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 bacterial genetic adaptation often observed in CF isolates is *mucA* mutations. MucA inhibits the sigma factor AlgU. Mutations in *mucA* lead to AlgU misregulation, resulting in a mucoid phenotype that is associated with poor CF disease outcomes. Due to its ability to be mutated, *mucA* is assumed to be dispensable for bacterial viability. Here we show that, paradoxically, a portion of *mucA* is essential in *P. aeruginosa*. We demonstrate that *mucA* is no longer required in a strain lacking *algU*, that *mucA* alleles encoding for proteins that do not bind to AlgU are insufficient for viability, and that *mucA* is no longer essential in mutant strains containing AlgU variants with reduced sigma factor activity. Furthermore, 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.

32 **INTRODUCTION**

33 The major cause of death in people with cystic fibrosis (CF), a human autosomal recessive
34 genetic disease, is respiratory failure due to chronic lung infection. *Pseudomonas aeruginosa* is
35 a prevalent CF respiratory pathogen (Cystic Fibrosis Foundation Patient Registry, 2019). The
36 CF lung environment selects for mucoid *P. aeruginosa* mutants, which overproduce the
37 exopolysaccharide alginate and are associated with poor disease prognosis (Douglas *et al.*,
38 2009, Emerson *et al.*, 2002, Farrell *et al.*, 2009, Henry *et al.*, 1992, Li *et al.*, 2005, Nixon *et al.*,
39 2001, Parad *et al.*, 1999, Pedersen *et al.*, 1992, Strateva *et al.*, 2010). Conversion to the mucoid
40 phenotype in clinical *P. aeruginosa* isolates, which is thought to be advantageous for chronic
41 infection, is often caused by *mucA* mutations (Martin *et al.*, 1993).

42

43 MucA is an anti-sigma factor to the alternative sigma factor AlgU (also known as AlgT, σ^E, or
44 σ^{22}), which responds to envelope stress (Damron & Goldberg, 2012, Govan & Deretic, 1996).
45 MucA is a transmembrane protein that sequesters AlgU away from RNA polymerase (RNAP)
46 via its N-terminus (Li *et al.*, 2019, Schurr *et al.*, 1996, Xie *et al.*, 1996). The C-terminus of MucA
47 is in the periplasm, where it is protected from proteolysis by MucB (Mathee *et al.*, 1997, Schurr
48 *et al.*, 1996). The envelope stress response is controlled via a regulated intramembrane
49 proteolysis cascade: MucB dissociates from MucA upon stress detection, allowing the proteases
50 AlgW and MucP to cleave MucA from the inner membrane (Qiu *et al.*, 2007, Wood & Ohman,
51 2009). In the cytoplasm, MucA is further degraded by ClpXP, releasing AlgU to interact with
52 RNAP and activate the AlgU regulon (Qiu *et al.*, 2008). AlgU regulates at least 350 genes,
53 including those responsible for the production of itself, MucA, and the alginate biosynthetic
54 enzymes (Firoved & Deretic, 2003, Schulz *et al.*, 2015, Wood & Ohman, 2009). This system is
55 homologous to the well-studied envelope stress response in *Escherichia coli*: MucA shares 28%
56 identity (72% similarity) to the anti-sigma factor RseA. The cognate sigma factor of the *E. coli*

57 RseA, called RpoE, is homologous to and functionally interchangeable with AlgU (Yu *et al.*,
58 1995), with 66% identity (93% similarity).
59
60 For several reasons, *mucA* is assumed to be dispensable for *P. aeruginosa* viability. First, after
61 being released from the cell membrane, MucA is presumed to be fully degraded in the
62 cytoplasm by ClpXP (Qiu *et al.*, 2008). Second, *mucA* mutations often arise in CF clinical
63 isolates (Boucher *et al.*, 1997, Candido Cacador *et al.*, 2018, Ciofu *et al.*, 2008, Martin *et al.*,
64 1993, Pulcrano *et al.*, 2012), and many laboratory *mucA* mutants exist in the literature
65 (Gallagher *et al.*, 2011, Liberati *et al.*, 2006, Skurnik *et al.*, 2013, Turner *et al.*, 2015). Third,
66 there are three published strains in which the entirety of *mucA* is removed from the genome
67 (Intile *et al.*, 2014, Jones *et al.*, 2010, Pritchett *et al.*, 2015). Paradoxically, three different whole-
68 genome studies in two different strains of *P. aeruginosa* identified *mucA* as an essential gene
69 (Lee *et al.*, 2015, Liberati *et al.*, 2006, Skurnik *et al.*, 2013). Additionally, it has been anecdotally
70 noted that *mucA* could not be deleted from the *P. aeruginosa* PAO1 genome (Panmanee *et al.*,
71 2019). To investigate this paradox, we systematically attempted to delete *mucA* from *P.*
72 *aeruginosa* using allelic exchange. Our results show that a portion of *mucA* was required for
73 bacterial viability in multiple *P. aeruginosa* strains, that *mucA* was no longer essential in a strain
74 lacking *algU*, and that *mucA* alleles that encode for proteins that do not interact with AlgU were
75 insufficient to rescue viability or led to a growth defect. We found that *algU* mutations encoding
76 for a less active sigma factor could relieve *mucA* essentiality, and that AlgU overproduction in
77 the absence of MucA was toxic. Interestingly, our works suggests that *mucA* essentiality can be
78 suppressed by increasing the levels of the housekeeping sigma factor RpoD. Together, our
79 results strongly suggest that the unregulated activity of AlgU itself, in the absence of MucA,
80 leads to bacterial cell death.
81

82 **RESULTS**

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

84 To determine if *mucA* is essential, we attempted to delete the gene from various *P. aeruginosa*
85 strains, using a modified allelic exchange protocol to turn it into a robust assay (Fig S1). An
86 allele of *mucA* missing >95% of the coding region was introduced into *P. aeruginosa*. Using
87 PCR-confirmed isolates containing both the endogenous and deletion alleles in the genome, we
88 counter-selected for loss of one allele and then determined which *mucA* allele isolates resolved
89 to via PCR. For non-essential genes, we should observe isolates that resolved to the
90 endogenous or deletion allele. However, for essential genes, only cells that resolved to the
91 endogenous allele should be isolated, as cells that resolved to the deletion allele should not
92 survive in the absence of the gene. For statistical power, we screened ≥125 isolates for strains
93 resolving only to the endogenous *mucA*. We performed this assay on a diverse set of wild-type
94 *P. aeruginosa* strains, including four laboratory, five clinical and two environmental isolates
95 (Table 1). These strains are not only isolated from diverse locations, but they also vary in their
96 colony morphology and their exopolysaccharide production profile (Colvin *et al.*, 2012). For all
97 strains tested, all observed isolates resolved to the endogenous *mucA* allele ($p < 0.0001$,
98 Fisher's exact test), strongly suggesting that *mucA* is essential in wild-type *P. aeruginosa*. Since
99 all tested strains required *mucA* for viability, we continued our experiments using PAO1 as a
100 representative strain.

101

102 **Depletion of MucA leads to cell death**

103 To evaluate the effect of nutrient conditions on *mucA* essentiality, we tested the effect of
104 depleting MucA on *P. aeruginosa* viability in different media. We engineered a strain lacking the
105 native *mucA* and containing a chromosomally-integrated rhamnose-inducible copy (Δ*mucA*
106 *att*Tn7::P_{rhaBAD}-*mucA*). We determined viability of this strain grown without rhamnose over time.
107 Since cells would cease producing MucA in the absence of rhamnose, the MucA present in the

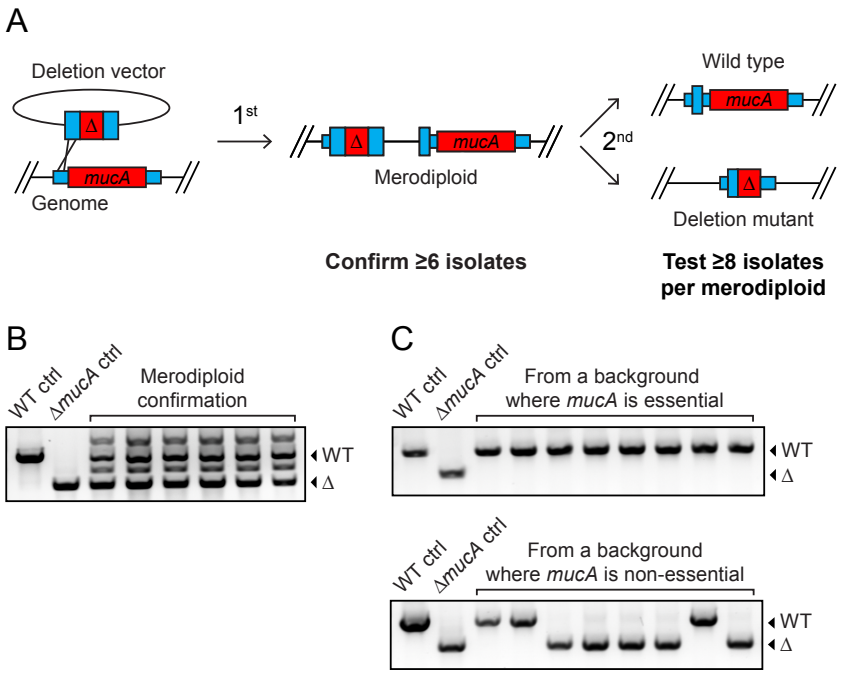


Figure S1. Allelic exchange assay.
(A) Schematic of assay. To delete the endogenous *mucA* (short red box with *mucA*) from the genome, the deletion vector pBT396, which contains an allele of *mucA* that is missing >95% of the coding region (tall red box with delta), was introduced into *P. aeruginosa* 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

