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The *Pseudomonas aeruginosa* Sensor Kinase KinB Negatively Controls Alginate Production through AlgW-Dependent MucA Proteolysis▽

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Mucoidy, or overproduction of the exopolysaccharide known as alginate, in *Pseudomonas aeruginosa* is a poor prognosticator for lung infections in cystic fibrosis. Mutation of the anti-σ factor MucA is a well-accepted mechanism for mucoid conversion. However, certain clinical mucoid strains of *P. aeruginosa* have a wild-type (wt) *mucA*. Here, we describe a loss-of-function mutation in *kinB* that causes overproduction of alginate in the wt *mucA* strain PAO1. KinB is the cognate histidine kinase for the transcriptional activator AlgB. Increased alginate production due to inactivation of *kinB* was correlated with high expression at the alginate-related promoters P_{algU} and P_{algD}. Deletion of alternative σ factor RpoN (σ^{54}) or the response regulator AlgB in *kinB* mutants decreased alginate production to wt nonmucoid levels. Mucoidy was restored in the *kinB algB* double mutant by expression of wt AlgB or phosphorylation-defective AlgB.D59N, indicating that phosphorylation of AlgB was not required for alginate overproduction when *kinB* was inactivated. The inactivation of the DegS-like protease AlgW in the *kinB* mutant caused loss of alginate production and an accumulation of the hemagglutinin (HA)-tagged MucA. Furthermore, we observed that the *kinB* mutation increased the rate of HA-MucA degradation. Our results also indicate that AlgW-mediated MucA degradation required *algB* and *rpoN* in the *kinB* mutant. Collectively, these studies indicate that KinB is a negative regulator of alginate production in wt *mucA* strain PAO1.

Cystic fibrosis (CF) patients are predisposed to bacterial respiratory infections due to the mucus buildup in their airways (17). Mutation of the chloride ion transporter called CFTR creates a hospitable environment for the opportunistic pathogen *Pseudomonas aeruginosa* (27). The emergence of mucoid, or alginate-overproducing, strains marks the beginning of chronic infection by *P. aeruginosa* (13). The presence of mucoid strains causes significant deterioration of lung function (40). Mucoid strains produce alginate by increasing transcription of the *algD* promoter of the alginate biosynthetic operon (Fig. 1) (11). The first molecular mechanism for the conversion to mucoidy elucidated was mutation of the *mucA* gene (32). MucA is the anti-σ factor that sequesters the alternative sigma factor AlgU (also called AlgT or σ^{22}) (Fig. 1) (33, 46). When MucA is not functional due to mutation, increased transcription directed by AlgU at the *algD* promoter (P_{algD}) activates alginate biosynthesis (Fig. 1) (57).

Activation of alginate production by AlgU is controlled at transcriptional and posttranslational levels (Fig. 1). Transcription of *algU* occurs from multiple promoters, two of which are AlgU dependent (12, 45), and therefore AlgU autoregulates its expression. Alginate production is also negatively controlled by MucB and MucD, which are encoded downstream of *algU* and *mucA*. MucB cooperates with MucA-AlgU sequestering, presumably by protecting the periplasmic portion of MucA from degradation and thus stabilizing the MucA-AlgU interaction (46). Inactivation of *mucB* in a wild-type (wt) *mucA* strain causes elevated alginate production (31). MucD is homologous to DegP of *Escherichia coli*, which degrades unfolded proteins in the periplasm (22) and also functions as a chaperone (49). In *P. aeruginosa*, *mucD* inactivation causes alginate overproduction and sensitivity to H_2O_2 and heat (6).

There is a high level of conservation between *E. coli* σ^E-RseA and *P. aeruginosa* AlgU-MucA. Activation of σ^E occurs after sequential proteolytic cleavage of the anti-σ factor RseA, first by activated DegS and finally by RseP proteases (3). DegS is a serine protease that is activated in response to unfolded outer membrane proteins via a conserved C-terminal sequence (51, 52). This conserved signal transduction pathway is referred to as regulated intramembrane proteolysis (1). In *P. aeruginosa*, AlgU is associated with the inner membrane and MucA in wt, nonmucoid strains (44). Recently, the *P. aeruginosa* DegS homologue, AlgW, has been shown to activate alginate production through regulated proteolysis of MucA in response to increased expression of *mucE* (Fig. 1) (43). Also, the cell wall inhibitor D-cycloserine can activate the AlgU stress response in *P. aeruginosa*, dependent upon AlgW (Fig. 1) (54).

When MucA does not repress AlgU, transcriptional activation at P_{algD} and alginate overproduction occurs. Significant research has focused on the multitude of regulators that bind and/or regulate transcriptional activity at P_{algD}. Most P_{algD} transcriptional regulators are AlgU dependent, such as AlgR, AmrZ, and AlgB (Fig. 1). The response regulator AlgR binds multiple sites within P_{algD} and is required for P_{algD} expression (21, 38). Additionally, the alginate and motility regulator Z (AmrZ) also promotes activity at P_{algD} (5, 50). The NtrC family response regulator AlgB has recently been shown to bind at P_{algD} and cause transcriptional activation (26). Beyond AlgU and the AlgU-dependent transcription factors, a second alternative sigma factor, RpoN, has been suggested to have dual roles as both a positive and a negative regulator at P_{algD} (Fig. 1) (7).

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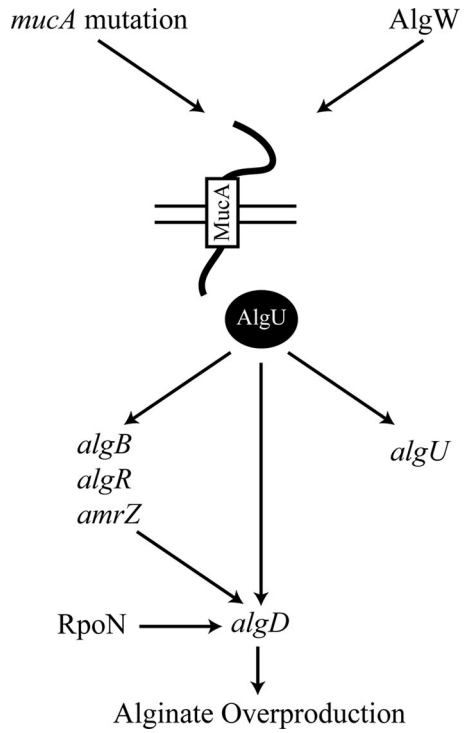

FIG. 1. MucA-AlgU is the central regulatory pathway controlling the expression of the mucoid phenotype in *P. aeruginosa*. In *mucA* mutants, AlgU is not repressed (32) and activates transcription of downstream promoters. The *algU* gene is transcribed by five promoters, two of which (P1 and P3) are dependent on AlgU (46). AlgU activates transcription of *algB*, *algR*, and *amrZ*, whose gene products participate in transcriptional activation of P_{algD} (4, 38, 56). In a *muc-23* mucoid mutant, RpoN has also been shown to bind to P_{algD} and activate or repress transcription under certain environmental conditions (7). AlgU also activates transcription of P_{algD} (57). Activation of transcription at P_{algD} results in alginate overproduction and a mucoid phenotype. MucA is the anti-σ-factor that sequesters AlgU(T) (46). The predicted protease AlgW can cleave MucA, which results in derepression of AlgU. Overexpression of the periplasmic peptide MucE results in mucoidy due to activation of AlgW (43), which leads to degradation of MucA. Cell wall inhibitors such as D-cycloserine have been shown to upregulate AlgW-dependent transcription at P_{algD} (54).

The P_{algD} transcriptional regulator, AlgB (15, 26, 56), is a response regulator of a two-component signal transduction system. Typically, two-component signal transduction systems are comprised of a response regulator and a sensor kinase. Upon phosphorylation of the response regulator by the sensor kinase, the response regulator binds specific DNA sequences near a promoter and modulates transcription. The *E. coli* homologue of AlgB, known as NtrC, activates phosphorylation-dependent transcription at target promoters with the σ^{54}-holoenzyme (24). σ^{54} (RpoN) is required for mucoidy in a *P. aeruginosa* prototype strain (*muc-23*) (7), but *rpoN* is not required for alginate synthesis in several different *mucA* mutant strains (7, 37, 38). AlgB is an NtrC family response regulator that mediates alginate biosynthesis in *mucA* mutants (16). The primary role of AlgB that has been elucidated thus far has been transcriptional activation of P_{algD} (26). KinB is the cognate sensor kinase of AlgB (29), and furthermore, KinB is capable of autophosphorylation and transfer of phosphate to AlgB (29). Interestingly, phosphorylation of AlgB is not required for P_{algD} activation (28). Unlike *algB*, *kinB* is not required for alginate production in a *mucA22* mutant (28).

Previous extensive research has focused on regulation of alginate production in *mucA* mutant strains. However, recent data show that *algD* expression can occur independent of *mucA* mutations by regulated proteolysis of MucA (43, 54). Studies have shown that expression of *algD* is increased under anaerobic conditions (9, 19), which may occur in the CF lung (39). Given the data that *P. aeruginosa* can produce alginate irrespective of *mucA* mutation, we sought to further characterize mucoidy in wt *mucA* strain PAO1. In this report, we show that inactivation of *kinB* in nonmucoid *P. aeruginosa* strain PAO1 results in alginate overproduction that requires the predicted protease AlgW. We observed that *algB* and *rpoN* are also required in *kinB* mutants for alginate production and high P_{algU} and P_{algD} expression. We also show evidence of regulated MucA degradation in *P. aeruginosa*. A novel role for AlgB and RpoN in signal transduction of regulated proteolysis to release AlgU from sequestering by MucA in the *kinB* mutant background is proposed. Our results support a model in which KinB negatively regulates the AlgU signal transduction pathway in *P. aeruginosa* strain PAO1.

