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MucA-Mediated Coordination of Type III Secretion and Alginate Synthesis in *Pseudomonas aeruginosa*

Weihui Wu,[1] Hassan Badrane,[1] Shiwani Arora,[2] Henry V. Baker,[1] and Shouguang Jin[1]*

Department of Molecular Genetics and Microbiology[1] and Department of Medicine,[2] University of Florida College of Medicine, Gainesville, Florida

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The type III secretion system (T3SS) of *Pseudomonas aeruginosa* is an important virulence factor. The T3SS of *P. aeruginosa* can be induced by a low calcium signal or upon direct contact with the host cells. The exact pathway of signal sensing and T3SS activation is not clear. By screening a transposon insertion mutant library of the PAK strain, mutation in the *mucA* gene was found to cause repression of T3SS expression under both type III-inducing and -noninducing conditions. Mutation in the *mucA* gene is known to cause alginate overproduction, resulting in a mucoid phenotype. Alginate production responds to various environmental stresses and plays a protective role for *P. aeruginosa*. Comparison of global gene expression of *mucA* mutant and wild-type PAK under T3SS-inducing conditions confirmed the down regulation of T3SS genes and up regulation of genes involved in alginate biosynthesis. Further analysis indicated that the repression of T3SS in the *mucA* mutant was AlgU and AlgR dependent, as double mutants *mucA*/*algU* and *mucA*/*algR* showed normal type III expression. An *algR*::*Gm* mutant showed a higher level of type III expression, while overexpression of the *algR* gene inhibited type III gene expression; thus, it seems that the AlgR-regulated product inhibits the expression of the T3SS genes. It is likely that *P. aeruginosa* has evolved tight regulatory networks to turn off the energy-expensive T3SS when striving for survival under environmental stresses.

Pseudomonas aeruginosa is a versatile gram-negative bacterium that is present in soil, marshes, tap water, and coastal marine habitats. Patients with cystic fibrosis (CF), severe burns, or immunosuppression are at particularly high risk of *P. aeruginosa* infection (41, 45). Among CF patients, *P. aeruginosa* colonizes inside the thick mucus layer of the airway. In this anaerobic environment, *P. aeruginosa* overproduces the exopolysaccharide alginate and forms a biofilm which protects the bacterium from reactive oxygen intermediates and inhibits phagocytosis (15). More than 90% of *P. aeruginosa* strains isolated from CF patients show the mucoid phenotype, due to the overproduction of alginate (37). Clearly, alginate overproduction is a strategy to overcome environmental stresses and a number of stress signals trigger the overproduction of alginate, converting the bacterium to the mucoid phenotype (30).

The genes encoding enzymes for alginate synthesis form an operon (*algD* operon), and the expression of this operon is under the tight control of several regulators. The key regulatory gene of this operon is the *algU* gene (also called *algT*), encoded in an *algU* operon which consists of *algU-mucA-mucB-mucC-mucD*. The *algU* gene encodes a sigma factor, σ^{22}, which autoregulates its own promoter and activates many other genes, including those for alginate biosynthesis (31). The second gene in the *algU* operon, the *mucA* gene, encodes a transmembrane protein with a cytoplasmic portion binding to and inactivating AlgU (σ^{22}). The third gene of the *algU* operon, the *mucB* gene, encodes a periplasmic protein, possibly sensing certain environmental signals. Upon sensing certain environmental signals, MucB transduces the signal to MucA, which in turn releases the bound form of AlgU (σ^{22}), resulting in activation of alginate production (31). The majority of *P. aeruginosa* isolates from the lungs of older CF patients carry mutations in the *mucA* or *mucB* gene and display a mucoid phenotype (29). In the AlgU regulon, two-component regulatory systems AlgB-FimS (27) and AlgR-AlgZ (46) and regulators AlgP (8) and AlgQ (25) are required for alginate synthesis. Among them, AlgR was also shown to be essential for *P. aeruginosa* pathogenesis (26). An *algR* mutant is less virulent than a wild-type strain in an acute septicemia infection mouse model (26), and AlgR is also required for twitching motility (42, 43). Proteomic analysis of the *algR* mutant suggested that AlgR is a global regulator, affecting expression of multiple genes (26).

P. aeruginosa also possesses a type III secretion system (T3SS), including a protein translocation apparatus and effector proteins. Upon establishing intimate contact with host cells, effector proteins are injected into host cells (22). T3SS mediates the killing of macrophages, polymorphonuclear phagocytes, and epithelial cells (4, 5). The T3SS regulon consists of a total of 32 genes in four operons encoding the protein secretion and translocation apparatus and four effector molecules, ExoS, ExoT, ExoU, and ExoY (22). However, so far no strain has been reported to encode all four effectors. Expression of the T3SS regulon can be stimulated by direct contact with the host cell or by growth under a low Ca^{2+} environment (22). ExsA, an AraC-type transcriptional regulator, is the master regulator of the T3SS regulon. ExsA binds to a consensus sequence present upstream of the operons belonging to the T3SS regulon (21). The mechanism by which ExsA senses low Ca^{2+} signal is not known. Recently, it was shown that under T3SS-inducing conditions (low Ca^{2+}), the cyclic AMP level

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, FL 32610. Phone: (352) 392-8323. Fax: (352) 392-3133. E-mail: sjin@mgm.ufl.edu.

increased and a CRP homologue, Vfr, was also required for T3SS activation (44). Vfr is a global regulator which mediates activation of quorum sensing (35), twitching motility (1), type II secretion (44), and repression of flagellum synthesis (7).

In this study, a transposon (Tn) insertional mutant bank of the PAK strain was screened for mutants that are defective in T3SS expression. We found that mutation in the *mucA* gene suppresses the expression of T3SS genes, greatly reducing the response to low Ca^{2+} signal. Furthermore, the suppression is dependent on the AlgU and AlgR functions. Comparison of global gene expression of the *mucA* mutant and wild-type PAK under type III-inducing conditions confirmed the above observation. Several groups of genes have been found to be differently expressed in the *mucA* mutant and PAK, and their possible roles in T3SS expression are discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Plasmids and bacterial strains used in this work are listed in Table 1. Bacteria were gown in Luria broth (LB) at 37°C. Antibiotics were used at the following concentrations: for *Escherichia coli*, ampicillin at 100 μg/ml, gentamicin at 10 μg/ml, tetracycline at 10 μg/ml, and kanamycin at 50 μg/ml; for *P. aeruginosa*, carbenicillin at 150 μg/ml, gentamicin at 100 μg/ml, tetracycline at 100 μg/ml, spectinomycin at 200 μg/ml, streptomycin at 200 μg/ml, and neomycin at 400 μg/ml. For β-galactosidase assays, an overnight culture was reinoculated into LB at 1% and supplemented with 5 mM EGTA. Bacteria were grown to an optical density at 600 nm (OD_{600}) between 1.0 and 2.0 before β-galactosidase assays (33).

Construction of Tn insertional mutant bank. The *P. aeruginosa* PAK strain containing the *exoT-lacZ* fusion plasmid (pHW0006) was grown overnight at 42°C, while *E. coli* donor strain BW20767/pRL27 was cultured to mid-log phase at 37°C. Cells of the two types of bacteria were washed with LB once to remove antibiotics in the culture medium. About 5×10^8 PAK/pHW0006 cells were mixed with 10^9 donor *E. coli* cells, and the mixture was filtered onto a sterile nitrocellulose membrane (pore size, 0.22 μm). The membrane was laid on top of nutrient agar and incubated at 37°C for 7 to 9 h before washing off the bacterial mixture from the membrane with LB. The bacterial suspension was serially diluted with LB and spread on L-agar plates containing spectinomycin at 100 μg/ml, streptomycin at 100 μg/ml, tetracycline at 50 μg/ml, neomycin at 400 μg/ml, and 20 μg of 5-bromo-4-chloro-3-indolyl-β-L-thiogalactopyranoside (X-Gal)/ml, and 2.5 mM EGTA for colony counting as well as mutant screening.

