Genetic basis for the essentiality of a bacterial anti-sigma factor involved in virulence

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17 **ABSTRACT**

18 During decades-long infections in the cystic fibrosis (CF) airway, *Pseudomonas aeruginosa*
19 undergoes selection. One such bacterial genetic adaptation frequently observed in CF isolates
20 is mutation of *mucA.* MucA inhibits the sigma factor AlgU. In clinical isolates, *mucA* mutations
21 lead to AlgU misregulation, resulting in a mucoid phenotype that is associated with poor CF
22 disease outcomes. Here we show that, paradoxically, a portion of the *mucA* gene is essential for
23 *P. aeruginosa* viability. We demonstrate that *mucA* is no longer essential in a strain lacking
24 *algU*, that *mucA* alleles that encode for proteins that do not bind to AlgU are insufficient for
25 bacterial viability, and that *mucA* is no longer essential in mutant strains containing AlgU
26 variants with reduced sigma factor activity. Finally, we found that overexpression of *algU*
27 prevents cell growth in the absence of MucA, and that this phenotype can be rescued by
28 overproduction of RpoD, the housekeeping sigma factor. Together, these results suggest that in
29 the absence of MucA, the inability to regulate AlgU activity may inhibit essential housekeeping
30 functions, resulting in the loss of bacterial viability. Finally, we speculate that essentiality of anti-
31 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 one of the most prevalent respiratory pathogens of CF patients (Cystic Fibrosis Foundation
36 Patient Registry, 2019). The CF lung environment selects for mucoid *P. aeruginosa* mutants,
37 which overproduce the exopolysaccharide alginate and are associated with poor disease
38 prognosis (Emerson *et al.*, 2002, Henry *et al.*, 1992, Li *et al.*, 2005, Parad *et al.*, 1999, Pedersen
39 *et al.*, 1992). Conversion to the mucoid phenotype in clinical *P. aeruginosa* isolates, which is
40 thought to be advantageous for chronic infection, is frequently caused by *mucA* mutations
41 (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 the sigma factor away from RNA polymerase
46 (RNAP) via its N-terminal interaction with AlgU (Li *et al.*, 2019, Schurr *et al.*, 1996, Xie *et al.*,
47 1996). The C-terminus of MucA is in the periplasm, where it is protected from proteolysis via an
48 interaction with MucB (Mathee *et al.*, 1997, Schurr *et al.*, 1996). The envelope stress response
49 is controlled via a regulated intramembrane proteolysis cascade: MucB dissociates from MucA
50 upon detection of stress, allowing the proteases AlgW and MucP to cleave MucA from the inner
51 membrane (Qiu *et al.*, 2007, Wood & Ohman, 2009). In the cytoplasm, MucA is further
52 degraded by ClpXP, releasing AlgU to interact with RNAP and activate the AlgU regulon (Qiu *et*
53 *al.*, 2008). AlgU regulates at least 350 genes, including those responsible for the production of
54 itself, its cognate anti-sigma factor MucA, and the alginate biosynthetic enzymes (Firoved &
55 Deretic, 2003, Schulz *et al.*, 2015, Wood & Ohman, 2009). This system is homologous to the
56 well-studied envelope stress response in *Escherichia coli*: MucA shares 28% identity (72%

57 similarity) to the anti-sigma factor RseA, and the cognate sigma factor of the *E. coli* RseA, called
58 RpoE, is homologous to and functionally interchangeable with AlgU (Yu *et al.*, 1995), with 66%
59 identity (93% similarity).
60
61 For several reasons, *mucA* is assumed to be dispensable for *P. aeruginosa* viability. First, after
62 being released from the cell membrane, MucA is presumed to be fully degraded in the
63 cytoplasm by ClpXP (Qiu *et al.*, 2008). Second, *mucA* mutations commonly arise in CF clinical
64 isolates (Boucher *et al.*, 1997, Candido Cacador *et al.*, 2018, Ciofu *et al.*, 2008, Martin *et al.*,
65 1993, Pulcrano *et al.*, 2012), and many laboratory *mucA* mutants exist in the literature
66 (Gallagher *et al.*, 2011, Liberati *et al.*, 2006, Skurnik *et al.*, 2013, Turner *et al.*, 2015). Third,
67 there are three published strains in which the entirety of *mucA* is removed from the genome
68 (Intile *et al.*, 2014, Jones *et al.*, 2010, Pritchett *et al.*, 2015). Paradoxically, three different whole-
69 genome studies of essential genes in two different strains of *P. aeruginosa* identified *mucA* as
70 an essential gene, based on the low observed frequency of transposon insertions in this gene
71 (Lee *et al.*, 2015, Liberati *et al.*, 2006, Skurnik *et al.*, 2013). To investigate this paradox, we
72 systematically attempted to delete *mucA* from *P. aeruginosa* using allelic exchange. Our results
73 show that a portion of *mucA* is required for bacterial viability in multiple *P. aeruginosa* strains.
74 Furthermore, our work shows that *mucA* was no longer essential in a strain lacking *algU*, and
75 that *mucA* alleles that encode for proteins that do not interact with AlgU were insufficient to
76 rescue viability or led to a growth defect. We found that *algU* mutations, which encode for a less
77 active sigma factor, can relieve *mucA* essentiality, and that overproduction of AlgU in the
78 absence of MucA is lethal. Interestingly, our works suggests that *mucA* essentiality can be
79 suppressed by increasing the levels of the housekeeping sigma factor RpoD. Together, our
80 results strongly suggest that the unregulated activity of AlgU itself, in the absence of MucA,
81 leads to bacterial cell death, which is in part due to sigma factor competition with RpoD.
82

83 **RESULTS**

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

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

105

106 **Alginate biosynthesis is not solely responsible for *mucA* essentiality**

107 Clinical isolates containing *mucA* mutations are mucoid due to their overproduction of alginate
108 (Martin *et al.*, 1993). To determine if alginate overproduction is responsible for *mucA*

109 essentiality, we attempted to delete *mucA* from a strain lacking *algD*, a key alginate biosynthesis
110 gene (Deretic *et al.*, 1987). Using our allelic exchange assay, we were unable to delete *mucA* in
111 this background (Table S1).
112
113 Expression of the alginate biosynthesis genes is controlled by three AlgU-regulated transcription
114 factors that are active in mucoid cells: AlgB, AlgR, and AmrZ (Martin *et al.*, 1994, Wozniak &
115 Ohman, 1994, Wozniak *et al.*, 2003). Since these transcription factors regulate a suite of genes
116 in addition to those involved in alginate biosynthesis (Huang *et al.*, 2019, Jones *et al.*, 2014,
117 Kong *et al.*, 2015, Leech *et al.*, 2008), we tested if overexpression of these three regulons
118 underlies *mucA* essentiality by attempting to delete *mucA* from strains lacking these
119 transcription factors, either individually or all together. We found that we could not delete *mucA*
120 in any of these backgrounds (Table S1). Altogether, our data support the conclusion that
121 eliminating alginate biosynthesis and the expression of large parts of the AlgU regulon do not
122 alleviate *mucA* essentiality.
123
124 **The first 50 amino acids of MucA are necessary and sufficient for cell viability**
125 While we were unable to delete *mucA* using an allele lacking >95% of the coding region, *mucA*
126 is commonly mutated in clinical isolates and many *mucA* transposon mutants exist (Fig S2).
127 These data suggest that only a portion of *mucA* is essential. To determine the essential portion
128 of *mucA*, we first constructed a series of strains containing an ectopic chromosomally-integrated
129 *mucA*, driven by its native promoter. We then attempted to delete the endogenous wild-type
130 *mucA* from these strains. Although we designed these ectopic alleles to encode for a protein
131 with a His-tag, we were unable to probe whether equivalent amounts of protein were produced
132 across our strains, since truncated versions of MucA are cleaved in the cytosol, as shown in
133 previous work (Qiu *et al.*, 2008). However, we expect that the ectopic alleles produce similar
134 protein levels, as they are all under the same promoter and located in the same genomic site.

