Control of AlgU, a Member of the σ^E -Like Family of Stress Sigma Factors, by the Negative Regulators MucA and MucB and *Pseudomonas aeruginosa* Conversion to Mucoidy in Cystic Fibrosis

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The alternative sigma factor AlgU (*Pseudomonas aeruginosa* σ^E) is required for full resistance of *P. aeruginosa* to oxidative stress and extreme temperatures. AlgU also controls conversion of *P. aeruginosa* to the mucoid, alginate-overproducing phenotype associated with lethal infections in cystic fibrosis patients. Mutations that cause conversion to mucoidy in cystic fibrosis isolates occur frequently in *mucA*, the second gene within the *algU mucABCD* gene cluster. Here we analyze the biochemical basis of conversion to mucoidy. MucA was shown to act as an anti-sigma factor by binding to AlgU and inhibiting its activity. MucB, another negative regulator of AlgU, was localized in the periplasm. MucB exerts its function from this compartment, since deletion of the leader peptide and the cytoplasmic location of MucB abrogated its ability to inhibit mucoidy. These data support a model in which a multicomponent system, encompassing an anti- σ factor and elements in the periplasmic compartment, modulates activity of AlgU. Since factors controlling AlgU are conserved in other gram-negative bacteria, the processes controlling conversion to mucoidy in *P. aeruginosa* may be applicable to the regulation of AlgU (σ^E) equivalents in other organisms.

The *algU mucABCD* cluster (Fig. 1) controls conversion of *Pseudomonas aeruginosa* to the mucoid, alginate-overproducing phenotype (4, 9, 29–31) associated with the establishment of lethal respiratory infections in cystic fibrosis (CF) patients (17). The uncovering of the genetic elements controlling conversion to mucoidy in *P. aeruginosa* has also facilitated the recognition of a new class of bacterial alternative σ factors (9, 27) distantly related to the σ^{70} family (27). This new group of alternative σ factors, termed ECF (27) or the σ^E -like family (9, 10), includes members with an average identity of approximately 25%. Within this diverse superfamily, *P. aeruginosa* AlgU (also known as AlgT [11] or Pa σ^E [9]), the second or extreme heat shock sigma factor σ^E (RpoE) in *Escherichia coli* (13) and *Salmonella typhimurium* (32), and several more recently described homologs (Fig. 1) found in diverse gram-negative organisms make up a more homogeneous subset of highly conserved (62 to 93% identity with AlgU) and most likely functionally related alternative σ factors.

P. aeruginosa AlgU and *E. coli* σ^E are functionally interchangeable (49). Biochemical studies in *E. coli* have indicated that σ^E functions as an alternative sigma factor (13, 39, 40). Recent work has demonstrated that AlgU is an RNA polymerase subunit and that it directs transcription of cognate promoters in vitro (22, 43). Interestingly, the conservation of the primary structure and function of AlgU homologs extends beyond the genes encoding these σ factors. The known and putative equivalents of *algU* are followed by elements closely

resembling the *mucABCD* genes (Fig. 1). It has been proposed that these genes play a role in the regulation of cognate σ factors on the basis of the observation that spontaneous mutations in *mucA* (31) or experimental inactivation of *mucB* (30), also known as *algN* (18), causes up-regulation of AlgU-dependent promoters. In this work, using purified components (AlgU, MucA, and MucB), we demonstrate that MucA interacts directly with AlgU and inhibits its activity while MucB exerts its inhibitory effect remotely from the periplasm.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. PAO1 is the standard genetic strain of *P. aeruginosa*. CF1 and CF23 were mucoid isolates from cystic fibrosis patients (31). PAO6857 was constructed via gene replacements by using pDMB100 and previously described methods (30). *E. coli* was grown in Luria broth (LB) supplemented with chloramphenicol (30 μ g/ml), ampicillin (40 μ g/ml), or kanamycin (25 μ g/ml) when required. *P. aeruginosa* was grown on pseudomonas isolation agar supplemented when necessary with carbenicillin (300 μ g/ml).

Genetic methods and alginate assay. Plasmid transfer by triparental conjugation and scoring of suppression of the mucoid phenotype were carried out as previously described (29). Alginate production was assayed according to the method of Knutson and Jeanes as previously described (25).

Protein overproduction and purification. To overproduce the oligohistidine-MucA fusion construct (His₁₀-MucA), wild-type MucB, and MucB Δ LP (MucB from which the leader peptide [LP] has been deleted), the appropriately modified *mucA* and *mucB* genes were subcloned behind T7 expression signals. An *NdeI* site was generated at the 5' end of each gene by PCR with the following oligonucleotides (with *NdeI* sites underlined): 5'-GTATCCATATGAGTCGTGAAGCCC-3' (for His₁₀-MucA), 5'-CTGAGGAGACATATGCGCACCA-3' (for MucB⁺), and 5'-ACTCAGCATATGGACGCTTCCGACTGG-3' (for MucB Δ LP). The modified *mucA* was cloned as an *NdeI*-*BglII* fragment into pET-16b (Novagen), and the resulting plasmid was termed pET*mucA*. To generate *pmucB*^{wt} and *pmucB* Δ LP, *mucB*⁺ and *mucB* Δ LP were cloned as *NdeI*-*EcoRI* fragments into pT7-7, which placed the coding sequence behind T7 translational signals (ribosomal binding site and initiation codon), and modified genes were excised as *XbaI* fragments and inserted behind the *tac* promoter of pVDtac39. The identities of all constructs were verified by DNA sequence analysis.

