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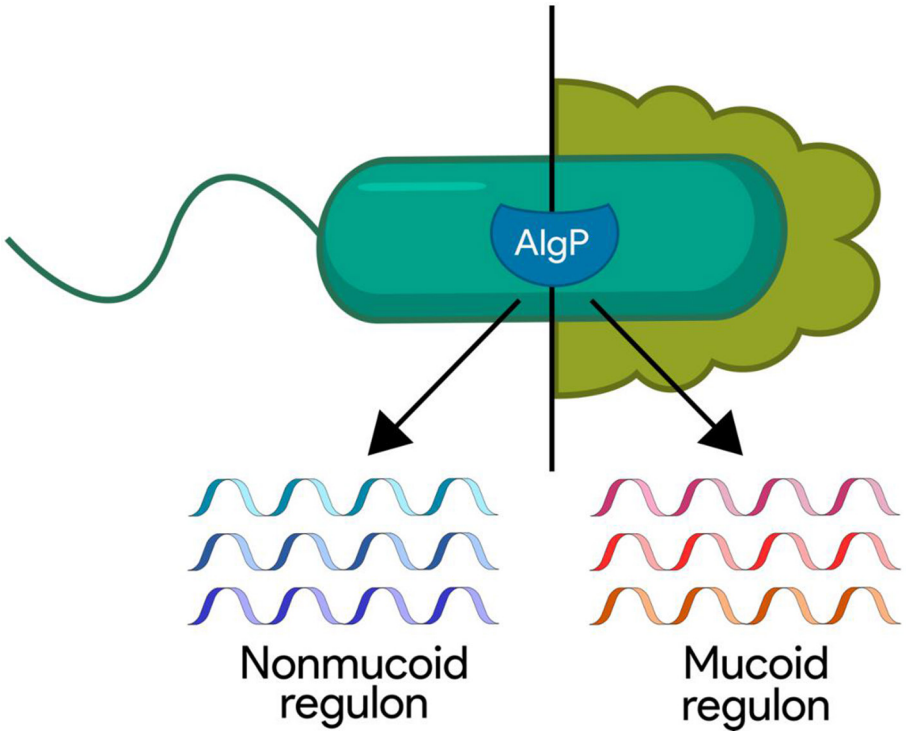
EDITOR'S CHOICE

The histone-like protein AlgP regulon is distinct in mucoid and nonmucoid *Pseudomonas aeruginosa* and does not include alginate biosynthesis genes

Ashley R. Cross[1,2], Erika E. Csatary[2,3], Vishnu Raghuram[1,2], Frances L. Diggle[2,4,5], Marvin Whiteley[2,4,5], William M. Wuest[2,3] and Joanna B. Goldberg[1,2,*]



Graphical abstract

Transition of *Pseudomonas aeruginosa* from a nonmucoid to a mucoid phenotype, characterized by the overproduction of alginate, correlates with the establishment of a chronic lung infection in people with cystic fibrosis. The histone-like protein AlgP has been proposed to regulate alginate production. Here, we define the AlgP transcriptome in both nonmucoid and mucoid *P. aeruginosa* and find that the AlgP regulon is distinct in each background, but that alginate genes are not a significant component.

Abstract

The opportunistic bacterial pathogen *Pseudomonas aeruginosa* causes acute and chronic infections that are notoriously difficult to treat. In people with cystic fibrosis, *P. aeruginosa* can cause lifelong lung infections, and isolation of mucoid *P. aeruginosa*, resulting from the overproduction of alginate, is associated with chronic infection. The histone-like protein AlgP has previously been implicated in the control of alginate gene expression in mucoid strains, but this regulation is unclear. To explore AlgP in further detail, we deleted *algP* in mucoid strains and demonstrated that the deletion of *algP* did not result in a nonmucoid phenotype or a decrease in alginate production. We showed that the *algP* promoter is expressed by both the nonmucoid strain PAO1 and the isogenic mucoid strain PDO300, suggesting that there may be genes that are differentially regulated between these

strains. In support of this, using RNA sequencing, we identified a small AlgP regulon that has no significant overlap between PAO1 and PDO300 and established that alginate genes were not differentially regulated by the deletion of *algP*. Of note, we found that deleting *algP* in PAO1 increased expression of the nitric oxide operon *norCBD* and the nitrous oxide reductase genes *nosRZ* and subsequently promoted growth of PAO1 under anaerobic conditions. Altogether, we have defined a narrow regulon of genes controlled by AlgP and provided evidence that alginate production is not greatly affected by AlgP, countering the long-standing premise in the field.

