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Membrane-to-cytosol redistribution of ECF sigma factor AlgU and conversion to mucoidy in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients

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Summary

The conversion to mucoid phenotype in *Pseudomonas aeruginosa* during chronic infections in cystic fibrosis (CF) is due to mutations in the *algU mucABCD* gene cluster. This cluster encodes an extreme stress response system conserved in Gram-negative bacteria. The system includes an ECF sigma factor, AlgU (σ^E), an inner membrane protein, MucA, which inhibits AlgU activity, and MucB, a periplasmic protein that negatively controls AlgU. In this work, we investigated whether and how these factor interact to transduce signals between different cellular compartments. The mutation *mucAΔG440*, which renders a large fraction of *P. aeruginosa* CF isolates mucoid, did not abrogate AlgU–MucA interactions, although it eliminated MucA–MucB interactions in the yeast two-hybrid system. The *mucAΔG440* truncation of the periplasmic C-terminal tail of MucA destabilized the molecule resulting in low or undetectable steady-state levels in *P. aeruginosa*. Somewhat reduced levels of MucA were also seen in cells with inactivated *mucB* or with the *mucACF53* allele carrying the missense P184S mutation, which mildly affected interactions with MucB. The events downstream from MucA destabilization were also investigated. AlgU was found to associate with inner membranes in *mucA*$^+$ cells. In mutants destabilizing MucA, a limited redistribution of AlgU from the membrane to the cytosol was observed. The redistribution was spontaneous in *mucAΔG440* cells, while in *mucB* and *mucACF53* mutants it required additional signals. Despite a large reduction in MucA levels in *mucAΔG440* cells, only a small fraction of AlgU was redistributed to the cytosol and a significant portion of this σ factor remained membrane bound and behaved as a peripheral inner membrane protein. The fraction of AlgU that depended on MucA for association with the membrane also brought RNA polymerase into this compartment. These results are consistent with a model in which MucB–MucA–AlgU–RNA polymerase interactions at the membrane allow transduction of potentially lethal stress signals with both rapid reaction times of the preassembled complexes and efficient resupply at the membrane from the prebound components.

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Introduction

The conversion to mucoid phenotype in *Pseudomonas aeruginosa*, associated with the establishment of chronic respiratory infections in cystic fibrosis (CF) (Deretic *et al*., 1995; Govan and Deretic, 1996), is caused by mutations within the *algU mucABCD* gene cluster (Martin *et al*., 1993a,b,c; Boucher *et al*., 1997a,b), which encodes the sigma factor AlgU (Martin *et al*., 1993), also known as AlgT (DeVries and Ohman, 1994), and four other regulatory proteins (Martin *et al*., 1993a,b,c; Boucher *et al*., 1996; 1997a). AlgU is required for transcriptional initiation of alginate biosynthetic genes which when upregulated cause the mucoid phenotype (Martin *et al*., 1993a,b,c; DeVries and Ohman, 1994; Martin *et al*., 1994). MucA is believed to associate with AlgU and inhibit its activity (Schurr *et al*., 1996; Xie *et al*., 1996). Consequently, mutations in *mucA* are associated with increased expression of AlgU-dependent genes (Martin *et al*., 1993c).

The *algU mucABCD* gene cluster is conserved, with some variations, in Gram-negative bacteria (Deretic *et al*., 1995; Missiakas and Raina, 1998). Besides controlling alginate production, AlgU is the *P. aeruginosa* equivalent of the extreme heat shock sigma factor σ^E of *Escherichia coli* (Yu *et al*., 1995). Both AlgU (Schurr *et al*., 1995a; Schurr and Deretic, 1997) and σ^E (Erickson and Gross, 1989; Raina *et al*., 1995; Rouviére *et al*., 1995) control extreme stress response in these organisms. MucA and its *E. coli* homologue RseA have recently been shown to be located in the inner membrane via a single transmembrane domain with its amino terminus in the cytoplasm and its carboxy terminus in the periplasm (De Las Penas *et al*., 1997; Mathee *et al*., 1997; Missiakas *et al*., 1997). MucB (Schurr *et al*., 1996; Mathee *et al*., 1997) and its

E. coli equivalent homologue RseB (De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997) are located in the periplasm. Inactivation of *mucB* is conducive to alginate overproduction under certain growth conditions (Martin *et al.*, 1993b). Although the precise mechanism of MucB action is not known, its periplasmic location is essential for its function (Schurr *et al.*, 1996). MucC and its homologues contain two putative transmembrane domains and are thought to be located in the inner membrane (Boucher *et al.*, 1997a). MucC displays only a mild negative effect on AlgU detectable exclusively in synergy with MucA or MucB (Boucher *et al.*, 1997a). MucD (Boucher *et al.*, 1996) is homologous to the periplasmic serine protease HtrA or DegP (Lipinska *et al.*, 1988; Strauch and Beckwith, 1988) and is believed to act as an extreme stress response effector via the removal of denatured or damaged proteins.

Because the *algU mucABCD* system is highly conserved in Gram-negative organisms, analyses of the regulation of AlgU by MucA and MucB may serve as a model for understanding recognition and signal transduction in response to extreme stress conditions. A recent study of a collection of mucoid *P. aeruginosa* isolates from CF patients, revealed that over 80% of the alginate-overproducing clinical strains have mutations in *mucA* (Boucher *et al.*, 1997b). This pool of mutants provides a resource to help dissect the regulation of AlgU by MucA. The most frequent lesion type (Boucher *et al.*, 1997b) in mucoid CF isolates is represented by the *mucAΔG440* allele (Fig. 1), which is a frameshift mutation located within the last third of the gene encoding the periplasmic domain of MucA. The importance of the periplasmic domain of MucA is underscored by the findings that loss of MucB, which acts in the periplasm (Schurr *et al.*, 1996), also leads to the mucoid phenotype (Martin *et al.*, 1993b). It is not known how periplasmic MucB and periplasmic domains of MucA affect AlgU in the cytoplasm. Moreover, the activity of MucA remains to be explained in the context of its membrane localization. In one model, AlgU could be sequestered by MucA in the membrane, but the membrane association of AlgU has not been investigated. In this work, we have initiated studies of how MucA and MucB affect AlgU localization. Our results suggest that mutations in the carboxy terminus of MucA typically found in clinical isolates affect both the stability of this protein and its interactions with MucB. In addition, we present the unexpected phenomenon that AlgU associates with the inner membrane even in strains containing undetectable amounts of MucA, albeit a subpopulation of AlgU molecules redistributes from the membrane to the cytosol in the absence of MucA. These observations and additional findings are consistent with a model in which MucA affects translocation from the membrane of a fraction of AlgU associated with RNA polymerase. This system, which is designed to provide response to stress conditions, is deregulated due to *mucA* mutations in mucoid *P. aeruginosa*.

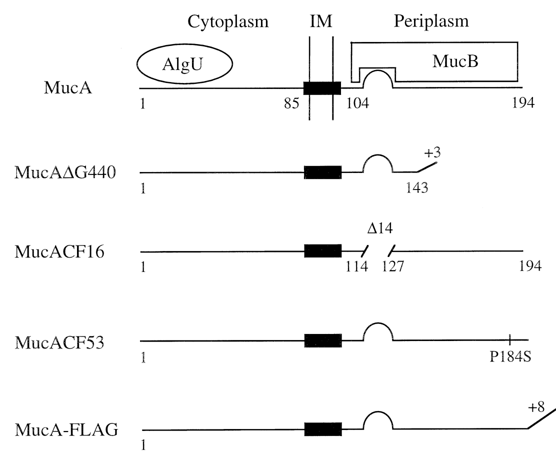

Fig. 1. Schematic representation of mutant MucA forms. Filled rectangle, the putative transmembrane domain (residues 85–104) of MucA. Semi-circle within the periplasmic domain of MucA, putative region that destabilizes interactions with MucB. Oval (AlgU) and rectangle (MucB) are depicted as interacting with the cytoplasmic N-terminal domain and the C-terminal periplasmic domain of MucA respectively. The mutation *mucAΔG440* is common among CF strains. This frameshift mutation causes the loss of the C-terminal 50-amino-acid residues of MucA with termination four triplets downstream resulting in the addition of three amino acids (as indicated by the raised tail). MucACF16 has an in-frame deletion that results in a loss of the residues 114–127. MucACF53 has a missense Pro_{184}→Ser_{184} mutation (P184S). MucA–FLAG has the eight-amino-acid FLAG epitope added to the MucA C-terminus.

