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Two Distinct Loci Affecting Conversion to Mucoidy in *Pseudomonas aeruginosa* in Cystic Fibrosis Encode Homologs of the Serine Protease HtrA

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Conversion to a mucoid, exopolysaccharide alginate-overproducing phenotype in *Pseudomonas aeruginosa* is associated with chronic respiratory infections in cystic fibrosis. Mucoidy is caused by *muc* mutations that derepress the alternative σ factor AlgU, which in turn activates alginate biosynthetic and ancillary regulatory genes. Here we report the molecular characterization of two newly identified genes, *algW* and *mucD*, that affect expression of mucoidy. The *algW* gene, mapping at 69 min, was isolated on the basis of its ability to suppress mucoidy and reduce transcription of the alginate biosynthetic gene *algD*. The predicted primary structure of AlgW displayed similarity to HtrA (DegP), a serine protease involved in proteolysis of abnormal proteins and required for resistance to oxidative and heat stress in enteric bacteria. Inactivation of *algW* on the chromosome of the wild-type nonmucoid strain PAO1 caused increased sensitivity to heat, H_2O_2, and paraquat, a redox cycling compound inducing intracellular levels of superoxide. This mutation also permitted significant induction of alginate production in the presence of subinhibitory concentrations of paraquat. Two new genes, *mucC* and *mucD*, were identified immediately downstream of the previously characterized portion (*algU mucA mucB*) of the gene cluster at 67.5 min encoding the alternative σ factor AlgU and its regulators. Interestingly, the predicted gene product of *mucD* also showed similarities to HtrA. Inactivation of *mucD* on the PAO1 chromosome resulted in conversion to the mucoid phenotype. The mutation in *mucD* also caused increased sensitivity to H_2O_2 and heat killing. However, in contrast to *algW* mutants, no increase in susceptibility to paraquat was observed in *mucD* mutants. These findings indicate that *algW* and *mucD* play partially overlapping but distinct roles in *P. aeruginosa* resistance to reactive oxygen intermediates and heat. In addition, since mutations in *mucD* and *algW* cause conversion to mucoidy or lower the threshold for its induction by reactive oxygen intermediates, these factors may repress alginate synthesis either directly by acting on AlgU or its regulators or indirectly by removing physiological signals that may activate this stress response system.

Pseudomonas aeruginosa is an opportunistic pathogen causing acute infections in individuals with compromised defense mechanisms, such as burned or neutropenic patients. Another notorious and in many aspects unique infection caused by *P. aeruginosa* is its characteristic association with the respiratory tract of patients with cystic fibrosis (CF) (8, 41, 53). This chronic infection differs from the acute disseminated disease by being localized almost exclusively to the lumen of the respiratory tract (1, 26, 53). Furthermore, the protracted colonization of the respiratory tract is accompanied by a distinct set of phenotypic changes of the bacterium (8, 53). For example, *P. aeruginosa* strains colonizing CF patients often convert to a mucoid, exopolysaccharide alginate-overproducing phenotype (17), lose lipopolysaccharide O side chains (18), display reduced motility (33), show lowered susceptibility to nonopsonic phagocytosis (53), and are inferior in mouse models of bacteremic virulence (33). These phenotypic alterations, referred to as the CF phenotype (53) or chronic infection phenotype (8), are believed to be the cumulative product of a series of genetic changes which occur during the protracted course of colonization of the CF respiratory tract (8, 41).

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Recently, the genetic mechanism of conversion to mucoidy in *P. aeruginosa* has been partially elucidated (35, 37). This work had its foundation in the seminal studies by Fyfe and Govan, who have established that mutations (termed *muc* for mucoidy), mapping in at least two different loci on the *P. aeruginosa* chromosome (Fig. 1A), are responsible for alginate overproduction (14). One group of *muc* mutations is cotransducible with *pruAB* at 67.5 min, while the mapping data for the other locus are consistent with its position between *hisI* at 69 min and *proB* at 71 min (14, 17). More recent work has confirmed the location for a subset of *muc* mutations at 67.5 min (13, 34) and their effects on expression of alginate-specific genes (6). The molecular characterization of the genes corresponding to *muc* alleles cotransducible with *pruAB* has been achieved only recently (35–38). These genes comprise a cluster consisting of *algU*, *mucA*, and *mucB* (35, 36) and extended here to include the newly identified *mucC* and *mucD* genes (Fig. 1A). The *algU mucABCD* cluster is the major site for *muc* mutations mapping at 67.5 min (37). Several specific *mucA* alleles from CF and laboratory isolates have been sequenced and shown to cause mucoidy by gene replacements in wild-type nonmucoid strains (37). The *mucA* gene, along with the downstream gene *mucB* (also known as *algN* [16]), plays a negative regulatory role (16, 36). Inactivation of the *mucA* or *mucB* gene (36, 37) results in a high-level transcription of alginate biosynthetic and ancillary regulatory genes, such as *algD* and *algR* (6), respectively, leading to the mucoid phenotype (36, 37). This activation is due to the initiation of transcription

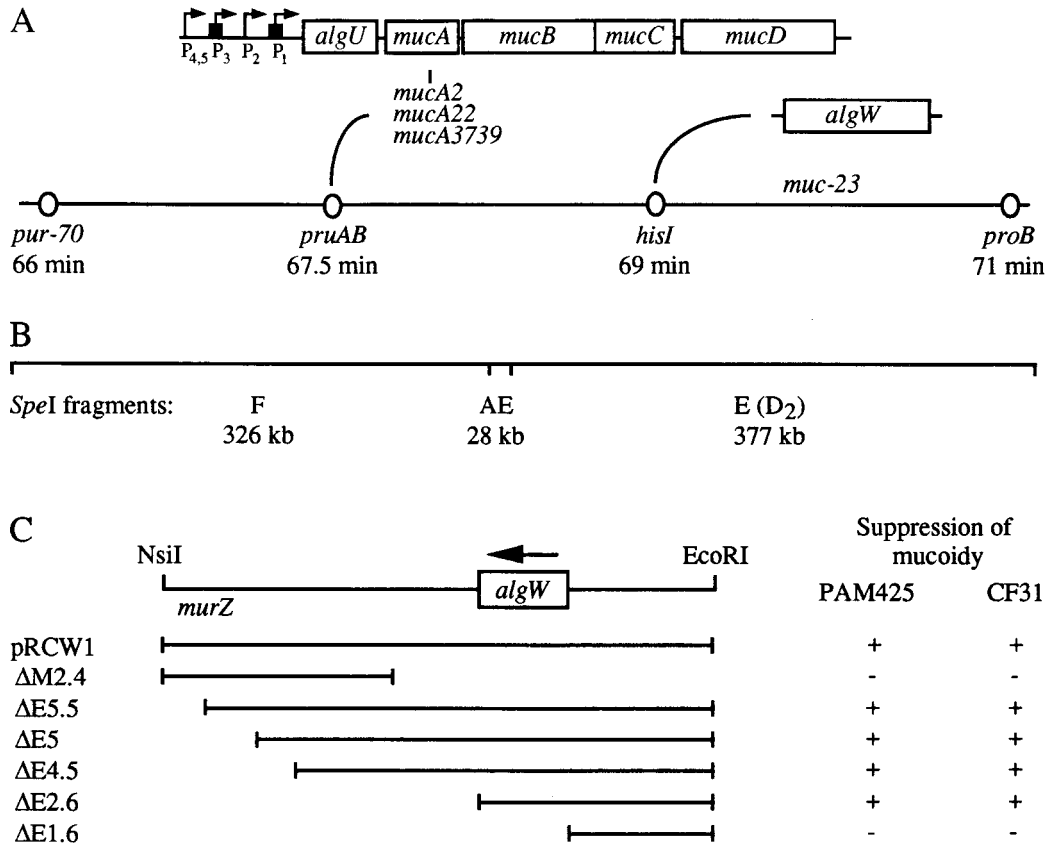

FIG. 1. Genetic loci in the late region of the *P. aeruginosa* chromosomal map that control conversion to mucoidy and subcloning of *algW*. (A) Organization of the *algU mucABCD* gene cluster and genetic linkage of the *mucA* and *muc-23* mutations to auxotrophic markers at 67.5 and 69 min. P_1 to P_5, promoters upstream of the *algU* gene. P_1 and P_3 are transcribed by AlgU, an alternative σ factor 66% identical to and functionally interchangeable with the *E. coli* σ^E (7, 58). The previously genetically mapped *muc* mutations (14) are shown; additional *mucA* mutations identified in CF strains (37) are not included, but some of them are identical to the *mucA22* mutation. In *mucA* (36, 37), *mucB* (36), or, as shown in this work, *mucD* mutants, transcription from AlgU-dependent promoters of alginate biosynthetic and regulatory genes is increased, resulting in the mucoid phenotype. (B) Physical map (*Spe*I fragments) of the late region. The genetic and physical maps are not to scale; *algU mucABCD* and *pruAB* are on *Spe*I fragment F, while *algW* and *hisI* are on fragment E (D_2). (C) Deletion subcloning of *algW*. A set of overlapping deletions was generated as described in Materials and Methods. The indicated fragments were subcloned into the broad-host-range vector pVDZ′2, the resulting constructs were conjugated into the mucoid strains PAM425 and CF31, and exconjugants were scored for suppression of the mucoid phenotype. +, suppression (nonmucoid phenotype); −, no suppression (mucoid phenotype). *murZ*, position of a close homolog of *murZ* involved in peptidoglycan synthesis in *E. coli* (GenBank accession numbers M92358 and M76452).

directed by the alternative sigma factor AlgU (35, 38, 58), also known as AlgT (9, 57), which is encoded by the first gene in the cluster at 67.5 min (Fig. 1A).

