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

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ABSTRACT

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

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

*MucA* is an anti-sigma factor to the alternative sigma factor AlgU (also known as AlgT, $\sigma^E$, or $\sigma^{22}$), which responds to envelope stress (Damron & Goldberg, 2012, Govan & Deretic, 1996). *MucA* is a transmembrane protein that sequesters the sigma factor away from RNA polymerase (RNAP) via its N-terminal interaction with AlgU (Li *et al.*, 2019, Schurr *et al.*, 1996, Xie *et al.*, 1996). The C-terminus of *MucA* is in the periplasm, where it is protected from proteolysis via an interaction with *MucB* (Mathee *et al.*, 1997, Schurr *et al.*, 1996). The envelope stress response is controlled via a regulated intramembrane proteolysis cascade: *MucB* dissociates from *MucA* upon detection of stress, allowing the proteases AlgW and MucP to cleave *MucA* from the inner membrane (Qiu *et al.*, 2007, Wood & Ohman, 2009). In the cytoplasm, *MucA* is further degraded by ClpXP, releasing AlgU to interact with RNAP and activate the AlgU regulon (Qiu *et al.*, 2008). AlgU regulates at least 350 genes, including those responsible for the production of itself, its cognate anti-sigma factor *MucA*, and the alginate biosynthetic enzymes (Firoved & Deretic, 2003, Schulz *et al.*, 2015, Wood & Ohman, 2009). This system is homologous to the well-studied envelope stress response in *Escherichia coli*: *MucA* shares 28% identity (72%
similarity) to the anti-sigma factor RseA, and the cognate sigma factor of the *E. coli* RseA, called RpoE, is homologous to and functionally interchangeable with AlgU (Yu et al., 1995), with 66% identity (93% similarity).

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

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

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

Alginate biosynthesis is not solely responsible for *mucA* essentiality

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

Expression of the alginate biosynthesis genes is controlled by three AlgU-regulated transcription factors that are active in mucoid cells: AlgB, AlgR, and AmrZ (Martin et al., 1994, Wozniak & Ohman, 1994, Wozniak et al., 2003). Since these transcription factors regulate a suite of genes in addition to those involved in alginate biosynthesis (Huang et al., 2019, Jones et al., 2014, Kong et al., 2015, Leech et al., 2008), we tested if overexpression of these three regulons underlies mucA essentiality by attempting to delete mucA from strains lacking these transcription factors, either individually or all together. We found that we could not delete mucA in any of these backgrounds (Table S1). Altogether, our data support the conclusion that eliminating alginate biosynthesis and the expression of large parts of the AlgU regulon do not alleviate mucA essentiality.

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

While we were unable to delete mucA using an allele lacking >95% of the coding region, mucA is commonly mutated in clinical isolates and many mucA transposon mutants exist (Fig S2). These data suggest that only a portion of mucA is essential. To determine the essential portion of mucA, we first constructed a series of strains containing an ectopic chromosomally-integrated mucA, driven by its native promoter. We then attempted to delete the endogenous wild-type mucA from these strains. Although we designed these ectopic alleles to encode for a protein with a His-tag, we were unable to probe whether equivalent amounts of protein were produced across our strains, since truncated versions of MucA are cleaved in the cytosol, as shown in previous work (Qiu et al., 2008). However, we expect that the ectopic alleles produce similar protein levels, as they are all under the same promoter and located in the same genomic site.
Since we expected that the resultant \( \Delta \text{mucA} \) strain would be mucoid in some cases, we used a \( \Delta \text{algD} \) background to eliminate alginate production, which made the strains easier to manipulate.

Mutant MucA proteins of varying lengths were ectopically expressed to determine the necessary portion of MucA. Full-length MucA is 194 amino acids (aa). Based on the co-crystal structures of MucA with AlgU and MucB (Li et al., 2019), the first 78 residues of MucA interact with AlgU, and the last 48 residues of MucA interact with MucB. We engineered mucA alleles that encode for various truncated proteins (Fig 1A). We then attempted to delete the endogenous mucA from these strains. Since the flanking regions of these two alleles differ, our deletion allele specifically targets the native allele. We were able to easily recover isolates that resolved to the mucA deletion allele from strains containing ectopic alleles that encode for the full length MucA (aa 1-194), as well as MucA aa 1-143, aa 1-110, aa 1-75, and aa 1-62 (Fig 1B). These results show that the requirement for mucA can be complemented in trans, and suggest that the gene product of mucA is required for viability, irrespective of its genomic location.