108 cells at the start of the experiment would be depleted as the cells divided. In all four media we
109 tested, if rhamnose was not removed, the cells increased in density by ~2-logs over time. In
110 comparison, when rhamnose was removed, the cells lost viability over time in all four media (Fig
111 1), showing that *mucA* is essential in *P. aeruginosa* in various nutritional environments.
112
113 **Alginate biosynthesis is not solely responsible for *mucA* essentiality**
114 Clinical isolates with *mucA* mutations are mucoid due to the overproduction of alginate (Martin
115 *et al.*, 1993). To determine if alginate overproduction is responsible for *mucA* essentiality, we
116 attempted to delete *mucA* from a strain lacking *algD*, a key alginate biosynthesis gene (Deretic
117 *et al.*, 1987). We were unable to delete *mucA* in this background (Table S1).
118
119 Expression of the alginate biosynthesis genes is controlled by three AlgU-regulated transcription
120 factors that are active in mucoid cells: AlgB, AlgR, and AmrZ (Martin *et al.*, 1994, Wozniak &
121 Ohman, 1994, Wozniak *et al.*, 2003). Since these transcription factors regulate many genes in
122 addition to those involved in alginate biosynthesis (Huang *et al.*, 2019, Jones *et al.*, 2014, Kong
123 *et al.*, 2015, Leech *et al.*, 2008), we tested if overexpression of these three regulons underlies
124 *mucA* essentiality. We could not delete *mucA* from strains lacking these transcription factors
125 (Table S1). We conclude that eliminating alginate biosynthesis and the expression of ~50%
126 AlgU regulon do not alleviate *mucA* essentiality.
127
128 **The first 50 amino acids of MucA are necessary and sufficient for cell viability**
129 While we were unable to delete *mucA* using an allele lacking >95% of the coding region, *mucA*
130 is often mutated in clinical isolates and many *mucA* transposon mutants exist (Fig S2),
131 suggesting that only a portion of *mucA* is essential. Based on the co-crystal structures of MucA
132 with AlgU and MucB (Li *et al.*, 2019), the first 78 residues of MucA interact with AlgU, and the
133 last 48 residues of MucA interact with MucB. We attempted to delete the endogenous *mucA*

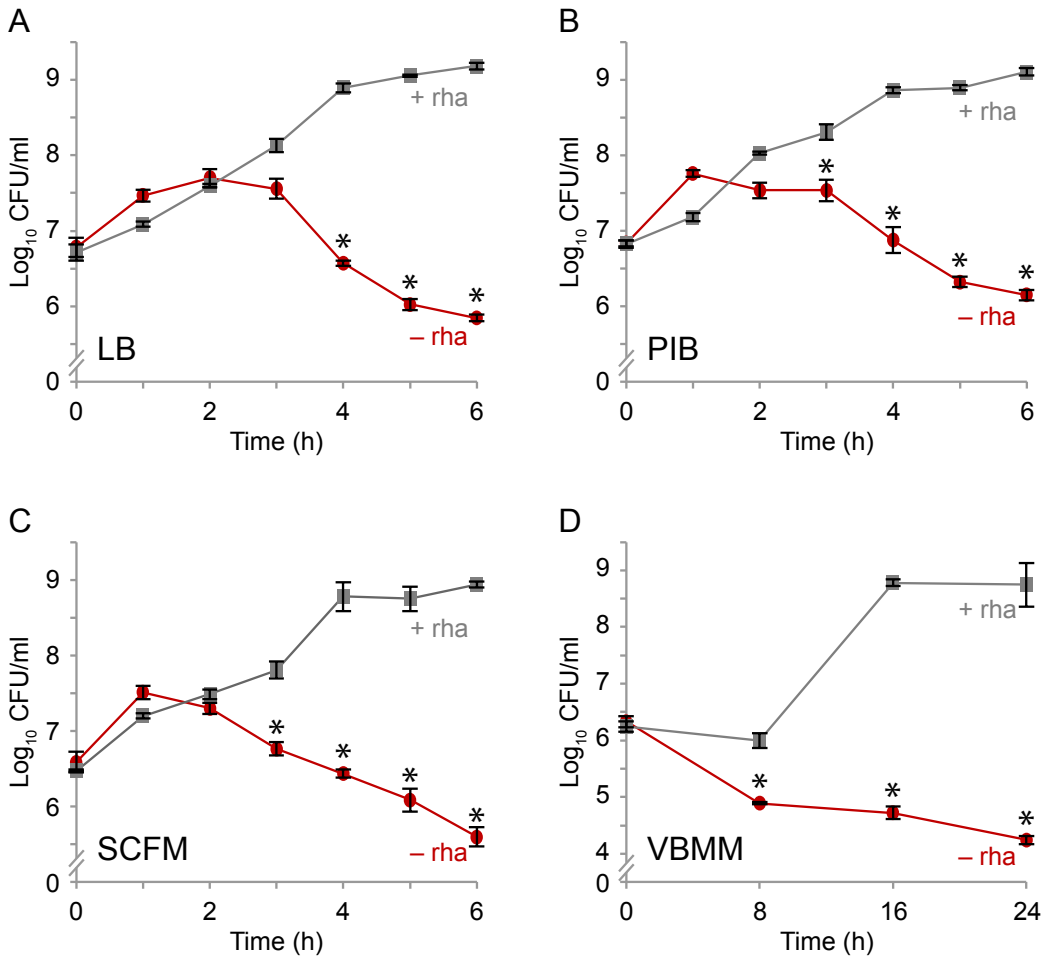


Figure 1. Depletion of MucA results in cell death.
Viable colony counts of PAO1 Δ*mucA* attTn7::P_{rhaBAD}-*mucA* over time in **(A)** LB, **(B)** PIB, **(C)** SCFM and **(D)** VBMM with (+ rha; gray squares) or without (– rha; red circles) 0.05% rhamnose. Viable colony counts after incubation in the 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).

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

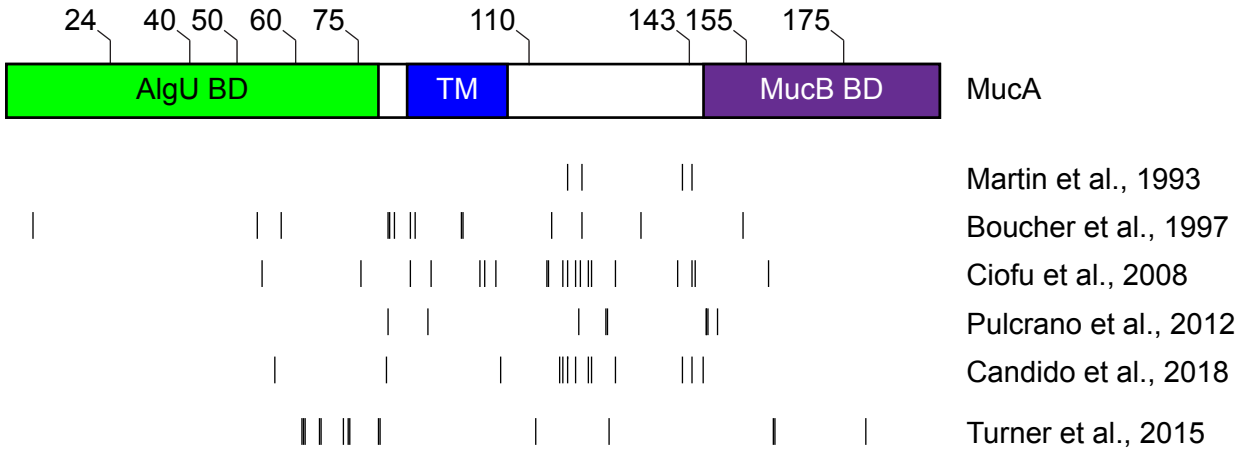


Figure S2. Published mutations in *mucA*.
Top, schematic of MucA with the regions indicated encoding for the AlgU binding domain (green, AlgU BD), the transmembrane domain (blue, TM), and the MucB binding domain (purple, MucB BD). Hash marks on top indicate the residue number in MucA. Bottom, sites of *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.