Determination of Tn insertion sites. To locate the Tn insertion sites of the isolated mutants, the Tn with flanking DNA was rescued as a plasmid from the mutant chromosome. Plasmid rescue was carried out as described elsewhere (24). Briefly, genomic DNA of the Tn insertion mutants was isolated with the Wizard genomic DNA purification kit (Promega) and digested with PstI. The digested DNA was subjected to self-ligation with T4 DNA ligase and electroporated into DH5α/λ*pir*. Plasmids were isolated from the transformants and sequenced with primers tpnRL17-1 (5′-AAC AAG CCA GGG ATG TAA CG-3′) and tpnRL13-2 (5′-CAG CAA CAC CTT CTT CAC GA-3′) for the DNA flanking the two ends of the Tn. The DNA sequences were then compared with the *P. aeruginosa* genomic sequence by using BLASTN (39).

Generation of knockout mutants. Chromosome gene knockout mutants were generated as described previously (19). The target genes were amplified by PCR and cloned into pCR-TOPO2.1 (Invitrogen). After subcloning the PCR product into pEX18Tc or pEX18Ap, the target gene was disrupted by insertion of a gentamicin resistance cassette, leaving about 1 kb upstream and downstream of the insertion-mutation site. The plasmids were electroporated into wild-type PAK and single-crossover mutants were selected on LB plates containing gentamicin at 150 μg/ml and tetracycline at 100 μg/ml or carbenicillin at 150 μg/ml. Double-crossover mutants were selected by plating single-crossover mutants on LB plates containing 5% sucrose and gentamicin at 150 μg/ml. In the case of the *mucA22* mutant, a 1.8-kb fragment of the *mucA* gene region was amplified from FRD1 (mucoid strain) (27) genomic DNA, and the fragment was cloned into the HindIII site of pEX18Gm. The plasmid was transformed into *P. aeruginosa* to select for single crossover mutants on LB agar plates containing gentamicin 150 μg/ml. Single-crossover mutants were plated on L-agar plates containing 5% sucrose to select for double-crossover mutants. The double-crossover mutants were mucoid, and the introduction of the *mucA22* mutation was confirmed by sequencing of the *mucA* gene.

Plasmid constructs for complementation and overexpression. Construction of reporter fusions between the *exsA*, *exoT*, *exoS*, and *pscN* genes and promoterless *lacZ* on pDN19*lacZ* was described elsewhere (14a). For *mucA* gene complementation, the *mucA* gene was amplified from PAK genomic DNA by PCR with primers MucA-1 (5′-CGG ATC CTC CGC GCT CGT GAA GCA ATC G-3′) and MucA-2 (5′-TAC TGC GGC GCA CGG TCT CGA CCC ATA C-3′). The PCR product was cloned into pCR-TOPO2.1 and transformed into *E. coli* TOP10F′. The obtained plasmid was digested with HindIII-XmnI and cloned into the HindIII-SmaI sites of pUCP19. The *mucA* gene in the resulting plasmid, pWW021, is driven by a *lac* promoter on the vector. To generate a *mucA* gene driven by the *algU* promoter, the *mucA* gene on the pCR-TOPO2.1 plasmid was subcloned into the BamHI and XmnI sites of pEX18Tc, resulting in *mucA*-pEX18Tc. To obtained the *algU* gene promoter, an 800-bp DNA fragment upstream of the *algU* gene open reading frame (ORF) was amplified by PCR with primers AlgT1 (5′-CCT TCG CGG GTC AGG TGG TAT TCG AAG C-3′) and AlgT2 (5′-TTG GAT CCG CGC TGT ACC CGT TCA ACC A-3′) and cloned into pCR-TOPO2.1. Then, this fragment was ligated into the EcoRI and BamHI sites upstream of the *mucA* gene on the plasmid *mucA*-pEX18Tc. The obtained plasmid was digested with EcoRI-XmnI, and the *algU* promoter and *mucA* gene ORF fragment were cloned into the EcoRI-SmaI sites of pUCP19. On the resulting plasmid (pWW020), the *mucA* gene is driven by the *algU* promoter and the transcriptional direction is opposite to that of the *lac* promoter on the vector.

For *algR* complementation, the *algR* gene was amplified from PAK genomic DNA by PCR with primers algR1 (5′-GGT CTA GAG GCC GAG CCC CTC GGG AAA G-3′) and algR2 (5′-GTG GAT CCT ACT GCT CTC GGC GGC GCT G-3′). The PCR product was initially cloned into pCR-TOPO2.1. The resulting plasmid was digested with ClaI, blunted ended with Klenow enzyme, and digested with XbaI. The *algR* gene-containing fragment was ligated into XbaI-SmaI sites of plasmid pMMB67EH, resulting in pWW022, on which the *algR* gene is driven by the *tac* promoter on the vector. For *algU* gene overexpression, the *algU* gene ORF was amplified from PAK genomic DNA by PCR with primers algU1 (5′-GGG AAA GCT TTT GCA AGA AGC CCG AGT C-3′) and algU2 (5′-GCT TCG TTA TCC ATC ACA GCG GAC AGA G-3′). The *algU* gene was cloned into HindIII-EcoRI sites of pUCP19, where the expression of the *algU* gene in the resulting plasmid pWW025 was driven by *lac* promoter on the vector.

Western blotting. Overnight cultures of PAK and PAK*mucA22* were subcultured (diluted to 1%) into LB or LB plus 5 mM EGTA. When the OD_{600} of the culture reached 2.0 to 3.0, bacterial cultures were centrifuged at $10{,}000 \times g$ for 3 min. The supernatants were directly mixed with loading buffer. The pellets were resuspended with phosphate-buffered saline and then mixed with loading buffer. Samples from equivalent numbers of bacterial cells were loaded and separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane, hybridized with anti-ExoS polyclonal antibody, and detected by the ECL method (Amersham).

RNA isolation and microarray analysis. For RNA isolation, three single colonies of PAK and isogenic mutant PAK*mucA22* were inoculated into 3 ml of LB and grown overnight. PAK and PAK*mucA22* were subcultured into LB containing 5 mM EGTA. PAK started with an OD_{600} of 0.03, and the *mucA22* mutant started with an OD_{600} of 0.06. After 3 to 4 h of culture, bacteria were harvested at an OD_{600} of 1.0 to 1.2. Total RNA was isolated using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. The purity and quantity were determined by spectrometry and electrophoresis. Fifteen micrograms of RNA of each sample was used for cDNA synthesis. cDNA fragmentation and biotin terminal labeling were carried out as instructed (Affymetrix). Microarray analysis was performed with the Affymetrix GeneChip *P. aeruginosa* genome array. The experimental procedure followed the manufacturer's instructions. Data were acquired and analyzed with Microarray Suite version 5.0 (Affymetrix). Significance analysis of microarrays (40) was used to detect differentially expressed ORFs. Then, a cutoff of 5% false discovery rate and a change greater than threefold were chosen to analyze the data.

