# Inhibition of AlgU by MucA is required for viability in *Pseudomonas aeruginosa*

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**ABSTRACT**

*Pseudomonas aeruginosa* causes chronic lung infections in people with cystic fibrosis (CF), and this bacterium undergoes selection in the CF lung environment over the course of these life-long infections. One genetic adaptation frequently observed in CF *P. aeruginosa* isolates is mutation of *mucA.* MucA inhibits the sigma factor AlgU. Clinical *mucA* mutations lead to misregulation of AlgU, resulting in a mucoid bacterial 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*, and *mucA* alleles that encode for proteins that do not bind to AlgU are insufficient for bacterial viability. Furthermore, we found that *mucA* is no longer essential in mutant strains containing AlgU variants with reduced sigma factor activity, suggesting that reducing AlgU activity can suppress the requirement for *mucA*. Finally, we found that overexpression of *algU* from an inducible promoter 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 leads to sigma factor competition, preventing the expression of essential housekeeping genes and resulting in the loss of bacterial viability.

**AUTHOR SUMMARY**

Clinical *Pseudomonas aeruginosa* isolates with mutations in the gene encoding the anti-sigma factor MucA have long been observed, and loss of MucA function is thought to be advantageous for chronic infection in the cystic fibrosis (CF) airway. Surprisingly, our work shows that MucA is required for bacterial viability in various *P. aeruginosa* strains, due to its inhibition of the sigma factor AlgU. Our results suggest that without MucA, the unchecked activity of AlgU competes with housekeeping functions, leading to bacterial cell death. Therefore, there is a potential benefit of targeting this MucA-AlgU interaction for those with CF, as our results show that eliminating this interaction either kills *P. aeruginosa* or selects for bacteria with *algU* mutations that have a reduced capacity for mucoid conversion.

44 **INTRODUCTION**

45 The major cause of death in people with cystic fibrosis (CF), a human autosomal recessive
46 genetic disease, is respiratory failure due to chronic lung infection. *Pseudomonas aeruginosa* is
47 one of the most prevalent respiratory pathogens of CF patients [1]. The CF lung environment
48 selects for mucoid *P. aeruginosa* mutants, which are associated with poor disease prognosis
49 and the overproduction of the exopolysaccharide alginate [2-6]. Conversion to the mucoid
50 phenotype in clinical *P. aeruginosa* isolates is frequently caused by *mucA* mutations [7].
51
52 MucA is an anti-sigma factor to the alternative sigma factor AlgU (also known as AlgT, $\sigma^E$, or
53 $\sigma^{22}$), which responds to envelope stress [8, 9]. MucA is a transmembrane protein that
54 sequesters the sigma factor away from RNA polymerase (RNAP) via its N-terminal interaction
55 with AlgU [10-12]. The C-terminus of MucA is in the periplasm, where it is protected from
56 proteolysis via an interaction with MucB [10, 13]. The envelope stress response is controlled via
57 a regulated intramembrane proteolysis cascade: MucB dissociates from MucA upon detection of
58 stress, allowing the proteases AlgW and MucP to cleave MucA from the inner membrane [14,
59 15]. In the cytoplasm, MucA is further degraded by ClpXP, releasing AlgU to interact with RNAP
60 and activate the AlgU regulon [16]. AlgU regulates at least 350 genes, including those
61 responsible for the production of itself and its cognate anti-sigma factor MucA, as well as the
62 biosynthesis of the exopolysaccharide alginate [15, 17, 18]. This system is homologous to the
63 well-studied envelope stress response in *Escherichia coli*: MucA shares 28% identity (72%
64 similarity) to the anti-sigma factor RseA, and the cognate sigma factor of the *E. coli* RseA, called
65 RpoE, is homologous to and functionally interchangeable with AlgU [19], with 66% identity (93%
66 similarity).
67

68 For several reasons, *mucA* is assumed to be dispensable for *P. aeruginosa* viability. First, after
69 being released from the cell membrane, MucA is presumed to be fully degraded in the
70 cytoplasm by ClpXP [16]. Second, *mucA* mutations commonly arise in clinical CF isolates [7,
71 20-23], and many laboratory *mucA* mutants exist in the literature [24-27]. Third, there are three
72 published strains in which the entirety of *mucA* is removed from the genome [28-30].
73 Paradoxically, three different whole-genome studies of essential genes in two different strains of
74 *P. aeruginosa* identified *mucA* as an essential gene, based on the low observed frequency of
75 transposon insertions in this gene [25, 26, 31]. To investigate this paradox, we attempted to
76 delete *mucA* from *P. aeruginosa* using allelic exchange. Our results show that a portion of *mucA*
77 is required for bacterial viability in multiple *P. aeruginosa* strains. Furthermore, our work shows
78 that *mucA* was no longer essential in a strain lacking *algU*, and that *mucA* alleles that encode
79 for proteins that do not interact with AlgU were insufficient to rescue viability or lead to a growth
80 defect. We found that *algU* mutations, which encode for a less active sigma factor, can relieve
81 *mucA* essentiality, and that overproduction of AlgU in the absence of MucA is lethal.
82 Interestingly, our works suggests that *mucA* essentiality can be suppressed by increasing the
83 levels of the housekeeping sigma factor RpoD. Together, our results strongly suggest that the
84 unregulated activity of AlgU itself, in the absence of MucA, leads to bacterial cell death, which is
85 in part due to sigma factor competition with RpoD.
86

87 **RESULTS**

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

89 To determine if *mucA* is essential, we attempted to delete the gene from various *P. aeruginosa*
90 strains using a modified allelic exchange protocol to turn it into a robust assay (Fig S1). We
91 introduced a deletion allele of *mucA*, which is missing >95% of the coding region, into *P.*
92 *aeruginosa* and selected for merodiploids. Via PCR, we confirmed that isolates contained both
93 the endogenous and deletion alleles of *mucA* in the genome. Using six confirmed merodiploids,

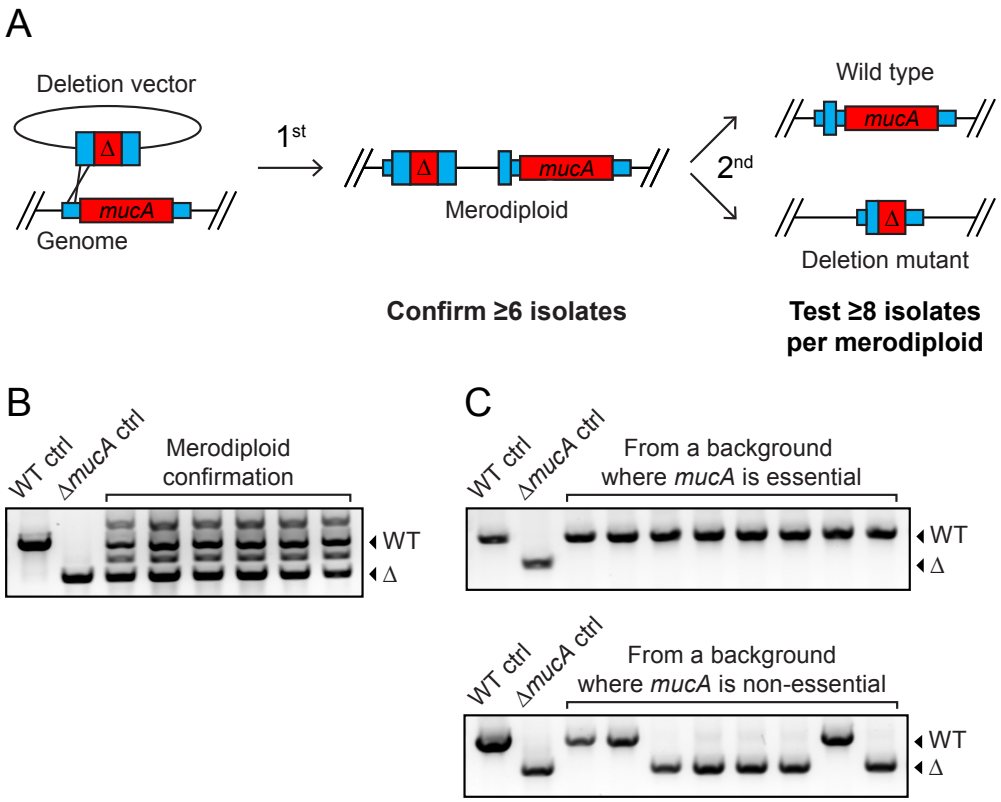



**Figure S1. Allelic exchange assay.**
**(A)** Schematic of assay. To delete the endogenous *mucA* (short red box with *mucA*) from the genome, the deletion vector pBT396, which contains an allele of *mucA* that is missing >95% of the coding region (tall red box with delta), was introduced into *P. aeruginosa*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