135 Since we expected that the resultant Δ*mucA* strain would be mucoid in some cases, we used a
136 Δ*algD* background to eliminate alginate production, which made the strains easier to
137 manipulate.
138
139 Mutant MucA proteins of varying lengths were ectopically expressed to determine the necessary
140 portion of MucA. Full-length MucA is 194 amino acids (aa). Based on the co-crystal structures of
141 MucA with AlgU and MucB (Li *et al.*, 2019), the first 78 residues of MucA interact with AlgU, and
142 the last 48 residues of MucA interact with MucB. We engineered *mucA* alleles that encode for
143 various truncated proteins (Fig 1A). We then attempted to delete the endogenous *mucA* from
144 these strains. Since the flanking regions of these two alleles differ, our deletion allele specifically
145 targets the native allele. We were able to easily recover isolates that resolved to the *mucA*
146 deletion allele from strains containing ectopic alleles that encode for the full length MucA (aa 1-
147 194), as well as MucA aa 1-143, aa 1-110, aa 1-75, and aa 1-62 (Fig 1B). These results show
148 that the requirement for *mucA* can be complemented *in trans*, and suggest that the gene
149 product of *mucA* is required for viability, irrespective of its genomic location.
150
151 Interestingly, while we were able to delete the endogenous *mucA* from strains containing an
152 ectopic *mucA* that encoded for aa 1-50 (Fig 1B), isolates that resolved to the deletion allele
153 grew up more slowly than those that resolved to the wild-type allele. This suggests that while
154 this allele is sufficient for viability, it is not well tolerated by the cells. All tested isolates of strains
155 carrying *mucA* alleles encoding shorter products (aa 1-40 and aa 1-24) resolved to the wild-type
156 allele. These results show that while an allele encoding the first 50 residues of MucA is sufficient
157 for cell viability, alleles encoding for shorter lengths are not.
158
159 To determine if the first 50 residues of MucA are necessary for viability, we constructed a strain
160 with an ectopic *mucA* that encodes for aa 51-194. We were unable to delete the native *mucA*

161 from this strain (Fig 1B). Together, these results suggest that the first 50 amino acids of MucA
162 are necessary and sufficient for cell viability in *P. aeruginosa*. Consistent with these results,
163 while many clinical and engineered *mucA* mutations have been reported (Boucher *et al.*, 1997,
164 Candido Cacador *et al.*, 2018, Ciofu *et al.*, 2008, Martin *et al.*, 1993, Pulcrano *et al.*, 2012,
165 Turner *et al.*, 2015), no mutations fall within the region of *mucA* that encodes for the first 50
166 residues (Fig S2). Furthermore, this region of MucA corresponds to the AlgU binding domain,
167 suggesting that the physical interaction between the two proteins is important for cell viability.
168

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

170 To evaluate the effect of the nutrient conditions on *mucA* essentiality, we tested the effect of
171 depleting MucA on *P. aeruginosa* cell viability in different media. To deplete MucA from cells, we
172 engineered a strain lacking the native *mucA* and containing a rhamnose-inducible copy (Δ*mucA*
173 *att*Tn7::P_{rhaBAD}-*mucA*). We removed rhamnose from growing Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* cells
174 and determined the viability of cells over time based on their ability to recover on medium
175 containing rhamnose. Since cells would cease producing MucA in the absence of rhamnose, the
176 MucA present in the cells at the start of the experiment would deplete as the cells divided. In all
177 four media we tested, if rhamnose was not removed at the beginning of the experiment, the
178 cells increased in density by ~2-logs over the duration of the experiment. In comparison, when
179 rhamnose was removed, the Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* cells lost viability over time in all four
180 media (Fig 2). Together, these results suggest that *mucA* is essential in *P. aeruginosa*
181 independent of the nutrient environment.
182

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

184 Since the only described function for MucA is to inhibit AlgU, we hypothesized that *mucA*
185 essentiality is rooted in its regulation of AlgU. In such a case, *mucA* essentiality should require
186 *algU*. Therefore, we attempted to delete *mucA* from a strain lacking *algU* (Δ*algU*). We found that

187 we were able to delete *mucA* in the absence of *algU*, with 16 of the tested isolates resolving to
188 the deletion allele out of the 48 colonies tested (see Fig 4A), showing that *mucA* essentiality is
189 *algU*-dependent.
190
191 Because our data suggest that the AlgU-binding domain of MucA is required for viability (Fig
192 1B), we hypothesized that the physical interaction between MucA and AlgU is necessary for cell
193 survival. In the co-crystal structure of MucA and AlgU (Li *et al.*, 2019), four residues in MucA
194 make more than one hydrogen bond with AlgU: D15, E22, R42, and E72 (Fig 3A). Although E72
195 likely contributes to the binding energy between these two proteins, it is unlikely that E72 is
196 critical to the MucA-AlgU interaction, since the first 50 amino acids of MucA are sufficient for
197 viability (Fig 1B). Therefore, we engineered alleles that encode for MucA D15A, E22A, or R42A
198 to maximally affect the hydrogen bonding while limiting the effects on overall protein structure.
199 We used MucA aa 1-75 as a base because the co-crystal structure includes the first 78 residues
200 of MucA. We tested the effect of these substitutions on the MucA-AlgU interaction via a yeast-
201 two hybrid assay. Using beta-galactosidase activity as a proxy for their interaction, wild-type
202 MucA aa 1-75 strongly interacted with AlgU (Fig 3B). This interaction was dependent on the
203 presence of both MucA and AlgU, and the effect was not directional (Fig S3). In comparison, the
204 D15A, E22A, and R42A MucA mutants failed to interact with AlgU (Fig 3B). To determine the
205 effects of these mutations on viability, we engineered strains carrying an ectopic *mucA* encoding
206 these substitutions and then attempted to delete the native *mucA*. As described above, an allele
207 encoding MucA aa 1-75 was sufficient for viability (Fig 1B). However, we were unable to delete
208 the endogenous *mucA* from strains carrying alleles encoding the D15A and R42A substitutions,
209 suggesting that the mutant protein is not sufficient for viability (Fig 3C). For the strain carrying a
210 *mucA* allele encoding the E22A substitution, we were able to delete the endogenous *mucA*.
211 However, isolates that resolved to the deletion allele in this strain grew up slower than those
212 that resolved to the wild-type allele. Similar to the *mucA* allele encoding only the first 50 amino

