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Pseudomonas aeruginosa MucD Regulates the Alginate Pathway through Activation of MucA Degradation via MucP Proteolytic Activity▽

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Alginate overproduction in *Pseudomonas aeruginosa* can be caused by the proteolysis of the anti-sigma factor MucA regulated by the AlgW protease. Here, we show that inactivation of MucD, an HtrA/DegP homolog and alginate regulator, can bypass AlgW, leading to an atypical proteolysis of MucA that is dependent on the MucP protease.

Pseudomonas aeruginosa is capable of producing an exopolysaccharide known as alginate. Alginate overproduction, or mucoidy, allows *P. aeruginosa* to persist in the cystic fibrosis (CF) lung and contribute to the disease complications (7). Alginate is a linear copolymer comprised of β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) linked by a β-(1-4)-glycosidic bond. AlgU (σ^{22}) is an extracytoplasmic function (ECF) sigma factor that drives alginate production. AlgU is under negative regulation by the cognate anti-sigma factor MucA (10). When the *mucA* gene is mutated, AlgU activates alginate production (10) by promoting expression of the alginate biosynthetic operon at the *algD* promoter (20).

Mutation of the *mucA* gene is a common mechanism for conversion to mucoidy in *P. aeruginosa*. The degradation of MucA by the envelope protease AlgW is another mechanism for alginate overproduction (14). This appears to follow the model of regulated intramembrane proteolysis (RIP). RIP of the anti-sigma factors, such as MucA (*Escherichia coli* RseA), is typically a two-step process mediated by proteases. Site 1 RIP proteases, such as AlgW (*E. coli* DegS), can be activated to degrade the anti-sigma factors when envelope proteins, such as MucE, accumulate to a certain level, or under cell wall stress conditions (3, 14). This model further predicts that after AlgW initiates MucA degradation, the site 2 metalloprotease protease MucP (*E. coli* RseP) (14) would then be activated to cleave MucA. Cytoplasmic ClpXP proteases are then necessary to finish the proteolytic cascade of MucA degradation, thus activating AlgU (13).

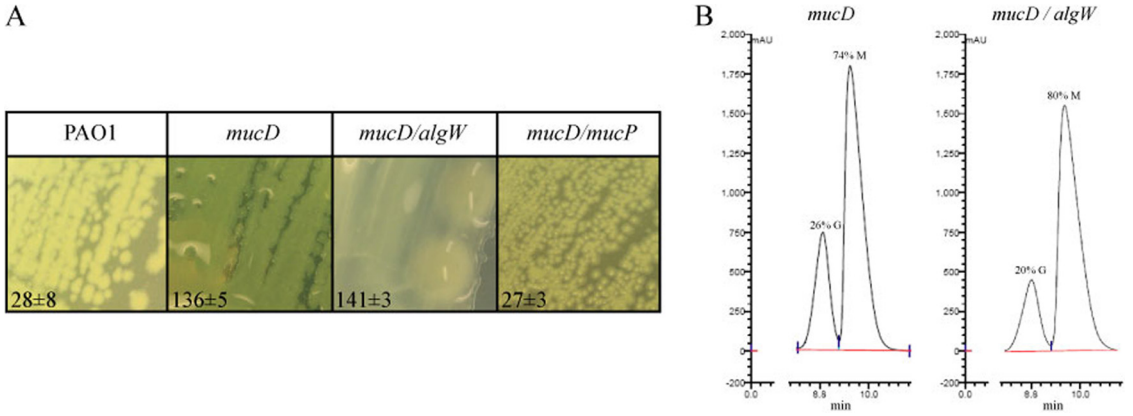

FIG. 1. Mucoidy of the *mucD* mutant requires MucP, a metalloprotease ortholog of *E. coli* RseP. (A) The phenotypes of PAO1, PAO1*mucD*, PAO1*mucDalgW*, and PAO1*mucDmucP* are shown, and the amounts of alginate produced by each strain are indicated as μg/ml/optical density at 600 nm (OD_{600}) units ± standard deviations (SD). Strains were cultured on PIA for 24 h at 37°C. (B) HPLC analysis of exopolysaccharides produced by PAO1*mucD* and PAO1*mucDalgW* reveals that the PAO1*mucDalgW* polysaccharide is alginate.