RESULTS

Activation of T3SS requires a functional *mucA* gene. To identify *P. aeruginosa* genes that affect the expression of T3SS, a Tn insertion mutant bank was constructed in PAK containing an *exoT*::*lacZ* (transcriptional fusion) reporter plasmid (pHW0006) (see Materials and Methods). On plates containing X-Gal and EGTA, the density of the blue color of each

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strains		
BW20767/pRL27	RP4-2-Tc::Mu-1 Kan::Tn7 integrant *leu-63*::IS*10 recA1 zbf-5 creB510 hsdR17 endA1 thi uidA* (ΔMluI::*pir*)/pRL27	24
DH5α/λ*pir*	ϕ80*dlacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*/λ*pir*	24
P. aeruginosa strains		
PAK	Wild-type *P. aeruginosa* strain	David Bradley
PAK *exsA*::Ω	PAK with *exsA* disrupted by insertion of Ω cassette	12
PAK A44	PAK *mucA*::Tn*5* mutant isolate	This study
PAK A61	PAK *mucA*::Tn*5* mutant isolate	This study
PAK *mucA22*	Point mutation (ΔG440) in *mucA* gene of PAK	This study
mucA22 algU::Gm	*mucA22* with *algU* disrupted by insertion of Gm cassette	This study
mucA22 algR::Gm	*mucA22* with *algR* disrupted by insertion of Gm cassette	This study
PAK *algU*::Gm	PAK with *algU* disrupted by insertion of Gm cassette	This study
PAK *algR*::Gm	PAK with *algR* disrupted by insertion of Gm cassette	This study
Plasmids		
pCR2.1-TOPO	Cloning vector for the PCR products	Invitrogen
pHW0005	*exoS* promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ	Unhwan Ha
pHW0006	*exoT* promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ	Unhwan Ha
pHW0024	*pscN* promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ	Unhwan Ha
pHW0032	*exsA* promoter of PAK fused to promoterless *lacZ* on pDN19lacZΩ	Unhwan Ha
pUCP19	Shuttle vector between *E. coli* and *P. aeruginosa*	38
pWW020	*mucA* gene on pUCP19 driven by *algU* promoter	This study
pWW021	*mucA* gene on pUCP19 driven by *lac* promoter	This study
pWW025	*algU* gene on pUCP19 driven by *lac* promoter	This study
pMMB67EH	Low-copy-number broad-host-range cloning vector	13
pWW022	*algR* gene on pMMB67EH driven by *lac* promoter	This study
pEX18Tc	Gene replacement vector, Tc^r; $oriT^+$ $sacB^+$	19
pEX18Ap	Gene replacement vector, Ap^r; $oriT^+$ $sacB^+$	19
pPS856	Source of Gm^r cassette; Ap^r Gm^r	19
algU::Gm-pEX18Tc	*algU* disrupted by insertion of Gm^r cassette on pEX18Tc	This study
algR::Gm-pEX18Ap	*algR* disrupted by insertion of Gm^r cassette on pEX18Ap	This study

colony indicated the expression level of the *exoT* gene in that particular Tn insertion mutant. To identify optimal screening conditions, combinations of different concentrations of X-Gal and EGTA were tested. In the presence of 20 μg of X-Gal/ml and 2.5 mM EGTA, wild-type PAK and the type III-defective PAK*exsA* mutant harboring pHW0006 showed the greatest visual difference in colony color (blue) and thus these concentrations were adopted for the screening conditions. The mutant bank cells were grown on the screen plates, and we looked for colonies with lighter blue color. About 15,000 Tn insertion mutants were screened. Among four colonies with lighter blue color, two of them showed a mucoid phenotype. The mucoid mutants were picked to test their T3SS activity by β-galactosidase assay. As shown in Fig. 1A, the *exoT* gene promoter activity was three- to fourfold lower in the mutants than in the parent strain PAK/pHW0006. To confirm this observation, the *exoT-lacZ* reporter plasmid was cured from the Tn insertion mutants by passage in the absence of antibiotic selection and the *pscN*::*lacZ* reporter plasmid (pHW0024) was reintroduced. The resulting strain was subjected to a β-galactosidase assay. The assay results shown in Fig. 1B indicated that the expression of the *pscN* gene was also repressed in these mucoid mutants under both T3SS-inducing and -noninducing conditions. Similar results were also obtained by introducing *exsA*::*lacZ* (pHW0032) and *exoS*::*lacZ* (pHW0005) reporter plasmids and testing β-galactosidase activities (data not shown), confirming that the two Tn mutants were indeed defective in T3SS expression.

The Tn and flanking DNA were rescued from the mutant strains and subjected to sequencing analysis (see Materials and Methods). Sequencing results showed that the Tn was inserted into two different positions on the *mucA* gene in these two mutants, explaining the mucoid phenotype of the isolates. Mutation in the *mucA* gene is commonly observed among *P. aeruginosa* isolates from CF patients, such as *mucA22*, where a nucleotide G was deleted within five G residues between positions 429 and 433 of the *mucA* coding region, causing protein truncation (3, 37). The identical *mucA22* mutant was constructed in the background of PAK by allelic replacement with a *mucA* fragment amplified from FRD1 (27), which bears the *mucA22* mutation (see Materials and Methods). Expression of the effector genes *exoS* and *exoT* in the resulting mutant strain PAK*mucA22* was compared to that in PAK by Western blot analysis of the secreted and cell-associated proteins by using anti-ExoS antibody, which also cross-recognizes ExoT due to a high sequence homology between the ExoS and ExoT proteins. As shown in Fig. 2A, expression of ExoS and ExoT in the resulting PAK*mucA22* was greatly reduced in comparison to that in wild-type PAK when grown under type III-inducing conditions. Reporter plasmids pHW0032 (*exsA*::*lacZ*) and pHW0005 (*exoS*::*lacZ*) were further introduced into PAK*mucA22* and tested for β-galactosidase activity. Similar to the original isolates of the *mucA* Tn insertional mutants, expression of the *exsA* and *exoS* genes in PAK*mucA22* was almost nonresponsive to low Ca^{2+} signal, compared to a three- to fourfold induction in the wild-type PAK background (Fig. 2B