213 acids, these results suggest that while MucA E22A is sufficient for viability, the cells do not
214 tolerate it well.
215
216 **Mutations that reduce AlgU activity alleviate the requirement for MucA**
217 Since MucA inhibits AlgU from binding to RNAP, we hypothesized that *mucA* essentiality is due
218 to its inhibition of the AlgU regulon, which includes *algU* itself. To start to test this hypothesis,
219 we reasoned that strains containing a mutant AlgU with lower affinity for DNA would alleviate
220 *mucA* essentiality, since expression of the AlgU regulon would inherently be lower in such
221 mutants. Therefore, we engineered Δ*algU* strains with an ectopic chromosomally-integrated
222 *algU* allele encoding such DNA-binding mutants. Based on homology to *E. coli* RpoE mutants
223 that have decreased *in vitro* transcriptional activity (Campagne *et al.*, 2014), we engineered
224 *algU* alleles that encode for K57A and N81A. As described above, we were able to delete *mucA*
225 from a Δ*algU* strain (Fig 4A). This phenotype could be rescued via the ectopic addition of a wild-
226 type *algU* allele, as we were no longer able to delete *mucA* from such a strain. In contrast,
227 *mucA* could be readily deleted from strains carrying alleles encoding AlgU K57A or N81A,
228 showing that such mutant alleles failed to rescue the Δ*algU* phenotype (Fig 4A). To determine
229 the effect of these substitutions on AlgU activity, we induced envelope stress using D-
230 cycloserine (Wood *et al.*, 2006) and measured AlgU activity using a plasmid-borne *gfp* reporter
231 driven under the AlgU-regulated *algD* promoter (Damron *et al.*, 2009). Similar to what is seen for
232 RpoE (Campagne *et al.*, 2014), these mutant strains had reduced AlgU activity upon induction
233 of envelope stress, relative to the strain containing a wild-type *algU* (Fig S4A,B). These results
234 suggest that AlgU mutants with reduced sigma factor activity can suppress *mucA* essentiality.
235
236 As mentioned above, others have reported strains in which the entirety of *mucA* is deleted from
237 *P. aeruginosa* PAO1, PAK, and PA103 (Intile *et al.*, 2014, Jones *et al.*, 2010, Pritchett *et al.*,
238 2015). Since we were unable to delete *mucA* from these three strain backgrounds (Table 1), we

239 hypothesized that these Δ*mucA* strains may contain suppressor mutations that allowed for their
240 survival in the absence of *mucA*. To identify these suppressors, we sequenced the genomes of
241 these Δ*mucA* isolates. In the PAO1 Δ*mucA* strain (Pritchett *et al.*, 2015), we found that while the
242 native *mucA* was deleted, the strain contained a second full-length copy of *algU* and a *mucA*
243 allele that encoded for MucA aa 1-155 elsewhere in the genome. As expected from our data
244 (Fig 1B), we were able to delete the native *mucA* allele from a PAO1 strain containing an
245 ectopic *mucA* allele that encoded for MucA aa 1-155 (with 5 of the 28 tested isolates resolving
246 to the deletion allele), confirming that the ectopic *mucA* allele in the published PAO1 Δ*mucA*
247 strain is sufficient for viability in the absence of the endogenous *mucA*. In comparison, the PAK
248 and PA103 Δ*mucA* strains (Intile *et al.*, 2014, Jones *et al.*, 2010) contain missense mutations in
249 *algU,* which results in AlgU A58T and E46G, respectively. We replicated these mutations in
250 PAO1 by inserting *algU* alleles encoding these substitutions in a Δ*algU* strain. We were able to
251 delete *mucA* from these strains (Fig 4A). These results confirm that the *algU* alleles in the
252 published PAK and PA103 Δ*mucA* strains suppress *mucA* essentiality. To examine the activity
253 of these mutant proteins, we used our reporter assay. Similar to what we saw with the AlgU
254 DNA-binding mutants, we found that strains carrying these AlgU substitutions had reduced
255 sigma factor activity relative to that of strains carrying wild-type AlgU (Fig S4B). We note that
256 our reporter assay does not appear to be very sensitive to low levels of AlgU activity. Both the
257 published PAK and PA103 Δ*mucA* strains are mucoid (Intile *et al.*, 2014, Jones *et al.*, 2010),
258 suggesting that AlgU A58T and E46G are not completely inactive. While we cannot distinguish
259 this low level of activity from the null case, our results show that nevertheless these AlgU
260 mutants have significantly less transcriptional activity than the wild-type protein (Fig S4A,B).
261
262 To identify additional suppressors of *mucA* essentiality, we used the Δ*mucA att*Tn7::P_{rhaBAD}-
263 *mucA* strain. This strain was not viable when grown in the absence of rhamnose, but natural
264 revertants arose at a frequency of less than 1 in 10^9 colony forming units. We sequenced the

265 *algU* gene in 25 of these revertants, since our work shows that AlgU mutants can suppress
266 *mucA* essentiality (Fig 4A). All 25 isolates contained mutations in *algU* that are expected to
267 encode for sigma factors with reduced activity (Fig 4B, Table S2). There were 10 revertants with
268 deletions or nonsense mutations of *algU*, encoding either no product or a truncated product
269 completely lacking Region 4 of the sigma factor. There were 6 revertants that contained multi-
270 base pair duplications in *algU* that would lead to the insertion of 3 or 4 amino acids in Region 2
271 helix 3 of the sigma factor. There were 9 revertants containing missense mutations, 8 of which
272 were unique, encoding the following substitutions: D18G, A21V, Y29C, A47T, D49G, Y59C,
273 N81D, and R174G. Using a model of σ^E in complex with the RNAP core and the promoter
274 element, we expect these insertions and substitutions to affect sigma factor folding, RNAP core
275 interactions, or promoter element interactions (Fig S5). To determine the effect of these 25
276 mutations on AlgU activity, we then tested AlgU function using our *algD* reporter assay in these
277 natural revertants. As expected, we saw that all 25 revertants had much lower AlgU activity than
278 the parental strain upon induction of envelope stress with D-cycloserine (Fig S4C). Together,
279 these data strongly suggest that *algU* mutations that encode for a protein with reduced
280 transcriptional activity allow *P. aeruginosa* to survive in the absence of *mucA*.
281

282 **Overexpression of *algU* in the absence of *mucA* is lethal**

283 Collectively, our results suggest that *mucA* essentiality is due to unregulated AlgU activity. We
284 therefore reasoned that overexpression of *algU* should be lethal. Supporting this hypothesis, the
285 toxic effects of *algU* overexpression have been previously noted (Cross *et al.*, 2020,
286 Hershberger *et al.*, 1995, Schurr *et al.*, 1994). However, studies have shown that *algU* can be
287 overexpressed in wild-type *P. aeruginosa* (Qiu *et al.*, 2008, Schulz *et al.*, 2015). It is important to
288 note that these wild-type strains contain *mucA*, which is positively regulated by AlgU. We
289 therefore examined the effect of overproducing AlgU in WT, Δ*algU*, and Δ*algU* Δ*mucA* strains,
290 using a chromosomally integrated arabinose-inducible *algU*. Of note, the Δ*algU* and Δ*algU*

291 Δ*mucA* strains lack the positive feedback of AlgU on its own expression, and the expression of
292 *algU* is completely dependent on the inducer. Our results show that in the absence of inducer
293 when AlgU is not overexpressed, all three strains had similar growth rates (Fig 5A). In the
294 presence of 1% arabinose (i.e. high expression of *algU*), all three strains had a growth defect.
295 However, in comparison to the other two strains, the strain lacking *mucA* failed to grow at all in
296 the presence of this high inducer concentration.
297
298 To determine if the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* strain can grow under lower levels of *algU*
299 induction, we tested a range of inducer concentrations (Fig 5B, Table S3). We found that
300 although there was a growth defect, Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* was able to grow in the
301 presence of 0.1% arabinose, suggesting that this strain was able to tolerate low levels of *algU*
302 induction. Furthermore, as expected, the drop in growth rate in comparison to the no arabinose
303 condition was statistically larger for the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* than for the other two
304 strains ($N=3$, $p < 0.05$, ANOVA with post hoc Tukey HSD), since this strain lacks the ability to
305 produce any MucA to reduce AlgU activity. Together, these results strongly suggest that in the
306 absence of *mucA*, while a certain level of AlgU activity is tolerated, high AlgU activity is fatal to
307 the cell.
308
309 The above experiments were performed using only one medium (LB). To determine if
310 overexpression of *algU* in the absence of *mucA* is lethal under other nutrient conditions, we
311 determined the growth rate of the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* strain in other media.
312 Similar to the results in LB (Fig 5), Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU* strain failed to grow in the
313 presence of 1% arabinose for the three other media we tested (Fig S6), suggesting that AlgU
314 overproduction is toxic independent of the nutrient conditions.
315

