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# Activation of the *Pseudomonas aeruginosa* AlgU Regulon through *mucA* Mutation Inhibits Cyclic AMP/Vfr Signaling▽†

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***Pseudomonas aeruginosa* is an opportunistic pathogen that causes acute, invasive infections in immunocompromised individuals and chronic, persistent respiratory infections in individuals with cystic fibrosis (CF). The differential progression of acute or chronic infections involves the production of distinct sets of virulence factors. *P. aeruginosa* strains isolated from patients with acute respiratory infection are generally nonencapsulated and express a variety of invasive virulence factors, including flagella, the type III secretion system (T3SS), type IV pili (TFP), and multiple secreted toxins and degradative enzymes. Strains isolated from chronically infected CF patients, however, typically lack expression of invasive virulence factors and have a mucoid phenotype due to the production of an alginate capsule. The mucoid phenotype results from loss-of-function mutations in *mucA*, which encodes an anti-sigma factor that normally prevents alginate synthesis. Here, we report that the cyclic AMP/Vfr-dependent signaling (CVS) pathway is defective in *mucA* mutants and that the defect occurs at the level of *vfr* expression. The CVS pathway regulates the expression of multiple invasive virulence factors, including T3SS, exotoxin A, protease IV, and TFP. We further demonstrate that *mucA*-dependent CVS inhibition involves the alternative sigma factor AlgU (AlgT) and the response regulator AlgR but does not depend on alginate production. Our findings show that a single naturally occurring mutation leads to inverse regulation of virulence factors involved in acute and persistent infections. These results suggest that mucoid conversion and inhibition of invasive virulence determinants may both confer a selective advantage to *mucA* mutant strains of *P. aeruginosa* in the CF lung.**

*Pseudomonas aeruginosa* is a Gram-negative bacterium capable of causing acute invasive infections such as pneumonia in ventilated patients and chronic, life-long infections in the airways of individuals with the genetic disease cystic fibrosis (CF) (15). In CF, *P. aeruginosa* infection rarely spreads beyond the airways and mortality is primarily associated with unrelenting airway inflammation and progressive loss of lung function. Early in life, most CF patients suffer recurrent but transient airway infection by *P. aeruginosa* bacteria acquired from the environment (3, 53). Eventually the airways become permanently colonized by a single strain that persists for years or decades (32). The colonizing strains appear to harbor a complete repertoire of virulence factors associated with invasive infections (32, 58). During the chronic phase of infection, *P. aeruginosa* adopts a biofilmlike lifestyle, existing as matrix-encased microcolonies within thickened airway mucus (25, 50). In the CF lung, *P. aeruginosa* strains undergo phenotypic diversification (32, 51), including conversion to a mucoid colony phenotype due to overproduction of the capsular polysaccharide alginate (18). Alginate-producing biofilms are more resistant to phagocytic- and antibiotic-mediated killing (20, 27). The appearance of mucoid *P. aeruginosa* in CF patients is regarded as a hallmark of chronic infection and correlates with an accelerated decline in lung function and poor patient prognosis (28, 44).

Mucoid conversion of *P. aeruginosa* in the CF lung results primarily from loss-of-function mutations in *mucA*, which encodes an anti-sigma factor (2, 36, 49). In the absence of MucA, the alternative sigma factor AlgU (also named AlgT) activates the transcription of multiple genes, including the gene encoding the AlgR response regulator (18, 60). Together, AlgU and AlgR activate the transcription of genes encoding the biosynthetic enzymes for alginate production, resulting in the mucoid phenotype (34, 39). In addition, *mucA* mutation is associated with reduced expression of the type III secretion system (T3SS) and the tissue-damaging proteases LasA and elastase, as well as reduced flagellar motility (16, 35, 40, 61). These observations suggest that in addition to playing a role in mucoid conversion, the alginate pathway also regulates virulence gene expression.

The expression of many invasive virulence factors, including T3S, elastase, exotoxin A (ETA), type IV pili (TFP), and protease IV, is dependent on the second messenger 3′,5′-cyclic

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AMP (cAMP), which is synthesized by two adenylate cyclase enzymes (CyaA and CyaB), and the cAMP-dependent transcription factor Vfr (1, 12, 56, 59). In the present study, we demonstrate that in addition to inhibiting T3SS gene expression, *mucA* mutation inhibits protease IV and ETA secretion and TFP production and that the downregulation of these virulence factors is independent of alginate production. We show that *mucA* mutation blocks the production of invasive virulence factors by inhibiting cAMP/Vfr-dependent signaling (CVS) at the level of *vfr* expression and that this inhibition involves AlgU and the response regulator AlgR. Our results demonstrate that a single naturally occurring mutation, common to most CF isolates, results in inverse regulation of invasive and chronic virulence factors and may have implications for the pathogenesis of persistent CF airway infection.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The strains used in this study are listed in Table 1. *P. aeruginosa* was routinely grown in LB broth at 37°C, except where noted. The mucoid phenotype was evaluated by growth on Pseudomonas isolation agar (Difco). Strains containing pMMB-based or pJN-based expression plasmids were grown in the presence of 150 μg/ml carbenicillin (Cb) or 75 μg/ml gentamicin (Gm), respectively, during routine passage; strains were grown in 30 μg/ml Cb or 15 μg/ml Gm, respectively, during virulence factor expression and CVS assays.

The oligonucleotides used in strain construction are listed in Table S1 in the supplemental material. The plasmid containing the *mucA22* allele (pDONRX*mucA22*) was constructed by amplifying the *mucA* gene from the mucoid CF isolate FRD1, which contains a frameshift due to the deletion of a single G at position 430 of the open reading frame (33). The PCR product was tailed with *attB1* and *attB2* sequences for Gateway cloning (Invitrogen) and cloned into the Gateway-adapted suicide vector pDONRX (13). The *algU* and *mucA* deletion allele plasmids (pDONRXΔ*algU* and pDONRXΔ*mucA*) were constructed from *P. aeruginosa* strain PAK chromosomal DNA via splicing by overlap extension PCR as previously described (59), using the oligonucleotides indicated in Table S1 in the supplemental material. The *algD* and *algZR* deletion allele plasmids (pEXGmΔ*algD* and pEXGmΔ*algZR*) were constructed by amplifying upstream and downstream regions (~1,000 bp) flanking the target genes; the resulting PCR products were digested with restriction endonucleases (detailed in Table S1 in the supplemental material) and then sequentially ligated into pEX18Gm. For all constructs, mutant alleles were introduced onto the chromosome of the appropriate *P. aeruginosa* strains as described previously (21) and confirmed by DNA sequencing of PCR products.