134 from a series of strains containing an ectopic chromosomally-integrated *mucA*, driven by its
135 native promoter and encoding for truncation products (Fig 2A). We were unable to probe
136 whether equivalent amounts of protein were produced across our strains, since our truncated
137 versions of MucA are cleaved out of the membrane and degraded in the cytosol, as previously
138 shown (Qiu *et al.*, 2008). However, we expect that the ectopic alleles are expressed at similar
139 levels prior to endogenous *mucA* deletion, as they are under the same promoter and at the
140 same genomic site. These strains with two *mucA* alleles were non-mucoid, showing that the
141 endogenous *mucA* allele, encoding full-length protein, is dominant over the ectopic alleles that
142 encode for truncated MucA. We used a Δ*algD* background to make strains easier to manipulate,
143 as deleting the endogenous *mucA* would result in alginate overproduction in some strains.
144
145 We then attempted to delete the endogenous *mucA* from these strains. Since the flanking
146 regions of the two alleles differ, our deletion allele specifically targets the native allele. We were
147 able to easily recover isolates that resolved to the *mucA* deletion allele from strains containing
148 ectopic alleles encoding for the full length MucA (aa 1-194), as well as MucA aa 1-143, aa 1-
149 110, aa 1-75, and aa 1-62 (Fig 2B). Interestingly, while the endogenous *mucA* could be deleted
150 from strains containing an ectopic *mucA* that encoded for aa 1-50 (Fig 2B), isolates resolving to
151 the deletion allele grew up more slowly than those resolving to the native allele. This suggests
152 that while this allele is sufficient for viability, it is not well tolerated. All tested isolates of strains
153 carrying *mucA* alleles encoding shorter products (aa 1-40 and aa 1-24) resolved to the native
154 allele. Furthermore, we were unable to delete the native *mucA* from a strain with an ectopic
155 *mucA* that encodes for aa 51-194 (Fig 2B). These results suggest that the first 50 amino acids
156 of MucA are necessary and sufficient for *P. aeruginosa* cell viability. Consistent with these
157 results, reported *mucA* mutations (Boucher *et al.*, 1997, Candido Cacador *et al.*, 2018, Ciofu *et*
158 *al.*, 2008, Martin *et al.*, 1993, Pulcrano *et al.*, 2012, Turner *et al.*, 2015) almost entirely fall
159 outside the region of *mucA* encoding the first 50 residues (Fig S2).

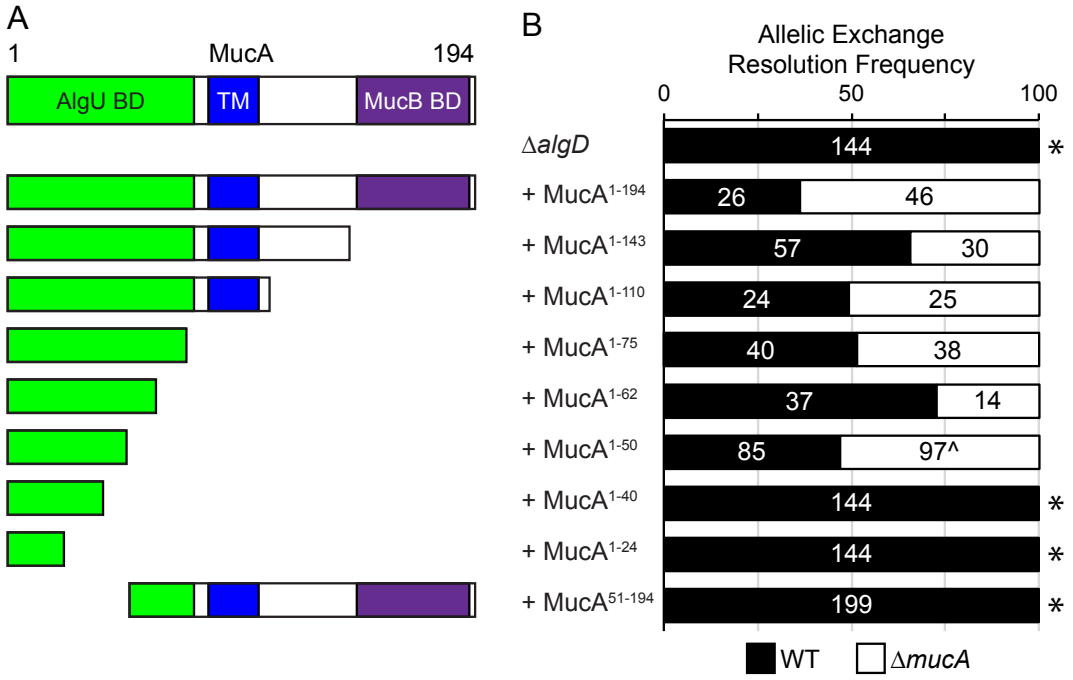


Figure 2. The first 50 amino acids of MucA are necessary and sufficient for viability. **(A)** Schematic of MucA encoded by the ectopic *mucA* alleles in PAO1 Δ*algD* strains tested for *mucA* essentiality in (B). MucA aa 1-143 lacks the MucB binding domain and is the product of the common *mucA22* allele. 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 *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.

160

161 **The interaction of MucA with AlgU is required for cell survival**

162 Since the only described function for MucA is to inhibit AlgU, we hypothesized that *mucA*
163 essentiality is rooted in its regulation of AlgU. To test this, we attempted to delete *mucA* from a
164 strain lacking *algU* (Δ*algU*). We were able to do so, with 16 of the tested isolates resolving to the
165 deletion allele out of the 48 colonies tested (see Fig 4A), showing that *mucA* essentiality is *algU*-
166 dependent.

167

168 Because our data suggest that the AlgU-binding domain of MucA is required for viability (Fig
169 2B), we hypothesized that the physical interaction between MucA and AlgU is necessary for cell
170 survival. In the co-crystal structure (Li *et al.*, 2019), four residues in MucA make more than one
171 hydrogen bond with AlgU: D15, E22, R42, and E72 (Fig 3A). We engineered alleles that encode
172 for MucA D15A, E22A, R42A, or E72A to maximally affect the hydrogen bonding while limiting
173 the effects on overall protein structure. We used MucA aa 1-75 as a base because the co-
174 crystal structure includes the first 78 residues of MucA. As described above, an allele encoding
175 MucA aa 1-75 was sufficient for viability (Fig 2B). We were unable to delete the native *mucA*
176 from strains carrying alleles encoding the D15A and R42A substitutions (Fig 3B). While we were
177 able to delete the native *mucA* from the strain containing MucA E22A, isolates that resolved to
178 the deletion allele grew up slower than those that resolved to the wild-type allele, suggesting
179 that the cells do not tolerate this allele well. As expected, due to being outside the required
180 region of MucA (Fig 2B), we were able to delete the native *mucA* from a strain containing MucA
181 E72A with 9 of 48 tested isolates resolving to the deletion allele.

182

183 To determine if the D15A, E22A, and R42A substitutions affect the ability of MucA to interact
184 with AlgU, we used a yeast-two hybrid assay. Using beta-galactosidase activity as a proxy for
185 their interaction, wild-type MucA aa 1-75 strongly interacted with AlgU (Fig 3C). This interaction

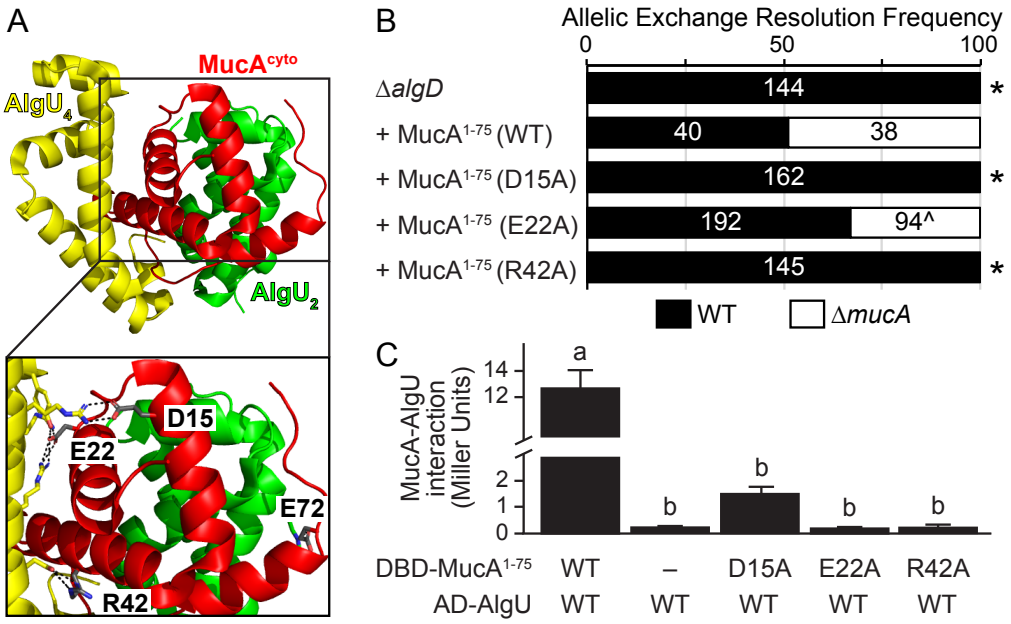
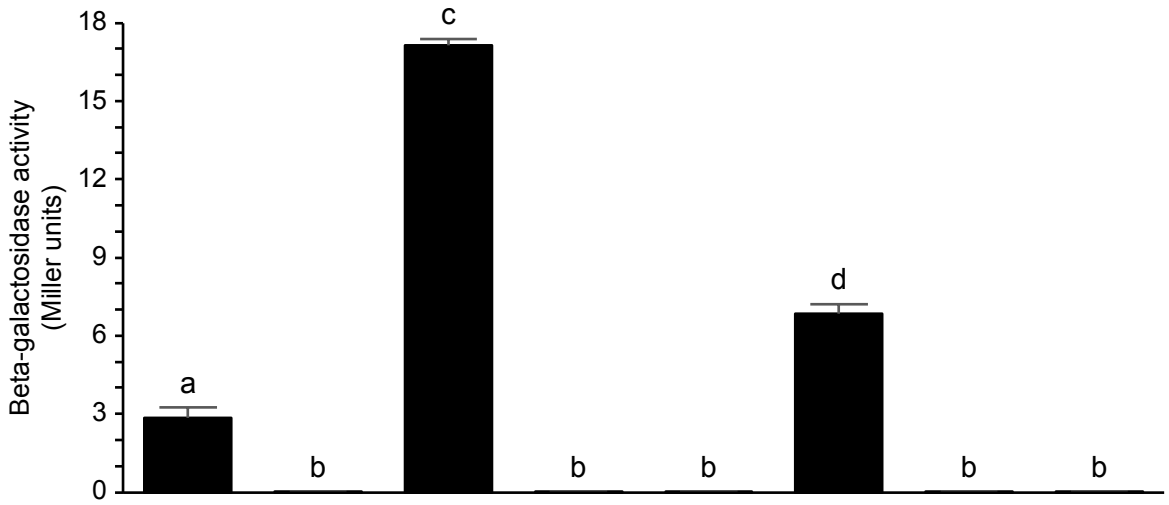