Results and discussion

Interactions of mutant forms of MucA with AlgU and MucB

Most mucoid clinical or laboratory isolates of *P. aeruginosa* have mutations causing truncation of the carboxy terminal domain of MucA (Boucher *et al.*, 1997b). One possibility is that these mutations affect the ability of MucA to bind AlgU. Alternatively, because these mutations affect the putative periplasmic domain of MucA, interactions between MucA and the periplasmic regulatory protein MucB may be disturbed. We examined the ability of representative forms of MucA to interact with AlgU and MucB. We focused our studies on three different *mucA* alleles found in clinical isolates (Fig. 1): (i) *mucAΔG440,* the most common frameshift mutation in CF isolates, which deletes the last 50 amino acids of the protein (Martin *et al.*, 1993c; Boucher *et al.*, 1997b); (ii) *mucACF53*, an allele carrying the missense mutation Pro_{184}→Ser_{184}; and (iii) *mucACF16,* an allele that has an in frame deletion of 42 bp that causes the loss of amino acid residues 114–127 within the periplasmic domain of the MucA protein. The rationale for choosing these *mucA* forms is based on

the *mucAΔG440* allele being the most common type of mutation found in CF isolates and *mucACF53 and mucACF16* representing the rare mutations or polymorphisms that do not cause premature termination of *mucA* and thus could be potentially informative about the structure–function of MucA.

We first tested the effects of the *mucA* alleles on alginate production/mucoid status of *P. aeruginosa* by gene replacements in the standard genetic strain PAO1: *mucAΔG440* and *mucACF53* alleles rendered cells mucoid (producing 383.2 $\pm$ 25.3 μg and 99.8 $\pm$ 9.4 μg of uronic acid per mg wet weight of cells, respectively, compared with PAO1 levels of 1.0 $\pm$ 0.1 μg of uronic acid per mg wet weight of cells) when grown on the medium routinely used to assess mucoid status of *P. aeruginosa* (PIA; see *Experimental procedures*). The levels of alginate production in *mucACF53* cells were somewhat less than in *mucAΔG440* cells, consistent with the possibility that the missense mutation P184S in *mucACF53* was less severe than the *mucAΔG440* truncation. The replacement of *mucA*$^+$ with *mucACF16* on the chromosome of PAO1 did not increase alginate production and the strain remained non-mucoid (0.6 $\pm$ 0.1 μg of uronic acid per mg wet weight of cells). Thus, the truncation mutation *mucAΔG440* and the missense mutation P184S are responsible for the mucoid phenotype in the respective CF isolates. In contrast, *mucACF16* is most likely a naturally occurring polymorphism.

To investigate interactions with AlgU and MucB in the yeast two hybrid system, the *mucA* alleles were fused to the GAL4 activation domain (AD) in plasmid pACT2, while the *algU* and *mucB* genes were fused to the GAL4 DNA binding domain (BD) of plasmid pAS2-1. Interactions between the chimeric proteins were examined by monitoring the expression of both the *GAL1–lacZ* and $GAL1_{UAS}$*–HIS3* reporter genes in diploid yeast cells generated from the mating of the *Saccharomyces cerevisiae* strains Y187 and Y190 carrying either the BD plasmids or the AD plasmids respectively. Although the obtained values for β-galactosidase were relatively low, this assay permitted quantitative comparisons (Table 1). The wild-type MucA indeed interacted with MucB (Table 1). Consistent with the model in which the periplasmic domain of MucA interacts with the periplasmic protein MucB, mutations within the carboxy terminus of *mucA* affected this interaction. No interactions between MucAΔG440 and MucB were detected, although MucAΔG440 interacted with AlgU. The missense mutation P184S (*mucACF53*) within the C-terminus of MucA showed slightly reduced interactions with MucB ($P = 0.04$, *t*-test). Similarly, addition of the FLAG epitope to the C-terminal end of MucA (Fig. 1) reduced interactions with MucB (Table 1). The *mucACF16* allele (deletion of amino acids 114–127) displayed increased interactions with MucB. Surprisingly, all tested MucA mutant forms showed equally strong interactions with AlgU compared with the wild-type MucA in the two-hybrid system (Table 1). These results suggest that alterations within the C-terminus of MucA influence the capacity of MucA to interact with MucB while not interfering with its affinity for AlgU.

Truncation of MucA C-terminal domain affects its stability in P. aeruginosa

The two-hybrid analyses and the previous observations that the loss of MucB causes mucoidy (Martin *et al.*, 1993b) suggest that the interaction of MucB with MucA may play a role in enabling MucA to inhibit AlgU. We next considered whether MucB–MucA interactions either helped stabilize the MucA protein (e.g. by preventing its degradation) or affected functions of its cytosolic domain by exerting effects on AlgU via conformational changes. We first examined MucA levels in the three prototypical *mucA* mutants described in the previous section using antibodies raised against a portion of the amino terminal cytoplasmic domain of full-size MucA (residues 38–56; see *Experimental procedures*). Cells with the *mucA* truncation *ΔG440* showed absence of a full-size MucA (Fig. 2A) and its putative truncation product of $\approx$16 kDa (Fig. 2B) was at the levels corresponding to 15% relative to the *mucA*$^+$ cells as judged by Western blot signal intensity (Fig. 3). The P184S missense mutation (*mucACF53*) showed somewhat reduced levels of MucA (74 $\pm$ 7% of wild-type levels; $P = 0.05$, ANOVA) (Fig. 2A). The *mucACF16* in frame 14 amino acid deletion, which does not cause mucoidy, showed increased levels of MucA (CF16 variant with M_r of 19 kDa) relative PAO1. Because mutations within the carboxy terminus of MucA affected interactions with MucB, we also examined the levels of MucA in *P. aeruginosa* with inactivated *mucB.* We observed a 57 $\pm$ 6% ($P = 0.0001$, ANOVA) reduction in MucA levels (Fig. 2A, lane *B*$^-$ and Fig. 3). A small amount of potential degradation intermediates could be sporadically detected in some samples from *mucACF53 and mucB* mutant strains. These results suggest that the loss or reduction of interactions between MucA and MucB leads to a destabilization of MucA diminishing its steady-state levels. In the case of the truncation of the MucA C-terminal domain, the destabilization of this protein appeared to be more complete (Fig. 3). The absence of a full-size MucA in *mucAΔG440* cells coincided with either the presence of small amounts (15% relative to the wild type levels) of a 16 kDa band, which corresponded to the expected size of the truncated version of MucA. Figure 2B illustrates the highest levels of the 16 kDa band reacting with anti-MucA antibodies that could be detected. In the majority of extracts, the band could not be distinguished from background bands or from a similar band (Fig. 2B, lane

Table 1. Effect of representative alterations in *mucA* on interactions of MucA with AlgU and MucB in the yeast two-hybrid system.

	BD fusion[b] β-Galactosidase activity (U/OD_{600})[c]		
AD fusion[a]	BD vector	AlgU	MucB
AD-MucA	<0.01[d]	2.47 ± 0.34	3.69 ± 1.11
AD-MucAΔG440	ND	4.03 ± 1.65	<0.01
AD-MucACF53	ND	2.45 ± 0.27	1.99 ± 0.22
AD-MucACF16	ND	3.52 ± 0.10	13.83 ± 2.27
AD-MucA−FLAG	ND	1.72 ± 0.06	1.33 ± 0.34
AD vector	ND	0.05 ± 0.01	<0.01

a. MucA variants were fused to the Gal4 activation domain (AD) of plasmid pAS2-1. A summary of the characteristics of the MucA variants is given in Fig. 1.
b. BD, Gal4 binding domain fusions with AlgU, MucB. BD-vector, control with no fusion.
c. Interactions were assessed by measuring the expression of the *GAL1–lacZ* reporter gene in Y187/Y190 diploid yeast cells carrying the indicated pairs of plasmids. β-Galactosidase activity in at least three independent cultures grown in SDC -Leu/Trp/His media was determined as described in *Experimental procedures*. The mean values ± SE are reported in Miller units normalized for optical density of the culture at 600 nM. A competitive inhibitor of imidazole glycerol-phosphate dehydrogenase (His3), 3-amino-triazole, was added to cultures showing positive interaction (β-galactosidase activity > 1.0) to ensure greater retention of the plasmids and more consistent β-galactosidase activity. ND, activity not determined; these samples showed β-galactosidase staining in colony filter assays comparable to samples with activities below the limit of detection.
d. Below detection limit (0.01 U/OD_{600}).