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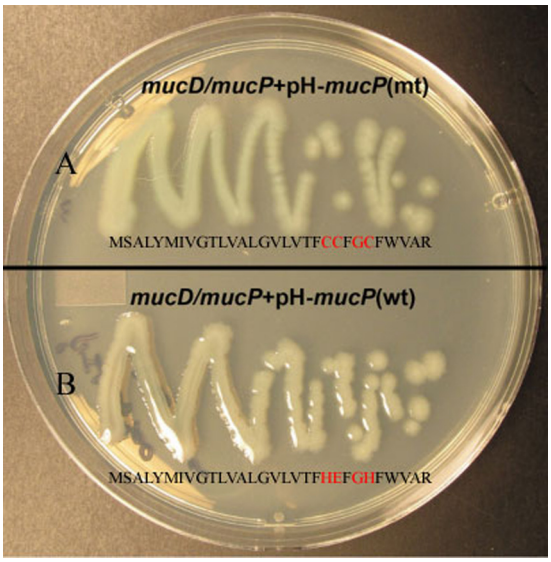

FIG. 2. MucP contains a conserved motif of HEXXH required for mucoidy in the *mucD* mutant. pHERD20T with the mutant (mt) *mucP* gene encoding the CCFGC motif (A) or the wild-type (wt) MucP containing the HEXXH motif (B) were mobilized into the double *mucD mucP* mutant. Strains were cultured on PIA with 300 μg/ml carbenicillin plus 0.1% (wt/vol) arabinose to induce the expression of the wild-type or mutant *mucP* gene from the P_{BAD} promoter of pHERD20T. The N-terminal amino acid sequence of MucP is shown for the locations of the wild-type and mutant motifs of MucP. Red type indicates amino acid changes.

The HtrA/DegP homologue of *P. aeruginosa*, known as MucD, is a serine protease that is a negative regulator of alginate production and a positive regulator of heat shock stress (2). Inactivation of the *mucD* gene and mutation of the proteolytic domain of the *mucD* gene both result in alginate overproduction (2, 18, 22). Based on the function of DegP in *E. coli*, MucD may act on misfolded envelope proteins to block the activation of RIP of MucA. Supporting this model, when the envelope protein MucE is induced, MucD can suppress MucE-mediated activation of AlgW (14). However, the relationship between MucD and AlgW in the activation of the proteolytic cascade of MucA degradation is not clear. Here, we used the inactivation of the *mucD* gene as a model to characterize RIP of MucA. We report that AlgW is not essential for alginate overproduction in the *mucD* mutant. Furthermore, in the absence of the *mucD* gene, the MucP protease is required for the degradation of MucA.

Alginate overproduction due to inactivation of the *mucD* gene requires MucP but not AlgW. We previously noticed that the *algW* gene is not required for mucoidy in the *mucD* mutant (14). Even though a visual difference between the phenotypes is noted (Fig. 1A), no statistical difference in the amount of alginate produced between the *mucD* and *mucD algW* mutants is observed as measured using the carbazole assay (4, 5, 12–14). The carbazole assay utilizes sulfuric acid to hydrolyze the polysaccharide. The hydrolyzed sugar monomer is then reacted with the carbazole reagent for detection (9). However, some neutral sugars, such as hexoses and pentoses, as well as the acyl groups of uronic acids, can interfere with the specificity of the reaction (1, 6, 8). Furthermore, even DNA has been shown to affect this assay (21). These points led us to resort to a high-performance liquid chromatography (HPLC) protocol, similar to that used for the analysis of seaweed alginate (16), to confirm that the *mucD algW* phenotype is due to the accumulation of alginate and not another polymer. Exopolysaccharides from the *mucD* and *mucD algW* mutants were ethanol precipitated and dried by speed vacuum overnight. Seven milligrams of collected exopolysaccharide was hydrolyzed in 1 ml of 3 M trifluoroacetic acid (TFA) for 2 h at 110°C. TFA was removed from the samples by drying. The hydrolyzed sample was suspended in 300 μl of water, and the pH was adjusted to 9 with 0.3 M NaOH. The hydrolyzed alginate was then derivatized by adding 150 μl of 1-phenyl-3-methyl-5-pyrazolone (PMP) at a