AlgU is the *P. aeruginosa* equivalent (7, 38, 58) of σ^E, the extreme heat shock sigma factor from enteric bacteria (11). AlgU and σ^E are functionally interchangeable (58). σ^E controls a number of stress-responsive genes, e.g., *rpoH*, which encodes the major heat shock sigma factor σ^{32} (11), and *htrA* (*degP* [55]), a gene necessary for resistance to heat killing in *Escherichia coli* (29, 30) and for full virulence and resistance to reactive oxygen intermediates in *Salmonella typhimurium* (22). σ^E was initially characterized only at the protein level as an alternative sigma factor present in cells exposed to extreme temperatures (11). However, very little was known about the control of its activity; this lack of information can be partially attributed to the fact that the gene encoding σ^E (*rpoE*) was not identified until very recently (7, 20, 32, 38, 43, 47). In contrast, recent progress permitted uncovering of several elements directly controlling AlgU (35–38). Its regulation is complex, and it is likely that factors in addition to those in the *algU mucABCD* cluster affect its function. For example, not all *muc* mutations map in the region linked to *pruAB* at 67.5 min (14). In the initial report on *algU* sequence analysis (35), in addition to the cosmid carrying the corresponding wild-type locus, nine other cosmids isolated from the reference genomic library of the nonmucoid standard genetic strain PAO1 were found to be capable of affecting mucoidy in a strain-dependent manner. One of the cosmids from this collection, pMO011809, carries an insert originating from the chromosomal region linked to *hisI* at 69 min (Fig. 1A). To continue our systematic analysis of conversion to mucoidy and further define elements regulating AlgU, we characterized the *algW* gene responsible for the suppression of alginate production by pMO011809. We also show that both *algW* and *mucD* encode elements homologous to the serine endopeptidase HtrA (DegP) (30, 55) and that both genes participate in the regulation of mucoidy.

MATERIALS AND METHODS

Media and growth conditions. *E. coli* was grown on LB supplemented with ampicillin (100 μg/ml), kanamycin (25 μg/ml), gentamicin (13 μg/ml), tetracycline (10 μg/ml), or paraquat when required. *P. aeruginosa* was grown on *Pseudomonas* isolation agar (PIA; Difco) or in LB supplemented with tetracycline (300 or 50 μg/ml, respectively), gentamicin (150 μg/ml), carbenicillin (300 μg/ml), or 1 mM isopropylthiogalactopyranoside (IPTG) when required. All incubations

TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Relevant genotype and properties[a]	Source or reference(s)
P. aeruginosa		
PAO1	Prototroph Alg^{-wt}	B. Holloway
PAO578(I)	*mucA22* Alg^{+c} (type I)	14, 48
PAO578(II)	*mucA22 sup-2* Alg^{+m} (type II)	48
PAO6854	PAO1 Alg^{+PO} *algW*::Tc^r	This work
PAO6859	PAO578(II) *algW*::Tc^r	This work
PAO6860	PAO1 Alg^{+m} *mucD*::Gm^r	This work
PAO6861	PAO578(II) $algD^+$ *algD-xylE*	This work
PAM425	*mucA3739* Alg^{+m}	This work
PAM426	PAM425 $algD^+$ *algD-xylE*	This work
CF31	Alg^{+m}, CF isolate	This work
Plasmids		
pVDZ′2	IncP1 mob^+ tra^+ *lacZ′* Tc^r	35
pCMobA	pHC79 mob^+	40
pKI11	pUC18 1.4-kb Tc^r cassette	S. Lory
pKI11G	pUC18 Gm^r cassette	S. Lory
pT7-5	ColE1 Ap^r ϕ10 promoter-*Eco*RI-polylinker-*Hin*dIII	S. Tabor
pT7-6	ColE1 Ap^r ϕ10 promoter-*Hin*dIII-polylinker-*Eco*RI	S. Tabor
pHYDX	pCMobA *algD-xylE* Ap^r (Cb^r)	58
pMO012046	$algU^+$ $mucABCD^+$ (cosmid clone in pLA2917)	35
pDMU4/76	$algU^+$ $mucA^+$ $mucB^+$ in pVDZ′2	35
pMO011809	$algW^+$ $hisI^+$ (cosmid clone in pLA2917)	35, 45
pRCW1	$algW^+$ (*Hin*dIII-*Nsi*I fragment in pVDZ′2)	35
pCBG4.5	$algW^+$ as ΔE4.5 in pVDZ′2 Gm^r	This work
pCBE2.6	$algW^+$ as ΔE2.6 in pVDZ′2	This work
pCBE1.6	$algW^+$ as ΔE1.6 in pVDZ′2	This work
pCBM2.4	$algW^+$ as ΔM2.4 in pVDZ′2	This work
pCB52.6	ΔE2.6 in pT7-5	This work
pCB51.6	ΔE1.6 in pT7-5	This work
pCB64.5	ΔE4.5 in pT7-6	This work
pCB62.6	ΔE2.6 in pT7-6	This work
pCB61.6	ΔE1.6 in pT7-5	This work
pCBX2.6	pUC12 mob^+ ΔE2.6 *algW*::Tc^r Ap^r (Cb^r)	This work
pJMSD1	pUC12 mob^+ *mucD*::Gm^r Ap^r (Cb^r)	This work
p*tac-mucD*$^+$	pVDtac39 IncQ/P4 mob^+ *tac-mucD*$^+$ $lacI^q$ Ap^r (Cb^r)	This work

[a] Alg^{-wt}, wild-type nonmucoid phenotype: Alg^{+c}, mucoid phenotype resulting from constitutive alginate production; Alg^{+m}, medium-dependent mucoid phenotype; Alg^{+PO}, increased alginate production in the presence of paraquat. ΔE1.6 to ΔE4.5 and ΔM2.4 are deletion products of the *Hin*dIII-*Nsi*I insert from pRCW1 (see Fig. 1C).

excluding those for heat killing assays were carried out at 30, 37, or 42°C. For *P. aeruginosa* virulence studies, bacteria were grown in LB to an optical density at 600 nm (OD_{600}) of 0.4. The culture was pelleted by centrifugation at 3,000 × *g* and washed twice in cold 0.01 M phosphate-buffered saline (PBS; pH 7.4). The final cell pellet was resuspended in 20 ml of 15% glycerol in 0.01 M PBS and frozen at −80°C in 1-ml aliquots. The stocks were thawed for inoculation of animals, and the viable cell counts were determined at the time of infection by plating on LB. For growth patterns at different temperatures, overnight cultures grown at 30°C were diluted to the same OD_{590}, and the OD was monitored during continuous incubation at the indicated temperatures.

Bacterial strains and plasmids. Strains of *P. aeruginosa* and plasmids used in this study are shown in Table 1. PAO6854 and PAO6859 were derivatives of PAO1 and PAO578(II) constructed by gene replacements of the chromosomal *algW* gene with *algW*::Tc^r. This was carried out via homologous recombination between the chromosome and the insert of the nonreplicative plasmid pCBX2.6. pCBX2.6 was introduced into strains to be mutated via triparental conjugation, and tetracycline-resistant (Tc^r) exconjugants were selected on PIA supplemented with tetracycline. Tc^r carbenicillin-sensitive (Cb^s) clones were identified by replica plating, and gene replacements were confirmed in candidate strains by Southern blot hybridizations. Strain PAO6860 was derived from PAO1 by using the same procedure to knock out the *mucD* gene, using plasmid pJMSD1. Strains PAM426 and PAO6861, carrying *algD-xylE* fusions integrated on the chromosomes of PAM425 and PAO578(II), respectively, were constructed by using plasmid pHYDX as previously described (58).

The cosmid clones pMO011809 and pMO012046 have been described previously (35, 44). All pCB plasmids contained deletion derivatives of the 6-kb *Hin*dIII-*Nsi*I fragment from pRCW1 (originating from pMO011809) generated by subcloning this fragment into M13 bacteriophage mTM010 and subjecting it to the deletion procedure. Plasmid pCBX2.6 was generated by transferring the ΔE2.6 *Hin*dIII-*Eco*RI deletion derivative from mTM010 into pUC12. The resulting plasmid, termed pCBU2.6, was digested with *Nru*I and linkered with *Not*I, and a *Not*I-modified Tc^r cassette from pKI11 was inserted. This construct was digested with *Eco*RI, and a 1.4-kb *Eco*RI fragment with *mob* from pCMobA (40) was inserted. Plasmids pCBE2.6, pCBE1.6, and pCBM2.4 were constructed by cloning the respective ΔE2.6, ΔE1.6, and ΔM2.4 deletion products (Fig. 1C) as *Hin*dIII-*Eco*RI fragments into pVDZ′2. For pCBG4.5, the 4.5-kb fragment from ΔE4.5 was cloned as a *Hin*dIII-*Eco*RI fragment into pVDZ′2. This construct was digested with *Eco*RI, and a 1.6-kb *Eco*RI-modified gentamicin resistance (Gm^r) cassette was inserted. To generate pCB52.6 and pCB51.6, the *Hin*dIII-*Eco*RI fragments ΔE2.6 and ΔE1.6, respectively, were cloned into pT7-5. pCB64.5, pCB62.6, and pCB61.6 were generated by cloning the ΔE4.5, ΔE2.6, and ΔE1.6 fragments, respectively, into pT7-6. To complete the sequence of *mucC* and *mucD*, a 5-kb *Eco*RI fragment from pMO011809 was subcloned into pUC12. For insertional inactivation of *mucD* on the chromosome of PAO1, a 0.8-kb PCR product generated with the primers UL-9 (5′GCGACCTGAGCCTGCG3′) and UR-28 (5′AGGACCCATTCGCCCAC3′) was subcloned into pCRII (InVitrogen), excised as an *Eco*RI fragment, and subcloned into pUC12. The resulting construct was further modified by inserting the Gm^r cassette from pKI11G into the *Xho*I site within *mucD*, followed by cloning of *mob* from pcMobA into the *Hin*dIII site, to generate pJMSD1. Plasmid p*tac-mucD*$^+$ was constructed by subcloning the 5-kb *Eco*RI fragment with *mucD* from pMO011809 into pVDtac39.