A^{+}) present in *mucA*$^{+}$ cells. The low levels or absence of MucA in *mucAΔG440* cells are in keeping with the strongest phenotypic effects of this *mucA* allele on alginate production, as described in previous sections.

To determine whether other representative *mucA* mutations found in CF isolates affect MucA stability, we also examined the levels of MucA in a panel of CF isolates with different truncation mutations (Fig. 2B). The predicted sizes of the truncated MucA versions in these strains varied from 15.5 to 17.0 kDa. Some cross-reactivity with the MucA antibody was observed in the region of the blot containing the low M_r polypeptides, but the bands did not correspond to the expected truncated version of MucA. The most consistently observed cross-reactive band of 16 kDa was also seen in *mucA*$^{+}$ cells and its intensity was lower in *mucA* truncation mutants compared with the full-size MucA in wild-type cells. These results are consistent with the possibility that the majority of mutations in *mucA* causing mucoidy in CF isolates destabilize MucA.

Mutant mucA *alleles are able to inhibit AlgU when overexpressed.*

Our studies suggest that mutations in *mucA* result in reduced levels of MucA (Figs 2 and 3). When overexpressed, the majority of the *mucA* alleles tested were able to reduce alginate production when overexpressed in the strain PAO578I (*mucAΔG440*) (Table 2), suggesting that all mutant forms retained some ability to inhibit AlgU. However, the *mucAΔG440* allele and the C-terminal tagged *mucA-FLAG* form, when overexpressed, were able to inhibit alginate production only partially (Table 2). In contrast, the *mucACF53* allele, was able to inhibit AlgU as efficiently as a *mucA*$^{+}$ clone. The *mucACF16* allele inhibited AlgU better than the wild-type *mucA*, as evidenced by the reduced alginate production and morphological changes (less pronounced mucoidy), even in the absence of the inducer IPTG. Immunoblots of extracts from these cells detected high levels of the MucA mutant forms except the C-terminal tagged MucA-FLAG and the truncated MucAΔG440 (data not shown). These results indicate that mutant MucA forms retain the ability to inhibit AlgU activity when present in sufficient amounts. The complementation of chromosomal *mucA* mutations with plasmid borne *mucA*$^{+}$ was also an illustration of the direct effects of *mucA* inactivation rather than polar effects on the downstream genes.

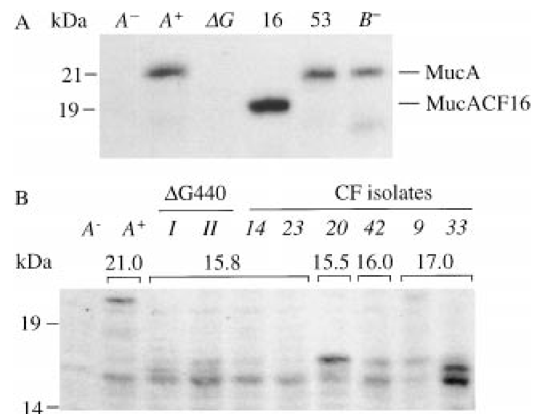

Fig. 2. MucA in wild type and mucoid cells.
A. Immunoblot analysis of total protein extracts from isogenic *mucA* variants constructed in the PAO1 background. The strains were grown in LB media and extracts prepared as described in *Experimental procedures*. Equal protein amounts were separated by SDS–PAGE, electroblotted to Immobilon P membranes, and probed with affinity purified antibody to the N-terminal domain of MucA. Predicted M_r of MucA and MucACF16 are indicated. *A*$^{-}$, PAO6852 (*algU*::Tc^{r}::*mucA*); *A*$^{+}$, PAO1 (*mucA*$^{+}$); *ΔG,* PAO578I (*mucAΔG440*); *16*, PAO6901 (*mucACF16*); *53*, PAO6902 (*mucACF53*); and *B*$^{-}$ PAO6857 (*mucB::*Tc^{r}).
B. Analysis of MucA in a panel of clinical isolates and laboratory strains with *mucA* truncation mutations. *ΔG440 I and II,* PAO578I *mucAΔG440 and* PAO578II *mucAΔG440 sup-2* respectively; CF isolates (indicated by numbers only) contained assorted frameshift or nonsense mutations (CF9, CF20, CF33 and CF42) or were identical to *mucAΔG440* (CF14 and CF23) (Boucher *et al.*, 1997). *A*$^{-}$ and *A*$^{+}$ as in panel A. The wild type and predicted M_r of truncated polypeptides are indicated above each lane. Bars, M_r standards (kDa).

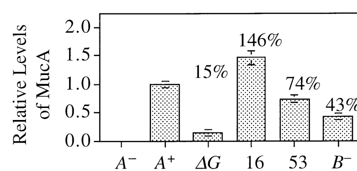

Fig. 3. Quantitation of MucA levels in *mucA* mutants. Levels of protein bands immunoreactive with MucA antibody (21 kDa band for full-size MucA in *mucA*$^+$, *mucACF53*, and *mucB*::Tc^r cells; 16 kDa band in *mucAΔG440*; and 19 kDa in *mucACF16*) were determined by densitometric analysis of immunoblots using three separate samples for each strain (except *mucAΔG440* cells where the number of independent samples was six) and expressed relative to *mucA*$^+$ cells (set as 100%). The 16 kDa band was not detected in all samples from *mucAΔG440* cells and may include some intensity contributed by cross-reacting products. Background density observed in *mucA* null cells (PAO6852) was subtracted from all values. Symbols as in Fig. 2A.

Localization of AlgU in wild type and mucA *mutant cells*

Based on the two-hybrid analysis, MucA, which spans the inner membrane, most likely forms a complex with AlgU via its N-terminal cytoplasmic domain and with MucB via its C-terminal periplasmic domain. In one model of how MucA works, its complex with AlgU (possibly stabilized by MucA–MucB interaction) may sequester AlgU to the membrane, thereby limiting its availability to initiate transcription of subordinate promoters. In this model, a redistribution of AlgU from membranes to the cytosol could serve as a readout of its reduced association with MucA. We tested the localization of AlgU in wild type and *mucA* mutant cells. For these experiments, cellular membranes were separated from soluble cytoplasmic proteins by high-speed centrifugation of total cellular extracts prepared as described in *Experimental procedures*. The levels of AlgU in membrane (M) and cytosolic (C) fractions were determined by immunoblot analysis using monoclonal antibodies specific for AlgU (Fig. 4). The relative levels of AlgU in each sample were quantified by densitometry (Fig. 4, lower panel).

In the wild-type PAO1 cells, AlgU was found predominantly in the membrane fraction (67 ± 1%). Surprisingly, examination of the localization of AlgU in *mucAΔG440* cells revealed that a large proportion of AlgU was still associated with the membrane (54 ± 4%) (Fig. 4). The presence of AlgU in the membrane fraction did not result from its sedimentation as part of a large aggregate or complex as AlgU was detected in inner membranes fractions further purified by isopycnic centrifugation in sucrose gradients from both wild-type and *mucAΔG440* cells (IM in Fig. 4). Nevertheless, some redistribution of AlgU from the membrane to the cytosolic fraction was observed in *mucAΔG440* cells (33 ± 1% to 46 ± 4% in the cytoplasm for PAO1 and PAO578I respectively; $P = 0.02$, ANOVA). The overall level of AlgU was 1.6-fold higher ($P = 0.004$, ANOVA) in *mucAΔG440* cells relative to the *mucA*$^+$ strain (Fig. 4). The increase in the overall levels of AlgU can be attributed to increased transcription of *algU* from its autoregulatory, AlgU-dependent promoters (Schurr *et al.*, 1995). The AlgU redistribution results are consistent with the interpretation that MucA sequesters a portion of AlgU to the membrane, but also suggest that appreciable association of AlgU with the membrane occurs even when the C-terminal domain of MucA is truncated. Because the levels of truncated MucA are reduced in *mucAΔG440* cells to less than 15% of the wild-type levels (Fig. 4), it is likely that AlgU association with the membrane takes place even in the absence of MucA. Nevertheless, the *mucAΔG440* mutation did cause a redistribution of AlgU from membranes to the cytosol in addition to increasing the absolute amounts of AlgU in the cell.