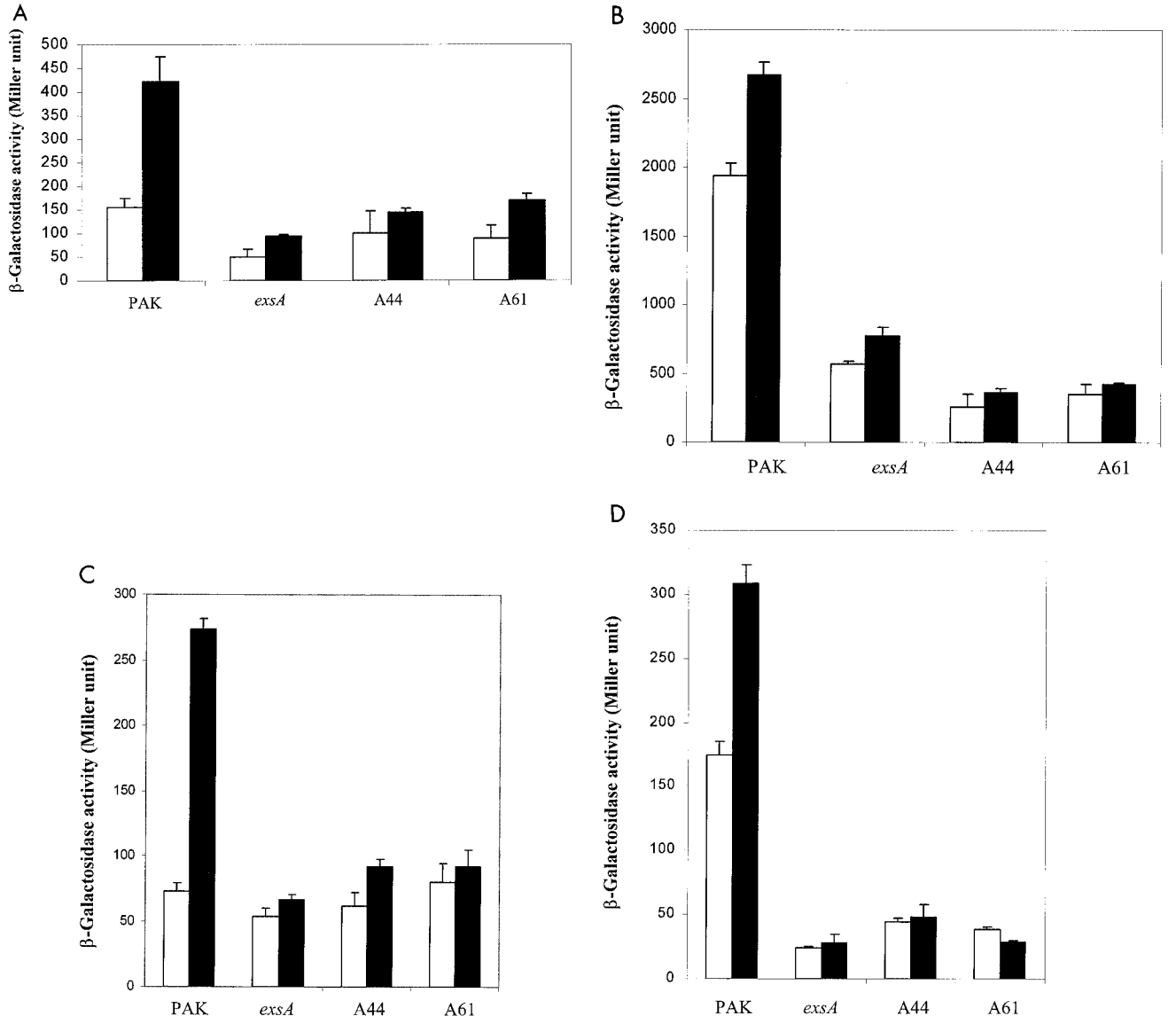


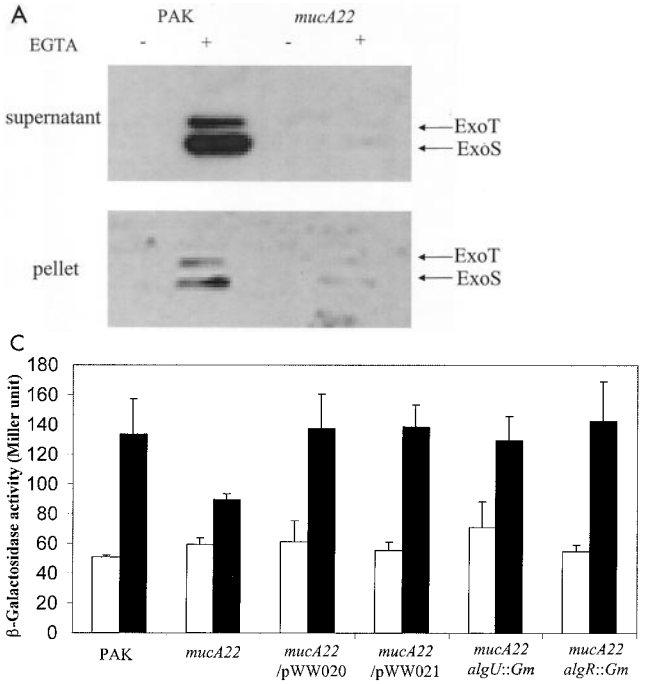
FIG. 1. Expression of type III secretion genes in Tn insertional mutants of *mucA*. PAK, PAK*exsA*, and *mucA* mutants A44 and A61 harboring pHW0006 containing *exoT*::*lacZ* (A), pHW0032 containing *exsA*::*lacZ* (B), pHW0005 containing *exoS*::*lacZ*(C), or pHW0024 containing *pscN*::*lacZ* (D) were tested for β-galactosidase activities. Bacteria were grown in LB (white bars) or LB containing 5 mM EGTA (black bars) to an OD_{600} of 1 to 2 before β-galactosidase assays. Each assay was done in triplicate, and the error bars indicate standard deviations.

and C). Upon complementation of the PAK*mucA22* mutant with the *mucA* gene in pUCP19, either driven by the *algU* promoter (pWW020) or *lac* promoter (pWW021), expression of the *exsA* and *exoS* genes in the resulting strains was restored to that of the wild type (Fig. 2C). These results clearly demonstrate that expression of the T3SS genes requires a functional *mucA* gene.

Microarray analysis of gene expression in the *mucA* mutant. To further understand the mechanism of MucA-mediated regulation of T3SS genes, global gene expression profiles were compared between PAK*mucA22* and its wild-type parent strain PAK grown under T3SS-inducing conditions. Previously, a microarray analysis compared global gene expression patterns between mucoid (*mucA* mutant) and wild-type *P. aeruginosa* under non-T3SS-inducing conditions (10). Under these conditions, the T3SS activity in both strains was low; thus, no obvious effect of the *mucA* gene on the T3SS was observed.

Results of our gene array analysis were consistent with the published data (15, 31); genes under the control of AlgU are up regulated in a PAK*mucA22* mutant background compared to that in wild-type PAK, including genes for alginate biosynthesis (operon PA3540-3551) and regulation (Table 2). Also up regulated were an operon, PA4468-4471, consisting of the *sodM* gene (PA4468) encoding manganese superoxide dismutase, whose production is known to be higher in mucoid than nonmucoid *P. aeruginosa* (18), and the *fumC* gene (PA4470) encoding a tricarboxylic acid cycle enzyme fumarase C, which is essential for alginate production (17). Their results validated our gene array data.

Meanwhile, the expression levels of *exoS*, *exoT*, *exoY*, and



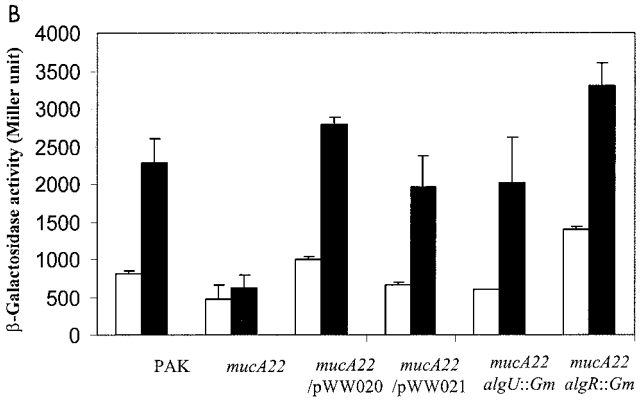


FIG. 2. Expression and secretion of ExoS protein. (A) Comparison of cellular and secreted forms of ExoS in strains PAK and PAK*mucA22* grown in LB or LB plus 5 mM EGTA. Supernatants and pellets from equivalent bacterial cell numbers were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and immunoblotted with anti-ExoS antibody. Both ExoS and ExoT are indicated by the arrow. Anti-ExoS polyclonal antibody also recognizes ExoT due to high homology between these two proteins. (B) Expression of *exsA*::*lacZ* (pHW0032) in the backgrounds of PAK, PAK*mucA22*, and PAK*mucA22algR*::*Gm* with or without the *mucA* clone driven by an *algU* promoter (pWW020) or *lac* promoter (pWW021). (C) Expression of *exoS*::*lacZ* (pHW0005) in the same backgrounds as described above. Bacteria were grown to an OD_{600} of 1 to 2 in LB with (black bars) or without (white bars) EGTA before β-galactosidase assays.