Genetic manipulations and DNA methods. Triparental bacterial conjugations using pRK2013 as a helper plasmid were carried out as previously described (40). Generation of unidirectional deletion clones in M13 bacteriophage was as previously described (35). Nucleotide sequencing was performed by a 7-deaza-dGTP modification of the chain termination method, using deletion clones with universal M13 or custom-made primers as previously described (35).

T7 RNA polymerase/promoter system and gene product visualization. The polypeptide products of the cloned genes were visualized by expression in a T7 system in *E. coli*. A temperature-inducible T7 expression system was used (plasmid vectors pT7-5 and pT7-6 and T7 RNA polymerase encoded by pGP1-2), and proteins were labeled with Expre^{35}S^{35}S protein labeling mix (1,000 Ci/mmol; DuPont NEN) with previously described modifications (35). Proteins were separated on sodium dodecyl sulfate–12% polyacrylamide gels. ^{14}C-labeled methylated proteins (Amersham) were used as molecular weight standards. Gels were fixed in 10% acetic acid, washed with H_2O, and impregnated with 1 M salicyclic acid, and bands representing radiolabeled polypeptides were detected by autofluorography at −70°C.

Susceptibility to killing with reactive oxygen intermediates and heat. Sensitivity to paraquat and H_2O_2 was determined by measuring the diameter of the zone of killing surrounding disks (6-mm diameter; BBL) impregnated with 10 μl of either 2% paraquat or 6% H_2O_2. Disks were placed on 2 ml of soft agar overlay (0.6%) containing 100 μl of *P. aeruginosa* overnight cultures and solidified on top of 25 ml of 1.5% agar in LB. For heat killing, bacterial cultures were grown to an A_{595} of 0.35 and then incubated at 50°C for the indicated times. Appropriate cell dilutions were plated on LB in triplicates, and viable cells were scored as CFU. Survival was expressed as the percentage of input cells which retained viability. Three independent samples were used for each time point.

Enzyme and alginate assays. Catechol 2,3-dioxygenase, the gene product of *xylE*, was assayed in sonic extracts as previously described (36). Alginate production was assayed by using previously published methods (35).

Determination of LD_{50} for *P. aeruginosa* in the neutropenic mouse model. The animal model of fatal *P. aeruginosa* sepsis in experimentally induced neutropenia in mice was used with previously described modifications (19). C56BL/6 mice (5 to 6 weeks old; average weight, 22.7 g) were rendered neutropenic by three intraperitoneal injections of 200 μg of cyclophosphamide per ml every other day. Two to four days following the final dose of cyclophosphamide, the mice were challenged by intraperitoneal injection of bacteria in 0.2 ml of PBS. Mice in groups of five for each infectious dose tested were injected with inocula ranging from 10 to 10,000 CFU in 10-fold increments. The 50% lethal dose (LD_{50}) was determined by the Reed-Muench method (45).

Nucleotide sequence accession numbers. The sequence of *algW* shown in Fig. 3 has been assigned GenBank accession number U29172. The *mucCD* sequence presented in Fig. 8 is deposited under GenBank accession number U32853. The *mucA3739* mutation has been deposited under GenBank accession number U33272.

RESULTS

Suppression of mucoidy and subcloning of *algW*. In the initial communication on the isolation and characterization of *algU*, we also reported that several other cosmids from the reference genomic library of the wild-type nonmucoid strain

TABLE 2. Suppression of mucoidy in *P. aeruginosa* by plasmid-borne *algW*

Strain[a]	Phenotype[b] (genotype)	Colony morphology[c]		Suppression of mucoidy[d] with plasmid[e]:			
		PIA	LB	pMO011809	pCBE2.6	pDMU4/76	pVDZ′2
PAM425	Alg^{+m} (*mucA3739*)	M	NM	+	+	+	−
PAO578(I)	Alg^{+c} (*mucA22*)	M	M	−	−	+	−
PAO578(II)	Alg^{+m} (*mucA22 sup-2*)	M	NM	+	+	+	−
CF31	Alg^{+c}	M	M	+	+	+	−

[a] PAM425 is a cross between a mucoid clinical *P. aeruginosa* isolate (Ps3739) and PAO. The *mucA* mutation in PAM425 (*mucA3739*) mapped in this work is described in the text and the legend to Fig. 6. PAO578(I) is identical to PAO578 (14, 48), a strain carrying the previously characterized mutation *muc-23*. PAO578(II) is a spontaneous derivative of PAO578(I) with an attenuated mucoid phenotype. CF31 is a mucoid isolate from a patient with CF.

[b] Alg^{+c}, mucoid phenotype resulting from constitutive alginate production; Alg^{+m}, medium-dependent mucoid phenotype.

[c] Scored as mucoid (M) or nonmucoid (NM) on PIA and LB.

[d] Scored on PIA as loss of mucoid phenotype (+) or no effect (−).

[e] pMO011809, cosmid with the *algW* locus from PAO1; pCBE2.6, 2.6-kb fragment with *algW* in pVDZ′2; pDMU4/76, 2.5-kb fragment with the wild-type *algU mucA mucB* cluster in pVDZ′2; pVDZ′2, broad-host-range subcloning vector.

PAO1 affect alginate production in mucoid *P. aeruginosa* isolates. One of the cosmids, pMO011809, contains an insert originating from the 377-kb *Spe*I fragment E (alternative name, D_2) (21) tightly linked to the *hisI* marker at 69 min (Fig. 1A). The *algW* locus carried on pMO011809 suppresses mucoidy in a subset of mucoid *P. aeruginosa* strains which include PAM425 (carrying a *muc* mutation transferred from a mucoid CF isolate, Ps3739, into the PAO background [34]) and a mucoid clinical isolate, CF31 (Table 2 and Fig. 1C). These strains were used as indicator strains to delimit the region responsible for suppression of mucoidy. The *algW* region was first subcloned on the broad-host-range vector pVDZ′2 as a 6-kb *Hin*dIII-*Nsi*I fragment from pMO011809 (35, 44). This insert was subjected to deletion analysis (see Materials and Methods) by cloning the region into an M13 bacteriophage and generating a series of unidirectional overlapping deletions by using a previously described strategy (35). The deletion products were then subcloned into the broad-host-range vector pVDZ′2 and conjugated into PAM425 and CF31. The resulting exconjugants were scored for alginate production and mucoid phenotype. The results of these studies (Fig. 1C) placed one end of the complementing ability between the subclones ΔE2.6 (the last clone suppressing mucoidy) and ΔE1.6 (a deletion that abrogated the ability to suppress mucoidy). No suitable deletions from the other end could be generated in repeated attempts.

Expression analysis of *algW* and detection of its gene product. To determine the number of potential genes in the region corresponding to *algW* and to further delimit this locus, different deletion fragments were subcloned into the vectors pT7-5 and pT7-6, and gene products were detected by expression analysis in a T7 system (see Materials and Methods). The results of these analyses are shown in Fig. 2. The 4.5-kb region containing *algW* and the adjacent genes encoded polypeptides of 43.3 kDa (P43.3), 40.8 kDa (P40.8), 38 kDa (P38), and 29.5 kDa (P29.5). The deletion product ΔE2.6, which still suppresses mucoidy, produced only one detectable polypeptide (P43.3) when cloned in pT7-5 (Fig. 2A, lane 1; Fig. 2B, construct 1). P43.3 disappeared when the deletion product ΔE1.6, no longer capable of suppressing mucoidy (Fig. 1C), was used instead of ΔE2.6 (Fig. 2A, lane 2; Fig. 2B, construct 2). Further expression analysis of the constructs shown in Fig. 2 confirmed assignment of P43.3 to *algW*, allowed determination of the direction of *algW* transcription, and permitted its localization between the genes encoding P40.8 and P38 on one side and P29.5 on the other.