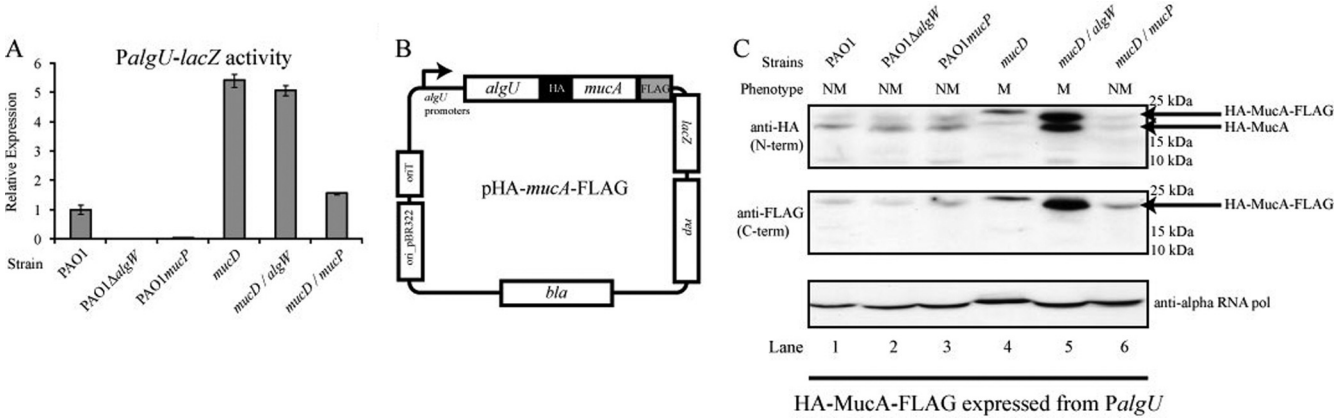

FIG. 3. P*algU* activity and HA-MucA-FLAG Western blotting suggest that MucA degradation is dependent on MucP in the *mucD* mutant. (A) The β-galactosidase activity from pP*algU-lacZ* was determined after 24 h of growth on PIA. Values are normalized to PAO1 carrying the P*algU-lacZ* reporter construct and are indicated as means ± SD from three independent experiments. (B) Schematic diagram of the construction of pHA-*mucA*-FLAG. The P*algU* promoters are used to drive the expression of *algU*-HA-*mucA*-FLAG. Since they are expressed from autoregulated P*algU*, mucoid strains will have larger amounts of HA-MucA-FLAG. (C) Western blotting shows the effects of *mucD*, *algW*, and *mucP* gene inactivation on the degradation of HA-MucA-FLAG. Total protein lysates were prepared from strains cultured on PIA and blotted with both anti-HA (Roche, Mannheim, Germany) and anti-FLAG (Sigma). The relative positions of the peptides visualized are aligned with their molecular weights on the right of the panel. Shown is one representative of five experiments.

TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Genotype, phenotype, locus, or sequence[a]	Source
P. aeruginosa strains		
PAO1	Prototroph, NM	P. Phibbs
PAO1Δ*algW*	PAO1 in-frame deletion of *algW* gene (PA4446), NM	This study
PAO1*mucP*	PAO1 *mucP*::Tc^r (PA3649), NM	14
PAO1*mucD*	PAO1 *mucD*::Gm^r (PA0766), M	14
PAO1*mucDalgW*	PAO1 *mucD*::Gm^r *algW*::Tc^r, M	14
PAO1*mucDmucP*	PAO1 *mucD*::Gm^r *mucP*::Tc^r, NM	14
E. coli strain TOP10	DH5α derivative	Invitrogen
Plasmids		
pCR4	TOPO cloning vector	Invitrogen
p*PalgU-lacZ*	P*algU* from miniCTX-P*algU-lacZ* (5) fused to *lacZ* in pLP170 EcoRI/HindIII	This study
pCR4-P*algU-algU*-HA-*mucA*	TOPO-cloned P*algU-algU*-HA-*mucA*	This study
pCR4-P*algU-algU*-HA-*mucA*-FLAG	TOPO-cloned P*algU-algU*-HA-*mucA*-FLAG	This study
pHA-*mucA*-FLAG	1.8-kb AflII/HindIII fragment containing P*algU-algU*-HA-*mucA*-FLAG in pHERD20T, resulting in the replacement of P_{BAD} promoter and *araC*	This study
pEX100T-Δ*algW*	1.4-kb fragment flanking the *algW* gene fused with pEX100T-NotI with in-frame deletion of *algW*	14
pHERD20T-HA-*mucA*	N-terminally tagged HA-*mucA* in pHERD20T EcoRI/HindIII	5
Oligonucleotides		
ncoI-mucP-F	ATACCCATGGATGAGTGCGCTTTACATGAT	This study
ncoI-CCFGC-mucP-F	ATACCCATGGATGAGTGCGCTTTACATGATCGTCGGCACCCTGGTAGCC CTGGGTGTGCTGGTGACGTTCTGCTGCTTCGGCTGCTTC	This study
mucP-R-hindIII	CAAGCTTTCACAGACGACTCAGATCGTTGA	This study
hin-PalgU-F	CAAGCTTAGGTCGAGCCCTGCGACAGT	This study
eco-PalgU-R	GAATTCGTGCACGAACCGCACGATCAA	This study
co-HAmucA-F	GCCAAGAGAGGTATCGCTATGTACCCATACGATGTTCCAGATTACGCTA GTCGTGAAGCCCTGCA	This study
co-HAmucA-R	TGCAGGGCTTCACGACTAGCGTAATCTGGAACATCGTATGGGTACATAG CGATACCTCTCTTGGC	This study
hin-mucA-R	CAAGCTTTCAGCGGTTTTCCAGGCTGGCTGCC	This study
aflII-HAmucA-F	TCTTAAGAGTAGGTCGAGCCCTGCGACAGTTCGCCCTT	This study
hin-mucAFLAG-R	CAAGCTTTCATTTGTCGTCGTCGTCTTTGTAGTCGCGGTTTTCCAGGCTG GCTGCC	This study

[a] NM, nonmucoid phenotype; M, mucoid phenotype.

concentration of 0.5 mol/liter. The reaction mixture was incubated at 70°C for 90 min. The pH was then adjusted to 7 with 350 μl of 0.3 M HCl. To remove residual PMP, the samples were extracted with 1 ml of chloroform. The PMP-labeled alginate monomers were separated in a phosphate-acetonitrile mobile phase at pH 6.7 and pumped by a Dionex P480 HPLC pump through an Agilent Eclipse XDB-C18 4.6- by 150-mm column. The PMP-labeled alginate monomers were detected at 245 nm by a Dionex PDA-1000 UV-Vis detector. Chromatograms were generated for known alginate standards (alginic acid from brown algae [61% M/39% G]; Sigma-Aldrich catalog no. A7003) to establish the retention times of PMP-tagged M and G. Under these conditions, PMP-derivatized M and G were detected at 8.8 ± 0.1 min and 9.5 ± 0.1 min, respectively.

A small difference in the G/M ratio was observed, with the *mucD* strain producing 26%:74% G/M and the *mucD algW* strain producing 20%:80% G/M (Fig. 1B). To corroborate these data, samples of the same ethanol-precipitated alginate from the *mucD* and *mucD algW* mutants were submitted to the ASTM international standard test method for determining the chemical composition and sequence of alginate by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy (designation F2259-03). NMR analysis precisely confirmed the G/M ratios that were observed via HPLC analysis (data not shown). Alginate is produced in the *mucD algW* mutant, which indicates that in the absence of *mucD*, the AlgW protease is not required for activating the alginate pathway.

MucP contains a protease motif required for mucoidy in the *mucD* mutant. As previously reported (11), the *mucP* gene (PA3649) encodes an ortholog of metalloprotease of *E. coli* RseP. Alignment of *E. coli* RseP to *P. aeruginosa* MucP revealed a highly conserved catalytic domain of zinc metalloprotease with an HEXXH motif in the N terminus of MucP (Fig. 2). The conserved motif HEXXH of MucP was mutated from HEFGH to CCFGC, and the resulting *mucP* mutant was cloned into pHERD20T (12). The wild-type *mucP* gene restored the *mucD mucP* double mutant to a mucoid phenotype, but the mutant *mucP* gene with the CCFGC motif could not restore mucoidy (Fig. 2). This result indicates that MucP has a protease active site and further suggests that *mucP* encodes a RseP-like protease as other data have indicated (14, 19).