To overproduce and purify MucA, bacterial lawns of *E. coli* K381 (pET*mucA*) were resuspended in Luria broth to an optical density at 600 nm of 0.5 and

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FIG. 1. Conservation of gene clusters encoding AlgU and σ^E (RpoE) homologs and their regulators. *P. aeruginosa* AlgU controls alginate production and resistance to reactive oxygen intermediates and extreme temperatures (4, 9, 49). *Photobacterium* sp. SS9 RpoE controls adaptation to high pressure and cold in this deep-sea bacterium (7). *E. coli* RpoE (σ^E) has been termed the extreme or second heat shock sigma factor and was initially identified as a minor RNA polymerase sigma factor (13, 39, 40). Equivalent genes are represented by arrows and boxes with identical patterns. Percent identities (predicted gene products) relative to *P. aeruginosa* AlgU, MucA, MucB, MucC, and MucD are given for genes within the clusters. There are three known types of arrangements. (i) *P. aeruginosa* (Pa) (4, 9) and *Azotobacter vinelandii* (Av) (33) have five-gene clusters. An additional homolog of *mucD*, the *algW* gene, has been identified elsewhere on the *P. aeruginosa* chromosome (4). (ii) *Photobacterium* sp. SS9 (Ph) (7) and *E. coli* (Ec) (49) (also GenBank entry D64044) have four-gene clusters; these clusters contain equivalents of *algU mucABC*. *E. coli* *htrA* (*degP*), a likely *mucD* equivalent, is elsewhere on the chromosome (26). Additional *htrA* homologs in *E. coli*, *hhoA* (*degQ*) and *hhoB* (*degS*), have been recently identified (2, 46). Only the *rpoE* gene encoding σ^E has been characterized (32) in *S. typhimurium* (St); the dashed line indicates that the sequences downstream of *rpoE* have not been examined. (iii) *H. influenzae* (Hi) has a three-gene cluster. The *rpoE mclA mucB* cluster in *H. influenzae* (GenBank entries U32745 and U32746) lacks the fourth and the fifth genes; however, this organism has two unlinked *mucC* homologs (HI0589 and HI0850) of unknown function and a *mucD* homolog (HI0945, *htrH*) elsewhere on the chromosome (GenBank entries U32740, U32766, and U32775). In *P. aeruginosa*, *mucA*, *mucB*, *mucD*, and *algW* play a negative regulatory role in AlgU expression. Mutations in *mucA* are responsible for mucoid colony morphology in a significant number of CF isolates (31); experimental inactivation of *mucB* (30) or *mucD* (4) can also cause conversion to mucoidy, while inactivation of *algW* lowers the threshold for induction of alginate production by reactive oxygen intermediates (4).

expression was induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) and a shift in temperature from 30 to 42°C. After incubation for 45 min at 42°C and then 3 h at 37°C, the cells were harvested and lysed in a French press. The crude lysate was applied to a 4-ml nickel-nitrilotriacetic acid (Ni-NTA)-agarose column in the presence of 8 M urea and 40 mM imidazole. The bound His₁₀-MucA was eluted with 80 to 125 mM imidazole in 0.1 M NaH₂PO₄-10 mM Tris-HCl, pH 8.0. To characterize the purified protein, the band corresponding to His₁₀-MucA was transferred to an Immobilon-P membrane (Millipore), and after digestion with cyanogen bromide, the amino acid sequence of the cleavage products was determined by Edman degradation. The sequence obtained matched the predicted residues following the five methionines at different posi-

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Genotype and relevant properties ^a	Reference or source
<i>P. aeruginosa</i>		
PAO1	Alg ^{-wt}	B. Holloway
PAO578(I)	Alg ⁺ , <i>mucA22</i>	15, 41
PAO671	Alg ⁺ , PAO381 <i>mucB::Tc^r</i>	30
PAO6857	Alg ⁺ , PAO1 <i>mucB::Tc^r</i>	This work
CF1	Alg ⁺ , <i>mucA</i> mutant (Δ A-371)	31
CF23	Alg ⁺ , <i>mucA</i> mutant (Δ G-440)	31
<i>E. coli</i>		
BL21(DE3)	<i>lacUV5</i> promoter-controlled T7 RNA polymerase	45
Plasmids		
pET-16b	Expression vector	Novagen
pVDTac39	IncQ/P4 <i>mob⁺ tac lacI^q</i>	8
pT7-5	ColE1 Ap ^r ϕ 10	S. Tabor
pT7-7	ColE1 Ap ^r ϕ 10	S. Tabor
pT7-33	pT7-5 <i>mucA⁺</i>	29
pT7-39	pT7-5 <i>algU⁺ mucA⁺</i>	29
pETU1610	pET-16b <i>algU⁺</i> (His ₁₀ -AlgU)	43
pET <i>mucA</i>	pET-16b <i>mucA⁺</i> (His ₁₀ -MucA)	This work
<i>pmucB⁺</i>	pVDTac39 <i>tac-mucB⁺</i>	This work
<i>pmucBΔLP</i>	pVDTac39 <i>tac-mucBΔLP</i>	This work

^a Alg^{-wt}, wild-type nonmucoid phenotype; Alg⁺, mucoid phenotype.

tions in wild-type MucA: M-1, SREALQE; M-14, DNEADE; M-50, HREPTL; M-85, VGRLAV; and M-115, AQQGT. An additional Met residue (M-134) was followed by the sequence LAXYXEEQ (with the X indicating that no one amino acid could be unambiguously identified at that position), which is compatible with the predicted MucA primary structure at that location but indicative of the presence of a Met residue at position 135 instead of the Val predicted to be at that position from the *mucA* DNA sequence data.

