The anti-sigma factor MucA is required for viability in *Pseudomonas aeruginosa*

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ABSTRACT

During decades-long infections in the cystic fibrosis (CF) airway, *Pseudomonas aeruginosa* undergoes selection. One such bacterial genetic adaptation frequently observed in CF isolates is mutation of *mucA.* MucA inhibits the sigma factor AlgU. In clinical isolates, *mucA* mutations lead to AlgU misregulation, resulting in a mucoid phenotype that is associated with poor CF disease outcomes. Here we show that, paradoxically, a portion of the *mucA* gene is essential for *P. aeruginosa* viability. We demonstrate that *mucA* is no longer essential in a strain lacking *algU*, that *mucA* alleles that encode for proteins that do not bind to AlgU are insufficient for bacterial viability, and that *mucA* is no longer essential in mutant strains containing AlgU variants with reduced sigma factor activity. Finally, we found that overexpression of *algU* prevents cell growth in the absence of MucA, and that this phenotype can be rescued by overproduction of RpoD, the housekeeping sigma factor. Together, these results suggest that in the absence of MucA, the inability to regulate AlgU activity results in the loss of bacterial viability. Finally, we speculate that essentiality of anti-sigma factors that regulate envelope function may be a widespread phenomenon in bacteria**.**

INTRODUCTION

The major cause of death in people with cystic fibrosis (CF), a human autosomal recessive genetic disease, is respiratory failure due to chronic lung infection. *Pseudomonas aeruginosa* is one of the most prevalent respiratory pathogens of CF patients (Cystic Fibrosis Foundation Patient Registry, 2019). The CF lung environment selects for mucoid *P. aeruginosa* mutants, which overproduce the exopolysaccharide alginate and are associated with poor disease prognosis (Emerson *et al.*, 2002, Henry *et al.*, 1992, Li *et al.*, 2005, Parad *et al.*, 1999, Pedersen *et al.*, 1992). Conversion to the mucoid phenotype in clinical *P. aeruginosa* isolates, which is thought to be advantageous for chronic infection, is frequently caused by *mucA* mutations (Martin *et al.*, 1993).

MucA is an anti-sigma factor to the alternative sigma factor AlgU (also known as AlgT, σ^E, or σ^{22}), which responds to envelope stress (Damron & Goldberg, 2012, Govan & Deretic, 1996). MucA is a transmembrane protein that sequesters the sigma factor away from RNA polymerase (RNAP) via its N-terminal interaction with AlgU (Li *et al.*, 2019, Schurr *et al.*, 1996, Xie *et al.*, 1996). The C-terminus of MucA is in the periplasm, where it is protected from proteolysis via an interaction with MucB (Mathee *et al.*, 1997, Schurr *et al.*, 1996). The envelope stress response is controlled via a regulated intramembrane proteolysis cascade: MucB dissociates from MucA

upon detection of stress, allowing the proteases AlgW and MucP to cleave MucA from the inner membrane (Qiu *et al.*, 2007, Wood & Ohman, 2009). In the cytoplasm, MucA is further degraded by ClpXP, releasing AlgU to interact with RNAP and activate the AlgU regulon (Qiu *et al.*, 2008). AlgU regulates at least 350 genes, including those responsible for the production of itself, its cognate anti-sigma factor MucA, and the alginate biosynthetic enzymes (Firoved & Deretic, 2003, Schulz *et al.*, 2015, Wood & Ohman, 2009). This system is homologous to the well-studied envelope stress response in *Escherichia coli*: MucA shares 28% identity (72% similarity) to the anti-sigma factor RseA. Furthermore, the cognate sigma factor of the *E. coli* RseA, called RpoE, is homologous to and functionally interchangeable with AlgU (Yu *et al.*, 1995), with 66% identity (93% similarity).

For several reasons, *mucA* is assumed to be dispensable for *P. aeruginosa* viability. First, after being released from the cell membrane, MucA is presumed to be fully degraded in the cytoplasm by ClpXP (Qiu *et al.*, 2008). Second, *mucA* mutations commonly arise in CF clinical isolates (Boucher *et al.*, 1997, Candido Cacador *et al.*, 2018, Ciofu *et al.*, 2008, Martin *et al.*, 1993, Pulcrano *et al.*, 2012), and many laboratory *mucA* mutants exist in the literature (Gallagher *et al.*, 2011, Liberati *et al.*, 2006, Skurnik *et al.*, 2013, Turner *et al.*, 2015). Third, there are three published strains in which the entirety of *mucA* is removed from the genome (Intile *et al.*, 2014, Jones *et al.*, 2010, Pritchett *et al.*, 2015). Paradoxically, three different whole-genome studies in two different strains of *P. aeruginosa* identified *mucA* as an essential gene, based on the low observed frequency of transposon insertions in this gene (Lee *et al.*, 2015, Liberati *et al.*, 2006, Skurnik *et al.*, 2013). Additionally, it has been anecdotally noted that *mucA* could not be deleted from the *P. aeruginosa* PAO1 genome (Panmanee *et al.*, 2019). To investigate this paradox, we systematically attempted to delete *mucA* from *P. aeruginosa* using allelic exchange. Our results show that a portion of *mucA* is required for bacterial viability in multiple *P. aeruginosa* strains. Furthermore, our work shows that *mucA* was no longer essential in a strain lacking *algU*, and that *mucA* alleles that encode for proteins that do not interact with AlgU were insufficient to rescue viability or led to a growth defect. We found that *algU* mutations encoding for a less active sigma factor could relieve *mucA* essentiality, and that overproduction of AlgU in the absence of MucA was toxic. Interestingly, our works suggests that *mucA* essentiality can be suppressed by increasing the levels of the housekeeping sigma factor RpoD. Together, our results strongly suggest that the unregulated activity of AlgU itself, in the absence of MucA, leads to bacterial cell death.

RESULTS

mucA is essential for viability in a diverse set of *P. aeruginosa* isolates

To determine if *mucA* is essential, we attempted to delete the gene from various *P. aeruginosa* strains, using a modified allelic exchange protocol to turn it into a robust assay (Fig S1). We introduced a deletion allele of *mucA*, which is missing >95% of the coding region, into *P. aeruginosa* via conjugation and selected for merodiploids. Via PCR, we confirmed that isolates contained both the endogenous and deletion alleles of *mucA* in the genome. Using six confirmed merodiploids, we then counter-selected for loss of one of the two alleles. Isolates were then tested via PCR to determine which *mucA* allele the isolate resolved to. If a gene is non-essential, isolates that resolved to either the wild-type or deletion allele should be observed. However, if a gene is essential, then only the cells that resolved to the wild-type allele should be isolated, as cells that resolved to the deletion allele would not survive due to the absence of the gene. To give statistical power to our assay, we performed three biological replicates for strains from which we were unable to delete *mucA*, for a minimum total of 125 isolates screened. We performed this assay on a diverse set of wild-type *P. aeruginosa* strains, including four common laboratory isolates as well as seven clinical and environmental isolates (Table 1). These strains are not only isolated from diverse locations, but they also vary in their colony morphology and their exopolysaccharide production profile (Colvin *et al.*, 2012). For all strains tested, all observed isolates resolved to the wild-type *mucA* allele ($p < 0.0001$, Fisher's exact test), strongly suggesting that *mucA* is essential in a variety of wild-type *P. aeruginosa* strains. Since all tested strains required *mucA* for viability, we continued our experiments using the laboratory isolate PAO1 as a representative strain.

Alginate biosynthesis is not solely responsible for *mucA* essentiality

Clinical isolates containing *mucA* mutations are mucoid due to their overproduction of alginate (Martin *et al.*, 1993). To determine if alginate overproduction is responsible for *mucA* essentiality, we attempted to delete *mucA* from a strain lacking *algD*, a key alginate biosynthesis gene (Deretic *et al.*, 1987). Using our allelic exchange assay, we were unable to delete *mucA* in this background (Table S1).

Expression of the alginate biosynthesis genes is controlled by three AlgU-regulated transcription factors that are active in mucoid cells: AlgB, AlgR, and AmrZ (Martin *et al.*, 1994, Wozniak & Ohman, 1994, Wozniak *et al.*, 2003). Since these transcription factors regulate a suite of genes in addition to those involved in alginate biosynthesis (Huang *et al.*, 2019, Jones *et al.*, 2014,

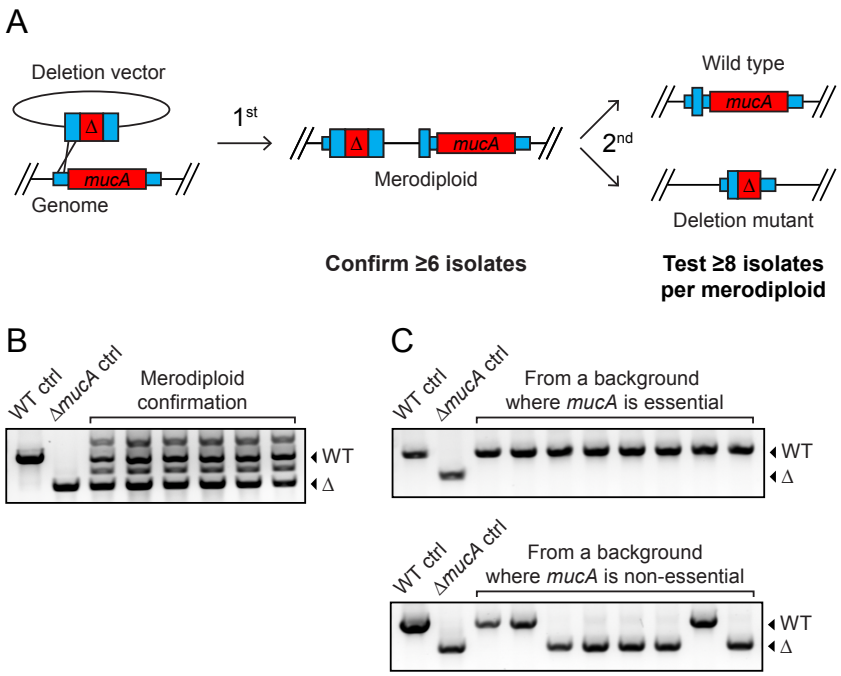


Figure S1. Allelic exchange assay.
(A) Schematic of assay. To delete the endogenous *mucA* (short red box with *mucA*) from the genome, the deletion vector pBT396, which contains an allele of *mucA* that is missing >95% of the coding region (tall red box with delta), was introduced into *P. aeruginosa* via conjugation. Regions of homology (tall blue boxes) to the genome (short blue boxes) approximately 400 base pairs in length flank the deletion allele and allow for recombination into the genome (X) to create a merodiploid. This first recombination event (1st) can be selected for using antibiotics due to integration of the vector backbone, which contains a gentamicin resistance marker. At least six merodiploids were confirmed via PCR to ensure the genome contained both the deletion and endogenous *mucA* allele. Merodiploids can then undergo a second recombination event (2nd) that will lead to the loss of one of the two alleles, resolving to either the endogenous or deletion allele, which results in a cell that is either wild-type (top) or a deletion mutant (bottom), respectively. For each of the six confirmed merodiploids, we counter-selected for the second recombination event via the loss of *sacB*, a vector backbone marker. Using PCR, eight isolates per merodiploid were tested to determine which allele each isolate resolved to. If a gene is non-essential, we expect to observe both wild type or deletion mutants. However, if a gene is essential, we expect to isolate only those cells that resolved to wild type, as the cells cannot survive with the deletion allele. If we are unable to delete *mucA* from the first replicate of this experiment, we perform two additional biological replicates, for a minimum total of 125 colonies screened. If all isolates resolve to wild-type, we deem *mucA* to be essential in that strain background ($p < 0.0001$, Fisher's exact test). **(B)** Representative image of merodiploid confirmation. The PCR products corresponding to the endogenous allele and the deletion allele are indicated with an arrow labelled "WT" and "Δ," respectively. Positive (WT ctrl; PAO1) and negative (Δ*mucA* ctrl; BTPa355) controls are included. PCR products from six representative merodiploids are shown. **(C)** Representative image of PCR products from isolates after the second recombination. Image is labeled as in (B). Top, the PCR products of 8 isolates from a strain in which *mucA* was deemed essential. Bottom, the PCR products of 8 isolates from a strain in which *mucA* was not essential.