The pMMB-based *mucA* and *algZR* expression plasmids (pPa-*mucA* and pPa-*algZR*, respectively) were constructed by PCR amplification followed by Gateway cloning into the pMMBV1GW plasmid as previously described (59). pPa-*mucA* contains the wild-type *mucA* open reading frame from strain PAK, and pPa-*algZR* contains the region extending from the *algZ* start codon (ATG) to the *algR* stop codon (TGA). All pMMB-based expression plasmids were introduced into *P. aeruginosa* via conjugation (14) followed by plating on LB agar with 150 μg/ml Cb and 25 μg/ml irgasan (Irg).

Plasmid pJN-*algZ* was constructed by PCR amplification of *algZ* from *P. aeruginosa* strain PA103 genomic DNA using the primer pair NheI-UP-algZR and SmaI-DN-algZ (see Table S1 in the supplemental material). The NheI-SmaI restriction fragment was cloned into pJN105, resulting in an arabinose-inducible *algZ* expression vector. Plasmids pJN-*algR* and pJN-*algZR* were constructed by PCR amplification of *algR* using the algR-XbaI-Up and algR-SacI-Dn primer pair and the pUS150 plasmid (31) as template. The XbaI-SacI restriction fragment (860 bp) was cloned into pJN105, resulting in the *algR* expression vector pJN-*algR*. An NheI-SpeI *algZ*-containing fragment from pJN-*algZ* was cloned into pJN-*algR*, resulting in the *algZR* expression plasmid pJN-*algZR*. All pJN-based expression plasmids were introduced into *P. aeruginosa* via conjugation (14) followed by plating on LB agar with 75 μg/ml Gm and 25 μg/ml Irg.

**Virulence factor and CVS assays.** Assays for β-galactosidase activity, protease IV activity, direct measurement of cAMP, and immunoblot analysis of Vfr and secreted ETA and ExoS were performed as previously described (12). The preparation of TFP sheared from the bacterial surface and analysis of pilin monomers by SDS-PAGE were performed as described previously (13). The CVS reporter is a synthetic version of the *Escherichia coli lac*P1 promoter fused to *lacZ* (*lac*P1Δ*lacI-lacZ*) (13). Anti-PcrV immunoblots were performed as described previously (46).

**Statistical analysis.** The two-tailed unpaired Student's *t* test was used for data comparison where appropriate. Statistical calculations were performed using Prism, version 5.0b (GraphPad Software).

## RESULTS

**Inactivation of *mucA* results in reduced expression of acute virulence factors regardless of mucoid status.** *P. aeruginosa* mutants lacking functional MucA have a mucoid colony phenotype and are defective for the expression of several virulence factors (T3S, LasA protease, and elastase) associated with tissue damage and invasive infection (35, 40, 61). Because each of these virulence factors was previously shown to be regulated by the CVS pathway (1, 12, 56, 59), we hypothesized that *mucA* mutation disrupts CVS. To test this hypothesis, we assessed the expression of several additional CVS-regulated virulence factors in a *mucA* mutant strain. We generated a mucoid derivative of *P. aeruginosa* strain PAK by replacing the wild-type copy of *mucA* with the *mucA22* allele, which carries a nonsense mutation and is the most common naturally occurring *mucA* mutation in CF isolates (2, 4). Consistent with the results of a previous study (61), the *mucA22* mutant was defective for the expression and secretion of the T3SS effector ExoS (Fig. 1A). In addition, protease IV activity in culture supernatants and secreted ETA were reduced in the *mucA22* mutant relative to their levels in the wild-type parent (Fig. 1B and C). Because the alginate capsule prevented the analysis of TFP production in the *mucA22* mutant, we constructed a nonmucoid derivative of *mucA22* by inactivating *algD*, which encodes a GDP-mannose dehydrogenase that is required for alginate biosynthesis (9). The nonmucoid *algD mucA22* double mutant yielded substantially less surface pilin than an isogenic *algD* mutant (Fig. 1D). The virulence phenotypes displayed by the *mucA22* mutant (ExoS, protease IV, and ETA) and the *algD mucA22* mutant (TFP) were restored by plasmid-expressed *mucA* (see Fig. S1 in the supplemental material). The expression of ExoS, protease IV, ETA, and TFP was also reduced in mutants lacking the ability to synthesize cAMP (*cyaAB*) or the cAMP-responsive Vfr regulator. The similarity in the phenotypes of the *mucA22*, *cyaAB*, and *vfr* mutants is consistent with our hypothesis that *mucA22* mutants are CVS defective.

To eliminate the possibility that the alginate capsule prevented efficient secretion of ExoS, protease IV, and ETA in the *mucA22* mutant, we examined the production of these virulence factors in the *algD mucA22* double mutant that cannot synthesize alginate but retains the other elements of the alginate regulatory pathway (i.e., AlgU and AlgZ/AlgR). The virulence phenotypes of the *mucA22* and *algD mucA22* mutants were equivalent (Fig. 1A to C), suggesting that virulence factor regulation by *mucA22* is independent of alginate production. In addition, the *algD* mutant was indistinguishable from wild-type PAK with respect to each of the virulence phenotypes tested (Fig. 1A to D), indicating that *algD* inactivation does not interfere with virulence factor expression.

