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Posttranslational Control of the *algT* (*algU*)-Encoded σ^{22} for Expression of the Alginate Regulon in *Pseudomonas aeruginosa* and Localization of Its Antagonist Proteins MucA and MucB (AlgN)

KALAI MATHEE, CRAIG J. MCPHERSON, AND DENNIS E. OHMAN*

Department of Microbiology and Immunology, University of Tennessee, and Veterans Affairs Medical Center, Memphis, Tennessee 38163

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Pseudomonas aeruginosa **strains associated with cystic fibrosis are often mucoid due to the copious production of alginate, an exopolysaccharide and virulence factor. Alginate gene expression is transcriptionally controlled by a gene cluster at 68 min on the chromosome: *algT* (*algU*)-*mucA*-*mucB* (*algN*)-*mucC* (*algM*)-*mucD* (*algY*). The *algT* gene encodes a 22-kDa alternative sigma factor (σ^{22}) that autoregulates its own promoter (P*algT*) as well as the promoters of *algR*, *algB*, and *algD*. The other genes in the *algT* cluster appear to regulate the expression or activity of σ^{22}. The goal of this study was to better understand the functional interactions between σ^{22} and its antagonist regulators during alginate production. Nonmucoid strain PAO1 was made to overproduce alginate (indicating high *algD* promoter activity) through increasing σ^{22} in the cell by introducing a plasmid clone containing *algT* from *mucA22*(Def) strain FRD1. However, the bacterial cells remained nonmucoid if the transcriptionally coupled *mucB* on the clone remained intact. This suggested that a stoichiometric relationship between σ^{22} and MucB may be required to control sigma factor activity. When the transcription and translational initiation of *algT* were measured with *lacZ* fusions, alginate production correlated with only about a 1.2- to 1.7-fold increase in *algT-lacZ* activity, respectively. An *algR-lacZ* transcriptional fusion showed a 2.8-fold increase in transcription with alginate production under the same conditions. A Western blot analysis of total cell extracts showed that σ^{22} was approximately 10-fold higher in strains that overproduced alginate, even though *algT* expression increased less than 2-fold. This suggested that a posttranscriptional mechanism may exist to destabilize σ^{22} in order to control certain σ^{22}-dependent promoters like *algD*. By Western blotting and *phoA* fusion analyses, the MucB antagonist of σ^{22} was found to localize to the periplasm of the cell. Similar experiments suggest that MucA localizes to the inner membrane via one transmembrane domain with amino- and carboxy-terminal domains in the cytoplasm and periplasm, respectively. These data were used to propose a model in which MucB-MucA-σ^{22} interact via an inner membrane complex that controls the stability of σ^{22} protein in order to control alginate biosynthesis.**

Patients with cystic fibrosis (CF) are frequently afflicted with pulmonary infections by *Pseudomonas aeruginosa*. Following colonization of the CF patient's respiratory tract, mucoid variants of *P. aeruginosa* emerge, become predominant, and lead to chronic pulmonary disease (32, 54). This mucoid phenotype confers an increased capacity to resist phagocytosis (1, 66, 70) and a mechanism of adherence (39, 44, 58). *P. aeruginosa* isolates from non-CF patients rarely show this phenotype, and so the lung environment of the CF patient apparently selects for such mucoid variants. The mucoid phenotype, combined with the high tolerance of this opportunistic pathogen for many antibiotics, appears to give *P. aeruginosa* a selective advantage in the lungs of CF patients.

The mucoid phenotype is due to the overproduction of alginate, a linear polymer composed of O-acetylated D-mannuronate and L-guluronate (18, 27). The gene products of *algA*, *algC*, and *algD* are required for the formation of the main precursor of alginate, GDP-mannuronate (for a review, see reference 50). A periplasmic polymer is subject to the action of C5-epimerase encoded by *algG* (6, 23), to that of an alginate lyase encoded by *algL* (3, 53, 61), and to O acetylation by proteins encoded by *algIJF* (24, 25, 68). The product of *algE* may be involved in polymer export to the bacterial surface (8, 59). The genes encoding enzymes for alginate biosynthesis are in an 18-kb operon at 34 min on the chromosome (7), except for *algC*, which is located at 10 min (76). Located upstream of *algD*, the promoter for the alginate biosynthetic operon (P*algD*) is nearly silent in typical nonmucoid *P. aeruginosa* but shows high activity in alginate-overproducing (Alg^+) strains (7, 11, 56, 73).

Alginate overproduction requires a 22-kDa alternative sigma factor (σ^{22}) encoded by *algT* (also known as *algU*) (15, 34, 47). The *algT* gene was originally identified as a *trans*-acting positive regulator for alginate biosynthesis located at about 68 min on the chromosome (21). *P. aeruginosa* σ^{22} shows homology to σ^{E} of *Escherichia coli* (15, 47). Encoded by *rpoE*, σ^{E} controls σ^{32} expression as part of an extreme heat shock response (17, 35, 57, 60). A number of proteins have now been shown to be related to σ^{E} and σ^{22}, and they were recently described as the extracytoplasmic function (ECF) subfamily of eubacterial RNA polymerase sigma factors involved in the regulation of ECFs (40).

A hierarchy of alginate gene regulation which is under σ^{22} (*algT* or *algU*) control exists in *P. aeruginosa* (73). An *algT*::

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Tennessee and VA Medical Center, 858 Madison Ave., Room 601, Memphis, TN 38163. Phone: (901) 448-8094. Fax: (901) 448-8462. E-mail: dohman@utmem1.utmem.edu.

Tn*501* mutation blocks expression of P*algD* and reduces expression of the alginate regulatory genes *algR* (*algR1*) and *algB* (71, 73). The *algR* gene (10) at 9 min and the *algB* gene (28, 72) at about 13 min encode proteins that have homology to environmentally responsive two-component regulatory proteins. Tn*501* insertional mutations in *algB* and *algR* reduce expression of P*algD* by 28- and 17-fold, respectively, in Alg^+ *P. aeruginosa* FRD1 (73). Mutation in *algT* also blocks transcription at *algT*, indicating that its own promoter (P*algT*) is under σ^{22} control (15, 47). The promoter regions preceding the transcriptional initiation sites of *algT*, *algR*, *algB*, and *algD* all have significant homology to promoters recognized by σ^E (15, 34, 47, 56). In vitro transcription studies have shown that RNA polymerase with σ^{22} can autoinitiate transcription from P*algT* (34).

The activation of genes for alginate overproduction in *P. aeruginosa* appears to occur through the deregulation of *algT* or its product, σ^{22} (15, 47). Four genes downstream of *algT* (see Fig. 1) called *mucA*, *mucB* (or *algN*), *mucC* (or *algM*), and *mucD* (or *algY*) play a role in the normal control of alginate gene expression (2, 12, 21, 29, 56). Mucoid *P. aeruginosa* isolates from CF patients frequently have a defective *mucA* allele, and inactivation of *mucA* in *P. aeruginosa* PAO1 results in Alg^+; this indicates that MucA functions as a negative regulator of alginate production (12, 46). MucA has been shown to be an anti-sigma factor by its affinity for σ^{22} and its inhibition of transcriptional activity in vitro (64, 74). The *mucB* gene was identified as a negative regulator by Tn*501* mutagenesis; typical nonmucoid strains like PAO1 carrying a plasmid with *algT* (cloned from the *mucA*-defective CF isolate FRD1) became Alg^+ if *mucB* (i.e., *algN*) on the clone was inactivated (29). Also, it has been shown that a clone of the *mucAB* genes from PAO1 can suppress the mucoid phenotype in certain mucoid strains (45). The product of *mucC* (*algM*) may act as a positive regulator (56). The product of *mucD* appears to act as a negative regulator of alginate production and has homology to the HtrA (DegP) heat shock periplasmic protease of *E. coli* (2, 56). The Alg^+ phenotype due to *mucA* mutation is unstable under laboratory conditions, and spontaneous reversion to Alg^- is often due to suppressor mutations in *algT* (15, 62).