Table 1. *mucA* is essential in a variety of wild-type *P. aeruginosa* strains.

Strain background	Description†	Number of isolates resolved to	
		WT	Δ*mucA*
PAO1	Laboratory strain, Class II	168	0*
PA14	Laboratory strain, Class I	147	0*
PA103	Laboratory strain, unclassified	361	0*
PAK	Laboratory strain, unclassified	191	0*
CF127	Mucoid CF isolate, unclassified	136	0*
CF18	Non-mucoid CF isolate, unclassified	129	0*
CF27	Rugose CF Isolate, Class IV	152	0*
X13273	Blood isolate, Class II	142	0*
X24509	UTI isolate, Class II	221	0*
MSH10	Water isolate, Class III	146	0*
E2	Tomato plant isolate, Class II	141	0*

† Class identification is based on the exopolysaccharide expression profile as described in (Colvin *et al.*, 2012): Class I, Pel-dominant matrix; Class II, Psl-dominant matrix; Class III, exopolysaccharide redundant matrix users; and Class IV, matrix overproducers. CF, cystic fibrosis; UTI, urinary tract infection.

* $p < 0.0001$, Fischer's exact test

Table S1. Eliminating alginate biosynthesis does not alleviate *mucA* essentiality.

PAO1	Number of isolates resolved to	
	WT	Δ*mucA*
WT	168	0*
Δ*algD*	144	0*
Δ*algB*	158	0*
Δ*algR*	153	0*
Δ*amrZ*	131	0*
Δ*algB* Δ*algR* Δ*amrZ*	133	0*

* $p < 0.0001$, Fischer's exact test

Kong *et al.*, 2015, Leech *et al.*, 2008), we tested if overexpression of these three regulons underlies *mucA* essentiality by attempting to delete *mucA* from strains lacking these transcription factors, either individually or all together. We found that we could not delete *mucA* in any of these backgrounds (Table S1). Altogether, our data support the conclusion that eliminating alginate biosynthesis and the expression of large parts of the AlgU regulon do not alleviate *mucA* essentiality.

The first 50 amino acids of MucA are necessary and sufficient for cell viability

While we were unable to delete *mucA* using an allele lacking >95% of the coding region, *mucA* is commonly mutated in clinical isolates and many *mucA* transposon mutants exist (Fig S2). These data suggest that only a portion of *mucA* is essential. To determine the essential portion of *mucA*, we first constructed a series of strains containing an ectopic chromosomally-integrated *mucA*, driven by its native promoter. We then attempted to delete the endogenous wild-type *mucA* from these strains. Although we designed these ectopic alleles to encode for a protein with a His-tag, we were unable to probe whether equivalent amounts of protein were produced across our strains, since truncated versions of MucA that lack the MucB binding domain are cleaved out of the membrane and degraded in the cytosol, as shown in previous work (Qiu *et al.*, 2008). However, we expect that the ectopic alleles have similar expression levels prior to endogenous *mucA* deletion, as they are all under the same promoter and located in the same genomic site. Furthermore, these strains that contain two *mucA* alleles were non-mucoid in all cases, showing that the endogenous *mucA* allele, which encodes for the full-length protein, is dominant over the ectopic alleles that encode for truncated MucA.

Mutant MucA proteins of varying lengths were ectopically expressed to determine the necessary portion of MucA. Full-length MucA is 194 amino acids (aa). Based on the co-crystal structures of MucA with AlgU and MucB (Li *et al.*, 2019), the first 78 residues of MucA interact with AlgU, and the last 48 residues of MucA interact with MucB. We engineered *mucA* alleles that encode for various truncated proteins (Fig 1A). Since we expected that the resultant strain where the endogenous *mucA* is deleted would be mucoid in some cases, we used a Δ*algD* background to eliminate alginate production, which made the strains easier to manipulate. We then attempted to delete the endogenous *mucA* from these strains. Since the flanking regions of these two alleles differ, our deletion allele specifically targets the native allele. We were able to easily recover isolates that resolved to the *mucA* deletion allele from strains containing ectopic alleles that encode for the full length MucA (aa 1-194), as well as MucA aa 1-143, aa 1-110, aa 1-75,

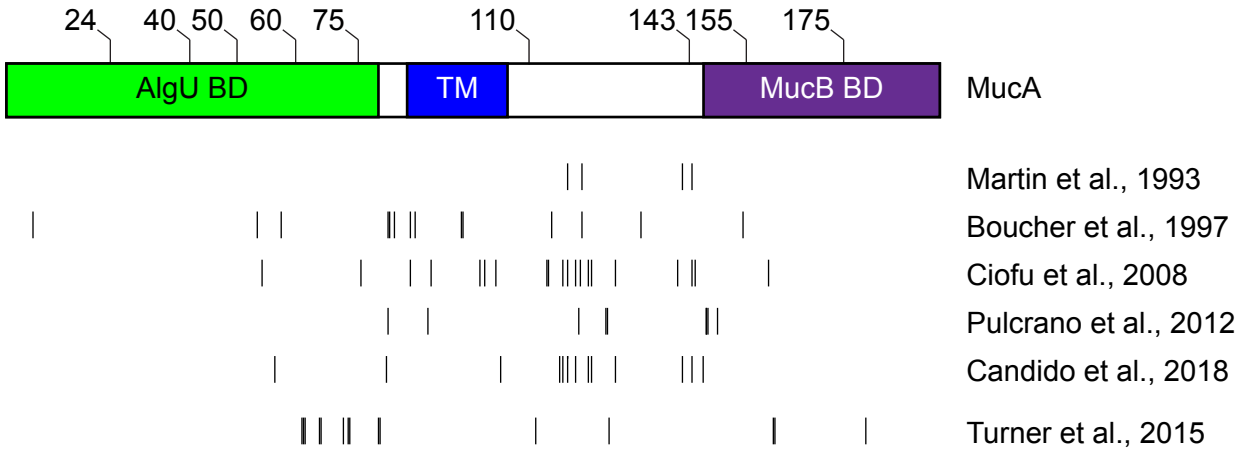


Figure S2. Published mutations in *mucA*.
Top, schematic of MucA with the regions indicated encoding for the AlgU binding domain (green, AlgU BD), the transmembrane domain (blue, TM), and the MucB binding domain (purple, MucB BD). Hash marks on top indicate the residue number in MucA. Bottom, sites of *mucA* mutations that lead to the production of a truncated protein in published isolates. Each line represents one or more individual isolates described in the references on the right. The mutations for the upper five references (Boucher *et al.*, 1997, Candido Cacador *et al.*, 2018, Ciofu *et al.*, 2008, Martin *et al.*, 1993, Pulcrano *et al.*, 2012) are in cystic fibrosis clinical isolates of *P. aeruginosa*, while the mutations in the last reference (Turner *et al.*, 2015) are random transposon insertions in the laboratory PAO1 and PA14 strains.

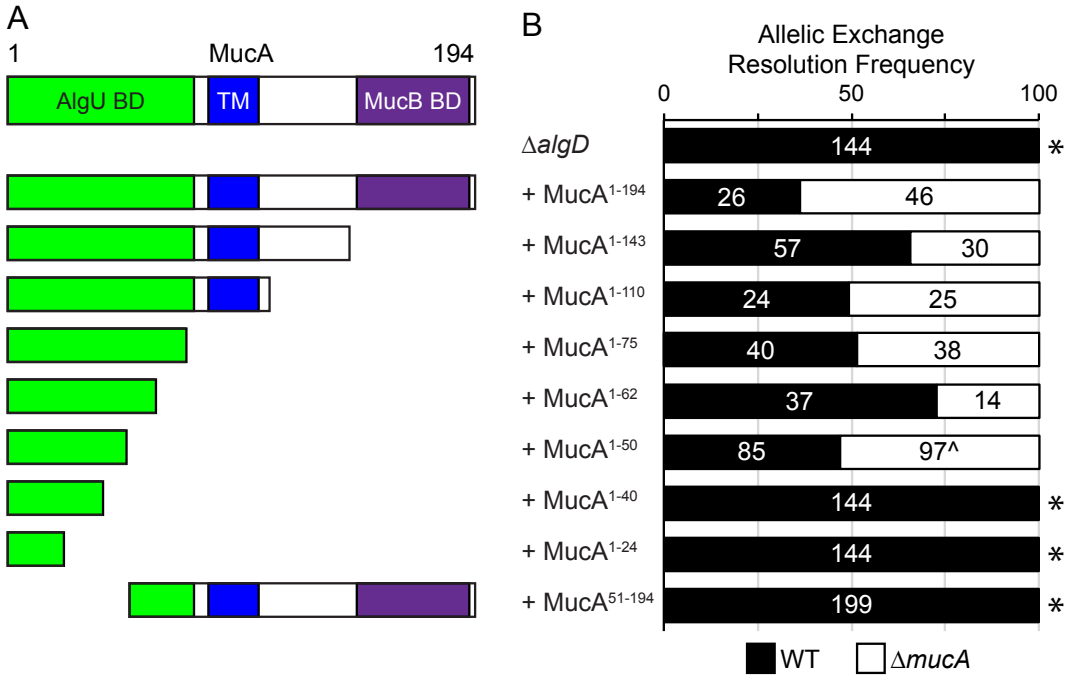


Figure 1. The first 50 amino acids of MucA are necessary and sufficient for viability. **(A)** Schematic of MucA encoded by the ectopic *mucA* alleles in the PAO1 Δ*algD* strains tested for *mucA* essentiality in (B). MucA aa 1-143 lacks the MucB binding domain and is the product of the common *mucA22* allele. MucA 1-110 lacks the entire periplasmic domain. MucA aa 1-75 contains most of the AlgU binding domain, while shorter truncations contain only parts of the AlgU binding domain (MucA aa 1-62, 1-50, 1-40, and 1-24). Green, AlgU binding domain (AlgU BD); blue, the transmembrane domain (TM); and purple, the MucB binding domain (MucB BD). **(B)** Frequency of observed isolates resolving to the endogenous wild-type *mucA* allele (WT, black) or the deletion allele (Δ*mucA*, white) in the allelic exchange assay. Super-imposed on the bars are the number of isolates that were tested in each category. Asterisk, $p < 0.0001$; Fisher's exact test. Caret, slower growing isolates.

and aa 1-62 (Fig 1B). These results show that the requirement for *mucA* can be complemented *in trans*, and suggest that the gene product of *mucA* is required for viability, irrespective of its genomic location.

Interestingly, while we were able to delete the endogenous *mucA* from strains containing an ectopic *mucA* that encoded for aa 1-50 (Fig 1B), isolates that resolved to the deletion allele grew up more slowly than those that resolved to the wild-type allele. This suggests that while this allele is sufficient for viability, it is not well tolerated by the cells. All tested isolates of strains carrying *mucA* alleles encoding shorter products (aa 1-40 and aa 1-24) resolved to the wild-type allele. These results show that while an allele encoding the first 50 residues of MucA is sufficient for cell viability, alleles encoding for shorter lengths are not.