To overproduce and purify MucB, PAO1 (*pmucB^{wt}*) was grown to an optical density at 600 nm of 0.6 and expression was induced with 1 mM IPTG for 4 h. Cells were French pressed in a solution containing 20 mM Tris-HCl, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5% glycerol, and 1 μ g of pancreatic DNase I per ml and centrifuged at 38,720 $\times$ g for 20 min. After two consecutive protein precipitations with ammonium sulfate (50 and 65% saturation), the protein pellet (50 to 65% ammonium sulfate fraction) was resuspended in and dialyzed against a solution containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM PMSF; the proteins were then chromatographed on a MonoQ HR 5/5 ion-exchange column by using a fast protein liquid chromatography (FPLC) system (Pharmacia) with a linear gradient of 0.1 to 1 M NaCl in 20 mM Tris-HCl (pH 8.0)-1 mM PMSF. Fractions containing MucB were pooled and loaded on a 6-ml heparin-Sepharose CL-6B bed equilibrated with 20 mM Tris (pH 7.5)-1 mM PMSF, and MucB was eluted with a linear 0.1 to 1 M NaCl gradient. Fractions containing MucB were pooled and subjected to gel filtration on a Superose 12 column in 20 mM Tris (pH 8.0)-0.1 M NaCl-1 mM PMSF.

Monoclonal antibodies against AlgU and MucB and Western blotting (immunoblotting) analysis. Monoclonal antibodies against His₁₀-AlgU and MucB were generated by immunizing BALB/c mice with purified proteins and generating hybridomas as previously described (37). Hybridomas producing AlgU- or MucB-reactive antibodies were subcloned by the method of limiting cell dilution and cryopreserved. Western blotting was performed with 6C6 (AlgU-specific) or 2A7 (MucB-specific) monoclonal antibodies as described previously (43).

Chemical cross-linking reactions. Chemical cross-linking was carried out according to the method of Duncan and Losick (12). Five-milliliter cultures of *E. coli* K381 carrying pT7-39 (*algU⁺ mucA⁺*) or pT7-33 (*mucA⁺*) (29) were radio-labeled with 55 μ Ci of [³⁵S]Met and [³⁵S]Cys (1,175 Ci/mmol; Express³⁵S protein labeling mix, Du Pont) as described previously (29, 49). Cells were resuspended in 500 μ l of lysis buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 100 mM NaCl, 0.1 mM DTT, 0.5% Triton X-100) and lysed by bead beating for 2 min in a MiniBeadbeater-8 (Biospec Products). Cell extract (15 μ g of total protein in 20 μ l) was incubated for 30 min at 30°C with 1-mg/ml disuccinimidyl suberate (DSS; Pierce) in a final reaction volume of 50 μ l which contained 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 10% glycerol, 0.5 mg of bovine serum albumin (BSA) per ml, 150 mM NaCl, and 1 mM DTT. Reactions were terminated by adding 20 mM lysine and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dye. The samples were then boiled for 3 min, and proteins were separated by SDS-PAGE and transferred to an Immobilon-P membrane for autoradiography and Western blotting.

AlgU transcriptional runoff assay. The in vitro runoff assay using AlgU with

the P_1 promoter of *algU* has been previously described (43). His₁₀-AlgU was renatured, in the presence or absence of His₁₀-MucA, by dilution (1:10) into renaturation buffer (total volume, 30 μ l) consisting of 100 mM Tris-HCl (pH 7.5), 33% glycerol, 10 mM KCl, 10 mM MgCl₂, 0.3 mM ATP, 0.33 mg of GroEL per ml, and 0.2 mg of GroES per ml (Epicentre Technologies) and incubation for 20 min at 25°C. His₁₀-MucA was renatured separately by dialysis against renaturation buffer without GroEL and GroES. A standard σ factor-core RNA polymerase preincubation reaction mixture (6 μ l) consisted of 3 μ l of core RNA polymerase (0.6 pmol; Epicentre Technologies) freshly diluted with 3 μ l on renaturation buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM β -mercaptoethanol, 1 mM EDTA, 0.4 mg of BSA per ml, 0.1% Triton X-100, and 0.72 pmol of His₁₀-AlgU (with 1.4 to 14 pmol of MucA when indicated). After 10 min on ice, DNA template (0.1 pmol) was added to the RNA polymerase-sigma factor mixture, and the resultant mixture was incubated in transcription buffer (40 mM Tris-HCl [pH 7.5], 50 mM KCl, 5 mM MgCl₂, 1 mM DTT) for 10 min at 37°C. Transcription was carried out in a final volume of 20 μ l and was initiated by addition of 200 μ M (each) ATP, CTP, and GTP and 20 μ M [α -³²P]UTP (8 Ci/mmol) to the DNA template-core RNA polymerase-sigma factor mix; samples were incubated for 15 min at 37°C. The reaction was stopped, and products were separated on a sequencing gel. The *algU* P_1 154-bp DNA template was generated as previously described (43).