**The CVS pathway is disrupted in the *mucA22* mutant.** To determine whether the *mucA22* mutation interferes with the CVS pathway, we used a cAMP/Vfr-dependent transcriptional reporter system (CVS reporter). The CVS reporter consists of an artificial promoter derived from the *Escherichia coli lac*P1

TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Description and relevant characteristics[a] | Source or reference |
|---|---|---|
| *P. aeruginosa* strains | | |
| PAK | *P. aeruginosa* strain K, wild type | 54 |
| PAK::P*exoS-lacZ* | PAK with chromosomal *exoS* promoter reporter | 12 |
| PAK*mucA22*::P*exoS-lacZ* | *mucA22* with chromosomal *exoS* promoter reporter | This study |
| PAKΔ*algD*::P*exoS-lacZ* | *algD* with chromosomal *exoS* promoter reporter | This study |
| PAKΔ*algDmucA22*::P*exoS-lacZ* | *algD mucA22* with chromosomal *exoS* promoter reporter | This study |
| PAKΔ*cyaAB*::P*exoS-lacZ* | *cyaAB* with chromosomal *exoS* promoter reporter | 12 |
| PAKΔ*vfr*::P*exoS-lacZ* | *vfr* with chromosomal *exoS* promoter reporter | This study |
| PAK::*lac*P1Δ*lacI-lacZ* | PAK with chromosomal CVS reporter | 13 |
| PAK*mucA22*::*lac*P1Δ*lacI-lacZ* | *mucA22* with chromosomal CVS reporter | This study |
| PAKΔ*mucA*::*lac*P1Δ*lacI-lacZ* | *mucA* with chromosomal CVS reporter | This study |
| PAKΔ*algD*::*lac*P1Δ*lacI-lacZ* | *algD* with chromosomal CVS reporter | This study |
| PAKΔ*algDmucA22*::*lac*P1Δ*lacI-lacZ* | *algD mucA22* with chromosomal CVS reporter | This study |
| PAKΔ*cyaAB*::*lac*P1Δ*lacI-lacZ* | *cyaAB* with chromosomal CVS reporter | 13 |
| PAKΔ*vfr*::*lac*P1Δ*lacI-lacZ* | *vfr* with chromosomal CVS reporter | 13 |
| PAKΔ*algZR*::*lac*P1Δ*lacI-lacZ* | *algZR* with chromosomal CVS reporter | This study |
| PAKΔ*cyaABmucA22*::*lac*P1Δ*lacI-lacZ* | *cyaAB mucA22* with chromosomal CVS reporter | This study |
| PAKΔ*vfrmucA22*::*lac*P1Δ*lacI-lacZ* | *vfr mucA22* with chromosomal CVS reporter | This study |
| PAKΔ*algU*::*lac*P1Δ*lacI-lacZ* | *algU* with chromosomal CVS reporter | This study |
| PAKΔ*algUmucA22*::*lac*P1Δ*lacI-lacZ* | *algU mucA22* with chromosomal CVS reporter | This study |
| PAKΔ*algZR*::*lac*P1Δ*lacI-lacZ* | *algZR* with chromosomal CVS reporter | This study |
| PAKΔ*algZRmucA22*::*lac*P1Δ*lacI-lacZ* | *algZR mucA22* with chromosomal CVS reporter | This study |
| PAK::P*vfr-lacZ* | PAK with chromosomal *vfr* promoter reporter | 12 |
| PAKΔ*vfr*::P*vfr-lacZ* | *vfr* with chromosomal *vfr* promoter reporter | 12 |
| PAK*mucA22*::P*vfr-lacZ* | *mucA22* with chromosomal *vfr* promoter reporter | This study |
| FRD1 | Mucoid CF clinical isolate with *mucA22* allele | 42 |
| PAO1 | *P. aeruginosa* laboratory-passaged strain, wild type | 23 |
| PAO1*mucA22* | *mucA22* mutation in PAO1 | This study |
| PA103 | *P. aeruginosa* laboratory-passaged strain, wild type | 29 |
| PA103*mucA22* | *mucA22* mutation in PA103 | This study |
| E2 | *P. aeruginosa* environmental isolate, wild type | 58 |
| E2*mucA22* | *mucA22* mutation in E2 | This study |
| MSH3 | *P. aeruginosa* environmental isolate, wild type | 58 |
| MSH3*mucA22* | *mucA22* mutation in MSH3 | This study |
| Plasmids | | |
| pDONR201 | Gateway cloning vector; $Km^r$ | Invitrogen |
| pEX18Gm | Suicide vector; $Gm^r$ | 21 |
| pDONRX | Gateway-adapted suicide vector; $Km^r$ | 13 |
| pDONRX*mucA22* | Suicide vector containing *mucA22* allele; $Gm^r$ | This study |
| pDONRXΔ*mucA* | Suicide vector containing *mucA* deletion allele; $Gm^r$ | This study |
| pDONRXΔ*algU* | Suicide vector containing *algU* deletion allele; $Gm^r$ | This study |
| pEXGmΔ*algD* | Suicide vector containing *algD* deletion allele; $Gm^r$ | This study |
| pEXGmΔ*algZR* | Suicide vector containing *algZR* deletion allele; $Gm^r$ | This study |
| pEXGmΔ*vfr* | Suicide vector containing *vfr* deletion allele; $Gm^r$ | 59 |
| pEXGmΔ*cyaA* | Suicide vector containing *cyaA* deletion allele; $Gm^r$ | 59 |
| pEXGmΔ*cyaB* | Suicide vector containing *cyaB* deletion allele; $Gm^r$ | 59 |
| pMMB67EH | Empty *P. aeruginosa* expression vector; $Ap^r$ | 14 |
| pMMBV1GW | Gateway-adapted version of pMMB67EH; $Ap^r$ | 13 |
| pPa-*cyaB* | *P. aeruginosa cyaB* carried on pMMBV2; $Ap^r$ | 13 |
| pPa-*vfr* | *P. aeruginosa vfr* carried on pMMBV1; $Ap^r$ | 12 |
| pPa-*algZR* | *P. aeruginosa algZR* carried on pMMBV1; $Ap^r$ | This study |
| pPa-*mucA* | *P. aeruginosa mucA* carried on pMMBV1; $Ap^r$ | This study |
| pUS150 | Plasmid containing wild-type *algR*; $Ap^r$ | 31 |
| pJN105 | Arabinose-inducible expression plasmid; $Gm^r$ | 41 |
| pJN-*algR* | *P. aeruginosa algR* carried on pJN105; $Gm^r$ | This study |
| pJN-*algZ* | *P. aeruginosa algZ* carried on pJN105; $Gm^r$ | This study |
| pJN-*algZR* | *P. aeruginosa algZR* carried on pJN105; $Gm^r$ | This study |