Localization of AlgU and levels of MucA in cells exposed to environmental stress

Because adverse environmental conditions, including elevated temperature can increase AlgU activity (Schurr *et al.*, 1995), we examined effects of heat shock on MucA and AlgU levels and localization, to address the mechanism of activation of the system under physiological conditions. When wild-type *mucA*$^+$ cells were exposed to 50°C for 20 min, a redistribution of AlgU to the cytosol was observed (Fig. 5A). The fraction of AlgU in the cytosol changed from 33 ± 1% to 44 ± 4% ($P = 0.03$, ANOVA) when compared with untreated cells. The total levels of AlgU were increased by 1.4-fold ($P = 0.07$, ANOVA) relative to the untreated cells. The redistribution of AlgU was similar to that observed in mucoid

Table 2. Overexpression of mutant *mucA* can suppress mucoidy in *P. aeruginosa*.

Plasmid[a]	IPTG[b] (1 mM)	Phenotype[c]	Alginate[d] (μg/wcw)
Vector	–	M	79.2 ± 7.6
Vector	+	M	109.0 ± 14.3
p*tac*–*mucA*$^+$	–	M	142.6 ± 26.4
p*tac*–*mucA*$^+$	+	NM	4.3 ± 0.3
p*tac*–*mucAΔG440*	–	M	168.9 ± 16.0
p*tac*–*mucAΔG440*	+	M*	43.4 ± 12.8
p*tac*–*mucACF53*	–	M	93.0 ± 18.5
p*tac*–*mucACF53*	+	NM	1.3 ± 0.2
p*tac*–*mucACF16*	–	M*	37.5 ± 1.8
p*tac*–*mucACF16*	+	NM	1.2 ± 0.1
p*tac*–*mucA–FLAG*	–	M	260.6 ± 6.5
p*tac*–*mucA–FLAG*	+	M	149.2 ± 15.5

a. Plasmids were transferred into the *mucAΔG440* strain PAO578I by triparental conjugation.
b. Exconjugants were grown on PIA medium supplemented with carbenicillin (300 mg ml^{-1}) and 1 mM isopropylthiogalactopyranoside (IPTG) where indicated.
c. M, mucoid phenotype; M*, mucoid after 48 h; NM, non-mucoid phenotype.
d. Production of alginate is expressed in mg of uronic acid per mg (wet weight) of cells ± SE.

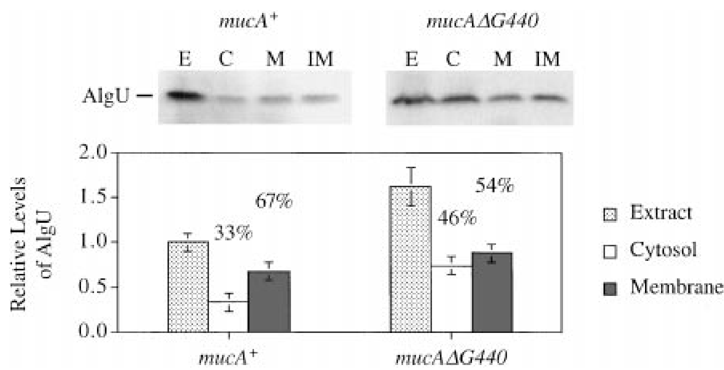

Fig. 4. Localization of AlgU in wild-type and *mucAΔG440* cells. Immunoblot of total extract (E), cytoplasmic (C), total membrane (M) and inner membrane (IM) fractions prepared from the strains PAO1 ($mucA^+$), PAO578I (*mucAΔG440*) and relative levels of AlgU in each fraction relative to levels in PAO1 ($mucA^+$) total extract determined by densitometric analysis. Equivalent amounts of the protein were separated by SDS–PAGE, electroblotted to Immobilon P membranes, and probed with monoclonal antibodies to AlgU. Because the bar graph in the lower panel represents quantitation of at least three independent sample sets some of the values may not absolutely match the appearance of the Western blot in the top panel.

mucAΔG440 strain (Fig. 4). However, the relocation of AlgU to the cytosol in cells exposed to heat shock did not appear caused chiefly by the degradation of MucA as the levels of MucA increased (Fig. 5B) relative to untreated cells (the apparent increase in MucA levels can be attributed to elevated transcription of the *algU mucABCD* gene cluster). However, an increase in MucA turnover cannot be excluded during the process as putative degradation intermediates of MucA became detectable, suggestive of possible MucA degradation (Fig. 5B; lower M_r bands).

We also examined whether a shift in temperature can release AlgU from isolated membranes *in vitro*. Membrane samples prepared from PAO1 ($mucA^+$) and PAO578I (*mucAΔG440*) were incubated at elevated temperature (50°C). This treatment caused an increase in the amount of AlgU found in the supernatant after resedimentation of the membranes in samples from PAO1 (Fig. 5C). No similar redistribution of AlgU to the supernatant was observed in membranes from *mucAΔG440* cells. These results suggest that exposure to stress causes redistribution of AlgU if MucA is present, and is consistent with the existence of two populations of membrane-bound AlgU: a MucA-associated fraction and a MucA-independent fraction, as noted in our *in vivo* studies with *mucA* mutant cells.

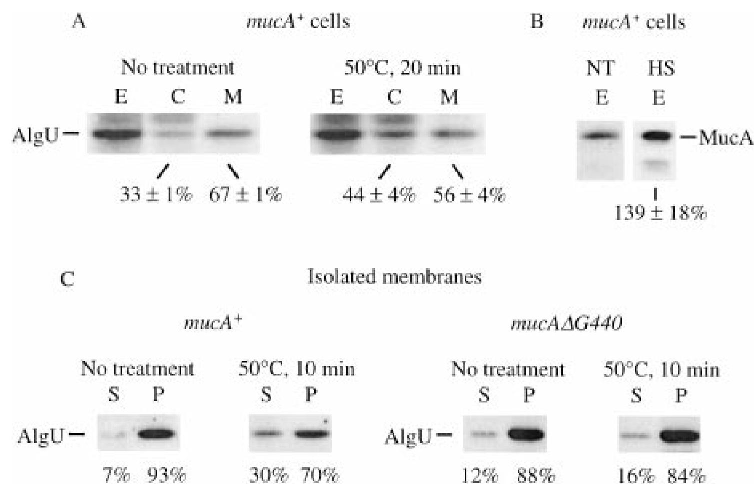

Fig. 5. Effect of extreme heat shock on the association of AlgU with the membrane.
A. Immunoblots probed with monclonal antibodies for AlgU of total extract (E), cytoplasmic (C), and total membrane (M) fractions prepared from PAO1 $mucA^+$ cells before and after exposure to 50°C for 20 min. The portion of AlgU in the cytosol and membrane fractions was determined by densitometric analysis and expressed relative to total extract levels.
B. Immunoblots of total extract of PAO1 cells prepared before or after incubation at 50°C for 20 min and probed with MucA antibodies.
C. Isolated total membrane samples from PAO1 $mucA^+$ and PAO578I (*mucAΔG440*) cells were subjected to either incubation on ice (No treatment) or incubation at 50°C for 10 min. The membranes were resedimented and equal volumes of the supernatant (S) and resuspended pellet (P) were separated by SDS–PAGE, electroblotted to Immobilon P membranes and probed with monoclonal antibodies to AlgU. The percentage values were calculated by dividing the intensity of bands in individual lanes by the sum of intensities of the bands in both lanes.