316 **Expression of *rpoD* can rescue lethality of *algU* overexpression.**

317 It has been suggested that AlgU competes for RNAP binding with RpoD, the essential primary
318 sigma factor (Yin *et al.*, 2013). If high concentrations of AlgU outcompetes RpoD for RNAP, this
319 could reduce the expression of essential housekeeping genes and lead to toxicity in AlgU-
320 overexpressing cells. If this is true, we hypothesized that overexpressing *rpoD* with *algU* could
321 ameliorate the growth defect of high *algU* expression in the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU*
322 strain. We inserted into this strain a copy of *rpoD* under the same arabinose-inducible promoter.
323 We chose to test growth in the presence of 2% arabinose, since this new strain contains two
324 arabinose-inducible promoters, in contrast to the strains described above in Fig 5. Growth with
325 2% arabinose caused a growth defect for all strains tested, even in a strain lacking an ectopic
326 arabinose-inducible gene (Fig 6). As expected from our results (Fig 5), Δ*algU* Δ*mucA*
327 *att*Tn7::P_{araBAD}-*algU* failed to grow in the presence of 2% arabinose. This lethality defect,
328 however, was rescued in the Δ*algU* Δ*mucA att*Tn7::P_{araBAD}-*algU att*CTX::P_{araBAD}-*rpoD* strain.
329 Furthermore, the growth rate of this strain in the presence of arabinose was indistinguishable
330 from that of Δ*algU* ΔmucA and Δ*algU* Δ*mucA att*CTX::P_{araBAD}-*rpoD* (Table S4). Since increasing
331 RpoD levels rescued the lethality associated with high AlgU levels, these results suggest that in
332 the absence of MucA, the ensuing sigma factor competition of AlgU with RpoD for RNAP
333 contributes to the viability defect, and that MucA may be required to rein in AlgU in order to
334 reduce the interference with the essential function of RpoD.

335

336 **DISCUSSION**

337 Although it has been assumed that *mucA* can be deleted without affecting bacterial viability, our
338 work shows that *mucA* is essential in a variety of *P. aeruginosa* wild-type strains and under
339 various nutrient conditions. Our results strongly suggest that the interaction of MucA with its
340 cognate sigma factor AlgU is required for viability, and that AlgU mutants with reduced sigma
341 factor activity can suppress *mucA* essentiality. Furthermore, overproduction of AlgU is

342 detrimental to cell growth, which can be rescued by increasing RpoD production. Together, our
343 data strongly suggest that unchecked AlgU activity in the absence of MucA leads to cell death.
344
345 Our data point to the negative regulation of AlgU being necessary for cell survival in *P.*
346 *aeruginosa* (Fig 7). Under non-stress conditions, AlgU is bound to and inhibited by a full-length
347 MucA. Under envelope stress or in strains containing *mucA* mutations, the anti-sigma factor is
348 cleaved. We propose that while this cleaved cytosolic form of MucA does not inhibit AlgU to the
349 same extent as the full-length protein, it is still able to interact with and inhibit AlgU to some
350 degree. Although AlgU is active under such conditions, because *mucA* is positively regulated by
351 AlgU, this negative feedback allows the cell to keep AlgU activity under control. In comparison,
352 in the absence of MucA, this ability to control AlgU is lost. We propose that the strong positive
353 feedback of AlgU on its own expression is what leads to cell death and *mucA* essentiality.
354 Supporting this model are several lines of evidence. First, *mucA* essentiality is rooted in its
355 interaction with *algU* (Figs 1 and 3), strongly suggesting that the ability of MucA to inhibit AlgU is
356 required for viability. Second, deletion of AlgU-regulated genes, including those encoding the
357 well-characterized transcription factors AlgB (Wozniak & Ohman, 1994), AlgR (Martin *et al.*,
358 1994, Wozniak & Ohman, 1994), and AmrZ (Wozniak *et al.*, 2003), do not allow for *mucA*
359 deletion (Table S1), supporting that *algU* itself is the lethal component of the AlgU regulon.
360 Third, AlgU mutants can suppress *mucA* essentiality, and all 25 natural revertants that we
361 screened contained mutations in *algU* (Fig 4). The strains carrying these mutations had
362 decreased AlgU transcriptional activity (Fig S4), supporting the idea that decreasing the positive
363 feedback of AlgU on its own expression allows the cell to survive in the absence of MucA.
364 Lastly, overexpression of *algU* leads to a growth defect, which is lethal at high levels in the
365 absence of *mucA* (Fig 5) and can be rescued by overexpression of *rpoD* (Fig 6), suggesting that
366 sigma factor competition between AlgU and RpoD may be partially responsible for lethality in
367 the absence of MucA.

368

369 While our data supports our model that the AlgU-regulated gene critical for *mucA* essentiality is
370 *algU* itself, we cannot exclude the possibility that the regulation of another single gene or a
371 combination of genes within the AlgU regulon is responsible for the *mucA* requirement. This
372 possibility, however, seems less likely for two reasons. First, all the revertants we tested that
373 could grow in the absence of *mucA* expression contained *algU* mutations (Table S2). If another
374 single AlgU-regulated gene were responsible for *mucA* essentiality, we would have expected to
375 find a revertant with such a mutation. Second, we were unable to delete *mucA* from a strain
376 lacking three AlgU-regulated transcription factors (Table S1). Genes regulated by AlgB, AlgR, or
377 AmrZ can account for approximately fifty percent of the AlgU regulon (Huang *et al.*, 2019, Jones
378 *et al.*, 2014, Leech *et al.*, 2008, Schulz *et al.*, 2015), suggesting that it is unlikely that deleting a
379 combination of AlgU-regulated genes can alleviate the necessity for *mucA*. Nonetheless, we are
380 actively searching for non-*algU* suppressors of *mucA* essentiality.

381

382 Our data show that the anti-sigma factor MucA is required for viability in various *P. aeruginosa*
383 strains (Table 1). However, the requirement of an anti-sigma factor for bacterial viability is not
384 unique to *P. aeruginosa*. While there do not appear to be any essential anti-sigma factors in *E.*
385 *coli*, as all of them are individually deleted in the Keio collection (Baba *et al.*, 2006), there are
386 many occurrences in other organisms. There are two anti-sigma factors, YhdL and YxlC, in
387 *Bacillus subtilis* that are necessary for bacterial viability (Horsburgh & Moir, 1999, Koo *et al.*,
388 2017, Mendez *et al.*, 2012). The essentiality of *yhdL* is notable as this anti-sigma factor
389 negatively regulates SigM, a sigma factor involved in the envelope stress response (Horsburgh
390 & Moir, 1999). Similarly, in *Mycobacterium tuberculosis*, the gene encoding the anti-sigma factor
391 RslA is identified as essential due to an underrepresentation of interruptions in a genome-wide
392 transposon insertional analysis (Griffin *et al.*, 2011). However, similar to our results (Fig 4), *rslA*
393 can be deleted from *M. tuberculosis* lacking the gene encoding its cognate sigma factor SigL,