Subcellular fractionation. Preparation of periplasmic extracts (6), the alkaline phosphatase assay (6), conversion to spheroplasts (24), separation of membranes into inner membrane, outer membrane, and mixed membrane fractions by isopycnic sucrose density centrifugation (21), and characterization and designation of membrane fractions (21) were carried out by established methods with minor modifications. The periplasmic fraction was obtained by extraction with 0.2 M MgCl₂ as previously described (6). Bacterial pellets from 100 ml of culture were resuspended in 1 ml of extraction buffer (0.2 M MgCl₂, 10 mM Tris-HCl [pH 8.4]) and incubated for 20 min at 4°C. For spheroplast conversion, 0.5 mg of lysozyme was added per ml of extraction buffer. After centrifugation, the 0.2 M MgCl₂ extract was considered to be the periplasmic fraction; for spheroplasts, the cell pellet was resuspended in 10 mM MgCl₂–10 mM Tris-HCl (pH 8.4) and incubated for 20 min on ice, and the resulting spheroplasts were pelleted and lysed by sonication for analysis.

RESULTS

Complementation of *mucA* and *mucB* mutations. Inactivation of *mucA*, *mucB*, or *mucD* on the chromosome of *P. aeruginosa* causes transcriptional activation of *algD*, a key alginate biosynthetic gene dependent on AlgU, resulting in conversion of *P. aeruginosa* to mucoidy (4, 30, 31). Before performing biochemical analyses, we wanted to rule out the possibility that *mucA* or *mucB* mutations cause conversion to mucoidy because of their polar effects on the downstream genes. In order to test this possibility, we investigated whether *mucA* and *mucB* mutations can be complemented in *trans* with the respective genes. When plasmid-borne *mucA*⁺ was introduced into a laboratory mucoid strain, PAO578(I), carrying a frameshift mutation in *mucA* (4, 15, 41) and its transcription was induced with IPTG, a 28-fold reduction in alginate production and nonmucoid colony morphology resulted (Table 2). In order to test whether *mucA* mutations can be complemented in mucoid CF isolates with characterized *mucA* mutations, two strains, CF1 and CF23 (31), were selected. In each case investigated, the strain lost its strong mucoid character and alginate production was reduced to the amounts usually seen in nonmucoid cells, although a low level of residual alginate production was still seen in strain CF23. These experiments support the notion that *mucA* mutations cause conversion to mucoidy primarily because of the absence of MucA. In a similar type of experiment using two *mucB* mutant strains, PAO671 and PAO6857, plasmid-borne *mucB*⁺ was able to suppress mucoidy and alginate production (Table 3). These findings are consistent with the interpretation that the effects on the expression of the alginate system caused by inactivation of *mucA* or *mucB* are primarily due to the absence of the corresponding gene products.

Association of MucA and AlgU. To investigate the mechanisms underlying the negative effect of MucA on AlgU (30, 41), the possibility that MucA binds directly to AlgU was tested. The *algU* and *mucA* genes were coexpressed in *E. coli*

TABLE 2. Complementation of *mucA* mutations in *P. aeruginosa*

Strain ^a	<i>mucA</i> mutation ^b (allele)	Plasmid ^c	IPTG (1 mM)	Alginate produced ^d	Phenotype ^e
PAO578(I)	Δ G-440 (<i>mucA22</i>)	None	–	85.5 $\pm$ 3.1	M
		<i>ptac-mucA</i> ⁺	+	8.1 $\pm$ 0.6	M
CF1	Δ A-371	<i>ptac-mucA</i> ⁺	+	3.0 $\pm$ 0.4	NM
		None	–	22.5 $\pm$ 1.0	M
CF23	Δ G-440	<i>ptac-mucA</i> ⁺	–	12.2 $\pm$ 1.4	M
		<i>ptac-mucA</i> ⁺	+	2.0 $\pm$ 0.5	NM
		None	–	52.8 $\pm$ 4.4	M
		<i>ptac-mucA</i> ⁺	+	37.4 $\pm$ 1.9	M
		<i>ptac-mucA</i> ⁺	+	7.4 $\pm$ 0.3	M**

^a PAO578(I) is a laboratory mucoid strain carrying the *mucA22* mutation (31, 41). CF1 and CF23 are mucoid CF isolates with previously characterized *mucA* alleles.

^b The positions of the frameshift mutations in *mucA* are given relative to the beginning of the structural gene.

^c *ptac-mucA*⁺ is a broad-host-range plasmid with wild-type *mucA* gene cloned behind the *tac* promoter controlled by *lacI*^q (31).