Examination of the association of AlgU with the membrane.

The observation that AlgU is able to associate with the membrane by a mechanism independent of MucA cannot be explained by the previous models envisioning MucA as the tether for potential association of AlgU with the membrane. We next considered the possibility that AlgU could bind to the membrane via interactions with MucC, another regulator of AlgU activity predicted to reside in the membrane (Boucher *et al*., 1997a). Western blots of cytosolic and membrane samples prepared from the double *mucA2 mucC*::*Gm*^{r} mutant (strain PAO6876) revealed no change in the association of AlgU with the membrane (data not shown), ruling out the possibility that association with MucC could explain AlgU partitioning with the membrane in the absence of MucA.

To examine biochemically the nature of the association of AlgU with the membrane, samples were treated with agents that disrupt different types of protein bonding with membranes. These experiments were performed on membrane samples from wild-type cells (PAO1) and cells containing the truncation mutation *mucAΔG440* (PAO578I). Following treatments, the membranes were repelleted by centrifugation, and the levels of AlgU in the supernatant (S) and in the resuspended pellet (P) were determined by Western blots. As a control, we also determined the levels of MucA in each fraction. Treatment of membrane samples from wild-type strain PAO1 and the *mucAΔG440* strain PAO578I with 1 M NaCl or NaCl plus 25 mM EDTA which disrupt electrostatic interactions had little or no effect on the association of AlgU or MucA with the membrane in wild-type PAO1 cells but caused detectable release of AlgU from membranes isolated from PAO578 (*mucAΔG440*) (Fig. 6A). It is important to note that recovery of total AlgU or MucA proteins varied in some of the experiments and that the following considerations are based on relative amounts between paired S and P fractions only. Treatment of membranes with 0.15 M Na_2CO_3 to change the pH to 11 or under denaturing conditions with 4 M urea caused significant release of AlgU from the membrane (Fig. 6A). The treatment with urea apparently affected membrane integrity as it also caused the release of a portion of MucA presumed to be an integral membrane protein. We also examined the ability of AlgU to be released or solubilized from the membrane by the zwitterionic detergent CHAPS. The level of AlgU in the supernatant fraction began to increase at a CHAPS concentration of 0.1% and was nearly complete with 0.5% CHAPS (Fig. 6B and C). The release of AlgU from the membrane began at a lower detergent concentration than expected for an integral membrane protein as judged by the overall change in the protein concentration in the samples or compared with MucA release (Fig. 6B and C). In addition, the release of AlgU was similar to the peripherally associated inner membrane protein D-lactate dehydrogenase (Genis and Stewart, 1996), as judged by the changes in the distribution of the corresponding enzymatic activity (Fig. 6C). Based on these results, it appears that AlgU is peripherally associated with the membrane and is most likely bound by non-ionic interactions. Interestingly, no major difference in the ability to remove AlgU from the membrane by physical–chemical treatment were observed in preparations from *mucA*$^+$ and *mucA* mutant cells in keeping with the possibility that a significant fraction of membrane associated AlgU is bound independently of interactions with MucA. In some of the experiments described here, the recovery of proteins after various treatments was reduced but the yields were similar in *mucA*$^+$ and *mucA* mutant cells.

Colocalization of membrane-bound AlgU and RNA polymerase α subunit

The observations presented in the previous sections are inconsistent with a simple model of AlgU inhibition by MucA-dependent sequestration in the membrane. Thus, we considered a more complex role for MucA and hypothesized that the portion of AlgU that is controlled by MucA may be affected at the stage of translocation from the membrane during interactions with RNA polymerase. To test this possibility, we examined whether RNA polymerase was associated with the membrane in wild-type *P. aeruginosa* cells. Western blot analyses of cytoplasmic and membrane fractions with sera against the α subunit of RNA polymerase revealed that this RNA polymerase subunit was indeed found in the membrane fraction of *mucA*$^+$ cells and in much lower (albeit detectable) amounts in *mucAΔG440* cells (Fig. 7A). The greater association of RNA polymerase specifically with membranes from *mucA*$^+$ cells was further confirmed by examining samples containing the inner membrane fraction purified by isopycnic centrifugation in sucrose density gradients (Fig. 7B). RNA polymerase is most likely not associated with the MucA-independent fraction of membrane-bound AlgU as there were no differences in the amounts of α subunit in the inner-membrane preparation from *mucA* mutant and double *algU mucA* mutant cells (Fig. 7B, lanes *U*$^-$ *A*$^-$ *B*$^+$ and *U*$^+$ *AΔG B*$^+$). Exposure of wild-type cells to elevated temperatures also caused a decrease in the levels of RNA polymerase α subunit associated with the inner membrane (Fig. 7B, compare HS with NT). These results fit a model in which MucA acts not by simply inhibiting the association of AlgU with RNA polymerase, but instead affects this complex on

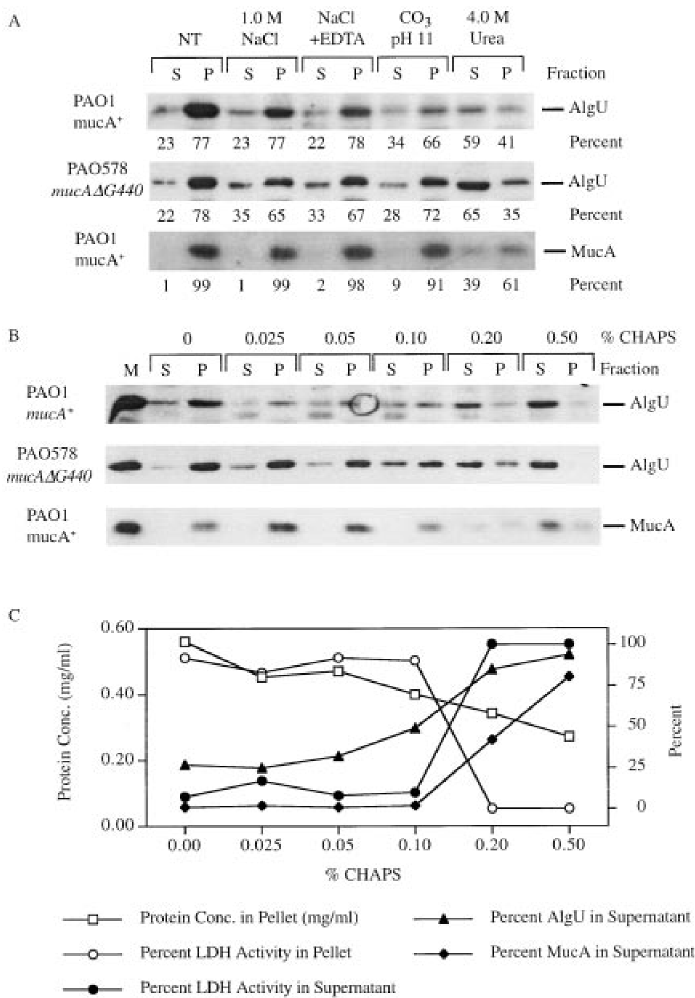

Fig. 6. Nature of AlgU association with membranes. Total membrane samples from PAO1 *mucA*$^+$ and PAO578I *mucA*Δ*G440* were treated with agents which disrupt protein interactions with membranes. After resedimentation of the membranes, equal volumes of the supernatant (S) and resuspended membrane pellet (P) sample were separated by SDS–PAGE, electroblotted to Immobilon P membranes and probed with monoclonal antibodies to AlgU and affinity purified antibodies to MucA. The distribution (expressed as percentage of total) of AlgU or MucA in each fraction was calculated by dividing the intensity of bands in individual lanes by the sum of intensities of the bands in both lanes as determined by densitometric analysis; values are given below each lane.
A. Membrane samples were incubated with buffer (no treatment, NT); or buffer containing 1.0 M NaCl; 1.0 M NaCl + 25 mM EDTA (NaCl + EDTA); 100 mM Na_2CO_3 (pH 11.0); or 4.0 M Urea.
B. Membranes from different strains were extracted with a buffer containing the indicated final concentration of the zwittergent CHAPS.
C. Protein concentration in the pellet (squares), fractions of total D-lactate dehydrogenase (LDH) activity in the pellet (open circles) or supernatant (filled circles), and fractions of total AlgU (filled triangles) and total MucA (filled diamonds) in supernatants of membranes samples from PAO1 extracted with CHAPS detergent (shown in panel B).

the membrane with AlgU already complexed with RNA polymerase or being in a loading position.