Figure 3. The physical interaction of MucA and AlgU is required for survival.
(A) Four residues of MucA make greater than one predicted hydrogen bond with AlgU. The MucA-AlgU co-crystal structure (PDB 6IN7; Li et al., 2019) with the cytosolic domain of MucA (aa 1-78; red) and Regions 2 and 4 of AlgU (green, $AlgU_2$; yellow, $AlgU_4$) is shown. The residues of MucA that are predicted to make more than one hydrogen bond with AlgU (grey) are labeled in the inset. Black dotted lines, predicted hydrogen bonds; red atoms, oxygen; blue atoms, nitrogen. **(B)** Frequency of observed isolates resolving to the endogenous *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. **(C)** 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 groupings ($p < 0.01$; biological triplicate with technical quadruplicates; ANOVA with post-hoc Tukey HSD).

186 was dependent on the presence of both MucA and AlgU, and the effect was not directional (Fig
187 S3). In comparison, the D15A, E22A, and R42A MucA mutants failed to interact with AlgU (Fig
188 3C). We conclude that the interaction of MucA and AlgU are required for viability.
189
190 **Mutations that reduce AlgU activity alleviate the requirement for MucA**
191 If *mucA* essentiality is due to its inhibition of the AlgU regulon, strains containing a mutant AlgU
192 with lower affinity for DNA should alleviate *mucA* essentiality because the regulon expression is
193 reduced in such mutants. We engineered Δ*algU* strains with an ectopic chromosomally-
194 integrated *algU* allele encoding such DNA-binding mutants (K57A and N81A), based on
195 homology to *E. coli* RpoE mutants that have decreased *in vitro* transcriptional activity
196 (Campagne *et al.*, 2014). As described above, we were able to delete *mucA* from a Δ*algU* strain
197 (Fig 4A). This phenotype could be rescued via the ectopic addition of a wild-type *algU* allele, as
198 we were no longer able to delete *mucA* from such a strain. In contrast, *mucA* could be deleted
199 from strains carrying alleles encoding AlgU K57A or N81A, showing that these alleles failed to
200 rescue the Δ*algU* phenotype (Fig 4A). To determine the effect of these substitutions on AlgU
201 activity, we induced envelope stress using D-cycloserine (Wood *et al.*, 2006) and measured
202 AlgU activity using a plasmid-borne *gfp* reporter driven under the AlgU-regulated *algD* promoter
203 (Damron *et al.*, 2009). Similar to what is seen for RpoE (Campagne *et al.*, 2014), these mutant
204 strains had reduced AlgU activity upon induction of envelope stress (Fig 5A,B).
205
206 Strains with the entirety of *mucA* deleted from *P. aeruginosa* PAO1, PAK, and PA103 exist
207 (Intile *et al.*, 2014, Jones *et al.*, 2010, Pritchett *et al.*, 2015). Since we were unable to delete
208 *mucA* from these strain backgrounds (Table 1), we sequenced the genomes of these Δ*mucA*
209 strains to determine if they contain suppressor mutations that allowed for their survival in the
210 absence of *mucA*. In PAO1 Δ*mucA* (Pritchett *et al.*, 2015), while the native *mucA* was deleted,
211 the strain contained a second full-length copy of *algU* and a *mucA* allele that encoded for aa 1-



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).

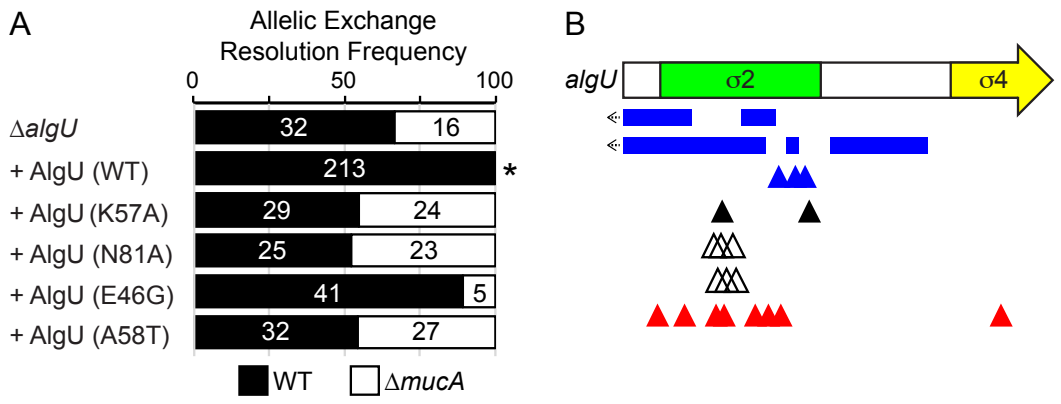


Figure 4. Mutations in AlgU can suppress *mucA* essentiality.
(A) Frequency of observed isolates resolving to the endogenous *mucA* allele (WT, black) or the deletion allele (Δ*mucA*, white) in the allelic exchange assay, using PAO1 Δ*algU att*Tn7::P_{algU}-*algU*, where *algU* encodes for the indicated 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.

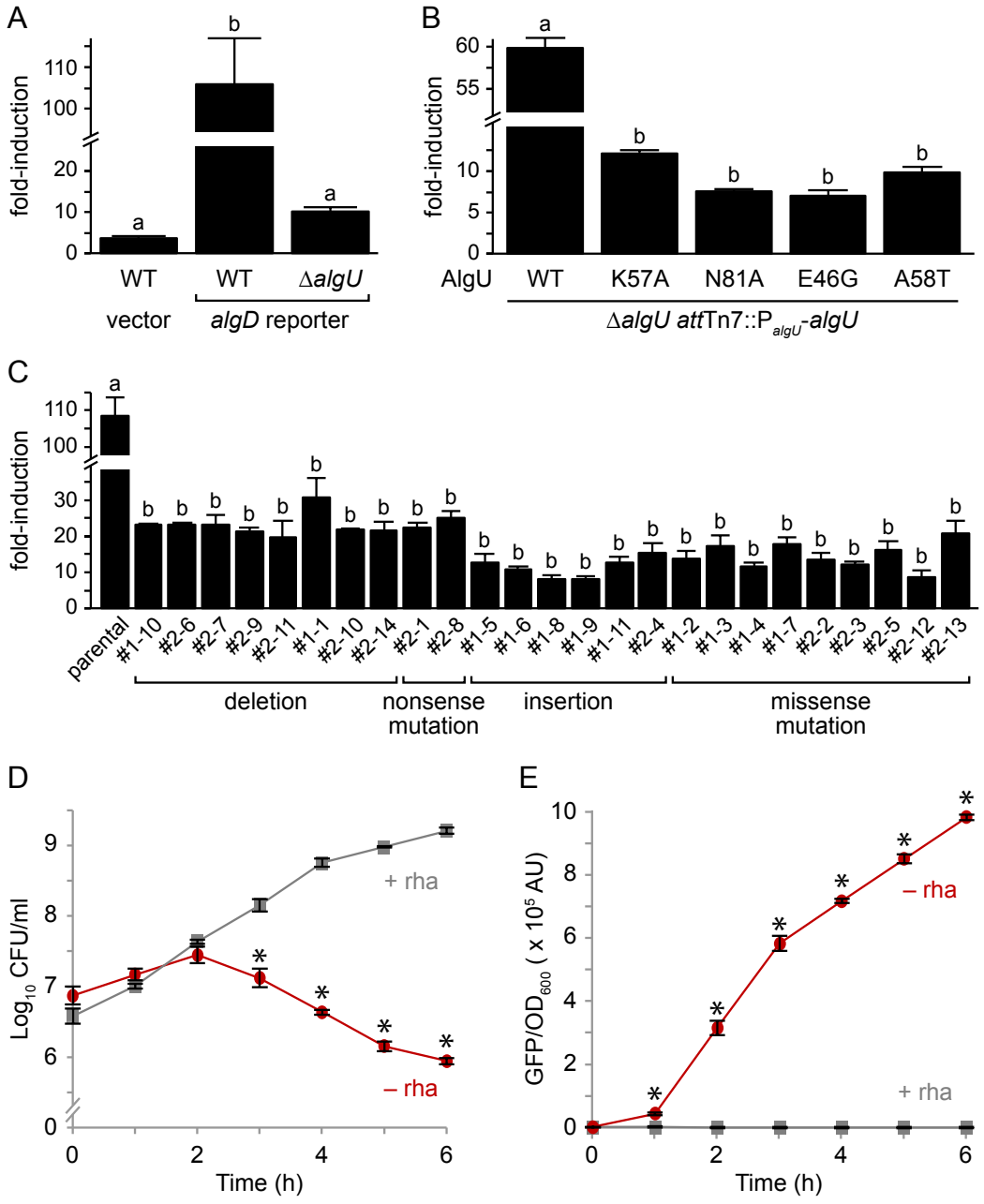


Figure 5. AlgU mutants that suppress *mucA* essentiality have decreased activity.
(A) Fold-induction of GFP in PAO1 wild-type (WT) and Δ*algU* carrying a plasmid with either a promoter-less *gfp* (vector) or a *gfp* driven by the *algD* promoter (*algD* reporter), which is positively regulated by AlgU, 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 groupings ($p < 0.01$; biological triplicate with technical quadruplicates; ANOVA with post-hoc Tukey HSD). **(B)** Fold-induction of GFP in 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 isolate identification numbers are shown and are grouped based on their *algU* mutation. **(D)** Viable colony counts of PAO1 Δ*mucA* attTn7::P_{rhaBAD}-*mucA* 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).