^d Alginate produced was measured in terms of micrograms of uronic acid produced per milligram (wet weight) of cells $\pm$ standard error. *P* values (*t* test) for strains harboring *ptac-mucA*⁺ with and without IPTG were 2.08×10^{-3} , 2.08×10^{-3} , and 2.08×10^{-3} for PAO578(I), CF1, and CF23, respectively.

^e M, mucoid; NM, nonmucoid; M**, slightly mucoid after 48 h of growth.

(Fig. 2A, lane 1) by using the plasmid pT7-39, which encodes both *algU* and *mucA* cloned behind a T7 promoter. The extracts exclusively containing radiolabeled AlgU and MucA (expressed from the T7 promoter) were incubated with DSS, a homobifunctional amine-reactive cross-linking reagent. As shown in Fig. 2A, lane 3, a new radiolabeled species was observed upon incubation with DSS. The radioactive band was broad, typical of cross-linking reactions, and similar to the previously reported cross-linking products of another σ factor–anti- σ factor complex (1). The apparent molecular mass of the center of this radioactive band was 46 kDa, corresponding to the molecular mass expected for a 1:1 complex between AlgU (apparent molecular mass, 27 kDa [29]) and MucA (apparent molecular mass, 20 kDa [29]). In order to confirm that the 46-kDa cross-linked complex contained AlgU as one of its components, monoclonal antibodies were generated against purified His₁₀-AlgU (43). Western blot analysis of the cross-linking products with coexpressed and radiolabeled AlgU and MucA indicated that the 46-kDa product was recognized by the monoclonal antibody 6C6, which is specific for AlgU (Fig. 2B). The center of the ³⁵S-labeled, cross-linked band coincided

TABLE 3. Abrogation of negative effects of MucB on alginate production by deletion of leader peptide

Plasmid ^a	Alginate production ^b (phenotype ^c) of strain:	
	PAO671 (PAO381 <i>mucB</i> ::Tc ^r)	PAO6857 (PAO1 <i>mucB</i> ::Tc ^r)
pVDtac39	6.6 $\pm$ 0.4 (M)	15.4 $\pm$ 2.9 (M)
<i>pmucB</i> ⁺	2.3 $\pm$ 0.1 (NM)	2.3 $\pm$ 0.3 (NM)
<i>pmucB</i> Δ LP	8.3 $\pm$ 0.6 (M)	10.4 $\pm$ 0.8 (M)

^a pVDtac39, broad-host-range vector; *pmucB*⁺, expression clone of wild-type *mucB* in pVDtac39; *pmucB* Δ LP, expression clone of a modified *mucB* gene lacking the region encoding the leader peptide of MucB (see Fig. 4B).

^b Plasmids were introduced into *P. aeruginosa* strains by triparental conjugation, and resulting exconjugants were tested for alginate production (in terms of micrograms of uronic acid produced per milligram (wet weight) of cells [$\pm$ standard error]) after growth for 48 h at 37°C as previously described (4, 31). *P* values (*t* test) for *pmucB*⁺ and *pmucB* Δ LP were 5.21×10^{-4} (PAO671) and 1.28×10^{-6} (PAO6857).

^c M, mucoid; NM, nonmucoid.

FIG. 2. Detection of MucA and AlgU complexes by cross-linking. (A) Autoradiogram of crude extracts containing [³⁵S]Met- and [³⁵S]Cys-labeled AlgU (filled triangles) and MucA (open triangles) separated by SDS-PAGE and blotted onto an Immobilon-P membrane. (B) Western blot analysis of the gel in panel A with anti-AlgU monoclonal antibody C6C. Stippled triangles, AlgU-MucA complex (U/A). Lanes: 1, *E. coli*(pT7-39) with *algU* and *mucA* in the direction of T7 promoter transcription; 2, same as lane 1, incubated in the cross-linking buffer without DSS; 3, same as lane 2 with the addition of the cross-linking agent DSS; 4, *E. coli*(pT7-33) with *mucA* in the direction of T7 promoter (*algU* was deleted; see panel D for overexposed DSS-treated sample). (C) Autoradiogram of crude extracts containing [³⁵S]Met- and [³⁵S]Cys-labeled AlgU (filled triangle). Lanes: 1, *E. coli*(pETU1610) (43) with radiolabeled His₁₀-AlgU, incubated in the cross-linking buffer without DSS; 2, same as lane 1, with the addition of the cross-linking agent DSS. (D) Autoradiogram of crude extracts containing [³⁵S]Met- and [³⁵S]Cys-labeled MucA (open triangle). Lanes: 1, *E. coli*(pT7-33) with radiolabeled MucA, incubated in the cross-linking buffer without DSS; 2, same as lane 1, with the addition of DSS.

perfectly with the center of the protein band detected by antibody 6C6. In additional control experiments, we investigated whether the 46-kDa complex could form in extracts with radiolabeled AlgU or MucA alone. We had difficulty obtaining a suitable construct expressing AlgU in the absence of MucA since plasmid constructs with *algU* alone were highly unstable, as noted previously (41). However, it was possible to express

FIG. 3. Inhibition of AlgU by MucA. (A) Lanes: 1 and 2, σ^{70} -dependent transcription from the λP_R promoter; 3 and 4, AlgU-dependent transcription from the P₁ promoter. Lanes 2 and 3 had equal amounts of MucA added to the reaction mixture (see Materials and Methods). Filled arrow, λP_R transcript; open arrow, *algU* P₁ transcript. (B) Lanes: 1, 2, and 3, *algU* P₁ transcripts produced in independent reactions with equal amounts of AlgU (7.2 pmol); 4, 5, and 6, same as lanes 1 to 3, respectively, with the addition of a 10:1 molar excess MucA (three independent reactions); 7, 8, and 9, same as lanes 1 to 3, respectively, with a 20:1 molar excess of MucA.