To better understand the mechanism of σ^{22} control and its association with alginate overproduction, we tested whether the promoter for its structural gene (P*algT*) undergoes transcriptional activation in Alg^+ strains. Transcriptional and translational fusions of *algT* to *lacZ* were constructed and tested in nonmucoid and mucoid variants of the PAO1 strain background. High activation of *algD* transcription (under σ^{22} control) has previously been observed to correlate with the Alg^+ phenotype (12, 73). In this study, we observed only a small increase in *algT-lacZ* transcription and translational initiation when strain PAO1 was made Alg^+ by carrying multiple copies of *algT* in *trans*. In contrast, we found evidence for a posttranscriptional control mechanism which appears to affect the stability of σ^{22} in the cell. We also performed localization studies of the antagonist proteins MucA and MucB (AlgN) to better understand the mechanism by which they control σ^{22} activity and stability.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains, plasmids, and primers used in this study are shown in Table 1. *E. coli* and *P. aeruginosa* were routinely cultured in L broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter). LA-PIA, used in triparental matings, was a 1/1 mixture of *Pseudomonas* isolation agar (Difco) and L agar. Antibiotics when used were at the following concentrations (per milliliter) unless indicated otherwise: ampicillin at 50 μg, kanamycin at 30 μg, carbenicillin at 300 μg, and tetracycline at 20 μg for *E. coli* and 100 μg for *P. aeruginosa.*

DNA manipulations. General DNA manipulations were performed as described previously (41). Klenow DNA polymerase was used to fill in 3′ protrusions from restriction digests as described by the manufacturer. Plasmid DNA was extracted from *E. coli* by a method described previously (49). Large-scale preparations of plasmid DNA were made with the QIAprep plasmid Midi kit (Qiagen). DNA fragments for cloning were purified from agarose gels with the QIAEX II gel extraction kit (Qiagen). Single-stranded oligonucleotide primers were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B). DNA was amplified by PCR with *Taq* DNA polymerase (Perkin-Elmer Cetus), and the products were cloned with a TA cloning kit (Invitrogen). Transformation of *E. coli* strains with ligated DNA mixtures and intact plasmids was performed by the methods of Dagert and Ehrlich (9) and Kushner (38), respectively. Triparental matings were used as described elsewhere (30) to mobilize plasmids from *E. coli* HB101 to *P. aeruginosa* with the conjugation helper plasmid pRK2013 (20). Transconjugants following triparental matings were selected on LA-PIA with appropriate antibiotics.

Electroporation. Electroporation of *P. aeruginosa* PAO1 was performed by a procedure adapted from the work of Diver et al. (16). Briefly, 0.2 ml of a *P. aeruginosa* PAO1 overnight culture was used to inoculate 20 ml of fresh L broth and incubated at 37°C with shaking. When the culture reached an optical density at 600 nm (OD_{600}) of 0.6 to 0.8, the cells were pelleted by centrifugation (10,000 × *g*, 10 min at 4°C), washed once with 20 ml and twice with 10 ml of cold SMH buffer (300 mM sucrose, 1 mM $MgCl_2$, 1 mM HEPES, pH 7.0), and resuspended in 1 ml of cold SMH. Plasmid DNA (1 to 2 μg) was added to a 100-μl aliquot of these electrocompetent cells, mixed, and incubated on ice for 1 min. The bacterium-DNA mixture was transferred to a chilled electroporation cuvette (Bio-Rad Gene Pulser/*E. coli* Pulser cuvette) with a 0.2-cm electrode gap. The electroporation was performed at 800 Ω, 25 μF, and an 8-kV/cm gap on a Gene Pulser electroporator (Bio-Rad). Immediately after electroporation, 900 μl of cold SOC medium (2.0% tryptone, 0.5% yeast extract, 10 mM $MgCl_2$, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 20 mM glucose) was added to the cells and incubated on ice for 30 min, followed by another 30 min of incubation at 37°C. Samples (200 μl) of the electroporated cells were spread onto L agar plates containing the appropriate antibiotics followed by incubation for ~36 h at 37°C.

Gene fusions with *lacZ*. To construct the *algT-lacZ* transcriptional fusion, a 926-bp *Fsp*I fragment from pJF15 containing the *algT* promoter region was ligated to *Sma*I-cut pQF50 to give rise to pKMG37 (see Fig. 1). To construct the *nadB-lacZ* transcriptional fusion, the same *Fsp*I fragment was cloned in the opposite orientation to form pKMG36 (see Fig. 1). To construct an *algT-lacZ* translational fusion, a 0.49-kb PCR product containing sequences internal to *algT* was amplified from pJF15 with primers CMP9 with an *Eco*RI site and CMP10 with a *Bam*HI site. This PCR product was ligated to pCRII, generating pCM97. An *Eco*RI-*Bam*HI '*algT* ' fragment from pCM97 was ligated into *Eco*RI-*Bam*HI-cut *lacZ* vector pMLB1034, giving rise to pCM117, containing an in-frame fusion with *lacZ*. A 1.3-kb *Eco*RI fragment from pSF3 containing *oriT* was ligated into *Eco*RI-cut pCM117 to generate pCM162 (see Fig. 1). This plasmid was mobilized into *P. aeruginosa* PAO1 by triparental mating. As this plasmid cannot be maintained in *P. aeruginosa*, it integrates into the chromosome via homologous recombination upon selection with carbenicillin (150 μg/ml) and generates a chromosomal *algT-lacZ* protein fusion (see Fig. 1). To construct an *algR-lacZ* transcriptional fusion, a 2.3-kb *Eco*RI fragment containing the *algR* promoter region from pAL1 was ligated to *Eco*RI-cut pBluescript (KS^-), creating pKMG189. Subsequently, a 2.3-kb *Bam*HI-*Hin*dIII fragment from pKMG189 containing the *algR* promoter was purified and ligated to *Bam*HI-*Hin*dIII-cut pQF50 to give rise to pKMG197 (see Fig. 1).

Colonies containing active fusions were detected by their blue color on L agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) at 40 μg/ml. Assays for *lacZ* products (β-galactosidase) in *P. aeruginosa* were done essentially as previously described (48). Briefly, a 100-μl sample of bacterial culture at an OD_{600} of 1.0 was transferred to a tube containing 50 μl of 0.1% sodium dodecyl sulfate (SDS) and 25 μl of chloroform, vortexed for 15 s, and then maintained on ice for at least 20 min but no more than 50 min. Samples in duplicate were then incubated at 28°C for 5 min and assayed for β-galactosidase activity as described by Miller (52).

Antibodies. Rabbit anti-PhoA antibody was obtained from 5 Prime→3 Prime, Inc. Rabbit anti-AlgT (σ^{22}) and anti-MucB (AlgN) were prepared against synthetic peptides representing hydrophilic segments of each protein. The σ^{22} peptide was identical to residues 90 to 111 of its inferred amino acid sequence (NH_2-RGRRPPDSDVTAEDAEFFEGDHC-OH) (15). The MucB peptide was identical to residues 141 to 162 of its inferred amino acid sequence (NH_2-PRDQHRYGFELHLDRDTGLPLKC-OH) (29). Both were synthesized with an automated peptide synthesizer (Beckman) and then conjugated to a carrier protein. The conjugates were used for commercial (HRP, Inc.) elicitation of polyclonal antisera in New Zealand White rabbits. Antibodies against σ^{22} and MucB were typically used in immunoblots at dilutions of 1:500 with a 1:50,000 dilution of peroxidase-conjugated goat anti-rabbit antibody (Sigma) as the secondary antibody. The specificity of immunoblot results was confirmed by preadsorption of the antibody for 1 h with 50 μg of its respective peptide per ml and then by repeated Western blot analyses.