other T3SS-related genes were clearly down regulated in the *mucA* mutant background compared to those in wild-type PAK under T3SS-inducing conditions (Table 3), which confirmed our β-galactosidase assay and the Western blotting results. However, no significant changes in the expression of the *exsA* gene and a few other T3SS genes were observed. A previous gene array study also showed that expression of the *exsA* gene and the *exsD-pscL* operon is relatively nonresponsive to Ca^{2+}

TABLE 2. Expression of AlgU regulon genes in PAK*mucA22*

Group and ID no.	Name	Function	Fold change in *mucA22* vs wild type
Alginate biosynthesis genes			
PA3540	*algD*	Alginate biosynthesis	64.2
PA3541	*alg8*	Alginate biosynthesis	29.9
PA3542	*alg44*	Alginate biosynthesis	28.9
PA3543	*algK*	Alginate biosynthesis	81.2
PA3544	*algE*	Alginate biosynthesis	47.9
PA3545	*algG*	Alginate biosynthesis	38.0
PA3546	*algX*	Alginate biosynthesis	86.0
PA3547	*algL*	Alginate biosynthesis	43.7
PA3548	*algI*	Alginate biosynthesis	55.2
PA3549	*algJ*	Alginate biosynthesis	27.2
PA3550	*algF*	Alginate biosynthesis	70.5
PA3551	*algA*	Phosphomannose isomerase	38.7
Alginate biosynthesis regulatory genes			
PA0762	*algU*	Sigma factor	2.6
PA0763	*mucA*	Anti-sigma factor	2.4
PA0764	*mucB*	Negative regulator for alginate biosynthesis	1.3
PA5261	*algR*	Alginate biosynthesis; two-component system	1.5
PA5483	*algB*	Alginate biosynthesis; two-component system	2.0
PA5484	*kinB*	Two-component sensor	2.1
Genes known to be up regulated in *mucA* mutant			
PA0059	*osmC*	Osmotically inducible protein	3.8
PA0376	*rpoH*	Sigma factor	1.3
PA4876	*osmE*	Osmotically inducible lipoprotein	3.0
PA5489	*dsbA*	Thiol:disulfide interchange protein	1.3

depletion (44), yet a clear difference in the β-galactosidase activities could be observed when PAK harboring *exsA*::*lacZ* (pHW0032) was grown in LB with or without EGTA. Similarly, we have seen differences in the β-galactosidase activities between PAK(pHW0032) and PAK*mucA22*(pHW0032) under type III-inducing conditions without observing such differences in gene array data, suggesting possible involvement of post-transcriptional control of the *exsA* gene.

From the microarray analysis, genes that are differentially expressed more than threefold between PAK*mucA22* and PAK are listed in Tables 4 and 5. A number of genes known to be inducible under iron deprivation were also elevated in the *mucA22* mutant, including the sigma factor PvdS and genes regulated by PvdS for pyoverdine synthesis (17), the operon PA4468-4471 (17), and the probable two-component regulatory genes PA1300 and PA1301, encoding the extracytoplasmic function sigma-70 factor and a transmembrane sensor, respectively (34). Compared to the global gene expression profile of PAK grown under T3SS-inducing or -noninducing conditions, none of the above genes seem to be affected by Ca^{2+} depletion (44). The mechanism by which these genes are activated is not clear.

T3SS repression in the *mucA* mutant is AlgU dependent. MucA is an anti-sigma factor which represses the activity of AlgU (σ^{22}). In the *mucA* mutant, AlgU is derepressed and activates the expression of genes for alginate synthesis, resulting in a mucoid phenotype. AlgU can also activate the expression of itself and downstream genes (*mucA-B-C-D*) in the same operon. To determine the role of AlgU in the repression of T3SS in the *mucA* mutant, the *algU* gene was knocked out in the background of PAK*mucA22*, resulting in a PAK*mucA22algU*::*Gm* double mutant. Under T3SS-inducing conditions, expression of the *exsA* and *exoS* genes in this double mutant was similar to that in the wild type (Fig. 2B and C), indicating that AlgU is required for the T3SS repression in the *mucA* mutant. An *algU*::*Gm* mutant was further generated in the background of PAK, and T3SS activity in the resulting mutant was compared with that in PAK. As shown in Fig. 3, expression of the *exsA* and *exoS* genes was the same in the PAK*algU*::*Gm* mutant and wild-type PAK under both T3SS-inducing and -noninducing conditions, suggesting that the basal level of AlgU in wild-type *P. aeruginosa* does not play a significant role in the regulation of T3SS genes. When the *algU* gene was overexpressed in wild-type PAK by introducing pWW025, T3SS activity was partially repressed under type III-inducing conditions (Fig. 3). Since AlgU mediates the activation of the *algU-mucA* operon, an extra copy of *algU* also increased the expression of its repressor MucA; thus, overexpression of the *algU* gene could not repress T3SS expression to the level seen in the *mucA* mutant.

AlgR has a negative regulatory function on T3SS. *algR* is a regulatory gene required for alginate synthesis and is under the control of AlgU (27, 46). To investigate the role of AlgR in the regulation of T3SS, the *algR* gene was knocked out in the background of PAK*mucA22*. In the PAK*mucA22algR*::*Gm* double mutant, the expression of the *exsA* and *exoS* genes was restored to that of the wild type (Fig. 2A and B), suggesting that the repression of T3SS in the *mucA* mutant is also AlgR dependent. To test the function of AlgR on T3SS in wild-type *P. aeruginosa*, an *algR*::*Gm* mutant was generated in the PAK background. The expression of the *exoS* gene was consistently higher in the resulting PAK*algR*::*Gm* mutant than in PAK under both type III-inducing and -noninducing conditions (Fig. 4B). However, the expression of the *exsA* gene was similar in the PAK*algR*::*Gm* mutant and wild-type PAK. Complementation of the *algR* mutant with an *algR*-expressing clone (pWW022) decreased *exsA* and *exoS* expression under both inducing and noninducing conditions (Fig. 4). However, higher expression of *algR* induced by increasing the amount of isopropyl-β-D-thiogalactopyranoside (IPTG) could not further decrease *exsA* and *exoS* expression (Fig. 4). These results indicate that AlgR has a negative regulatory effect on the T3SS, but the up regulation of AlgR alone might not be sufficient to repress T3SS activity to the level seen in the *mucA* mutant. It is likely that in the *mucA* mutant, *algR* gene expression is activated by AlgU, which in turn represses T3SS activity.