To determine if the first 50 residues of MucA are necessary for viability, we constructed a strain with an ectopic *mucA* that encodes for aa 51-194. We were unable to delete the native *mucA* from this strain (Fig 1B). Together, these results suggest that the first 50 amino acids of MucA are necessary and sufficient for cell viability in *P. aeruginosa*. Consistent with these results, while many clinical and engineered *mucA* mutations have been reported (Boucher *et al.*, 1997, Candido Cacador *et al.*, 2018, Ciofu *et al.*, 2008, Martin *et al.*, 1993, Pulcrano *et al.*, 2012, Turner *et al.*, 2015), almost all fall outside the region of *mucA* that encodes for the first 50 residues (Fig S2). Furthermore, this region of MucA corresponds to the AlgU binding domain, suggesting that the physical interaction between the two proteins is important for cell viability.

Depletion of MucA leads to cell death

To evaluate the effect of the nutrient conditions on *mucA* essentiality, we tested the effect of depleting MucA on *P. aeruginosa* cell viability in different media. To deplete MucA from cells, we engineered a strain lacking the native *mucA* and containing a chromosomally-integrated rhamnose-inducible copy (Δ*mucA att*Tn7::P_{rhaBAD}-*mucA*). We removed rhamnose from growing Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* cells and determined the viability of cells over time based on their ability to recover on medium containing rhamnose. Since cells would cease producing MucA in the absence of rhamnose, the MucA present in the cells at the start of the experiment would deplete as the cells divided. In all four media we tested, if rhamnose was not removed at the beginning of the experiment, the cells increased in density by ~2-logs over the duration of the experiment. In comparison, when rhamnose was removed, the Δ*mucA att*Tn7::P_{rhaBAD}-*mucA*

cells lost viability over time in all four media (Fig 2). Together, these results show that *mucA* is essential in *P. aeruginosa* in various nutritional environments.

The interaction of MucA with AlgU is required for cell survival

Since the only described function for MucA is to inhibit AlgU, we hypothesized that *mucA* essentiality is rooted in its regulation of AlgU. In such a case, *mucA* essentiality should require *algU*. Therefore, we attempted to delete *mucA* from a strain lacking *algU* (Δ*algU*). We found that we were able to delete *mucA* in the absence of *algU*, with 16 of the tested isolates resolving to the deletion allele out of the 48 colonies tested (see Fig 4A), showing that *mucA* essentiality is *algU*-dependent.

Because our data suggest that the AlgU-binding domain of MucA is required for viability (Fig 1B), we hypothesized that the physical interaction between MucA and AlgU is necessary for cell survival. In the co-crystal structure of MucA and AlgU (Li *et al.*, 2019), four residues in MucA make more than one hydrogen bond with AlgU: D15, E22, R42, and E72 (Fig 3A). Although E72 likely contributes to the binding energy between these two proteins, it is unlikely that E72 is critical to the MucA-AlgU interaction, since the first 50 amino acids of MucA are sufficient for viability (Fig 1B). Therefore, we engineered alleles that encode for MucA D15A, E22A, or R42A to maximally affect the hydrogen bonding while limiting the effects on overall protein structure. We used MucA aa 1-75 as a base because the co-crystal structure includes the first 78 residues of MucA. We tested the effect of these substitutions on the MucA-AlgU interaction via a yeast-two hybrid assay. Using beta-galactosidase activity as a proxy for their interaction, wild-type MucA aa 1-75 strongly interacted with AlgU (Fig 3B). This interaction was dependent on the presence of both MucA and AlgU, and the effect was not directional (Fig S3). In comparison, the D15A, E22A, and R42A MucA mutants failed to interact with AlgU (Fig 3B).

To determine the effects of substituting these MucA residues on viability, we engineered strains carrying an ectopic *mucA* encoding these substitutions and then attempted to delete the native *mucA*. As described above, an allele encoding MucA aa 1-75 was sufficient for viability (Fig 1B). However, we were unable to delete the endogenous *mucA* from strains carrying alleles encoding the D15A and R42A substitutions, suggesting that the mutant protein is not sufficient for viability (Fig 3C). For the strain carrying a *mucA* allele encoding the E22A substitution, we were able to delete the endogenous *mucA*. However, isolates that resolved to the deletion allele in this strain grew up slower than those that resolved to the wild-type allele. Similar to the *mucA*

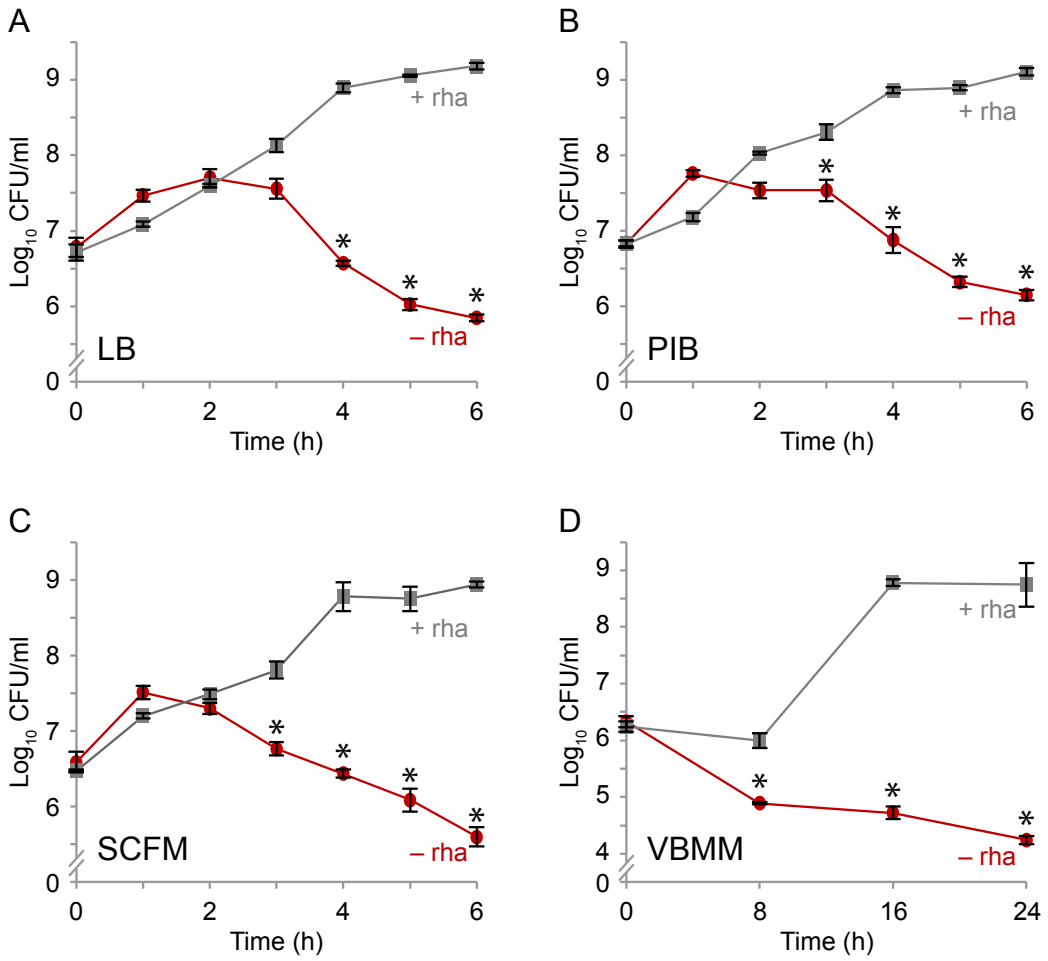


Figure 2. Depletion of MucA results in cell death.
Viable colony counts of PAO1 Δ*mucA* attTn7::P_{rhaBAD}-*mucA*-10His over time in **(A)** LB, **(B)** PIB, **(C)** SCFM and **(D)** VBMM with (+ rha; gray squares) or without (– rha; red circles) 0.05% rhamnose. Viable colony counts after incubation in the indicated condition and time was determined by plating the cells on LB with 0.05% rhamnose to allow cells to recover and grow. Hash, broken y-axis; error bars, SEM (N=3). Asterisk, statistically different from that at the same time point grown in the presence of rhamnose ($p < 0.01$, N=3, mixed model ANOVA with post-hoc Bonferroni test).

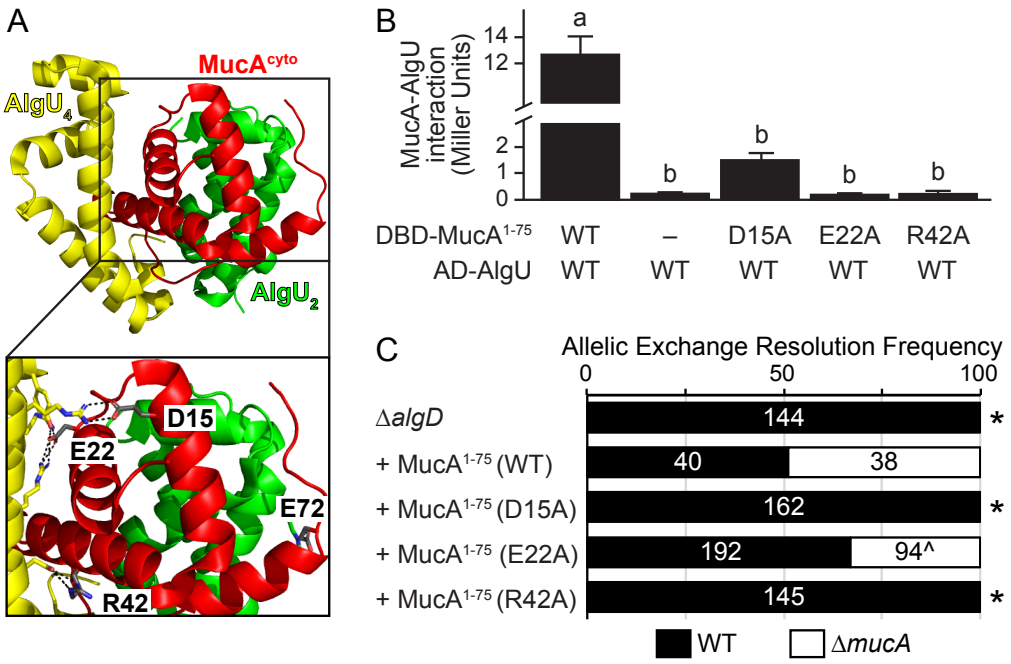
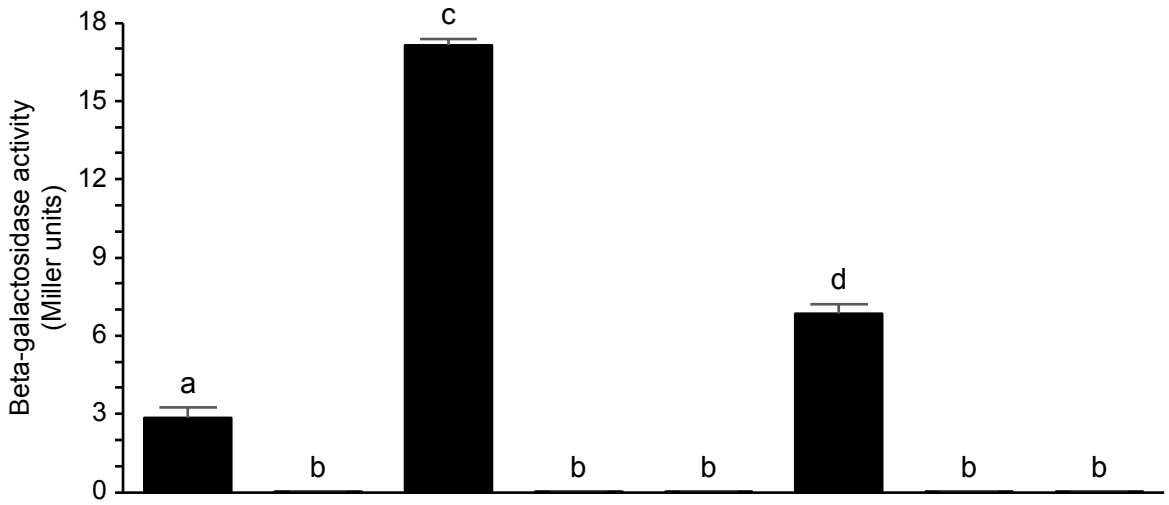