Physiological activation of the system independent of MucA degradation

In experiments with RNA polymerase α subunit association with the membranes, we noticed that its levels on membranes from the *mucB* mutant were not different from levels in *mucA*$^+$ strains (Fig. 7A and B). Consistent with this finding was the observation that AlgU did not redistribute to the cytosol in *mucB*::*Tc*^{r} cells as the partitioning of 68 ± 5% of AlgU in the membrane versus 32 ± 5% in the cytosol fraction was comparable to *mucA*$^+$ cells (Fig. 8A). It is important to note, however, that *mucA and mucB* mutant strains differ in their phenotypic expression of mucoidy. While *mucA*Δ*G440* cells are constitutively mucoid, *mucB* mutants are non-mucoid under conditions used in experiments described in the previous sections and become mucoid only during

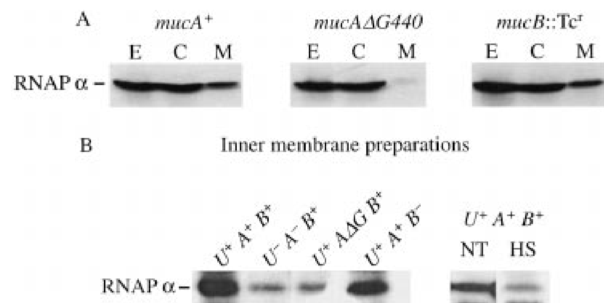

Fig. 7. Association of a subunit of RNA polymerase with membranes.
A. Immunoblots of total protein extract (E), cytoplasmic (C), total membrane (M) fractions prepared from the strains PAO1 (*mucA*$^+$), PAO578I (*mucA*Δ*G440*), and PAO6857 (*mucB*::Tc^r) and probed with antibodies to the RNA polymerase α subunit (RNAP α) from *E. coli*.
B. Immunoblots of purified inner membrane samples probed with antibodies to the α subunit of RNA polymerase. U^+ A^+ B^+, PAO1 (*mucA*$^+$); U^- A^- B^+, PAO6852 (*algU*::Tc^r::*mucA*); U^+ *AÆG* B^+, PAO578I (*mucA*Δ*G440*); U^+ A^+ B^-, PAO6857 (*mucB::Tc*^{r}); U^+ A^+ B^+ NT, and HS, samples prepared from PAO1 (*mucA*$^+$) cells before and after treatment with heat shock at 50°C, 20 min.

growth on special media that supply additional signals conducive to overproduction of alginate (Boucher *et al.*, 1997a). When *mucB*::Tc^r cells were grown on media promoting mucoidy, redistribution of AlgU similar to or exceeding that seen in *mucA*Δ*G440* cells was observed in the *mucB*::Tc^r strain (Fig. 8B). Furthermore, similar phenomena were observed with *mucACF53* cells (Fig. 8A and B). Significantly, no reduction in MucA levels was detected in *mucB*::Tc^r or *mucACF53* cells when mucoidy-inducing (Fig. 8C, PIA) and non-inducing (Fig. 8C, LB) media were compared (Fig. 8C). These results suggest that a redistribution of AlgU to the cytosol, which appears now to always correlate with the mucoid phenotype, can occur via a mechanism independent of MucA degradation, possibly via a conformational change in MucA. Although an alteration of some MucA-independent property of the membrane under conditions that promote mucoidy cannot be excluded, it is likely that AlgU redistribution depends on MucA or MucB as no change in AlgU localization was detected in wild-type, *mucA*$^+$ *mucB*$^+$ cells when similar conditions were tested.

Model of AlgU regulation by MucA and MucB

Based on the findings presented in this study, we propose a model (Fig. 9) of how AlgU, MucA, MucB and RNA polymerase cooperate at the membrane. Because this system is highly conserved (Missiakas and Raina, 1998), and MucA–AlgU interactions appear to correspond to RseA–σ^E interactions revealed both by biochemical means (Schurr *et al.*, 1996; Xie *et al.*, 1996; De Las Penas *et al.*, 1997; Missiakas *et al.*, 1997) and by two-hybrid analyses (Missiakas *et al.*, 1997), this model may be applicable to most if not all extreme stress response systems in Gram-negative organisms. Variations on this theme could also be extended to at least some ECF sigma factors in Gram-positive organisms (Gorham *et al.*, 1996).

AlgU associates with the bacterial inner membrane. In *Bacillus subtilis*, two sigma factors, unrelated in sequence to AlgU, are also membrane associated as an integral part of their regulation (Haldenwang, 1995; Ju *et al.*, 1997; Hofmeister, 1998; Zhang *et al.*, 1998), suggesting that compartmentalization and control of alternative sigma factors by processes at the membranes is a common method of regulation in bacteria. In the case of the extreme stress response system controlling AlgU, its localization to the membrane most likely provides a sensory system positioned at the frontier of the cell, poised to recognize and respond to potentially lethal changes in the extracellular environment.

Our results indicate that AlgU can associate with the inner membrane in two forms: (i) as a MucA-dependent fraction and (ii) as a MucA-independent fraction. Only the

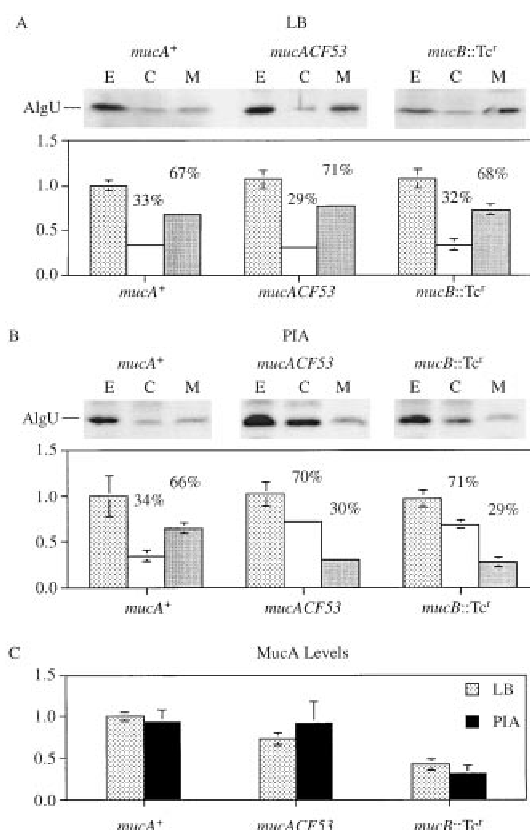

Fig. 8. **C**hanges in AlgU localization in *mucB* null and *mucA* missense mutants require additional signals. Total protein extract (E), cytoplasmic (C), and membrane (M) fractions were prepared from the strains PAO1 (*mucA*$^+$), PAO6902 (*mucACF53*), and PAO6857 (*mucB::Tc*^{r}) grown either (A) in LB culture (conditions promoting non-mucoid phenotype) or (B) on *Pseudomonas* isolation agar (PIA) (conditions promoting mucoid phenotype).
A. Immunoblots from samples grown in LB media probed with monoclonal antibodies to AlgU, and relative levels of AlgU determined by densitometric analysis of the immunoblots.
B. Immunoblots from samples grown on *Pseudomonas* isolation agar (PIA) probed with monoclonal antibodies to AlgU, and relative levels of AlgU determined by densitometric analysis of immunoblots.
C. Relative levels of MucA determined by densitometric analysis of MucA immunoblots of total extracts from cells grown on LB or PIA media. The level of MucA in each sample is expressed relative to the levels of MucA found in samples of PAO1 *mucA*$^+$ grown on LB media As the bar graph panels represent quantitation of at least three independent sample sets, some of the values may not absolutely match the appearance of the Western blots in the corresponding top panels.