The predicted gene product of AlgW shows homology to HtrA(DegP) and other HtrA-like proteins. The region encompassing the endpoints of deletions ΔE2.6 and ΔE1.6 and extending downstream and upstream of these positions was subjected to sequence analysis. Figure 3 shows the complete nucleotide sequence of the *algW* gene with the coding capacity for a predicted polypeptide of 41,558 Da. This is in a relatively good agreement with the apparent molecular mass of AlgW (43.3 kDa) determined from its electrophoretic mobility. Using global similarity analyses, AlgW was found to be 38% identical (50% similar) to HtrA (DegP), a serine protease from *E. coli*

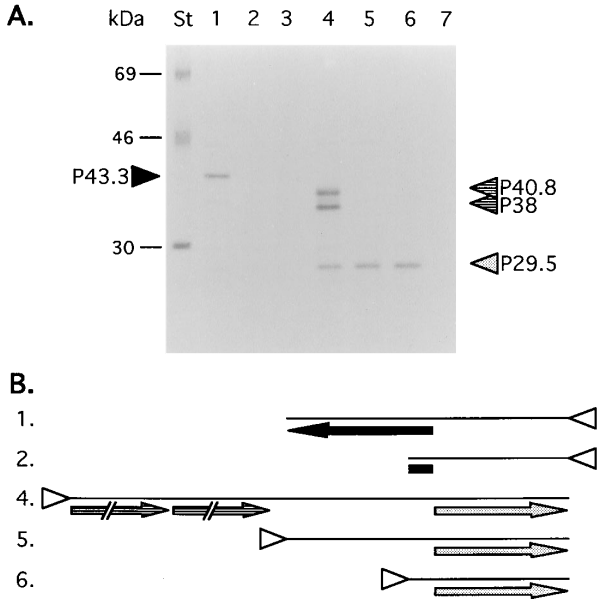

FIG. 2. T7 expression analysis of *algW* and adjacent genes. (A) Polypeptides encoded by different deletion derivatives of the *algW* region. Different deletion products (see Fig. 1C) were subcloned into pT7-5 or pT7-6, and expression analysis was carried out as described in Materials and Methods. Lanes: St, ^{14}C-labeled protein standard (Amersham); 1, ΔE2.6 cloned in pT7-5; 2, Δ1.6 cloned in pT7-5; 3, pT7-5 vector alone; 4, ΔE4.5 cloned in pT7-6; 5, ΔE2.6 cloned in pT7-6; 6, ΔE1.6 cloned in pT7-6; 7, pT7-6 vector alone. The lanes in panel A and constructs in panel B have matching numbers. Gene products indicated by triangles have matching patterns with the genes assigned in panel B. (B) Inserts and their orientations relative to the T7 promoter (open triangle) used for constructs represented in panel A. Filled box and arrow, *algW*; stippled arrows, gene encoding P29. The order of genes encoding P38 and P49.8 is not known (broken striped arrows).

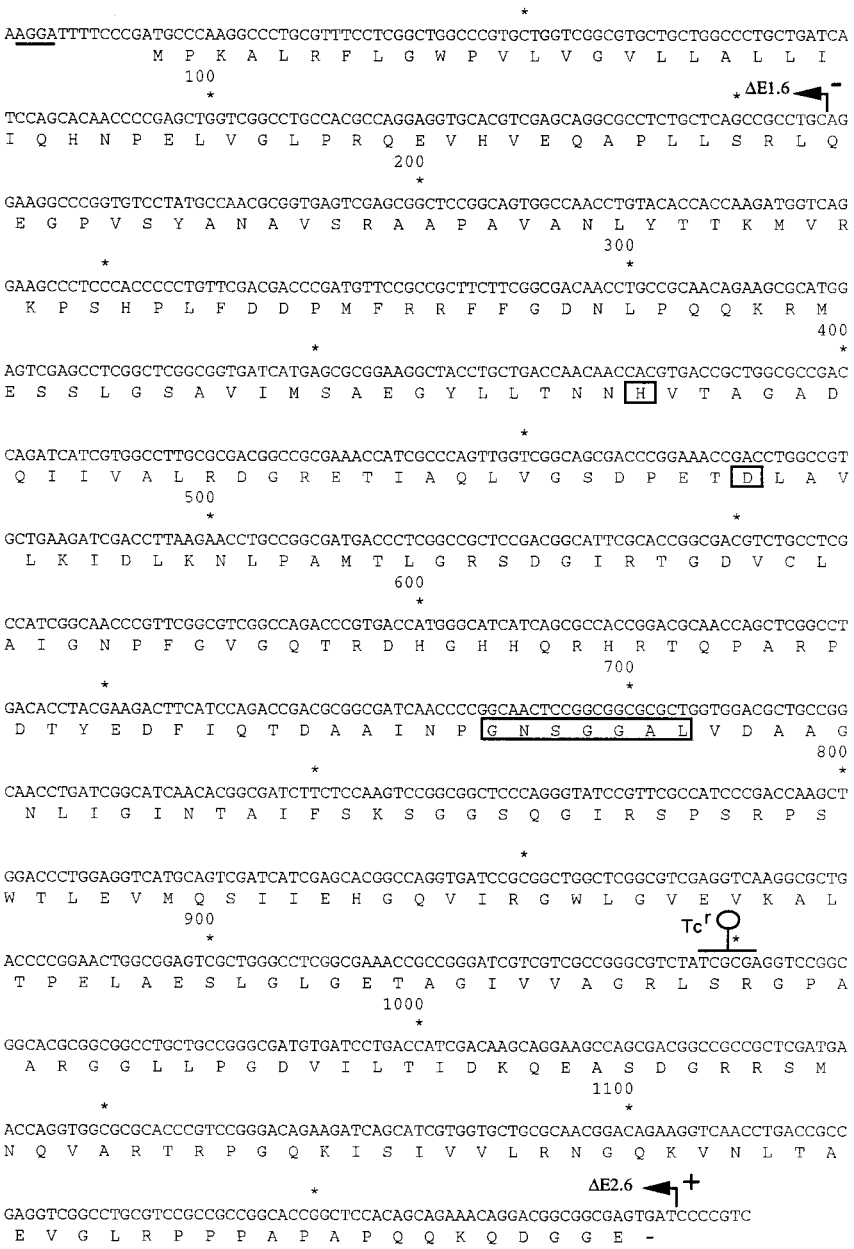

FIG. 3. Complete nucleotide sequence of the *algW* gene encoding a homolog of HtrA (DegP) from *E. coli*. Boxed are residues corresponding to the regions comprising the putative active center of the serine protease HtrA (3, 31). The putative ribosomal binding site is underlined. Arrows, endpoints of deletions corresponding to the constructs shown in Fig. 1C; +, suppression of mucoidy in PAM425; −, loss of suppression activity; balloon, point of insertion of the Tc^r cassette used to insertionally inactivate *algW* on *P. aeruginosa* chromosome.

and *S. typhimurium* (22, 30). Other close homologs of AlgW are the two newly described HtrA-like proteins from *E. coli* (HhoA and HhoB; GenBank accession number U15661), the HtrA and HtrA-like proteins from *Brucella abortus* (10, 46, 56), and further homologs in different bacterial pathogens: *Mycobacterium avium* (3), *Mycobacterium leprae* (GenBank accession number U15180), *Rochalimaea henselae* (GenBank accession number L20127), *Chlamydia trachomatis* (23), *Campylobacter jejuni* (GenBank accession number U27271), *Rickettsia tsutsugamushi* (GenBank accession number L11697), and the two predicted HtrA homologs from *Haemophilus influenzae* (12). The alignment of the highly conserved portions of AlgW and its closest homologs is shown in Fig. 4. Of these homologs, HtrA (DegP) from *E. coli* has been purified and shown to have a serine endopeptidase activity (31), in keeping with the presence of the conserved serine protease catalytic domain (the motif GNSGGAL) (3, 30, 31) which is also present in AlgW. Furthermore, all members of this family of proteins, including AlgW, contain several highly conserved regions, two of which contain invariant His residues and an Asp residue (Fig. 3). These sequences, along with the serine motif, have been proposed to comprise a His-Asp-Ser active site in HtrA homologs (3).

Analysis of the effects of *algW* inactivation on *P. aeruginosa* sensitivity to heat and reactive oxygen intermediates and on its virulence in neutropenic mice. To investigate whether se-

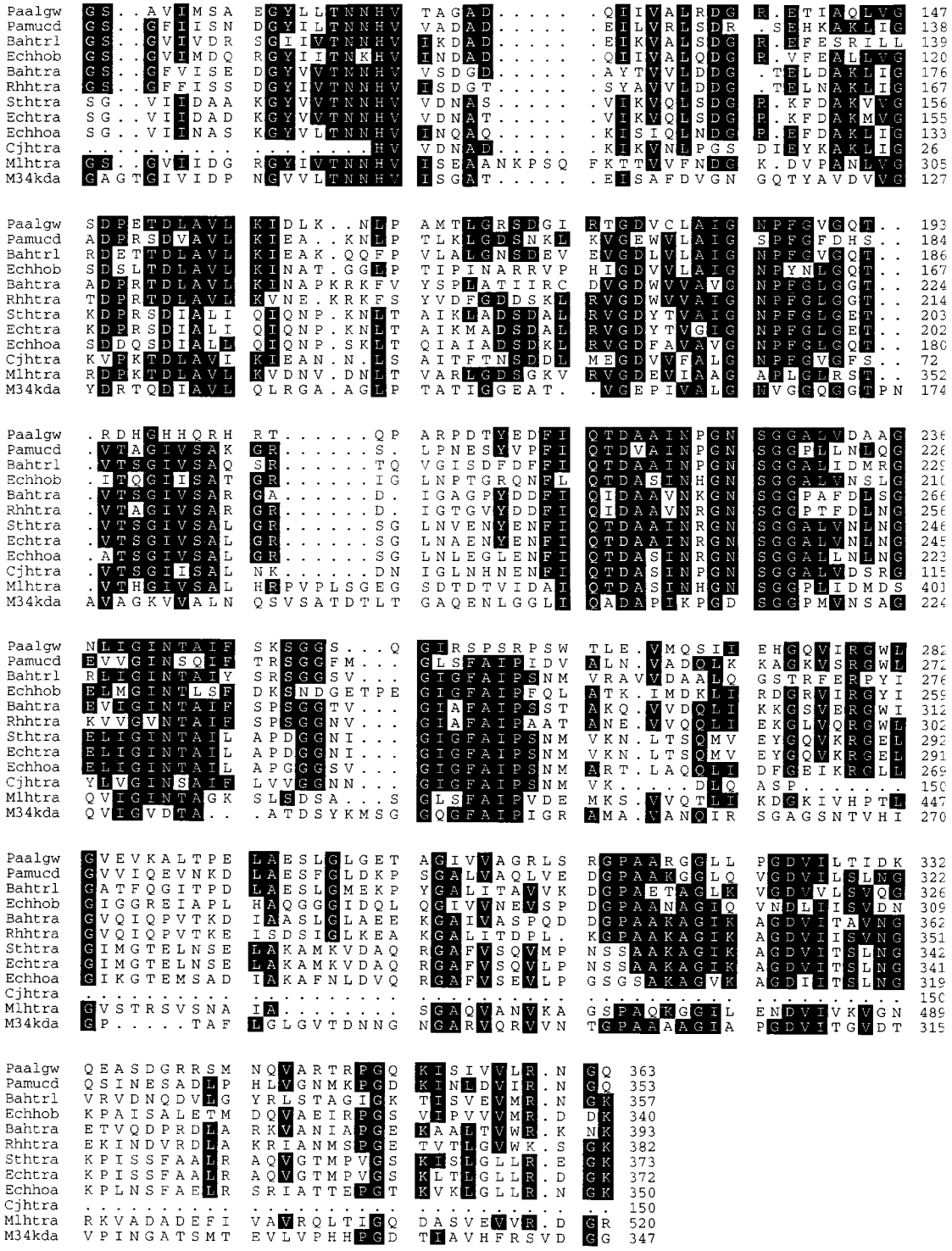

FIG. 4. Alignment of *P. aeruginosa* AlgW, MucD, and other members of the HtrA family of proteins. The most conserved regions shared by all proteins are shown; in the cases of AlgW (Paalgw) and MucD (Pamucd), these included regions starting at amino acid positions 107 (AlgW) and 97 (MucD) and extending to 363 (AlgW) and 353 (MucD). Other proteins are described in the text.