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

To determine if the first 50 residues of MucA are necessary for viability, we constructed a strain with an ectopic mucA that encodes for aa 51-194. We were unable to delete the native mucA
from this strain (Fig 1B). Together, these results suggest that the first 50 amino acids of MucA are necessary and sufficient for cell viability in *P. aeruginosa*. Consistent with these results, while many clinical and engineered mucA mutations have been reported (Boucher *et al.*, 1997, Candido Cacador *et al.*, 2018, Ciofu *et al.*, 2008, Martin *et al.*, 1993, Pulcrano *et al.*, 2012, Turner *et al.*, 2015), no mutations fall within the region of mucA that encodes for the first 50 residues (Fig S2). Furthermore, this region of MucA corresponds to the AlgU binding domain, suggesting that the physical interaction between the two proteins is important for cell viability.

**Depletion of MucA leads to cell death**

To evaluate the effect of the nutrient conditions on mucA essentiality, we tested the effect of depleting MucA on *P. aeruginosa* cell viability in different media. To deplete MucA from cells, we engineered a strain lacking the native mucA and containing a rhamnose-inducible copy (ΔmucA attTn7::P_{rhaBAD}-mucA). We removed rhamnose from growing ΔmucA attTn7::P_{rhaBAD}-mucA cells and determined the viability of cells over time based on their ability to recover on medium containing rhamnose. Since cells would cease producing MucA in the absence of rhamnose, the MucA present in the cells at the start of the experiment would deplete as the cells divided. In all four media we tested, if rhamnose was not removed at the beginning of the experiment, the cells increased in density by ~2-logs over the duration of the experiment. In comparison, when rhamnose was removed, the ΔmucA attTn7::P_{rhaBAD}-mucA cells lost viability over time in all four media (Fig 2). Together, these results suggest that mucA is essential in *P. aeruginosa* independent of the nutrient environment.

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

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

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

**Mutations that reduce AlgU activity alleviate the requirement for MucA**

Since MucA inhibits AlgU from binding to RNAP, we hypothesized that *mucA* essentiality is due to its inhibition of the AlgU regulon, which includes *algU* itself. To start to test this hypothesis, we reasoned that strains containing a mutant AlgU with lower affinity for DNA would alleviate *mucA* essentiality, since expression of the AlgU regulon would inherently be lower in such mutants. Therefore, we engineered ∆*algU* strains with an ectopic chromosomally-integrated *algU* allele encoding such DNA-binding mutants. Based on homology to *E. coli* RpoE mutants that have decreased *in vitro* transcriptional activity (Campagne *et al.*, 2014), we engineered *algU* alleles that encode for K57A and N81A. As described above, we were able to delete *mucA* from a ∆*algU* strain (Fig 4A). This phenotype could be rescued via the ectopic addition of a wild-type *algU* allele, as we were no longer able to delete *mucA* from such a strain. In contrast, *mucA* could be readily deleted from strains carrying alleles encoding AlgU K57A or N81A, showing that such mutant alleles failed to rescue the ∆*algU* phenotype (Fig 4A). To determine the effect of these substitutions on AlgU activity, we induced envelope stress using D-cycloserine (Wood *et al.*, 2006) and measured AlgU activity using a plasmid-borne *gfp* reporter driven under the AlgU-regulated *algD* promoter (Damron *et al.*, 2009). Similar to what is seen for RpoE (Campagne *et al.*, 2014), these mutant strains had reduced AlgU activity upon induction of envelope stress, relative to the strain containing a wild-type *algU* (Fig S4A,B). These results suggest that AlgU mutants with reduced sigma factor activity can suppress *mucA* essentiality.