AlgU as a fusion protein (His₁₀-AlgU) even in the absence of MucA by using the construct pETU1610 (42) (Fig. 2C). When extracts containing radiolabeled His₁₀-AlgU were exposed to the cross-linking agent, a band corresponding to the 46-kDa complex was not detected (Fig. 2C). When extracts of cells harboring pT7-33 (a derivative of pT7-39, from which *algU* has been deleted but which produces MucA) was used in cross-linking experiments, the 46-kDa complex was not observed even on overexposed autoradiograms (Fig. 2D). This indicated that the formation of the 46-kDa complex depended on the presence of AlgU. Collectively, these observations are consistent with the interpretation that the 46-kDa material represents a specific 1:1 complex between AlgU and MucA.

Inhibition of AlgU-directed transcription by MucA. In order to investigate whether MucA can inhibit AlgU-directed transcription from a cognate promoter, we used the His₁₀-AlgU (43) and the AlgU-dependent promoter P₁ (32, 42) previously characterized in transcriptional runoff experiments (22, 43). In order to purify MucA, a fusion construct (His₁₀-MucA) of an N-terminal oligohistidine tag and MucA was generated and the fusion protein was overproduced. His₁₀-MucA was purified (see Materials and Methods) by metal chelate affinity chromatography on Ni-NTA resin and characterized by protein sequencing as described in Materials and Methods. When the purified MucA was added to the purified His₁₀-AlgU, it inhibited transcription from the AlgU-dependent promoter P₁ (Fig. 3A); MucA had no significant effect on σ^{70} -directed transcription from a σ^{70} promoter, λP_R (Fig. 3A). When purified His₁₀-AlgU was permitted to refold in the presence of increasing amounts of MucA (Fig. 3B), a concentration-dependent inhibition of AlgU-directed transcription from the P₁ promoter of *algU* was observed. Under conditions of molar MucA-to-AlgU ratios of 20:1, less than 10% of the incorporation into the P₁

FIG. 4. Purification and localization of MucB. (A) Overproduction and purification of MucB. Lanes: 1, cell extract from PAO1; 2, PAO1 harboring plasmid *pmucB*⁺, uninduced; 3, PAO1 harboring plasmid *pmucB*⁺, induced with 1 mM IPTG; 4, purified MucB. (B) The predicted amino acid sequence of the first 31 residues of MucB, including the leader sequence (before vertical arrow). MucB^{wt}, amino terminus (arrow) of purified wild-type MucB determined by protein sequencing (underlined residues); MucBΔLP, product of *mucB*ΔLP mutant, with a deletion removing the coding region for the leader peptide (the first 21 amino acids). Ala (A) at position 22 of MucBΔLP was changed to Met (boldfaced M). (C) Western blot analysis of *P. aeruginosa* subcellular fractions with anti-MucB monoclonal antibody 2A7. Lanes: 1, total extract from PAO671 (*mucB*::Tc^r) harboring *pmucB*⁺; 2, PAO671 (*pmucB*⁺) spheroplasts; 3, periplasmic fraction from PAO671 (*pmucB*⁺); 4, total extract from PAO671 harboring *pmucB*ΔLP, which encodes MucBΔLP, lacking the leader peptide; 5, PAO671 (*pmucB*ΔLP) spheroplasts; 6, periplasmic fraction from PAO671 (*pmucB*ΔLP). Protein amounts loaded represent equivalent portions of each subcellular fraction. Cross-reactive bands with *M_s* lower than that of MucB are MucB degradation products as determined by protein sequencing. Filled triangles, MucB; open triangle, MucBΔLP.

transcript remained compared with the samples with MucA. These experiments, together with the genetic and cross-linking evidence, suggest that MucA inhibits AlgU activity most likely by binding to this protein, a phenomenon referred to in other systems as anti- σ factor activity (12, 36).

MucB is in the periplasmic compartment. In order to initiate investigations of the nature of MucB-mediated inhibition of AlgU-dependent functions (30, 41), MucB was purified (Fig. 4A) in its native form using the wild-type gene (except for the modification of translational initiation signals; see Materials and Methods). MucB was overproduced in *P. aeruginosa* and purified (Fig. 4A, lane 4) by conventional protein purification techniques (see Materials and Methods). When the purified MucB was subjected to N-terminal sequence analysis, the primary structure (10 cycles) did not match the predicted N terminus of MucB based on its DNA sequence. However, it matched the 10 amino acids beginning at position 22 of MucB (Fig. 4B). The 21-amino-acid sequence preceding the mature MucB conformed with the rules for leader peptides (38), and global homology searches revealed strong similarities to several characterized leader peptides (data not shown).