MATERIALS AND METHODS

Bacterial strains, plasmids, transposons, growth conditions, and oligonucleotides. The bacterial strains, plasmids, and transposons used in this study are listed in Table 1. *P. aeruginosa* strains were grown at 37°C in Lennox broth (LB), on LB agar, or on *Pseudomonas* isolation agar (PIA) plates (Difco, Sparks, MD). PIA plates were prepared with 20 ml of glycerol per liter as recommended by the manufacturer. When necessary, PIA medium was supplemented with carbenicillin, tetracycline, or gentamicin at a concentration of 300 μg/ml. The sequences of the primers used in this study are available upon request.

Transposon mutagenesis. The mariner transposon-containing plasmid pFAC (53) was introduced into PAO1 by biparental conjugations. The locations of the transposon insertion in the mucoid mutants were determined by inverse PCR (42, 43). The chromosomal DNAs of these strains were digested with SalI and ligated to generate circular closed DNA molecules (Fast-Link DNA ligation kit; Epicentre, Madison, WI). The ligated DNA was then used as the template for inverse PCR with primers (Gm3OUT and Gm5OUT) as previously described (42), which anneal to the gentamicin resistance (Gm^r) gene. The resulting amplicons were sequenced by the Marshall University Genomics Core Facility.

Mutant strain construction. For in-frame deletion of specific genes (*algU*, *algB*, *algB-kinB*, *algW*, *kinB*, and *rpoN*), the upstream and downstream sequence fragments (500 to 1,000 bp) flanking the target gene were PCR amplified and fused by using the crossover PCR method. The PCR products with the in-frame deletion of target gene were digested and ligated into pEX100T-NotI vector. A two-step allelic exchange procedure was employed with the pEX100T constructs for in-frame deletion. The single-crossover merodiploid exconjugants were selected based on carbenicillin resistance and sensitivity on PIA supplemented with 10% (wt/vol) sucrose (*sacB*). After overnight incubation of the merodiploids in LB broth at 37°C, the double-crossover recombinants were isolated on PIA with 10% sucrose. The in-frame deletion of the target gene was confirmed by antibiotic resistance assays and then PCR amplification of the flanking region of target gene with multiple sets of primers and amplicon sequencing.

Plasmid construction and complementation analyses. Alleles were cloned into the shuttle vector pHERD20T (41) for complementation with gene expression driven by the P_{BAD} arabinose-inducible promoter. For *lacZ* reporter analysis, *algU* and *algD* promoters were fused with *lacZ* in miniCTX-*lacZ* (20) (see Fig. 3A and C, respectively). All plasmid constructs containing PCR products were sequenced, and this confirmed that no mutations occurred.

Alginate assay. *P. aeruginosa* strains were grown at 37°C on PIA plates supplemented with carbenicillin and 0.1% (wt/vol) arabinose for 24 h. Bacterial growth was removed from plates with phosphate-buffered saline (PBS) and suspended in 50 ml of PBS per plate. The optical density at 600 nm (OD_{600}) of the bacterial suspension in PBS was measured and adjusted. Cell suspensions containing bacterial alginates were used for assay of the amounts of the uronic

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Phenotype, genotype, and/or description[a]	Source or reference
P. aeruginosa strains		
PAO1	Alg$^-$, prototroph	P. Phibbs
PAO1 Δ*algB*	Alg$^-$, PAO1 in-frame deletion of *algB* (PA5483)	This study
PAO1 Δ*algU*	Alg$^-$, PAO1 in-frame deletion of *algU* (PA0762)	This study
PAO1 Δ*kinB*	Alg$^+$, PAO1 in-frame deletion of *kinB* (PA5484)	This study
PAO1 Δ*rpoN*	Alg$^-$, PAO1 in-frame deletion of *rpoN* (PA4462)	This study
PAO1 *kinB*::*aacC1*	Alg$^+$, PAO1 *kinB*::Gm^r	This study
PAO1 *kinB*::*aacC1* Δ*algU*	Alg$^-$, PAO1 *kinB*::*aacC1* in-frame deletion of *algU* (PA0762)	This study
PAO1 Δ*algB* Δ*kinB*	Alg$^-$, PAO1 in-frame deletion of *algB* (PA5483) and *kinB* (PA5484)	This study
PAO1 *kinB*::*aacC1*Δ*algW*	Alg$^-$, PAO1 *kinB*::*aacC1* in-frame deletion of *algW* (PA4446)	This study
PAO1 *kinB*::*aacC1* Δ*rpoN*	Alg$^-$, PAO1 *kinB*::*aacC1* in-frame deletion of *rpoN* (PA4462)	This study
E. coli strains		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Lab strain
TOP10	DH5α derivative	Invitrogen
SM10/λpir	*thi recA thr leu tonA lacY supE* RP4–2-Tc::Mu1::*pir* Km^r	Lab strain
Plasmids		
pRK2013	Km^r Tra Mob ColE1	14
pFAC	Mini-*himar1* mariner transposon in *Pseudomonas* suicide plasmid; Ap^r Gm^r	53
pCR4-TOPO	TA cloning vector; 3.9 kb; Ap^r Km^r	Invitrogen
pUS56	*algB45* in pTrcHisA BamHI-EcoRI	28
pHERD20T	pUCP20T P_{lac} replaced by 1.3-kb AflII-EcoRI fragment of *araC*-P_{BAD} cassette	41
pHERD20T-*algU*	*algU* (PA0762) from PAO1 in pHERD20T EcoRI/HindIII	This study
pHERD20T-*algB*	*algB* from PAO1in pHERD20T EcoRI/HindIII	This study
pHERD20T-*algB45*	*algB45* from pUS56 in pHERD20T EcoRI/HindIII	This study
pHERD20T-*algW*	*algW* (PA4446) from PAO1in pHERD20T EcoRI/HindIII	This study
pHERD20T-MPDZ*algW*	*algW* with partial PDZ domain in pHERD20T EcoRI/HindIII	This study
pHERD20T-ΔPDZ*algW*	*algW* with complete deletion of PDZ domain in pHERD20T EcoRI/HindIII	This study
pHERD20T-*kinB*	*kinB* from PAO1 in pHERD20T KpnI/HindIII	This study
pHERD20T-HA-*mucA*	N-terminally tagged HA-*mucA* in pHERD20T EcoRI/HindIII	This study
pUCP20T-P_{BAD}-*rpoN*	*araC*-P_{BAD}-*rpoN* fusion in pUCP20 XbaI/HindIII	This study
MiniCTX-*lacZ*	Gene delivery vector for inserting genes at the CTX phage *att* site on the *P. aeruginosa* chromosome; Tc^r	20
MiniCTX-P_{algU}-*lacZ*	Complete P_{algU} promoter (541 bp upstream of ATG) EcoRI/HindIII fused with *lacZ* for integration at the CTX phage *att* site in *P. aeruginosa*	This study
MiniCTX-P_{algD}-*lacZ*	Complete P_{algD} promoter (1,525 bp upstream of ATG) HindIII/BamHI fused with *lacZ* for integration at the CTX phage *att* site in *P. aeruginosa*	This study
pEX100T	*Pseudomonas* suicide vector, *sacB oriT* Cb^r	47
pEX100T-NotI	*Pseudomonas* suicide vector with NotI restriction site fused into SmaI of pEX100T, *sacB oriT* Cb^r	This study
pEX100T-Δ*algU*	A 2.5-kb fragment flanking *algU* gene fused with pEX100T-NotI with in-frame deletion of *algU* with only 24 bp left coding for 8 amino acids of *algU*	This study
pEX100T-Δ*algB*	A 1.5-kb fragment flanking *algB* gene fused with pEX100T-NotI with in-frame deletion of *algB*	This study
pEX100T-Δ*algB*Δ*kinB*	A 1.5-kb fragment flanking *algB* gene fused with pEX100T-NotI with in-frame deletion of *algB* and *kinB*	This study
pEX100T-Δ*algW*	A 1.4-kb fragment flanking *algW* gene fused with pEX100T-NotI with in-frame deletion of *algW*	43
pEX100T-Δ*kinB*	A 2.5-kb fragment flanking *kinB* gene fused with pEX100T-NotI with in-frame deletion of *kinB*	This study
pEX100T-Δ*rpoN*	A 1.9-kb fragment flanking *rpoN* gene fused with pEX100T-NotI with in-frame deletion of *rpoN*	This study

[a] Alg$^-$, nonmucoid phenotype; Alg$^+$, mucoid phenotype.

acid using a standard curve made with D-mannuronic acid lactone (Sigma-Aldrich, St. Louis, MO) in the range of 0 to 100 μg/ml as described previously (23).

β-Galactosidase activity assay. The promoter fusion constructs miniCTX-P_{algU}-*lacZ*, and miniCTX-P_{algD}-*lacZ* were integrated onto the *P. aeruginosa* chromosome at the CTX phage *att* site (20). The β-galactosidase activity assay was based on the method originally described by Miller (36), with the modification that the cells were grown on PIA plates in triplicate for 24 h at 37°C and harvested in PBS, and the β-galactosidase activity was assayed after toluene permeabilization of the cells. The reported values represent the averages in triplicate from three independent experiments. The values displayed are normalized to PAO1 pHERD20T for each respective promoter fusion.