Western blot analysis. Cells from 1.0 ml of a culture at an OD_{600} of 1.0 were collected by centrifugation, washed in 1.0 ml of saline, and then resuspended in 1.0 ml of ice-cold saline. To examine supernatant proteins, 1.5 ml of culture was

TABLE 1. Bacterial strains and plasmids

Strain, plasmid, or primer	Genotype, phenotype, or sequence[a]	Source or reference
E. coli		
HB101	*proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20*	This laboratory
JM109	F′ *traD36 lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$ *B*$^+$/*e14*$^-$ (McrA$^-$) Δ(*lac-proAB*) *thi gyrA96*(Nal^r) *endA1 hsdR17*(r_K^- m_K^+) *relA1 supE44 recA1*	This laboratory
CC118	*araD139* Δ(*ara, leu*)*7697* Δ*lacX74 phoA*Δ*20 galE galK thi rpsE rpoB argE(Am) recA1*	42
P. aeruginosa		
PAO1	Prototrophic wild type (nonmucoid)	36
PDO120	PAO1::pCM162 (*algT-lacZ* protein fusion)	This study
FRD1	CF isolate (Alg$^+$)	55
FRD440	*hisI algT*::Tn*501-33* (Alg$^-$)	22
Plasmids		
pBluescript II (KS$^-$)	ColE1 *oriV lacZ*α(Ap^r)	Stratagene
pCR II	ColE1 *oriV* F1 *ori lacZ*α (Ap^r Km^r)	Invitrogen Corp.
pKK233-2	ColE1 *oriV* (Ap^r) with P*trc*	Pharmacia Biotech
pLAFR1	IncP1 *cos oriT* (Tc^r)	26
pAL1	pCP19 with 20-kb *Hin*dIII fragment from FRD1 containing *algR*	73
pMF54	pKK233-2 (P*trc* Ap^r) with $oriV_{SF}$ *oriT*(RK2) *lacI*^{q}	23
pMLB1034	pBR322 derivative (Ap^r) MCS-*lacZ* (without a promoter or ribosome binding site)	69
pPHO7	P15A *oriV* (Tc^r) with MCS-*phoA* (without a promoter or ribosome binding site)	33
pQF50	ColE1 *ori-oriR* (Ap^r) with MCS-*lacZ* (promoterless)	19
pRK2013	ColE1 *ori*-Tra[RK2]$^+$ (Km^r)	20
pSELECT-1	ColE1 *ori lacZ*α(Tc^r Ap^s)	Promega Corp.
pSF3	P15A *oriV* (Tc^r) *oriT*(RK2)	67
pTrc99A	ColE1 *ori* (Ap^r) with *lacI*^{q} P*trc*	Pharmacia
pTrcMP	pTrc99A with a 1.3-kb *Eco*RI fragment containing *oriT* from pSF3 and a 1.8-kb *Pst*I fragment from pMF17 containing $oriV_{SF}$	M. Franklin
pJF15	pLAFR1 with 7.6-kb *Eco*RI DNA from FRD1 containing *algT mucA22*(Def) *mucB* (*algN*)	21
pJF20	pLAFR1 with 2.9-kb *Eco*RI DNA from pJF15::Tn*501-45* containing *algT mucA22*(Def) *mucB* (*algN*)	21
pJF20.25	pJF20 with *mucB* (*algN*)::Tn*501-25* (Hg^r)	29
pJF20.33	pJF20 with *algT* (*algU*)::Tn*501-33* (Hg^r)	29
pCM97	pCR II with the cloned 0.49-kb internal fragment of *algT* PCR amplified with primers CMP9 and CMP10	This study
pCM117	pMLB1034 with a 0.49-kb *Eco*RI-*Bam*HI fragment from pCM97 to form an *algT-lacZ* protein fusion	This study
pCM162	pCM117 with 1.3-kb *Eco*RI fragment from pSF3 containing *oriT*	This study
pCM157	pSELECT-1 with a 2.1-kb *Eco*RI fragment from pJF20.33 containing *mucB* (*algN*)	This study
pCM217	pCM157 with an *Eco*RI site created 53 bp upstream of the start codon for *mucB* (*algN*)	This study
pCM220	pTrcMP with *mucB* (*algN*) on a 1.2-kb *Eco*RI fragment from pCM217	This study
pKMG36	pQF50 containing (in *Sma*I site) a 0.93-kb *Fsp*I fragment from pJF15 to form P*nadB-lacZ*	This study
pKMG37	pQF50 containing (in *Sma*I site) a 0.93-kb *Fsp*I fragment from pJF15 to form P*algT-lacZ*	This study
pKMG126	pMF54 with the 5′ end of *mucA* on a 0.50-kb *Nco*I-*Xba*I fragment PCR amplified with primers KAL25 and KAL26	This study
pKMG159	pMF54 with the 5′ end of *mucA* on a 0.22-kb *Nco*I-*Xba*I fragment PCR amplified with primers KAL25 and KAL39	This study
pKMG160	pMF54 with the 5′ end of *mucA* on a 0.30-kb *Nco*I-*Xba*I fragment PCR amplified with primers KAL25 and KAL40	This study
pKMG161	pMF54 with the 5′ end of *mucA* on a 0.42-kb *Nco*I-*Xba*I fragment PCR amplified with primers KAL25 and KAL41	This study
pKMG170	pKMG159 with a *mucA*(1-54)-*phoA* protein fusion by insertion of a 2.6-kb *Xba*I fragment from pPHO7	This study
pKMG172	pKMG160 with a *mucA*(1-82)-*phoA* protein fusion by insertion of a 2.6-kb *Xba*I fragment from pPHO7	This study
pKMG174	pKMG161 with a *mucA*(1-120)-*phoA* protein fusion by insertion of a 2.6-kb *Xba*I fragment from pPHO7	This study
pKMG176	pKMG126 with a *mucA*(1-143)-*phoA* protein fusion by insertion of a 2.6-kb *Xba*I fragment from pPHO7	This study
pKMG188	pMF54 with the 5′ end of *mucB* (*algN*) on a 0.78-kb *Bsp*HI-*Xba*I fragment PCR amplified with primers KAL42 and KAL46	This study
pKMG189	pBluescript (KS$^-$) with a 2.3-kb *Eco*RI fragment from pAL1 containing P*algR*	This study
pKMG194	pKMG188 with a *mucB* (*algN*) (1-233)-*phoA* protein fusion by insertion of a 2.6-kb *Xba*I fragment from pPHO7	This study
pKMG197	pQF50 with a 2.3-kb *Bam*HI-*Hin*dIII fragment from pKMG189 to form P*algR-lacZ*	This study
Primers		
CJMO1	5′-GTGGTACAGAGAATTCGCTGCCCTACG-3′ (*Eco*RI site)	
CMP9	5′-GAATTCAGCAACTGGTTGAACGGGT-3′ (*Eco*RI site)	
CMP10	3′-GGCACTACGTCACAGGCCACCTAGG-5′ (*Bam*HI site)	

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TABLE 1—*Continued*

Strain, plasmid, or primer	Genotype, phenotype, or sequence[a]	Source or reference
KAL25	5′-CGAATTCCATGGAAGCCTGACACAGCGGCAAATGCC-3′ (*Eco*RI and *Nco*I sites)	
KAL26	3′-TCGCTTCTCGTCCCCGCGGCGTCCACAGATCTCGTTCGAAGGG-5′ (*Xba*I and *Hin*dIII sites)	
KAL39	3′-CCGCGCCAGGCAGTACGTGGCGCTCGGAGATCTCG-5′ (*Xba*I site)	
KAL40	3′-GGCGAGGCGGCTTTCGCCTCTTCCCTGGAGATCTCG-5′ (*Xba*I site)	
KAL41	3′-GACGGCGTTTACCGCGTTGTCCCCTGAGATCTCG-5′ (*Xba*I site)	
KAL42	3′-GGGACTGGGCGTCAAAGTACGCAGCGTCAGATCTCG-5′ (*Xba*I site)	
KAL46	5′-CATGTCATGACCGTCAGTGGTACAGAGAGCGCGCTGCCC-3′ (*Bsp*HI site)	
Peptides used to elicit antibody		
AlgT (AlgU)	NH_2-RGRRPPDSDVTAEDAEFFEGDHC-OH	
MucB (AlgN)	NH_2-PRDQHRYGFELHLDRDTGLPLKC-OH	

[a] Primers include the restriction site indicated in addition to *P. aeruginosa* sequences. Abbreviations for phenotypes: Tc^r, tetracycline resistance; Hg^r, mercury resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cb^r, carbenicillin resistance; Tra^+, transfer by conjugation; *oriT*, transferable by conjugation; Alg^+, alginate overproducing; MCS, multiple cloning site; *oriV*, origin of vegetative replication; Def, encodes defective gene product.

centrifuged and 1.0 ml of the supernatant was collected. Proteins in 1.0-ml samples were precipitated by adding 240 μl of 80% trichloroacetic acid (TCA; 15% final concentration), incubated on ice for 15 min, and centrifuged (14,000 × *g* for 15 min). Pellets were washed twice with ice-cold acetone, dried, and resuspended in sample buffer (1% SDS, 125 mM Tris-HCl [pH 6.8], 1% β-mercaptoethanol, 10% sucrose, 0.025% bromophenol blue) with 500 μl for cells and 100 μl for supernatants. Western blotting was performed as previously described (51). Briefly, the samples in sample buffer were boiled for 5 min and subjected to electrophoresis on SDS–12.5% polyacrylamide gels. The separated proteins were electrotransferred to a membrane (Trans-Blot transfer medium, 0.45-μm pore size; Bio-Rad) in a Trans-Blot apparatus (Bio-Rad) in Tris-glycine buffer (0.3025% Tris-HCl [pH 6.8], 1.442% glycine, 20% methanol) at 90 V and 4°C for 1 h. The membrane was then blocked in Tris-buffered saline (TBS) (100 mM Tris-HCl [pH 7.0], 0.9% NaCl) containing 1% bovine serum albumin (Sigma) for 1 h. The blot was incubated with shaking overnight at room temperature with a 1:500 dilution of primary antibody in antibody buffer (TBS, 0.05% Tween 20, 1% bovine serum albumin). Then, the membrane was washed three times with TTBS (TBS, 0.05% Tween 20) and probed with a 1:50,000 dilution of peroxide-conjugated goat anti-rabbit immunoglobulin G (Sigma) in antibody buffer for 2 h. Following three more washes with TTBS, the reactive proteins were detected by chemiluminescence (ECL kit; Amersham) and exposed to Kodak X-Omat autoradiography film for 1 to 2 min.