Figure 3. The physical interaction of MucA and AlgU is required for survival.
(A) Four residues of MucA make greater than one predicted hydrogen bond with AlgU. The MucA-AlgU co-crystal structure (PDB 6IN7; Li et al., 2019) with the cytosolic domain of MucA (aa 1-78; red) and Regions 2 and 4 of AlgU (green, $AlgU_2$; yellow, $AlgU_4$) is shown. The residues of MucA (grey) that are predicted to make more than one hydrogen bond with AlgU are labeled in the inset. Black dotted lines, predicted hydrogen bonds; red atoms, oxygen; blue atoms, nitrogen. **(B)** Substitution of MucA residues at its interface with AlgU abolish their binding via yeast two-hybrid. The first 75 residues of MucA were fused to the Gal4 DNA binding domain (DBD-$MucA^{1-75}$) and AlgU was fused to the Gal4 activation domain (AD-AlgU). Interaction of MucA and AlgU led to *lacZ* expression. Beta-galactosidase activity (in Miller units) was used as a proxy for the protein interaction strength. WT, wild-type protein sequence; –, no fusion protein included; hash, broken y-axis; error bars, SEM (N=3); letters, statistical groups with the different letters representing statistically different groups ($p < 0.01$; biological triplicate with technical quadruplicates; ANOVA with post-hoc Tukey HSD). **(C)** Frequency of observed isolates resolving to the endogenous wild-type *mucA* allele (WT, black) or the deletion allele (Δ*mucA*, white) in the allelic exchange assay, using PAO1 Δ*algD att*Tn7::P_{algU}-*mucA*, where *mucA* encodes for the indicated substitution. Super-imposed on the bars are the number of isolates that were tested in each category. Asterisk, $p < 0.0001$; Fisher's exact test. Caret, slower growing isolates.



DBD	Krev1	Krev1	$MucA^{1-75}$	$MucA^{1-75}$	–	AlgU	–	AlgU
AD	Ral^{WT}	Ral^{Mut}	AlgU	–	AlgU	$MucA^{1-75}$	$MucA^{1-75}$	–
Avg	2.832	0.017	17.142	0.008	0.010	6.833	0.017	0.009
SEM	0.402	0.010	0.221	0.001	0.004	0.355	0.006	0.003

Figure S3. MucA aa 1-75 and AlgU interact via yeast two-hybrid assay.
The indicated proteins were fused to the Gal4 DNA binding domain (DBD; “bait”) or the Gal4 activation domain (AD; “prey”). Interaction of the bait and prey proteins drive the expression of *lacZ*. Beta-galactosidase activity (in Miller units) was used as a proxy for the protein interaction strength. The average (Avg) and standard error of the mean (SEM) of each bar are indicated (N=3). As positive and negative controls, Krev1 is known to interact with wild-type RalGDS (Ral^{WT}), but not the mutant RalGDS (Ral^{Mut}) via yeast two-hybrid. AlgU, construct encoding full-length wild-type AlgU; $MucA^{1-75}$, construct encoding only the first 75 residues of wild-type MucA; –, no fusion protein included; error bars, SEM (N=3); letters, statistical groups with the different letters representing statistically different groups ($p < 0.01$; biological triplicate with technical quadruplicates; ANOVA with post-hoc Tukey HSD).

allele encoding only the first 50 amino acids, these results suggest that while MucA E22A is sufficient for viability, the cells do not tolerate it well. As predicted above, we were able to delete the endogenous *mucA* from a strain carrying an allele encoding the E72A substitution with 9 of 48 tested isolates resolving to the deletion allele, suggesting MucA E72A is sufficient for viability.

Mutations that reduce AlgU activity alleviate the requirement for MucA

Since MucA inhibits AlgU from binding to RNAP, we hypothesized that *mucA* essentiality is due to its inhibition of the AlgU regulon, which includes *algU* itself. To start to test this hypothesis, we reasoned that strains containing a mutant AlgU with lower affinity for DNA would alleviate *mucA* essentiality, since expression of the AlgU regulon would inherently be lower in such mutants. Therefore, we engineered Δ*algU* strains with an ectopic chromosomally-integrated *algU* allele encoding such DNA-binding mutants. Based on homology to *E. coli* RpoE mutants that have decreased *in vitro* transcriptional activity (Campagne *et al.*, 2014), we engineered *algU* alleles that encode for K57A and N81A. As described above, we were able to delete *mucA* from a Δ*algU* strain (Fig 4A). This phenotype could be rescued via the ectopic addition of a wild-type *algU* allele, as we were no longer able to delete *mucA* from such a strain. In contrast, *mucA* could be readily deleted from strains carrying alleles encoding AlgU K57A or N81A, showing that such mutant alleles failed to rescue the Δ*algU* phenotype (Fig 4A). To determine the effect of these substitutions on AlgU activity, we induced envelope stress using D-cycloserine (Wood *et al.*, 2006) and measured AlgU activity using a plasmid-borne *gfp* reporter driven under the AlgU-regulated *algD* promoter (Damron *et al.*, 2009). Similar to what is seen for RpoE (Campagne *et al.*, 2014), these mutant strains had reduced AlgU activity upon induction of envelope stress, relative to the strain containing a wild-type *algU* (Fig 5A,B). These results suggest that AlgU mutants with reduced sigma factor activity can suppress *mucA* essentiality.

As mentioned above, others have reported strains in which the entirety of *mucA* is deleted from *P. aeruginosa* PAO1, PAK, and PA103 (Intile *et al.*, 2014, Jones *et al.*, 2010, Pritchett *et al.*, 2015). Since we were unable to delete *mucA* from these three strain backgrounds (Table 1), we hypothesized that these Δ*mucA* strains may contain suppressor mutations that allowed for their survival in the absence of *mucA*. To identify these suppressors, we sequenced the genomes of these Δ*mucA* isolates. In the PAO1 Δ*mucA* strain (Pritchett *et al.*, 2015), we found that while the native *mucA* was deleted, the strain contained a second full-length copy of *algU* and a *mucA* allele that encoded for MucA aa 1-155 elsewhere in the genome. As expected from our data

(Fig 1B), we were able to delete the native *mucA* allele from a PAO1 strain containing an ectopic allele that encoded for MucA aa 1-155 (with 5 of the 28 tested isolates resolving to the deletion allele), confirming that the ectopic *mucA* allele in the published PAO1 Δ*mucA* strain is sufficient for viability in the absence of the endogenous *mucA*. In comparison, the PAK and PA103 Δ*mucA* strains (Intile *et al.*, 2014, Jones *et al.*, 2010) contain missense mutations in *algU,* which results in AlgU A58T and E46G, respectively. We replicated these mutations in PAO1 by inserting *algU* alleles encoding these substitutions in a Δ*algU* strain. We were able to delete *mucA* from these strains (Fig 4A). These results confirm that the *algU* alleles in the published PAK and PA103 Δ*mucA* strains suppress *mucA* essentiality. To examine the activity of these mutant proteins, we used our reporter assay. Similar to what we saw with the AlgU DNA-binding mutants, we found that strains carrying these AlgU substitutions had reduced sigma factor activity relative to that of strains carrying wild-type AlgU (Fig 5B). We note that our reporter assay does not appear to be very sensitive to low levels of AlgU activity. Both published PAK and PA103 Δ*mucA* strains are mucoid (Intile *et al.*, 2014, Jones *et al.*, 2010), suggesting that AlgU A58T and E46G are not completely inactive. While we cannot distinguish this low level of activity from the null case, our results show that nevertheless these AlgU mutants have significantly less transcriptional activity than the wild-type protein (Fig 5A,B).

To identify additional suppressors of *mucA* essentiality, we used the Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* strain. This strain was not viable when grown in the absence of rhamnose, but natural revertants arose at a frequency of less than 1 in 10^9 colony forming units. We sequenced the *algU* gene in 25 of these revertants, since our work shows that AlgU mutants can suppress *mucA* essentiality (Fig 4A). All 25 isolates contained mutations in *algU* that are expected to encode for sigma factors with reduced activity (Fig 4B, Table S2). There were 10 revertants with deletions or nonsense mutations of *algU*, encoding either no product or a truncated product completely lacking Region 4 of the sigma factor. There were 6 revertants that contained multi-base pair duplications in *algU* that would lead to the insertion of 3 or 4 amino acids in Region 2 helix 3 of the sigma factor. There were 9 revertants containing missense mutations, 8 of which were unique, encoding the following substitutions: D18G, A21V, Y29C, A47T, D49G, Y59C, N81D, and R174G. Using a model of σ^E in complex with the RNAP core and the promoter element, we expect these insertions and substitutions to affect sigma factor folding, RNAP core interactions, or promoter element interactions (Fig S4). To determine the effect of these 25 mutations on AlgU activity, we tested AlgU function using our *algD* reporter assay in these natural revertants. As expected, we saw that all 25 revertants had much lower AlgU activity than

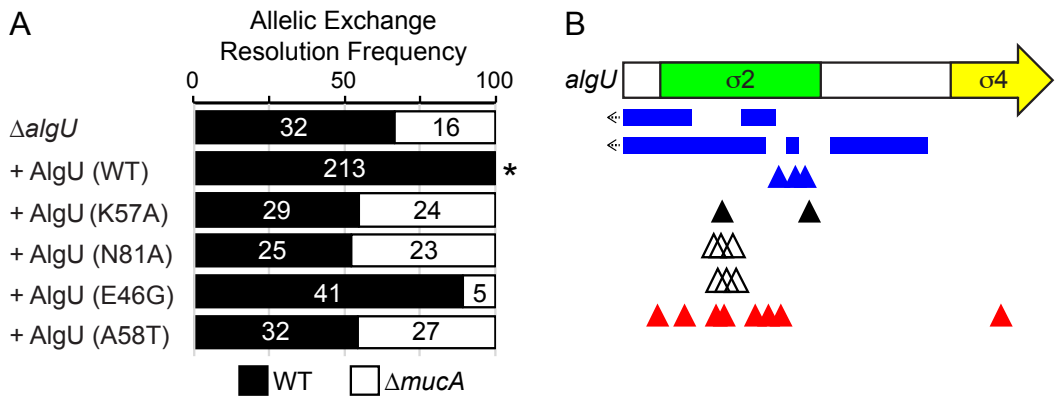


Figure 4. Mutations in AlgU can suppress *mucA* essentiality.
(A) Frequency of observed isolates resolving to the endogenous wild-type *mucA* allele (WT, black) or the deletion allele (Δ*mucA*, white) in the allelic exchange assay, using PAO1 Δ*algU att*Tn7::P_{algU}-*algU*, where *algU* encodes for the indicated substitution. Super-imposed on the bars are the number of isolates that were tested in each category. Asterisk, $p < 0.0001$; Fisher's exact test. **(B)** Schematic of mutations seen in revertants that could grow in the absence of *mucA*. Revertants were selected by growing PAO1 Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* on media lacking rhamnose. Blue rectangles, multi-base pair deletions; left arrow, deletion extends into the promoter; blue triangles, single base pair deletions; black triangles, nonsense mutations; white triangles, duplications resulting in 3 or 4 amino acid insertions; red triangles, missense mutations. See Table S2 for a full description of the *algU* mutations.