quence similarities of AlgW with HtrA and HtrA-like proteins extend to physiological roles, we insertionally inactivated *algW* on the chromosome of PAO1 (see Materials and Methods). Inactivation of *htrA* and its homologs in *E. coli*, *S. typhimurium*, and *B. abortus* causes separate or combined increase in sensitivity to high temperatures and superoxide- or hydroxyl radical-generating compounds (10, 22, 29, 56). When PAO1 (*algW*$^+$) and its isogenic derivative PAO6854 (*algW*::Tc^r) were tested for survival at elevated temperatures, PAO6854 showed a significant reduction in viability compared with the wild type (Fig.

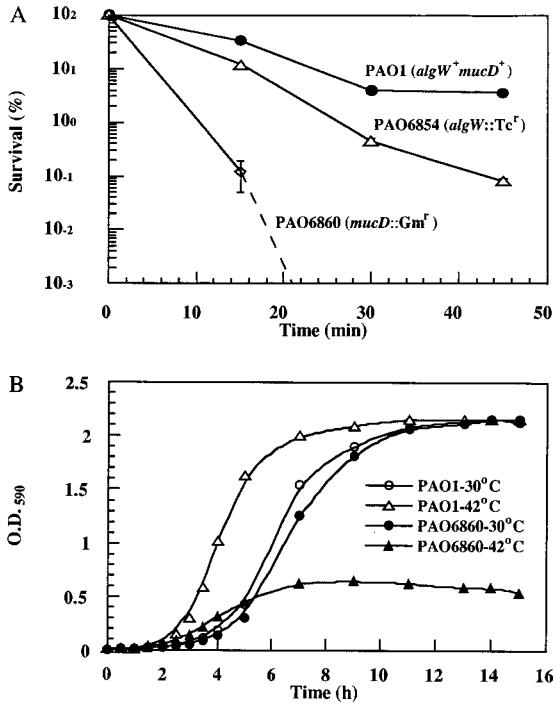

FIG. 5. (A) Heat killing curves for the standard genetic strain *P. aeruginosa* PAO1 (*algW*$^+$ *mucD*$^+$) and its *algW*::Tc^r (PAO6854) and *mucD*::Gm^r (PAO6860) derivatives. The strains were incubated at 50°C for the indicated times, and surviving cells were counted as CFU. Bars indicate standard errors. Survival is expressed as percentage of input CFU at time zero. The dashed line indicates that the 30-min point for PAO6860 did not give any viable colonies out of the input of 1.46×10^7 cells. (B) Growth curves (monitored by OD_{590}) of PAO1 and PAO6860 at the indicated temperatures.

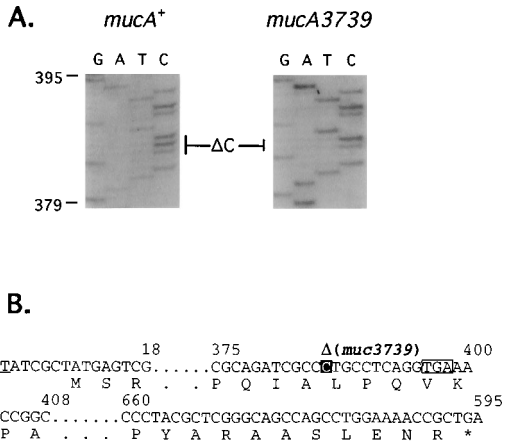

FIG. 6. The *mucA3739* mutation in PAM425. This mutation has been transferred by genetic means from a mucoid CF isolate (Ps3739) into the PAO background via a genetic cross (34). (A) DNA sequence (from positions 379 to 395) of the wild-type *mucA*$^+$ and *mucA3739* alleles. ΔC, missing nucleotide in *mucA3739*. (B) The region of *mucA* with the *mucA3739* mutation. Δ and boldface nucleotide, deletion of a C residue resulting in the frameshift mutation and truncation of *mucA* at the stop codon (boxed) 10 nucleotides downstream from the lesion. This truncation occurs at the same location as the mutation previously reported for CF1 (37), but the actual missing nucleotide is different. Dots, regions of *mucA* not shown. The complete *mucA* sequence has been published (36).

5A). Inactivation of *algW* in other *P. aeruginosa* strains [e.g., PAO578(II)] caused similar effects (data not shown). PAO6854 also displayed significantly increased susceptibility to H_2O_2 and paraquat, a redox cycling compound that increases intracellular superoxide levels (28) (Table 3). The reduced resistance to reactive oxygen intermediates of *algW*::Tc^r strains was complemented to the wild-type levels by introducing the plasmid pCBG4.5 carrying the functional *algW* gene (Table 3). This was the case with other isogenic pairs of strains tested, e.g., PAO578(II) and its *algW*::Tc^r derivative PAO6859 (Table 3). These results are consistent with the sequence similarities between AlgW and HtrA homologs and suggest the possibility that AlgW plays a role in protection against thermal and oxidative stress insults.

S. typhimurium htrA was initially isolated as a gene required for full virulence of this organism in mice (22). By analogy, and because of the lowered resistance to reactive oxygen intermediates of *algW* mutants (Table 3), it seemed possible that inactivation of *algW* could have an effect on virulence of *P. aeruginosa*. To initiate studies of the effects of *htrA*-like factors on *P. aeruginosa* virulence, the LD_{50}s for isogenic *P. aeruginosa* PAO1 (*algW*$^+$) and PAO6854 (*algW*::Tc^r) were compared in a

TABLE 3. Altered sensitivity to paraquat and H_2O_2 in *P. aeruginosa algW* and *mucD* mutants

Strain[a]	Genotype	Plasmid[b]	IPTG[c]	Growth inhibition zone (mean diam [mm] ± SE)[d]	
				2% PQ	6% H_2O_2
PAO1	*muc*$^+$ *algW*$^+$ *mucD*$^+$	—	NA	10.82 ± 0.29	8.5 ± 0.23
PAO6854	PAO1 *algW*::Tc^r	—	NA	18.08 ± 0.30	13.3 ± 0.18
PAO6854	PAO1 *algW*::Tc^r	pCBG4.5 (*algW*$^+$)	NA	11.13 ± 0.50	9.0 ± 0.29
PAO578(II)	*mucA22 sup-2 algW*$^+$	—	NA	13.25 ± 0.20	7.8 ± 0.25
PAO6859	PAO578(II) *algW*::Tc^r	—	NA	18.88 ± 0.20	15.5 ± 0.29
PAO6859	PAO578II *algW*::Tc^r	pCBG4.5 (*algW*$^+$)	NA	16.29 ± 0.37	9.4 ± 0.25
PAO6860	PAO1 *mucD*::Gm^r	—	−	8.72 ± 0.22	18.8 ± 0.19
PAO6860	PAO1 *mucD*::Gm^r	p*tac-mucD*$^+$	−	12.75 ± 0.47	16.9 ± 0.15
PAO6860	PAO1 *mucD*::Gm^r	p*tac-mucD*$^+$	+	13.58 ± 0.63	8.5 ± 0.23

[a] PAO1 is the standard genetic wild-type nonmucoid *P. aeruginosa* strain. PAO6854 is an *algW*::Tc^r derivative of PAO1. PAO578(II) is a derivative of PAO578 with attenuated mucoid phenotype due to the suppressor mutation (*sup-2*), and PAO6859 is its *algW*::Tc^r mutant.

[b] pCBG4.5 is a subclone of the 4.5-kb fragment with *algW* in pVDZ′2, modified by the addition of a Gm^r cassette. p*tac-mucD*$^+$ is a subclone of *mucD* from pMO011809 in pVDtac39. —, no plasmid.

[c] For experiments with p*tac-mucD*$^+$, plates were supplemented with 1 mM IPTG (+) or no IPTG was added (−). NA, not applicable.

[d] Sensitivity to killing by paraquat (PQ) and H_2O_2 is expressed as zones of inhibited growth (in mean diameter) around filter disks impregnated with 10 μl of the indicated solutions. All values are derived from at least 12 samples. *P* values (*t* test) were as follows: for PQ, 2.3×10^{-18} (PAO1 and PAO6854) and 1.17×10^{-15} [PAO578(II) and PAO6859]; for H_2O_2, 3.6×10^{-13} (PAO1 and PAO6854), 1.2×10^{-15} [PAO578(II) and PAO6859], and 5.42×10^{-20} (PAO1 and PAO6860).