[a] $Ap^r$, ampicillin resistance marker; $Km^r$, kanamycin resistance marker; $Gm^r$, gentamicin resistance marker.

promoter fused to the β-galactosidase-encoding *lacZ* gene and is integrated in single copy at the ΦCTX phage attachment site on the PAK chromosome. We have previously shown that this reporter is a general indicator of CVS function, since the reporter activity reflects both intracellular cAMP levels and a requirement for Vfr (13). Similar to the *cyaAB* and *vfr* control strains, the mucoid *mucA22* mutant had reduced CVS reporter activity relative to that of the wild-type strain ($P < 0.0001$) (Fig. 2A). The CVS reporter activity in the nonmucoid *algD mucA22* mutant was indistinguishable from that in the *mucA22* mutant, and the activity in the *algD* mutant was indistinguishable from that in the wild-type strain. In addition, the reduced

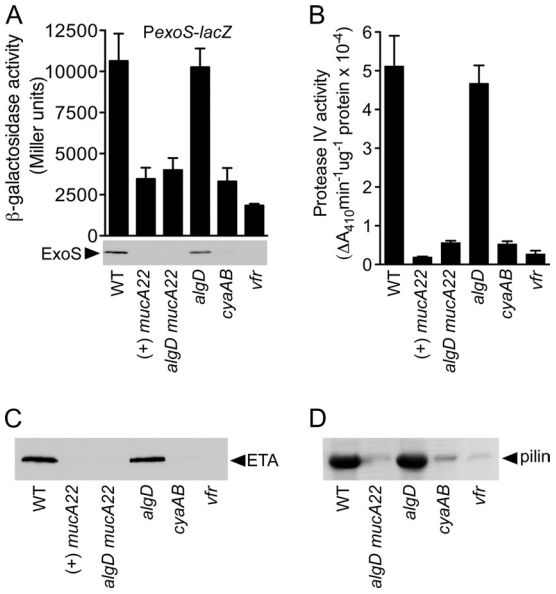


FIG. 1. The *P. aeruginosa mucA22* mutant has reduced expression of the T3SS (A), protease IV (B), ETA (C), and surface pilin (D). Assays were conducted using the wild-type strain (WT) or the indicated single (*mucA22*, *algD*, and *vfr*) or double (*algD mucA22* and *cyaAB*) mutant. All strains used in these analyses were nonmucoid except *mucA22* (+). (A) Bacteria were grown in LB containing 5 mM EGTA and 5 mM $MgCl_2$ to an optical density at 600 nm ($OD_{600}$) of 2.0 and assayed for expression of the P*exoS-lacZ* reporter (graph, top) and for ExoS protein levels in culture supernatant by immunoblotting (bottom). The reported values are the means ± standard errors of the means (SEM) ($n = 3$). The P*exoS-lacZ* reporter activity was significantly different ($P \leq 0.022$) from that in the WT in each of the mutants except the *algD* mutant. (B) Culture supernatants from bacteria grown in LB medium to early stationary phase ($OD_{600} = 4.0$) were assayed for protease IV activity using the substrate *N*-(*p*-tosyl)-Gly-Pro-Lys-4-nitroanilide. Activity is represented as the change in absorbance over time, normalized by protein concentration, and the reported values are the means ± SEM ($n = 3$). The protease IV activity was significantly different ($P \leq 0.028$) from that in the WT in each of the mutants except the *algD* mutant. (C) ETA secretion detected by immunoblotting TCA-precipitated culture supernatants from strains grown to an $OD_{600}$ of 2.0 under ETA-inducing conditions (low iron) in deferrated tryptic soy broth. (D) TFP were sheared from the surface of bacteria grown overnight on LB agar plates. Samples were normalized based on bacterial cell numbers, resolved by SDS-PAGE, and stained with GelCode blue stain (Thermo Scientific). Pilin, the major structural subunit of TFP, is indicated. TFP production was examined in the nonmucoid *algD mucA22* double mutant due to technical difficulties associated with the alginate capsule.

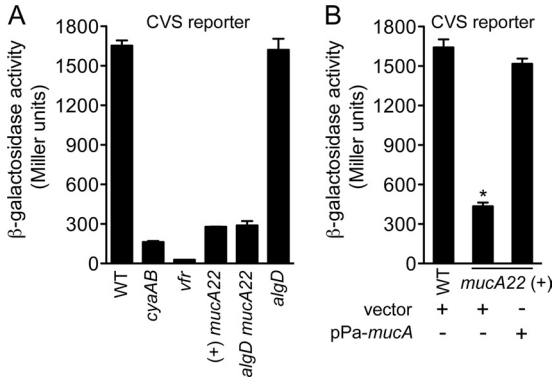


FIG. 2. The *mucA22* mutant is defective for expression of the CVS reporter. (A) The wild-type strain (WT) or indicated mutants bearing the cAMP/Vfr-dependent CVS reporter were grown in LB broth to mid-log growth phase ($OD_{600} = 1.0$) and assayed for β-galactosidase activity. The mucoid phenotype of the *mucA22* mutant is indicated (+). The reported values are the means ± SEM ($n = 3$), and all values except that for the *algD* mutant were significantly different ($P < 0.0001$) from the value for the WT. (B) Complementation of the CVS defect in the *mucA22* mutant. The WT and the *mucA22* mutant bearing the CVS reporter and an empty vector or a *mucA* expression plasmid (pPa-*mucA*) were grown as described above in the presence of 250 μM IPTG and assayed for β-galactosidase activity. The reported values are the means ± SEM ($n = 3$). *, value was significantly different ($P \leq 0.02$) from all other values in pairwise comparisons. Complementation of the mucoid *mucA22* mutant resulted in a nonmucoid phenotype.