As mentioned above, others have reported strains in which the entirety of *mucA* is deleted from *P. aeruginosa* PAO1, PAK, and PA103 (Intile *et al.*, 2014, Jones *et al.*, 2010, Pritchett *et al.*, 2015). Since we were unable to delete *mucA* from these three strain backgrounds (Table 1), we
hypothesized that these ∆mucA strains may contain suppressor mutations that allowed for their survival in the absence of mucA. To identify these suppressors, we sequenced the genomes of these ∆mucA isolates. In the PAO1 ∆mucA strain (Pritchett et al., 2015), we found that while the native mucA was deleted, the strain contained a second full-length copy of algU and a mucA allele that encoded for MucA aa 1-155 elsewhere in the genome. As expected from our data (Fig 1B), we were able to delete the native mucA allele from a PAO1 strain containing an ectopic mucA allele that encoded for MucA aa 1-155 (with 5 of the 28 tested isolates resolving to the deletion allele), confirming that the ectopic mucA allele in the published PAO1 ∆mucA strain is sufficient for viability in the absence of the endogenous mucA. In comparison, the PAK and PA103 ∆mucA strains (Intile et al., 2014, Jones et al., 2010) contain missense mutations in algU, which results in AlgU A58T and E46G, respectively. We replicated these mutations in PAO1 by inserting algU alleles encoding these substitutions in a ∆algU strain. We were able to delete mucA from these strains (Fig 4A). These results confirm that the algU alleles in the published PAK and PA103 ∆mucA strains suppress mucA essentiality. To examine the activity of these mutant proteins, we used our reporter assay. Similar to what we saw with the AlgU DNA-binding mutants, we found that strains carrying these AlgU substitutions had reduced sigma factor activity relative to that of strains carrying wild-type AlgU (Fig S4B). We note that our reporter assay does not appear to be very sensitive to low levels of AlgU activity. Both the published PAK and PA103 ∆mucA strains are mucoid (Intile et al., 2014, Jones et al., 2010), suggesting that AlgU A58T and E46G are not completely inactive. While we cannot distinguish this low level of activity from the null case, our results show that nevertheless these AlgU mutants have significantly less transcriptional activity than the wild-type protein (Fig S4A,B).

To identify additional suppressors of mucA essentiality, we used the ∆mucA attTn7::P_mabAD-mucA strain. This strain was not viable when grown in the absence of rhamnose, but natural revertants arose at a frequency of less than 1 in 10⁹ colony forming units. We sequenced the
algU gene in 25 of these revertants, since our work shows that AlgU mutants can suppress mucA essentiality (Fig 4A). All 25 isolates contained mutations in algU that are expected to encode for sigma factors with reduced activity (Fig 4B, Table S2). There were 10 revertants with deletions or nonsense mutations of algU, encoding either no product or a truncated product completely lacking Region 4 of the sigma factor. There were 6 revertants that contained multi-base pair duplications in algU that would lead to the insertion of 3 or 4 amino acids in Region 2 helix 3 of the sigma factor. There were 9 revertants containing missense mutations, 8 of which were unique, encoding the following substitutions: D18G, A21V, Y29C, A47T, D49G, Y59C, N81D, and R174G. Using a model of $\sigma^E$ in complex with the RNAP core and the promoter element, we expect these insertions and substitutions to affect sigma factor folding, RNAP core interactions, or promoter element interactions (Fig S5). To determine the effect of these 25 mutations on AlgU activity, we then tested AlgU function using our algD reporter assay in these natural revertants. As expected, we saw that all 25 revertants had much lower AlgU activity than the parental strain upon induction of envelope stress with D-cycloserine (Fig S4C). Together, these data strongly suggest that algU mutations that encode for a protein with reduced transcriptional activity allow P. aeruginosa to survive in the absence of mucA.

Overexpression of algU in the absence of mucA is lethal

Collectively, our results suggest that mucA essentiality is due to unregulated AlgU activity. We therefore reasoned that overexpression of algU should be lethal. Supporting this hypothesis, the toxic effects of algU overexpression have been previously noted (Cross et al., 2020, Hershberger et al., 1995, Schurr et al., 1994). However, studies have shown that algU can be overexpressed in wild-type P. aeruginosa (Qiu et al., 2008, Schulz et al., 2015). It is important to note that these wild-type strains contain mucA, which is positively regulated by AlgU. We therefore examined the effect of overproducing AlgU in WT, $\Delta$algU, and $\Delta$algU $\Delta$mucA strains, using a chromosomally integrated arabinose-inducible algU. Of note, the $\Delta$algU and $\Delta$algU
ΔmucA strains lack the positive feedback of AlgU on its own expression, and the expression of 
algU is completely dependent on the inducer. Our results show that in the absence of inducer 
when AlgU is not overexpressed, all three strains had similar growth rates (Fig 5A). In the 
presence of 1% arabinose (i.e. high expression of algU), all three strains had a growth defect. 
However, in comparison to the other two strains, the strain lacking mucA failed to grow at all in 
the presence of this high inducer concentration.