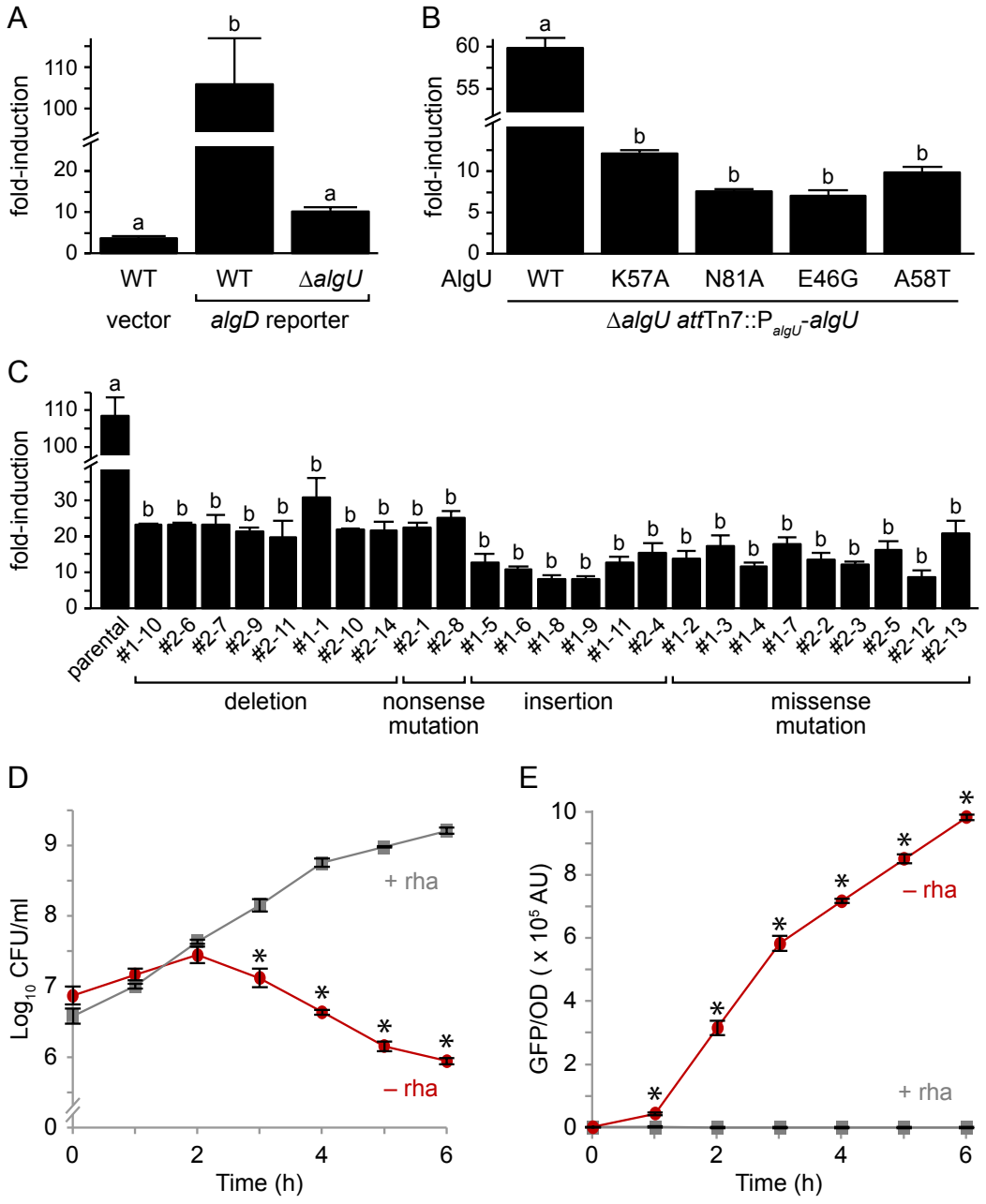


Figure 5. AlgU mutants that suppress *mucA* essentiality have decreased sigma factor activity.
(A) Fold-induction of GFP in PAO1 wild-type (WT) and Δ*algU* strains carrying a plasmid with either a promoter-less *gfp* (vector) or a *gfp* driven by the *algD* promoter (*algD* reporter), which is positively regulated by AlgU, after a 2h D-cycloserine treatment to activate envelope stress. GFP fluorescence was normalized to cell density and was then divided by the signal from

untreated cells to determine the fold-induction. Hash, broken y-axis; error bars, SEM (N=3); letters, statistical groups with the different letters representing statistically different groups ($p < 0.01$; biological triplicate with technical quadruplicates; ANOVA with post-hoc Tukey HSD). **(B)** Fold-induction of GFP in strains of PAO1 Δ*algU att*Tn7::P_{algU}-*algU*, where *algU* encodes for the indicated substitution. The experiment and statistics are as described in (A). WT, the wild-type AlgU sequence. **(C)** Fold-induction of GFP in the revertants isolated from PAO1 Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* (parental) that can grow in the absence of MucA. The experiment and statistics are as described in (A), except all cells were grown in the presence of 0.05% rhamnose to allow for comparison to the parental strain, which grows only in the presence of rhamnose. The revertant isolate numbers are shown and are grouped based on their *algU* mutation. **(D)** Viable colony counts of PAO1 Δ*mucA* attTn7::P_{rhaBAD}-*mucA*-10His carrying the *algD* reporter over time in LB with (+ rha; gray squares) or without (– rha; red circles) 0.05% rhamnose. Hash, broken y-axis; error bars, SEM (N=3). Asterisk, statistically different from that at the same time point grown in the presence of rhamnose ($p < 0.01$, N=3, mixed model ANOVA with post-hoc Bonferroni test). **(E)** Normalized GFP fluorescence of the samples in (D). GFP fluorescence was normalized to cell density. The statistical analysis is as described in (D).

Table S2. Description of changes found in *algU* of PAO1 Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* revertants that can grow in the absence of *mucA* expression.

	Isolate	Genomic Change to *algU*[†]	Effect on AlgU[‡]
Multi-base pair deletions			
	#1-10	831242-831394 del	No product made
	#2-9	831298-831491 del	No product made
	#2-7	831467-831473 del	I56fs X97
	#2-11	831469-831502 del	K57fs X88
	#2-6	831595-831715 del	V99fs X107
Single base pair deletions			
	#2-14	831467 del	I56fs X99
	#1-1	831510 del	Y72fs X99
	#2-10	831538 del	I80fs X99
Nonsense mutations			
	#2-1	831433 C>T	Q45X
	#2-8	831521 G>A	W74X
Multi-base pair insertions			
	#1-9	831427-831435 ins GACGCCCAG	D43-A44 ins DAQ
	#1-8	831430-831438 ins GCCCAGGAA	A44-Q45 ins AQE
	#1-11	831436-831444 ins GAAGCCCAG	E46-A47 ins EAQ
	#2-4	831436-831444 ins GAAGCCCAG	E46-A47 ins EAQ
	#1-5	831445-831456 ins GACGTAGCCCAG	D49-V50 ins DVAQ
	#1-6	831451-831459 ins GCCCAGGAA	A51-Q52 ins AQE
Missense mutations			
	#1-3	831353 A>G	D18G
	#2-12	831362 C>T	A21V
	#2-3	831386 A>G	Y29C
	#2-2	831439 G>A	A47T
	#1-2	831446 A>G	D49G
	#2-13	831446 A>G	D49G
	#1-7	831476 A>G	Y59C
	#2-5	831541 A>G	N81D
	#1-4	831820 C>G	R174G

[†] genomic location based on the Pseudomonas Genome Database (Winsor *et al.*, 2016) for PAO1; bp, base pair; del, deletion; ins, insertion.
[‡] fs, frameshift; ins, insertion; X, stop codon.

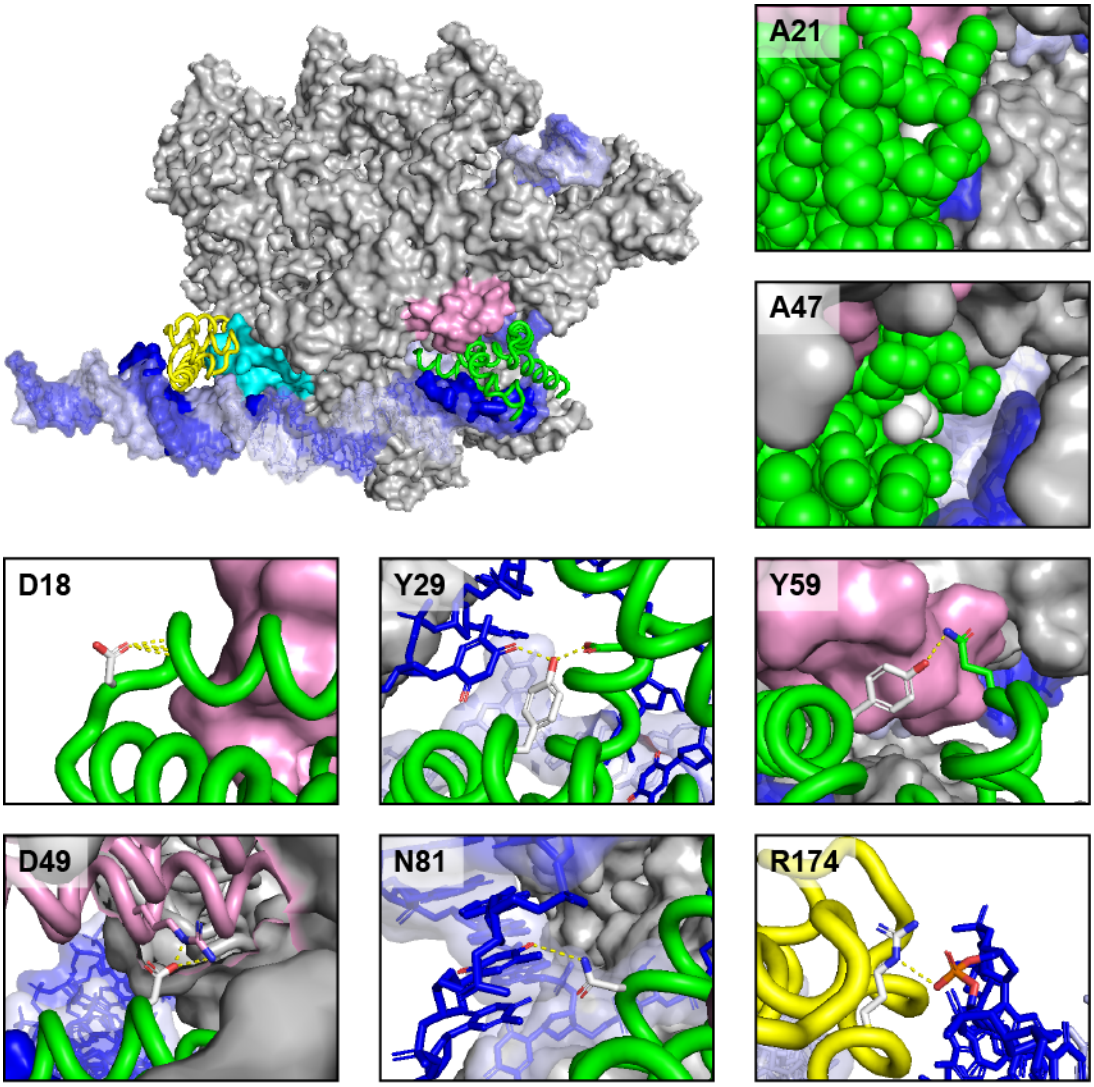


Figure S4. Location of affected AlgU residues in revertants that can grow in the absence of MucA.