CVS reporter activity seen in the *mucA22* mutant was complemented by plasmid-expressed *mucA* (Fig. 2B). These results demonstrate that the *mucA22* mutation results in a defect in the CVS pathway that is independent from alginate production. To determine whether the CVS defect is specific for the *mucA22* allele, we constructed an in-frame *mucA* deletion mutant, which, like the *mucA22* mutant, was mucoid and displayed reduced CVS reporter activity relative to that of its parent strain (see Fig. S2 in the supplemental material). This result suggests that any of the numerous mutations that naturally occur in *mucA* (2, 4) are likely to affect CVS.

**The *mucA22* defect in CVS occurs at the level of *vfr* expression.** The data presented thus far cannot distinguish whether the *mucA22* effect on the CVS pathway is specific for cAMP and/or Vfr. To address the individual contributions of Vfr and cAMP, we used plasmid-based expression of *vfr* (pPa-*vfr*) from the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter and expression of the primary *P. aeruginosa* adenylate cyclase CyaB (pPa-*cyaB*) to generate cAMP. Vfr expression restored CVS reporter activity in a *mucA22 vfr* double mutant, indicating that Vfr contributes to the CVS defect caused by *mucA22* (Fig. 3A). In contrast, CyaB overexpression failed to restore full CVS reporter activity in the *mucA22* mutant lacking adenylate cyclase activity (*cyaAB mucA22*) when induced to the same level that complemented the *cyaAB* mutant. This result is consistent with our finding that intracellular cAMP levels are similar in the *mucA22* and wild-type strains (Fig. 3B). Taken together, these results support the conclusion that the reduced CVS reporter activity in the *mucA22* mutant is due to reduced *vfr* expression and not reduced cAMP synthesis.

Because the *mucA22* effect was specific for the Vfr component of the CVS pathway, we determined whether the *mucA22* mutation decreased *vfr* promoter activity. To measure *vfr* transcription, we used a chromosomal reporter consisting of the *vfr* promoter region fused to the *lacZ* gene; the activity of this reporter was previously shown to be Vfr dependent (12). *vfr* promoter reporter activity was reduced in the *mucA22* mutant and could be complemented by plasmid-based expression of wild-type *mucA* (pPa-*mucA*) (Fig. 3C). Furthermore, the defect in *vfr* promoter reporter activity displayed by the *mucA22* mutant was restored by pPa-*vfr* using the same induction conditions that resulted in complementation of the *vfr* mutant.

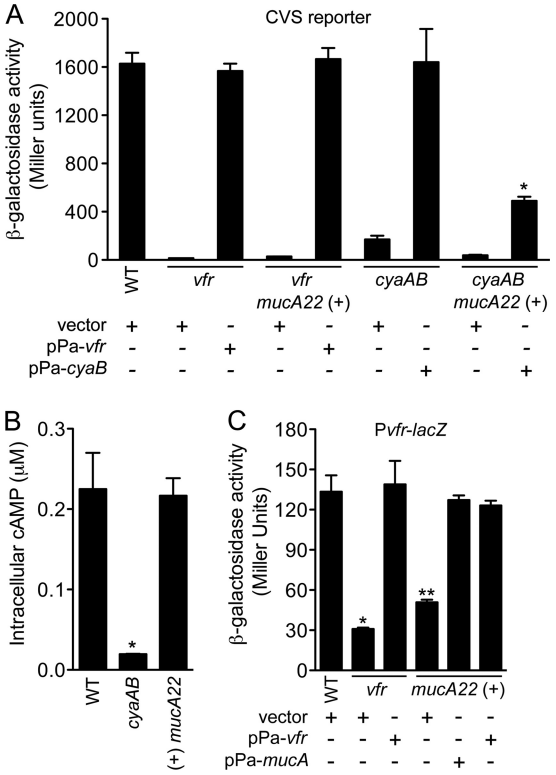


FIG. 3. The *mucA22* mutant is specifically defective for the Vfr component of CVS at the level of *vfr* expression. (A) Wild-type PAK (WT) and the indicated mutants bearing the CVS reporter and carrying an empty vector, a *vfr* expression plasmid (pPa-*vfr*), or a *cyaB* expression plasmid (pPa-*cyaB*) were grown to mid-log growth phase ($OD_{600}$ = 1.0) in LB broth containing 40 μM (pPa-*vfr*) or 100 μM (pPa-*cyaB*) IPTG and assayed for β-galactosidase activity. Reported values are the means ± SEM ($n \geq 3$). *, value was significantly different ($P = 0.014$) from the value for the *cyaAB* mutant carrying pPa-*cyaB*. (B) Intracellular cAMP levels in the WT, the cAMP-lacking double adenylate cyclase mutant (*cyaAB*), and the *mucA22* mutant. The mucoid *mucA22* mutant is indicated (+); all other strains are nonmucoid. Bacteria were grown in LB broth to late-log growth phase ($OD_{600}$ = 3.0), and lysates were assayed for cAMP by enzyme immunoassay. Values are reported as the means ± SEM ($n = 3$). *, value was significantly different ($P \leq 0.044$) from all other values in pairwise comparisons. (C) WT PAK and the *vfr* and *mucA22* mutants bearing a chromosomal *vfr* promoter reporter (P*vfr-lacZ*) and carrying an empty vector, a *mucA* expression plasmid (pPa-*mucA*), or a *vfr* expression plasmid (pPa-*vfr*) were grown to mid-log growth phase ($OD_{600}$ = 1.0) in LB broth containing 40 μM (pPa-*vfr*) or 250 μM (pPa-*mucA*) IPTG and assayed for β-galactosidase activity. The reported values are the means ± SEM ($n \geq 4$). In pairwise comparisons, there were significant differences between the values for the *vfr* mutant carrying the vector and the *vfr* mutant carrying pPa-*vfr* or the WT carrying the vector (*, $P < 0.0001$) and between the values for the *mucA22* mutant carrying the vector and the *mucA22* mutant carrying pPa-*mucA* or pPa-*vfr* or the WT carrying the vector (**, $P \leq 0.0006$). The *mucA22* strain complemented with pPa-*mucA* is nonmucoid.