Cell fractionation. To obtain fractions enriched for cytoplasmic and periplasmic proteins from *P. aeruginosa*, the procedure of Cheng et al. (4) was used to convert cells to spheroplasts, which releases the contents of the periplasm. Briefly, cells in 10 ml of culture were collected by centrifugation, washed with spheroplast solution (200 mM $MgCl_2$, 0.1 M Tris-HCl, pH 8.0), resuspended in spheroplast solution containing 0.5 mg of lysozyme per ml, and then incubated at 25°C for 30 min. Following centrifugation (4,000 × *g*, 15 min), the pelleted spheroplasts were resuspended in sample buffer. The periplasmic proteins in the supernatant fraction were precipitated with 15% TCA and resuspended in 100 μl of sample buffer.

Overproduction of MucB. A 2.1-kb *Eco*RI fragment from pJF20.33 (see Fig. 1) containing *mucB* was cloned into the *Eco*RI site of pSELECT-1 to create pCM157. By Altered Site mutagenesis (Promega) as described by the manufacturer, primer CJM01 was used to create an *Eco*RI site 53 bp upstream of the *mucB* start codon, yielding pCM217. The 1.2-kb *Eco*RI fragment from pCM217 containing *mucB* was purified and ligated to *Eco*RI-cut pTrcMP, downstream of the *trc* promoter (P*trc*), to create pCM220 (see Fig. 3). This plasmid can be conjugally transferred and maintained in *P. aeruginosa*. Expression of P*trc-mucB* on pCM220 in *P. aeruginosa* was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) to the growth medium.

Gene fusions with *phoA*. To construct a *mucB-phoA* translational fusion, an 0.78-kb *Bsp*HI-*Xba*I-cut PCR product generated from PAO1 chromosomal DNA with KAL46-KAL42 primers was cloned into *Nco*I-*Xba*I-cut pMF54 to form pKMG188. A 2.6-kb *Xba*I fragment containing *phoA* from pPHO7 was cloned into *Xba*I-cut pKMG188 to fuse *phoA* in frame with *mucB* at residue 233 and form pKMG194 (see Fig. 3). To construct plasmids containing *mucA-phoA* translational fusions, *Nco*I-*Xba*I-cut PCR products from pJF15 were generated with primer pairs KAL25-KAL39, KAL25-KAL40, KAL25-KAL41, and KAL25-KAL26 and cloned into *Nco*I-*Xba*I-cut pMF54 to create pKMG159, pKMG160, pKMG161, and pKMG126, respectively. The 2.6-kb *Xba*I fragment containing *phoA* from pPHO7 was cloned into *Xba*I-cut pKMG159, pKMG160, pKMG161, and pKMG126, creating pKMG170, pKMG172, pKMG174, and pKMG176, in which alkaline phosphatase was fused in frame with MucA residues 54, 82, 120, and 143, respectively (see Fig. 4). Protein fusions with *phoA* were verified by Western blot analysis with rabbit anti-alkaline phosphatase (5 Prime→3 Prime, Inc.). Colonies containing active fusions were screened for blue color on L agar containing 5-bromo-4-chloro-3-indolyl phosphate (XP) at 40 μg/ml.

RESULTS

P*algT-lacZ* transcriptional and translational activities show small increases with alginate overproduction. Expression of P*algD* is σ^{22} (i.e., *algT* or *algU* gene product) dependent and shows high transcriptional activation when strains overproduce alginate (Alg^+) (11, 73). Here we tested whether the *algT* promoter (P*algT*), which is also σ^{22} dependent, undergoes transcriptional activation in Alg^+ cells. Measurements of promoter activity were done in the wild-type Alg^- strain, PAO1. This strain background can be readily converted to Alg^+ by pJF20.25 (Fig. 1A), which carries 2.9 kb of DNA from the mucoid CF strain FRD1 encoding *algT*, *mucA22*(Def), and *mucB*::Tn*501* (29). This plasmid (six to eight copies per cell) increases the copy number of σ^{22} in the cell but not that of the downstream negative regulators. Cells carrying the same plasmid but with *mucB* intact (pJF20 [Fig. 1A]) remain Alg^- (21, 29). To probe for P*algT* activity, a P*algT-lacZ* transcriptional fusion reporter was constructed (pKMG37 [Fig. 1B]) by cloning a 926-bp *Fsp*I fragment containing the P*algT* promoter region into a broad-host-range *lacZ* vector (pQF50) that is compatible with pJF20 (i.e., IncP1) derivatives. β-Galactosidase activity was used as a measure of P*algT-lacZ* activity. Although we anticipated that high *algT* transcription might correlate with the Alg^+ phenotype, we found that P*algT-lacZ* activity in Alg^- PAO1(pJF20, pKMG37) was still 84% of the activity in Alg^+ PAO1(pJF20.25, pKMG37) (Table 2). When the plasmid-encoded *algT* was insertionally inactivated in Alg^- PAO1(pJF20.33, pKMG37), the level of σ^{22} in the cell was reduced to that produced by the single chromosomal *algT* gene, and P*algT-lacZ* activity from the reporter plasmid fell to 27% of that seen in the Alg^+ strain (Table 2). This was comparable to the P*algT-lacZ* activity seen in the vector-containing strain, Alg^- PAO1(pLAFR3, pKMG37) (i.e., 32% [Table 2]). For comparison, a control plasmid carrying P*nadB-lacZ* (pKMG36 [Fig. 1B]) was constructed by cloning the same P*algT* fragment in the opposite orientation. The *nadB* gene is adjacent to *algT* and is divergently transcribed (14). P*nadB-lacZ* activity was approximately three- to fourfold lower than P*algT-lacZ* activity in PAO1(pJF20.25, pKMG37) and was unaffected by the mucoid status of the cells (data not shown).