Model of the RNAP holoenzyme containing σ^E bound to the promoter. Gray, RNAP core; cyan, β flap; pink, β' coiled coil; green, σ^E Region 2; yellow, σ^E Region 4; blue/light blue, promoter element. Insets, location of residues that are substituted in the revertants with missense *algU* mutations. The side chain of the affected residue is in white. Substitution of A21 and A47 (V in model) likely affect protein packing and folding of AlgU. Substitution of D18, Y29, and Y59 would reduce predicted intra- and inter-molecular hydrogen bonding and likely affect AlgU folding. Substitution of D49, N81, and R174 would likely affect the interaction of AlgU with the RNAP core or the promoter. Dashed yellow lines, hydrogen bonds; red atoms, oxygen; blue atoms, nitrogen; orange, phosphorus.

the parental strain upon induction of envelope stress with D-cycloserine (Fig 5C). Together, these data strongly suggest that *algU* mutations that encode for a protein with reduced transcriptional activity allow *P. aeruginosa* to survive in the absence of *mucA*.

Since we hypothesize that *mucA* essentiality is due to AlgU inhibition, we reasoned that we should see increased AlgU activity as MucA is depleted from cells, which would be concomitant with the decrease in viability. To test this, we repeated our MucA depletion assay in a Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* strain containing our reporter plasmid to measure AlgU activity. In addition to monitoring cell viability, we measured the GFP fluorescence (as a proxy for AlgU activity) over time. As expected, this strain had a similar viability to that seen in Figure 2 over time (Fig 5D), and as MucA was depleted, the AlgU activity increased (Fig 5E). These results show the cell death observed upon MucA depletion is correlated with a dramatic increase in AlgU activity.

Overexpression of *algU* in the absence of *mucA* leads to a growth defect

Collectively, our results suggest that *mucA* essentiality is due to unregulated AlgU activity. We therefore reasoned that overexpression of *algU* should be toxic. Supporting this hypothesis, the toxic effects of *algU* overexpression have been previously noted (Cross *et al.*, 2020, Hershberger *et al.*, 1995, Schurr *et al.*, 1994). However, studies have shown that *algU* can be overexpressed in wild-type *P. aeruginosa* (Qiu *et al.*, 2008, Schulz *et al.*, 2015). It is important to note that these wild-type strains contain *mucA*, which is positively regulated by AlgU. We therefore examined the effect of overproducing AlgU in WT, Δ*algU*, and Δ*algU* Δ*mucA* strains, using a chromosomally integrated arabinose-inducible *algU*. Of note, the Δ*algU* and Δ*algU* Δ*mucA* strains lack the positive feedback of AlgU on its own expression, and the expression of *algU* is completely dependent on the inducer in these two strains. Our results show that in the absence of inducer when AlgU is not overexpressed, all three strains had similar growth rates (Fig 6A). In the presence of 1% arabinose (i.e. high expression of *algU*), all three strains had a growth defect. However, in comparison to the other two strains, the strain lacking *mucA* failed to grow at all in the presence of this high inducer concentration.

To determine if the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* strain can grow under lower levels of *algU* induction, we tested a range of inducer concentrations (Fig 6B, Table S3). We found that although there was a growth defect, Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* was able to grow in the presence of 0.1% arabinose, suggesting that this strain was able to tolerate low levels of *algU* induction. Furthermore, as expected, the drop in growth rate in comparison to the no arabinose

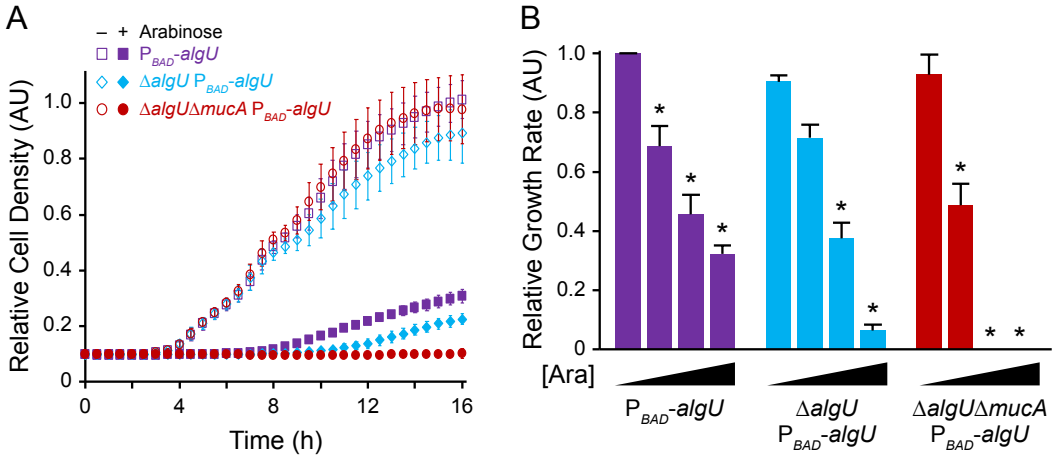


Figure 6. Overexpression of *algU* leads to a growth defect.
(A) Growth curves of PAO1 strains containing an arabinose-inducible copy of *algU* in wild-type (purple squares), Δ*algU* (blue diamonds), and Δ*algU* Δ*mucA* (red circles) backgrounds in LB. Readings are normalized to wild-type PAO1 (lacking an inducible *algU* gene). Open symbols represent conditions in the absence of arabinose; closed symbols, with 1% arabinose. Error bars, SD (n=18). **(B)** Growth rate of strains as described in (A), with increasing induction of *algU* (relative to PAO1 *att*Tn7::P_{araBAD}-*algU* grown in the absence of arabinose). Strains were grown in LB with 0%, 0.1%, 0.25%, and 1% arabinose ([Ara]). Error bars, SEM (N=3). Asterisk, statistically different from the same strain grown in the absence of arabinose ($p < 0.01$, N = 3, two-way ANOVA with post-hoc Bonferroni). See Table S3 for full statistical comparisons.

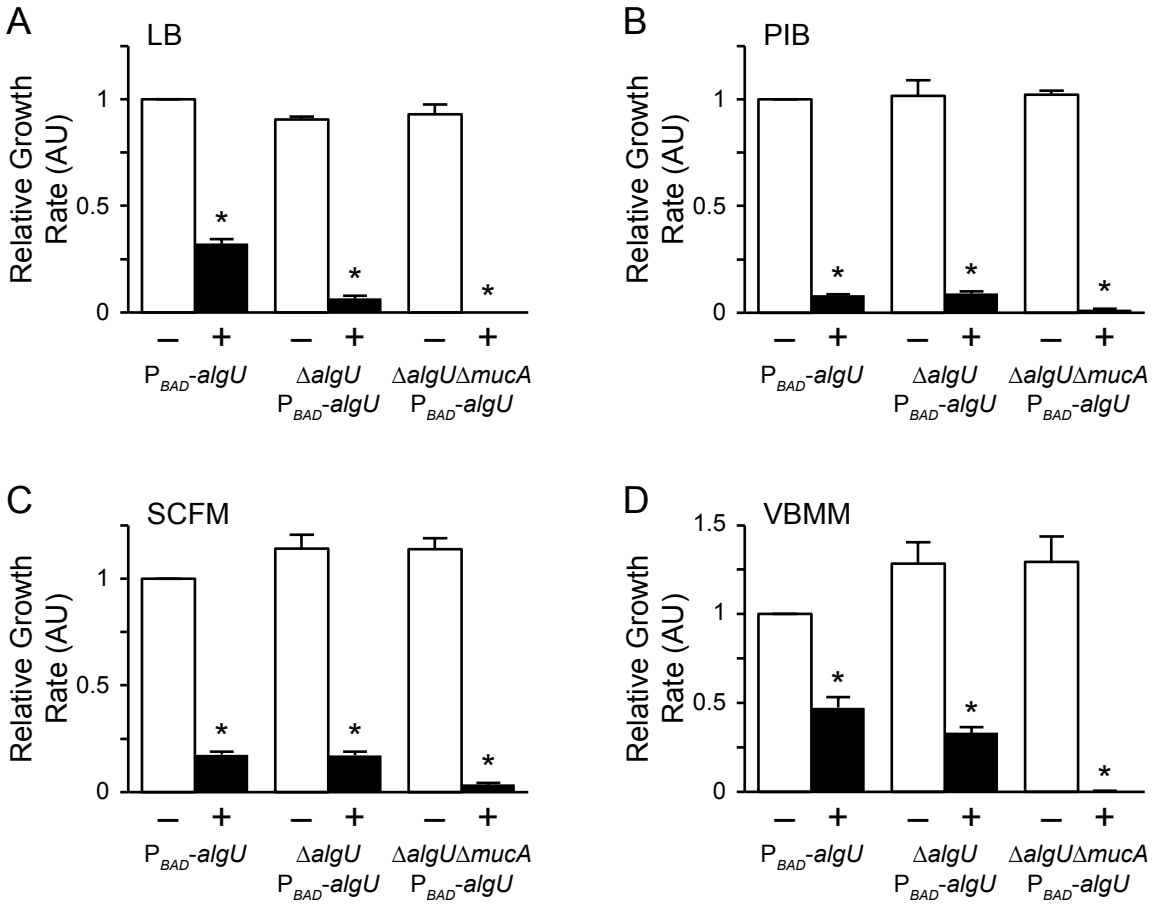


Figure S5. Overexpression of *algU* in the absence of *mucA* causes a growth defect in various media.
Growth rate of indicated strains grown in (A) LB, (B) PIB, (C) SCFM, and (D) VBMM with (+) or without (–) 1% arabinose (relative to PAO1 *att*Tn7::P_{araBAD}-*algU* grown in the absence of arabinose). Error bars, SEM (N=3). Asterisk, statistically different from the same strain grown in the absence of arabinose ($p < 0.01$, N = 3, two-way ANOVA with post-hoc Bonferroni). See Table S3 for full statistical comparisons. LB contains tryptone and yeast extract, both of which provide protein hydrolates, as the main carbon source. The main carbon source of PIB is also protein hydrolates, but from pancreatic gelatin digest. While PIB also contains glycerol, which can serve as a carbon source, it is not a preferred carbon source for *Pseudomonas aeruginosa*. SCFM is a defined medium that contains amino acids as the primary carbon source, and mimics the nutrients found in the CF lung environment. VBMM is a minimal medium that contains citrate as the sole carbon source.

Table S3. Statistical differences in growth rate among groups under conditions of *algU* induction.