94 we then counter-selected for loss of one of the two alleles. Isolates were then tested via PCR to
95 determine which *mucA* allele the isolate resolved to. If a gene is non-essential, isolates that
96 resolved to either the wild-type or deletion allele should be observed. However, if a gene is
97 essential, then only the cells that resolved to the wild-type allele should be isolated, as cells
98 cannot survive in the absence of the gene. To give statistical power to our assay, we performed
99 three biological replicates for strains from which we were unable to delete *mucA*, for a minimum
100 total of 125 isolates screened. We performed this assay on a diverse set of wild-type *P.*
101 *aeruginosa* strains, including four common laboratory isolates as well as seven other clinical
102 and environmental isolates (Table 1). These strains are not only isolated from diverse locations,
103 but they also vary in their colony morphology and their exopolysaccharide production profile
104 [32]. For all strains tested via our allelic exchange assay, all observed isolates had the wild-type
105 *mucA* allele ($p < 0.0001$, Fisher's exact test), strongly suggesting that *mucA* is essential in a
106 variety of wild-type *P. aeruginosa* strains. Paradoxically, among the *P. aeruginosa* isolates we
107 tested were three laboratory strains (PAO1, PAK, and PA103) for which Δ*mucA* mutants have
108 previously been published [28-30]. Since we were unable to delete *mucA* from these strains, it
109 suggests that the published Δ*mucA* strains in these *P. aeruginosa* backgrounds contain
110 secondary site mutations that allow for their viability, as later described. Nonetheless, since all
111 tested strains required *mucA* for viability, we continued our experiments using the laboratory
112 isolate PAO1 as a representative strain.
113
114 **Alginate biosynthesis is not solely responsible for *mucA* essentiality**
115 Clinical *P. aeruginosa* isolates containing *mucA* mutations are mucoid due to their
116 overproduction of alginate [7]. To determine if alginate overproduction is responsible for *mucA*
117 essentiality, we attempted to delete *mucA* from a strain lacking *algD*, a key alginate biosynthesis
118 gene [33]. Using our allelic exchange assay, we were unable to delete *mucA* in this background
119 (Table S1), suggesting that alginate production alone is not responsible for *mucA* essentiality.

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

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

[a] Class identification is based on the exopolysaccharide expression profile as described in [32]: 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 | |
|---|---|---|
| | Δ*mucA* | WT |
| WT | 0* | 168 |
| Δ*algD* | 0* | 144 |
| Δ*algB* | 0* | 158 |
| Δ*algR* | 0* | 153 |
| Δ*amrZ* | 0* | 131 |
| Δ*algB* Δ*algR* Δ*amrZ* | 0* | 133 |

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

120

121 Expression of the alginate biosynthesis genes is controlled by three AlgU-regulated transcription
122 factors that are active in mucoid cells: AlgB, AlgR, and AmrZ [34-36]. Since these transcription
123 factors regulate a suite of genes in addition to those involved in alginate biosynthesis [37-40],
124 we tested if overexpression of these three regulons underlies *mucA* essentiality by attempting to
125 delete *mucA* from strains lacking these transcription factors, either individually or all together.
126 We found that we could not delete *mucA* in any of these backgrounds (Table S1). Altogether,
127 our data support the conclusion that eliminating alginate biosynthesis and the expression of
128 large parts of the AlgU regulon do not alleviate *mucA* essentiality.

129

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

131 While we were unable to delete *mucA* using an allele in which >95% of the coding region is
132 missing, *mucA* is commonly mutated in clinical isolates of *P. aeruginosa* and many *mucA*
133 transposon mutants exist (Fig S2). These data suggest that only a portion of *mucA* is essential,
134 instead of the entire gene. To determine the necessary portion of *mucA*, we first constructed a
135 series of strains containing an ectopic *mucA* at the neutral *att*Tn7 site in the genome, driven by
136 its native promoter. We then attempted to delete the endogenous wild-type *mucA* from these
137 strains. Although we designed these ectopic alleles to encode for a protein with a His-tag, we
138 were unable to probe whether equivalent amounts of protein were produced across our strains,
139 since truncated versions of MucA are cleaved in the cytosol, as shown in previous work [16].
140 However, we expect that the ectopic alleles produce similar protein levels, as they are all under
141 the same promoter and located in the same genomic site. Since we expected that the resultant
142 Δ*mucA* strain would be mucoid in some cases, we used a Δ*algD* background to eliminate
143 alginate production, which made the strains easier to manipulate. As described above, *mucA*
144 was still essential in the Δ*algD* background (Table S1).

145

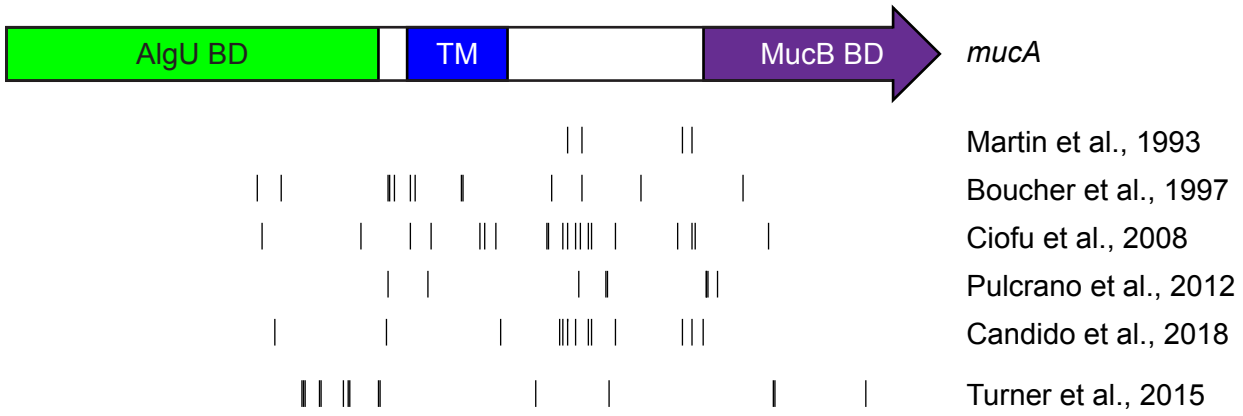



**Figure S2. Published mutations in *mucA*.**
Top, schematic of *mucA* with the regions indicated encoding for the AlgU binding domain (green, AlgU BD), the transmembrane domain (blue, TM), and the MucB binding domain (purple, MucB BD). Bottom, sites of *mucA* mutations that lead to the production of 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 [32-36] are in cystic fibrosis clinical isolates of *P. aeruginosa*, while the mutations in the last reference [37] are random transposon insertions in the laboratory PAO1 and PA14 strains.

146 Mutant MucA proteins of varying lengths were ectopically expressed to determine the necessary
147 portion of MucA. Full-length MucA is 194 amino acids (aa) in length. Based on the co-crystal
148 structures of MucA with AlgU and MucB [12], the first 78 residues of MucA interact with AlgU,
149 and the last 48 residues of MucA interact with MucB (Fig 1A). We engineered *mucA* alleles that
150 encode for proteins lacking the MucB binding domain (aa 1-143), the entire periplasmic domain
151 (aa 1-110), and the transmembrane and periplasmic domains (aa 1-75), as well as alleles that
152 encode for only parts of the AlgU binding domain (aa 1-62, 1-50, 1-40, and 1-24)(Fig 1A). Using
153 our allelic exchange assay, we attempted to delete the endogenous *mucA* from strains
154 containing these ectopic *mucA* alleles. Since the flanking regions of these two alleles differ, our
155 deletion allele specifically targets the native allele. We were able to easily recover isolates that
156 resolved to the *mucA* deletion allele from strains containing ectopic alleles that encode for the
157 full length MucA (aa 1-194), as well as MucA aa 1-143, aa 1-110, aa 1-75, and aa 1-62. These
158 results show that the requirement for *mucA* can be complemented *in trans*, and suggest that the
159 gene product of *mucA* is required for viability, irrespective of its genomic location.
160
161 Interestingly, while we were able to delete the endogenous *mucA* from strains containing an
162 ectopic *mucA* that encoded for aa 1-50 (Fig 1B), isolates that resolved to the deletion allele
163 grew up more slowly than those that resolved to the wild-type allele. This suggests that while
164 this allele is sufficient for viability, it is not well tolerated by the cells. All tested isolates of strains
165 carrying *mucA* alleles encoding shorter products (aa 1-40 and aa 1-24) resolved to the wild-type
166 allele. These results show that while an allele encoding the first 50 residues of MucA is sufficient
167 for cell viability, alleles encoding for shorter lengths are not.
168
169 To determine if the first 50 residues of MucA are necessary for viability, we constructed a strain
170 with an ectopic *mucA* that encodes for aa 51-194. We were unable to delete the native *mucA*
171 from this strain, suggesting that this allele is not sufficient for cell viability (Fig 1B). Together,