To determine if the ΔalgU ΔmucA attTn7::ParaBAD-algU strain can grow under lower levels of algU 
induction, we tested a range of inducer concentrations (Fig 5B, Table S3). We found that 
although there was a growth defect, ΔalgU ΔmucA attTn7::ParaBAD-algU was able to grow in the 
presence of 0.1% arabinose, suggesting that this strain was able to tolerate low levels of algU 
induction. Furthermore, as expected, the drop in growth rate in comparison to the no arabinose 
condition was statistically larger for the ΔalgU ΔmucA attTn7::ParaBAD-algU than for the other two 
strains (N=3, p < 0.05, ANOVA with post hoc Tukey HSD), since this strain lacks the ability to 
produce any MucA to reduce AlgU activity. Together, these results strongly suggest that in the 
absence of mucA, while a certain level of AlgU activity is tolerated, high AlgU activity is fatal to 
the cell.

The above experiments were performed using only one medium (LB). To determine if 
overexpression of algU in the absence of mucA is lethal under other nutrient conditions, we 
determined the growth rate of the ΔalgU ΔmucA attTn7::ParaBAD-algU strain in other media. 
Similar to the results in LB (Fig 5), ΔalgU ΔmucA attTn7::ParaBAD-algU strain failed to grow in the 
presence of 1% arabinose for the three other media we tested (Fig S6), suggesting that AlgU 
overproduction is toxic independent of the nutrient conditions.
Expression of rpoD can rescue lethality of algU overexpression.

It has been suggested that AlgU competes for RNAP binding with RpoD, the essential primary sigma factor (Yin et al., 2013). If high concentrations of AlgU outcompetes RpoD for RNAP, this could reduce the expression of essential housekeeping genes and lead to toxicity in AlgU-overexpressing cells. If this is true, we hypothesized that overexpressing rpoD with algU could ameliorate the growth defect of high algU expression in the ΔalgU ΔmucA attTn7::P_{araBAD}algU strain. We inserted into this strain a copy of rpoD under the same arabinose-inducible promoter. We chose to test growth in the presence of 2% arabinose, since this new strain contains two arabinose-inducible promoters, in contrast to the strains described above in Fig 5. Growth with 2% arabinose caused a growth defect for all strains tested, even in a strain lacking an ectopic arabinose-inducible gene (Fig 6). As expected from our results (Fig 5), ΔalgU ΔmucA attTn7::P_{araBAD}algU failed to grow in the presence of 2% arabinose. This lethality defect, however, was rescued in the ΔalgU ΔmucA attTn7::P_{araBAD}algU attCTX::P_{araBAD}rpoD strain. Furthermore, the growth rate of this strain in the presence of arabinose was indistinguishable from that of ΔalgU ΔmucA and ΔalgU ΔmucA attCTX::P_{araBAD}rpoD (Table S4). Since increasing RpoD levels rescued the lethality associated with high AlgU levels, these results suggest that in the absence of MucA, the ensuing sigma factor competition of AlgU with RpoD for RNAP contributes to the viability defect, and that MucA may be required to rein in AlgU in order to reduce the interference with the essential function of RpoD.

DISCUSSION

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

Our data point to the negative regulation of AlgU being necessary for cell survival in *P. aeruginosa* (Fig 7). Under non-stress conditions, AlgU is bound to and inhibited by a full-length MucA. Under envelope stress or in strains containing *mucA* mutations, the anti-sigma factor is cleaved. We propose that while this cleaved cytosolic form of MucA does not inhibit AlgU to the same extent as the full-length protein, it is still able to interact with and inhibit AlgU to some degree. Although AlgU is active under such conditions, because *mucA* is positively regulated by AlgU, this negative feedback allows the cell to keep AlgU activity under control. In comparison, in the absence of MucA, this ability to control AlgU is lost. We propose that the strong positive feedback of AlgU on its own expression is what leads to cell death and *mucA* essentiality.