Medium: LB	Strain	*att*Tn7::P_{araBAD}-*algU*				Δ*algU* *att*Tn7::P_{araBAD}-*algU*				Δ*algU* Δ*mucA* *att*Tn7::P_{araBAD}-*algU*			
Strain	Ara	0	0.1	0.25	1	0	0.1	0.25	1	0	0.1	0.25	1
*att*Tn7::P_{araBAD}-*algU*	0		*	*	*								
	0.1				*								
	0.25											*	
	1								*				*
Δ*algU* *att*Tn7::P_{araBAD}-*algU*	0							*	*				
	0.1							*	*		*		
	0.25								*			*	
	1												
Δ*algU* Δ*mucA* *att*Tn7::P_{araBAD}-*algU*	0										*	*	*
	0.1											*	*
	0.25												
	1												

Medium: PIB	Strain	*att*Tn7::P_{araBAD}-*algU*		Δ*algU* *att*Tn7::P_{araBAD}-*algU*		Δ*algU* Δ*mucA* *att*Tn7::P_{araBAD}-*algU*	
Strain	Ara	0	1	0	1	0	1
*att*Tn7::P_{araBAD}-*algU*	0		*				
	1						
Δ*algU* *att*Tn7::P_{araBAD}-*algU*	0				*		
	1						
Δ*algU* Δ*mucA* *att*Tn7::P_{araBAD}-*algU*	0						*
	1						

Medium: SCFM	Strain	*att*Tn7::P_{araBAD}-*algU*		Δ*algU* *att*Tn7::P_{araBAD}-*algU*		Δ*algU* Δ*mucA* *att*Tn7::P_{araBAD}-*algU*	
Strain	Ara	0	1	0	1	0	1
*att*Tn7::P_{araBAD}-*algU*	0		*				
	1						
Δ*algU* *att*Tn7::P_{araBAD}-*algU*	0				*		
	1						
Δ*algU* Δ*mucA* *att*Tn7::P_{araBAD}-*algU*	0						*
	1						

Medium: VBMM	Strain	*att*Tn7::P_{araBAD}-*algU*		Δ*algU* *att*Tn7::P_{araBAD}-*algU*		Δ*algU* Δ*mucA* *att*Tn7::P_{araBAD}-*algU*	
Strain	Ara	0	1	0	1	0	1
*att*Tn7::P_{araBAD}-*algU*	0		*			*	
	1						*
Δ*algU* *att*Tn7::P_{araBAD}-*algU*	0				*		
	1						*
Δ*algU* Δ*mucA* *att*Tn7::P_{araBAD}-*algU*	0						*
	1						

Significance was determined via a two-way ANOVA with post-hoc Bonferroni. Ara, concentration of arabinose (%). Asterisk, $p < 0.05$. Open box, no statistical difference.

condition was statistically larger for the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* than for the other two strains (N=3, $p < 0.05$, ANOVA with post hoc Tukey HSD), since this strain lacks the ability to produce any MucA to reduce AlgU activity. Together, these results strongly suggest that in the absence of *mucA*, while a certain level of AlgU activity is tolerated, high AlgU activity is fatal to the cell.

The above experiments were performed using only one medium (LB). To determine if overexpression of *algU* in the absence of *mucA* leads to a growth defect under other nutrient conditions, we determined the growth rate of the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* strain in other media. Similar to the results in LB (Fig 6), Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* strain failed to grow in the presence of 1% arabinose for the three other media we tested (Fig S5). Overall, our results show that AlgU overproduction is toxic under various nutrient conditions.

Expression of *rpoD* can rescue the growth defect of *algU* overexpression.

It has been suggested that AlgU competes for RNAP binding with RpoD, the essential primary sigma factor (Yin *et al.*, 2013). We, therefore, hypothesized that overexpressing *rpoD* with *algU* could ameliorate the growth defect of high *algU* expression in the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* strain. We inserted into this strain a copy of *rpoD* under the same arabinose-inducible promoter. We chose to test growth in the presence of 2% arabinose, since this new strain contains two arabinose-inducible promoters, in contrast to the strains described above in Figure 6. We confirmed that RpoD is overproduced in these strains containing an ectopic inducible *rpoD* allele via Western blot (Fig S6). Growth with 2% arabinose caused a growth defect for all strains tested, even in a strain lacking an ectopic arabinose-inducible gene (Fig 7). As expected from our results (Fig 6), Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* failed to grow in the presence of 2% arabinose. The Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU att*CTX::P_{araBAD}-*rpoD* strain, however, was able to grow in the presence of arabinose. Furthermore, the growth rate of this strain in the presence of arabinose was indistinguishable from that of Δ*algU* Δ*mucA att*CTX::P_{araBAD}-*rpoD* (Table S4). These results show that the toxicity associated with high AlgU levels in the absence of MucA can be reduced via overproduction of RpoD.

DISCUSSION

Although it has been assumed that *mucA* can be deleted without affecting bacterial viability, our work shows that *mucA* is essential in a variety of *P. aeruginosa* wild-type strains and under various nutrient conditions. Our results strongly suggest that the interaction of MucA with its

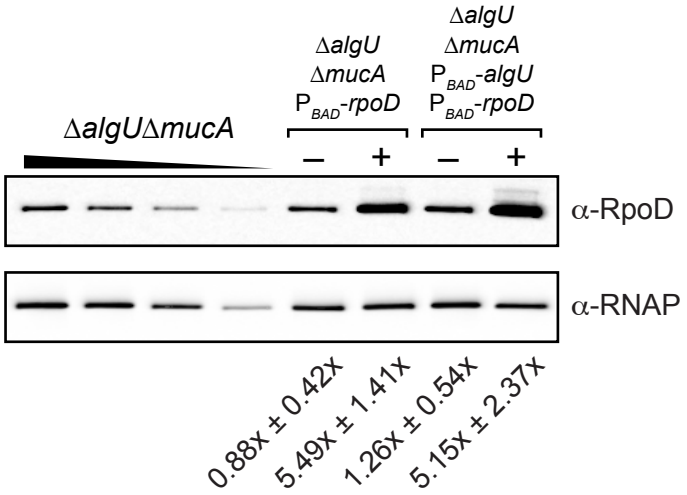


Figure S6. RpoD production in strains with an ectopic inducible *rpoD* allele.
Expression of *rpoD* was inferred via semi-quantitative Western blot in the indicated strains. The lysates of the parental strain (PAO1 Δ*mucA* Δ*algU*) at different concentrations (100%, 75%, 50%, and 25%) is shown to establish baseline cellular RpoD levels (top). RNAP was used as a loading control (bottom). Strains were grown in LB with (+) or without (–) 2% arabinose. The relative amount of RpoD is indicated, where 1.00x represents RpoD levels in the parental strain (± SD; N=3). While the strains produce significantly more RpoD in the presence of arabinose relative to the same strain in the absence of arabinose ($p < 0.01$), there is no statistical difference in the amount of RpoD produced from the two induced strains (two-way ANOVA with post-hoc Bonferroni).

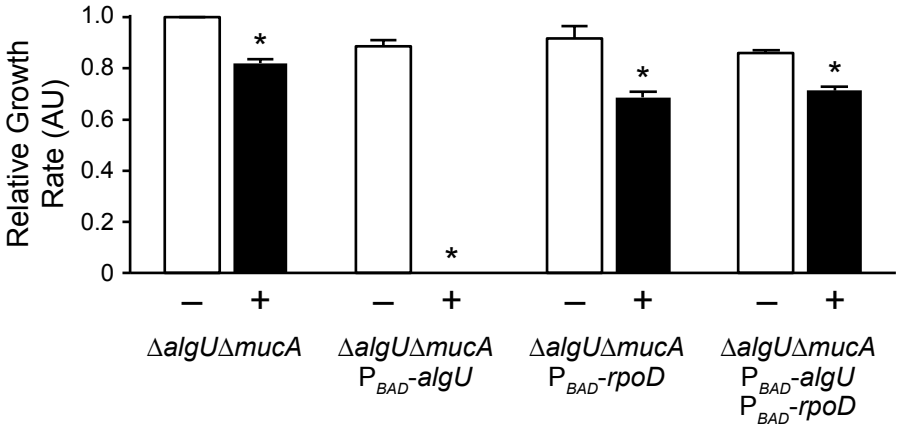


Figure 7. Growth defect caused by *algU* overexpression can be rescued via *rpoD* overexpression.
Growth rate of indicated strains grown in LB with (+) or without (–) 2% arabinose (relative to PAO1 Δ*algU* Δ*mucA* grown in the absence of arabinose). Error bars, SEM (N=3). Asterisk, statistically different from the same strain grown in the absence of arabinose ($p < 0.01$, N = 3, two-way ANOVA with post-hoc Bonferroni). See Table S4 for full statistical comparisons.

Table S4. Statistical differences in growth rate among groups overexpressing *algU* and/or *rpoD*.

	Strain	Δ*algU*Δ*mucA*		Δ*algU*Δ*mucA* *att*Tn7::P_{BAD}-*algU*		Δ*algU*Δ*mucA* *att*CTX::P_{BAD}-*rpoD*		Δ*algU*Δ*mucA* *att*Tn7::P_{BAD}-*algU* *att*CTX::P_{BAD}-*rpoD*	
Strain	Ara	0	2	0	2	0	2	0	2
Δ*algU*Δ*mucA*	0		*	*				*	
	2				*		*		*
Δ*algU*Δ*mucA* *att*Tn7::P_{BAD}-*algU*	0				*				
	2						*		*
Δ*algU*Δ*mucA* *att*CTX::P_{BAD}-*rpoD*	0						*		
	2								
Δ*algU*Δ*mucA* *att*Tn7::P_{BAD}-*algU* *att*CTX::P_{BAD}-*rpoD*	0								*
	2								

Significance was determined via a two-way ANOVA with post-hoc Bonferroni. Ara, concentration of arabinose (%). Asterisk, $p < 0.05$. Open box, no statistical difference.

cognate sigma factor AlgU is required for viability, and that AlgU mutants with reduced sigma factor activity can suppress *mucA* essentiality. Furthermore, overproduction of AlgU is detrimental to cell growth, which can be rescued by increasing RpoD production. Together, our data strongly suggest that unchecked AlgU activity in the absence of MucA leads to cell death.

Our data point to the negative regulation of AlgU being necessary for cell survival in *P. aeruginosa* (Fig 8). Under non-stress conditions, AlgU is bound to and inhibited by a full-length MucA. Under envelope stress or in strains containing *mucA* mutations, the anti-sigma factor is cleaved. We propose that while this cleaved cytosolic form of MucA does not inhibit AlgU to the same extent as the full-length protein, it is still able to interact with and inhibit AlgU to some degree. Although AlgU is active under such conditions, because *mucA* is positively regulated by AlgU, this negative feedback allows the cell to keep AlgU activity under control. In comparison, in the absence of MucA, this ability to control AlgU is lost. We propose that the strong positive feedback of AlgU on its own expression and activity is what leads to cell death and *mucA* essentiality. Supporting this model are several lines of evidence. First, *mucA* essentiality is rooted in its interaction with *algU* (Figs 1 and 3), strongly suggesting that the ability of MucA to inhibit AlgU is required for viability. Second, deletion of AlgU-regulated genes, including those encoding the well-characterized transcription factors AlgB (Wozniak & Ohman, 1994), AlgR (Martin *et al.*, 1994, Wozniak & Ohman, 1994), and AmrZ (Wozniak *et al.*, 2003), do not allow for *mucA* deletion (Table S1), supporting that *algU* itself is the lethal component of the AlgU regulon. Third, AlgU mutants can suppress *mucA* essentiality, and all 25 natural revertants that we screened contained mutations in *algU* (Fig 4). The strains carrying these mutations had decreased AlgU transcriptional activity (Fig 5), supporting the idea that decreasing the positive feedback of AlgU on its own expression allows the cell to survive in the absence of MucA. Lastly, overexpression of *algU* leads to a growth defect, which is lethal at high levels in the absence of *mucA* (Fig 6). This toxicity of *algU* overexpression was be rescued by overexpression of *rpoD* (Fig 7), presumably via decreased AlgU activity. Together, our work strongly suggests that growth defect in the absence of *mucA* is caused by unchecked AlgU activity (Figs 5-7), in agreement with previous studies suggesting that overproduction of AlgU is toxic (Cross *et al.*, 2020, Hershberger *et al.*, 1995, Schurr *et al.*, 1994).