212 155 elsewhere in the genome. As expected (Fig 2B), we were able to delete the native *mucA*
213 allele from a PAO1 strain containing an ectopic allele that encoded for MucA aa 1-155 (with 5 of
214 the 28 tested isolates resolving to the deletion allele), confirming that the ectopic *mucA* allele in
215 the published PAO1 Δ*mucA* strain is sufficient for viability in the absence of the endogenous
216 *mucA*. The PAK and PA103 Δ*mucA* strains (Intile *et al.*, 2014, Jones *et al.*, 2010) contain
217 missense mutations in *algU,* which result in A58T and E46G, respectively. We replicated these
218 mutations in PAO1 by inserting *algU* alleles encoding these substitutions in a Δ*algU* strain. We
219 were able to delete *mucA* from these strains (Fig 4A). These results confirm that the *algU* alleles
220 in the published PAK and PA103 Δ*mucA* strains suppress *mucA* essentiality. Using our reporter
221 assay, we found that strains carrying these AlgU substitutions had reduced sigma factor activity
222 (Fig 5B). We note that our reporter assay is not very sensitive to low levels of AlgU activity. Both
223 PAK and PA103 Δ*mucA* strains are mucoid (Intile *et al.*, 2014, Jones *et al.*, 2010), suggesting
224 that AlgU A58T and E46G are not completely inactive. Nevertheless, our results show that
225 these AlgU mutants have significantly less transcriptional activity than the wild-type protein (Fig
226 5A,B).
227
228 To identify additional suppressors of *mucA* essentiality, we used the Δ*mucA att*Tn7::P_{rhaBAD}-
229 *mucA* strain. This strain was not viable in the absence of rhamnose, but natural revertants arose
230 at a frequency of less than 1 in 10^9 colony forming units. We sequenced the *algU* gene in 25
231 revertants, since AlgU mutants can suppress *mucA* essentiality (Fig 4A). All 25 isolates
232 contained mutations in *algU* that are predicted to be hypomorphic (Fig 4B, Table S2). There
233 were 10 revertants with deletions or nonsense mutations of *algU*, encoding either no product or
234 a truncated product completely lacking Region 4 of the sigma factor. There were 6 revertants
235 that contained multi-base pair duplications in *algU* that would lead to the insertion of 3 or 4
236 amino acids in Region 2 helix 3 of the sigma factor. There were 9 revertants containing
237 missense mutations, 8 of which were unique, encoding the following substitutions: D18G, A21V,

238 Y29C, A47T, D49G, Y59C, N81D, and R174G. Using a model of σ^E in complex with the RNAP
239 core and the promoter element, we expect these insertions and substitutions to affect sigma
240 factor folding, RNAP core interactions, or promoter interactions (Fig S4). Using our *algD*
241 reporter assay in these natural revertants, we saw that all 25 revertants had much lower AlgU
242 activity than the parental strain upon induction of envelope stress (Fig 5C), as predicted.
243 Together, these data strongly suggest that *algU* mutations that encode for a protein with
244 reduced transcriptional activity allow *P. aeruginosa* to survive in the absence of *mucA*.
245
246 If *mucA* essentiality is due to AlgU inhibition, AlgU activity should increase as MucA is depleted
247 from cells, concomitant with the decrease in viability. To test this, the viability and fluorescence
248 (as a proxy for AlgU activity) of a Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* strain containing our *algD*
249 reporter plasmid was measured over time in the absence of rhamnose. This strain had a similar
250 viability to that seen in Figure 2 (Fig 5D), and as MucA was depleted, the AlgU activity
251 increased (Fig 5E). These results show the cell death observed upon MucA depletion is
252 correlated with a dramatic increase in AlgU activity.
253
254 **Overexpression of *algU* in the absence of *mucA* leads to a growth defect**
255 Our results suggest that *mucA* essentiality is due to unregulated AlgU activity. We reasoned that
256 overexpression of *algU* should be toxic. However, *algU* can be overexpressed in wild-type *P.*
257 *aeruginosa* that contain *mucA* (Qiu *et al.*, 2008, Schulz *et al.*, 2015). We therefore examined the
258 effect of overproducing AlgU in wild type, Δ*algU*, and Δ*algU* Δ*mucA* strains, using a
259 chromosomally integrated arabinose-inducible *algU*. Of note, the expression of *algU* is
260 completely dependent on the inducer in the Δ*algU* and Δ*algU* Δ*mucA* strains, which lack positive
261 feedback of AlgU on its own expression. Our results show that in the absence of inducer when
262 AlgU is not overexpressed, all three strains had similar growth rates (Fig 6A). In the presence of

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.

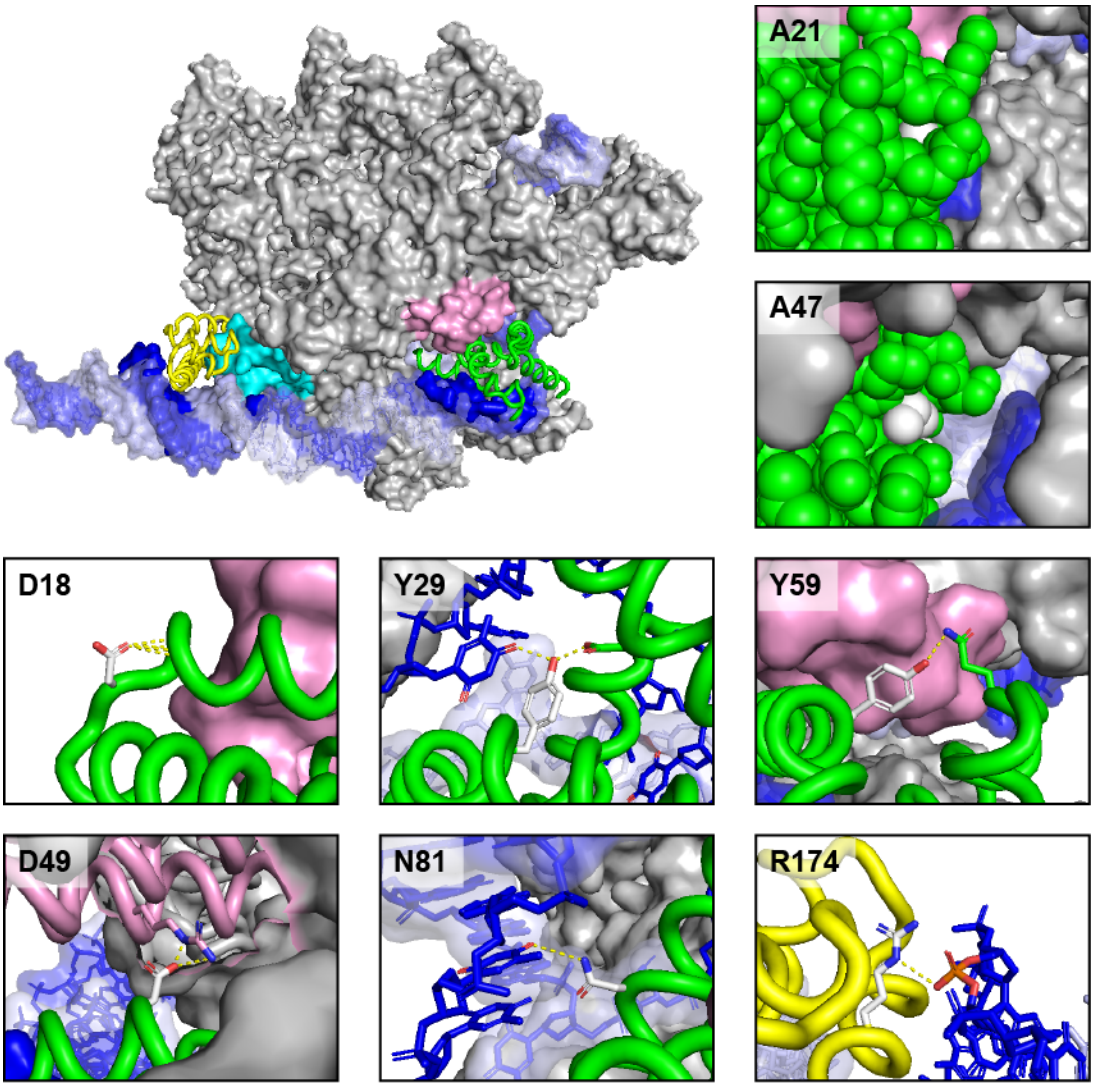


Figure S4. Location of affected AlgU residues in revertants that can grow in the absence of MucA.

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.