Western blot analysis. Cell lysates were prepared with Ready-Preps (Epicentre, Madison, WI) by the manufacturer's protocol. Cell lysates were quantified by D_C assay (Bio-Rad, Hercules, CA). Forty micrograms of protein was boiled in sodium dodecyl sulfate loading buffer. The samples were electrophoresed on 12% polyacrylamide gels or 15% ProteaGel (Protea, Morgantown, WV) polyacrylamide and then electroblotted (Trans-Blott cell; Bio-Rad, Hercules, CA) onto 0.45-μm nitrocellulose. The membrane was blocked with 3% nonfat dry

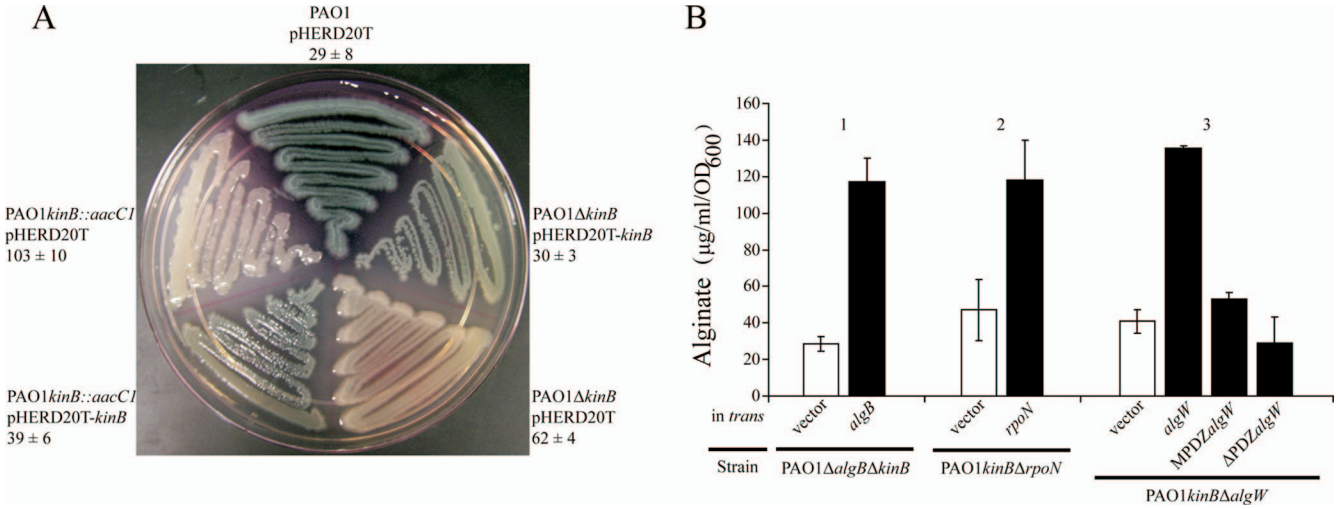

FIG. 2. Mutation of *kinB* in PAO1 results in a mucoid phenotype dependent upon *algB*, *rpoN*, and *algW*. (A) Colony morphologies of *P. aeruginosa* PAO1 and mucoid *kinB* mutants with or without *kinB* expressed in *trans.* For complementation, *kinB* was expressed from the P_{BAD} promoter of pHERD20T. Strains were grown on a PIA-carbenicillin plate supplemented with 0.1% arabinose at 37°C for 24 h and at room temperature for 24 h. Alginate production was assayed by the carbazole assay (23) after 24 h at 37°C. The amount of alginate is indicated as μg/ml/OD_{600} unit. Values are expressed as means ± standard deviations from three independent experiments. (B) *kinB* mutants require *algB*, *rpoN*, and *algW* for alginate overproduction. Each mutant strain was assayed for alginate production with a vector control (pHERD20T) or with the gene indicated in *trans* expressed from the P_{BAD} promoter of pHERD20T. The strains were grown for 24 h at 37°C on PIA supplemented with carbenicillin and 0.1% arabinose.

milk in PBS (pH 7.4). Primary antibodies were diluted 1:1,000 in 3% nonfat dry milk in PBS. The membranes were probed with mouse monoclonal antibodies against AlgU (46), RpoN (Neoclone, Madison, WI), alpha RNA subunit polymerase subunit (Neoclone), rabbit polyclonal antibody against AlgB (28), or rat monoclonal antibody against hemagglutinin (HA) (Roche, Mannheim, Germany) overnight at 4°C with shaking. Horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin G (IgG) or HRP-labeled anti-rabbit IgG was diluted 1:5,000 in 3% nonfat dry milk in PBS and used as the secondary antibodies. Advanced ECL or ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ) was used for detecting HRP-labeled goat anti-mouse IgG or anti-rabbit IgG (Roche) by the manufacturer's procedure. The signals were detected with an EC3 imaging system (UVP, Upland, CA) by capturing with a BioChemi HR camera. For reprobing, membranes were stripped with 62.5 mM Tris-HCl (pH 6.8)–2% sodium dodecyl sulfate–100 mM β-mercaptoethanol for 15 min at 50°C and then washed in PBS.

HA-MucA steady-state and kinetic concentration Western blot analysis. To assay the HA-MucA degradation profile after 48 h of growth at 37°C, HA-MucA was expressed in *trans* in mucoid and nonmucoid strains from pHERD20T with 0.1% arabinose on PIA-carbenicillin plates. Cells were scraped from the plate, suspended in PBS (pH 7.4), and pelleted by centrifugation. Proteins were isolated and prepared as described for Western blot analysis.

To observe the rate of degradation of HA-MucA in PAO1 and PAO1 *kinB*::*aacC1*, we analyzed cell lysates over a time course. We utilized the conditional expression of HA-MucA from pHERD20T to compare the rates of degradation. Cultures of PAO1 and PAO1 *kinB*::*aacC1* were incubated overnight at 37°C in LB-carbenicillin (100 μg/ml). The cultures were OD matched, and equal numbers of cells were inoculated into 500 ml of LB-carbenicillin (100 μg/ml) supplemented with 1% (wt/vol) arabinose to induce expression of HA-*mucA*. At an OD_{600} of 0.2, the cells were harvested by centrifugation at 7,000 × *g* for 10 min. The cells were then resuspended in 500 ml of M9 broth with 0.4% glucose supplemented with 100 μg/ml carbenicillin. Samples were taken at 10-min intervals by harvesting 50 ml of culture at 4,000 × *g* and 4°C for 10 min. Pellets were immediately stored at −80°C until proteins were prepared with Ready-Preps (Epicentre, Madison, WI) by the manufacturer's protocol and subjected to Western blot analysis with anti-HA and anti-alpha RNA polymerase subunit antibodies.

RESULTS

Inactivation of *kinB* in *P. aeruginosa* strain PAO1 results in alginate overproduction. To discover novel negative regulators of alginate biosynthesis, the standard genetic strain PAO1 was subjected to mariner transposon mutagenesis (53). Stable mucoid gentamicin-resistant mutants were isolated. Mucoid mutants were verified for single transposon insertions by Southern hybridization (data not shown), and the pFAC transposon insertions were mapped by inverse PCR and sequencing as previously described (42, 43). Numerous mucoid mutants with insertions into the well-characterized negative regulator genes *mucA*, *mucB*, and *mucD* were identified. Interestingly, an insertion into *kinB* converted PAO1 to the mucoid phenotype (GenBank accession number for the *kinB* insertion in PAO1, FJ209363) (Fig. 2A). To show that mucoidy due to *kinB* inactivation was not caused by polar effects on nearby genes, we constructed an in-frame deletion of *kin*B in PAO1. Alginate overproduction resulted when *kinB* was deleted (Fig. 2A). However, PAO1 Δ*kinB* produced less alginate, i.e., 62 ± 4 μg/ml/OD_{600} unit, versus 103 ± 10 μg/ml/OD_{600} unit for PAO1 *kinB*::*aacC1* (Fig. 2A). The mucoid phenotypes of PAO1 *kinB*::*aacC1* and PAO1 Δ*kinB* were complemented by conditional expression of *kinB* (Fig. 2A). Expression of *kinB* in *trans* in PAO1 *kinB*::*aacC1* and PAO1 Δ*kinB* decreased alginate production to wt PAO1 levels, as expected (Fig. 2A). Furthermore, sequencing analysis confirmed that the *mucA* gene of PAO1 *kinB*::*aacC1* did not harbor mutations (GenBank accession number, FJ209362). Thus, inactivation or deletion of *kinB* in a wt *mucA* background causes alginate overproduction. This suggests that KinB is a negative regulator of alginate in *P. aeruginosa* strain PAO1.

Alginate production in *kinB* mutants requires *algB* and *rpoN*. Alginate overproduction in *mucA* mutants requires AlgB, an NtrC-type transcriptional activator (56). The *algB* gene is located immediately upstream of *kinB* in the genome. The *kinB* gene encodes the cognate kinase that has been shown to phosphorylate AlgB (29). Deletion of both *algB* and *kinB* together results in wt nonmucoid alginate production (Fig. 2B,

bars 1). Alginate production was restored in the PAO1 Δ*algB* Δ*kinB* double mutant by expression of *algB* in *trans* (Fig. 2B, bars 1). Since *rpoN* has been shown to be required for alginate production in a mucoid strain with an undefined *muc-23* mutation (7), we examined whether *rpoN* was required in PAO1 *kinB*::*aacC1*. Deletion of *rpoN* from PAO1 *kinB*::*aacC1* resulted in loss of mucoidy and could be complemented with *rpoN* expressed in *trans* (Fig. 2B, bars 2).

Alginate production in *kinB* mutants requires *algW*. Since the *mucA* gene is not mutated in PAO1 *kinB*::*aacC1*, one possible explanation for the mucoid phenotype is that MucA is being degraded. AlgW has been shown to be required for activation of the alginate biosynthetic operon by D-cycloserine (54), and AlgW mediates regulated proteolysis of MucA during overexpression of *mucE* (43). We next tested whether mucoidy due to loss of *kinB* was dependent upon AlgW-regulated proteolysis. Deletion of *algW* from PAO1 *kinB*::*aacC1* resulted in a nonmucoid phenotype and lowered alginate production (Fig. 2B, bars 3). Expression of *algW* in *trans* restored alginate production (Fig. 2B, bars 3). The PDZ domain of AlgW is required for MucE-mediated signal transduction (43). Therefore, to show that PAO1 *kinB*::*aacC1* utilizes activated AlgW for derepression of MucA, we introduced an *algW* allele with the PDZ domain truncated and an *algW* allele with the PDZ domain completely deleted. When these mutant *algW* alleles were expressed in *trans* in the double mutant PAO1 *kinB*::*aacC1* Δ*algW*, alginate overproduction was not restored (Fig. 2B, bars 3). These data suggest that activation of AlgW is required for alginate overproduction in PAO1 *kinB*::*aacC1*.