AlgW is dispensable for *algU* activity in the *mucD* mutant. Since two of the five promoters of the *algU* gene are dependent upon AlgU (15), it is possible to measure the activity of AlgU with a promoter-reporter system. A construct was assembled by fusing the *algU* promoters (P*algU*) to *lacZ* on the multicopy vector pLP170 (11). A Miller assay was performed as previously described (5), and data were normalized with respect to

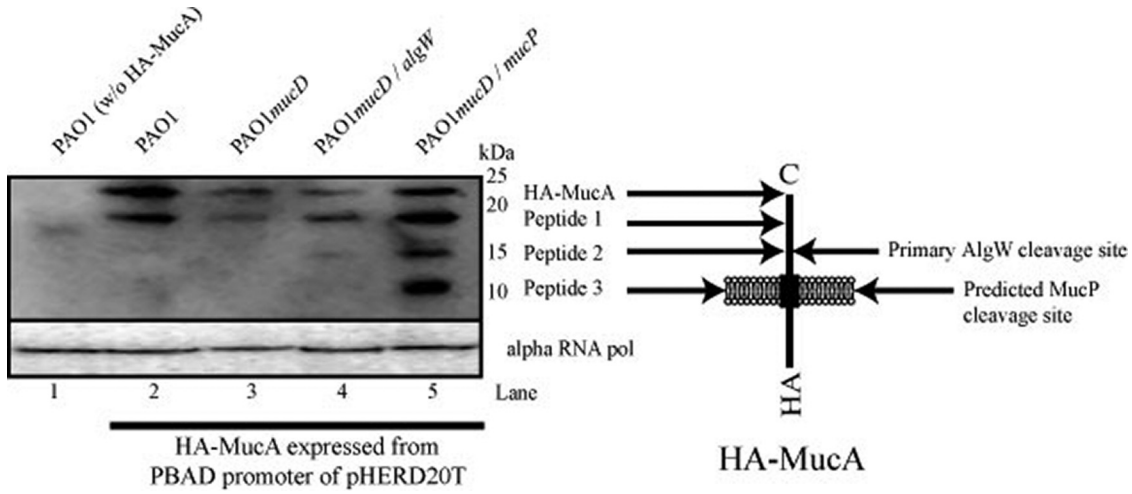

FIG. 4. Expression of HA-*mucA* from P_{BAD} promoter shows the accumulation of the HA-MucA peptides in the *mucD mucP* mutant. Western blotting shows the effects of *mucD*, *algW*, and *mucP* gene inactivation on the degradation of HA-MucA. The relative positions of the peptides visualized are aligned with the predicted regions of HA-MucA. Shown is one representative of three experiments. An accumulation of full-length HA-MucA indicates less MucA degradation, whereas a decreased level of HA-MucA would suggest increased degradation of MucA. As a control for cross-reactivity, total protein samples from PAO1 without (w/o) plasmid, which lacks HA-tagged peptides, were used (lane 1). The bottom panel shows anti-alpha RNA polymerase (alpha RNA pol) (RpoA; Neoclone, Madison, WI) blotting for the loading control. The highly regulated P_{BAD} promoter has a basal expression in the absence of arabinose (12). Degradation of HA-MucA occurs in the *mucD* and *mucD algW* mutants, but in the double *mucD mucP* mutant, an accumulation of three truncated HA-MucA peptides indicates incomplete proteolysis of HA-MucA.

PAO1. To test the effects of the *algW* gene in the control of AlgU activity, PAO1Δ*algW* was generated with pEX100T-Δ*algW* (4, 5). Compared to PAO1, the PAO1Δ*algW* and PAO1*mucP* strains did not have a detectable promoter activity (Fig. 3A). This is expected, because without these presumptive proteases degrading MucA, AlgU is still sequestered by MucA to the inner membrane; therefore, *algU* transcription is not initiated. When *mucD* is inactivated, a high level of *algU* promoter activity was noted (Fig. 3A), suggesting that the RIP activities are negatively regulated by MucD. However, in the *mucD algW* double mutant, a high level of *algU* activity is still observed (Fig. 3A). This indicates that AlgW is not essential for the initiation of *algU* expression in the *mucD* strain and correlated with alginate production between the strains (Fig. 1A). Inactivation of the *mucP* gene in the *mucD* mutant decreased the *algU* promoter activity to the level seen for PAO1 (Fig. 3A). The *algU* activity levels suggest that the MucP protease may directly cleave MucA independent of AlgW in the *mucD* mutant.