TABLE 4. AlgW suppression of mucoidy at the level of *algD* transcription

Strain[a]	Genotype	Plasmid	Phenotype[b]	CDO sp act[c] (U/mg)
PAM426	*mucA3739 algD*$^+$ *algD-xylE*	None	M	4.88 ± 0.54
PAM426	*mucA3739 algD*$^+$ *algD-xylE*	pCBG4.5 (*algW*$^+$)	NM	0.21 ± 0.04
PAO6861	*mucA22 sup-2 algD*$^+$ *algD-xylE*	None	M	9.69 ± 0.86
PAO6861	*mucA22 sup-2 algD*$^+$ *algD-xylE*	pCBG4.5 (*algW*$^+$)	NM	2.67 ± 0.35

[a] All strains harbored an *algD-xylE* transcriptional fusion integrated on the chromosome in addition to an active *algD* gene.
[b] Scored as mucoid (M) or nonmucoid (NM) on PIA.
[c] Transcription of *algD* expressed as specific activity (in units per milligram of protein) of catechol 2,3-dioxygenase (CDO), the product of *xylE* used as a reporter gene.

previously described neutropenic mouse model of experimental *P. aeruginosa* infection (19). No significant difference in LD_{50} was observed (LD_{50}s were 2.3×10^1 for PAO1 and 1.8×10^1 for PAO6854). This finding suggests that the *algW* lesion may not be critical for acute sepsis and bacteremic virulence of *P. aeruginosa* in this animal model.

Characterization of the *muc-3739* mutation as a lesion in *mucA*. To further address the role of *algW* in mucoidy, strain-dependent patterns (Table 2) of suppression of mucoidy with plasmid-borne *algW* were compared with those of pDMU4/76 carrying the wild-type *algU mucA mucB* genes. The *algW* gene suppresses mucoidy in PAM425, carrying the mutation *muc-3739* which is responsible for its mucoid phenotype, and in a number of mucoid CF isolates tested (35). However, *algW* did not affect mucoidy in PAO578 [Table 2, data for PAO578(I)], a strain which carries the previously characterized *mucA22* mutation within the gene cluster at 67.5 min (37). In contrast, pDMU4/76, which carries the wild-type *algU mucA mucB* locus from PAO1, in addition to complementing the *mucA22* mutation in PAO578, also suppressed mucoidy in PAM425 (Table 2). One explanation for the complementation of PAM425 with pDMU4/76 was that the *muc-3739* mutation might be within the *algU mucA mucB* cluster. To test this possibility, the corresponding region from PAM425 was cloned and sequenced, and a frameshift mutation was identified in the *mucA* gene (Fig. 6). The *mucA3739* allele is a newly characterized mutation in *mucA*, a negative regulator of the sigma factor AlgU. The *mucA* gene is the site of previously identified mutations (37) in several mucoid CF isolates (e.g., CF1, CF8, CF14, CF23, and FRD1). The net effect of the frameshift mutation in *mucA3739* is a truncation of MucA at the same position (Fig. 6) as in strain CF1, although the actual nucleotides that have been altered or are missing are different (37). The identification of *muc-3739* as a mutation in *mucA* is in a good agreement with the previously reported genetic mapping data (34) showing a close linkage of *muc-3739* with *pruAB* at 67.5 min.

AlgW can suppress alginate production in *mucA* mutants after attenuation of mucoid phenotype via a suppressor mutation. The finding that the mutation in PAM425 was in *mucA* posed a question regarding the inability of *algW* to suppress mucoidy in some other *mucA* mutants, e.g., PAO578, that also carry frameshift mutations within *mucA*. A partial answer to this paradox came from the previous extensive analyses of PAO578 (48). It has been noted that mucoid strains frequently accumulate second-site suppressor mutations which render them less mucoid (attenuated mucoid phenotype) or completely nonmucoid (9, 48). In this context, strain PAO578 has been subjected to detailed analysis (48), and four classes of spontaneous derivatives of this strain with altered mucoid character have been observed: (i) PAO578 type I [designated PAO578(I)], the original *mucA22* mutant, which is mucoid on all media and under all conditions tested (Table 2); (ii) PAO578 type II [a representative strain has been designated PAO578(II)], the most common derivative with the mucoid phenotype indistinguishable from that of PAO578(I) on PIA but nonmucoid on LB (Table 2) and thus considered to have an attenuated mucoid phenotype; (iii) type III strains, nonmucoid pseudorevertants which in the majority of cases have mutations in *algU* (48); and (iv) type IV derivatives, which show some alginate production but only upon prolonged incubation times. Of these strains, PAO578(II) was used to examine suppression of mucoidy by *algW*. Interestingly and in contrast to its parental strain PAO578(I), which was refractory to the effects of plasmid-borne *algW*, the introduction of pMO011809 or the *algW* subclone pCBE2.6 caused a nonmucoid phenotype in PAO578(II) even when it was grown on PIA (Table 2). These findings suggest that a given refractory *mucA* strain can become susceptible to suppression by plasmid-borne *algW* upon secondary mutations [designated as *sup-2* in PAO578(II)] that attenuate the mucoid phenotype.

AlgW affects expression of mucoidy at the level of *algD* transcription. The similarity of AlgW to a known serine protease suggested that its gene product could act by directly degrading alginate biosynthetic or polymerization enzymes. However, *algW* did not suppress mucoidy in the majority of mucoid strains tested, suggesting that its mode of action could be indirect. One model that seemed compatible with these considerations was the possibility that *algW* interferes with the activation of *algD* transcription, a key regulatory point in expression of the mucoid phenotype (49). To test this hypothesis, we generated strains PAM426 and PAO6861, derivatives of PAM425 and PAO578(II), respectively, with chromosomally encoded *algD-xylE* fusions (see Materials and Methods). When plasmid pCBG4.5 was introduced into PAM426 and PAO6861, it significantly repressed *algD* transcription, an effect which was accompanied by nonmucoid colony morphology (Table 4). These results are consistent with the interpretation that *algW* interferes with the function or activation of the regulatory systems controlling *algD* expression.

Inactivation of *algW* in the wild-type nonmucoid strain PAO1 allows induction of alginate production in the presence of paraquat. The suppression of the mucoid phenotype and *algD* transcription by plasmid-borne *algW* in attenuated derivatives of *mucA* mutants suggests that *algW* may play a negative role in expression of the mucoid phenotype. In keeping with this notion, insertional inactivation of *algW* on the chromosomes of *mucA* mutants [e.g., PAO578(II); strain PAO6859] did not cause a loss of the mucoid phenotype. In a converse experiment, inactivation of *algW* on the chromosome of the wild-type nonmucoid strain PAO1 (strain PAO6854) was not sufficient to visibly alter alginate production, as judged by the nonmucoid phenotype of PAO6854 on standard media (PIA or LB). However, when PAO6854 was grown on LB supplemented with paraquat, this resulted in a significant increase in alginate production and mucoid colony morphology after 48 h of growth (Table 5). This effect was complemented by plasmid-borne *algW*, which prevented induction with paraquat (Table 5, PAO6854 harboring pCBG4.5). In the absence of *algW* muta-