Pseudomonas aeruginosa is an opportunistic pathogen with the ability to persist in many different environments due to a large genome encoding an extensive repertoire of virulence factors, toxins and exopolysaccharides [1]. *P. aeruginosa* causes a variety of acute and chronic illnesses and is one of the most common bacterial pathogens colonizing the lungs of people with cystic fibrosis (CF) [2–4]. People living with CF have impaired lung function and a reduced ability to clear inhaled bacteria that can result in the establishment of chronic infections [5–8]. During long-term respiratory infection in CF patients, *P. aeruginosa* can accumulate genetic mutations that rewire regulatory circuits to be better suited to this environment. Some mutations result in dramatic phenotypic changes, such as the transition from a nonmucoid to a mucoid phenotype. Mucoid conversion is characterized by the overproduction of the exopolysaccharide alginate [9–13]. The emergence of mucoid colonies correlates with the establishment of a chronic infection, increased inflammation and worsening lung disease [14–16].

Transcription of the alginate biosynthesis operon is highly regulated and has been well studied [12, 17–22]. Histone-like proteins are unique transcriptional regulators that control virulence expression in *P. aeruginosa* and regulate alginate production [23–25]. One such histone-like protein is AlgP (also called AlgR3 and Hp-1). Originally, *algP* was recognized by its ability to partially restore a nonmucoid clinical *P. aeruginosa* isolate to a mucoid phenotype by providing *algP* in trans. However, other studies showed that this complementation required the expression of an additional gene, *algQ* [26, 27], suggesting that the nonmucoid phenotype of the clinical isolate may not be due to *algP* disruption. Further, unlike other known regulators of alginate biosynthesis that are highly expressed in only mucoid strains, *algP* mRNA has been shown to be produced by both nonmucoid and mucoid strains from a sigma 70-like promoter [26, 28]. Sequence analysis of *algP* revealed that the carboxyl-terminal domain contains multiple KPAA amino acid repeat units and that AlgP is highly basic, resulting in a high affinity for DNA [26, 27, 29]. Konyecsni and Deretic purified a short repeat peptide from the lysine-rich region of *algP* and demonstrated that this peptide bound multiple upstream regions of the alginate promoter using a gel mobility shift assay [29]. While AlgP had long been described for its ability to bind to the alginate promoter, the AlgP peptide used for these studies also bound nonspecific DNA, indicating a lack of specificity [29]. Therefore, the exact role for AlgP in the regulation of the mucoid phenotype is still unclear, and the goal of this work was to re-examine the relationship between AlgP and alginate.

Based on published data, we hypothesized that deletion of *algP* in the mucoid *P. aeruginosa* PAO1 laboratory strain PDO300 strain would result in a nonmucoid phenotype due to decreased alginate production [11, 27, 29]. To test this hypothesis, we deleted *algP* in PDO300 and assessed alginate levels. When visualized on an agar plate, PDO300 Δ*algP* was mucoid and was indistinguishable from PDO300 (Fig. 1a). We considered that small differences in alginate production might not be visible, so we quantified how much alginate was produced by each strain and observed no significant difference in the amount of alginate produced by PDO300 and PDO300 Δ*algP* in a minimal MOPS succinate medium (Fig. 1a) as well as nutrient-rich lysogeny broth (LB) (Fig. S1, available in the online version of this article).