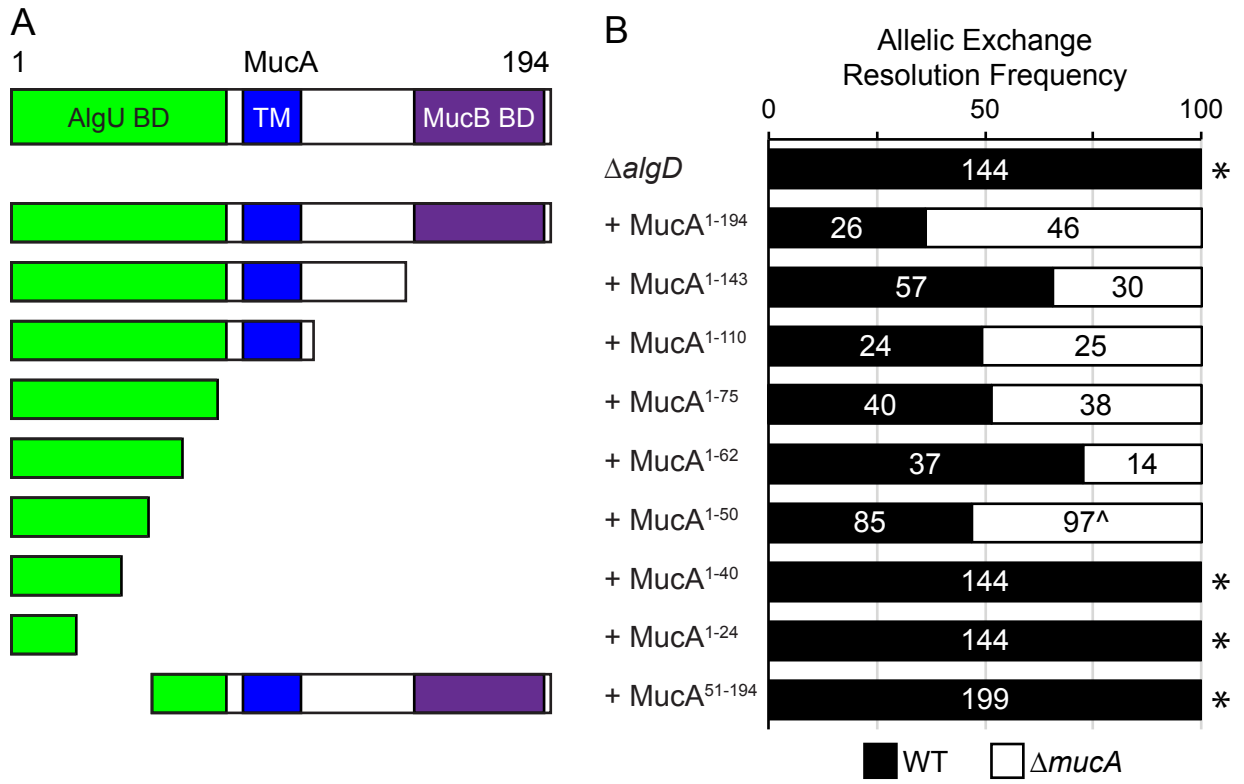



**Figure 1. The first 50 amino acids of MucA are necessary and sufficient for viability.** **(A)** Schematic of MucA encoded by the ectopic *mucA* alleles in the PAO1 Δ*algD* strains tested for *mucA* essentiality in (B). MucA aa 1-143 is the product of the common *mucA22* allele. MucA 1-110 lacks the entire periplasmic domain. MucA aa 1-75 contains almost all 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 DB); 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.

172 these results suggest that the first 50 amino acids of MucA are necessary and sufficient for cell
173 viability in *P. aeruginosa*. Consistent with these results, while many clinical and engineered
174 *mucA* mutations have been reported [7, 20-23, 27], no mutations fall within the region of *mucA*
175 that encodes for the first 50 residues (Fig S2). Furthermore, this region of MucA corresponds to
176 the AlgU binding domain, suggesting that the physical interaction between the two proteins is
177 important for cell viability.
178
179 **The interaction of MucA with AlgU is required for cell survival**
180 Since the only described function for MucA is to inhibit AlgU, we hypothesized that *mucA*
181 essentiality is rooted in its regulation of AlgU. In such a case, *mucA* essentiality should require
182 *algU*. Therefore, we attempted to delete *mucA* from a strain lacking *algU* (Δ*algU*), using our
183 allelic exchange assay. We found that we were able to delete *mucA* in the absence of *algU*, with
184 16 of the tested isolates resolving to the deletion allele out of the 48 colonies tested (see Fig
185 3A), showing that *mucA* essentiality is *algU*-dependent.
186
187 Because our data suggest that the AlgU-binding domain of MucA is required for viability (Fig
188 1B), we hypothesized that the physical interaction between MucA and AlgU is necessary for cell
189 survival. In the co-crystal structure of MucA and AlgU [12], there are four amino acids in MucA
190 with side chains that make more than one hydrogen bond with AlgU: D15, E22, R42, and E72
191 (Fig 2A). Although E72 likely contributes to the binding energy between these two proteins, it is
192 unlikely that E72 is critical to the MucA-AlgU interaction, since the first 50 amino acids of MucA
193 are sufficient for viability (Fig 1B). Therefore, we engineered alleles that encode for MucA D15A,
194 E22A, or R42A to maximally affect the hydrogen bonding while limiting the effects on overall
195 protein structure. We used an allele encoding MucA aa 1-75 as a base for these mutations
196 because the co-crystal structure includes the first 78 residues of MucA and such an allele is
197 sufficient for viability without growth issues (Fig 1B). To determine the effect of these

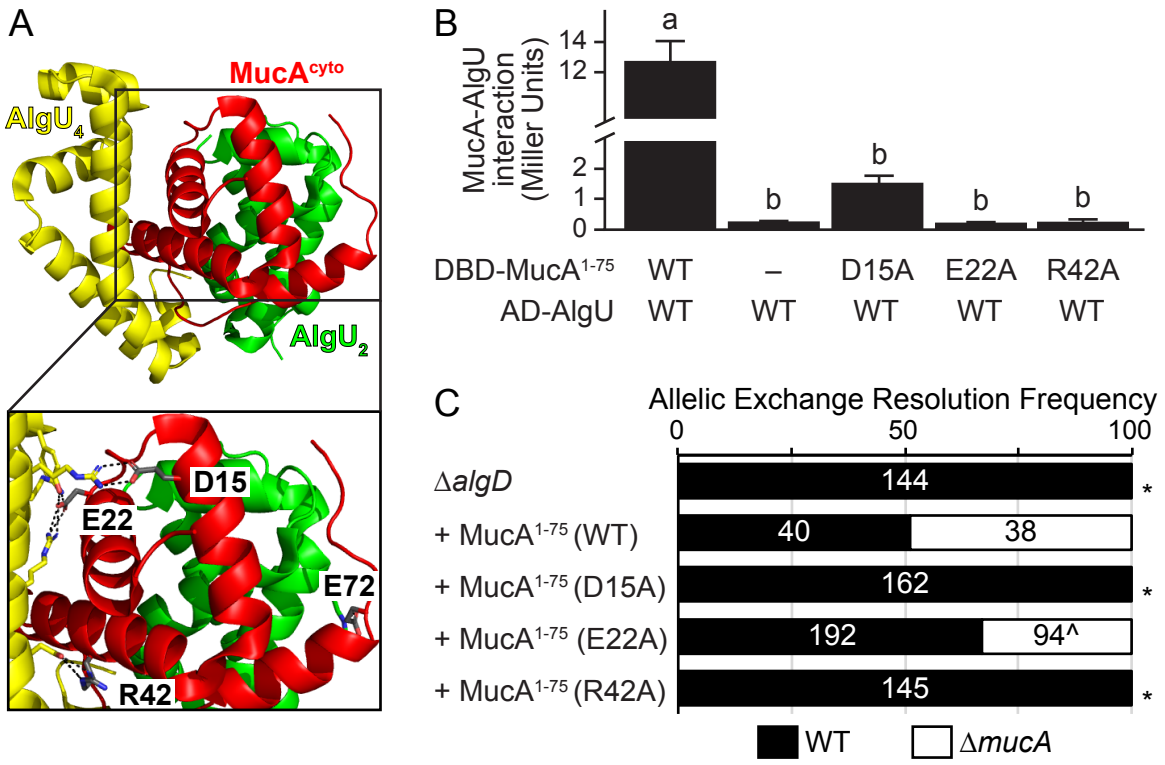
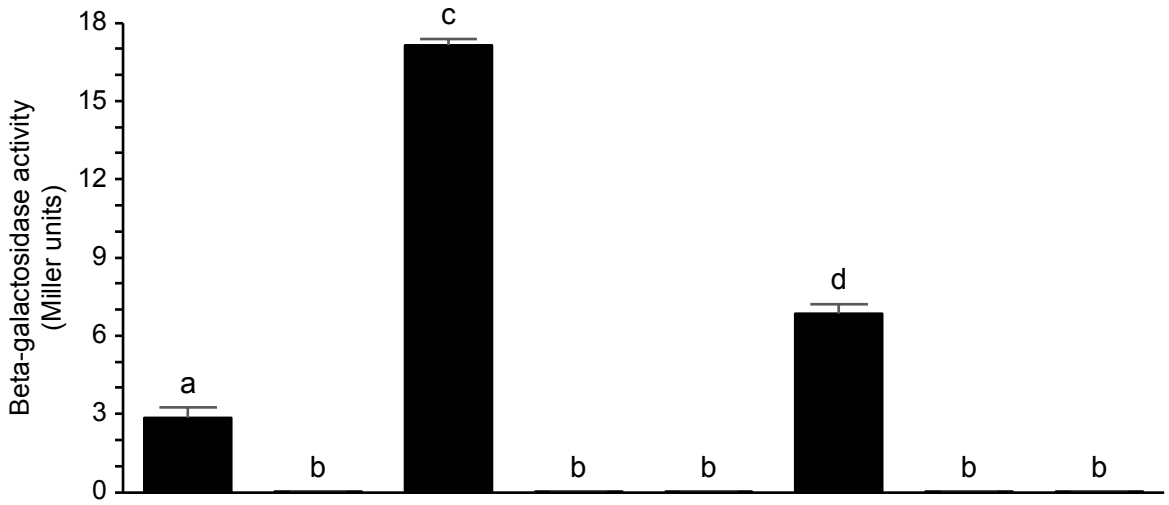