HA-MucA-FLAG expressed from the P*algU* promoters shows that the *mucP* gene is required for the MucA degradation that leads to the activation of AlgU. Since the transmembrane anti-sigma factor MucA can be targeted by multiple proteases at distinct locations, we designed a dually tagged MucA expression construct. We reasoned that using the *algU* promoters would better simulate MucA expression that occurs *in vivo*. To create this construct, PAO1 genomic DNA was used as a template to amplify the *algU* promoters, the *algU* gene, and the hemagglutinin (HA)-*mucA* gene via splicing by overlap extension (SOE) PCR with the primer sequences indicated in Table 1. This amplicon was TOPO cloned and used as a template for PCR to add an AflII restriction site in front of P*algU*, a FLAG sequence, and a HindIII site to the 3′ end of the *mucA* gene. The resulting amplicon, AflII-P*algU-algU*-HA-*mucA*-FLAG-HindIII, was TOPO cloned. The dually tagged construct was then digested and ligated to replace the P_{BAD}-*araC* region in pHERD20T. pHA-*mucA*-FLAG (Fig. 3B) was mobilized into the strains of this study. Cells were cultured on *Pseudomonas* isolation agar (PIA) supplemented with 300 μg/ml carbenicillin to select for plasmid maintenance. Cell lysates were prepared with a ProteaPrep bacterial cell lysis kit (Protea, Morgantown, WV) and quantified by using a DC assay (Bio-Rad, Hercules, CA). Fifty micrograms of total protein was electrophoresed on 15% polyacrylamide gels (SDS-PAGE) and then electroblotted (Trans-Blott cell; Bio-Rad, Hercules, CA) onto 0.45-μm nitrocellulose filters and probed with the antibodies indicated (Fig. 3C).

Nonmucoid strains PAO1, PAO1Δ*algW*, and PAO1*mucP* all have small amounts of HA-MucA-FLAG (Fig. 3C). Blotting for the N-terminal HA epitope reveals two peptides; however, only full-length HA-MucA-FLAG peptide is detected via the C-terminal FLAG tag (Fig. 3C, lanes 1 to 3). The mucoid PAO1*mucD* strain shows a slightly increased amount of HA-MucA-FLAG (Fig. 3C, lane 4), indicating that free AlgU was available to transcribe the P*algU* promoters, thus expressing HA-MucA-FLAG. However, the mucoid *mucD algW* strain reveals large amounts of HA-MucA-FLAG (Fig. 3C, lane 5) and it appears that HA-MucA-FLAG is cleaved into a lower-molecular-weight HA-MucA when HA and FLAG blots are compared (Fig. 3C, lane 5). This result suggests that the degradation of MucA occurred in the absence of the *mucD* gene, even without the *algW* gene. The nonmucoid *mucD mucP* strain had small amounts of HA-MucA-FLAG, similar to those for PAO1 (Fig. 3C, lane 6), suggesting that the level of free AlgU is not sufficient enough to drive the P*algU* promoters.