Supporting this model are several lines of evidence. First, *mucA* essentiality is rooted in its interaction with *algU* (Figs 1 and 3), strongly suggesting that the ability of MucA to inhibit AlgU is required for viability. Second, deletion of AlgU-regulated genes, including those encoding the well-characterized transcription factors AlgB (Wozniak & Ohman, 1994), AlgR (Martin *et al.*, 1994, Wozniak & Ohman, 1994), and AmrZ (Wozniak *et al.*, 2003), do not allow for *mucA* deletion (Table S1), supporting that *algU* itself is the lethal component of the AlgU regulon. Third, AlgU mutants can suppress *mucA* essentiality, and all 25 natural revertants that we screened contained mutations in *algU* (Fig 4). The strains carrying these mutations had decreased AlgU transcriptional activity (Fig S4), supporting the idea that decreasing the positive feedback of AlgU on its own expression allows the cell to survive in the absence of MucA. Lastly, overexpression of *algU* leads to a growth defect, which is lethal at high levels in the absence of *mucA* (Fig 5) and can be rescued by overexpression of *rpoD* (Fig 6), suggesting that sigma factor competition between AlgU and RpoD may be partially responsible for lethality in the absence of MucA.
While our data supports our model that the AlgU-regulated gene critical for mucA essentaility is algU itself, we cannot exclude the possibility that the regulation of another single gene or a combination of genes within the AlgU regulon is responsible for the mucA requirement. This possibility, however, seems less likely for two reasons. First, all the revertants we tested that could grow in the absence of mucA expression contained algU mutations (Table S2). If another single AlgU-regulated gene were responsible for mucA essentaility, we would have expected to find a revertant with such a mutation. Second, we were unable to delete mucA from a strain lacking three AlgU-regulated transcription factors (Table S1). Genes regulated by AlgB, AlgR, or AmrZ can account for approximately fifty percent of the AlgU regulon (Huang et al., 2019, Jones et al., 2014, Leech et al., 2008, Schulz et al., 2015), suggesting that it is unlikely that deleting a combination of AlgU-regulated genes can alleviate the necessity for mucA. Nonetheless, we are actively searching for non-algU suppressors of mucA essentaility.

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

Our work strongly suggests that lethality in the absence of mucA is caused by unchecked AlgU activity (Figs 5 and 6), in agreement with previous studies suggesting that overproduction of AlgU is toxic (Cross et al., 2020, Hershberger et al., 1995, Schurr et al., 1994). Our data suggests that sigma factor competition may play a role in this lethality (Fig 6). Competition between AlgU and the primary sigma factor RpoD has been previously suggested (Yin et al., 2013). Overexpression of RpoD suppresses alginate biosynthesis in a mucoid CF isolate. Additionally, the activity of AlgU increases in a mutant with decreased RpoD activity (Yin et al., 2013). Expanding to other organisms, in B. subtilis, the lethality defect associated with deleting yhdL, which encodes for an anti-sigma factor, can be rescued via overproduction of the primary sigma factor SigA, similar to our results (Fig 6). Zhao and colleagues suggest that the lethality of this strain is due to the YhdL-cognate sigma factor, SigM, outcompeting SigA for RNAP (Zhao et al., 2019). Based on these findings, and our data showing that RpoD can rescue the lethality of high AlgU levels (Fig 6), we speculate that sigma factor competition may be involved in other instances where anti-sigma factors that regulate cell envelope functions have been found to be essential.
The interaction of MucA and AlgU is of interest, as mucA mutations are common in clinical CF isolates of *P. aeruginosa* (Boucher *et al.*, 1997, Candido Cacador *et al.*, 2018, Ciofu *et al.*, 2008, Martin *et al.*, 1993, Pulcrano *et al.*, 2012). Our results (Fig 4) agree with the literature showing that mucoid isolates with mucA mutations revert to a non-mucoid state via changes to algU when the cells are grown under standard laboratory conditions (Ciofu *et al.*, 2008, DeVries & Ohman, 1994, Sautter *et al.*, 2012, Schurr *et al.*, 1994). While these algU secondary site mutations are found in non-mucoid CF isolates with mucA mutations, such isolates are detected at a lower frequency (Ciofu *et al.*, 2008). As suggested by Ciofu and associates, this may be due to the importance of AlgU for the survival of *P. aeruginosa* in the CF lung environment, suggesting that reducing AlgU activity may increase *P. aeruginosa* eradication from the CF airway. Based on our data and the published literature, we propose that the MucA-AlgU interaction may serve as a good therapeutic target. Our results show that destabilizing the MucA-AlgU interaction results in bacterial cell death or mutations in algU that likely result in reduced sigma factor activity and reduced mucoid conversion, of which either outcome could be beneficial in the treatment of *P. aeruginosa* CF lung infections.