**Figure 2. The physical interaction of MucA and AlgU is required for survival.**
**(A)** Four residues of MucA make greater than one predicted hydrogen bond with AlgU. The MucA-AlgU co-crystal structure (6IN7) with the cytosolic domain of MucA (aa 1-78; red) and Regions 2 and 4 of AlgU (green, $AlgU_2$; 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 lead 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.

198 substitutions on the MucA-AlgU interaction, we used a yeast-two hybrid assay. Using beta-
199 galactosidase activity as a proxy for their interaction, wild-type MucA aa 1-75 strongly interacted
200 with AlgU (Fig 2B). This interaction was dependent on the presence of both MucA and AlgU,
201 and the effect was not directional, as we saw a similar response when the binding partners were
202 switched (Fig S3). In comparison to the wild-type MucA, the D15A, E22A, and R42A MucA
203 mutants failed to interact with AlgU (Fig 2B). To determine the effects of these mutations on *P.*
204 *aeruginosa* viability, we engineered strains carrying an ectopic *mucA* encoding these
205 substitutions and then attempted to delete the native *mucA*. As described above, an allele
206 encoding MucA aa 1-75 was sufficient for viability (Fig 1B). However, we were unable to delete
207 the endogenous *mucA* from strains carrying alleles encoding the D15A and R42A substitutions,
208 suggesting that the mutant protein is not sufficient for viability (Fig 2C). For a strain carrying a
209 *mucA* allele encoding the E22A substitution, we were able to delete the endogenous *mucA*.
210 However, isolates that resolved to the deletion allele in this strain grew up slower than those
211 that resolved to the wild-type allele. Similar to the *mucA* allele encoding only the first 50 amino
212 acids, these results suggest that while MucA E22A is sufficient for viability, the cells do not
213 tolerate it well.
214
215 **Mutations that reduce AlgU activity alleviate the requirement for MucA**
216 Since MucA inhibits AlgU from binding to RNA polymerase, we hypothesized that *mucA*
217 essentiality is due to the regulation of AlgU and the expression of its regulon, which includes
218 *algU* itself. To start to test this hypothesis, we reasoned that strains containing mutations
219 encoding a mutant AlgU with lower affinity for DNA would alleviate the requirement for *mucA*,
220 since the expression of the AlgU regulon would inherently be lower in such AlgU mutants and,
221 thus, may not require MucA. Therefore, we engineered strains where the native *algU* is deleted
222 and an *algU* allele under its native promoter encoding such DNA-binding mutants is inserted at
223 the neutral *att*Tn7 site on the chromosome. Based on homology to *E. coli* RpoE mutants that




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 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).

224 have decreased *in vitro* transcriptional activity [41], we engineered *algU* alleles that encode for
225 K57A and N81A. We then attempted to delete *mucA* from Δ*algU* strains containing these alleles.
226 As described above, we were able to delete *mucA* from a Δ*algU* strain (Fig 3A). This phenotype
227 could be complemented via the addition of a wild-type *algU* allele, as we were no longer able to
228 delete *mucA* from such a strain. In contrast, *mucA* could be readily deleted from strains carrying
229 alleles encoding AlgU K57A or N81A, showing that such mutant alleles failed to rescue the
230 Δ*algU* phenotype (Fig 3A). To determine the effect of these substitutions on AlgU activity, we
231 induced envelope stress using D-cycloserine [42] and measured AlgU activity using a plasmid-
232 borne *gfp* reporter driven under the AlgU-regulated *algD* promoter [43]. As expected and similar
233 to what is seen in RpoE [41], these mutant strains had reduced AlgU transcriptional activity
234 upon induction of envelope stress, relative to the strain containing a wild-type *algU* (Fig S4B).
235 Furthermore, the AlgU activity of these mutant strains was equivalent to that in a Δ*algU* strain
236 (Fig S4A). These results suggest that AlgU mutants with reduced sigma factor activity can
237 suppress *mucA* essentiality and support our hypothesis that *mucA* essentiality is rooted in its
238 inhibition of AlgU.
239
240 As mentioned above, others have reported strains in which the entirety of *mucA* is deleted from
241 the genomes of *P. aeruginosa* PAO1, PAK, and PA103 [28-30]. Since we were unable to delete
242 *mucA* from these three strain backgrounds, we hypothesized that these Δ*mucA* strains may
243 contain suppressor mutations that allowed for their survival in the absence of *mucA*. To identify
244 these suppressors, we sequenced the genomes of these Δ*mucA* isolates. In the PAO1 Δ*mucA*
245 strain [30], we found that while the native *mucA* was deleted, the strain contained a second full-
246 length copy of *algU* and a *mucA* allele that encoded for MucA aa 1-155 elsewhere in the
247 genome. As expected based on our data (Fig 1B), we were able to delete the native *mucA* allele
248 from a PAO1 strain containing an ectopic *mucA* allele that encoded for MucA aa 1-155 (with 5 of
249 the 28 tested isolates resolving to the deletion allele), confirming that the ectopic *mucA* allele in

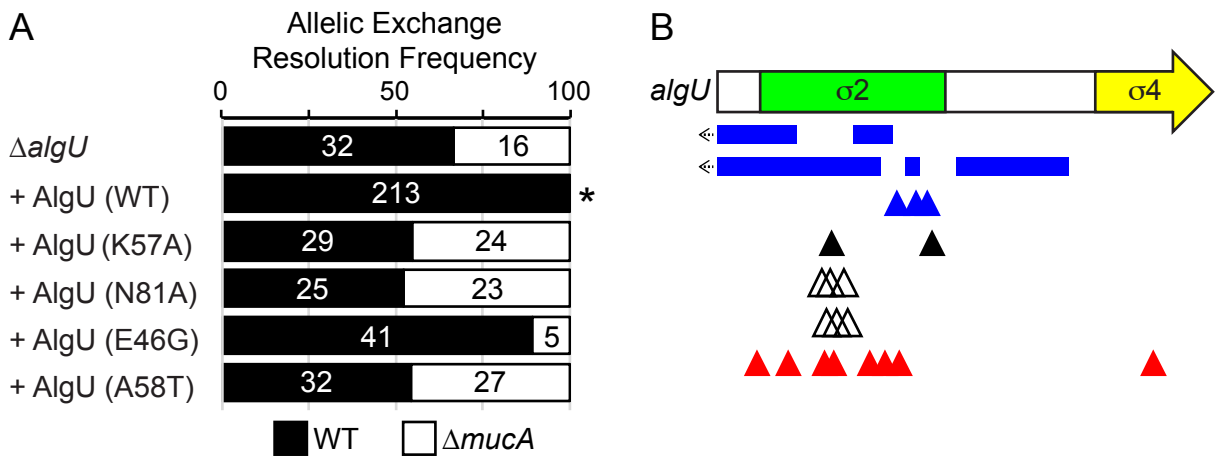



**Figure 3. AlgU mutants with reduced sigma factor activity suppress *mucA* essentiality.** **(A)** Frequency of observed isolates resolving to the endogenous wild-type *mucA* allele (WT, black) or the deletion allele (Δ*mucA*, white) in the allelic exchange assay, using PAO1 Δ*algU* *att*Tn7::$P_{algU}$-*algU*, 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.