394 which regulates genes involved in cell envelope processes (Dainese *et al.*, 2006). Furthermore,
395 in the defined transposon mutant library for *Vibrio cholerae*, an interruption in the *mucA*
396 homolog *rseA* does not exist, suggesting that this anti-sigma factor may be essential in *V.*
397 *cholerae*, similar to in *P. aeruginosa* (Cameron *et al.*, 2008). Finally, the *Pseudomonas syringae*
398 *mucA* homolog is deemed essential based on a reduced number of transposon insertions
399 (Helmann *et al.*, 2019). Therefore, although the *E. coli mucA* homolog *rseA* is not required for
400 cell viability (De Las Penas *et al.*, 1997, Missiakas *et al.*, 1997), these data, together with our
401 results, suggest that essentiality of anti-sigma factors that regulate cell envelope function may
402 be a widespread phenomenon in bacteria.
403
404 Our work strongly suggests that lethality in the absence of *mucA* is caused by unchecked AlgU
405 activity (Figs 5 and 6), in agreement with previous studies suggesting that overproduction of
406 AlgU is toxic (Cross *et al.*, 2020, Hershberger *et al.*, 1995, Schurr *et al.*, 1994). Our data
407 suggests that sigma factor competition may play a role in this lethality (Fig 6). Competition
408 between AlgU and the primary sigma factor RpoD has been previously suggested (Yin *et al.*,
409 2013). Overexpression of RpoD suppresses alginate biosynthesis in a mucoid CF isolate.
410 Additionally, the activity of AlgU increases in a mutant with decreased RpoD activity (Yin *et al.*,
411 2013). Expanding to other organisms, in *B. subtilis*, the lethality defect associated with deleting
412 *yhdL*, which encodes for an anti-sigma factor, can be rescued via overproduction of the primary
413 sigma factor SigA, similar to our results (Fig 6). Zhao and colleagues suggest that the lethality of
414 this strain is due to the YhdL-cognate sigma factor, SigM, outcompeting SigA for RNAP (Zhao *et*
415 *al.*, 2019). Based on these findings, and our data showing that RpoD can rescue the lethality of
416 high AlgU levels (Fig 6), we speculate that sigma factor competition may be involved in other
417 instances where anti-sigma factors that regulate cell envelope functions have been found to be
418 essential.
419

420 The interaction of MucA and AlgU is of interest, as *mucA* mutations are common in clinical CF
421 isolates of *P. aeruginosa* (Boucher *et al.*, 1997, Candido Cacador *et al.*, 2018, Ciofu *et al.*, 2008,
422 Martin *et al.*, 1993, Pulcrano *et al.*, 2012). Our results (Fig 4) agree with the literature showing
423 that mucoid isolates with *mucA* mutations revert to a non-mucoid state via changes to *algU*
424 when the cells are grown under standard laboratory conditions (Ciofu *et al.*, 2008, DeVries &
425 Ohman, 1994, Sautter *et al.*, 2012, Schurr *et al.*, 1994). While these *algU* secondary site
426 mutations are found in non-mucoid CF isolates with *mucA* mutations, such isolates are detected
427 at a lower frequency (Ciofu *et al.*, 2008). As suggested by Ciofu and associates, this may be
428 due to the importance of AlgU for the survival of *P. aeruginosa* in the CF lung environment,
429 suggesting that reducing AlgU activity may increase *P. aeruginosa* eradication from the CF
430 airway. Based on our data and the published literature, we propose that the MucA-AlgU
431 interaction may serve as a good therapeutic target. Our results show that destabilizing the
432 MucA-AlgU interaction results in bacterial cell death or mutations in *algU* that likely result in
433 reduced sigma factor activity and reduced mucoid conversion, of which either outcome could be
434 beneficial in the treatment of *P. aeruginosa* CF lung infections.
435

436 **METHODS AND MATERIALS**

437 **Bacterial strains and growth conditions**. The bacterial strains, plasmids, and oligonucleotides
438 used for this study are in Tables S5-S7. The construction of strains is described in Supporting
439 Information. Bacteria were grown at 37°C in LB with shaking or on semi-solid LB media, unless
440 otherwise noted.
441

442 **Allelic exchange assay.** The protocol, depicted in Fig S1, was modified from (Hmelo *et al.*,
443 2015). Briefly, after introduction of the *mucA* deletion vector, merodiploids were selected on
444 semi-solid VBMM (Vogel & Bonner, 1956) with 60 mg/L gentamicin. Using OMS118 and
445 OMS119, PCR was performed on at least six isolates to confirm that the presence of both the

446 wild type and deletion alleles of *mucA*. Confirmed merodiploids were then individually streaked
447 on NSLB (10 g/L tryptone, 5 g/L yeast extract) with 10% sucrose semi-solid media for
448 counterselection. PCR was performed on eight colonies per merodiploid, using OBT601 and
449 OBT602 to determine the resolution to either the WT or deletion allele.
450
451 **MucA depletion assay.** Cells were grown in LB with 0.05% rhamnose at 37°C with shaking to
452 an OD_{600} of 0.3. After washing, the culture was then divided in two, half resuspended with
453 rhamnose and half without in its respective media base (LB, PIB, SCFM, or VBMM). Cells were
454 incubated at 37°C with shaking. At the indicated time points, two aliquots were removed from
455 each culture, serially diluted and plated in triplicate onto LB agar plates with 0.05% rhamnose
456 for recovery. Colonies were counted and log_{10}-transformed CFU/mL of the culture was
457 calculated.
458
459 **Yeast two-hybrid assay.** The ProQuest Two-Hybrid System (Invitrogen) was used, per
460 manufacturer's instructions. Briefly, yeast containing the Gal4 activation domain-based prey and
461 the Gal4 DNA binding domain-based bait vectors were grown overnight in SD-Leu-Trp broth
462 (Clontech). The OD_{600} value was recorded. ONPG (VWR) was added to lysed cells, and the
463 mixture was incubated at 37°C until a light yellow color was achieved. The incubation time was
464 recorded. The OD_{420} of the supernatants was determined using a Synergy Hybrid HTX
465 Microplate Reader (Bio-Tek Instruments). Beta galactosidase activity was determined using
466 Miller units, based on the following equation: 1000 x OD_{420}/(time x culture volume x OD_{600}).
467
468 **Natural revertant assay.** *P. aeruginosa* Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* was grown in LB with
469 0.05% rhamnose at 37°C with shaking to an OD_{600} of 1. Cells were washed and plated on semi-
470 solid LB plates without rhamnose. Plates were incubated for 24-48 h at 37°C. For isolates that
471 grew on LB without rhamnose, *algU* was Sanger sequenced.

472

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480

481 **AUTHOR CONTRIBUTIONS**

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

485

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684

685 **TABLES**

686 **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*

687 † Class identification is based on the exopolysaccharide expression profile as described in

688 (Colvin *et al.*, 2012): Class I, Pel-dominant matrix; Class II, Psl-dominant matrix; Class III,

689 exopolysaccharide redundant matrix users; and Class IV, matrix overproducers. CF, cystic

690 fibrosis; UTI, urinary tract infection.

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

692

693 **FIGURE LEGENDS**

694 **Figure 1. The first 50 amino acids of MucA are necessary and sufficient for viability.**

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

705

706 **Figure 2. Depletion of MucA results in cell death.**

707 Viable colony counts of PAO1 Δ*mucA* attTn7:: *rhaSR*-P_{rhaBAD}-*mucA*-10His over time in **(A)** LB,
708 **(B)** PIB, **(C)** SCFM and **(D)** VBMM with (+ rha; red circles) or without (– rha; gray squares)
709 0.05% rhamnose. Viable colony counts after incubation in the indicated condition and time was
710 determined by plating the cells on LB with 0.05% rhamnose to allow cells to recover and grow.
711 Hash, broken y-axis; error bars, SEM (N=3). Asterisk, statistically different from that at the same
712 time point grown in the presence of rhamnose ($p < 0.01$, N=3, mixed model ANOVA with post-
713 hoc Bonferroni test).