MucA-dependent form redistributes to the cytosol if MucA is inactive or absent. As a substantial portion of the membrane-bound RNA polymerase shows similar dependence on MucA, it is possible that MucA prevents membrane-to-cytosol translocation of AlgU associated with transcriptionally competent RNA polymerase. Such preassembled complexes could ensure rapid response to potentially fatal damage. The second MucA-independent fraction of membrane-bound AlgU may serve as a pool from which transcriptionally competent complexes could

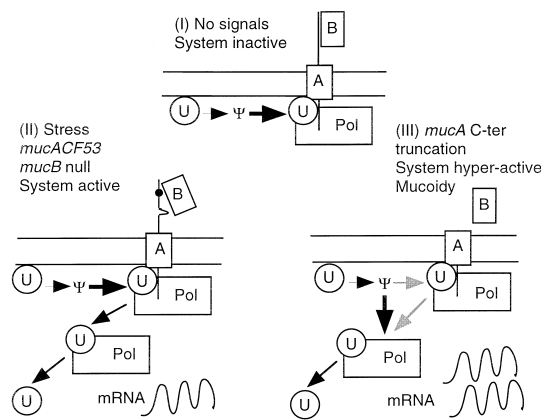

Fig. 9. Model of AlgU activation in *muc* mutants or under stress conditions. (i) In wild-type cells in the absence of signals, the system is arrested as a complex of AlgU with MucA and other components. (ii) Upon exposure to stress conditions in wild-type cells, or under growth conditions conducive to mucoidy in *mucB* null mutants or *mucA* mutants that interfere with MucA–MucB interactions or alter MucA function, the inhibitory activity of MucA is reduced and transcription initiation takes place. (iii) In *mucADG440*, MucA is unstable and the system is running full throttle. It is hypothesized that transcriptionally active complexes are recruited directly from the postulated intermediate Ψ (AlgU–RNA polymerase transiently associated at the membrane). Symbols: A, MucA; B, MucB; U, AlgU; Pol, RNA polymerase; Ψ, postulated AlgU-RNA polymerase loading intermediate at the membrane; horizontal lines, inner membrane; vertical lines, periplasmic (top) and cytoplasmic (bottom) domains of MucA; dot, P184S mutation within the C-terminal domain of MucA; bump on the periplasmic portion of MucA, MucB-interaction inhibitory domain; patterned arrow, reduced steady-state flow due to low levels of truncated MucA; thin arrow, low steady-state flow; thick arrow, high steady-state or increased flow. Free cytosolic AlgU represents spent sigma factor or sigma factor actively engaged in transcription.

be quickly replenished. As this population of AlgU does not spontaneously dissociate from the membrane, it is likely that AlgU translocation to the cytosol is an active process. Furthermore, RNA polymerase appears not to associate with the membrane-bound AlgU free of MucA (Fig. 7B), suggesting that the assembly of AlgU and RNA polymerase on the membrane may be a multistage process. We postulate the existence of an intermediate (Ψ; see Fig. 9) consisting of AlgU bound to other factors (possibly including RNA polymerase) that is normally sequestered by MucA. If MucA is absent or altered to a less-inhibitory form, the AlgU/RNA polymerase complex translocates to the cytosol.

The majority of *mucA* mutations causing constitutive expression of the system (e.g. *mucAΔG440*) destabilize MucA and reduce or significantly diminish its levels. Less prominent reductions in MucA amounts are seen in cells encoding MucAP184S (CF53) and those lacking MucB. It appears that, in such cells, additional environmental signals can induce further changes that promote AlgU-dependent transcription. Such changes are likely to be conformational, as no further reduction in MucA levels is detected. Similar changes may also function during conditions of extreme heat shock, as in cells exposed to 50°C the MucA-dependent fraction of AlgU redistributes to the cytosol without a concomitant reduction in MucA levels. The inhibitory conformation of MucA may be influenced by MucB, which interacts with the periplasmic domain of MucA. We were able to differentiate functionally at least two domains on MucA that play a role in interactions with MucB. The P184S mutation reduces MucA–MucB interactions, suggesting that this region is involved in or is necessary for efficient MucA binding to MucB. In contrast, deletion of a small region of the periplasmic domain of MucA closer to its transmembrane region improves MucB binding, suggestive of the existence of a domain in MucA that inhibits interactions with MucB. The positive and negative interaction domains of MucA may constitute a physiological toggle switch that can be translated into activation or inactivation of the extreme stress response system.

Experimental procedures

Bacterial strains and growth conditions

The laboratory strains of *P. aeruginosa* used in this study are listed in Table 3. These strains are derivatives of the standard non-mucoid genetic strain PAO1. The CF strains used have been previously described (Boucher *et al.*, 1997). *P. aeruginosa* was grown on Luria broth (LB) or on *Pseudomonas* isolation agar (PIA, Difco) supplemented with carbenicillin (300 mg ml^{-1}), gentamicin (150 mg ml^{-1}), tetracycline (300 mg ml^{-1}), 10% (w/v) sucrose or IPTG (1 mM) when required. For preparation of extracts and membranes, cultures were grown in liquid media to an A_{600} of 0.6–0.8 or on agar plates for 15–18 h.

Genetic manipulations, allelic replacements and complementation analysis

The strain PAO578I (*mucAΔG440*) has been previously described (Fyfe and Govan, 1980; Schurr *et al.*, 1994). For gene replacements with *mucA* alleles, the *mucA* sequences of CF16 and CF53 were amplified from genomic DNA by PCR using oligonucleotide UL5 (5′-GCCGCACGTCACG-AGC-3′) and UR20 (5′-CGCCAACCGCACCATCGCTC-3′). The resulting PCR product was cloned into pCR2.1 (Invitrogen), sequenced and subcloned into the non-replicative plasmid pCVD442 (similar to pCVD441; Donnesberg and Kaper, 1991) as an *Xba*I/*Sac*I fragment. These constructs were further modified by insertion of a Gm^r cassette (Boucher *et al.*, 1997) from pKI11G to generate pDR130 and pDR131 containing *mucACF16* and *mucACF53* respectively. The strain PAO6852 (the 3′ end of *algU* and 5′ end of *mucA* gene is deleted and disrupted by a Tc^r cassette; Martin *et al.*, 1994) was used as the recipient for a two-step allelic exchange procedure. First, exconjugants that had integrated the respective plasmids were selected on PIA medium supplemented with gentamicin. Then, Gm^r colonies were subjected to selection for a second recombination event that

Table 3. *P. aeruginosa* laboratory strains used in this study.

Strain	Genotype and relevant properties	Reference or source
PAO1	Prototroph Alg^{-wt}	B. Holloway
PAO578I	Alg$^+$, *mucAΔG440*	Fyfe and Govan (1980); Schurr *et al.* (1994)
PAO578II	Alg$^+$, *mucAΔG440 sup2*	Schurr *et al.* (1994)
PAO6852	Alg$^-$, *algU*::Tc^r::*mucA*	Martin *et al.* (1994)
PAO6857	Alg$^+$, *mucB*::Tc^r	Schurr *et al.* (1996)
PAO6876	Alg$^+$, *mucA2 mucC*::Gm^r	Boucher *et al.* (1997a)
PAO6901	Alg$^-$, *mucACF16*	This work
PAO6902	Alg$^+$, *mucACF53*	This work

caused the loss of the plasmid sequences on PIA medium containing 10% sucrose. The resulting colonies that were both Gm^s and Tc^s were identified by replica plating. The strain PAO578I (*mucAΔG440*) has been previously described (Fyfe and Govan, 1980; Schurr *et al.*, 1994). All gene replacements were confirmed by PCR amplification and sequencing.