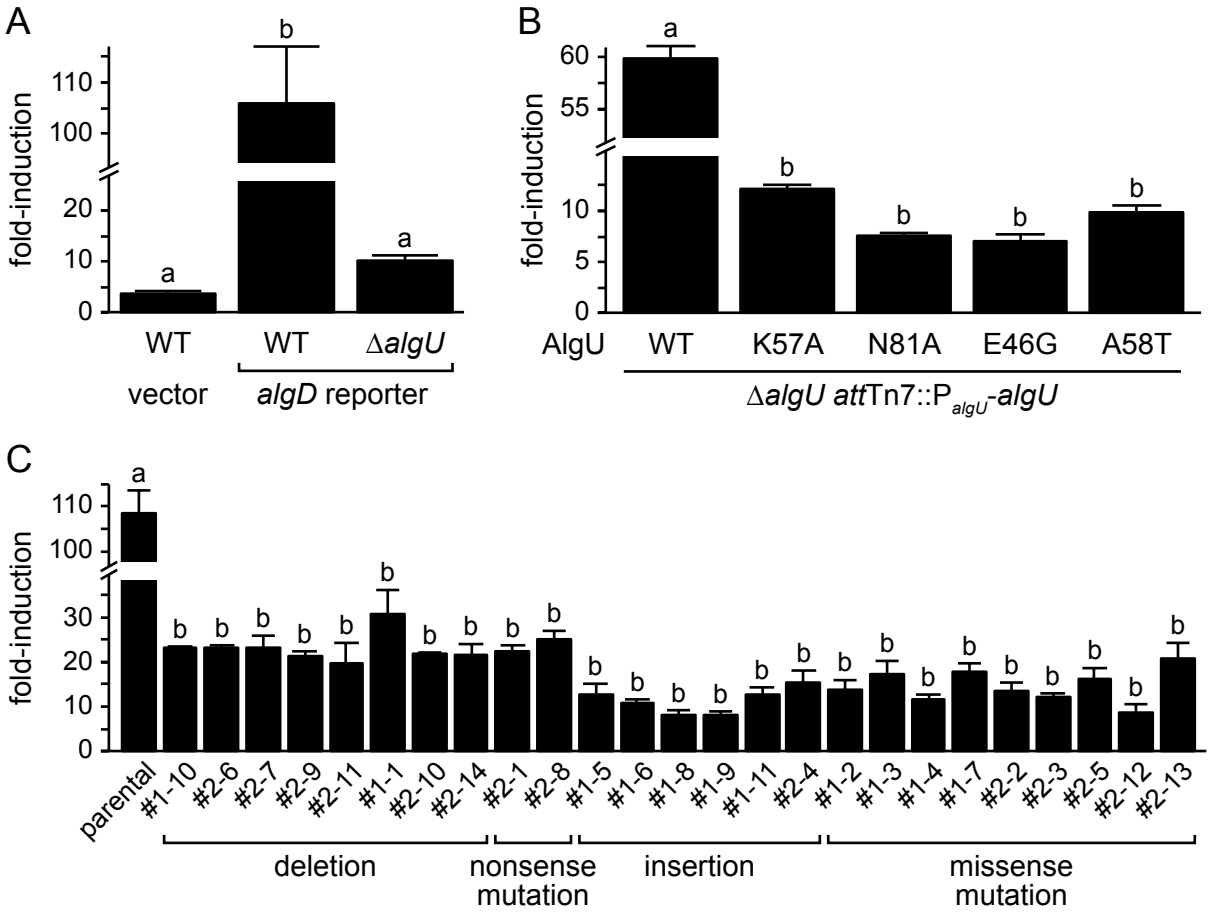



**Figure S4. AlgU mutants have decreased sigma factor activity.**
**(A)** Fold-induction of GFP in PAO1 wild-type (WT) and Δ*algU* strains carrying a plasmid with either a promoter-less *gfp* (vector) or a *gfp* driven by the *algD* promoter (*algD* reporter), which is positively regulated by AlgU, after 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.

250 the published PAO1 Δ*mucA* strain is sufficient for viability in the absence of the endogenous
251 *mucA*. In comparison, the PAK and PA103 Δ*mucA* strains [28, 29] contained missense
252 mutations in *algU,* which results in AlgU A58T and E46G, respectively. To determine the effect
253 of these AlgU mutants, we replicated them in PAO1 by inserting *algU* alleles encoding these
254 substitutions in a Δ*algU* strain. We were able to delete *mucA* from these strains (Fig 3A). These
255 results confirm that the *algU* alleles in the published PAK and PA103 Δ*mucA* strains suppress
256 *mucA* essentiality and show that these alleles do not rescue the Δ*algU* phenotype, unlike an
257 allele encoding the wild-type protein. Furthermore, these data suggest that, similar to the AlgU
258 DNA binding mutants, a mutant AlgU containing these substitutions is not functionally equivalent
259 to the wild-type protein. To examine the activity of these mutant proteins, we used our plasmid-
260 borne *algD* reporter to measure AlgU activity upon induction of envelope stress with D-
261 cycloserine. Similar to what we saw with the DNA-binding mutants of AlgU, we found that
262 strains carrying these AlgU substitutions had reduced *gfp* expression relative to that of strains
263 carrying wild-type AlgU (Fig S4B). We note that our reporter assay does not appear to be very
264 sensitive to low levels of AlgU activity. Both the published PAK and PA103 Δ*mucA* strains are
265 mucoid [28, 29], suggesting that AlgU A58T and E46G are not completely inactive. While we
266 cannot distinguish this low level of activity from the null case, our results show that nevertheless
267 these AlgU mutants have significantly less transcriptional activity than the wild-type protein (Fig
268 S4A,B).
269
270 To identify additional suppressors of *mucA* essentiality, we engineered a strain lacking the
271 native *mucA* and containing a rhamnose-inducible copy (Δ*mucA att*Tn7::$P_{rhaBAD}$-*mucA*). This
272 strain was not viable when grown in the absence of rhamnose, but natural revertants arose at a
273 frequency of less than 1 in $10^9$ colony forming units. We sequenced the *algU* gene in 25 of
274 these revertants, since our work shows that AlgU mutants can suppress *mucA* essentiality (Fig
275 3A). All 25 isolates contained mutations in *algU* (Fig 3B, Table S2). There were 10 revertants

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

| | Isolate | Genomic Change [a] | Product Change [b] |
|---|---|---|---|
| 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 |

[a] genomic location based on the Pseudomonas Genome Database [2] for PAO1; bp, base pair; del, deletion; ins, insertion.
[b] fs, frameshift; ins, insertion; X, stop codon.

276 with deletions or nonsense mutations of *algU*, encoding either no product or a truncated product
277 completely lacking Region 4 of the sigma factor. We expect that all of these mutations would
278 lead to a non-functional AlgU. There were 6 revertants that contained multi-base pair
279 duplications in *algU* that would lead to the insertion of 3 or 4 amino acids in Region 2 helix 3 of
280 the sigma factor, likely disrupting interactions with RNAP or the -10 promoter element and
281 reducing sigma factor activity. There were 9 revertants each containing one missense mutation,
282 8 of which were unique, encoding the following substitutions: D18G, A21V, Y29C, A47T, D49G,
283 Y59C, N81D, and R174G. Using a model of $\sigma^E$ in complex with the RNAP core and the
284 promoter element, we expect these substitutions to affect sigma factor folding, RNAP core
285 interactions, or promoter element interactions (Fig S5). To determine the effect of these 25
286 mutations on AlgU activity, we then tested AlgU function using our *algD* reporter assay in these
287 natural revertants. As expected, we saw that all 25 revertants had much lower AlgU activity than
288 the parental strain upon induction of envelope stress with D-cycloserine (Fig S4C). Together,
289 these data strongly suggest that *algU* mutations that encode for a protein with reduced
290 transcriptional activity allow *P. aeruginosa* to survive in the absence of *mucA*.
291
292 **Overexpression of *algU* in the absence of *mucA* is lethal**
293 Collectively, our results suggest that *mucA* essentiality is due to unregulated AlgU activity. We
294 therefore reasoned that overexpression of *algU* should be lethal. Supporting this hypothesis, the
295 toxic effects of *algU* overexpression have been previously noted [44-46]. However, studies have
296 shown that *algU* can be overexpressed in wild-type *P. aeruginosa* [16, 17]. It is important to note
297 that these wild-type strains contain *mucA*, which is positively regulated by AlgU. We therefore
298 examined the effect of overproducing AlgU in a strain lacking *mucA*, using a chromosomally
299 integrated arabinose-inducible *algU*. Since we could delete *mucA* only in a Δ*algU* background,
300 we tested the overexpression of *algU* in three strains: PAO1, PAO1 Δ*algU*, and PAO1 Δ*algU*
301 Δ*mucA*. Of note, the Δ*algU* and Δ*algU* Δ*mucA* strains lack the positive feedback of AlgU on its