714

715 **Figure 3. The physical interaction of MucA and AlgU is required for survival.**

716 **(A)** Four residues of MucA make greater than one predicted hydrogen bond with AlgU. The
717 MucA-AlgU co-crystal structure (PDB 6IN7; Li et al., 2019) with the cytosolic domain of MucA
718 (aa 1-78; red) and Regions 2 and 4 of AlgU (green, $AlgU_2$; yellow, $AlgU_4$) is shown. The

719 residues of MucA (grey) that are predicted to make more than one hydrogen bond with AlgU are
720 labeled in the inset. Black dotted lines, predicted hydrogen bonds; red atoms, oxygen; blue
721 atoms, nitrogen. **(B)** Substitution of MucA residues at its interface with AlgU abolish their binding
722 via yeast two-hybrid. The first 75 residues of MucA were fused to the Gal4 DNA binding domain
723 (DBD-MucA$^{1-75}$) and AlgU was fused to the Gal4 activation domain (AD-AlgU). Interaction of
724 MucA and AlgU led to *lacZ* expression. Beta-galactosidase activity (in Miller units) was used as
725 a proxy for the protein interaction strength. WT, wild-type protein sequence; –, no fusion protein
726 included; hash, broken y-axis; error bars, SEM (N=3); letters, statistical groups with the different
727 letters representing statistically different groups ($p < 0.01$; biological triplicate with technical
728 quadruplicates; ANOVA with post-hoc Tukey HSD). **(C)** Frequency of observed isolates
729 resolving to the endogenous wild-type *mucA* allele (WT, black) or the deletion allele (Δ*mucA*,
730 white) in the allelic exchange assay, using PAO1 Δ*algD att*Tn7::P_{algU}-*mucA*, where *mucA*
731 encodes for the indicated substitution. Super-imposed on the bars are the number of isolates
732 that were tested in each category. Asterisk, $p < 0.0001$; Fisher's exact test. Caret, slower
733 growing isolates.
734
735 **Figure 4. AlgU mutants with reduced sigma factor activity suppress *mucA* essentiality.**
736 **(A)** Frequency of observed isolates resolving to the endogenous wild-type *mucA* allele (WT,
737 black) or the deletion allele (Δ*mucA*, white) in the allelic exchange assay, using PAO1 Δ*algU*
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739 bars are the number of isolates that were tested in each category. Asterisk, $p < 0.0001$; Fisher's
740 exact test. **(B)** Schematic of mutations seen in revertants that could grow in the absence of
741 *mucA*. Revertants were selected by growing PAO1 Δ*mucA att*Tn7::P_{rhaBAD}-*mucA* on media
742 lacking rhamnose. Blue rectangles, multi-base pair deletions; left arrow, deletion extends into
743 the promoter; blue triangles, single base pair deletions; black triangles, nonsense mutations;

744 white triangles, duplications resulting in 3 or 4 amino acid insertions; red triangles, missense
745 mutations. See Table S2 for a full description of the *algU* mutations.
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747 **Figure 5. Overexpression of *algU* is lethal in the absence of *mucA*.**
748 **(A)** Growth curves of PAO1 strains containing an arabinose-inducible copy of *algU* in wild-type
749 (purple squares), Δ*algU* (blue diamonds), and Δ*algU* Δ*mucA* (red circles) backgrounds in LB.
750 Readings are normalized to wild-type PAO1 (lacking an inducible *algU* gene). Open symbols
751 represent conditions in the absence of arabinose; closed symbols, with 1% arabinose. Error
752 bars, SD (n=18). Asterisk, statistically different from the same strain grown in the absence of
753 arabinose ($p < 0.01$, N = 3, ANOVA with post-hoc Tukey HSD). **(B)** Growth rate of strains as
754 described in (A), with increasing induction of *algU* (relative to PAO1 *att*Tn7::P_{araBAD}-*algU* grown
755 in the absence of arabinose). Strains were grown in LB with 0%, 0.1%, 0.25%, and 1%
756 arabinose ([Ara]). Error bars, SEM (N=3). Asterisk, statistically different from the same strain
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758 Table S3 for full statistical comparisons.
759
760 **Figure 6. Lethality caused by *algU* overexpression can be rescued via *rpoD* expression.**
761 Growth rate of indicated strains grown in LB with (+) or without (–) 2% arabinose (relative to
762 PAO1 Δ*algU* Δ*mucA* grown in the absence of arabinose). Error bars, SEM (N=3). Asterisk,
763 statistically different from the same strain grown in the absence of arabinose ($p < 0.05$, N = 3,
764 ANOVA with post-hoc Tukey HSD). See Table S4 for full statistical comparisons.
765
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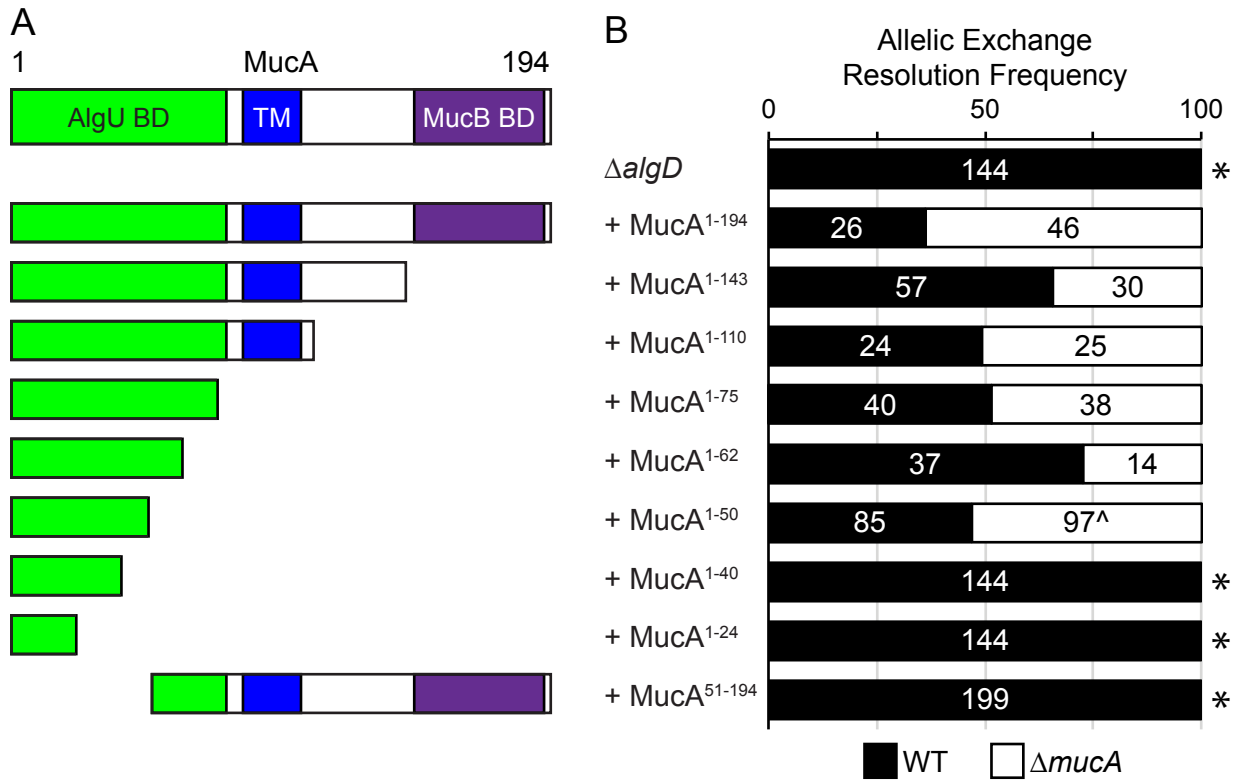
767 Under conditions of no envelope stress (left), the full-length MucA (red) binds strongly to AlgU
768 (green star), leaving very little free AlgU to interact with the RNAP (grey oval). Therefore, the
769 AlgU regulon (green arrow) is in the "off" state (red X). Under conditions of envelope stress or in

