effect of *algT* polar insertions (*algT*p) on detection of MucD in strains of PAO and FRD backgrounds. Cultures were grown in L broth to an optical density of 600 nm (OD_{600}) of 1.0 and lysed. Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were probed with rabbit MucD antibodies. Lanes: 1, PAO1 (wild type); 2, PDO-LS586 (*algT*::*aacCI*Ω) (29); 3, PDO300 (*mucA22*; mucoid) (18); 4, PDO350 (Δ*mucD*::*aacCI*Ω, mucoid); 5, FRD1 (wild type, *mucA22*, mucoid) (23); 6, FRD440 (*algT*::Tn*501*) (9). MucD protein was detected in all strains except PDO350 (Δ*mucD*). (B) Effect of a polar insertion in *mucB* on downstream *mucD* expression and MucD protein levels. Culturing and immunodetection methods were the same as described for panel A. Samples of PAO1 (10, 20, or 40 μg protein in lanes 1, 2, and 3, respectively) or PDO353 *mucB*::*aacCI*Ω (10, 20, or 40 μg protein in lanes 4, 5, and 6, respectively) were compared. Densitometry (performed in triplicate) was used to determine cellular levels of MucD protein.

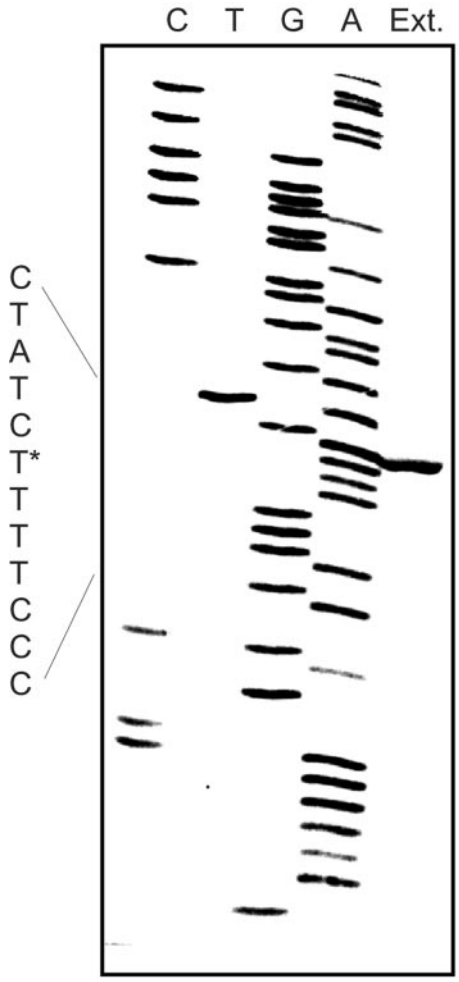

FIG. 2. Results of a primer extension (Ext.) analysis to determine the transcriptional initiation site of *mucD* transcripts from P*mucD*. Total cellular RNA was isolated (QIAGEN RNeasy) from PAO1 cultures (L broth; OD_{600} of 1.0). A primer (5′ACGATGATCAGAG GTTCGACAAGGCCCG) complementary to the region of DNA 165 bases upstream of the *mucD* ATG start codon was end labeled with [γ-^{32}P]ATP (Perkin-Elmer), annealed to 25 μg of RNA, and extended with AMV reverse transcriptase (New England Biolabs). The resulting extension products were loaded onto an 8% polyacrylamide–7 M urea gel. An adjacent sequencing ladder (CTGA) was prepared using a USB Thermo Sequenase ^{33}P-radiolabeled terminator cycle sequencing kit (USB Corp.) and the same oligonucleotide. Products were revealed by autoradiography. The 5′ end of the extension product for P*mucD* aligned with a T*, located at −245 bp and within *mucC*.