263 1% arabinose (i.e. high expression of *algU*), all three strains had a growth defect with the strain
264 lacking *mucA* failing to grow at all.
265
266 To determine if the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* strain can grow under lower levels of *algU*
267 induction, we tested a range of inducer concentrations (Fig 6B, Table S3). We found that
268 although there was a growth defect, Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* was able to grow in the
269 presence of 0.1% arabinose. The drop in growth rate in comparison to the no arabinose
270 condition was statistically larger for the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* than for the other two
271 strains (N=3, $p < 0.05$, ANOVA with post hoc Tukey HSD), since this strain lacks the ability to
272 produce any MucA to reduce AlgU activity. These results strongly suggest that in the absence of
273 *mucA*, while a certain low level of AlgU activity is tolerated, high AlgU activity is fatal to the cell.
274
275 The above experiments were performed using only one medium. To determine if *algU*
276 overexpression in the absence of *mucA* leads to a growth defect under other nutrient conditions,
277 we determined the growth rate of the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* strain in other media.
278 The Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* strain failed to grow in the presence of 1% arabinose for
279 all four media we tested (Fig S5), showing that AlgU overproduction is toxic under various
280 nutrient conditions.
281
282 **Expression of *rpoD* can rescue the growth defect of *algU* overexpression**
283 AlgU competes for RNAP binding with RpoD, the essential primary sigma factor (Yin *et al.*,
284 2013). We, therefore, tested if the growth defect of high *algU* expression in the Δ*algU* Δ*mucA*
285 background could be ameliorated by overexpressing *rpoD* under the same arabinose-inducible
286 promoter. We tested growth in the presence of 2% arabinose, since these new strains contain
287 two arabinose-inducible promoters, in contrast to the strains described in Figure 6. We
288 confirmed that RpoD was overproduced in these new strains at similar levels upon arabinose

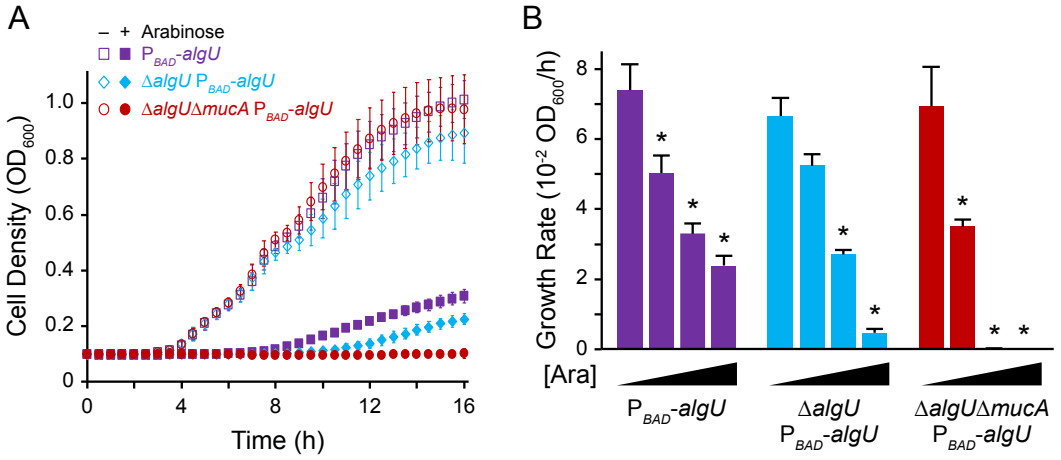


Figure 6. Overexpression of *algU* leads to a growth defect.
(A) Growth curves of PAO1 strains containing an arabinose-inducible copy of *algU* in wild-type (purple squares), Δ*algU* (blue diamonds), and Δ*algU* Δ*mucA* (red circles) backgrounds in LB. Open symbols represent conditions in the absence 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*. 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 without arabinose ($p < 0.01$, N = 3, two-way ANOVA with post-hoc Bonferroni). See Table S3 for full statistical comparisons.

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.

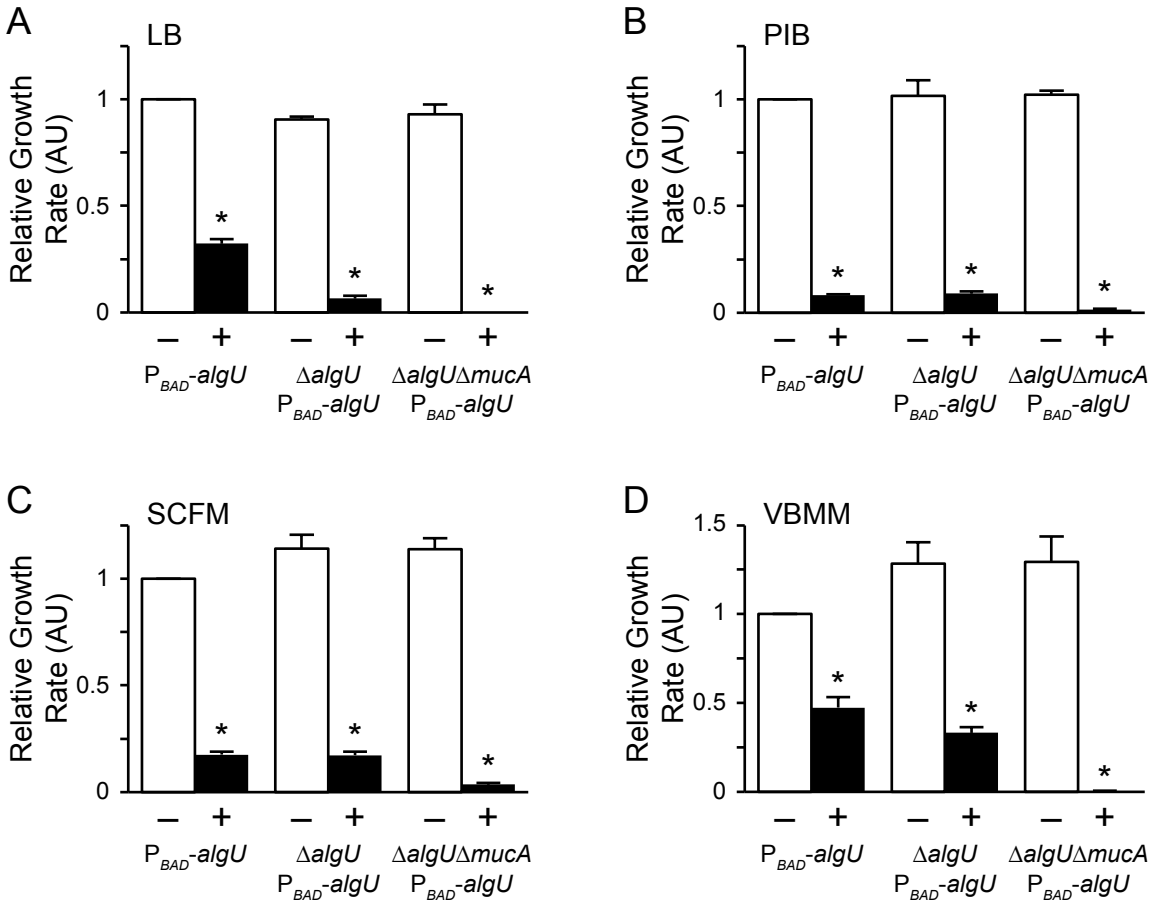


Figure S5. Overexpression of *algU* in the absence of *mucA* causes a growth defect in various media.

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.

289 induction (Fig S6). As expected (Fig 6), Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* failed to grow in the
290 presence of 2% arabinose. The Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU att*CTX::P_{araBAD}-*rpoD* strain,
291 however, was able to grow with arabinose. Furthermore, the growth rate of this strain with
292 arabinose was indistinguishable from that of Δ*algU* Δ*mucA att*CTX::P_{araBAD}-*rpoD* (Table S4).
293
294 There are two major explanations for how sigma factor competition may lead to toxicity during
295 *algU* overexpression. First, the AlgU overproduction may reduce housekeeping gene expression
296 due to the limited availability of RNAP to interact with RpoD. Second, *algU* overexpression
297 results in high AlgU activity, which may lead to toxic expression of the AlgU regulon. We,
298 therefore, constructed a strain containing an inducible *algU* encoding a K57A substitution in the
299 Δ*algU* Δ*mucA* background. AlgU K57A exhibited reduced sigma factor activity (Fig 5A,B) and
300 based on its location in the crystal structure (Campagne *et al.*, 2014), we do not expect this
301 substitution to affect RNAP affinity. In the presence of arabinose, the Δ*algU* Δ*mucA*
302 *att*Tn7::P_{araBAD}-*algU* K57A strain exhibited a growth rate greater than that of the strain
303 overexpressing wild-type AlgU, but significantly less than that of the parental Δ*algU* Δ*mucA*
304 strain (Fig 7, Table S4). RpoD overproduction in this background returned growth to a
305 statistically similar level as that of a strain overexpressing RpoD alone (Table S4). Assuming
306 that the K57A substitution does not affect AlgU affinity for RNAP, these results suggest that
307 AlgU overexpression may lead to toxic expression of the AlgU regulon.
308
309 **DISCUSSION**
310 Contrary to its assumed dispensability, our work shows that *mucA* is essential in a variety of *P.*
311 *aeruginosa* wild-type strains and under various nutrient conditions (Table 1, Fig 1). Our data
312 strongly suggest that unchecked AlgU activity in the absence of MucA leads to cell death (Fig
313 8). Under non-stress conditions, MucA inhibits AlgU. Under envelope stress or in strains
314 containing *mucA* mutations, the anti-sigma factor is cleaved. We propose that while this cleaved