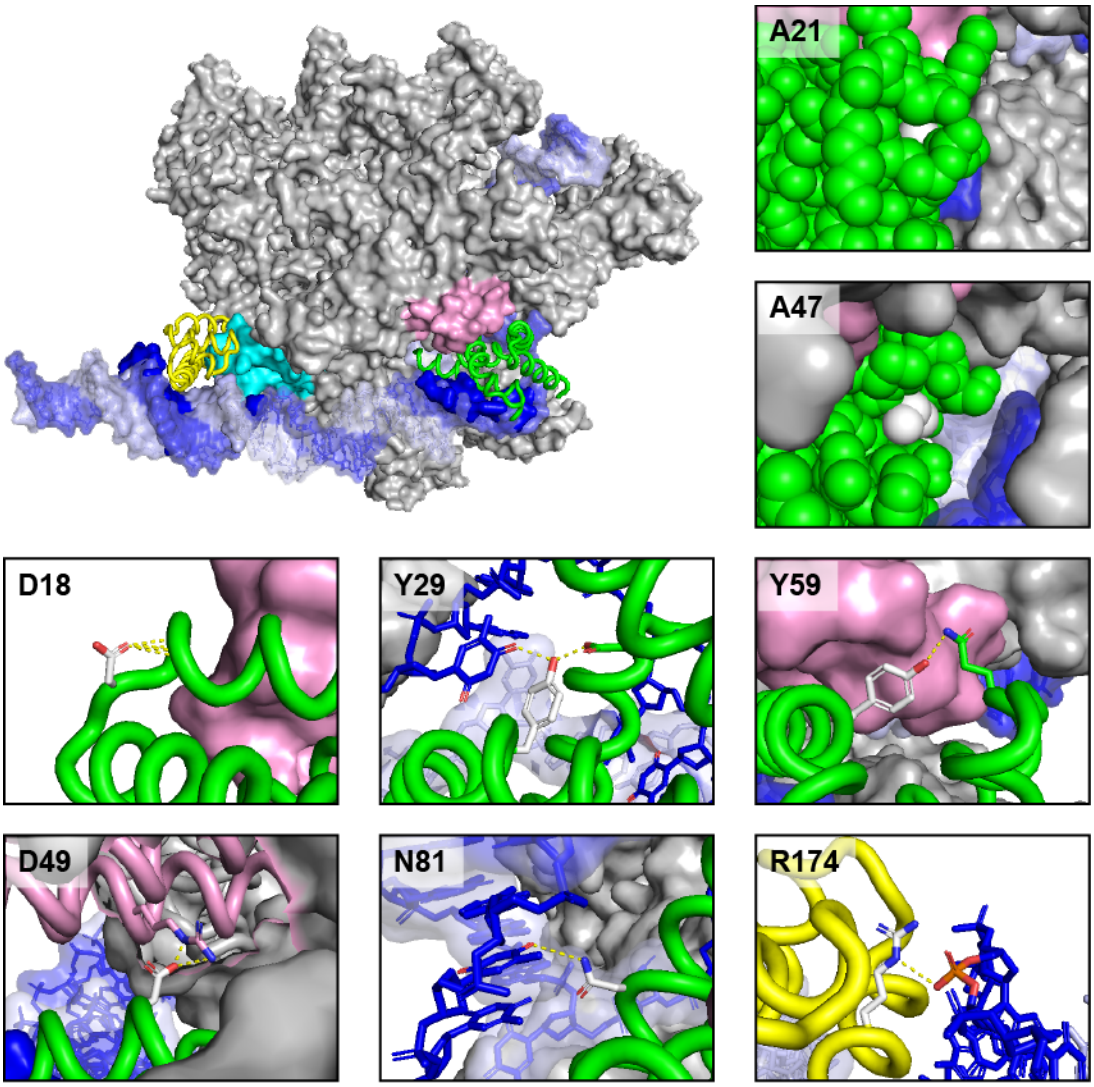



**Figure S5. Location of affected AlgU residues in revertants that can grow in the absence of MucA.**

Model of the RNAP holoenzyme containing $\sigma^E$ bound to the promoter. Gray, RNAP core; cyan, β flap; pink, β' coiled coil; green, $\sigma^E$ Region 2; yellow, $\sigma^E$ 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.

302 own expression, and the expression of *algU* is completely dependent on the inducer in these
303 strains. Our results show that in the absence of inducer when AlgU is not overexpressed, all
304 three strains had similar growth rates (Fig 4A). In the presence of 1% arabinose (i.e. high
305 expression of *algU*), all three strains had a growth defect in comparison to the same strain
306 grown in the absence of inducer. However, in comparison to the other two strains, the strain
307 lacking *mucA* failed to grow at all in the presence of this high inducer concentration.
308
309 To determine if the Δ*algU* Δ*mucA att*Tn7::$P_{araBAD}$-*algU* strain can grow under lower levels of *algU*
310 induction, we tested a range of inducer concentrations (Fig 4B, Table S3). We found that
311 although there was a growth defect, Δ*algU* Δ*mucA att*Tn7::$P_{araBAD}$-*algU* was able to grow in the
312 presence of 0.1% arabinose, suggesting that this strain was able to tolerate low levels of *algU*
313 induction. Furthermore, as expected, the drop in growth rate in comparison to the no arabinose
314 condition was statistically larger for the Δ*algU* Δ*mucA att*Tn7::$P_{araBAD}$-*algU* than for the other two
315 strains (N=3, $p < 0.05$, ANOVA with post hoc Tukey HSD), since this strain lacks the ability to
316 produce any MucA to reduce AlgU activity. Together, these results strongly suggest that in the
317 absence of *mucA*, while a certain level of AlgU activity is tolerated, a high AlgU activity level is
318 fatal to the cell.
319
320 **Expression of *rpoD* can rescue lethality of *algU* overexpression.**
321 It has been suggested that AlgU competes for RNAP binding with RpoD, the essential primary
322 sigma factor [47]. Therefore, we hypothesized that the toxicity of AlgU at high concentrations is
323 due to its competition with RpoD for RNAP, preventing the expression of essential
324 housekeeping genes. To test this hypothesis, we examined if overexpressing *rpoD* with *algU*
325 could ameliorate the growth defect of high *algU* expression in the Δ*algU* Δ*mucA att*Tn7::$P_{araBAD}$-
326 *algU* strain. We inserted into this strain a copy of *rpoD* under the same arabinose-inducible
327 promoter at the neutral *att*CTX site. We chose to test growth in the presence of 2% arabinose,

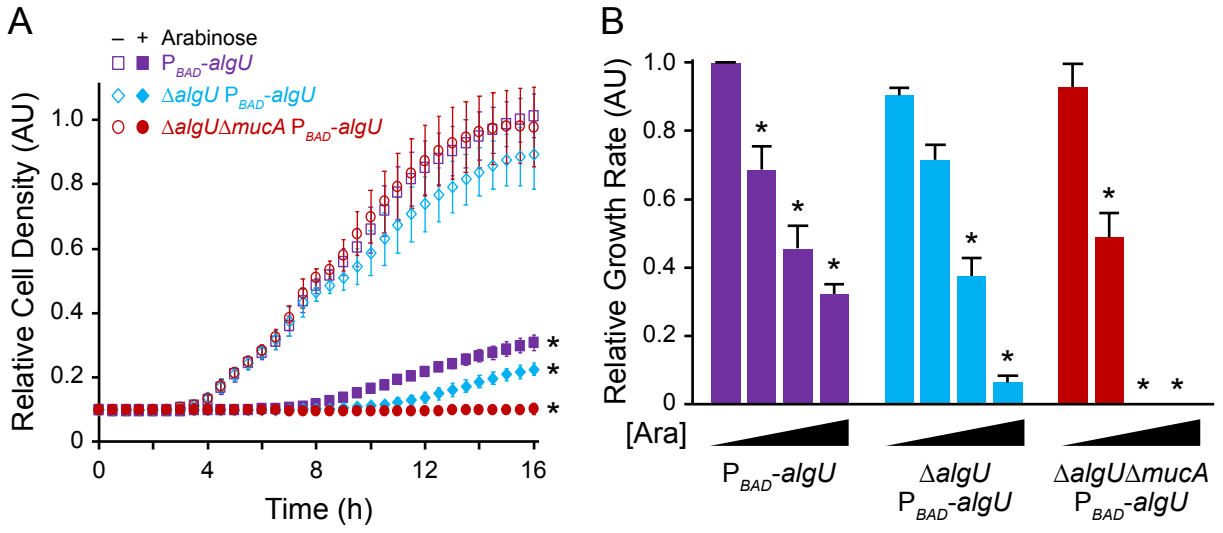



**Figure 4. Overexpression of *algU* is lethal in the absence of *mucA*.**
**(A)** Growth curves of PAO1 strains containing an arabinose-inducible copy of *algU* in wild-type (purple squares), Δ*algU* (blue diamonds), and Δ*algU* Δ*mucA* (red circles) backgrounds in LB. Readings are normalized to wild-type 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). Asterisk, growth rate statistically different from the same strain grown in the absence of arabinose ($p < 0.01$, N = 3, ANOVA with post-hoc Tukey HSD). **(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, growth rate statistically different from the same strain grown in the absence of arabinose ($p < 0.05$, N = 3, ANOVA with post-hoc Tukey HSD). See Table S3 for full statistical comparisons.