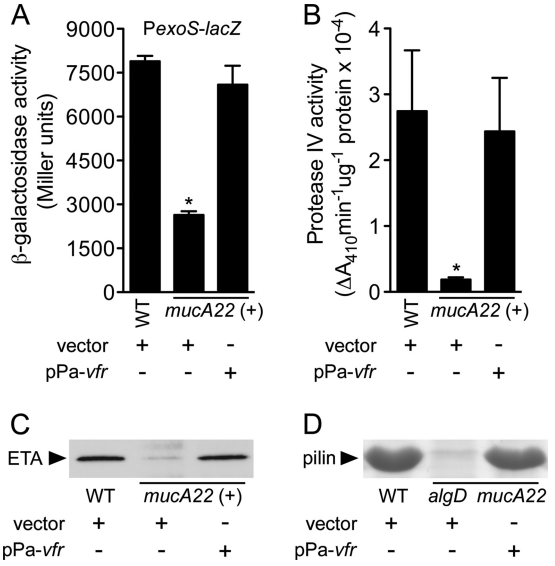


FIG. 4. Expression of *vfr* in *trans* restores acute virulence factor expression in a *mucA22* mutant. (A to C) The wild-type strain (WT) and the *mucA22* mutant carrying an empty vector or the *vfr* expression plasmid (pPa-*vfr*) were assayed for *exoS* promoter activity (A), protease IV activity (B), and ETA secretion (C). (D) The wild-type strain and the *algD mucA22* double mutant carrying the above-listed plasmids were assayed for TFP production. The mucoid nature of the *mucA22* mutant is indicated (+). All assays were performed as described in the legend for Fig. 1, except that bacteria were grown in the presence of 100 μM IPTG. *, value was significantly different [$P \leq 0.003$ (A); $P \leq 0.050$ (B)] from all other values in pairwise comparisons.

These results indicate that *mucA*22 mutation inhibits CVS by disrupting *vfr* transcription.

**Plasmid-expressed *vfr* restores acute virulence factor production in the *mucA22* mutant.** Since plasmid-encoded Vfr restored *vfr* promoter activity in the *mucA22* mutant, we determined whether it could also restore acute virulence factor production. The expression of pPa-*vfr* in the *mucA22* mutant resulted in the restoration of T3SS (*exoS*) gene expression, protease IV activity, and ETA secretion (Fig. 4A to C); TFP production was restored in the *algD mucA22* double mutant harboring pPa-*vfr* (Fig. 4D). These results demonstrate that the *mucA22* mutation affects CVS-dependent virulence systems through the regulation of *vfr*.

**The CVS defect in a *mucA22* mutant is mediated by AlgU and AlgZ/AlgR.** Wu et al. demonstrated that the AlgU sigma factor and AlgR response regulator are required for *mucA*-mediated inhibition of T3SS gene expression (61). Given that CVS controls T3SS gene expression (59), we hypothesized that AlgU and AlgR affect CVS activity. AlgU is a homolog of the *E. coli* heat shock sigma factor RpoE and is a mediator of the *P. aeruginosa* stress response (34, 47, 48). In the context of mucoid conversion, AlgU has a dual role of activating multiple genes (including *algR*) required for the expression of the *algD* operon and being a direct transcriptional activator of *algD* (18, 60). AlgR is a response regulator that binds the *algD* promoter and is part of a two-component system that includes the putative sensor kinase AlgZ (38, 39, 64).

To determine whether AlgU and/or the AlgZ/AlgR system contribute to the CVS defect, an *algU* or *algZR* mutation was introduced into the wild-type and *mucA22* backgrounds. We evaluated the effects of *algZ* and *algR* simultaneously because the genes are predicted to be cotranscribed and function together as part of the same signaling system (64). The introduction of the *algU* or *algZR* mutation into the *mucA22* mutant restored CVS activity to the wild-type level (Fig. 5A, top),

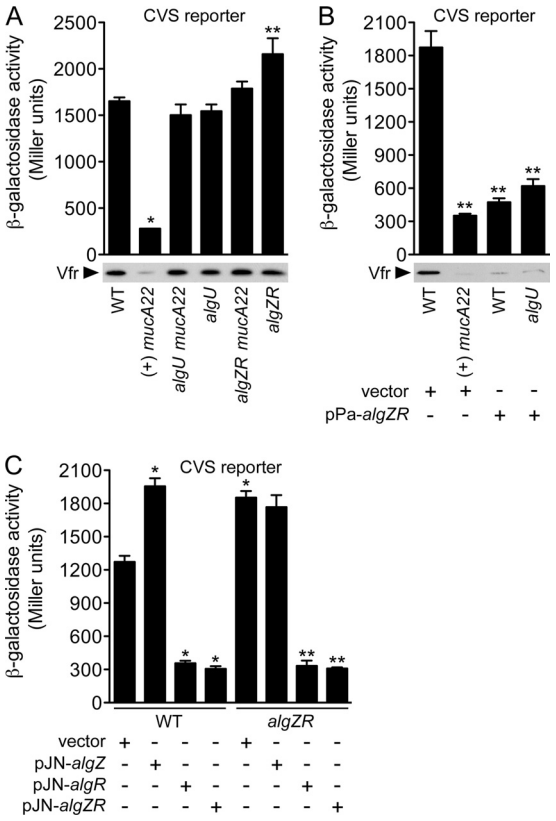


FIG. 5. The CVS defect in a *mucA22* mutant is mediated through AlgZ/AlgR. (A and B) Wild-type PAK (WT) and the indicated mutants bearing the CVS reporter were grown in LB broth to mid-log growth phase ($OD_{600}$ = 1.0) and assayed for β-galactosidase activity (graphs, top) or for Vfr protein levels by immunoblot of whole-cell lysates (bottom). (B) Strains carried an empty vector (vector) or an *algZR* expression plasmid (pPa-*algZR*) and were induced with 100 μM IPTG. The mucoid *mucA22* mutant is indicated (+). The reported values are the means ± SEM ($n \geq 3$). *, value was significantly different ($P < 0.0001$) from all other values in pairwise comparisons; **, value was significantly different [$P = 0.044$ (A); $P \leq 0.0007$ (B)] from the value for the WT strain. (C) The WT or an *algZ algR* double mutant (*algZR*) bearing the CVS reporter was grown to mid-log growth phase ($OD_{600}$ = 1.0) in LB broth containing 1% arabinose and assayed for β-galactosidase activity. Strains carried an empty vector or an *algZ* (pJN-*algZ*), *algR* (pJN-*algR*), or *algZR* (pJN-*algZR*) expression plasmid. The reported values are the means ± SEM ($n = 3$). * or **, value was significantly different ($P < 0.0001$) from the value for the WT or the *algZR* strain carrying the empty vector, respectively.