770 strains containing *mucA* mutations that lead to a truncated product (middle), a cleaved cytosolic
771 form of MucA is produced. This form can still interact with and inhibit AlgU. However, the
772 strength of the interaction is weaker than with the full-length MucA, allowing for a pool of free
773 AlgU that can then bind to and recruit RNAP to the promoters of its regulon. Under such
774 conditions, the AlgU regulon, which includes *algU* and *mucA*, is activated and in the "on" state
775 (green up arrows). However, because of the feedback on MucA, negative regulation of AlgU
776 activity is still present in the cell. In the absence of *mucA* (right), there is no MucA to bind to and
777 regulate AlgU. All of the AlgU is free to interact with the core RNAP. This leads to high
778 expression of the AlgU regulon and overproduction of AlgU itself. Under such strong positive
779 feedback and in the absence of the negative regulator, the unchecked activity of AlgU leads to
780 cell death.
781



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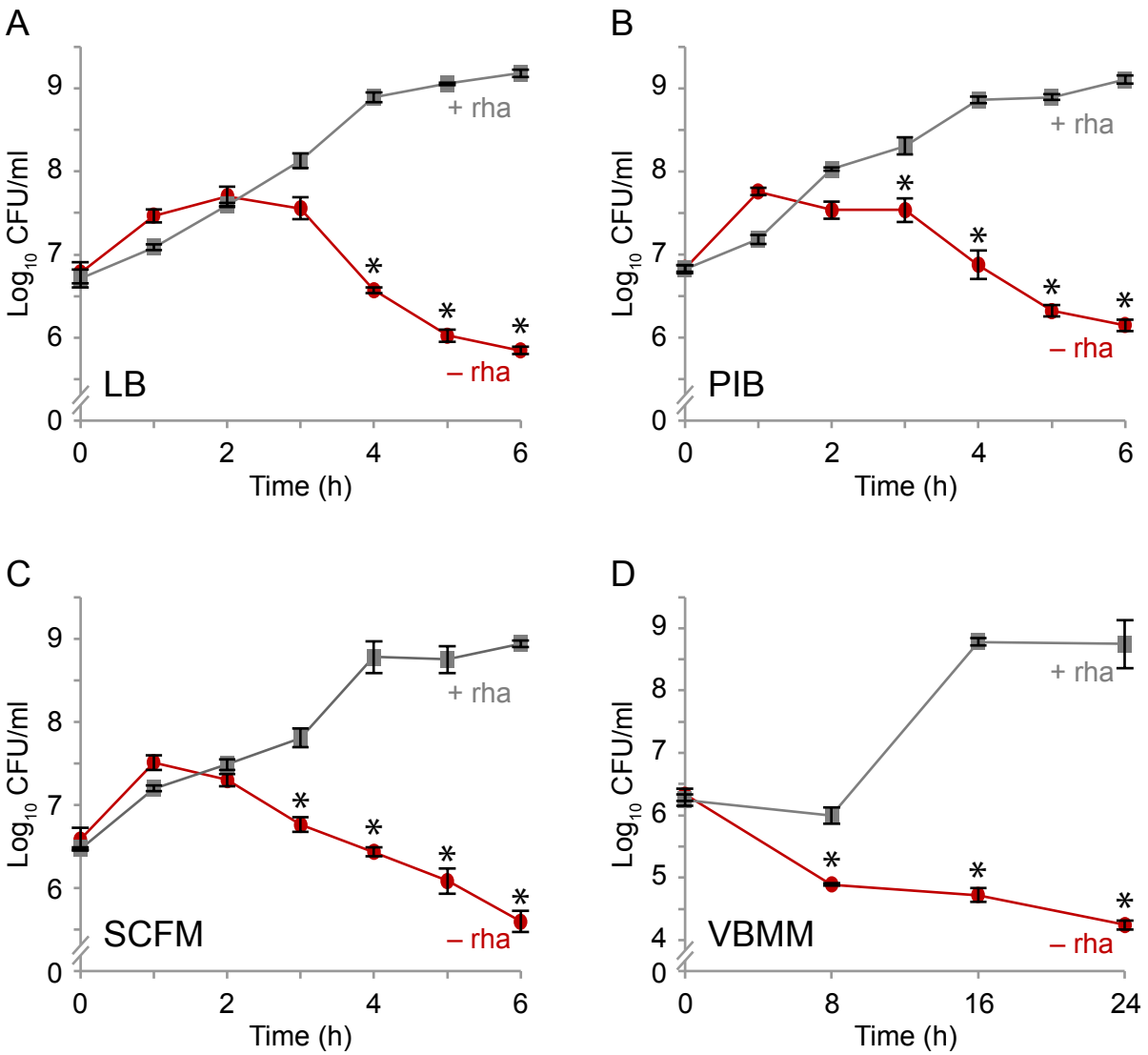

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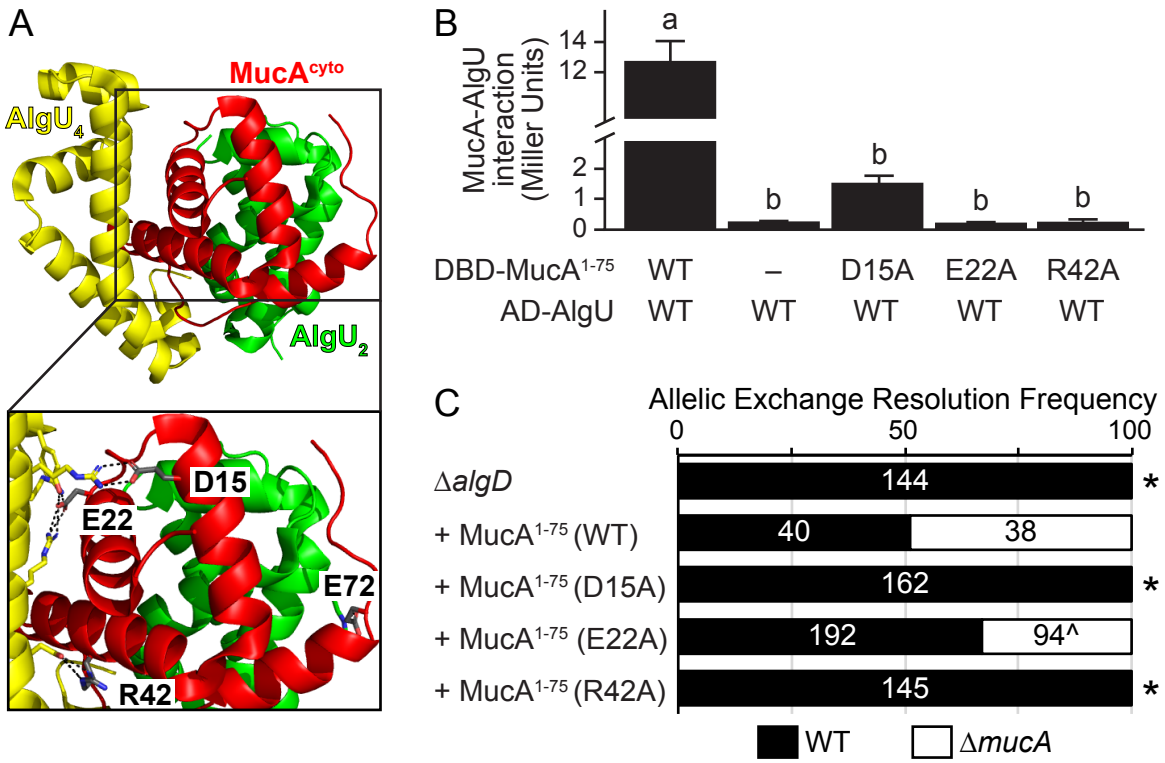