units) in PAO1. Each plasmid had the same 3′ fusion joint, 62 nucleotides into the *mucD* coding region, but with various lengths of DNA upstream (Fig. 3). The largest construct (pLW38a) contained *mucB-mucC-mucD′-lacZ* and showed high activity (~1,600 U), even though it did not contain the upstream P*algT*. With this fragment in reverse orientation to *lacZ*, β-galactosidase activity was less than 1% of that seen with pLW38a, indicating that the background was low. The constructs with just *mucB* deletions (pLW41, pLW40, pLW42, and pLW43) showed little or no change in maximum *mucD-lacZ* activity. Further deletions in the *mucC* gene (pLW44 and pLW70) showed some deleterious effects. Interestingly, having only 29 bp upstream of the P*mucD* transcriptional start site (pLW70) still allowed 39% of maximal P*mucD-lacZ* activity, but a deletion of the start site (pLW39) reduced β-galactosidase activity to 3% of the maximum. Thus, the DNA requirement for P*mucD* expression was small. When PAO-derived mutants with defects in known regulators for alginate production (e.g., *algT*, *mucA*, *mucB*, *mucD*, *algB*, *kinB*, and *rpoS*) were used as hosts for pLW43 (i.e., P*mucD-lacZ*), there was no observable effect on β-galactosidase activity, indicating that P*mucD* was not under their control (in L broth at log phase; data not shown). Since *mucD* mutants are temperature sensitive (2), the effect of heat stress on P*mucD* was also investigated. The growth temperature of PAO1(pLW43) was shifted from 37°C to 44°C, but again there was no measurable effect on the expression β-galactosidase activity for P*mucD-lacZ*, sug-

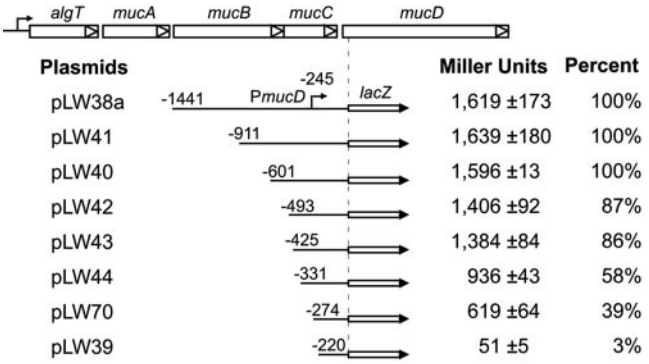

FIG. 3. Deletion analysis of DNA for relative P*mucD* expression. A series of *lacZ* transcriptional fusions were constructed using the *lacZ* transcriptional fusion vector pSS223 (34) with the 3′ end at +60 bp into the *mucD* coding sequence and with various amounts of upstream DNA. Plasmids were transferred to PAO1 by conjugation and grown in L broth to an OD_{600} of 1.0, and aliquots were tested for β-galactosidase activity (Miller units). Activity from pLW38a containing the *mucB-mucC* region upstream of *mucD* was assigned a value of 100%.

gesting that P*mucD* was not heat shock inducible (data not shown).

On the basis of the homology MucD shares with HtrA (DegP) and the temperature-sensitive phenotype in both mutant backgrounds, MucD is likely to be a protease involved in the proteolysis of misfolded periplasmic proteins, although this activity has not yet been directly demonstrated. We took advantage of the well-defined His, Asp, and Ser catalytic triad known for HtrA-like serine proteases (30) to construct the *mucD217* allele by oligonucleotide mutagenesis, which encoded MucD-S217A, substituted in the enzymatic motif. This was cloned into a suicide vector (pUC19) and crossed into the PDO350 chromosome so that the resulting strain (PDO354) would express *mucD217* in single copy numbers in the *algT* operon (Fig. 4), and MucD-S217A was observed using Western blot analysis (data not shown). When strains were compared for growth in L broth at 37°C, the $MucD^+$, $MucD^-$, and MucD-S217A strains grew equally well (Fig. 5A). In contrast, at 43°C the $MucD^-$ and MucD-S217A strains showed markedly reduced growth compared to PAO1 (Fig. 5B). Similar results were observed at 37°C and 43°C when the *mucD* defect in PDO350 was complemented from a multicopy plasmid with wild-type *mucD* (pLW37) or with *mucD217* (pLW67) (data not shown). These results were similar to those seen in *E. coli* (30), suggesting that MucD proteolytic activity was also required for normal growth at elevated temperatures.

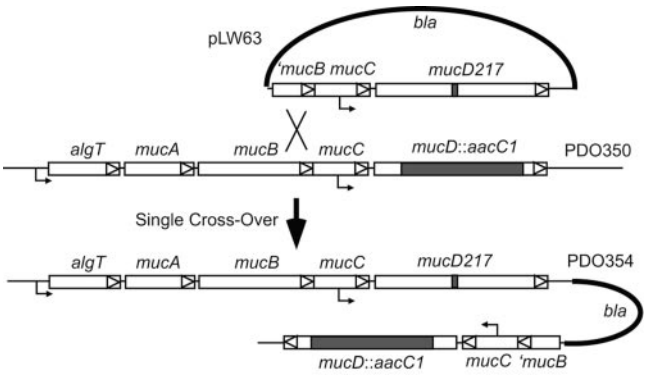

FIG. 4. Construction of PDO354 with *mucD217* expressing MucD-S217A with a defect in the conserved protease motif in single copy from the chromosome. Oligonucleotide mutagenesis as described previously (20) was used with pLW1 (pUC19 with "*mucB mucC mucD lep*" [2.5 kb]) to form pLW63 with the *mucD217* allele. Suicide plasmid pLW63 was transferred to PDO350 (Δ*mucD*::*aacCI*Ω, mucoid), with selection for carbenicillin (*bla*) resistance to mediate integration into the chromosome by homologous recombination. The resulting strain, PDO354, was verified by PCR for correct insertion in the chromosomal operon, and production of MucD-S217A was confirmed by Western analysis.