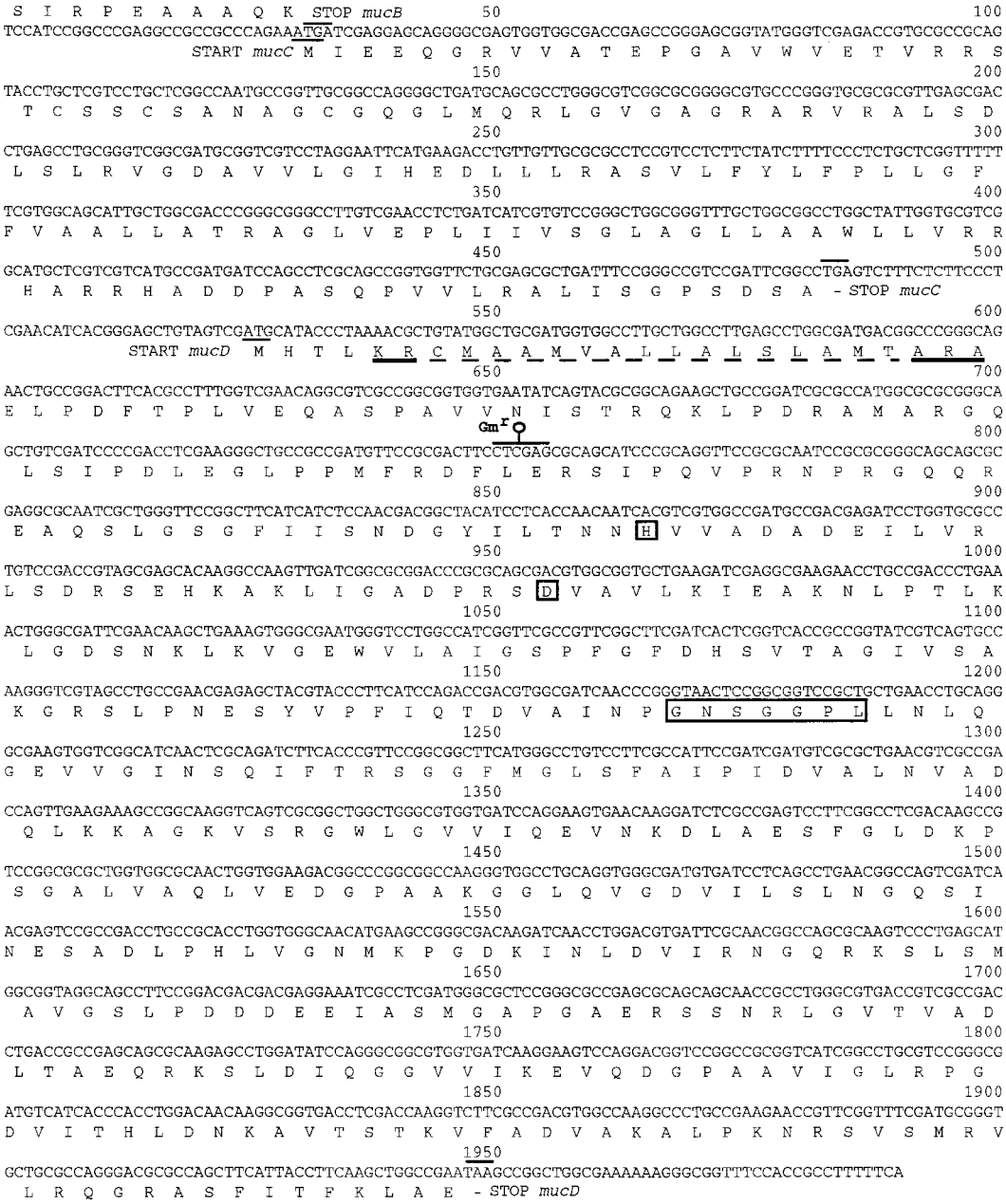

FIG. 7. Nucleotide sequence of the *mucC* and *mucD* genes from *P. aeruginosa* PAO1. Shown is the end of the previously published *mucB* sequence (36) that partially overlaps with the initiation codon of *mucC*. Underlined nucleotides, initiation codons; overlined nucleotides, stop codons; underlined amino acids, the putative leader peptide in *mucD* (short solid line, two positively charged residues; longer solid line, possible signal peptidase site; dashed line, hydrophobic region); balloon, site of insertion of the Gm^r cassette used to insertionally inactivate *mucD*. Other markings are as in Fig. 3.

tion (strain PAO1), there was no visible change under identical conditions (Table 5). These findings are consistent with the interpretation that *algW* plays a negative role in alginate production. Furthermore, inactivation of *algW* in the wild-type nonmucoid background allows increased expression of the alginate system under conditions of oxidative stress which alone do not cause a morphologically detectable change in *algW*$^+$ strains.

Identification of two new genes, *mucC* and *mucD*, located downstream of *algU mucA mucB*: MucD is similar to HtrA (DegP) and AlgW. In previously published studies (35–37), we presented the complete sequence of the first three genes (*algU mucA mucB*) in the cluster encoding the alternative sigma factor AlgU and its regulators. In the process of sequencing this region in *P. aeruginosa* (36) and *Azotobacter vinelandii* (39), we also noticed the presence of a potential homolog of *algW* located downstream of the *algU mucA mucB* genes. To further investigate this possibility, we determined the complete nucleotide sequence of two genes, termed *mucC* and *mucD*, located immediately downstream of *mucB* (Fig. 7). The predicted gene product of *mucC* showed significant homology (Fig. 8) with the product of the fourth gene (ORF4) in the cluster consisting of an *algU* (*rpoE*) analog and its regulators recently identified in *Photobacterium* sp. strain SS9 (4). The entire system is impor-

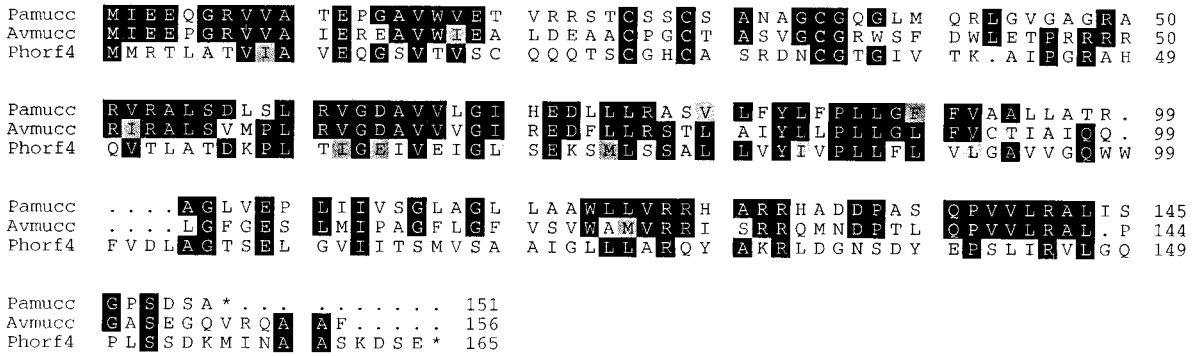

FIG. 8. Alignments of the predicted *P. aeruginosa mucC* gene product (Pamucc) and the products of the corresponding genes from *A. vinelandii* (39) (Avmucc) and *Photobacterium* sp. (Phorf4). ORF4 is required for the cold-temperature-adapted growth of *Photobacterium* sp. strain SS9 (4).

tant for adaptation to high pressures and extreme cold in this deep-sea bacterium, while ORF4 is required for the cold-temperature-adapted growth of *Photobacterium* sp. strain SS9 (4). A similar factor has been identified in *A. vinelandii*, another bacterium that makes alginate and has a gene cluster highly homologous to *algU mucABCD* (39). The predicted gene product of *P. aeruginosa mucD* (corresponding to the 53.9-kDa polypeptide detected in the T7 expression system; data not shown) displayed significant similarities to *algW* and other members of the HtrA and HtrA-like superfamily of proteins (Fig. 4). These observations indicate that the gene cluster at 67.5 min, in addition to the alternative sigma factor AlgU and its putative negative regulators *mucA* and *mucB*, contains two additional genes, including an element similar to *algW* and *htrA*.

Insertional inactivation of *mucD* causes conversion to the mucoid phenotype, increased sensitivity to H_2O_2, and decreased viability at high temperatures. The role of *algW* as a negative regulator of mucoidy and the similarities between MucD and AlgW suggested to us that *mucD* could have a similar effect on alginate production. To test this hypothesis, *mucD* was inactivated on the chromosome of PAO1 (see Materials and Methods). This effect was accompanied by conversion to the mucoid phenotype on PIA without any additional manipulation (Table 5). This finding indicated that *mucD* had an even stronger effect than *algW* on suppression of alginate synthesis, since additional stimuli (e.g., exposure to oxidative stress mediators), required to visibly stimulate alginate synthesis in the case of *algW* inactivation, were not needed for *mucD*::Gm^r (Table 5). Nevertheless, the *mucD* mutant strain PAO6860 produced less alginate than freshly isolated *mucA* or *mucB* mutants and required somewhat longer incubation times to assume a fully mucoid appearance. It was also nonmucoid on LB. In keeping with the similarities of *mucD* to *htrA* and *algW*, PAO6860 showed increased susceptibility to killing with H_2O_2 (Table 3), but unlike the *algW* mutant PAO6854, it was not more sensitive to paraquat than the parental strain PAO1. Both the mucoid phenotype and sensitivity to H_2O_2 of PAO6860 could be complemented by plasmid-borne *mucD*$^+$ (Tables 3 and 5, data for strains harboring p*tac-mucD*$^+$). However, different levels of *mucD* expression were required, since suppression of alginate synthesis occurred without IPTG (Table 5), while this inducer of transcription from the *tac* promoter was needed to restore the resistance to H_2O_2 to its wild-type levels (Table 3).

Inactivation of *mucD* also had a more profound effect on susceptibility to heat killing than did inactivation of *algW*::Tc^r (Fig. 5A). While the difference in survival after incubation for 30 min at 50°C between the wild-type (PAO1) and *algW*::Tc^r (PAO6854) strains was approximately 1 log unit, no surviving cells (sensitivity of detection was 1.46×10^{-7}) were scored in the corresponding sample with PAO6860 (*mucD*::Gm^r), suggesting that this strain was several log units more sensitive to heat killing. This was also noticeable at milder temperatures, as exemplified by the growth patterns of PAO1 and PAO6860 at 42°C (Fig. 5B). While PAO1 displayed accelerated growth at 42°C, PAO6860 reached an OD_{590} equal to one-fourth of that of the wild-type control and showed evidence of lysis upon continued incubation at 42°C. Interestingly, a similar growth profile has been reported for *htrA* mutants in *E. coli* (29).

TABLE 5. Induction of alginate production by paraquat in *algW*::Tc^r and conversion to mucoidy in *mucD*::Gm^r *P. aeruginosa*

Strain	Plasmid[a]	Genotype	Alginate production[b] (μg of uronic acid/mg [wet wt] of cells ± SE) (phenotype[c])			
			37°C		42°C	
			−PQ	+PQ	−PQ	+PQ
PAO1	—	*muc*$^+$ *algW*$^+$ *mucD*$^+$	1.63 ± 0.16 (NM)	1.22 ± 0.18 (NM)	1.81 ± 0.20 (NM)	2.71 ± 0.24 (NM)
PAO6854	—	PAO1 *algW*::Tc^r	3.69 ± 0.29 (NM)	4.37 ± 0.29 (M**)	3.61 ± 0.27 (NM)	8.52 ± 0.45 (M*)
PAO6854	pCBG4.5	PAO6854 *algW*$^+$	2.99 ± 0.53 (NM)	3.13 ± 0.11 (NM)	1.82 ± 0.6 (NM)	2.46 ± 0.15 (NM)
PAO6860	—	PAO1 *mucD*::Gm^r	16.12 ± 2.25 (M)	ND	ND	ND
PAO6860	p*tac-mucD*$^+$	PAO6860 *mucD*$^+$	1.10 ± 0.07 (NM)	ND	ND	ND

[a] Plasmids pCBG4.5 (*algW*$^+$) and p*tac-mucD*$^+$ are described in Table 3, footnote *b*. —, no plasmid.