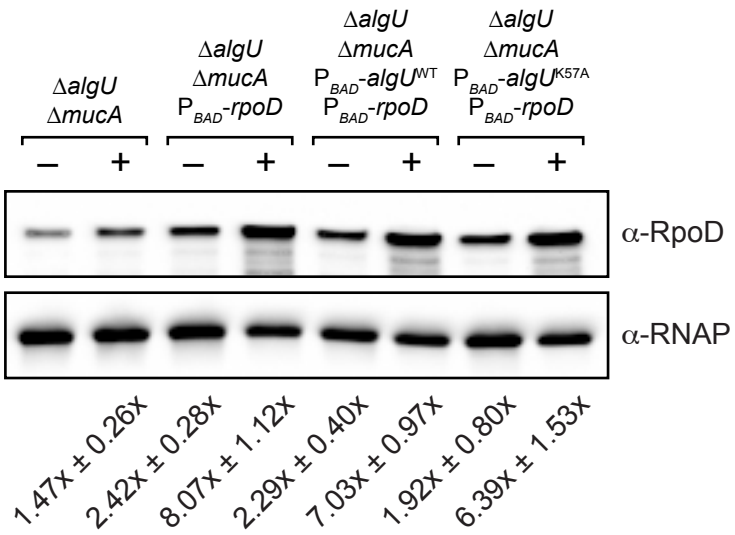


Figure S6. RpoD production in strains with an ectopic inducible *rpoD* allele.
Expression of *rpoD* was inferred via semi-quantitative Western blot in the indicated strains (top). RNAP was used as a loading control (bottom). Strains were grown in LB with (+) or without (–) 2% arabinose. The relative amount of RpoD is indicated, where 1.00x represents RpoD levels in the parental strain without arabinose (± 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 induced strains containing an ectopic *rpoD* allele (two-way ANOVA with post-hoc Bonferroni).

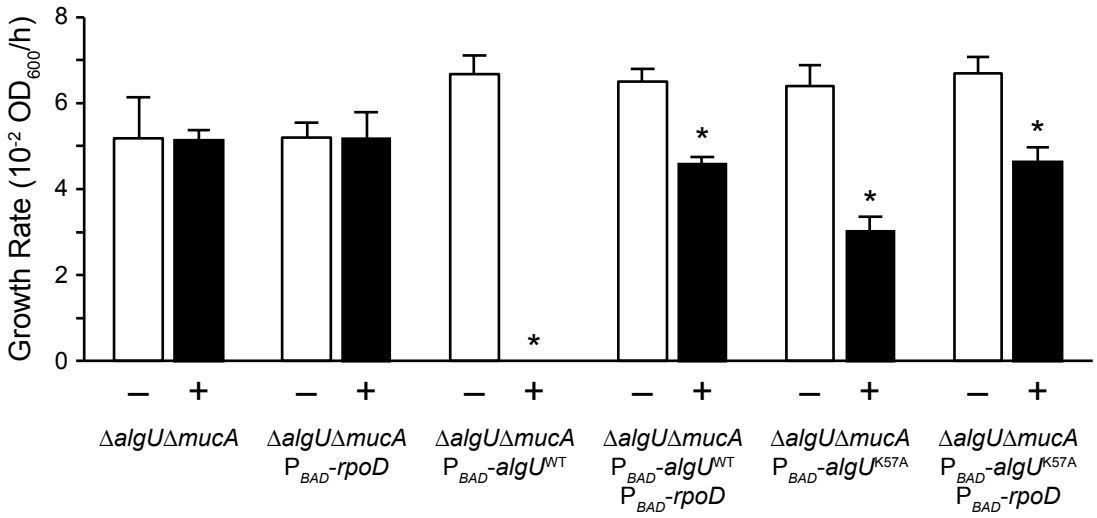


Figure 7. Growth defect caused by *algU* overexpression can be rescued via *rpoD* overexpression.
Growth rate of indicated strains grown in LB with (+) or without (–) 2% arabinose. Error bars, SEM (N=3). Asterisk, statistically different from the same strain grown without arabinose ($p < 0.01$, N = 3, 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* P_{BAD}-*rpoD*		Δ*algU*Δ*mucA* P_{BAD}-*algU*^{WT}		Δ*algU*Δ*mucA* P_{BAD}-*algU*^{WT} P_{BAD}-*rpoD*		Δ*algU*Δ*mucA* P_{BAD}-*algU*^{K57A}		Δ*algU*Δ*mucA* P_{BAD}-*algU*^{K57A} P_{BAD}-*rpoD*	
Strain	Ara	0	2	0	2	0	2	0	2	0	2
Δ*algU*Δ*mucA*	0										
	2				*				*		
Δ*algU*Δ*mucA* P_{BAD}-*rpoD*	0										
	2				*				*		
Δ*algU*Δ*mucA* P_{BAD}-*algU*^{WT}	0				*						
	2						*		*		*
Δ*algU*Δ*mucA* P_{BAD}-*algU*^{WT} P_{BAD}-*rpoD*	0						*				
	2										
Δ*algU*Δ*mucA* P_{BAD}-*algU*^{K57A}	0								*		
	2										
Δ*algU*Δ*mucA* P_{BAD}-*algU*^{K57A} 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.

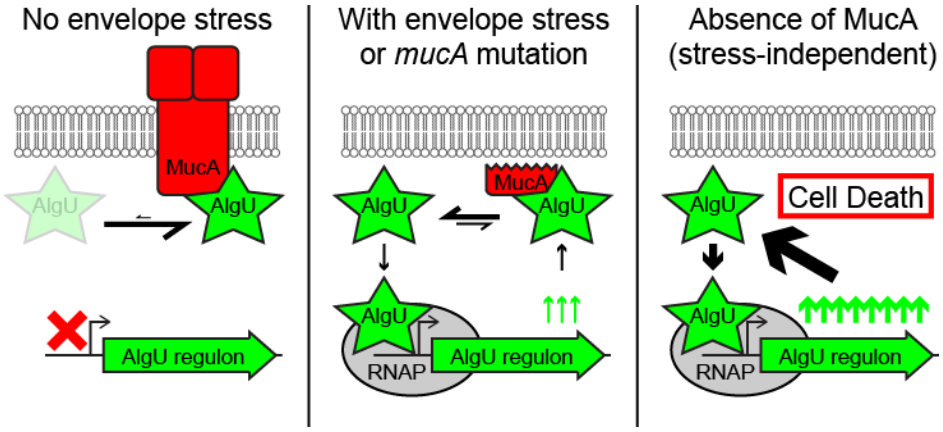


Figure 8. Unchecked AlgU activity in the absence of MucA leads to bacterial cell death. Under conditions of no envelope stress (left), the full-length MucA (red) binds strongly to AlgU (green star), leaving very little free AlgU to interact with the RNAP (grey oval). Therefore, the AlgU regulon (green 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 absence of *mucA* (right), there is no MucA to inhibit 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 AlgU activity leads to cell death.

315 cytosolic form of MucA does not inhibit AlgU to the same extent as the full-length protein, it is
316 still able to inhibit AlgU to some degree. Although AlgU is active under such conditions, because
317 *mucA* is positively regulated by AlgU, this negative feedback keeps AlgU activity under control.
318 In comparison, this ability to control the positive feedback of AlgU on its own expression and
319 activity is lost in the absence of MucA, which we propose is the cause of cell death. Supporting
320 this model are several lines of evidence. First, *mucA* essentiality is rooted in its interaction with
321 *algU* (Fig 2-3), strongly suggesting that the ability of MucA to inhibit AlgU is required for viability.
322 Second, AlgU mutants with decreased transcriptional activity can suppress *mucA* essentiality
323 (Fig 4-5), supporting the idea that decreasing the positive feedback of AlgU on its own
324 expression allows for survival in the absence of MucA. Lastly, *algU* overexpression led to a
325 growth defect, which was lethal at high levels in the absence of *mucA* (Fig 6), suggesting that
326 high AlgU activity leads to cell death when MucA is not present. Overall, our work strongly
327 suggests that *mucA* essentiality is caused by unchecked AlgU activity, in agreement with
328 previous studies suggesting that overproduction of AlgU is toxic (Cross *et al.*, 2020,
329 Hershberger *et al.*, 1995, Schurr *et al.*, 1994).
330
331 AlgU overproduction may be toxic because of reduced housekeeping gene expression (due to
332 sigma factor competition with RpoD) and/or the increased expression of the AlgU regulon.
333 Increasing RpoD levels alleviate the toxicity in both cases, by increasing the RNAP-RpoD
334 complexes and reducing the RNAP-AlgU complexes. Our data does not definitively distinguish
335 between these two mechanisms, which may not be mutually exclusive. Supporting a role for
336 decreased housekeeping functions in AlgU toxicity, *mucA* was still essential in a strain lacking
337 the three major AlgU-regulated transcription factors AlgB, AlgR, and AmrZ (Table S1), which
338 regulate ~50% of the AlgU regulon (Huang *et al.*, 2019, Jones *et al.*, 2014, Leech *et al.*, 2008,
339 Schulz *et al.*, 2015). However, we cannot exclude that a combination of genes in the other half
340 of the AlgU regulon may be responsible for *mucA* essentiality. Supporting the hypothesis that

