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

F. Heath Damron, 1 Dongru Qiu, 1 and Hongwei D. Yu 1,2,3*

Departments of Biochemistry and Microbiology 1 and Pediatrics, 2 Joan C. Edwards School of Medicine at Marshall University, Huntington, West Virginia 25755-9320, and Progenesis Technologies, LLC, Bldg. 740, Rm. 4136, Dow Technology Park, 3200 Kanawha Turnpike, South Charleston, West Virginia 25303 3

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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 PA_{algU} and PA_{algD}. Deletion of alternative σ factor RpoN (σE 5) or the response regulator AlgB in *kinB* mutants decreased alginate production to wt nonmucoid levels. MucA 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 σ22) (Fig. 1) (33, 46). When MucA is not functional due to mutation, increased transcription directed by AlgU at the *algD* promoter (PA_{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 H2O2 and heat (6).

There is a high level of conservation between *E. coli* σE-RseA and *P. aeruginosa* AlgU-MucA. Activation of σE 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 t-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 PA_{algD} and alginate overproduction occurs. Significant research has focused on the multitude of regulators that bind and/or regulate transcriptional activity at PA_{algD}. Most PA_{algD} transcriptional regulators are AlgU dependent, such as AlgR, AmrZ, and AlgB (Fig. 1). The response regulator AlgR binds multiple sites within PA_{algD} and is required for PA_{algD} expression (21, 38). Additionally, the alginate and motility regulator Z (AmrZ) also promotes activity at PA_{algD} (5, 50). The NtrC family response regulator AlgB has recently been shown to bind at PA_{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 PA_{algD} (Fig. 1) (7).
The AlgU 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 σ54-holoenzyme (24). σ54 (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 PalgD (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 PalgD 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 PalgD and PalgB 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.

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 PalgD (4, 38, 56). In a muc-23 mucoid mutant, RpoN has also been shown to bind to PalgD and activate or repress transcription under certain environmental conditions (7). AlgU also activates transcription of PalgD (57). Activation of transcription at PalgD 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 MucG 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 PalgD (54).

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 Lennnox 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 carbencillin, 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 (55) was introduced into PAO1 by bipartional 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") gene. The resulting ampiclons were sequenced by the Marshall University Genomics Core Facility.

Mutant strain construction. For in-frame deletion of specific genes (algU, 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% sucrose (sacB). 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 (OD600) 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 acids.
P. aeruginosa strains

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E. coli strains

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<td>TA cloning vector; 3.9 kb; Ap′ Km′</td>
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<td>MiniCTX-PalgU-lacZ</td>
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TABLE 1. Bacterial strains and plasmids used in this study

* Alg−, nonmucoid phenotype; Alg+, mucoid phenotype.

β-Galactosidase activity assay. The promoter fusion constructs miniCTX-PalgU-lacZ and miniCTX-PalgU-lacZ were integrated onto the P. aeruginosa chromosome at the CTX phase 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 tolune 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 D2 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 milk 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).

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).
A

PA01
pHERD20T
29 ± 8

PA01
kinB::aacC1
pHERD20T
103 ± 10

PA01
kinB
pHERD20T
62 ± 4

PA01
kinB::aacC1
 PDO

PA01
kinB::aacC1
 PDO

PA01
kinB::aacC1
 PDO

PA01
kinB::aacC1
 PDO

B

FIG. 2. Mutation of kinB in PA01 results in a mucoid phenotype dependent upon algB, rpoN, and algW. (A) Colony morphologies of P. aeruginosa PA01 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.

RESULTS

Inactivation of kinB in P. aeruginosa strain PA01 results in alginate overproduction. To discover novel negative regulators of alginate biosynthesis, the standard genetic strain PA01 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 PA01 to the mucoid phenotype (GenBank accession number for the kinB insertion in PA01, FI209363) (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 kinB in PA01. Alginate overproduction resulted when kinB was deleted (Fig. 2A). However, PA01 ∆kinB produced less alginate, i.e., 62 ± 4 µg/ml/OD_{600} unit, versus 103 ± 10 µg/ml/OD_{600} unit for PA01 kinB::aacC1 (Fig. 2A). The mucoid phenotypes of PA01 kinB::aacC1 and PA01 ∆kinB were complemented by conditional expression of kinB (Fig. 2A). Expression of kinB in trans in PA01 kinB::aacC1 and PA01 ∆kinB decreased alginate production to wt PA01 levels, as expected (Fig. 2A). Furthermore, sequencing analysis confirmed that the mucA gene of PA01 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 PA01.

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). Alginic production was restored in the PAO1 ΔalgB ΔkinB 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).

Alginic 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-cycloloserine (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 Muse-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 ΔalgW, 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 PBAD promoter in the presence of arabinosine complemented the PAO581 algB::aacC1 (mucA25 algB::Gm') 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 ΔalgB ΔkinB. Alginate overproduction occurred when algB45 was expressed in PAO1 ΔalgB ΔkinB (Table 2). These data suggest that 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 ΔalgB, 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.