The finding that MucB has a leader peptide and that this peptide is processed suggested the possibility that MucB may be located in the cell envelope. In order to further investigate subcellular localization of MucB, monoclonal antibodies against the purified protein were generated and *P. aeruginosa* cells (PAO671 [*mucB*::Tc^r] harboring plasmid-borne wild-type

mucB) were fractionated into periplasmic extracts, spheroplasts, and inner and outer membrane fractions. The majority of MucB was found in the periplasmic fraction (Fig. 4C) along with the alkaline phosphatase activity (used as a control; data not shown). Only traces of MucB were detectable in spheroplasts, as was the case with alkaline phosphatase. When *P. aeruginosa* membranes were separated into different fractions (21), a small proportion of MucB could be detected in samples corresponding to the inner membrane (data not shown). Only traces of MucB were seen in the other membrane fractions (previously designated as outer membrane 1 [OM1], OM2, and M [21]). When periplasmic proteins were extracted (6) prior to membrane preparation, no MucB could be detected in any of the membrane fractions, suggesting that the MucB detected in inner membranes was due to either contamination or weak interactions sensitive to osmotic shock.

Processing and compartmentalization of MucB are essential for its negative effects on AlgU activity. Since MucB affects AlgU (41), a σ factor which functions in the cytoplasm, it was of interest to test whether proper subcellular compartmentalization of MucB plays a role in its activity. We wanted to discern between the following two possibilities: (i) MucB affects AlgU prior to its processing and export into the periplasm, and (ii) MucB exerts its function from a periplasmic location. The *mucB* gene was mutated by removing the leader peptide (Fig. 4B). The deletion product, MucBΔLP, was expressed in *mucB*::Tc^r *P. aeruginosa* (harboring *pmucB*ΔLP) at

levels similar to those of the wild-type MucB (Fig. 4C). However, MucB Δ LP no longer partitioned in the periplasm and was found preferentially in spheroplasts (Fig. 4C), suggesting a cytoplasmic location. Moreover, mucB Δ LP could no longer complement the *mucB* mutation on the chromosome of *P. aeruginosa* and did not suppress mucoidy in *mucB::Tc^r* strains (Table 3). In further in vitro experiments, incubation of MucB with AlgU did not reveal detectable complexes between these two polypeptides when examined by cross-linking studies (data not shown). These results suggest that MucB affects AlgU activity from the periplasmic compartment.

DISCUSSION

In this work, the roles of two critical regulators of AlgU activity, MucA and MucB, have been investigated at the biochemical level. The focus of this study was on these two regulators with the following rationale: (i) mutations inactivating *mucA* are the cause of conversion to mucoidy of a significant portion of *P. aeruginosa* isolates from CF patients (4, 31), and (ii) the *mucA* and *mucB* homologs are present in all gene clusters encoding sigma factors from the AlgU-RpoE family and their putative regulators (Fig. 1), which is indicative of the possibility that *algU*, *mucA*, and *mucB* represent a core regulatory unit in these systems.

An anti- σ factor activity for MucA has been demonstrated by biochemical approaches and functional assays. MucA binds to AlgU and inhibits AlgU-dependent transcription in vitro. In recent years, control by anti-sigma factors has been described in several systems (1, 3, 12, 16, 23, 36), and on the basis of homologies between MucA and its counterparts in other systems, such relationships are likely to be germane to the bona fide AlgU-RpoE sigma factors (Fig. 1). It is less clear whether analogous interactions may be applicable to the entire superfamily of sigma factors referred to as ECF (27) or the σ^E -like family (9, 10), of which the AlgU-RpoE family (48) is only a subset. For example, not all genes encoding the sigma factors of the σ^E -like (ECF) family are followed by negative regulators. The *hrpL* gene from *Pseudomonas syringae* (47), the *rpoE* gene of *Mycobacterium leprae* (GenBank accession number U15180), and an additional AlgU homolog (gene designation HI1459; 46% overall similarity with AlgU) in *Haemophilus influenzae* (14) are not followed by the equivalents of *mucA* or *mucB*. Interestingly, genetic evidence has been presented suggesting that CarQ (34), proposed to belong to the ECF (σ^E -like) family, is followed by a negative regulator, *carR* (19), suggestive of anti-sigma activity. However, no significant sequence similarities have been detected between CarR on one side and MucA and its homologs on the other, and these two systems are most likely different.

It is necessary to discuss the reasons that a high concentration of MucA was needed to cause high-level inhibition of AlgU activity in our experiments. Since the specific activity of MucA in the preparations used is not known, it is not possible to decide whether the necessity of excess MucA in our in vitro analyses reflects low affinity of MucA for AlgU or is a simple consequence of low recovery of MucA activity. It is worth noting that, on the basis of physiological studies, inhibition of AlgU activity in wild-type nonmucoid cells is not absolute and that some AlgU-dependent promoters have appreciable activity even in *mucA*⁺ cells (32, 42). Furthermore, MucA probably does not work alone in vivo, and it has been well established that additional factors (e.g., MucB and MucD) are necessary for inhibition of AlgU in wild-type nonmucoid cells. It is also possible that appropriate modifications of components or co-factors may be needed, as has been noted in other systems, e.g.,

with SpoIIAB and σ^F , where, without the addition of ATP, a high concentration of one of the components was needed to generate detectable SpoIIAB- σ^F complexes (1). However, the simplest explanation for the large excess of MucA needed to inhibit AlgU activity in vitro is that the recovery of the in vitro-renatured MucA (after its elution in urea from the Ni-NTA column) may be very low. MucA renaturation may be less efficient than that of AlgU, which would be in keeping with the notion that sigma factors may have higher capacities for regaining activity upon renaturation than other proteins (20, 28). Moreover, the efficiency of and specific interactions occurring during AlgU and MucA synthesis in vivo may be different from those during in vitro renaturation. Additional analyses will be needed to determine physiological requirements for formation of AlgU-MucA complexes in vitro and in vivo as a step toward investigating factors that may modulate AlgU-MucA interactions.