[b] Determined as previously described (25). All strains were grown on plates at the indicated temperatures without or with 12 μM paraquat (PQ). The *P* values (*t* test) for PAO1 and PAO6854 grown in the presence of 12 μM paraquat were 1.17×10^{-5} and 3.45×10^{-11} for 37 and 42°C, respectively.

[c] NM, nonmucoid colony morphology on LB; M, mucoid after overnight incubation (18 h); M*, mucoid after 48 h of growth; M**, slightly mucoid after 48 h of growth; ND, not done.

DISCUSSION

In this work we have identified three new genes, *algW*, *mucC*, and *mucD*, within two loci in the late region of the *P. aeruginosa* chromosome that control conversion to mucoidy in this organism. AlgW and MucD act as negative regulators of alginate production and belong to the family of HtrA-like proteins found in diverse bacterial species. *E. coli* HtrA has been purified and shown to have a serine endopeptidase activity (31). The homologies of AlgW and MucD with HtrA (DegP) suggest that they may have similar activities. For example, the presence of the essential motif (GNSGGAL) containing active serine and other extensive identities with HtrA have been noted in AlgW and MucD. Furthermore, inactivation of *algW* and *mucD* causes physiological effects similar to those observed in *htrA* mutants in *E. coli* (temperature sensitivity) and *S. typhimurium* (sensitivity to reactive oxygen intermediates) (22, 29).

HtrA (DegP) has been implicated in degradation of abnormal periplasmic proteins. According to some reports (51), HtrA may be identical to the previously described *E. coli* serine endopeptidase Do, which has been implicated in proteolysis of oxidatively damaged proteins (5). Given the physiological effects of *algW* and *mucD* inactivation, it is possible that these proteins contribute to the removal of denatured or oxidized proteins and thus may affect the viability of *P. aeruginosa* exposed to thermal or oxidative insults. In the case of the main heat shock sigma factor σ^{32} (2, 15, 24), abnormal proteins are a source of signals for its activation. If AlgU is induced by protein denaturation under conditions of heat shock or oxidative stress, inactivation of *mucD* and *algW* on the chromosome of wild-type nonmucoid cells could render the system hypersensitive to induction by endogenous protein misfolding or oxidation. This would permit activation of AlgU and induction of alginate production as observed in *mucD* mutants under standard growth conditions and in *algW* mutants upon further stimulation by exposure to paraquat.

AlgU and σ^E are 66% identical (7, 32, 38) and functionally interchangeable (58), and the promoters recognized by these σ factors have highly conserved -35 and -10 canonical sequences (GAACTT-16/17 bp-TCTgA [7, 8, 11, 30, 50]). Interestingly, σ^E controls *htrA* in *E. coli* (11, 30, 43, 47), and on the basis of indirect evidence, such as the conservation of promoter sequences (22) and the presence of σ^E in *S. typhimurium* (38), it may also be required for the expression of *htrA* in this enteric bacterium. There are no detectable σ^E-like promoter sequences immediately upstream of *algW*, although the possibility that a σ^E-dependent promoter is present far upstream cannot be excluded. There are also no obvious σ^E promoter sequences immediately upstream of *mucD*. However, the situation with *mucD* may be different from that with *algW*. Given the small intergenic distances between all members of the cluster and absence of any obvious termination signals, it is possible that *algU* and the downstream *muc* genes, including *mucD*, are cotranscribed. This would place *mucD* under the control of the AlgU-dependent (σ^E) promoters P_1 and P_3 (38, 50) transcribing *algU* (Fig. 1A), resulting in a situation partially equivalent to the control of *htrA* by σ^E in *E. coli* (11, 30, 43, 47).

If *mucD* is transcribed from AlgU-dependent promoters, e.g., under inducing conditions, this may provide a feedback mechanism for the return to the baseline level following induction of the system. Under stress conditions that activate AlgU, the gene product of *mucD*, present in larger amounts as the result of such induction from the AlgU-dependent promoters at the beginning of the cluster, could remove the signals (e.g., denatured proteins) or otherwise affect AlgU activity, causing return of the system to its uninduced level. In the complete absence of MucD, e.g., when *mucD* is inactivated, the system may be constitutively upregulated. This model extends the previously known relationships between σ^E and *htrA* in *E. coli* (11, 30, 43, 47) by including a negative feedback regulatory loop.

The presence of two HtrA-like factors in the same organism, such as AlgW and MucD in *P. aeruginosa*, is not without a precedent. For example, there are at least three such elements in *E. coli*: HtrA (22), HhoA, and HhoB (GenBank accession number U15661). In *B. abortus*, which has a functional equivalent of HtrA (DegP) (10, 46, 56), a second HtrA-like element has also been characterized (56). The significance of the presence of multiple HtrA homologs in the same bacterium is not known, but it is possible that some of them specialize for different subcellular compartments or have different substrate specificities or expression characteristics.

The predicted gene product of *mucD* displays typical leader peptide features (Fig. 7) conforming with the rules derived from the known leader peptides (42). It is possible that MucD is processed and is in the periplasmic compartment, similar to the situation with HtrA (DegP) in *E. coli* (29, 55). Although AlgW has a hydrophobic region preceded by a segment containing two positively charged residues close to the N terminus, there is no obvious leader peptidase site, and several amino acid residues in different locations seem to be in violation of the recognized rules for leader peptides (42). Interestingly, inactivation of *algW* and *mucD* causes differential sensitivity to reactive oxygen intermediates, which may be explained by the difference in compartmentalization of AlgW and MucD. PAO1 *algW*::Tc^r derivatives display increased sensitivity to both H_2O_2 and paraquat, while *mucD*::Tc^r strains show increased susceptibility only to H_2O_2. The redox cycling compound paraquat generates superoxide in an NADPH-dependent reaction with diaphorases (28) and is expected to have its strongest effect in the cytoplasm. This is in keeping with the observation that *mucD*-deficient cells are not sensitive to paraquat since the predicted location of MucD is in the periplasm. Exogenously added H_2O_2 exerts its effects in all compartments, which is consistent with the sensitivity of both *mucD*::Gm^r and *algW*::Tc^r cells to this agent. Interestingly, *mucD* mutants are more susceptible to H_2O_2 than *algW*::Tc^r derivatives, which could be ascribed to reduced effects of the cytoplasmic catalase and peroxidase activities on the periplasmic compartment.

The *htrA* gene is crucial for survival in macrophages and virulence of *S. typhimurium* (22). A similar role of *htrA* and *htrA*-like genes in *Brucella* virulence has been observed within the first and second weeks of infection (10, 56), although the situation in this organism is somewhat more complicated since at 60 days postinfection, the *htrA* mutants exceed the colony counts of the wild type (56). These characteristics have been linked to the sensitivity of *htrA* mutants to reactive oxygen intermediates, a condition which may render them more susceptible to the bactericidal effects of oxidants produced by phagocytic cells (22). Whether *algW* and *mucD* play a direct role in *P. aeruginosa* persistence in the inflamed CF lung, e.g., as a result of their contributions to the resistance to killing by reactive oxygen intermediates, remains to be tested in an adequate CF animal model of respiratory infection once it becomes possible to establish chronic *P. aeruginosa* colonization in transgenic mice.

As shown in this work, *algW* and *mucD* have effects on expression of the mucoid phenotype, which plays a potentially crucial role during chronic infection in CF. In this environment, alginate coating has been proposed to play roles in antiphagocytosis (17, 41, 53) and in biofilm formation (26, 54),

with the associated phenomena of recalcitrance to antibiotic treatments and resistance to natural clearance mechanisms further compounded by the adhesive properties and viscosity of alginate (41, 53). Furthermore, the ability of alginate to afford resistance to oxidants such as hypochlorite and superoxide (27, 52), likely to be generated by the numerous neutrophils and monocytes recruited into the inflamed CF lung (41), is considered to be of significance for *P. aeruginosa* persistence in the respiratory tracts of CF patients. Thus, the *htrA* homologs *algW* and *mucD* may provide an interesting example of a dual involvement in protection against oxidative stress, indirectly by their regulatory effects on alginate production and directly as factors improving viability of cells exposed to oxidants.

With regard to the lack of demonstrable effect of *algW* on bacteremic virulence in this work, it is also worth noting that the massive colonization of the respiratory tract in CF is strongly contrasted with the confinement of *P. aeruginosa* to the lumen of the airways with almost complete absence of bacteremia in CF patients (1, 41, 53). This phenomenon has been attributed to the reduced acute virulence of CF isolates (33). Future considerations and extended comparisons of the roles of AlgU (σ^E), the HtrA homologs AlgW and MucD, alginate production, and resistance to oxidative stress and other environmental insults, in both acute and chronic infection models will provide additional insights into the factors contributing to the contrasting types of host-pathogen interactions in infections caused by *P. aeruginosa.*

The effects of *algW* and *mucD* on alginate production are also of interest with respect to continuing characterization of *muc* mutations. Mutations characterized thus far in CF isolates have been identified only in the *mucA* gene (37), an additional example being the *mucA3739* mutation characterized in this work. Given the effects of the experimental inactivation of *mucB* (36) and *mucD*, with a potential contribution of *algW*, as shown in this study, these genes are candidate sites for mutations causing mucoidy in CF isolates in those strains in which *mucA* mutations were not observed (37). In addition, analysis of suppressor mutations attenuating the mucoid phenotype, such as those that rendered *mucA* mutants susceptible to the effects of plasmid-borne *algW*, may provide important information regarding additional regulatory factors and interactions controlling AlgU activity.

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