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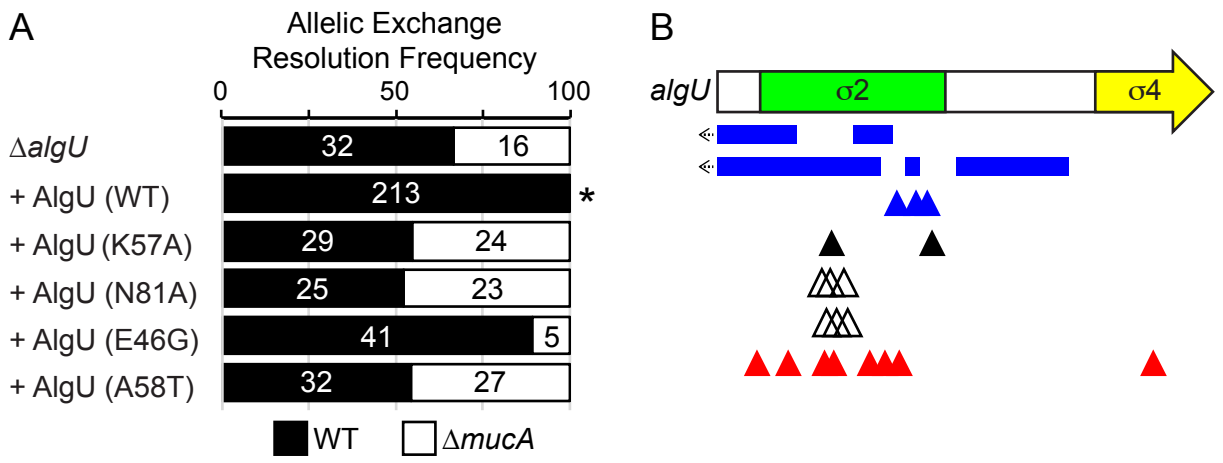

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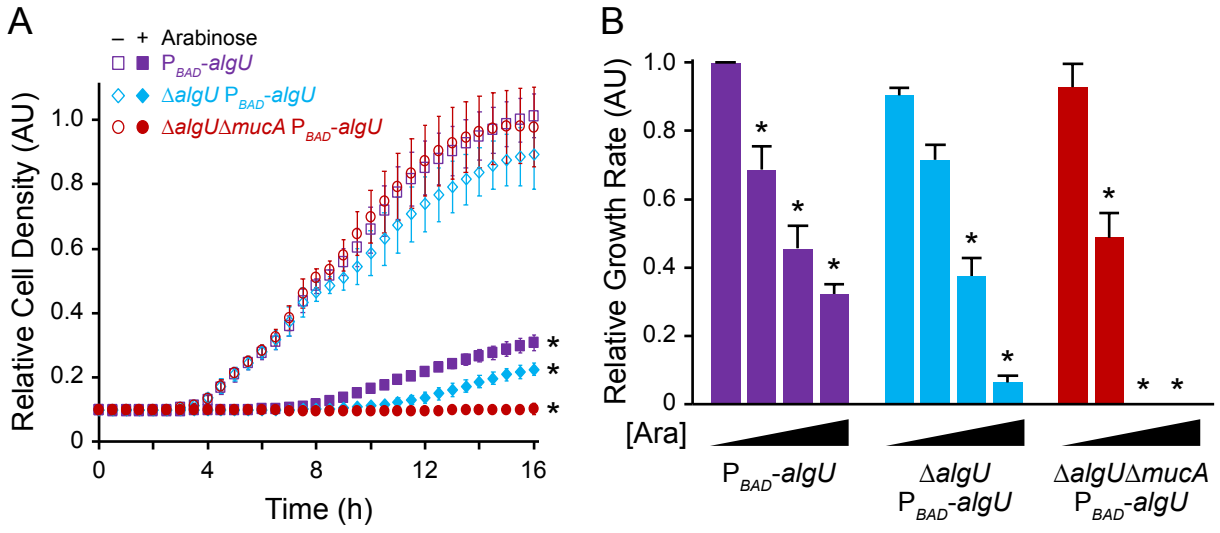

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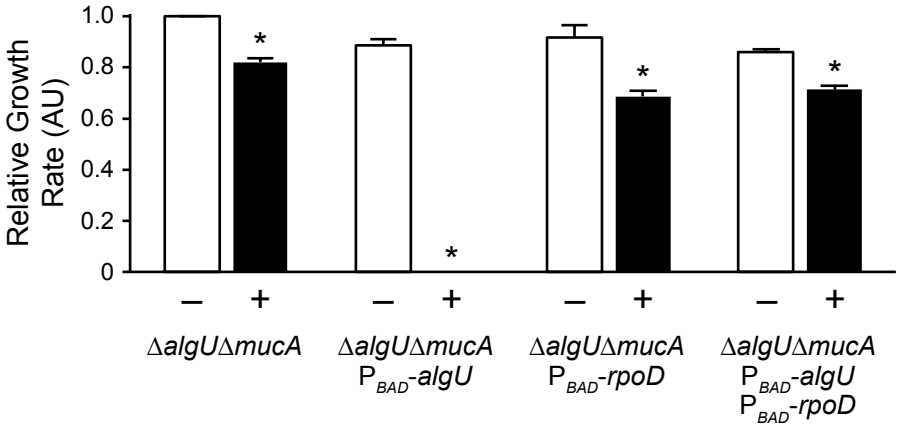

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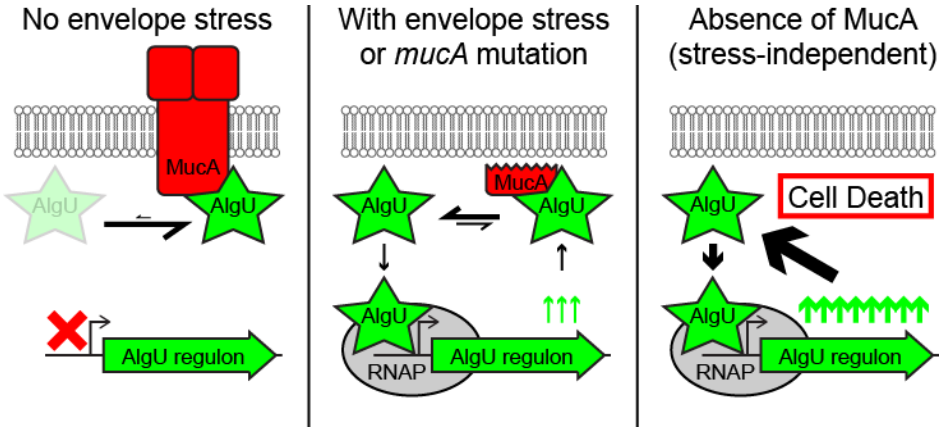

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