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

| | 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 | | | | | | | | | | | | |

Statistical significance was determined via a one-way ANOVA with post-hoc Tukey HSD test for pairwise comparisons. Ara, concentration of arabinose (%). Asterisk, $p < 0.05$. Open box, no statistical difference.

328 since this new strain contains two arabinose-inducible promoters, in contrast to the strains
329 described above in Fig 4. Growth with 2% arabinose caused a growth defect for all strains
330 tested, even in a strain lacking an exogenous arabinose-inducible gene (Fig 5), suggesting that
331 arabinose itself affects general bacterial physiology. As expected from our results in Fig 4,
332 Δ*algU* Δ*mucA att*Tn7::$P_{araBAD}$-*algU* failed to grow in the presence of 2% arabinose. This lethality
333 defect, however, was rescued in the Δ*algU* Δ*mucA att*Tn7::$P_{araBAD}$-*algU att*CTX::$P_{araBAD}$-*rpoD*
334 strain. Furthermore, the growth rate of this strain in the presence of arabinose was
335 indistinguishable from that of Δ*algU* Δ*mucA att*CTX::$P_{araBAD}$-*rpoD* (Table S4). Since increasing
336 RpoD levels rescued the lethality associated with high AlgU levels, these results suggest that in
337 the absence of MucA, the ensuing sigma factor competition of AlgU with RpoD for RNAP
338 contributes to the viability defect, and that MucA is required to rein in AlgU to prevent
339 interference with the essential function of RpoD.
340

341 **DISCUSSION**

342 Although it has been assumed that *mucA* can be deleted without affecting bacterial viability, our
343 work shows that *mucA* is essential in a variety of *P. aeruginosa* wild-type strains. Our results
344 strongly suggest that the interaction of MucA with its cognate sigma factor AlgU is required for
345 viability, and that AlgU mutants with reduced sigma factor activity can suppress *mucA*
346 essentiality. Furthermore, overproduction of AlgU is detrimental to cell growth, which can be
347 rescued by increasing RpoD production. Together, our data suggest that unchecked AlgU
348 activity in the absence of MucA leads to sigma factor competition and cell death.
349

350 Our data point to the negative regulation of AlgU being necessary for cell survival in *P.*
351 *aeruginosa* (Fig 6). Under non-stress conditions, AlgU is bound to and inhibited by a full-length
352 MucA. Under envelope stress or in strains containing *mucA* mutations, the anti-sigma factor is
353 cleaved. We propose that while this cleaved cytosolic form of MucA does not inhibit AlgU to the

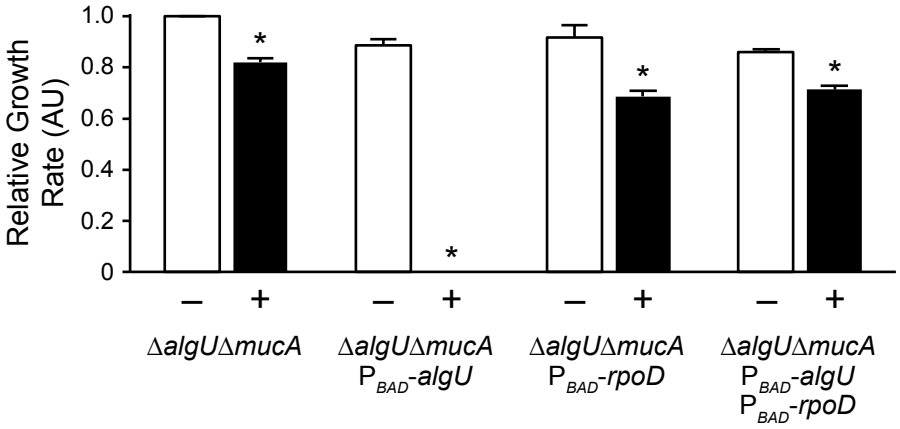



**Figure 5. Lethality caused by *algU* overexpression can be rescued via *rpoD* expression.** Growth rate of indicated strains grown in LB with (+) or without (–) of 2% arabinose (relative to PAO1 Δ*algU* Δ*mucA* grown in the absence of arabinose). Error bars, SEM (N=3). Asterisk, growth rate statistically different from same strain grown in the absence of arabinose ($p < 0.05$, N = 3, ANOVA with post-hoc Tukey HSD). 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 | | | | | | | | |

Statistical significance was determined via a one-way ANOVA with post-hoc Tukey HSD test for pairwise comparisons. Ara, concentration of arabinose (%). Asterisk, $p < 0.05$. Open box, no statistical difference.

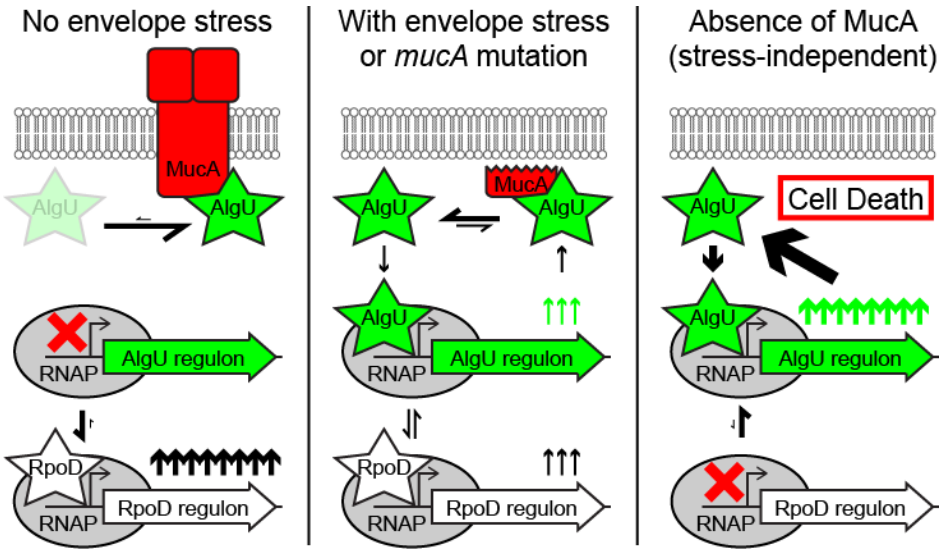



**Figure 6. Unchecked AlgU activity in the absence of MucA leads to bacterial cell death.** Under conditions of no envelope stress (left), the full-length MucA (red) binds strongly to AlgU (green star), leaving very little free AlgU to interact with the RNAP (grey oval). This skews the ratio of the holoenzyme to that with RpoD, the primary sigma factor (white star). Therefore, the AlgU regulon (green arrow) is in the "off" state (red X), while the RpoD regulon (white arrow) is in the "on" state (black up arrows). 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, leading to a decrease in the expression of the RpoD 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, outcompeting RpoD. 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 due to the inability of the cell to express essential housekeeping genes.