DISCUSSION

T3SS is an important virulence machinery for *P. aeruginosa*: it inhibits the host defense system by inducing apoptosis in macrophages, polymorphonuclear phagocytes, and epithelial cells. In our screen for mutants with lower T3SS activities, *mucA* mutants were found defective in *exoT* expression under type III-inducing conditions. Furthermore, the basal promoter activity of the type III master regulatory gene *exsA* was decreased two- to threefold in the *mucA* mutant compared to that in wild-type PAK, suggesting that the down regulation of T3SS genes occurs through repression of ExsA. Since ExsA is an autoactivator (21), the repression could be on the transcriptional or posttranscriptional level. Our microarray results showed that the transcript level of *exsA* in the *mucA* mutant was similar to that in wild-type PAK under type III-inducing conditions, which suggested that the activity of ExsA might be repressed at the posttranscriptional level. Similarly, a previous microarray study also showed that expression of the *exsA* gene was not affected by Ca^{2+} depletion (44). Further study is required to clarify the mechanism of *exsA* gene regulation.

MucA is a transmembrane protein, with its cytoplasmic domain binding to and repressing the sigma factor AlgU. Mutation in the *mucA* gene leads to derepression of AlgU, which in turn activates genes for alginate synthesis as well as others, such as *dsbA*, *oprF*, *osmE*, and *rpoH* (10, 28). In the *mucA* mutant, not only the sigma factor AlgU but also AlgQ, an anti-σ^{70} factor, are activated (9), thus posing the possibility that sigma factor competition by AlgU and AlgQ effectively decreases the availability of σ^{70}-containing RNA polymerase for the expression of T3SS genes (23). However, the observa-

TABLE 3. Expression of T3SS-related genes in PAK*mucA22*

ID no.	Gene	Function	Fold change in *mucA22* vs wild type
PA0044	*exoT*	Exoenzyme T	−2.0
PA2191	*exoY*	Adenylate cyclase	−1.3
PA3841	*exoS*	Exoenzyme S	−2.1
PA1707	*pcrH*	Regulatory protein	−1.4
PA1708	*popB*	Translocator protein	−1.6
PA1709	*popD*	Translocator outer membrane protein	−1.5
PA1718	*pscE*	Type III export protein	−1.4
PA1719	*pscF*	Type III export protein	−1.5

TABLE 4. Genes up regulated in PAK*mucA22*

ID no.[a]	Gene	Function	Fold change in *mucA22* vs wild type	TSB[b] (fold)	LB[b] (fold)
PA0059	*osmC*	Adaptation, protection	3.75	1.23	−1.29
PA2386	*pvdA*	Adaptation, protection	3.89	−1.50	1.07
#PA2397	*pvdE*	Adaptation, protection, membrane proteins, transport of small molecules	3.92	−3.00	−1.99
PA2401		Adaptation, protection	3.06	−1.00	−5.74
PA4468	*sodM*	Adaptation, protection	5.60	−1.30	1.02
PA2018		Antibiotic resistance and susceptibility, membrane proteins, transport of small molecules	3.88	−1.50	1.65
PA2019		Antibiotic resistance and susceptibility, transport of small molecules	4.16	−1.50	−1.36
PA1985	*pqqA*	Biosynthesis of cofactors, prosthetic groups, and carriers	2.99	−1.10	−1.24
PA1988	*pqqD*	Biosynthesis of cofactors, prosthetic groups, and carriers	3.18	−1.50	−1.28
PA1989	*pqqE*	Biosynthesis of cofactors, prosthetic groups, and carriers	3.00	1.44	−1.34
#PA2414		Carbon compound catabolism	5.17	−3.00	−1.29
PA3158	*wbpB*	Cell wall, LPS, and capsule; putative enzymes	5.76	−1.20	−1.08
PA0102		Central intermediary metabolism	3.38	−2.40	−1.15
PA2393		Central intermediary metabolism	3.27	−1.70	−4.76
PA2717	*cpo*	Central intermediary metabolism	4.75	1.21	−1.72
PA4470	*fumC1*	Energy metabolism	5.81	1.05	−1.62
PA5491		Energy metabolism	2.97	−1.30	1.08
#PA0320		Hypothetical	3.86	−7.80	−1.43
PA0586		Hypothetical	5.10	1.61	1.96
PA0587		Hypothetical	4.57	1.11	1.64
PA0588		Hypothetical	4.57	1.12	1.64
PA0613		Hypothetical	3.60	1.14	−1.55
#PA0737		Hypothetical	10.80	1.70	−1.00
PA0807		Hypothetical	3.97	1.21	−1.52
PA0990		Hypothetical	3.45	2.31	−1.48
PA1245		Hypothetical; membrane proteins	5.12	−1.10	−1.20
PA1323		Hypothetical	4.21	1.94	2.25
#PA1471		Hypothetical	12.4	1.46	1.07
#PA1784		Hypothetical	6.71	1.33	−1.31
PA1852		Hypothetical	3.34	1.90	−1.03
PA2159		Hypothetical	3.56	1.44	−2.46
PA2161		Hypothetical	2.95	1.50	−4.92
#PA2167		Hypothetical	9.54	1.16	−1.06
PA2168		Hypothetical	3.20	−1.20	−2.80
#PA2172		Hypothetical	3.86	−1.90	−1.99
PA2176		Hypothetical	7.30	1.79	2.10
PA2403		Hypothetical; membrane proteins	4.96	−1.30	−1.52
PA2404		Hypothetical; membrane proteins	5.83	1.27	−1.69
#PA2405		Hypothetical	10.00	1.10	−5.91
PA2406		Hypothetical	5.79	−1.10	−1.22
PA2412		Hypothetical	4.56	−1.30	−2.72
PA2485		Hypothetical	5.16	−1.20	5.51
PA2486		Hypothetical	6.30	1.17	1.41
PA2562		Hypothetical	3.30	1.27	2.38
PA3274		Hypothetical	5.30	1.61	−2.16
PA4154		Hypothetical	3.63	1.49	1.08
#PA4469		Hypothetical	8.78	−1.30	−1.64
PA4471		Hypothetical	3.00	−1.00	−3.49
PA5182		Hypothetical; membrane proteins	4.81	1.29	2.11
PA5183		Hypothetical; membrane proteins	3.88	1.20	2.02
PA5212		Hypothetical	3.24	1.32	1.83
PA2409		Membrane proteins, transport of small molecules	4.21	−1.20	−1.46
PA4876	*osmE*	Membrane proteins, adaptation, protection	3.00	1.36	1.27
PA2407		Motility and attachment	4.73	−1.20	−1.61
PA2385		Putative enzymes	3.19	1.05	−2.36
PA2394		Putative enzymes	3.56	1.00	−1.75
PA2402		Putative enzymes	3.18	−1.70	−2.00
PA2413		Putative enzymes	5.97	1.46	−2.32
PA4785		Putative enzymes	4.51	1.70	−1.98
PA0724		Related to phage, transposon, or plasmid	3.48	1.83	−2.39
PA1300		Transcriptional regulators	3.66	−1.70	−1.13
PA2426	*pvdS*	Transcriptional regulators	4.39	−1.40	−1.38
PA2408		Transport of small molecules	4.09	2.08	−3.14
PA3049	*rmf*	Translation, posttranslational modification, degradation	6.81	2.03	1.38
PA3188		Transport of small molecules	3.08	4.04	15.76
PA5470		Translation, posttranslational modification, degradation	3.31	−1.50	1.15
#PA2398	*fpvA*	Transport of small molecules	6.65	−2.10	−1.51

[a] #, up regulated in *mucA* mutant compared to PAK, but down regulated in PAK under type III-inducing conditions versus noninducing conditions, and vice versa. Not included are those known to be affected by the growth medium, such as those varied in TSB versus LB (44).

[b] Change in gene expression in PAK grown under T3SS-inducing conditions versus PAK grown under non-T3SS-inducing conditions (44). Bacteria were grown in TSB or LB.