In the absence of *kinB*, phosphorylation of AlgB at D59 is not required for alginate production. KinB has been shown to effectively phosphorylate AlgB in vitro (29). However, AlgB derivatives such as AlgB.D59N, which cannot be phosphorylated by KinB, still promote alginate production in *mucA* mutants (28). The *algB45* allele encodes AlgB.D59N, where the phosphorylation site (D59) has been mutated to asparagine (N) (28) We presumed that AlgB was not phosphorylated in the absence of the cognate histidine kinase KinB. To confirm that phosphorylation of AlgB at position 59 was not required for alginate production in the absence of KinB, we cloned the *algB45* allele into pHERD20T for conditional expression. The *algB45* gene was PCR amplified from pUS56 (28) and directionally cloned. The construct was sequenced to observe the expected D59N mutation and to ensure that no other mutations resulted. Expression of *algB45* from the P_{BAD} promoter in the presence of arabinose complemented the PAO581 *algB*::*aacC1* (*mucA25 algB*::Gm^r) mutant (Table 2) (42), which is consistent with the previous finding that the *algB45* allele can still promote alginate production in a *mucA22* mutant (28). Since the construct was functional, we introduced *algB45* into PAO1 Δ*algB* Δ*kinB*. Alginate overproduction occurred when *algB45* was expressed in PAO1 Δ*algB* Δ*kinB* (Table 2). These data suggests that in the absence of KinB, phosphorylation of AlgB at position 59 was not required for mucoidy. Interestingly, when we overexpressed *algB* or *algB45* in wt PAO1 and PAO1 Δ*algB*, we did not observe an increase in alginate production even when culture was on 1% arabinose (data not shown). It seems that deletion of *kinB* affects alginate production independent of the phosphorylation status of AlgB. Similar to the case for *mucA* mutants, phosphorylation of AlgB is not required for alginate overproduction in the *kinB* mutant with wt *mucA*.

TABLE 2. Complementation of alginate production by *algB* mutants with wt *algB* and phosphorylation-defective *algB45*

Strain	Plasmid	Arabinose (%, wt/vol)[a]	Phenotype[b]	Alginate, μg/ml/OD_{600} unit (mean ± SD)
PAO581 *algB*::*aacCC1* (*mucA25 algB*::Gm^r)	pHERD20T-*algB*	0	NM	49.0 ± 7.3
		1	M	285.7 ± 12.9
	pHERD20T-*algB45*	0	NM	51.9 ± 3.0
		1	M	228.2 ± 21.0
PAO1 Δ *algB*Δ*kinB*	pHERD20T-*algB*	0	NM	64.7 ± 10.9
		1	M	215.7 ± 13.5
	pHERD20T-*algB45*	0	NM	49.5 ± 2.9
		1	M	263.8 ± 2.5

[a] Strains were cultured for 24 h at 37°C on PIA supplemented with carbenicillin and arabinose.
[b] NM and M, nonmucoid and mucoid phenotypes, respectively.

P_{algU} and P_{algD} activities in *kinB* null mutants are dependent on *algU*, *algB*, *rpoN*, and *algW*. To examine the effect of the *kinB* mutation on the alginate-related promoters P_{algU} and P_{algD}, we integrated a single copy of the entire *algU* or *algD* promoter region (Fig. 3A and C, respectively) fused with *lacZ* onto the chromosomes of PAO1 and PAO1 *kinB*::*aacC1* as well as *kinB algU*, *kinB algB*, *kinB rpoN*, and *kinB algW* double mutants. The effect of each deletion or inactivated gene on the expression of the promoter fusions in the PAO1 and *kinB* backgrounds was assessed by complementation. The β-galactosidase activity was measured with vector alone (pHERD20T) and compared to that when the mutation was complemented with expression of the gene from the P_{BAD} promoter of pHERD20T (41) in the presence of 0.1% arabinose. As a control for these experiments, P_{algU} and P_{algD} expression was measured when *algU* was overexpressed (Fig. 3B, bars 1, and D, bars 1, respectively).

Previous studies have shown that only small changes in P_{algU} expression are required for mucoidy (33). Inactivation of *kinB* in PAO1 *kinB*::*aacC1* caused significantly increased P_{algU} expression compared to that in parent strain PAO1 (Fig. 3B, bars 2). The high P_{algU} expression in PAO1 *kinB*::*aacC1* can be reduced with *kinB* expressed in *trans* (Fig. 3B, bars 2). Deletion of *algU* eliminated detectable P_{algU} expression in PAO1 *kinB*::*aacC1* (Fig. 3B, bars 3). Since *algB* was observed to be required for alginate production in *kinB* mutants, we next examined whether *algB* was required for high levels of expression of P_{algU}. The high level of P_{algU} expression in the absence of *kinB* required *algB* (Fig. 3B, bars 4). AlgB has been established as a transcriptional activator at P_{algD} in the *mucA22* mutant FRD-1 (56). Here we show a possible new role for AlgB in addition to the role at P_{algD}. We also observed that *rpoN* has a role in influencing high expression of P_{algU} (Fig. 3B, bars 5) that can be restored with *rpoN* expressed in *trans*. This information shows a possible role of *rpoN* outside of characterized interactions at P_{algD} (7). As expected, P_{algU} expression is also influenced by the serine protease AlgW in PAO1 *kinB*::*aacC1* (Fig. 3B, bars 6). However, the level of expression