indicating that *algU* and *algZR* are each required for *mucA22*-mediated inhibition of CVS. Furthermore, the *algU* mutation had no effect on CVS reporter activity, while the *algZR* double mutant had significantly increased CVS reporter activity relative to that of the wild-type strain ($P = 0.045$) (Fig. 5A). These results indicate that basal *algZR* expression in the absence of *mucA* mutation is sufficient to partially inhibit CVS activity, consistent with the observation that *algR* inactivation results in increased T3SS gene expression (61). The results for CVS reporter activity parallel those obtained by Vfr immunoblot of whole-cell lysates (Fig. 5A, bottom), demonstrating that the suppression of the *mucA22* phenotype by *algU* and *algZR* deletion is specifically associated with the restoration of Vfr levels.

Since AlgZ/AlgR is downstream of AlgU in the alginate regulatory pathway (60), we tested whether the same is true for CVS regulation. Given that *algZ* and *algR* transcripts are elevated in mucoid strains (10) and that *algR* overexpression inhibits T3SS gene expression (61), we predicted that overexpressing *algZR* from a plasmid would phenocopy the *mucA22* mutant. We created an expression plasmid containing the *algZR* coding region driven by an IPTG-inducible *tac* promoter (pPa-*algZR*). When expressed in the wild-type or *algU* mutant backgrounds, pPa-*algZR* reduced CVS reporter activity ($P < 0.0007$) and Vfr protein to levels similar to those seen in the *mucA22* mutant (Fig. 5B). The fact that the effect of *algZR* overexpression was *algU* independent confirms that AlgU acts upstream of AlgZR with regard to CVS inhibition.

Although AlgZ and AlgR appear to comprise a two-component signal transduction system, AlgZ kinase activity has yet to be demonstrated and not all AlgR-dependent regulatory pathways in *P. aeruginosa* require AlgZ. For example, AlgZ is required for AlgR-dependent cyanide production and TFP-dependent twitching motility but not for alginate production (6, 57, 64). In fact, AlgZ appears to act as a repressor of alginate production, as deletion of *algZ* increases alginate synthesis in an otherwise mucoid strain (64). To determine the individual contributions of AlgZ and AlgR in regulating CVS, we overexpressed each gene in an otherwise wild-type strain and measured CVS reporter activity (Fig. 5C). With plasmid-based expression from an arabinose-inducible promoter, the overexpression of *algZ* alone resulted in a significant increase ($P < 0.0001$) in reporter activity, suggesting that AlgZ overexpression has an inhibitory effect on AlgR with regard to CVS control. In contrast, the overexpression of *algR* alone significantly reduced reporter activity ($P < 0.0001$), to a level indistinguishable from that of the same strain overexpressing both *algZ* and *algR*.

To further explore the relationship between CVS and AlgZ and AlgR, we overexpressed both genes in the *algZR* mutant, which has significantly elevated CVS reporter activity compared to that of its wild-type parent (Fig. 5A). The overexpression of *algZ* in the *algZR* mutant had no effect on reporter activity. In contrast, the overexpression of either *algR* alone or *algZ* and *algR* together resulted in a significant reduction ($P < 0.0001$) in CVS reporter activity. Taken together, these results suggest that AlgZ is not required for CVS control by AlgR and that AlgZ overexpression can inhibit AlgR activity.

**Mutation of *mucA* in a variety of *P. aeruginosa* isolates downregulates Vfr and virulence factor production.** To determine whether the effect of the *mucA22* mutation on the CVS pathway is specific to strain PAK or more generally applicable to other strains, we evaluated the effect of *mucA*22 in two common laboratory strains derived from non-CF human infections (PAO1 and PA103) and two environmental isolates (E2 and MSH3). The latter isolates were included since it is well established that individuals with CF are primarily infected by *P. aeruginosa* strains acquired from the environment (3, 53). Each of the resulting *mucA22* mutants displayed a mucoid phenotype and had reduced Vfr expression, and 3 of the 4 strains displayed decreased production of the conserved T3SS component PcrV relative to the levels in their respective non-mucoid parent strains (Fig. 6). Thus, the effect of the *mucA22*

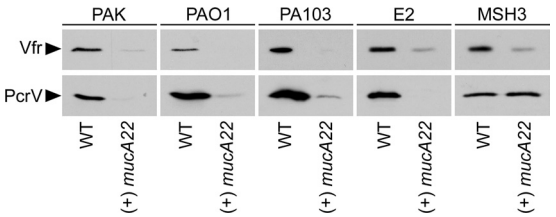


FIG. 6. Mutation of *mucA* results in downregulation of Vfr protein levels and T3SS components in multiple *P. aeruginosa* strains. Immunoblot of whole-cell lysates from the wild-type (WT) or isogenic *mucA22* mutant versions of the indicated laboratory and environmental strains probed with antibody specific for Vfr or PcrV, a T3SS component conserved among most *P. aeruginosa* strains. Mucoid *mucA22* mutants are indicated (+); all WT strains are nonmucoid.

mutation on CVS appears to be a common feature in *P. aeruginosa* isolates from diverse sources.