**METHODS AND MATERIALS**

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

**Allelic exchange assay.** The protocol, depicted in Fig S1, was modified from (Hmelo *et al.*, 2015). Briefly, after introduction of the mucA deletion vector, merodiploids were selected on semi-solid VBMM (Vogel & Bonner, 1956) with 60 mg/L gentamicin. Using OMS118 and OMS119, PCR was performed on at least six isolates to confirm that the presence of both the...
wild type and deletion alleles of \textit{mucA}. Confirmed merodiploids were then individually streaked on NSLB (10 g/L tryptone, 5 g/L yeast extract) with 10% sucrose semi-solid media for counterselection. PCR was performed on eight colonies per merodiploid, using OBT601 and OBT602 to determine the resolution to either the WT or deletion allele.

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

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

\textbf{Natural revertant assay.} \textit{P. aeruginosa ΔmucA attTn7::P_{rhaBAD}-mucA} was grown in LB with 0.05% rhamnose at 37°C with shaking to an \(OD_{600}\) of 1. Cells were washed and plated on semi-solid LB plates without rhamnose. Plates were incubated for 24-48 h at 37°C. For isolates that grew on LB without rhamnose, \textit{algU} was Sanger sequenced.
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AUTHOR CONTRIBUTIONS

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

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

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

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

* p < 0.0001, Fischer's exact test
**FIGURE LEGENDS**

**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 ΔalgD strains tested for mucA essentiality in (B). MucA aa 1-143 lacks the MucB binding domain and is the product of the common mucA22 allele. MucA 1-110 lacks the entire periplasmic domain. MucA aa 1-75 contains most of the AlgU binding domain, while shorter truncations contain only parts of the AlgU binding domain (MucA aa 1-62, 1-50, 1-40, and 1-24). Green, AlgU binding domain (AlgU 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 (ΔmucA, 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.

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

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

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

(A) Four residues of MucA make greater than one predicted hydrogen bond with AlgU. The MucA-AlgU co-crystal structure (PDB 6IN7; Li et al., 2019) with the cytosolic domain of MucA (aa 1-78; red) and Regions 2 and 4 of AlgU (green, AlgU2; yellow, AlgU4) 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\textsuperscript{1-75}) and AlgU was fused to the Gal4 activation domain (AD-AlgU). Interaction of MucA and AlgU led to lacZ expression. Beta-galactosidase activity (in Miller units) was used as a proxy for the protein interaction strength. WT, wild-type protein sequence; --, no fusion protein included; hash, broken y-axis; error bars, SEM (N=3); letters, statistical groups with the different letters representing statistically different groups (p < 0.01; biological triplicate with technical quadruplicates; ANOVA with post-hoc Tukey HSD). (C) Frequency of observed isolates resolving to the endogenous wild-type mucA allele (WT, black) or the deletion allele (\(\Delta\)mucA, white) in the allelic exchange assay, using PAO1 \(\Delta\)algD attTn7::P\textsubscript{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.

Figure 4. 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 (\(\Delta\)mucA, white) in the allelic exchange assay, using PAO1 \(\Delta\)algU attTn7::P\textsubscript{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 \(\Delta\)mucA attTn7::P\textsubscript{rhaBAD-mucA} on media lacking rhamnose. Blue rectangles, multi-base pair deletions; left arrow, deletion extends into the promoter; blue triangles, single base pair deletions; black triangles, nonsense mutations;
white triangles, duplications resulting in 3 or 4 amino acid insertions; red triangles, missense mutations. See Table S2 for a full description of the algU mutations.