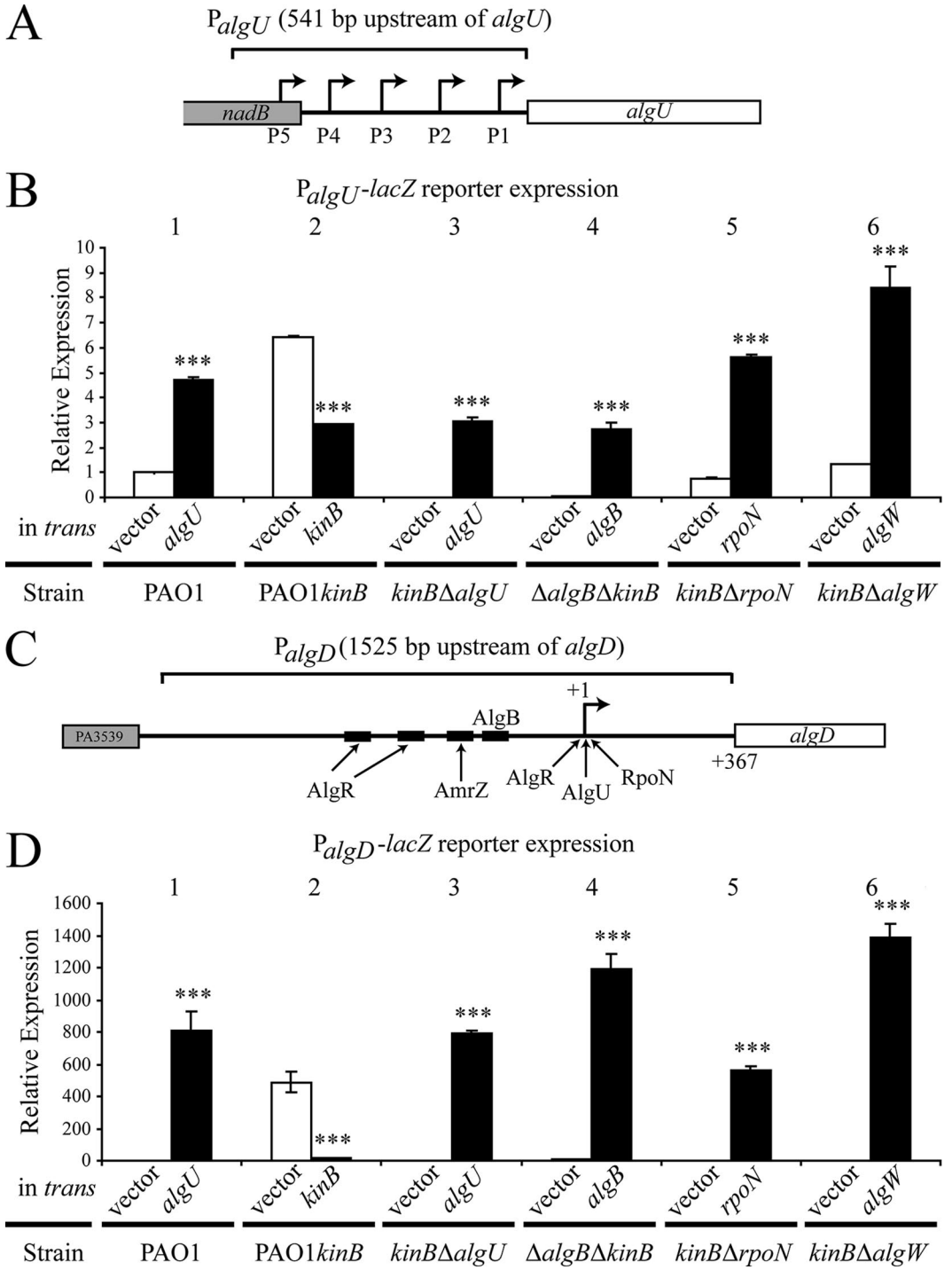

FIG. 3. Loss of *kinB* causes upregulation of both P_{algU} and P_{algD}. β-Galactosidase activity from P_{algU}-*lacZ* and P_{algD}-*lacZ* reporters on the chromosomes of PAO1, PAO1 *kinB*::*aacC1*, and PAO1 *kinB*::*aacC1* isogenic mutants was determined. P_{algU}-*lacZ* and P_{algD}-*lacZ* reporter constructs were integrated into the chromosomes of the indicated strains. Genes indicated were expressed in *trans* from the P_{BAD} promoter of pHERD20T. β-Galactosidase activities were determined after 24 h of growth on PIA with 0.1% arabinose. Values were normalized to PAO1 pHERD20T (empty vector) reporter expression and indicated as means ± standard deviations from three independent experiments. Student's *t* test was performed for comparison of activity of the strain with vector only or with the complementing gene in *trans*. Asterisks indicate significant differences (***, $P < 0.0001$). Strain PAO1 *kinB*::*aacC1* is indicated as PAO1 *kinB*. Note that expression of *algU* in PAO1 is a positive control for the analysis due to the AlgU-dependent nature of both P_{algU} and P_{algD}. (A) A schematic of the entire P_{algU} promoter region with the relative positions of the five promoters that were utilized for the *lacZ* promoter fusion. (B) P_{algU} activity in PAO1, PAO1 *kinB*::*aacC1*, and strains isogenic to PAO1 *kinB*::*aacC1*. High P_{algU} activity in PAO1 *kinB*::*aacC1* and PAO1 Δ*kinB* mutants requires *algU*, *algB*, *rpoN*, and *algW*. Note that *kinB* expression significantly lowers P_{algU} activity. (C) A schematic of the entire P_{algD} promoter region that was used for the *lacZ* promoter fusion. The relative binding sites of the P_{algD} transcriptional activators are indicated. (D) P_{algD} activity in PAO1, PAO1 *kinB*::*aacC1*, and strains isogenic to PAO1 *kinB*::*aacC1*. High P_{algD} activity in PAO1 *kinB*::*aacC1* and PAO1 Δ*kinB* requires *algU*, *algB*, *rpoN*, and *algW*. Note that *kinB* expression significantly lowers P_{algD} activity.

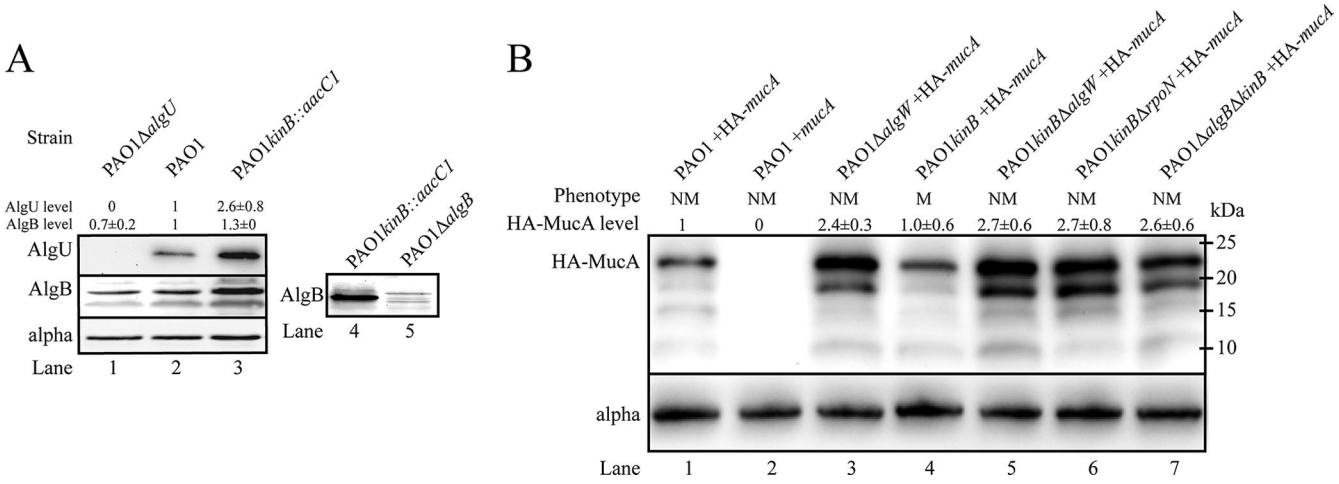

FIG. 4. PAO1 *kinB*::*aacC1* exhibits elevated levels of AlgB and AlgU, and HA-MucA degradation in PAO1 *kinB*::*aacC1* requires *algW*, *algB*, and *rpoN*. Shown are representative panels of blots from three independent experiments with 40 μg of total lysate. (A) A Western blot of total cell lysate of PAO1 *kinB*::*aacC1* shows elevated levels of AlgB and AlgU. Western blots of cell lysates were prepared from cells after 24 h growth on PIA. The membranes were probed with anti-AlgU, anti-AlgB, and anti-alpha subunit of RNA polymerase (loading control). Levels of each protein were adjusted for loading and then normalized to PAO1 levels and expressed as means ± standard deviations. Note that deletion of *algU* did not abolish AlgB expression. (B) Western blot analysis of N-terminally HA-tagged MucA in PAO1 and PAO1 *kinB*::*aacC1* isogenic backgrounds. Cell lysates were prepared after 48 h of growth on PIA-carbenicillin plates supplemented with 0.1% arabinose. The membranes were immunoblotted with rat anti-HA diluted 1:1,000 (Roche). Lane 2, PAO1 pHERD20T-*mucA* is a negative control for background and cross-reactivity. Lanes 1 and 3 to 7, HA-*mucA* expressed in *trans* from pHERD20T. Levels of each protein were adjusted for loading and then normalized to PAO1 pHERD20T-HA-*mucA* levels and expressed as means ± standard deviations. Apparent molecular masses are depicted. NM and M indicate nonmucoid and mucoid phenotypes, respectively.

of P_{algU} with *algW* in *trans* exceeded the P_{algU} expression level in PAO1 *kinB*::*aacC1*. A possible explanation for this is that *algW* expression from the arabinose promoter in the presence of 0.1% on a multicopy vector may exceed endogenous expression levels of *algW* in vivo.

P_{algD} expression was measured with the same strategy utilized for P_{algU}. Unlike P_{algU} activity, P_{algD} activity was minimally detectable in PAO1 (Fig. 3D, bars 1). The elevated level of P_{algD} expression in PAO1 *kinB*::*aacC1* was significantly reduced when *kinB* was expressed in *trans* (Fig. 3D, bars 2). The elevated level of P_{algD} in *kinB* mutants required *algU*, *algB*, *rpoN*, and *algW*, which correlates with the observations of P_{algU} expression. The *kinB* mutants with deletions of *algU*, *algB*, *rpoN*, and *algW* had minimally detectable P_{algD} (Fig. 3D, bars 3 to 6). When *algU*, *algB*, *algW*, and *rpoN* were expressed in *trans* to complement their respective gene deletions in *kinB* mutants, elevated P_{algD} expression was returned. Collectively these promoter fusions in the PAO1 and *kinB* backgrounds show that *algU*, *algB*, *algW*, and *rpoN* influence the P_{algU} and P_{algD} activity, which correlates with alginate production (Fig. 2B).

AlgU and AlgB expression is increased in PAO1 *kinB*::*aacC1*. Next we measured the expression of AlgU and AlgB in whole-cell lysates of PAO1 *kinB*::*aacC1* (Fig. 4A). To control for cross-reactivity of anti-AlgU and anti-AlgB, total lysates of PAO1 Δ*algU* and PAO1 Δ*algB* were blotted, and very low cross-reactivity was noted (Fig. 4A, lanes 1 and 5, respectively). Western blot analysis revealed that AlgU was upregulated 2.6- ± 0.8-fold in PAO1 *kinB*::*aacC1* compared to PAO1 (Fig. 4A, lanes 2 and 3). AlgB expression was also increased in PAO1 *kinB*::*aacC1*, which is consistent with a previous observation that *algB* transcription requires *algT*/*U* (57). Interestingly, AlgB was detected in PAO1 Δ*algU* cell lysate, which suggests that AlgB expression may also be controlled by another σ factor in addition to AlgU (Fig. 4A, lane 1).

MucA proteolytic degradation facilitates alginate overproduction in PAO1 *kinB*::*aacC1*. Since *mucA* is wt in PAO1 *kinB*::*aacC1*, MucA repression of AlgU must be relieved for activation of AlgU and alginate production. Based on the fact that alginate overproduction by PAO1 *kinB*::*aacC1* requires AlgW, our hypothesis is that alginate production in the *kinB* mutant occurs by regulated proteolysis of MucA. To test this model, we needed to observe MucA degradation. N-terminal HA-tagged MucA was expressed from pHERD20T-HA-*mucA* under induction of arabinose into nonmucoid and mucoid PAO1 derivative strains. The wt *mucA* gene without HA was expressed in *trans* as the negative control. Western blotting of PAO1 without HA-tagged *mucA* showed no background or cross-reactivity with other proteins (Fig. 4B, lane 2). In PAO1, full-length HA-MucA existed as well as other truncated degradation products (Fig. 4B lane 1). HA-MucA degradation in PAO1 is consistent with degradation of RseA in *E. coli*, which occurs in the absence of stress signals (2). Also, PIA contains triclosan, which has been shown to activate P_{algD} activity (54), suggesting that regulated proteolysis occurs in the presence of cell wall-inhibitory antibiotics. In PAO1 Δ*algW*, full-length HA-MucA is 2.4- ± 0.3-fold increased relative to PAO1 HA-MucA (Fig. 4B, lanes 1 and 3). This implies that HA-MucA is not as rapidly degraded in PAO1 Δ*algW* as in PAO1. However, PAO1 Δ*algW* also exhibited a truncated HA-MucA with an apparent molecular mass of 19 kDa (Fig. 4B, lane 3). The absence of this band in PAO1 suggests that deletion of *algW* inhibited efficient proteolysis of HA-MucA, resulting in accumulation of two major fragments of HA-MucA. Mucoid PAO1