For complementation analyses with different *mucA* alleles, the PCR-derived *mucA* sequences of CF16 and CF53 were subcloned into pVDtac24 (Deretic *et al.*, 1987) as a *Bst*YI fragment as previously described for the construction of ptac–mucA (Martin *et al.*, 1993c) to generate ptac–mucACF16 and ptac–mucACF53 respectively. The plasmid ptac–mucAΔG440 was created by cloning the *mucA* coding regions previously amplified from PAO578I (Martin *et al.*, 1993) into pBluescript SK+ (Stratagene) as a *Xho*I–*Bgl*II fragment and then subcloning into ptac–mucACF16 as a *Xho*I–*Sac*I fragment thereby replacing the *mucACF16* sequences. To create the *mucA-FLAG* allele, the 3′ end of *mucA* was modified to code for the eight residues recognized by the anti-FLAG monoclonal antibody (Eastman Kodak Company) by PCR amplification with oligonucleotides UL5 and MAFLAG (5′-TCACTTGTCATCGTCGTCCTTGTAGTCGCGGTTTTCC-AGGCTGGCT-3′). The resulting PCR product was ligated into pCR2.1 (Invitrogen) and then subcloned into pVDtac24 as a *Bst*YI–*Eco*RI fragment. Production of alginate was determined by assaying uronic acid by the method previously described by Knutson and Jeanes (1976) after growth on PIA medium for 48 h.

Yeast two-hybrid analysis

For the two-hybrid analysis, appropriately modified *algU*, *mucA and mucB* genes were fused to either the GAL4 DNA-binding domain (BD) or the GAL4 activation domain (AD) of plasmids pAS2-1 and pACT2 (Clontech) respectively. BD–AlgU and BD–MucB were created by subcloning the modified *algU* from pETU1610 (Schurr *et al.*, 1995) and the modified MucB minus the leader peptide from pmucBΔLP (Schurr *et al.*, 1996) as *Nde*I–*Bgl*II fragments into pAS2-1. For the construction of AD–MucA, a *Nco*I site was generated at the 5′ end of *mucA* by PCR amplification (the *Nco*I site is underlined) using oligonucleotides DR11 (5′-AGAT-CCATGG CTATGAGTGCTGAA-3′) and UR20, and the modified *mucA* was cloned into pACT2 as a *Nco*I–*Bgl*II fragment into pACT2. For the creation of AD–MucA–CF16, AD–MucA–CF53, AD–MucAΔG440 and AD–MucA–FLAG, the respective *mucA* sequences previously cloned into pCR2.1 or pBluescript SK+ (*mucAΔG440*) were subcloned into pAD–MucA as *Xho*I–*Sac*I fragments thereby replacing all but the 5′ end of the *mucA*$^+$ sequences. After creation, the DB plasmids were transformed into the yeast strain Y187 (*MATa, ura3–52, his3–200, ade2–101, trp1–901, leu2–3, 112, gal4Δ, met-, gal80Δ, URA3*::*GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*), and the AD plasmids were transformed into the yeast strain Y190 (*MATa, ura3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3, 112, gal4Δ, gal80Δ, cyh^{R}2, URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ, LYS2::GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3*) respectively (Clontech).

To determine the interaction between chimeric proteins, we monitored the expression of both the *GAL1–lacZ* and *GAL1$_{UAS}$–HIS3* reporter genes contained in diploid cells created from the mating of the yeast strains Y187 and Y190 carrying either the BD plasmids or the AD plasmids respectively. The β-galactosidase activity produced from the *GAL1–lacZ* gene was measured qualitatively by cleavage of X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) in colony-lift filter assays as described in the MATCHMAKER Two-Hybrid System 2 handbook (Clontech) and quantitatively by cleavage of ONPG by permeablized cells from liquid cultures (Ausubel *et al.*, 1989). The expression of *GAL1$_{UAS}$–HIS3* reporter gene was determined by scoring growth on media lacking histidine and containing 10 mM 3-amino-triazole (a competitive inhibitor of His3). Diploid cells were used because they were less affected by slow growth phenotype found in cells carrying both the BD–AlgU and AD–MucA plasmids that caused variable colony size and reporter expression in haploid cells.

Preparation of total protein extracts and subcellular fractionation

Harvested cells were resuspended in cold extract buffer [50 mM Tris-HCl, pH 7.5; 150 mM NaCl; and Complete™, EDTA-free protease inhibitor cocktail (Boehringer Mannheim)] and disrupted by either sonication (for smaller preparations from 10 to 25 ml liquid culture or cells grown on PIA plate) or by passage through a French press (for larger preparations from 500 ml of culture to isolated inner membrane and concentrated total membrane samples). For separation of soluble cytoplasmic proteins from total membranes, a 0.2 ml aliquot of the cell extract was subjected to centrifugation in a Sorvall RP100AT rotor at 100 000 *g* for 60 min at 4°C. The supernatant which contained the soluble cytoplasmic proteins (C) was removed, and the pellet containing total membranes (M) was washed once with

extract buffer and then resuspended in the original volume in extract buffer. The cytosolic fraction contained less than 5% of the total enzymatic activity of succinate dehydrogenase and D-lactate dehydrogenase (LDH) activity, two inner membrane proteins (Genis and Stewart, 1996). For the localization comparison, an equal volume of these samples was subjected to electrophoresis and the levels of proteins in immunoblots determined by densitometry. Inner membranes were purified by isopycnic centrifugation in sucrose gradients by established methods (Hancock and Nikaido, 1978). Protein concentrations were determined with Bio-Rad Bradford Reagent using BSA as a standard. Succinate dehydrogenase and D-lactate dehydrogenase activity was assayed by established methods (Kasahara and Anraku, 1974; Kaczorowski *et al*., 1978).

Treatment of isolated membranes with chemicals agents or elevated temperature

To examine the effect of chemical and physical treatments on the association of AlgU with the membrane, a concentrated total membrane sample was generated by centrifugation of 3.5 ml of cell extract in a Sorvall S100-AT5 rotor at 150 000 *g* for 90 min at 4°C and resuspending the pellet in one-seventh the original volume. This total cellular membrane preparation contained ≈97% of the total succinate dehydrogenase activity. An aliquot of the concentrated total membrane sample containing 0.2 mg protein in 0.1 ml was treated with an equal volume of extract buffer containing the chemical or detergent at 2% concentration. After incubation for 1 h at 4°C, the membranes were pelleted by centrifugation in a Sorvall RP100-AT rotor at 100 000 *g* for 60 min. The supernatant was removed and the pellet was washed once before resuspension in an equal volume of extract buffer. For treatment at elevated temperature, an aliquot of the concentrated membrane sample containing 200 mg protein was diluted to 0.2 ml and incubated at 50°C for 10 min. After cooling on ice, the membranes were collected by centrifugation as previously described. For gel electrophoresis and immunoblot analysis, one quarter of the sample was precipitated as described by Wessel and Flugge (1984).

Generation of peptide-specific polyclonal antibodies against MucA

A MucA-specific peptide, MucA-pep1 (CSTWSRYQLARSV-MHREPTL; residues 38–56 of the N-terminal, cytoplasmic domain of MucA) was synthesized due to its predicted hydrophilicity and antigenic index maxima (MacVector, IBI). The synthesis of the peptide, conjugation to a carrier protein and immunization of two rabbits was performed by Alpha Diagnostic International. Antibodies that specifically recognize the peptide were purified from sera by affinity chromatography. For this procedure, 0.2 mg of peptide was coupled to a 1 ml HiTrap NHS-activated column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The antibodies in the sera were concentrated by ammonium sulphate precipation, loaded onto the column and eluted with 0.1 M glycine-HCl, pH 2.5 as previously described (Page *et al*., 1994).

Gel electrophoresis and Western blot analysis.

For the localization comparisons, equal volumes of the extract (E), cytosolic (C) and membrane (M) fractions were subjected to SDS–PAGE. After SDS–PAGE, the proteins were transferred to Immobilon-P transfer membrane (Millipore Corporation) and the protein detected by immunoblot analysis using horseradish perioxidase-labelled secondary antibodies and Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products). AlgU was detected using murine ascites fluid 6C6 (Schurr *et al*., 1996) and the α subunit of RNA polymerase was detected with rabbit polyclonal sera generated against the α subunit of RNA polymerase from *E. coli* (M. J. Chamberlin, University of California, Berkeley) which also cross-reacts the α subunit of RNA polymerase from *P. aeruginosa*. The densitometric analysis was performed using NIH Image software (US National Institute of Health, http://rsb.info.nih.gov/nih-image).

Statistical analysis

Analysis of variance (ANOVA), posthoc pairwise comparisons with the Fisher's Protected LSD test or Student's *t*-test analyses were performed with SuperANOVA (version 1.11, Abacus Concepts).

Acknowledgements

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