341 increased AlgU regulon expression causes toxicity, mutations in *algU*, which is AlgU-regulated,
342 suppress *mucA* essentiality (Fig 4). While this autoregulation of AlgU is important for *mucA*
343 essentiality under physiological conditions, it does not lead to toxicity per se, since
344 overexpressing *algU* in a strain lacking the positive feedback led to a severe growth defect (Fig
345 6). Under such conditions, overproduction of AlgU K57A, which has reduced sigma factor
346 activity (Fig 5B), caused a less severe growth defect than wild-type AlgU overproduction (Fig 7).
347 Assuming that RNAP affinity is not affected by this AlgU substitution, this suggests that high
348 AlgU activity and the ensuing regulon expression may be the mechanism underlying toxicity.
349 Work in *Bacillus subtilis* has shown that the toxicity of unregulated SigM, an alternative sigma
350 factor, is due specifically to membrane protein overproduction, which is alleviated by a gain-of-
351 function mutation to the membrane insertase YidC1 (Zhao *et al.*, 2019b). Since only ~80 of the
352 ~200 AlgU-regulated predicted membrane proteins are regulated by AlgB, AlgR, and AmrZ
353 (Huang *et al.*, 2019, Jones *et al.*, 2014, Leech *et al.*, 2008, Schulz *et al.*, 2015), it is possible that
354 a gain-of-function YidC mutant may suppress *mucA* essentiality in *P. aeruginosa*. Such an
355 experiment would help distinguish between the potential mechanisms underlying AlgU toxicity.
356
357 Anti-sigma factor essentiality is not unique to *P. aeruginosa*. In the defined transposon mutant
358 library for *Vibrio cholerae*, an interruption in the *mucA* homolog *rseA* does not exist, suggesting
359 that this anti-sigma factor may be essential (Cameron *et al.*, 2008). Similarly, the *Pseudomonas*
360 *syringae mucA* homolog is deemed essential based on transposon insertion analysis (Helmann
361 *et al.*, 2019). Furthermore, in *Mycobacterium tuberculosis*, the gene encoding the anti-sigma
362 factor RslA is identified as essential (Griffin *et al.*, 2011). Similar to our results (Fig 4), *rslA* can
363 be deleted from *M. tuberculosis* lacking the gene encoding its cognate sigma factor SigL, which
364 regulates genes involved in cell envelope processes (Dainese *et al.*, 2006). Finally, there are
365 two anti-sigma factors, YhdL and YxlC, in *B. subtilis* that are necessary for bacterial viability
366 (Horsburgh & Moir, 1999, Koo *et al.*, 2017, Mendez *et al.*, 2012). The *yhdL* essentiality is

367 notable as this anti-sigma factor negatively regulates SigM, an envelope stress response sigma
368 factor (Horsburgh & Moir, 1999). Its essentiality could be suppressed by overproduction of SigA,
369 the *B. subtilis* housekeeping sigma factor (Zhao *et al.*, 2019a) and by a hyperactive form of the
370 membrane insertase YidC1 (Zhao *et al.*, 2019b). Interestingly, the YidC1 mutant ameliorated the
371 toxicity of unregulated SigM by reducing the secretion stress associated with high membrane
372 protein production (Zhao *et al.*, 2019b). Taken together with our data, while not universal (as
373 there are no essential anti-sigma factors in *E. coli* (Baba *et al.*, 2006)), we speculate that
374 essentiality of anti-sigma factors that regulate cell envelope function may be a widespread
375 phenomenon in bacteria and that their essentiality is due to the increased production of
376 membrane proteins when their sigma factors are not held in check.
377
378 In clinical CF isolates of *P. aeruginosa*, *mucA* mutations that lead to C-terminal truncations are
379 common (Fig S2) and are expected to result in proteins that retain partial AlgU-inhibitory
380 function. Interestingly, there is one *P. aeruginosa* CF isolate that is reported to have a *mucA*
381 mutation that would result in a truncation within the first 50 aa of the protein (Boucher *et al.*,
382 1997). Our sequencing data of the published Δ*mucA* strains (Intile *et al.*, 2014, Jones *et al.*,
383 2010, Pritchett *et al.*, 2015) suggests that this isolate may contain a suppressor mutation that
384 allows it to survive in the absence of a functional MucA. Nonetheless, in CF clinical isolates,
385 *mucA* mutations that lead to C-terminal truncations are the norm, while null mutations in *mucA*
386 with a presumed hypomorphic *algU* allele is rare (Fig S2). Furthermore, our results (Fig 4) agree
387 with the literature showing that mucoid isolates with *mucA* mutations revert to a non-mucoid
388 state via changes to *algU* (Ciofu *et al.*, 2008, DeVries & Ohman, 1994, Sautter *et al.*, 2012,
389 Schurr *et al.*, 1994). While these *algU* secondary site mutations are found in non-mucoid CF
390 isolates with *mucA* mutations, such isolates are detected at a lower frequency (Ciofu *et al.*,
391 2008). As suggested by Ciofu and associates, this may be due to the importance of AlgU for the
392 survival of *P. aeruginosa* in the CF lung environment, suggesting that reducing AlgU activity

393 may increase *P. aeruginosa* eradication from the CF airway. We propose that the MucA-AlgU
394 interaction may serve as a good therapeutic target. Our results show that destabilizing the
395 MucA-AlgU interaction results in bacterial cell death or mutations in *algU* that result in reduced
396 sigma factor activity and likely reduced mucoid conversion, of which either outcome could be
397 beneficial in the treatment of *P. aeruginosa* CF lung infections.
398

399 **METHODS AND MATERIALS**

400 **Bacterial strains and growth conditions**. The bacterial strains, plasmids, and oligonucleotides
401 used for this study are in Tables S5-S7. The construction of strains is described in
402 Supplementary Information. Bacteria were grown at 37°C in LB with shaking or on semi-solid LB
403 media, unless otherwise noted.
404

405 **Allelic exchange assay.** The protocol, depicted in Figure S1, was modified from (Hmelo *et al.*,
406 2015). Briefly, a vector containing the *mucA* deletion allele flanked by approximately equal
407 length homology regions is introduced via conjugation. Merodiploids were selected on semi-
408 solid VBMM (Vogel & Bonner, 1956) with 60 mg/L gentamicin. Using OMS118 and OMS119,
409 PCR was performed on at least six isolates to confirm that the presence of both the wild-type
410 and deletion alleles of *mucA*. Confirmed merodiploids were then individually streaked on NSLB
411 (10 g/L tryptone, 5 g/L yeast extract) with 10% sucrose semi-solid media for counterselection.
412 PCR was performed on eight colonies per merodiploid, using OBT601 and OBT602 to
413 determine the resolution to either the endogenous or deletion allele.
414

415 **MucA depletion assay.** Cells were grown in LB with 0.05% rhamnose at 37°C with shaking to
416 an OD_{600} of 0.3. After washing, the culture was then divided in two, half resuspended with
417 rhamnose and half without in its respective media base (LB, PIB, SCFM, or VBMM). Cells were
418 incubated at 37°C with shaking. At the indicated time points, two aliquots were removed from

419 each culture, serially diluted and plated in triplicate onto LB agar plates with 0.05% rhamnose
420 for recovery. Colonies were counted and log_{10}-transformed CFU/mL of the culture was
421 calculated.
422
423 **Yeast two-hybrid assay.** The ProQuest Two-Hybrid System (Invitrogen) was used, per
424 manufacturer's instructions. Briefly, yeast containing the Gal4 activation domain-based prey and
425 the Gal4 DNA-binding domain-based bait vectors were grown overnight in SD-Leu-Trp broth
426 (Clontech). The OD_{600} value was recorded. ONPG (VWR) was added to lysed cells, and the
427 mixture was incubated at 37°C until a light yellow color was achieved. The incubation time was
428 recorded. The OD_{420} of the supernatants was determined using a Synergy Hybrid HTX
429 Microplate Reader (Bio-Tek Instruments). Beta-galactosidase activity was determined using
430 Miller units, based on the following equation: 1000 x OD_{420}/(time x culture volume x OD_{600}).
431
432 **AlgU activity assay.** Strains of interest were transformed with a plasmid-borne *algD* reporter
433 (pBT435) via electroporation (Choi *et al.*, 2006). Strains were grown overnight in LSLB with 50
434 mg/L gentamicin and 0.05% rhamnose (where indicated) at 37°C with shaking. The overnight
435 culture was diluted 1:100 and grown to an OD_{600} of 0.3. Cells were then treated with fresh 400
436 mg/L D-cycloserine for 2 h at 37°C with shaking, as previously described (Wood *et al.*, 2006).
437 Cells were then pelleted and resuspended in PBS. GFP fluorescence and OD_{600} was
438 determined using a Synergy Hybrid HTX Microplate Reader (Bio-Tek Instruments). To
439 normalize the data, the GFP fluorescence was divided by the OD_{600} for each data point. To
440 determine the fold induction, the ratios for the treated samples were divided by the average of
441 that for the same strain not treated with D-cycloserine. The resulting ratios of the triplicate
442 samples were averaged.
443

444 **Natural revertant assay.** *P. aeruginosa* Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* was grown in LB with
445 0.05% rhamnose at 37°C with shaking to an OD_{600} of 1. Cells were washed and plated on semi-
446 solid LB plates without rhamnose. Plates were incubated for 24-48 h at 37°C. For isolates that
447 grew on LB without rhamnose, *algU* was Sanger sequenced.
448

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457

458 **AUTHOR CONTRIBUTIONS**

459 Conceptualization: MCS, BST; Acquisition, analysis, or interpretation of data: MCS, DR, AAK,
460 EKC, LAM, EAC, PAJ, BST; Writing – original draft: MCS, BST; and Writing – review and
461 editing: MCS, DR, LAM, EAC, PAJ, BST.
462

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