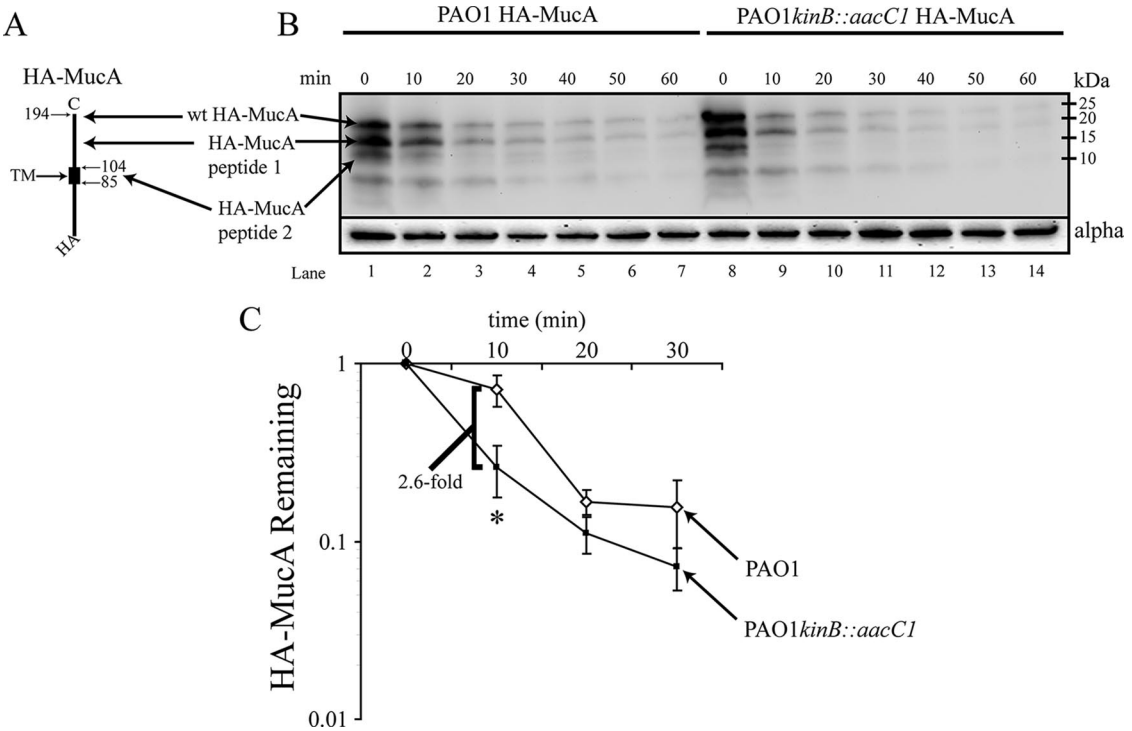

FIG. 5. Kinetic comparison of HA-MucA degradation in PAO1 and PAO1 *kinB*::*aacC1*. (A) Schematic diagram of HA-MucA. Indicated are the N-terminal HA tag, the transmembrane domain, and the relative cleavage sites resulting in the truncated HA-MucA peptides observed by Western blotting. (B) Western blotting analysis of a time course of HA-MucA degradation. PAO1 and PAO1 *kinB*::*aacC1* expressing HA-*mucA* from pHERD20T were grown at 37°C with shaking in LB supplemented with carbenicillin and arabinose until the OD_{600} reached 0.2. Cells were harvested and resuspended in M9 broth supplemented with 0.4% glucose. Glucose enhances repression of the P_{BAD} promoter. During the time course the OD_{600} was monitored and remained stable throughout. Equal numbers of cells were extracted at 10-min intervals and harvested at 4°C, and pellets were frozen at −80°C until cell lysates were prepared. Shown is a representative panel of blots with 40 μg of total lysate transferred and blotted with anti-HA from three independent experiments. Apparent molecular masses are indicated. (C) Quantitative measurement of wt HA-MucA in PAO1 and PAO1 *kinB*::*aacC1* from 0 min to 30 min. Levels of each protein were adjusted for loading and then normalized to PAO1 pHERD20T-HA-*mucA* or PAO1 *kinB*::*aacC1* pHERD20T-HA-*mucA* levels and expressed as means ± standard deviations from three independent experiment (*, $P < 0.01$).

kinB::*aacC1* lacks the secondary truncated peptide of HA-MucA (Fig. 4B, lane 4), and the concentration of full-length HA-MucA is lower than that in PAO1 *kinB*::*aacC1*Δ*algW* (Fig. 4B, lane 5). Interestingly, smaller degradation intermediates of HA-MucA (~15 kDa) were still observed when *algW* was deleted in PAO1 and PAO1 *kinB*::*aacC1* (Fig. 4B, lanes 3 and 5). These data suggest that regulated proteolysis of MucA may occur independent of AlgW, as has been shown when PAO1 *algW*::Tc^r converts to mucoidy in the presence of the reactive oxygen-producing paraquat (6). The presence of the truncated HA-MucA was apparent upon deletion of *algW*, *algB*, and *rpoN* in PAO1 *kinB* mutants (Fig. 4B, lanes 5, 6, and 7). Taken together, these observations suggest that *algB*, *algW*, and *rpoN* influence HA-MucA degradation and derepression of AlgU in PAO1 *kinB*::*aacC1*.

In order to better measure the differences in HA-MucA degradation between PAO1 and PAO1 *kinB*::*aacC1*, a time course was conducted to show in vivo depletion of HA-MucA in PAO1 and PAO1 *kinB*::*aacC1*. To quantify the degradation of HA-MucA, cells were grown in LB broth containing arabinose to express HA-MucA and then transferred to M9 minimal medium supplemented with 0.4% glucose lacking arabinose. The OD_{600} remained stable during the time course. Therefore, depletion of HA-MucA was due to in vivo proteolytic degradation and not to division of the cells. Samples were taken every 10 min for the cell lysis and Western blotting. We also performed the time course with 30-min intervals but found that degradation of HA-MucA even in PAO1 was rapid, indicating that shorter time points need to be used (data not shown). The HA-MucA levels were assayed by Western blotting of 40 μg of total cell lysate (Fig. 5B). The rate of HA-MucA degradation in PAO1 *kinB*::*aacC1* was 2.6-fold greater than that of PAO1 from 0 to 10 min (Fig. 5C). However, after 10 min the amounts of HA-MucA leveled off and were not significantly different between PAO1 and PAO1 *kinB*::*aacC1* (Fig. 5C). Assuming that the mobility of HA-MucA peptide 1 was not aberrant, it appears that there is a cleavage site between the transmembrane domain and the C terminus of MucA (Fig. 5A). The smaller major truncated MucA peptide recognized with an HA epitope (HA-MucA peptide 2) was likely created by cleavage near the transmembrane domain. This study indicates that HA-MucA degradation is rapid even in PAO1; however, inactivation of *kinB* causes an increased rate of degradation. The increased HA-MucA degradation likely contributes to the mucoid phenotype of *kinB* mutants.

DISCUSSION

We discovered that mutation of *kinB* in PAO1 results in overproduction of alginate (Fig. 2A). Alginate regulation in

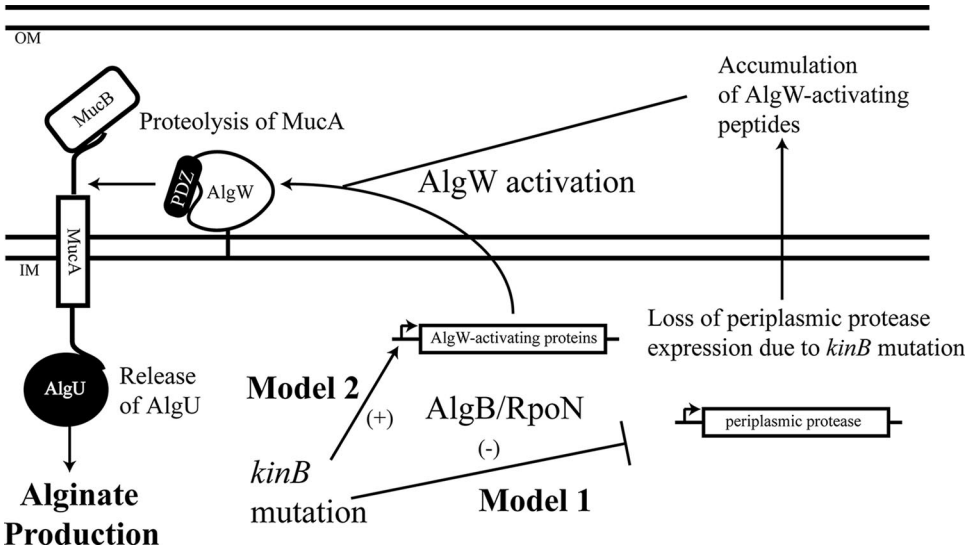

FIG. 6. Proposed models of negative regulation of alginate production by KinB in *P. aeruginosa*. Mutation of *kinB* in wt *mucA* strain PAO1 causes alginate overproduction. Alginate overproduction by *kinB* mutants requires *algB* and *rpoN*. In model 1, we propose that mutation of *kinB* causes loss of expression of a periplasmic protease(s), which leads to accumulation of AlgW-activating factors. In model 2, we propose that *algB* and *rpoN* control expression of factors which can activate the AlgW protease to release repression of MucA by proteolytic degradation and activate AlgU. In either case, derepression of MucA by regulated proteolysis causes AlgU activation, which facilitates *algU* expression, resulting in mucoidy by upregulating the alginate biosynthetic operon. OM and IM, outer membrane and inner membrane, respectively.

mucA mutant strains was the first characterized mode of conversion to mucoidy and is the best elucidated (32). However, recent studies have shown that regulated proteolysis mediated by AlgW is a mechanism for alginate production in *P. aeruginosa* (43, 54). Here we have presented data showing that inactivation of *kinB* causes mucoidy and is dependent upon *algB*, *algW*, and *rpoN* (Fig. 2B). We also observed through complementation analysis that phosphorylation of AlgB at the confirmed phosphorylation site is not required for alginate production in the *kinB* mutant. Our data suggest that the *kinB* mutation increases the rate of degradation of MucA by regulated proteolysis, which causes the mucoid phenotype of *kinB* mutants.