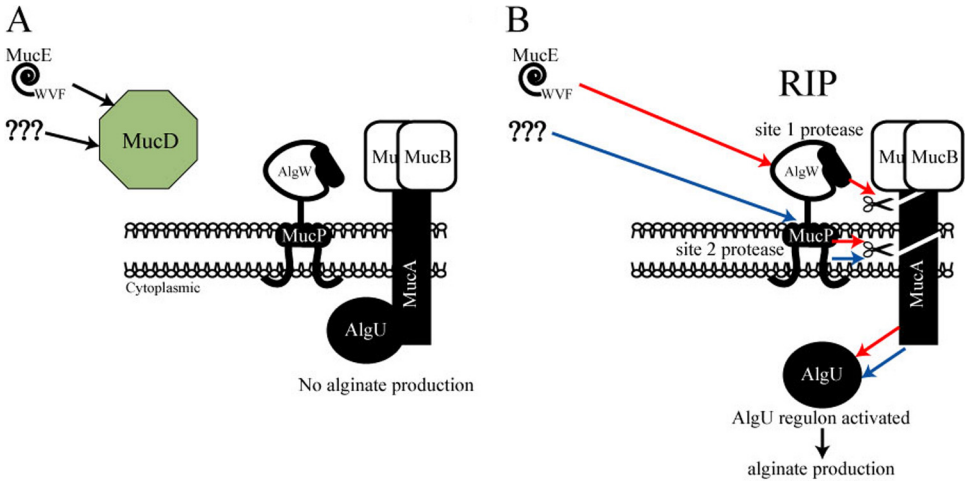

FIG. 5. MucD controls signals that activate proteolysis of MucA, leading to alginate overproduction. (A) AlgU is negatively regulated by anti-sigma factor MucA. MucB binds as a dimer to the C terminus of MucA, protecting it from proteolytic cleavage by AlgW (3). MucD is a chaperone/protease that controls the envelope protein quality. Poor protein quality in the envelope can serve as a signal to activate the degradation of MucA via RIP. (B) To simulate environmental stress, we used a *mucD* mutant strain. The absence of MucD would simulate stress conditions whereby envelope proteins could accumulate to a level which activates RIP of MucA. Some envelope proteins, including MucE, have a C-terminal sequence (such as WVF) that can activate AlgW proteolysis of MucA. When AlgW-activator proteins, such as MucE, accumulate to a certain level, RIP of MucA will occur sequentially (red arrows), first by AlgW (site 1 protease) and then by MucP (site 2 protease). However, as reported here, AlgW is not required for RIP of MucA in the absence of *mucD*, and unknown envelope protein signals (???) likely activate site 2 protease MucP directly (blue arrows). These models suggest that *P. aeruginosa* MucD mediates both AlgW-MucP and direct MucP RIP of MucA, which leads to alginate overproduction.

Detection of HA-MucA expressed from P_{BAD} suggests that MucP is activated in the *mucD* mutant for MucA degradation. One caveat to using the *algU* promoters to express tagged MucA is that the *algU* promoters are autoregulated. Therefore, mucoid strains will have higher levels of tagged MucA. One way to assess the degradation of MucA is to express the same amount of tagged MucA in each strain from a promoter independent of AlgU. To test this, the N-terminally HA-tagged MucA protein was expressed from P_{BAD} in pHERD20T (12) in PAO1, PAO1*mucD*, PAO1*mucDalgW*, and PAO1*mucDmucP* for Western blot analysis. pHERD20T has an arabinose-inducible P_{BAD} which has a basal expression in *P. aeruginosa* without induction (12). PAO1 cultured on PIA shows full-length HA-MucA and a truncated HA-MucA peptide 1 (Fig. 4), as expected based on previous data (5). Inactivation of the *mucD* gene caused a decreased concentration of full-length HA-MucA and a truncated HA-MucA peptide 1 compared to those for PAO1 (Fig. 4, lanes 2 and 3). Similar levels of HA-MucA and peptide 1 were observed for both PAO1*mucD* and PAO1*mucDalgW* mutant strains (Fig. 4, lanes 3 and 4), further suggesting that AlgW plays a minimal role in the MucA degradation in the *mucD* mutant. However, inactivation of the *mucP* gene caused an increased concentration of full-length HA-MucA and truncated HA-MucA peptides 1, 2, and 3 (Fig. 4, lane 5). This result suggests that without *mucP*, MucA is more stable in the *mucD* mutant.

Conclusions. MucA has been shown to be proteolytically degraded by AlgW in response to environmental signals and induction of envelope proteins (3, 14, 17, 19). Here, we show that in the absence of *mucD*, *algW* is not required for MucA degradation leading to AlgU activation. Our data suggest that MucP-dependent degradation of MucA may shorten the sequential degradation of MucA that is initiated by AlgW (Fig. 5). It is possible that activation of AlgW and that of MucP require divergent signals. The carboxyl terminus of MucE (WVF) is one type of substrate that activates AlgW for proteolysis of MucA (14). However, in the absence of MucD, there are uncharacterized substrates that may activate MucP-only proteolysis of MucA (Fig. 5). Acid stress has been shown to activate the σ^E stress response in *Salmonella enterica* serovar Typhimurium in an RseP-dependent manner, which does not require DegS (AlgW homologue). Our study and the aforementioned study suggest that RIP degradation of RseA-like anti-sigma factors, such as MucA, can occur not only at site 1, which is followed by site 2 cleavage, but also by direct site 2 cleavage. Distinct stress conditions, possibly those encountered in the CF lung, could activate alginate production by either typical AlgW-MucP proteolysis or direct MucP proteolysis of MucA.

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