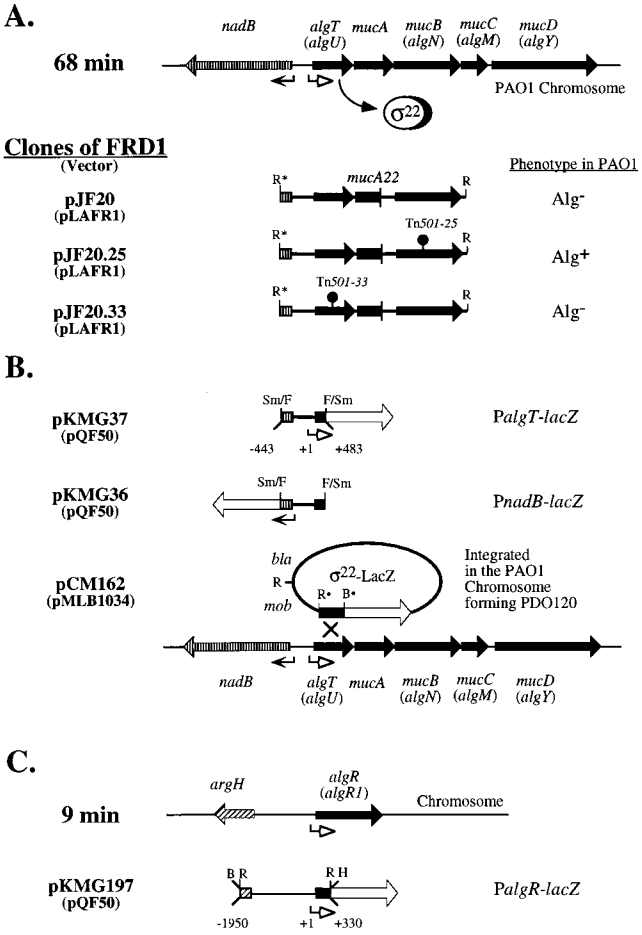

FIG. 1. (A) Segment of the 68-min region of the wild-type chromosome of *P. aeruginosa* PAO1 which contains the *algT* (*algU*) gene cluster encoding σ^{22} and its downstream control elements and the *nadB* (L-aspartate oxidase) gene upstream. Clone pJF20 contains 2.9 kb of DNA from the same region of the chromosome of mucoid strain FRD1, with a defect in *mucA* (*mucA22*). Derivatives of pJF20 show transposon insertion mutations *mucB*::Tn*501-25* and *algT*::Tn*501-33* in pJF20.25 and pJF20.33, respectively. Only pJF20.25 confers the mucoid (Alg^+) phenotype on PAO1 due to inactivation of *mucB* (*algN*). (B) *lacZ* transcriptional fusions to *algT* (pKMG37) and *nadB* (pKMG36) formed by cloning a 0.9-kb fragment containing the intervening DNA in both orientations into the broad-host-range vector pQF50. An *algT-lacZ* translational fusion (i.e., σ^{22}-LacZ) was formed by integrating pCM162, which contains an internal fragment of *algT* fused in frame to *lacZ*, into the chromosome of PAO1 by homologous recombination to form strain PDO120. (C) Segment of the 9-min region of the wild-type chromosome of *P. aeruginosa* that contains *algR* (*algR1*) and whose promoter is under σ^{22} control. An *algR-lacZ* transcriptional fusion was formed by cloning a 2.3-kb fragment containing the *algR* promoter into the broad-host-range vector pQF50. Restriction site designations: R, *Eco*RI; F, *Fsp*I; Sm, *Sma*I; B, *Bam*HI; H, *Hin*dIII. R* indicates a restriction site from the terminus of Tn*501*, and R · and B · indicate sites introduced in the primers used for PCR.

The analysis above of *algT-lacZ* activity from plasmids was potentially compromised by multicopy number effects. In order to discount this possibility, and to determine whether translational initiation at *algT* was different in Alg^+ cells, we constructed a chromosomal *algT-lacZ* translational fusion. Plasmid pCM162 (Fig. 1) contained a fragment (490 bp) internal to *algT* in vector pMLB1034 which put *algT* in frame with the vector-encoded *lacZ* gene. The subsequent cloning of a mobilization (*mob*) site into this narrow-host-range plasmid permitted transfer to PAO1 by triparental mating with selection for its *bla* (carbenicillin resistance) marker. This resulted in homologous recombination with the chromosome, thus forming strain PDO120 (Fig. 1B), which had a single-copy *algT-lacZ* translational fusion (i.e., σ^{22}-LacZ) under the control of P*algT*. This strain no longer contained an intact *algT* because of the *algT*::pCM162 insertion. The plasmids described above (pJF20, pJF20.25, pJG20.33, and pLAFR3) were then mated into the *algT-lacZ* strain PDO120. In previous studies, transfer of pJF20 to an *algT*::Tn*501* polar mutant (FRD440) resulted in the Alg^+ phenotype because no *mucA* was expressed in the cell (22). However, transfer of pJF20 to the *algT*::pCM162 strain (PDO120) resulted in an Alg^- phenotype; this suggests that the downstream *mucA* was expressed, possibly from a promoter in the vector. The combination of promoter and translation initiation activities of *algT-lacZ* was assayed by measuring β-galactosidase activities. Alg^- PDO120(pJF20) still showed about 61% of the σ^{22}-LacZ fusion activity of Alg^+ PDO120(pJF20.25). These results were similar to those described above for *algT-lacZ* transcription from plasmids (Table 2). In addition, PDO120 strains containing pJG20.33 or pLAFR3 did not contain an intact *algT* gene, and so no σ^{22}-LacZ fusion activity was detectable (Table 2). This indicated that P*algT* was almost totally dependent on σ^{22} for its activity. Overall, this data suggests that *algT* expression in *P. aeruginosa* (to produce σ^{22}) undergoes only a relatively small increase when cells are Alg^+.

Transcriptional activation of P*algR-lacZ* with alginate overproduction. A hierarchy of regulators in which σ^{22} expressed from *algT* controls expression of other regulatory genes, such as *algR* (*algR1*), which are required for alginate production exists (73). The *algR* promoter (P*algR*) has high sequence homology to σ^{22}-dependent promoters (15, 56), and *algT* mutation blocks *algR* expression (73). For comparison to the P*algT* measurements above, we tested P*algR* activity in *P. aeruginosa* and the effect of the Alg^+ phenotype obtained by expression of *algT* in *trans*. A P*algR-lacZ* transcriptional fusion probe, pKMG197 (Fig. 1C), was constructed by cloning a 2.3-kb *Eco*RI fragment containing P*algR* into the *lacZ* reporter plasmid, pQF50. In Alg^- PAO1(pJF20, pKMG197), P*algR-lacZ* activity was approximately 36% of that seen in Alg^+ PAO1 (pJF20.25, pKMG197) (Table 2). When the plasmid-encoded *algT* was insertionally inactivated in Alg^- PAO1(pJF20.33, pKMG197), there was only a single chromosomal copy of *algT* and P*algR-lacZ* activity fell to 27% of that seen in the Alg^+ strain (Table 2). Thus, the transcriptional activation of P*algR* was approximately twofold that seen with P*algT* (2.8-fold versus 1.2-fold, respectively) upon conversion to Alg^+.

High levels of σ^{22} protein are observed in Alg^+ strains. We examined the relative levels of σ^{22} protein in total cell extracts of nonmucoid and mucoid cells by Western blot analysis with a σ^{22} peptide antibody (Fig. 2). The Alg^+ CF isolate FRD1 displayed readily detectable levels of σ^{22} protein (lane 2). The migration of σ^{22} by polyacrylamide gel electrophoresis (PAGE) showed an apparent molecular mass of about 26 to 27 kDa, which was somewhat higher than its actual molecular mass of 22 kDa, but not an unusual property for sigma factors. The σ^{22} protein band was not seen in extracts of its *algT*::Tn*501-33* derivative, FRD440 (lane 1). To further confirm that this differentially recognized band represented σ^{22}, a Western blot analysis was performed with this antibody preparation after it was preabsorbed with the σ^{22} synthetic peptide originally used to elicit the antibody, which resulted in the loss of this band (data not shown). In wild-type nonmucoid strain PAO1 carrying vector pLAFR3, σ^{22} was barely detectable by Western blot analysis (Fig. 2, lane 6). The presence of σ^{22} was also barely detectable in Alg^- PAO1(pJF20), even though this strain expressed multiple copies of *algT* in *trans* (lane 5). In

TABLE 2. Effect on the transcriptional and translational activity of *algT* (*algU*) and transcriptional activity of *algR* (*algR1*) in *P. aeruginosa* PAO1 following conversion to mucoid by providing *algT* in *trans*[a]

algT$^+$ plasmid	Plasmid genotype	Alginate phenotype	β-Galactosidase activity (% relative to mucoid)		
			P*algT-lacZ*	σ^{22}-LacZ	P*algR-lacZ*
pJF20.25	*algT*$^+$ *mucA22 mucB*::Tn*501*	Alg$^+$	1,534 ± 177 (100)	190 ± 52 (100)	1,222 ± 199 (100)
pJF20	*algT*$^+$ *mucA22 mucB*$^+$	Alg$^-$	1,292 ± 229 (84)	115 ± 46 (61)	443 ± 101 (36)
pJF20.33	*algT*::Tn*501*	Alg$^-$	414 ± 51 (27)	<10 (<5)	333 ± 83 (27)
pLAFR3	Vector control	Alg$^-$	492 ± 81 (32)	<10 (<5)	395 ± 48 (32)