The *algU mucABCD* cluster offers a paradigm for studying the regulation of the extreme stress σ factors from the AlgU-RpoE family (10, 48), whose members appear to be ubiquitous among gram-negative organisms (7, 9, 14, 32, 33, 39, 40). While it is now known that the regulators of AlgU and its close homologs or equivalents exist in other organisms (7, 14, 27, 32, 33), the regulatory elements controlling activity of the sigma factors of this class were first uncovered in *P. aeruginosa*. The initial recognition of these factors came as an integral part of the description of the conversion of *P. aeruginosa* to mucoidy (4, 9, 10, 15, 18, 29–31). Figure 1 depicts the current summary of the known clusters encoding AlgU-RpoE sigma factors and their putative regulators. There are currently three known types of cluster organization: five-gene clusters in *P. aeruginosa* (4, 9) and *Azotobacter vinelandii* (33), four-gene clusters in *Photobacterium* sp. SS9 (7) and *E. coli* (49) (also GenBank entry D6404), and three-gene clusters in *H. influenzae* (14). Based on the fact that the smallest number of genes was observed in *H. influenzae* (*rpoE mclA mucB*), the minimum cluster encoding a bona fide AlgU-RpoE factor and its regulators appears to consist of only *algU*, *mucA*, and *mucB* equivalents. While homologs of *mucC* and *mucD* have been recognized elsewhere on the *H. influenzae* chromosome (14), it is not known whether they play a role in the control of σ^E in this organism. Interestingly, although a *mucD* equivalent is absent in the *E. coli* *rpoE mclA orf1 orf2* cluster, its close homolog, *htrA* (*degP*), has been described previously in *E. coli* by two independent groups (26, 44). However, it appears that mutations in *htrA* do not have a strong effect on σ^E activity in this organism (39). In addition to *mucABCD*, in which clustering of genes is indicative of their coordinated expression, other elements may affect the activities of AlgU and its homologs. For example, earlier studies (35) addressing the regulation of σ^E activity in enteric bacteria, which were performed before the homologs of MucA, MucB, and MucC had been described in *E. coli*, suggested that outer membrane proteins had a role in σ^E activity. Despite the fact that it is difficult to separate indirect effects from direct regulatory relationships in such studies, these reports are in keeping with the periplasmic location of MucB.

More work will be needed to define the activity of each of the regulators in the *algU mucABCD* cluster and uncover possible interactions between different elements. Preliminary investigations with several combinations of double mutants in the cluster suggest that the regulators of AlgU have additive effects in suppression of AlgU activity (5). While MucA appears to act as an anti- σ factor, MucB-dependent suppression of AlgU activity from a periplasmic location may require an intermediate factor. Alternatively, MucB may at least tran-

siently associate with the inner membrane, possibly gaining access to the systems that it may affect in the cytoplasm. MucC (4), all of whose homologs have two putative conserved transmembrane domains (5), could participate in these processes. However, the absence of *mucC* equivalents in some clusters (Fig. 1) suggests that *mucC* and its homologs may have an optional or independent modulatory function. Our genetic analyses (5) indicate that inactivation of *mucC* has no phenotypically appreciable effect on alginate production in a wild-type PAO1 background, thus ruling out the possibility that MucC mediates the effects of MucB on AlgU. However, under certain conditions, *mucC mucB* null double mutants show an enhanced mucoid phenotype compared with strains carrying a single *mucB::Tc^r* mutation, suggesting a role for MucC in the regulatory process (5).

The interaction of MucA and AlgU and the contribution of MucB from the periplasmic compartment, and possibly additional activities of other factors, may be of significance for (i) inhibition or sequestration of AlgU under normal growth conditions and (ii) recognition of stress signals and activation of the system under adverse environmental conditions. In *mucA* mutants (31), such as those selected in the lungs of CF patients, AlgU is most likely no longer complexed with MucA and is available to direct transcription of subordinate promoters, characterized by canonical σ^E (AlgU) -35 GAACCTT and -10 TCTgA sequences (13, 26), including those that are responsible for alginate production (9, 32, 42). In *P. aeruginosa*, this causes conversion to mucoidy (31). It is also likely that stress signals, e.g., temperature shifts and exogenously or endogenously generated reactive oxygen intermediates, affect this system and allow induction of AlgU activity, as has recently been described in *algU⁺ mucABCD⁺* cells (4, 41). These processes may be applicable to other members of this class of alternative σ factors (Fig. 1). How the stress signals are recognized and to what extent such processes are universal in different organisms are the subjects of current investigations.

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