PalgU and PalgD 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 PalgU and PalgD, 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::aacU, 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 PBAD promoter of pHERD20T (41) in the presence of 0.1% arabinose. As a control for these experiments, PalgU and PalgD 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 PalgU expression are required for mucoidy (33). Inactivation of kinB in PAO1 kinB::aacC1 caused significantly increased PalgU expression compared to that in parent strain PAO1 (Fig. 3B, bars 2). The high PalgU expression in PAO1 kinB::aacC1 can be reduced with kinB expressed in trans (Fig. 3B, bars 2). Deletion of algU eliminated detectable PalgU 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 PalgU. The high level of PalgU expression in the absence of kinB required algB (Fig. 3B, bars 4). AlgB has been established as a transcriptional activator at PalgD in the mucA22 mutant FRD1-1 (56). Here we show a possible new role for AlgB in addition to the role at PalgD. We also observed that rpoN has a role in influencing high expression of PalgU (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 PalgD (7). As expected, PalgU expression is also influenced by the serine protease AlgW in PAO1 kinB::aacC1 (Fig. 3B, bars 6). However, the level of expression

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Arabinose (% wt/vol)</th>
<th>Phenotype</th>
<th>Alginic, µg/ml/OD600 unit (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO581 algB::aacC1</td>
<td>pHERD20T-algB</td>
<td>0</td>
<td>NM</td>
<td>49.0 ± 7.3</td>
</tr>
<tr>
<td>(mucA25 algB::Gm')</td>
<td></td>
<td>1</td>
<td>M</td>
<td>285.7 ± 12.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>NM</td>
<td>51.9 ± 3.0</td>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>M</td>
<td>228.2 ± 21.0</td>
</tr>
<tr>
<td>PAO1ΔalgBΔkinB</td>
<td>pHERD20T- algB45</td>
<td>0</td>
<td>NM</td>
<td>64.7 ± 10.9</td>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>M</td>
<td>215.7 ± 13.5</td>
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<tr>
<td></td>
<td></td>
<td>0</td>
<td>NM</td>
<td>49.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>M</td>
<td>263.8 ± 2.5</td>
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* Strains were cultured for 24 h at 37°C on PIA supplemented with carbenicillin and arabinose.

NM and M, nonmucoid and mucoid phenotypes, respectively.
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 PBAD 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 ΔkinB 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 ΔkinB requires algU, algB, rpoN, and algW. Note that kinB expression significantly lowers P_{algD} activity.
of \( P_{\text{algD}} \) with \( P_{\text{algW}} \) in \( \text{trans} \) exceeded the \( P_{\text{algD}} \) expression level in PAO1 \( \text{kinB}:\text{aacC1} \). A possible explanation for this is that \( P_{\text{algW}} \) expression from the arabinose promoter in the presence of 0.1% on a multicopy vector may exceed endogenous expression levels of \( P_{\text{algW}} \) in vivo.

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

\textbf{AlgU and AlgB expression is increased in PAO1 \( \text{kinB}:\text{aacC1} \).}

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

\textbf{MucA proteolytic degradation facilitates alginate overproduction in PAO1 \( \text{kinB}:\text{aacC1} \).}

Since \( \text{mucA} \) is wt in PAO1 \( \text{kinB}:\text{aacC1} \), \( \text{MucA} \) repression of \( \text{AlgU} \) must be relieved for activation of \( \text{AlgU} \) and alginate production. Based on the fact that alginate overproduction by PAO1 \( \text{kinB}:\text{aacC1} \) requires \( \text{AlgW} \), our hypothesis is that alginate production in the \( \text{kinB} \) mutant occurs by regulated proteolysis of \( \text{MucA} \). To test this model, we needed to observe \( \text{MucA} \) degradation. N-terminal \( \text{TAG} \)-HA-tagged \( \text{MucA} \) was expressed from pHERD20T-\text{HA}-\text{mucA} under induction of arabinose into nonmucoid and mucoid PAO1 derivative strains. The wt \( \text{mucA} \) gene without HA was expressed in \( \text{trans} \) as the negative control. Western blotting of PAO1 without HA-tagged \( \text{mucA} \) 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 \( E. \text{coli} \), which occurs in the absence of stress signals (2). Also, \( \text{PIA} \) contains triclosan, which has been shown to activate \( P_{\text{algD}} \) activity (54), suggesting that regulated proteolysis occurs in the presence of cell wall-inhibitory antibiotics. In PAO1 \( \Delta\text{algW} \), 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 \( \Delta\text{algW} \) as in PAO1. However, PAO1 \( \Delta\text{algW} \) 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 \( \text{algW} \) inhibited efficient proteolysis of HA-MucA, resulting in accumulation of two major fragments of HA-MucA. Mucoid PAO1
**DISCUSSION**

We discovered that mutation of *kinB* in PAO1 results in overproduction of alginate (Fig. 2A). Alginate regulation in
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* 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 *PalgB* and *PalgD* promoter activity (Fig. 3B and D, respectively). Previously both *rpoN* (7) and *algB* (26) have been shown to affect transcription at *PalgD*. Conversely, our data show that these regulators, AlgB and RpoN, also affect *PalgE* transcription. Only relatively small changes in *PalgE* expression are required for mucoidy (33); however, PAO1 *kinB::aacC1* exhibits significantly elevated expression of both *PalgE* and *PalgD* (Fig. 3B and D, respectively). We also noted that deletion of *algU* from PAO1 *kinB::aacC1* resulted in complete loss of detectable *PalgE*-*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 *PalgD* 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 σE 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 to PalgD 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 PalgD, 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 PalgD (26). Since exhaustive studies have not been performed, we cannot dismiss the possibility that AlgB and/or RpoN may initiate transcription at PalgD. Based on our data, both the PalgD and the PalgE 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 PalgD and PalgE 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 Mg2+ 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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