[a] The copy number of σ^{22} was increased in *P. aeruginosa* PAO1 by pJF20 and pJF20.25 (Fig. 1), which provide excess σ^{22}, but only the latter was converted to Alg$^+$ due to loss of the plasmid-borne *mucB* (*algN*). The *mucA22* allele expresses a defective MucA product. Compatible P*algT-lacZ* (pKMG37 [Fig. 1B]) and P*algR-lacZ* (pKMG197 [Fig. 1C]) transcriptional fusion reporter plasmids were used to probe promoter activity via β-galactosidase expression. Abbreviations: Alg$^+$, alginate overproduction; Alg$^-$, nonmucoid. P*algT-lacZ* and P*algR-lacZ* represent transcriptional fusions, whereas σ^{22}-LacZ represents a translational fusion. Values shown represent the averages of three experiments (± standard deviations) producing comparable results. Percentages are relative to the respective gene fusion activity in the Alg$^+$ strain.

contrast, extracts of Alg$^+$ PAO1(pJF20.25) (lane 4), which contained the same clone but with *mucB* insertionally inactivated, showed high levels of σ^{22} protein in the cell. The material in lane 4 from Alg$^+$ PAO1(pJF20.25) required a 10-fold dilution to produce a band of approximately the same intensity as that in lane 5 from Alg$^-$ PAO1(pJF20) (data not shown). Thus, σ^{22} protein levels are approximately 10-fold higher in Alg$^+$ than in nonmucoid strains even though *algT* transcription and translation are only 1.7-fold higher in the former. This suggested that σ^{22} protein may be regulated by a posttranscriptional process that controls σ^{22} stability. The only genetic difference between Alg$^+$ PAO1(pJF20.25), which accumulated σ^{22} protein, and Alg$^-$ PAO1(pJF20), which did not accumulate σ^{22} protein, was the presence of *mucB* in *trans* in the latter. This suggested that MucB (AlgN), either directly or indirectly, played a role in destabilizing σ^{22}.

MucB (AlgN) is localized to the periplasm. To better understand how MucB controls intracellular accumulation of σ^{22}, studies were performed to localize this regulatory protein in *P. aeruginosa*. A hydrophilicity plot of the amino acid sequence predicted for MucB (34.5 kDa, 316 residues) showed a generally hydrophilic protein except for a strikingly hydrophobic amino terminus (Fig. 3A). Examination of the amino-terminal sequence (MRTTSLLLLLGSLMAVPATQAADA. . .) indicated that it resembled a signal sequence (37) of 21 amino acids with a charged amino terminus and hydrophobic core that was followed by a potential signal peptidase cleavage site (Ala-X-Ala ↓). This suggested that MucB (AlgN) may be localized to the periplasm.

To directly examine the presence of MucB in the periplasm, antibodies to a peptide of its sequence were elicited for use as a probe in immunoblot analyses of *P. aeruginosa* cell fractions (Fig. 3C). With strain PAO1, no band of the expected 32.5-kDa molecular mass (i.e., MucB with signal peptide removed) was

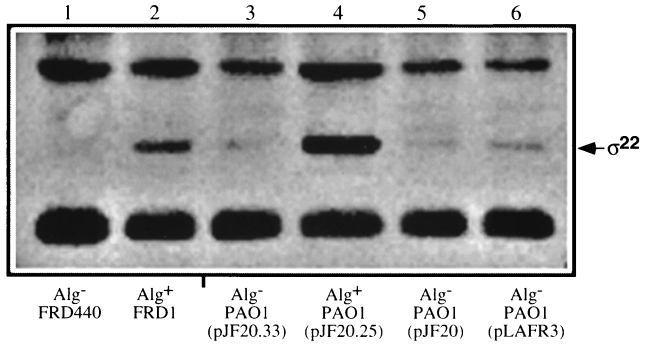

FIG. 2. Immunoblot analysis of σ^{22} (AlgT or AlgU)-related proteins in nonmucoid and mucoid *P. aeruginosa* strains. All strains were grown in L broth to an OD_{600} of 1.0, and washed cells were treated with 15% TCA to precipitate proteins which were subjected to SDS-PAGE followed by Western blotting with rabbit antipeptide represented in AlgT (AlgU) sequences to detect σ^{22} proteins. The position of σ^{22} is indicated.

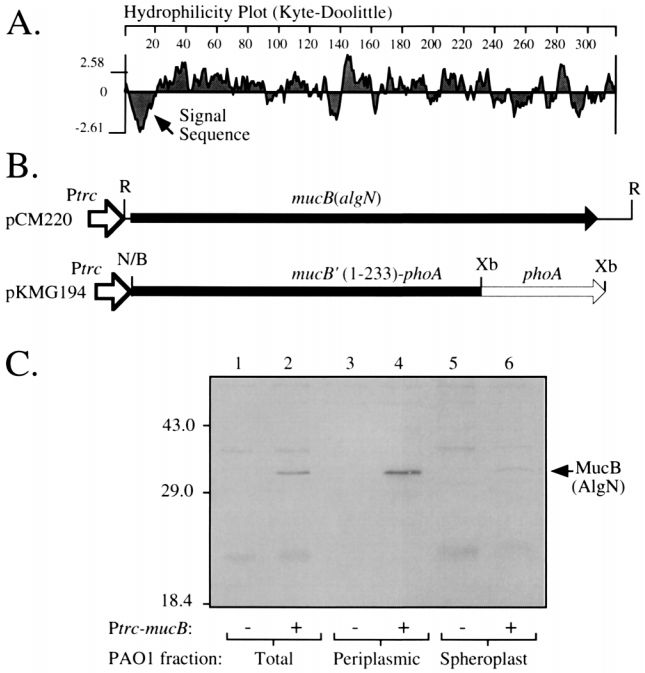

FIG. 3. Localization analysis of MucB (AlgN) in the periplasmic fraction of *P. aeruginosa*. (A) Hydrophilicity plot (Kyte-Doolittle) of the MucB (AlgN) amino acid sequence reveals a potential signal sequence. Positions of the residues are shown in the scale over the graph. (B) *P. aeruginosa* DNA in pCM220 which was constructed for the overexpression of *mucB* (*algN*) under the *trc* promoter. Plasmid pKMG194 was constructed to express a *mucB* (*algN*)-*phoA* translational fusion. Both plasmids were constructed from the broad-host-range P*trc* vectors. (C) Immunoblot analysis of MucB (AlgN)-related proteins in *P. aeruginosa* PAO1 or PAO1(pCM220) cell fractions. Strains were grown in L broth to an OD_{600} of 1.0, and washed cells were fractionated into the periplasmic and spheroplast (cytoplasmic) compartments. Proteins were precipitated with 15% TCA and subjected to SDS-PAGE followed by Western blotting with rabbit antipeptide represented in MucB (AlgN) sequences. Lanes: 1, total cells; 2, total cells overexpressing *mucB* (*algN*); 3, periplasm; 4, periplasm of cells overexpressing *mucB* (*algN*); 5, spheroplasts; 6, spheroplasts of cells overexpressing *mucB* (*algN*). The position of MucB (AlgN) is indicated. Numbers at left are molecular masses in kilodaltons.

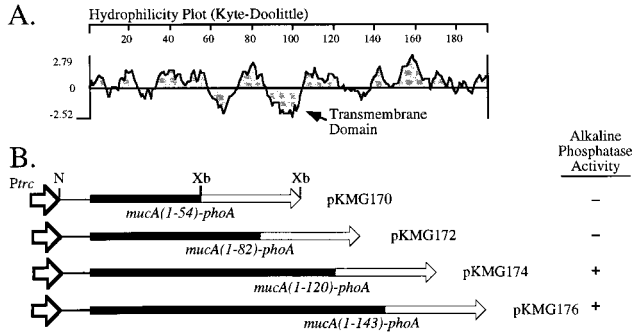

FIG. 4. Localization analysis of MucA in the inner membrane fraction of *P. aeruginosa*. (A) Hydrophilicity plot (Kyte-Doolittle) of the MucA amino acid sequence reveals a potential transmembrane domain. Positions of the residues are shown in the scale over the graph. (B) *P. aeruginosa* DNA in plasmids constructed to express *mucA-phoA* translational fusions and their expression of alkaline phosphatase activity. All plasmids were constructed from the broad-host-range P*trc* vector, pMF54, to permit expression in *P. aeruginosa*. Note that PhoA fusions on the C-terminal side of the transmembrane were positive (periplasmic localized) whereas fusions on the N-terminal side are negative (cytoplasmic membrane localized).

detected in total cell extracts (lane 1), in the periplasmic-enriched fractions (lane 3), or in spheroplasts (lane 5). Since the amount of MucB in the cell may be too low to detect by this method, we constructed pCM220 to overexpress *mucB* in *P. aeruginosa* (Fig. 3B). PAO1(pCM220) total cell extracts (lane 2) and periplasmic extracts (lane 4) both showed the presence of the MucB protein with the MucB antibodies, but the spheroplast-enriched fraction (lane 6) contained little detectable MucB. This comparative analysis suggested that MucB was predominantly in the periplasmic fraction.