While our data supports our model that the AlgU-regulated gene critical for *mucA* essentiality is *algU* itself, we cannot exclude the possibility that the regulation of another single gene or a combination of genes within the AlgU regulon is responsible for the *mucA* requirement. We have

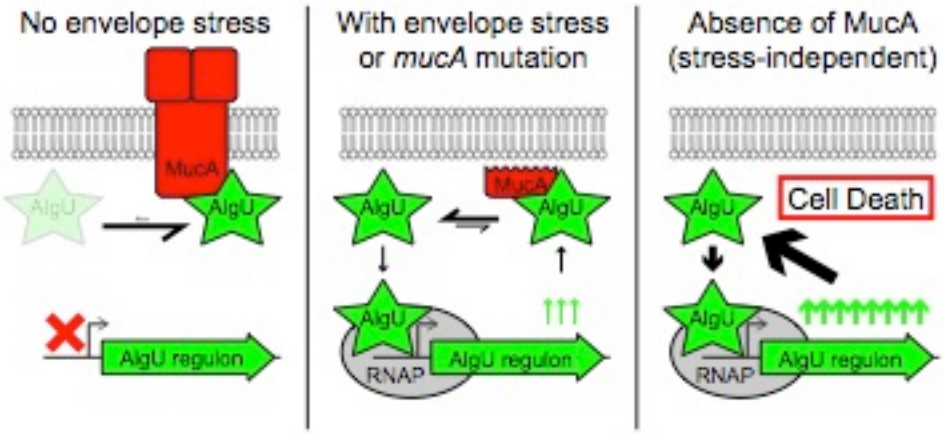


Figure 8. Unchecked AlgU activity in the absence of MucA leads to bacterial cell death. Under conditions of no envelope stress (left), the full-length MucA (red) binds strongly to AlgU (green star), leaving very little free AlgU to interact with the RNAP (grey oval). Therefore, the AlgU regulon (green arrow) is in the "off" state (red X). Under conditions of envelope stress or in strains containing *mucA* mutations that lead to a truncated product (middle), a cleaved cytosolic form of MucA is produced. This form can still interact with and inhibit AlgU. However, the strength of the interaction is weaker than with the full-length MucA, allowing for a pool of free AlgU that can then bind to and recruit RNAP to the promoters of its regulon. Under such conditions, the AlgU regulon, which includes *algU* and *mucA*, is activated and in the "on" state (green up arrows). However, because of the feedback on MucA, negative regulation of AlgU activity is still present in the cell. In the absence of *mucA* (right), there is no MucA to bind to and regulate AlgU. All of the AlgU is free to interact with the core RNAP. This leads to high expression of the AlgU regulon and overproduction of AlgU itself. Under such strong positive feedback and in the absence of the negative regulator, the unchecked activity of AlgU leads to cell death.

yet to find a revertant that can grow in the absence of *mucA* expression containing a mutation outside of *algU*. Additionally, we were unable to delete *mucA* from a strain lacking three AlgU-regulated transcription factors (Table S1). Genes regulated by AlgB, AlgR, or AmrZ can account for approximately fifty percent of the AlgU regulon (Huang *et al.*, 2019, Jones *et al.*, 2014, Leech *et al.*, 2008, Schulz *et al.*, 2015), suggesting that it is unlikely that deleting a combination of AlgU-regulated genes can alleviate the necessity for *mucA*. However, the AlgU regulon is large, and we are actively searching for non-*algU* suppressors of *mucA* essentiality.

Our data show that the anti-sigma factor MucA is required for viability in various *P. aeruginosa* strains (Table 1). However, the requirement of an anti-sigma factor for bacterial viability is not unique to *P. aeruginosa*. While there do not appear to be any essential anti-sigma factors in *E. coli*, as all of them are individually deleted in the Keio collection (Baba *et al.*, 2006), there are many occurrences in other organisms. There are two anti-sigma factors, YhdL and YxlC, in *Bacillus subtilis* that are necessary for bacterial viability (Horsburgh & Moir, 1999, Koo *et al.*, 2017, Mendez *et al.*, 2012). The essentiality of *yhdL* is notable as this anti-sigma factor negatively regulates SigM, a sigma factor involved in the envelope stress response (Horsburgh & Moir, 1999). In *Mycobacterium tuberculosis*, the gene encoding the anti-sigma factor RslA is identified as essential due to an underrepresentation of interruptions in a genome-wide transposon insertional analysis (Griffin *et al.*, 2011). Interestingly, similar to our results (Fig 4), *rslA* can be deleted from *M. tuberculosis* lacking the gene encoding its cognate sigma factor SigL, which regulates genes involved in cell envelope processes (Dainese *et al.*, 2006). Furthermore, in the defined transposon mutant library for *Vibrio cholerae*, an interruption in the *mucA* homolog *rseA* does not exist, suggesting that this anti-sigma factor may be essential in *V. cholerae* (Cameron *et al.*, 2008), similar to in *P. aeruginosa*. Finally, the *Pseudomonas syringae mucA* homolog is deemed essential based on a reduced number of transposon insertions (Helmann *et al.*, 2019). Therefore, although the *E. coli mucA* homolog *rseA* is not required for cell viability (De Las Penas *et al.*, 1997, Missiakas *et al.*, 1997), these data, together with our results, suggest that essentiality of anti-sigma factors that regulate cell envelope function may be a widespread phenomenon in bacteria.

The interaction of MucA and AlgU is of interest, as *mucA* mutations are common in clinical CF isolates of *P. aeruginosa*, resulting in mucoid cells (Boucher *et al.*, 1997, Candido Cacador *et al.*, 2018, Ciofu *et al.*, 2008, Martin *et al.*, 1993, Pulcrano *et al.*, 2012). Clinical *mucA* mutations that lead to C-terminal truncations are common (Fig S2) and, as implied by our results,

presumably result in a MucA protein that retains partial AlgU-inhibitory function. Interestingly, there is one *P. aeruginosa* CF isolate that is reported to have a *mucA* mutation that would result in a truncation within the first 50 aa of the protein (Boucher *et al.*, 1997). Our sequencing data of the published PAK and PA103 Δ*mucA* strains (Intile *et al.*, 2014, Jones *et al.*, 2010) suggests that this isolate may contain a suppressor mutation in *algU* that allows the clinical isolate to survive in the absence of a functional MucA. Nonetheless, in mucoid CF clinical isolates, *mucA* mutations that lead to C-terminal truncations are the norm, while null mutations in *mucA* with a presumed hypomorphic *algU* allele is rare (Fig S2). Furthermore, our results (Fig 4) agree with the literature showing that mucoid isolates with *mucA* mutations revert to a non-mucoid state via changes to *algU* when the cells are grown under standard laboratory conditions (Ciofu *et al.*, 2008, DeVries & Ohman, 1994, Sautter *et al.*, 2012, Schurr *et al.*, 1994). While these *algU* secondary site mutations are found in non-mucoid CF isolates with *mucA* mutations, such isolates are detected at a lower frequency (Ciofu *et al.*, 2008). As suggested by Ciofu and associates, this may be due to the importance of AlgU for the survival of *P. aeruginosa* in the CF lung environment, suggesting that reducing AlgU activity may increase *P. aeruginosa* eradication from the CF airway. Based on our data and the published literature, we propose that the MucA-AlgU interaction may serve as a good therapeutic target. Our results show that destabilizing the MucA-AlgU interaction results in bacterial cell death or mutations in *algU* that result in reduced sigma factor activity and likely reduced mucoid conversion, of which either outcome could be beneficial in the treatment of *P. aeruginosa* CF lung infections.

METHODS AND MATERIALS

Bacterial strains and growth conditions. The bacterial strains, plasmids, and oligonucleotides used for this study are in Tables S5-S7. The construction of strains is described in Supplementary Information. Bacteria were grown at 37°C in LB with shaking or on semi-solid LB media, unless otherwise noted.

Allelic exchange assay. The protocol, depicted in Fig S1, was modified from (Hmelo *et al.*, 2015). Briefly, a vector containing the *mucA* deletion allele flanked by approximately equal length homology regions is introduced via conjugation. Merodiploids were selected on semi-solid VBMM (Vogel & Bonner, 1956) with 60 mg/L gentamicin. Using OMS118 and OMS119, PCR was performed on at least six isolates to confirm that the presence of both the wild type and deletion alleles of *mucA*. Confirmed merodiploids were then individually streaked on NSLB (10 g/L tryptone, 5 g/L yeast extract) with 10% sucrose semi-solid media for counterselection.

PCR was performed on eight colonies per merodiploid, using OBT601 and OBT602 to determine the resolution to either the WT or deletion allele.

MucA depletion assay. Cells were grown in LB with 0.05% rhamnose at 37°C with shaking to an OD_{600} of 0.3. After washing, the culture was then divided in two, half resuspended with rhamnose and half without in its respective media base (LB, PIB, SCFM, or VBMM). Cells were incubated at 37°C with shaking. At the indicated time points, two aliquots were removed from each culture, serially diluted and plated in triplicate onto LB agar plates with 0.05% rhamnose for recovery. Colonies were counted and log_{10}-transformed CFU/mL of the culture was calculated.

Yeast two-hybrid assay. The ProQuest Two-Hybrid System (Invitrogen) was used, per manufacturer's instructions. Briefly, yeast containing the Gal4 activation domain-based prey and the Gal4 DNA binding domain-based bait vectors were grown overnight in SD-Leu-Trp broth (Clontech). The OD_{600} value was recorded. ONPG (VWR) was added to lysed cells, and the mixture was incubated at 37°C until a light yellow color was achieved. The incubation time was recorded. The OD_{420} of the supernatants was determined using a Synergy Hybrid HTX Microplate Reader (Bio-Tek Instruments). Beta-galactosidase activity was determined using Miller units, based on the following equation: 1000 x OD_{420}/(time x culture volume x OD_{600}).

AlgU activity assay. Strains of interest were transformed with a plasmid-borne *algD* reporter (pBT435) via electroporation. Strains were grown overnight in LSLB with 50 mg/L gentamicin and 0.05% rhamnose (where indicated) at 37°C with shaking. The overnight culture was diluted 1:100 and grown to an OD_{600} of 0.3. Cells were then treated with fresh 400 mg/L D-cycloserine for 2 h at 37°C with shaking, as previously described (Wood *et al.*, 2006). Cells were then pelleted and resuspended in PBS. GFP fluorescence and OD_{600} was determined using a Synergy Hybrid HTX Microplate Reader (Bio-Tek Instruments). To normalize the data, the GFP fluorescence was divided by the OD_{600} for each data point. To determine the fold induction, the ratios for the treated samples were divided by the average of that for the same strain not treated with D-cycloserine. The resulting ratios of the triplicate samples were averaged.

Natural revertant assay. *P. aeruginosa* Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* was grown in LB with 0.05% rhamnose at 37°C with shaking to an OD_{600} of 1. Cells were washed and plated on semi-

solid LB plates without rhamnose. Plates were incubated for 24-48 h at 37°C. For isolates that grew on LB without rhamnose, *algU* was Sanger sequenced.

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AUTHOR CONTRIBUTIONS

Conceptualization: MCS, BST; Acquisition, analysis, or interpretation of data: MCS, DR, AAK, EKC, LAM, EAC, PAJ, BST; Writing – original draft: MCS, BST; and Writing – review and editing: MCS, DR, LAM, EAC, PAJ, BST.

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