TABLE 5. Genes down regulated in PAK*mucA22*

ID no.[a]	Gene	Function	Fold change in *mucA22* vs wild type	TSB[b] (fold)	LB[b] (fold)
PA3450		Adaptation, protection	−3.5	1.84	1.17
PA2138		DNA replication, recombination, modification, and repair	−3.2	−2.50	−3.19
PA0523	*norC*	Energy metabolism	−3.0	−1.70	−1.7
#PA3445		Hypothetical	−3.9	2.66	1.59
#PA3446		Hypothetical	−5.1	1.36	1.57
PA3931		Hypothetical	−3.8	1.91	1.22
PA0281	*cysW*	Membrane proteins, transport of small molecules	−3.5	1.18	1.13
PA0282	*cysT*	Membrane proteins, transport of small molecules	−3.0	−1.10	−1.07
PA1601		Putative enzymes	−3.5	1.35	−1.44
PA3444		Putative enzymes	−5.1	1.11	−1.51
PA1246	*aprD*	Secreted factors (toxins, enzymes, alginate); protein secretion-export apparatus	−3.4	2.05	−2.64
PA1312		Transcriptional regulators	−3.1	−1.10	−1.55
PA3927		Transcriptional regulators	−5.1	−1.70	−1.67
PA0198	*exbB1*	Transport of small molecules	−5.5	2.71	−3.68
PA0280	*cysA*	Transport of small molecules	−6.0	−1.00	−1.18
PA2204		Transport of small molecules	−4.7	1.16	−1.16

[a] #, up regulated in *mucA* mutant compared to PAK but down regulated in PAK under type III-inducing conditions versus noninducing conditions and vice versa. Not included are those known to be affected by the growth medium, such as those varied in TSB versus LB (44).
[b] Change in gene expression in PAK grown under T3SS-inducing conditions versus PAK grown under non-T3SS-inducing conditions (44). Bacteria were grown in TSB or LB.

tion that AlgR, an AlgU-dependent transcriptional activator, is required for the T3SS suppression makes it unlikely that sigma factor competition leads to the type III gene suppression; instead, an AlgR-dependent repressor is likely involved. AlgR is a global regulator, affecting expression of multiple genes. Proteomics analysis of an *algR*::*Gm* mutant showed that more than 17 proteins were up regulated and 30 proteins were down regulated (26). In the present study, AlgR was also found to mediate the repression of type III secretion genes. In the PAK*algR*::*Gm* mutant background, expression of the *exoS* gene was higher than in wild-type PAK and, when complemented by an *algR* gene clone, expression of *exsA* and *exoS* genes decreased to about 50% of that seen in wild-type PAK (Fig. 4). The inability to suppress T3SS genes to the level seen in the *mucA* mutant by pWW022 was possibly due to a lower level of expression of the *algR* gene from pWW022 than that in the PAK*mucA* background, in which *algR* is activated through the MucA-AlgU pathway. pMMB67HE is a low-copy-number plasmid (13), and the *tac* promoter is not as strong a promoter in *P. aeruginosa* as it is in *E. coli*. Alternatively, other regulatory genes might be involved in the repression of T3SS. Further study is needed to understand this observation.

We propose a model for T3SS repression in the *mucA* mutant (Fig. 5). With the activation of AlgU, the regulatory genes *algP*, *algQ*, *algB*, and *algR* are activated, which up regulates the expression of the *algD* operon. AlgR is required for T3SS repression in the *mucA* mutant, but whether the repression function is directly on ExsA or not is unclear. The involvement of other regulatory genes (*algP*, *algQ*, and *algB*) in T3SS regulation awaits further study.

During chronic infection of CF patient airways, *P. aeruginosa* overproduces alginate and forms a biofilm (20). Alginate pro-

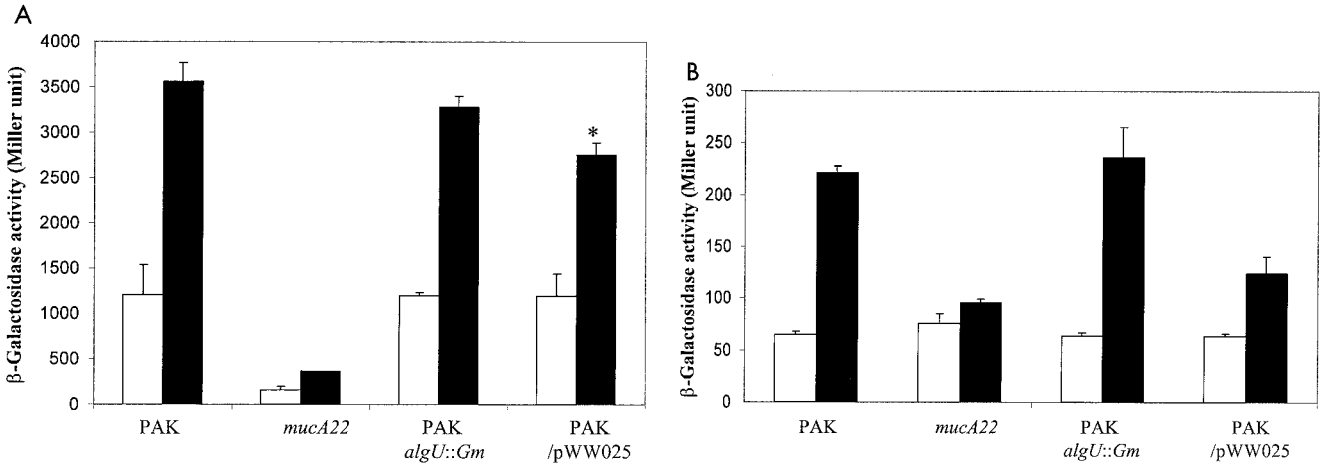


FIG. 3. Expression of *exsA*::*lacZ* (A) and *exoS*::*lacZ* (B) in strains PAK, PAK*mucA22*, PAK*algU*::*Gm*, and PAK harboring *algU* overexpression plasmid pWW025. Bacteria were grown in LB (white bars) or LB plus 5 mM EGTA (black bars) to an OD_{600} of 1 to 2 before β-galactosidase assays. *, $P < 0.05$.

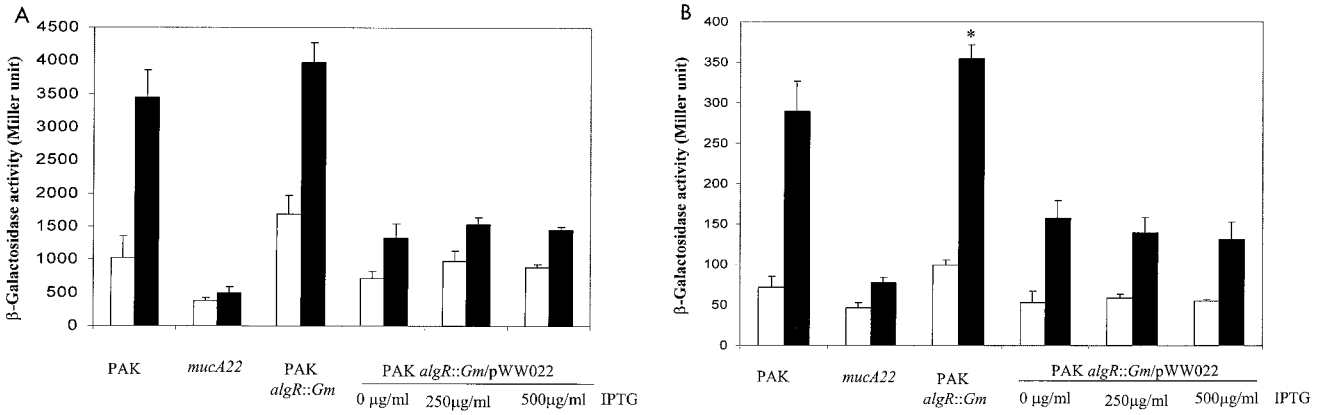


FIG. 4. Expression of *exsA*::*lacZ* (A) and *exoS*::*lacZ* (B) in the backgrounds of PAK, PAK*mucA22*, PAK*algR*::*Gm*, and PAK*algR*::*Gm* complemented with *algR*-expressing plasmid pWW022. For *algR* gene complementation, various concentrations of IPTG were added into the culture medium as indicated. Bacteria were grown in LB (white bars) or LB plus 5 mM EGTA (black bars) to an OD_{600} of 1 to 2 before β-galactosidase assays. *, $P < 0.05$.