Figure 5. 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), ΔalgU (blue diamonds), and ΔalgU ΔmucA (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, 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 attTn7::P_{araBAD}-algU grown in the absence of arabinose). Strains were grown in LB with 0%, 0.1%, 0.25%, and 1% arabinose ([Ara]). Error bars, SEM (N=3). Asterisk, statistically different from the same strain grown in the absence of arabinose (p < 0.05, N = 3, ANOVA with post-hoc Tukey HSD). See Table S3 for full statistical comparisons.

Figure 6. Lethality caused by algU overexpression can be rescued via rpoD expression.

Growth rate of indicated strains grown in LB with (+) or without (−) 2% arabinose (relative to PAO1 ΔalgU ΔmucA grown in the absence of arabinose). Error bars, SEM (N=3). Asterisk, statistically different from the same strain grown in the absence of arabinose (p < 0.05, N = 3, ANOVA with post-hoc Tukey HSD). See Table S4 for full statistical comparisons.

Figure 7. Unchecked AlgU activity in the absence of MucA leads to bacterial cell death.

Under conditions of no envelope stress (left), the full-length MucA (red) binds strongly to AlgU (green star), leaving very little free AlgU to interact with the RNAP (grey oval). Therefore, the AlgU regulon (green arrow) is in the “off” state (red X). Under conditions of envelope stress or in
strains containing mucA mutations that lead to a truncated product (middle), a cleaved cytosolic form of MucA is produced. This form can still interact with and inhibit AlgU. However, the strength of the interaction is weaker than with the full-length MucA, allowing for a pool of free AlgU that can then bind to and recruit RNAP to the promoters of its regulon. Under such conditions, the AlgU regulon, which includes algU and mucA, is activated and in the “on” state (green up arrows). However, because of the feedback on MucA, negative regulation of AlgU activity is still present in the cell. In the absence of mucA (right), there is no MucA to bind to and regulate AlgU. All of the AlgU is free to interact with the core RNAP. This leads to high expression of the AlgU regulon and overproduction of AlgU itself. Under such strong positive feedback and in the absence of the negative regulator, the unchecked activity of AlgU leads to cell death.
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 ΔalgD strains tested for mucA essentiality in (B). MucA aa 1-143 lacks the MucB binding domain and is the product of the common mucA22 allele. MucA 1-110 lacks the entire periplasmic domain. MucA aa 1-75 contains most of the AlgU binding domain, while shorter truncations contain only parts of the AlgU binding domain (MucA aa 1-62, 1-50, 1-40, and 1-24). Green, AlgU binding domain (AlgU BD); blue, the transmembrane domain (TM); and purple, the MucB binding domain (MucB BD).

(B) Frequency of observed isolates resolving to the endogenous wild-type mucA allele (WT, black) or the deletion allele (ΔmucA, 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.
Figure 2. Depletion of MucA results in cell death.
Viable colony counts of PAO1 ΔmucA attTn7:: rhaSR-P_{rhabAD-mucA-10His over time in (A) LB, (B) PIB, (C) SCFM and (D) VBMM with (+ rha; red circles) or without (– rha; gray squares) 0.05% rhamnose. Viable colony counts after incubation in the indicated condition and time was determined by plating the cells on LB with 0.05% rhamnose to allow cells to recover and grow. Hash, broken y-axis; error bars, SEM (N=3). Asterisk, statistically different from that at the same time point grown in the presence of rhamnose (p < 0.01, N=3, mixed model ANOVA with post-hoc Bonferroni test).
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(C) Frequency of observed isolates resolving to the endogenous wild-type mucA allele (WT, black) or the deletion allele (∆mucA, white) in the allelic exchange assay, using PAO1 ∆algD attTn7::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.
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Figure 6. Lethality caused by *algU* overexpression can be rescued via *rpoD* expression. Growth rate of indicated strains grown in LB with (+) or without (−) 2% arabinose (relative to PAO1 ∆*algU* ∆*mucA* grown in the absence of arabinose). Error bars, SEM (N=3). Asterisk, statistically different from the same strain grown in the absence of arabinose (p < 0.05, N = 3, ANOVA with post-hoc Tukey HSD). See Table S4 for full statistical comparisons.
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