To further test the hypothesis that MucB is exported from the cell, a translational fusion was constructed to express a MucB-PhoA hybrid protein. Such a hybrid should show alkaline phosphatase activity only if the fusion joint is exported through the inner membrane to the periplasm (43). To construct a translational *mucB-phoA* fusion, a DNA fragment containing the 5′ end of *mucB* (up to codon 233) was ligated in frame to a promoterless *phoA* to form pKMG194 (Fig. 3B). PAO1(pKMG194) was tested for alkaline phosphatase on L agar containing XP, and positive blue colonies were observed. This confirmed that MucB was exported to the periplasm. However, this raised the question as to how expression of the periplasmic protein MucB could affect the stability of the cytoplasmic sigma factor, σ^{22}.

MucA localization to the inner membrane. A possible mechanism by which MucB in the periplasm could affect σ^{22} in the cytoplasmic compartment was via their mutual interaction with an inner membrane protein. We hypothesized that this could be encoded by *mucA*. Mucoid isolates of *P. aeruginosa* from CF patients often have mutations in *mucA* (46), and the Alg^+ CF strain FRD1 used in this study has a *mucA22*-defective allele in which a deletion (ΔG after codon 143) results in the formation of a truncated MucA protein (15). In addition, the Alg^+ phenotype is unstable under laboratory conditions, and suppressor mutations of *mucA22* often occur in *algT* to restore the nonmucoid phenotype (15, 62). Such suppressor mutations which restore the wild-type Alg^- phenotype provide genetic evidence that σ^{22} and MucA interact directly.

The hydrophilicity plot of the predicted amino acid sequence for MucA (21 kDa, 195 residues) showed two hydrophobic regions between residues 61 to 71 and 84 to 103 that were potential transmembrane domains, although the latter showed greater hydrophobic character (Fig. 4A). To map the domains of MucA that may be exposed to the periplasm, four translational fusions to *phoA* were constructed. Fusions downstream of the second hydrophobic region at residues 143 (pKMG176) and 120 (pKMG174) gave rise to positive blue colonies in *E. coli* and in *P. aeruginosa* PAO1 when grown on agar medium containing XP, thus indicating exposure to the periplasmic compartment. However, the *mucA-phoA* fusion just downstream of the first hydrophobic region at 82 (pKMG172) was negative on XP (Fig. 4B), suggesting that this is not a transmembrane domain. The *mucA-phoA* fusion upstream of both hydrophobic regions at residue 54 (pKMG170) was also negative (Fig. 4B). An immunoblot analysis using antibody to alkaline phosphatase showed that all four *mucA-phoA* fusions were producing hybrid proteins. Further immunoblot analyses were performed on fractionated cells of *E. coli* CC118(pKMG176) and CC118(pKMG172), and these showed that MucA-PhoA in the former was predominantly in the membrane fraction, whereas MucA-PhoA in the latter was predominantly in the soluble cytoplasmic fraction (data not shown). Thus, these results suggest that MucA is an inner membrane protein, with a single transmembrane domain, which could interact with both cytoplasmic σ^{22} and periplasmic MucB (AlgN) via its amino- and carboxy-terminal domains, respectively.

DISCUSSION

The *algT* locus was originally identified in CF strain FRD1 at about 68 min on the *P. aeruginosa* chromosome and shown to be required for the Alg^+ phenotype (21, 22, 55). We now recognize that this alginate control region contains a cluster of at least five genes that may be cotranscribed: *algT* (*algU*)-*mucA-algB* (*algN*)-*mucC* (*algM*)-*mucD* (*algY*) (2, 15, 56). The first gene encodes an alternative sigma factor (σ^{22}) similar to σ^E in *E. coli*, and the other genes in the cluster encode proteins that may regulate expression or activity of this sigma factor. Production of wild-type σ^{22} is essential for normal transcription of *algR* (*algR1*), *algB*, and *algD*, as well as that of its own structural gene *algT* (15, 34, 47, 65, 73). These genes all have similar promoters that closely resemble those under σ^E control in *E. coli* (15, 34, 47, 56).

In this study, we began exploring the mechanism by which the accessory proteins expressed from genes downstream of *algT* control the cascade of σ^{22}-mediated gene expression that leads to alginate production. A clue to this was found in a previous study from this laboratory which examined the phenotypes produced by *algT* clones from the genome of mucoid CF strain FRD1 (29). The typical nonmucoid strain PAO1 demonstrates the Alg^+ phenotype when it expresses *algT* from a multicopy plasmid in *trans* without the downstream negative regulators (e.g., pJF20.25). In that clones from FRD1 are *mucA* defective (i.e., have the *mucA22* allele), the multicopy production of σ^{22} in the cell apparently overrides the single-copy production of MucA and MucB from the chromosome. However, PAO1 remains nonmucoid if the clone still carries the downstream negative regulator *mucB* (e.g., pJF20) (29). Thus, the multiple copies of *algT* in the cell could be controlled by multiple copies of *mucB*, and this prevented conversion to Alg^+, despite only a single copy of wild-type *mucA* in the cell.

Mucoid *P. aeruginosa* shows transcriptional activation of *algR*, *algB*, and especially *algD*, all of which are under *algT* (i.e., σ^{22}) control (73). To better understand the interplay among σ^{22}, MucA, and MucB in the control of σ^{22}-dependent promoters, we first examined the expression of P*algT* (an autoregulated σ^{22} or AlgT promoter) in PAO1 strains that were

Alg^+ or Alg^- due to pJF20.25 or pJF20, respectively. Using a transcriptional P*algT-lacZ* fusion probe on a second plasmid, we found that P*algT* transcription in Alg^+ PAO1(pJF20.25) was only about 16% (1.2-fold) higher than that in Alg^- PAO1 (pJF20). Thus, we questioned whether this small increase in *algT* transcription could account for activation of high P*algD*, which results in Alg^+. Since transcription of *algT* was not markedly different in Alg^+ and Alg^- cells, we examined whether translational initiation could be affected. However, an *algT-lacZ* protein fusion (i.e., σ^{22}-LacZ) was only about 39% (1.7-fold) higher in the mucoid strain. Thus, it appeared that the mechanism which controlled other σ^{22}-dependent promoters under these conditions was not at the level of *algT* transcription or translational initiation. However, this analysis of an *algT-lacZ* protein fusion does not negate the possibility of some other form of translational regulation.

It was also interesting to compare strains of PAO1 that carried *algT* in *trans* to those carrying just the vector. P*algT-lacZ* activity was about 2.6-fold higher in PAO1(pJF20) than in PAO1(pLAFR3), apparently because σ^{22} levels increased in the cell due to the higher copy number of *algT*. However, this increase in *algT* transcription did not lead to Alg^+ unless *mucB* on the plasmid was also inactivated (e.g., by Tn*501* mutation). We recently showed evidence that the genes in the cluster *algT mucA mucB* (*algN*) are cotranscribed (15). The *mucC* and *mucD* genes downstream may also be part of the same transcript. Thus, just increasing the rate of transcription of *algT* does not confer Alg^+ if *mucB* is coexpressed from the same transcript. This suggests that the σ^{22} control mechanism may involve a stoichiometric relationship between the levels of σ^{22} and MucB proteins. It is also noteworthy that the σ^{22}-LacZ fusion was silent when no active *algT* gene was present in the cell. This was consistent with our earlier observation in which a P*algT-cat* transcriptional fusion is nearly silent in an *algT*:: Tn*501* mutant (15). This requirement of σ^{22} for *algT* (*algU*) expression suggests that the three non-σ^{22} promoters recently mapped by RNase protection (63) played no role under the conditions used in this study.