duction is known to be activated by high osmolarity, nitrogen limitation, and membrane perturbation induced by ethanol (2); thus, the high salt concentration in the CF patient airway might be a signal for the overproduction of alginate. The biofilm mode of growth can help the bacterium survive in hostile environments and also render resistance against macrophages and polymorphonuclear cells (20). Our experimental data suggest that bacteria have evolved a mechanism to turn off T3SS when they need to synthesize alginate to overcome environmental stress. Such coordinated regulation of two energy-expensive processes is likely to render to the bacterium a survival advantage under environmental stress conditions. Indeed, the majority of *P. aeruginosa* isolates from CF patients at a late stage in the disease display the mucoid phenotype (11, 37) and are defective in type III gene expression (5). In a previous report, introduction of the wild-type *exsA* gene into type III secretion-defective clinical isolates restored type III secretion (5). However, our attempts to restore T3SS gene expression in 10 mucoid CF isolates by introducing a *mucA* gene clone failed, although all of the transformants were reverted back to the nonmucoid phenotype. It is possible that those mucoid clinical isolates may harbor additional mutations in the T3SS genes.

Known T3SS regulators include ExsA, Vfr, CyaA/B, and ExsD (21, 32, 44). Recently, DsbA and AceA and -B were also found to be necessary for the expression of T3SS. AceA and -B are subunits of pyruvate dehydrogenase, suggesting that metabolic imbalance influences the expression of T3SS (6, 36). DsbA is a periplasmic thiol-disulfide oxidoreductase and was shown to affect T3SS expression, twitching motility, and intracellular survival of *P. aeruginosa* upon infection of HeLa cells (14, 28). Interestingly, the *dsbA* gene is up regulated in the *mucA* mutant background, and its expression was shown to be affected by AlgU (28). However, the role of DsbA on the T3SS is believed to be through its general effect on protein disulfide bond formation in the periplasm, and up regulation of this gene may not be related to the MucA-AlgU-AglR-mediated suppression of the T3SS.

From the microarray analysis of the *mucA* mutant and wild-type strain under T3SS-inducing conditions, alginate synthesis genes and genes known to be under the control of AlgU were up regulated, while T3SS genes were down regulated in the *mucA* mutant (Tables 2 and 3). In addition, pyoverdine synthesis genes as well as an operon, PA4468-4471, which might be under the control of Fur (18), were up regulated in the *mucA* mutant under T3SS-inducing conditions (Table 4). These findings are consistent with published results, in which mucoid *P. aeruginosa* strains produced higher levels of pyoverdine, pyochelin, manganese superoxide dismutase (PA4468), and fumarase (PA4470) than wild-type strains (16, 17). However, pyochelin synthesis genes were not seen up regulated in our microarray data, suggesting again the possibility of trans-

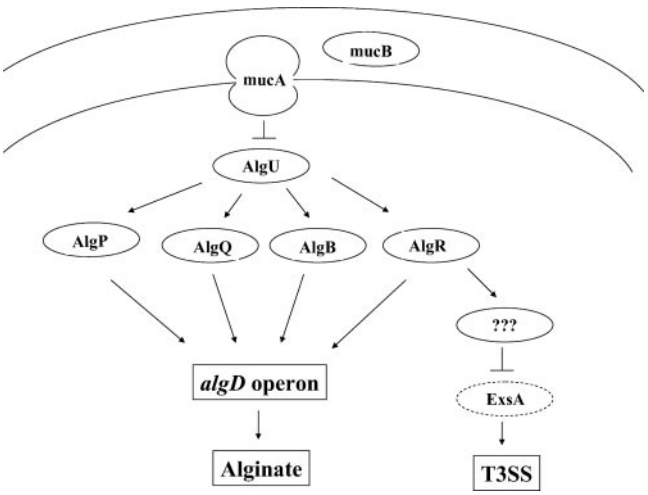


FIG. 5. Proposed model of MucA-mediated coordination of alginate production and T3SS expression. MucA is a transmembrane protein, with its cytoplasmic portion binding and inhibiting the sigma factor AlgU. Upon sensing of certain environmental stress signals by the periplasmic MucB, it signals MucA through the periplasmic domain to release the bound AlgU. Free AlgU is required for the expression of downstream transcriptional activators AlgP, AlgQ, AlgB, and AlgR, all of which contribute to the optimal expression of the *algD* operon, encoding enzymes for the synthesis of alginate. AlgR, on the other hand, also activates downstream genes which are responsible for the suppression of the type III secretion genes.

lational-level control for these genes. The mechanism by which these genes are up regulated in the *mucA* mutant background is not known.

The *mucA* gene mutation-mediated suppression of the T3SS genes requires AlgR, which is a transcriptional activator; thus, it is likely that an AlgR-regulated repressor mediates the suppression of T3SS genes. To identify such candidate genes from the gene array data, we initially identified genes that were differentially expressed in the *mucA* mutant compared to wild-type PAK under type III-inducing conditions. Those genes that were up regulated in the *mucA* mutant compared to PAK under type III-inducing conditions but that were down regulated in PAK under type III-inducing conditions versus noninducing conditions, and vice versa, were identified. We further eliminated those known to be affected by the growth medium, such as those with varied responses in tryptic soy broth (TSB) versus LB (44). Based on the above criteria, 13 genes were identified (Tables 4 and 5). For example, expression of the PA2172 gene in *mucA22* was up regulated about fourfold compared to wild-type PAK under T3SS-inducing conditions. From published data, the expression of this gene was down regulated twofold in wild-type PAK grown under type III-inducing conditions compared to that under noninducing conditions (44). Therefore, mutation in the *mucA* gene reversed the expression of PA2172 in response to the type III-inducing signal.

Among the 13 genes, *pvdE* and *fpvA* are involved in pyoverdine synthesis and absorption, respectively, and PA2414 is involved in carbon compound catabolism. The remaining 10 genes are all hypothetical genes. The expression of PA0737, PA2167, PA2176, and PA4785 seems to be ExsA dependent, since in the *exsA* mutant the expression of these genes was lower than in wild-type PAK under type III-inducing conditions and overexpression of *exsA* could activate expression of these genes under non-type III-inducing conditions (44). It is reasonable to hypothesize that one or more such differentially expressed genes mediate the repression of the T3SS in the *mucA* mutant. Attempts are under way to understand these possibilities by directed gene knockout of the identified candidate genes as well as screening of a random Tn insertional mutant bank generated in the background of PAK*mucA22* for those mutants with restored wild-type T3SS activity.

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