KinB is the cognate kinase of the alginate regulator AlgB (29), and alginate biosynthesis occurs independent of phosphorylation of AlgB (28). However, the role of *kinB* in alginate production has been examined only in mucoid *mucA*22 mutant strains such as FRD-1 (28). In *mucA* mutants, the requirement for regulated proteolysis to activate AlgU would likely be bypassed due to the *mucA* mutation. We observed that in *kinB* mutants, *algB* and *rpoN* are both required for alginate production (Fig. 2B) and increased P_{algU} and P_{algD} promoter activity (Fig. 3B and D, respectively). Previously both *rpoN* (7) and *algB* (26) have been shown to affect transcription at P_{algD}. Conversely, our data show that these regulators, AlgB and RpoN, also affect P_{algU} transcription. Only relatively small changes in P_{algU} expression are required for mucoidy (33); however, PAO1 *kinB*::*aacC1* exhibits significantly elevated expression of both P_{algU} and P_{algD} (Fig. 3B and D, respectively). We also noted that deletion of *algU* from PAO1 *kinB*::*aacC1* resulted in complete loss of detectable P_{algU}-*lacZ* activity as measured by β-galactosidase assay (Fig. 3B, bars 3). This has also been observed when *algU* is deleted from PAO1 (data not shown). Two of the *algU* promoters are AlgU dependent (12, 45); however, it is not clear which σ factors the other promoters depend upon. Therefore, it is possible that in vivo AlgU contributes the bulk of transcriptional activation of the AlgU promoters that is detectable by our reporter assay, but further analysis is required to fully understand the *algU* promoters.

Based on our data, we propose two alternative models for activation of alginate production through regulated proteolysis in *kinB* mutants (Fig. 6). In both models, regulated proteolysis of MucA by AlgW occurs, but the cause of the increased concentration of activating signals differs. The first model suggests that mutation of *kinB* affects expression of a protease or chaperone responsible for removal of misfolded proteins (Fig. 6). Mutation of an aminopeptidase gene, *phpA*, has been shown to cause increased P_{algD} activity and mucoidy (55). The second proposed model is that *algB* and *rpoN* directly control expression of peptide signals in the absence of *kinB* that activate AlgW and therefore increase proteolytic degradation of MucA (Fig. 6). Deletion of *algB* and *rpoN* in *kinB* mutants caused an accumulation of the major HA-MucA truncation product that was also observed when *algW* was deleted in PAO1 or PAO1 *kinB*::*aacC1* (Fig. 4B). We have also observed that *algB* and *rpoN* are not required for *algW* expression (data not shown), which suggested that loss of *algB* or *rpoN* may affect the proteolytic activity of AlgW. From this information, we hypothesize that *algB* and *rpoN* may be required for expression of signals that activate AlgW and regulated proteolysis. Our data suggest that increased regulated proteolysis occurs in *kinB* mutants of PAO1 (Fig. 4B and 5B and C). In *E. coli*, many outer membrane and periplasmic proteins have been shown to activate DegS protease activity through interaction with the PDZ domain (18). Interestingly, in *E. coli*, inactivation of the two-component histidine kinase EnvZ causes upregulation of the porin OmpC (48). Porins such as OmpC can activate regulated proteolysis (18). Analysis of the *P. aeruginosa* genome

shows no significant homologues to the DegS-activating peptides such as OmpC of *E. coli*. This is conceivable because *P. aeruginosa* and *E. coli* reside in different habitats, and therefore it is likely that activation of AlgU and activation of σ^E require different types of signals. However, proteins with probable activating sequences are encoded throughout the *P. aeruginosa* genome (43). Thus, *P. aeruginosa* likely has novel proteins that could potentially activate AlgW degradation of MucA. It is possible that RpoN, in tandem with response regulators such as AlgB, controls numerous genes with various functions which may be involved in signal transduction of the AlgU stress response.

AlgB and or RpoN could drive both *algU* and *algD* transcription. This is an alternative hypothesis to the models already described. Both AlgB and RpoN have been shown to bind at P_{algD} and are required for *algD* expression (7, 26). It has been suggested that AlgB may interact with other σ factors than RpoN (26). We have attempted to show AlgB binding with P_{algU} using a gel shift assay; however, interaction has not been observed (data not shown). Recent studies have employed special conditions to detect AlgB DNA binding at P_{algD} (26). Since exhaustive studies have not been performed, we cannot dismiss the possibility that AlgB and/or RpoN may initiate transcription at P_{algU}. Based on our data, both the P_{algU} and the P_{algD} promoters are highly upregulated in *kinB* mutants (Fig. 3B and D). Therefore, it is possible that in the absence of *kinB*, AlgB could activate transcription of both the P_{algU} and P_{algD} promoters.

Do *P. aeruginosa* CF isolates have *kinB* mutations? Most clinical observations have focused on surveying *mucA*, *mucB*, and *mucD* (8, 10, 32). Therefore, large-scale surveys looking for *kinB* mutants have not been performed. However, one recently sequenced epidemic CF isolate, C3719, does have a mutation that truncates the KinB protein to 526 amino acids instead of the wt 595 amino acids of PAO1 KinB (http://www.broad.mit.edu). Therefore, a CF isolate has been shown to have a *kinB* mutation, but C3719 is apparently nonmucoid (34). This suggests that either the mutation is not completely detrimental to KinB regulation or C3719 may have additional suppressor mutations in either known or novel alginate regulators. PAO579 is another strain that requires *rpoN* for mucoidy (7); however, the mucoid phenotype cannot be suppressed by complementation with *kinB* (data not shown). We are currently surveying for wt *mucA* CF isolates for *kinB* mutations. Many two-component signal systems can be activated by environmental conditions. The PhoP-PhoQ (30) and PmrA-PmrB (35) systems of *P. aeruginosa* are activated by low Mg^{2+} concentrations, whereas the conserved PhoB-PhoR system is activated by low phosphate concentrations (25). Therefore, elucidation of the environmental signals that relieve the negative regulation of KinB on alginate overproduction will be as interesting as finding *kinB* mutant CF isolates.

In this report we have characterized KinB as a negative regulator of alginate production and have proposed novel regulation of AlgW-dependent MucA derepression that is mediated by AlgB and RpoN. These data bring us a step closer toward understanding the molecular events leading to alginate production which preclude the classically described *mucA* mutations in *P. aeruginosa*. It will be interesting to further elucidate the unknown genes that may be under the negative control of the sensor kinase KinB and to determine the environmental stimulus that affects KinB regulation in *P. aeruginosa*.

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REFERENCES

1. **Ades, S. E.** 2004. Control of the alternative sigma factor sigmaE in *Escherichia coli*. Curr. Opin. Microbiol. **7:**157–162.
2. **Ades, S. E., I. L. Grigorova, and C. A. Gross.** 2003. Regulation of the alternative sigma factor sigma(E) during initiation, adaptation, and shutoff of the extracytoplasmic heat shock response in *Escherichia coli*. J. Bacteriol. **185:**2512–2519.
3. **Aiba, B. M., J. A. Leeds, C. Onufryk, C. Z. Lu, and C. A. Gross.** 2002. DegS and YaeL participate sequentially in the cleavage of RseA to activate the sigma(E)-dependent extracytoplasmic stress response. Genes Dev. **16:**2156–2168.
4. **Baynham, P. J., A. L. Brown, L. L. Hall, and D. J. Wozniak.** 1999. *Pseudomonas aeruginosa* AlgZ, a ribbon-helix-helix DNA-binding protein, is essential for alginate synthesis and *algD* transcriptional activation. Mol. Microbiol. **33:**1069–1080.
5. **Baynham, P. J., D. M. Ramsey, B. V. Gvozdyev, E. M. Cordonnier, and D. J. Wozniak.** 2006. The *Pseudomonas aeruginosa* ribbon-helix-helix DNA-binding protein AlgZ (AmrZ) controls twitching motility and biogenesis of type IV pili. J. Bacteriol. **188:**132–140.
6. **Boucher, J. C., J. Martinezsalazar, M. J. Schurr, M. H. Mudd, H. Yu, and V. Deretic.** 1996. Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease *htrA*. J. Bacteriol. **178:**511–523.
7. **Boucher, J. C., M. J. Schurr, and V. Deretic.** 2000. Dual regulation of mucoidy in *Pseudomonas aeruginosa* and sigma factor antagonism. Mol. Microbiol. **36:**341–351.
8. **Bragonzi, A., L. Wiehlmann, J. Klockgether, N. Cramer, D. Worlitzsch, G. Doring, and B. Tummler.** 2006. Sequence diversity of the mucABD locus in Pseudomonas aeruginosa isolates from patients with cystic fibrosis. Microbiology **152:**3261–3269.
9. **Bragonzi, A., D. Worlitzsch, G. B. Pier, P. Timpert, M. Ulrich, M. Hentzer, J. B. Andersen, M. Givskov, M. Conese, and G. Doring.** 2005. Nonmucoid *Pseudomonas aeruginosa* expresses alginate in the lungs of patients with cystic fibrosis and in a mouse model. J. Infect. Dis. **192:**410–419.
10. **Ciofu, O., B. Lee, M. Johannesson, N. O. Hermansen, P. Meyer, and N. Hoiby.** 2008. Investigation of the algT operon sequence in mucoid and non-mucoid Pseudomonas aeruginosa isolates from 115 Scandinavian patients with cystic fibrosis and in 88 in vitro non-mucoid revertants. Microbiology **154:**103–113.
11. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. Gene *algD* coding for GDPmannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. J. Bacteriol. **169:**351–358.
12. **DeVries, C. A., and D. E. Ohman.** 1994. Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternate sigma factor, and shows evidence for autoregulation. J. Bacteriol. **176:**6677–6687.
13. **Doggett, R. G., G. M. Harrison, R. N. Stillwell, and E. S. Wallis.** 1966. An atypical *Pseudomonas aeruginosa* associated with cystic fibrosis of the pancreas. J. Pediatr. **68:**215–221.
14. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
15. **Goldberg, J. B., and T. Dahnke.** 1992. *Pseudomonas aeruginosa* AlgB, which modulates the expression of alginate, is a member of the NtrC subclass of prokaryotic regulators. Mol. Microbiol. **6:**59–66.
16. **Goldberg, J. B., and D. E. Ohman.** 1984. Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. J. Bacteriol. **158:**1115–1121.
17. **Govan, J. R., and V. Deretic.** 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. **60:**539–574.
18. **Hasselblatt, H., R. Kurzbauer, C. Wilken, T. Krojer, J. Sawa, J. Kurt, R.**

Kirk, S. Hasenbein, M. Ehrmann, and T. Clausen. 2007. Regulation of the sigmaE stress response by DegS: how the PDZ domain keeps the protease inactive in the resting state and allows integration of different OMP-derived stress signals upon folding stress. Genes Dev. **21:**2659–2670.