When another σ^{22}-promoter fusion, P*algR-lacZ*, was examined under the same conditions to induce the mucoid phenotype, it showed about a 2.8-fold increase in activity in Alg^+ PAO1(pJF20.25) over Alg^- PAO1(pJF20). This activation was about twofold higher than that seen with the comparable P*algT-lacZ* fusion. In addition, there was no apparent increase in P*algR-lacZ* activity when PAO1 carried pLAFR1 (vector) or pJF20 (encoding *algT mucA22 mucB*), which is in contrast to P*algT-lacZ*, which showed approximately a threefold increase in activity due to the carriage of pJF20. That is, P*algR-lacZ* was activated only when cells were Alg^+. The explanation for these differences in the expression of two σ^{22}-dependent promoters is currently unknown. Perhaps the *algT* and *algR* promoters have different affinities for σ^{22} depending upon the conditions modulated by MucA and MucB.

We next looked for evidence that σ^{22} was under posttranscriptional control. Using a Western blot analysis, we found that σ^{22} could be readily detected in Alg^+ but not in Alg^- strains. When the Alg^+ CF strain FRD1 was tested, σ^{22} expressed from the chromosomal gene was readily detected, and it was absent as expected in an *algT*::Tn*501* mutant. In strain PAO1, σ^{22} was barely detected unless the Alg^+ phenotype was induced by providing only *algT* in *trans*. The amount of σ^{22} protein in Alg^+ PAO1(pJF20.25) was approximately 10-fold higher than that in nonmucoid PAO1(pJF20), even though the transcription and translational initiation at *algT* reporters increased by only 16 and 39%, respectively. These data suggest that σ^{22} could be regulated at the level of protein stability in some fashion by MucB. These results are reminiscent of studies of *E. coli* which showed that the control of the heat shock sigma factor σ^{32} also occurs via a posttranscriptional process. Although σ^{32} has a normal half-life of less than 1 min, increasing levels of this sigma factor are found in *E. coli* during stress (e.g., heat shock) due to both increased stabilization of the sigma factor and increased translation of mRNA (75). In *P. aeruginosa*, the apparent increase in σ^{22} levels could also be due to a combination of protein stability and mRNA translation efficiency, even though an *algT-lacZ* translational fusion showed only a low level of activation in mucoid cells. Experiments to measure the half-life of σ^{22}, in the presence and absence of MucAB, are in progress to verify the hypothesis that σ^{22} is subject to degradation by some mechanism. If correct, there may be specific environmental stress conditions that allow for the stabilization of σ^{22} to occur in *P. aeruginosa* and permit expression of the normally silent alginate genes.

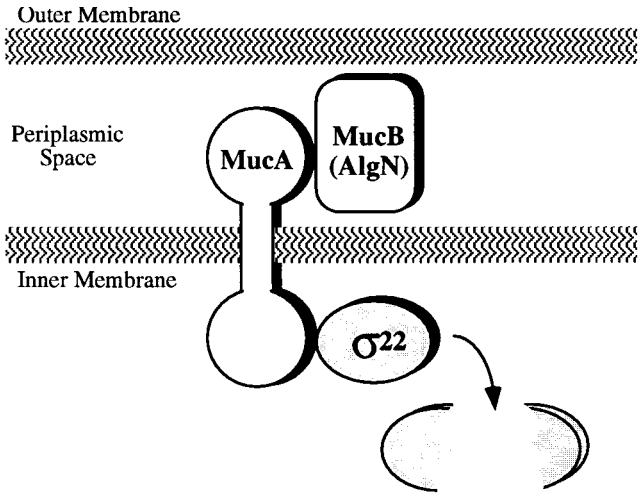

FIG. 5. Model for posttranslational control of σ^{22} in *P. aeruginosa*. MucA is a transmembrane protein that presumably interacts with MucB (AlgN) in the periplasmic space. This appears to transduce a signal to the cytoplasm that leads to rapid turnover of σ^{22}. Mucoid strains like FRD1 with *mucA* mutations are short circuited in the signal from MucB, and so σ^{22} is not destabilized. Increasing the copy number of σ^{22} in the cell (e.g., with pJF20.25) overrides the σ^{22} turnover process, and cells are mucoid. Increasing the copy number of σ^{22} in the cell with compensatory production of MucB (AlgN) (e.g., with pJF20) restores the σ^{22} destabilization process, and cells are nonmucoid.

The control of σ^{22} in *P. aeruginosa* by MucB (as described above) at first suggested an interaction between these proteins. However, we found several pieces of evidence to indicate that MucB is exported into the periplasm. The σ^{22} protein is presumably cytoplasmic, where it would be available to interact with RNA polymerase and subsequently with its specific promoters. Thus, it seems unlikely that MucB interacts directly with σ^{22} if they are in separate cellular compartments. However, a membrane protein could potentially transfer information from MucB to σ^{22}. Interestingly, further localization studies showed that MucA has a single transmembrane domain with significant portions of the amino and carboxy termini of the protein on each side of the inner membrane. Thus, MucA could provide the link between σ^{22} in the cytoplasm and MucB in the periplasm. Although still hypothetical, this arrangement suggests a signal transduction mechanism to control σ^{22} activity. Interaction between σ^{22} and MucA, an anti-sigma factor, could conceivably control σ^{22} destabilization depending upon interactions between MucA and MucB. A hypothetical model for this is shown in Fig. 5. In strains containing mutations in

mucA (such as in the mucoid CF strain FRD1), the signal would be short circuited and the destabilization of σ^{22} would be prevented.

A genetic argument may be made for a direct interaction between MucA and σ^{22} by the fact that adaptive mutations occur in *mucA* when *P. aeruginosa* colonizes the lungs of CF patients and becomes mucoid (46), and then secondary suppressor mutations often occur in *algT* (*algU*) to restore the nonmucoid phenotype (13, 15). Recent studies have directly addressed the role of MucA in the regulation of σ^{22} activity. Xie et al. (74) demonstrated that a protein-protein interaction takes place between σ^{22} and MucA and that this interaction prevents in vitro transcription as specified by σ^{22}. However, MucB protein did not show such a direct effect on σ^{22} activity. Similarly, Schurr et al. (64) showed that MucA acts as an anti-sigma factor by demonstrating that it binds σ^{22} (AlgU, σ^{E}) and inhibits σ^{22}-dependent transcription in vitro. Both of these studies required excess MucA (e.g., 10-fold on a molar basis) to demonstrate a high level of transcriptional inhibition, suggesting that other components in the reaction were missing or that MucA must be membrane bound to show activity. MucB was recently localized to the periplasm, and removal of its signal sequence abolished its ability to inhibit the Alg^{+} phenotype, suggesting that MucB may transiently associate with the inner membrane to gain access to the systems it affects in the cytoplasm (64). In our model proposed in Fig. 5, periplasmic MucB conveys its inhibitory activity through the transmembrane protein MucA; this may facilitate its interaction with cytoplasmic σ^{22}, leading to degradation of the sigma factor. This complements the above in vitro studies which showed a direct MucA-σ^{22} interaction.

A common regulatory mechanism in prokaryotes involves the use of alternative sigma factors to coordinately regulate their cognate promoters and regulons in response to a particular stimulus, and the σ^{22} control system of *P. aeruginosa* represents another example. Further studies are needed to characterize the half-life of σ^{22} and the specific roles of MucA and MucB in this process. The mechanism which controls σ^{22} at the level of its turnover is not yet known but could involve the action of the proteins encoded by *mucD* (*algY*) and *algW*, both of which bear resemblance to the HtrA (DegP) periplasmic protease in *E. coli* (2, 56). Perhaps these proteases cleave substrates that lead to σ^{22} destabilization. Members of the ECF subfamily of sigma factors, to which σ^{22} in *P. aeruginosa* belongs, have now been found in a wide array of bacteria (40). Both published and sequence database information suggest that the genes for ECF sigma factors are usually linked to genes encoding negative regulators, as is seen in *P. aeruginosa*. For example, in *Myxococcus xanthus*, it was recently shown that an ECF sigma factor (CarQ) involved in light-induced carotenogenesis is sequestered by an inner membrane protein (CarR) encoded by the adjacent gene in order to control the sigma factor's activity (31). Also, an ECF sigma factor (σ^{E}) in *Photobacterium* strain SS9 is involved in survival at cold temperatures and high pressures, and its gene is linked to three others that may control its activity (5). This suggests an evolutionarily conserved mechanism of controlling bacterial responses to environmental stimuli and the possibility of a common mechanism of interaction between the proteins.

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