## DISCUSSION

Non-CF and CF infections are similar in that the initiating step involves exposure to an environmental source of *P. aeruginosa* (3, 53). Despite initial colonization with nearly identical strains, non-CF and CF infections differ markedly in their progression. Non-CF infections generally involve rapidly growing bacteria that are highly virulent, invasive, and capable of causing systemic infection. In contrast, *P. aeruginosa* in the CF airway undergoes phenotypic and genotypic adaptations favoring minimally invasive, more slowly growing sessile bacteria that adopt a biofilm lifestyle (7, 19, 43). The adaptation of *P. aeruginosa* to the CF airway was initially thought to result from mutation, the classic example being the relationship between *mucA* mutation and mucoid conversion. There is now increasing evidence that active regulatory systems also contribute to this dichotomous lifestyle by inversely controlling the expression of distinct and often mutually exclusive virulence determinants (17, 24, 26, 55). However, the role of these pathways *in vivo* and the nature of the host signal(s) to which they respond have not been established. Our finding that *mucA* mutation disables the CVS pathway defines a naturally occurring mechanism for inverse regulation of acute and chronic virulence markers during infection (Fig. 7). Thus, a single mutation in *mucA* can now account for many of the phenotypes typically associated with CF isolates, including mucoidy and reduced expression of the acute virulence factors evaluated in this study (T3S, ETA, TFP, and protease IV) (Fig. 1), as well as other members of the Vfr virulence regulon, such as LasA protease and elastase (35, 40). It is likely that the effect of *mucA* inactivation is even more far-reaching, since the CVS regulon includes over 200 genes (59). Based on our previous findings that mutants lacking either Vfr or the capacity to synthesize cAMP are attenuated in cell culture and acute infection model systems (52, 59), the reduced virulence of mucoid *P. aeruginosa* mutants (45, 62) may now be explained by inhibition of CVS-dependent virulence factors. The connection between *mucA* inactivation and *vfr* is consistent with the hypothesis that acute virulence factors are detrimental during chronic *P. aeruginosa* infection in the CF lung. However, the finding that *vfr* mutants have a selective advantage over wild-type strains under static growth conditions (11) raises the possibility that CVS inhibition is also advantageous for growth in the chronically infected CF lung environment.

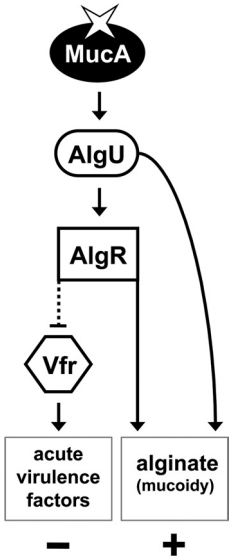


FIG. 7. Model for inverse regulation of acute virulence factors and alginate production by *mucA* inactivation in *P. aeruginosa*. Loss-of-function mutation of MucA (indicated by the white X) results in (i) a mucoid phenotype due to activation (+) of the alginate biosynthetic pathway and (ii) downregulation (−) of virulence factors associated with acute *P. aeruginosa* infection; both of these pathways are AlgU and AlgR dependent. Mutational inactivation of MucA, an anti-sigma factor, releases the AlgU sigma factor. AlgU activates the alginate biosynthesis operon both directly and indirectly via AlgU-dependent activation of *algR* expression. Our results suggest that elevated levels of AlgR repress *vfr* transcription, resulting in downregulation of the Vfr-dependent virulence factor regulon, including T3SS, ETA, TFP, and protease IV. The mechanism by which AlgR inhibits *vfr* expression (represented by dashed line) is unknown.

The onset of mucoid conversion in CF isolates correlates with a decline in lung function and a poorer clinical prognosis (28, 44). Our findings raise the possibility that the loss of CVS-regulated virulence traits in *mucA* mutants may correlate more closely with disease progression than mucoidy. Consistent with this idea is the finding that nonmucoid *mucA* mutant strains are isolated from chronically infected CF patients. In one study, 70% of the nonmucoid CF isolates carried a *mucA* mutation, suggesting that reversion from a mucoid to a nonmucoid phenotype had occurred *in vivo* (4). Further, second-site mutations in *algU* occurred at a lower frequency in *mucA* CF isolates than in spontaneous nonmucoid revertants isolated under laboratory conditions. This observation suggests that many CF isolates are constitutively active for AlgU irrespective of mucoid status and that there is selective pressure to maintain AlgU function in the CF airway. Maintenance of AlgU function would result in continued inhibition of CVS and acute virulence phenotypes, which is consistent with the findings that nonmucoid mutant strains in which AlgU is constitutively active have attenuated virulence (62) and that *algU* mutants have increased virulence in an acute infection mouse model (63). Based on the latter observation, Yu et al. (63) accurately predicted that the AlgU pathway might play opposing roles in the control of acute and chronic infections.

Because individual virulence genes and/or their regulators are also subject to mutational inactivation during CF airway infections (8, 37, 51), the *mucA*-dependent inhibition of virulence factor expression reported here is unlikely to be the sole mechanism for virulence factor downregulation. Indeed, mutations in *vfr* and the adenylate cyclase gene *cyaB* have been documented in CF isolates (51), both of which would lead to global virulence factor inhibition regardless of whether *mucA* was mutated. Also, a single strain can harbor a virulence gene mutation(s) in addition to a *mucA* mutation (22, 51). However, we hypothesize that virulence gene mutation largely occurs subsequent to *mucA* inactivation, since mucoid conversion has been shown to occur prior to inactivation of the antimutator *mutS* and *mutL* genes (5), which dramatically increases the rate of adaptive mutations in CF isolates (22, 37). This proposed sequence of events would result in the simultaneous downregulation of multiple virulence traits and may provide an advantage over the sequential acquisition of mutations within individual virulence genes. As such, mutation ultimately relieves the need for active repression. The overall redundancy of these mechanisms likely reflects the importance of virulence repression and/or the corresponding activation of other systems in the unique environment of the CF lung and suggests that there is powerful selection for particular attributes during chronic CF airway infection.

Our results demonstrate that *mucA*-dependent CVS inhibition is mediated by AlgR and Vfr. We explored the possibility that AlgR regulates *vfr* via recognition of the *vfr* promoter but failed to demonstrate AlgR binding in an electrophoretic mobility shift assay (see Fig. S3 in the supplemental material). These data and the lack of obvious AlgR binding sites (30) within the putative *vfr* promoter region are supportive of an indirect regulatory mechanism. While the mechanistic detail of CVS inhibition by AlgR remains to be determined, our findings provide the first example of a single commonly occurring mutation that leads to the inverse regulation of acute and chronic virulence traits during the pathogenesis of *P. aeruginosa* infection of CF airways.

## ACKNOWLEDGMENTS

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