354 same extent as the full-length protein, it is still able to interact with and inhibit AlgU to some
355 degree. Although AlgU is activated under such conditions, because *mucA* is positively regulated
356 by AlgU, this feedback allows the cell to keep AlgU activity under control. In comparison, in the
357 absence of MucA, this ability to control AlgU is lost. We propose that the strong positive
358 feedback of AlgU on its own expression is what leads to cell death and *mucA* essentiality, due
359 to sigma factor competition with the primary sigma factor RpoD. Supporting this model are
360 several lines of evidence. First, *mucA* essentiality is rooted in its interaction with *algU* (Figs 1
361 and 2), strongly suggesting that the ability of MucA to inhibit AlgU is required for viability.
362 Second, deletion of AlgU-regulated genes, including those encoding the well-characterized
363 transcription factors AlgB [35], AlgR [34, 35], and AmrZ [36], do not allow for *mucA* deletion
364 (Table S1), supporting that *algU* itself is the lethal component of the AlgU regulon. Third, AlgU
365 mutants can suppress *mucA* essentiality, and all 25 natural revertants that we screened
366 contained mutations in *algU* (Fig 3). The strains carrying these mutations had decreased AlgU
367 transcriptional activity (Fig S4), supporting the idea that decreasing the positive feedback of
368 AlgU on its own expression allows the cell to survive in the absence of MucA. Lastly,
369 overexpression of *algU* leads to a growth defect, which is lethal at high levels in the absence of
370 *mucA* (Fig 4) and can be rescued by overexpression of *rpoD* (Fig 5), suggesting that sigma
371 factor competition between AlgU and RpoD is responsible for lethality in the absence of MucA.
372
373 While our data supports our model that the AlgU-regulated gene critical for *mucA* essentiality is
374 *algU* itself, we cannot exclude the possibility that the regulation of another single gene or a
375 combination of genes within the AlgU regulon is responsible for the *mucA* requirement. This
376 possibility, however, seems less likely for two reasons. First, all tested revertants of Δ*mucA*
377 *att*Tn7::$P_{rhaBAD}$-*mucA* that could grow in the absence of *mucA* expression contained *algU*
378 mutations (Table S2). If another single AlgU-regulated gene were responsible for *mucA*
379 essentiality, we would have expected to find a revertant with such a mutation. Second, we were

380 unable to delete *mucA* from a strain lacking three AlgU-regulated transcription factors (Table
381 S1). Genes regulated by AlgB, AlgR, or AmrZ can account for approximately fifty percent of the
382 AlgU regulon [17, 37, 38, 40], suggesting that it is unlikely that deleting a combination of AlgU-
383 regulated genes can alleviate the necessity for *mucA*. Nonetheless, we are actively searching
384 for non-*algU* suppressors of *mucA* essentiality.
385
386 Our data show that the anti-sigma factor MucA is required for viability in various *P. aeruginosa*
387 strains (Table 1). However, the requirement of an anti-sigma factor for bacterial viability is not
388 unique to *P. aeruginosa*. While there do not appear to be any essential anti-sigma factors in *E.*
389 *coli*, as all of them are individually deleted in the Keio collection [48], there are many instances
390 in other organisms. For instance, the requirement for the anti-sigma factor that inhibits the
391 general stress response has been suggested in multiple Alphaproteobacteria [49]. In addition,
392 there are two anti-sigma factors, YhdL and YxlC, in *B. subtilis* that are necessary for bacterial
393 viability [50-52]. The essentiality of *yhdL* is notable as this anti-sigma factor negatively regulates
394 SigM, a sigma factor involved in the envelope stress response [51]. Similarly, in *Mycobacterium*
395 *tuberculosis*, the gene encoding the anti-sigma factor RslA is identified as essential due to an
396 underrepresentation of interruptions in a genome-wide transposon insertional analysis [53].
397 However, similar to our results (Fig 3), *rslA* has been deleted from *M. tuberculosis* lacking the
398 gene encoding its cognate sigma factor SigL, which regulates genes involved in cell envelope
399 processes [54]. Furthermore, in the defined transposon mutant library for *Vibrio cholerae*, an
400 interruption in the *mucA* homolog *rseA* does not exist, suggesting that this anti-sigma factor may
401 be essential in *V. cholerae*, similar to in *P. aeruginosa* [55]. Finally, the *P. syringae mucA*
402 homolog is deemed essential based on a reduced number of transposon insertions [56].
403 Therefore, although the *E. coli mucA* homolog *rseA* is not required for cell viability [57, 58],
404 these data, together with our results, suggest that essentiality of anti-sigma factors that regulate
405 cell envelope function may be a widespread phenomenon in bacteria.

406

407 Our work strongly suggests that lethality in the absence of *mucA* is caused by unchecked AlgU
408 activity (Figs 4 and 5), in agreement with previous studies suggesting AlgU overexpression is
409 toxic [44-46]. Our data suggests that sigma factor competition may play a role in this lethality
410 (Fig 5). The concentrations and affinities of individual sigma factors influence their competition
411 for the core RNAP [59, 60]. While sigma factor competition is best studied in *E. coli*, it has been
412 observed in other organisms, including *P. aeruginosa* [47, 59, 61-63]. One such example in *P.*
413 *aeruginosa* is between AlgU and RpoN (a sigma factor regulating nitrogen metabolism, as well
414 as other systems [64]), which have overlapping promoter sites upstream of the alginate
415 biosynthetic gene *algD* [65]. Shifts in the concentrations of these sigma factors affect *algD*
416 expression [66]. Furthermore, sigma factor competition between AlgU and the primary sigma
417 factor RpoD has also been suggested [47]. Yin and colleagues found that overexpression of
418 RpoD could suppress alginate biosynthesis in a mucoid CF isolate. Additionally, they saw that
419 the activity of both AlgU and RpoN was increased in a mutant with decreased RpoD activity
420 [47]. Expanding to other organisms, Zhao and colleagues found that in *B. subtilis*, the lethality
421 defect associated with deleting *yhdL*, which encodes for an anti-sigma factor, can be rescued
422 via overproduction of the primary sigma factor SigA, similar to our results (Fig 5). They suggest
423 that the lethality of this strain is due to the YhdL-cognate sigma factor, SigM, outcompeting SigA
424 for RNAP [61]. Based on these findings, and our data showing that RpoD can rescue the
425 lethality of high AlgU levels (Fig 5), we speculate that sigma factor competition may be involved
426 in other instances where anti-sigma factors that regulate cell envelope functions have been
427 found to be essential.

428

429 The interaction of MucA and AlgU is of interest, as *mucA* mutations are common in *P.*
430 *aeruginosa* isolated from the CF lung environment [7, 20-23]. Our results (Fig 3) agree with the
431 literature showing that mucoid CF isolates with *mucA* mutations revert to a non-mucoid state via

432 changes to *algU* when the cells are grown under standard laboratory conditions [21, 45, 67, 68].
433 While these *algU* secondary site mutations are found in non-mucoid CF isolates with *mucA*
434 mutations, such isolates are detected at a lower frequency [21]. As suggested by Ciofu and
435 associates, this may be due to the importance of AlgU for the survival of *P. aeruginosa* in the
436 CF lung environment, suggesting that reducing AlgU activity may increase *P. aeruginosa*
437 eradication from the CF airway. Based on our data and the published literature, we propose that
438 the MucA-AlgU interaction may serve as a good therapeutic target. Our results show that
439 destabilizing the MucA-AlgU interaction results in bacterial cell death or mutations in *algU* that
440 likely result in reduced sigma factor activity and reduced mucoid conversion, of which either
441 outcome could be beneficial in the treatment of *P. aeruginosa* CF lung infections.
442

443 **METHODS AND MATERIALS**

444 **Bacterial strains and growth conditions**. The bacterial strains, plasmids, and oligonucleotides
445 used for this study are in Tables S5-S7. The construction of strains is described in Supporting
446 Information. Bacteria were grown at 37°C in LB with shaking or on semi-solid LB media, unless
447 otherwise noted.
448

449 **Allelic exchange assay.** The protocol, depicted in Fig S1, was modified from [69]. Briefly, after
450 introduction of the *mucA* deletion vector, merodiploids were selected on semi-solid VBMM [70]
451 with 60 mg/L gentamicin. Using OMS118 and OMS119, PCR was performed on at least six
452 isolates to confirm that the presence of both the wild type and deletion alleles of *mucA*.
453 Confirmed merodiploids were then individually streaked on NSLB (10 g/L tryptone, 5 g/L yeast
454 extract) with 10% sucrose semi-solid media for counterselection. PCR was performed on eight
455 colonies per merodiploid, using OBT601 and OBT602 to determine the resolution to either the
456 WT or deletion allele.
457

458 **Yeast two-hybrid assay.** The ProQuest Two-Hybrid System (Invitrogen) was used, per
459 manufacturer's instructions. Briefly, yeast containing the Gal4 activation domain-based prey and
460 the Gal4 DNA binding domain-based bait vectors were grown overnight in SD-Leu-Trp broth
461 (Clontech). The $OD_{600}$ value was recorded. ONPG (VWR) was added to lysed cells, and the
462 mixture was incubated at 37°C until a light yellow color was achieved. The incubation time was
463 recorded. The $OD_{420}$ of the supernatants was determined using a Synergy Hybrid HTX
464 Microplate Reader (Bio-Tek Instruments). Beta galactosidase activity was determined using
465 Miller units, based on the following equation: 1000 x $OD_{420}$/(time x culture volume x $OD_{600}$).
466
467 **Natural revertant assay.** *P. aeruginosa* Δ*mucA att*Tn7::$P_{rhaBAD}$-*mucA* was grown in LB with
468 0.05% rhamnose at 37°C with shaking to an $OD_{600}$ of 1. Cells were washed and plated on semi-
469 solid LB plates without rhamnose. Plates were incubated for 24-48 h at 37°C. For isolates that
470 grew on LB without rhamnose, *algU* was Sanger sequenced.
471
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483 AAK, EAC, BST; Writing – original draft: MCS, BST; and Writing – review and editing: MCS, DR,
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485
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