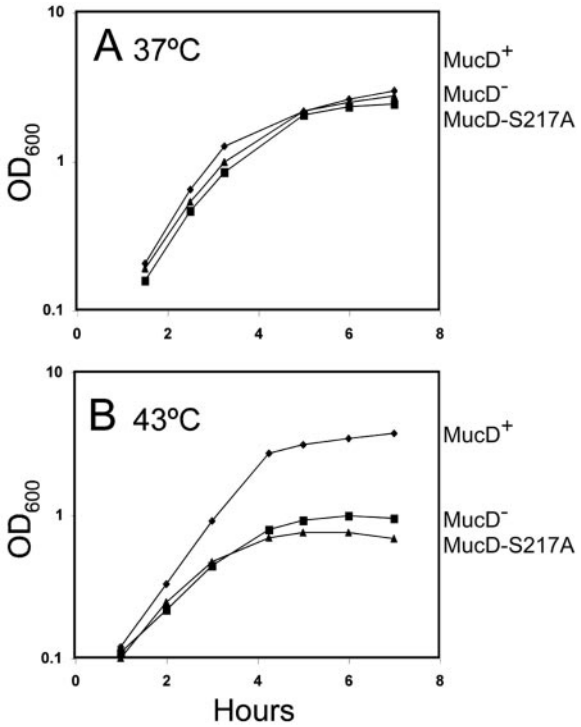

FIG. 5. Comparison of the growth of PAO isogenic strains at 37°C (A) or 43°C (B) expressing single-copy $mucD^+$, Δ*mucD*, or *mucD217* alleles. Growth was monitored by OD_{600}, and data shown are representative of three comparable experiments. Symbols indicate the following strains: ◆, PAO1 $mucD^+$; ■, PDO350 Δ*mucD*; and ▲, PDO354 *mucD217*.

A *mucD* null mutant also produces alginate when grown on *Pseudomonas* isolation agar (2). To determine whether MucD's proteolytic activity was involved in the control of alginate production, we measured the amount of alginate (13) produced by the $MucD^+$, $MucD^-$, and MucD-S217A strains at 37°C. A *mucA*::*aacCI* (nonpolar) mucoid mutant (PDO351) was also included for comparison. PAO1 produced no detectable alginate, but the *mucA*, *mucD*, and *mucD217* mutant strains all produced high levels of alginate (Table 1). Thus, MucD's proteolytic motif was important for its regulation of alginate production as well as temperature resistance. However, when *mucD217* was expressed from a high-copy-number plasmid in the *mucD* null mutant, the control of alginate gene expression was restored (Table 1), suggesting that MucD has a second function only observed at higher levels. This may be similar to

TABLE 1. Alginate production by *mucD* mutants[a]

Strain	Genotype	Alginate
PAO1	*mucD*$^+$	BD
PDO351	*mucA*::*aacCI* (nonpolar)	41.75 ± 12.8
PDO350	*mucD*::*aacCI*Ω	58.42 ± 6.79
PDO354	*mucD217*	44.82 ± 4.64
PDO350(pLW37)	*mucD*$^+$ (multicopy)	BD
PDO350(pLW67)	*mucD217* (multicopy)	BD

[a] Derivatives of *P. aeruginosa* PAO1 (wild type) were grown on PIA plates for 48 h at 37°C, and then bacteria and alginate were harvested. The *aacCI* (gentamicin resistance) cassettes were from Schweizer (28). Plasmids pLW37 and pLW67 were vector pASK-IBA3 (Sigma) containing *oriV*(SF) for multi-copy, broad-host-range replication (34) and either *mucD*$^+$ and *mucD217*, respectively. Alginate was measured in milligrams per milligram of wet cell weight by use of a colorimetric assay (13). BD means that alginate levels were below detection, indicating that the *mucD* allele was still functioning as a negative regulator.

HtrA in *E. coli*, which is known to have both proteolytic and chaperone functions (33).

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