19. **Hassett, D. J.** 1996. Anaerobic production of alginate by *Pseudomonas aeruginosa*: alginate restricts diffusion of oxygen. J. Bacteriol. **178:**7322–7325.
20. **Hoang, T. T., A. J. Kutchma, A. Becher, and H. P. Schweizer.** 2000. Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. Plasmid **43:**59–72.
21. **Kato, J., and A. M. Chakrabarty.** 1991. Purification of the regulatory protein AlgR1 and its binding in the far upstream region of the *algD* promoter in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **88:**1760–1764.
22. **Kim, K. I., S. C. Park, S. H. Kang, G. W. Cheong, and C. H. Chung.** 1999. Selective degradation of unfolded proteins by the self-compartmentalizing HtrA protease, a periplasmic heat shock protein in *Escherichia coli*. J. Mol. Biol. **294:**1363–1374.
23. **Knutson, C. A., and A. Jeanes.** 1968. A new modification of the carbazole reaction: application to heteropolysaccharides. Anal. Biochem. **24:**470–481.
24. **Kustu, S., A. K. North, and D. S. Weiss.** 1991. Prokaryotic transcriptional enhancers and enhancer-binding proteins. Trends Biochem. Sci. **16:**397–402.
25. **Lamarche, M. G., B. L. Wanner, S. Crepin, and J. Harel.** 2008. The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. FEMS Microbiol. Rev. **32:**461–473.
26. **Leech, A. J., A. Sprinkle, L. Wood, D. J. Wozniak, and D. E. Ohman.** 2008. The NtrC family regulator AlgB, which controls alginate biosynthesis in mucoid *Pseudomonas aeruginosa*, binds directly to the *algD* promoter. J. Bacteriol. **190:**581–589.
27. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. **15:**194–222.
28. **Ma, S., U. Selvaraj, D. E. Ohman, R. Quarless, D. J. Hassett, and D. J. Wozniak.** 1998. Phosphorylation-independent activity of the response regulators AlgB and AlgR in promoting alginate biosynthesis in mucoid *Pseudomonas aeruginosa*. J. Bacteriol. **180:**956–968.
29. **Ma, S., D. J. Wozniak, and D. E. Ohman.** 1997. Identification of the histidine protein kinase KinB in *Pseudomonas aeruginosa* and its phosphorylation of the alginate regulator *algB*. J. Biol. Chem. **272:**17952–17960.
30. **Macfarlane, E. L., A. Kwasnicka, M. M. Ochs, and R. E. Hancock.** 1999. PhoP-PhoQ homologues in Pseudomonas aeruginosa regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol. Microbiol. **34:**305–316.
31. **Martin, D. W., M. J. Schurr, M. H. Mudd, and V. Deretic.** 1993. Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: inactivation of *mucB* causes conversion to mucoidy. Mol. Microbiol. **9:**497–506.
32. **Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. Govan, B. W. Holloway, and V. Deretic.** 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc. Natl. Acad. Sci. USA **90:**8377–8381.
33. **Mathee, K., C. J. McPherson, and D. E. Ohman.** 1997. Posttranslational control of the *algT* (*algU*)-encoded sigma22 for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its antagonist proteins MucA and MucB (AlgN). J. Bacteriol. **179:**3711–3720.
34. **Mathee, K., G. Narasimhan, C. Valdes, X. Qiu, J. M. Matewish, M. Koehrsen, A. Rokas, C. N. Yandava, R. Engels, E. Zeng, R. Olavarietta, M. Doud, R. S. Smith, P. Montgomery, J. R. White, P. A. Godfrey, C. Kodira, B. Birren, J. E. Galagan, and S. Lory.** 2008. Dynamics of Pseudomonas aeruginosa genome evolution. Proc. Natl. Acad. Sci. USA **105:**3100–3105.
35. **McPhee, J. B., S. Lewenza, and R. E. Hancock.** 2003. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in Pseudomonas aeruginosa. Mol. Microbiol. **50:**205–217.
36. **Miller, J. H.** 1972. Beta-galactosidase assay, p. 352–355. *In* J. H. Miller (ed.), Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
37. **Mohr, C. D., and V. Deretic.** 1990. Gene-scrambling mutagenesis: generation and analysis of insertional mutations in the alginate regulatory region of *Pseudomonas aeruginosa*. J. Bacteriol. **172:**6252–6260.
38. **Mohr, C. D., N. S. Hibler, and V. Deretic.** 1991. AlgR, a response regulator controlling mucoidy in *Pseudomonas aeruginosa*, binds to the FUS sites of the *algD* promoter located unusually far upstream from the mRNA start site. J. Bacteriol. **173:**5136–5143.
39. **Palmer, K. L., S. A. Brown, and M. Whiteley.** 2007. Membrane-bound nitrate reductase is required for anaerobic growth in cystic fibrosis sputum. J. Bacteriol. **189:**4449–4455.
40. **Pedersen, S. S., N. Hoiby, F. Espersen, and C. Koch.** 1992. Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. Thorax **47:**6–13.
41. **Qiu, D., F. H. Damron, T. Mima, H. P. Schweizer, and H. D. Yu.** 2008. P_{BAD}-based shuttle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. Appl. Environ. Microbiol. **74:**7422–7426.
42. **Qiu, D., V. M. Eisinger, N. E. Head, G. B. Pier, and H. D. Yu.** 2008. ClpXP proteases positively regulate alginate overexpression and mucoid conversion in Pseudomonas aeruginosa. Microbiology **154:**2119–2130.
43. **Qiu, D., V. M. Eisinger, D. W. Rowen, and H. D. Yu.** 2007. Regulated proteolysis controls mucoid conversion in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **104:**8107–8112.
44. **Rowen, D. W., and V. Deretic.** 2000. Membrane-to-cytosol redistribution of ECF sigma factor AlgU and conversion to mucoidy in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Mol. Microbiol. **36:**314–327.
45. **Schurr, M. J., H. Yu, J. C. Boucher, N. S. Hibler, and V. Deretic.** 1995. Multiple promoters and induction by heat shock of the gene encoding the alternative sigma factor AlgU (σ^E) which controls mucoidy in cystic fibrosis isolates of *Pseudomonas aeruginosa*. J. Bacteriol. **177:**5670–5679.
46. **Schurr, M. J., H. Yu, J. M. Martinez-Salazar, J. C. Boucher, and V. Deretic.** 1996. Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. J. Bacteriol. **178:**4997–5004.
47. **Schweizer, H. P., and T. T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:**15–22.
48. **Slauch, J. M., S. Garrett, D. E. Jackson, and T. J. Silhavy.** 1988. EnvZ functions through OmpR to control porin gene expression in *Escherichia coli* K-12. J. Bacteriol. **170:**439–441.
49. **Spiess, C., A. Beil, and M. Ehrmann.** 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell **97:**339–347.
50. **Tart, A. H., M. J. Blanks, and D. J. Wozniak.** 2006. The AlgT-dependent transcriptional regulator AmrZ (AlgZ) inhibits flagellum biosynthesis in mucoid, nonmotile *Pseudomonas aeruginosa* cystic fibrosis isolates. J. Bacteriol. **188:**6483–6489.
51. **Walsh, N. P., B. M. Alba, B. Bose, C. A. Gross, and R. T. Sauer.** 2003. OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. Cell **113:**61–71.
52. **Wilken, C., K. Kitzing, R. Kurzbauer, M. Ehrmann, and T. Clausen.** 2004. Crystal structure of the DegS stress sensor: how a PDZ domain recognizes misfolded protein and activates a protease. Cell **117:**483–494.
53. **Wong, S. M., and J. J. Mekalanos.** 2000. Genetic footprinting with mariner-based transposition in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **97:**10191–10196.
54. **Wood, L. F., A. J. Leech, and D. E. Ohman.** 2006. Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: roles of sigma (AlgT) and the AlgW and Prc proteases. Mol. Microbiol. **62:**412–426.
55. **Woolwine, S. C., A. B. Sprinkle, and D. J. Wozniak.** 2001. Loss of *Pseudomonas aeruginosa* PhpA aminopeptidase activity results in increased *algD* transcription. J. Bacteriol. **183:**4674–4679.
56. **Wozniak, D. J., and D. E. Ohman.** 1991. *Pseudomonas aeruginosa* AlgB, a two-component response regulator of the NtrC family, is required for *algD* transcription. J. Bacteriol. **173:**1406–1413.
57. **Wozniak, D. J., and D. E. Ohman.** 1994. Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. J. Bacteriol. **176:**6007–6014.