Since the original role for AlgP was described in a mucoid clinical isolate [29], we reasoned that the regulation of alginate by AlgP might be dependent on strain background. To attempt to reconcile our findings with those in the literature, we examined whether deletion of *algP* in a mucoid clinical isolate would render this strain nonmucoid. Therefore we deleted *algP* from a mucoid strain, 2192, isolated from a person with CF [30]. 2192 Δ*algP* was still mucoid and produced alginate at levels similar to wild-type 2192 (Figs 1b and S1). We also found that the 2192 strains produced 40 times as much alginate as PDO300 when grown in MOPS succinate medium. However, in LB, the strains made similar levels of alginate

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Author affiliations: [1]Division of Pulmonary, Allergy and Immunology, Cystic Fibrosis and Sleep, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, USA; [2]Emory+Children's Center for Cystic Fibrosis and Airway Disease Research, Emory University School of Medicine, Atlanta, GA, USA; [3]Department of Chemistry, Emory University, Atlanta GA, USA; [4]School of Biological Sciences, Georgia Institute of Technology, Atlanta GA, USA; [5]Center for Microbial Dynamics and Infection, Georgia Institute of Technology, Atlanta GA, USA.
*Correspondence: Joanna B. Goldberg, joanna.goldberg@emory.edu
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Abbreviations: ANOVA, analysis of variance; CF, cystic fibrosis; c.f.u., colony-forming unit; h, hour; LB, lysogeny broth; MOPS, 3-(*N*-morpholino) propanesulfonic acid; ns, not significant; RNA, ribonucleic acid; SD, standard deviation.
Three supplementary figures and six supplementary tables are available with the online version of this article.

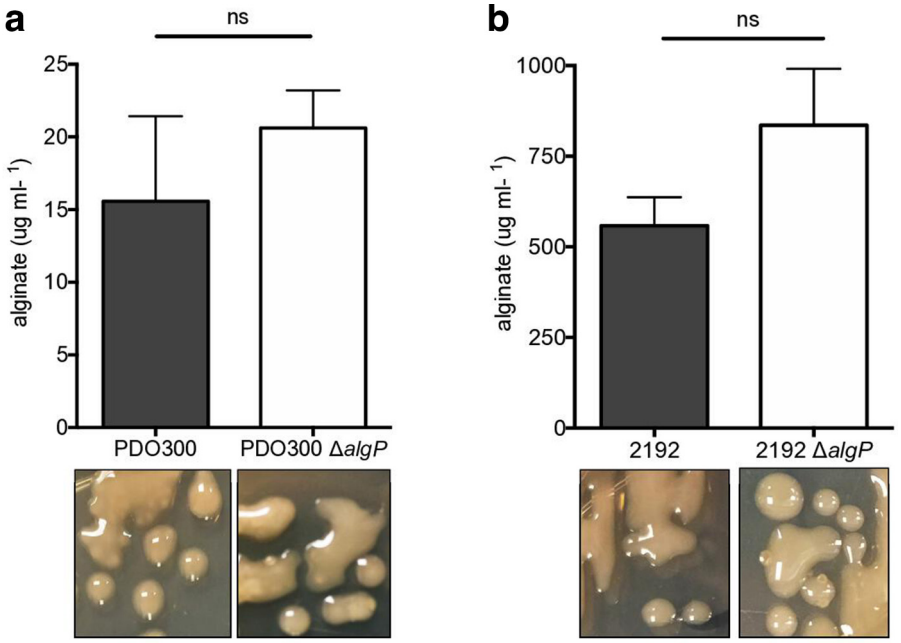

Fig. 1. Deletion of *algP* from mucoid strains does not decrease alginate levels. Alginate was purified from 24 h cultures grown in MOPS succinate minimal media and quantified using a carbazole assay. Deletion of *algP* from mucoid laboratory strain PDO300 (a) or mucoid cystic fibrosis isolate 2192 (b) does not result in decreased alginate production. Significance was determined using an unpaired *t*-test. Error bars represent the SD of three biological replicates. A picture of the mucoid phenotype of each strain grown on lysogeny agar (LA) is shown below each bar. NS, not significant.

(Fig. S1). In all cases, the Δ*algP* mutants produced similar amounts of alginate to the wild-type parental strains, whether grown on rich media or in minimal media.

Through mapping of the *algP* transcriptional start site, Konyecsni and Deretic observed that *algP* mRNA was produced by nonmucoid and mucoid isolates [26]. However, this expression was not quantified. To expand on this observation, we measured *algP* promoter activity in mucoid PDO300 and the isogenic nonmucoid strain PAO1. We constructed a transcriptional *lacZ* reporter by fusing the predicted *algP* promoter sequence to a promoterless *lacZ* (P_{algP}–*lacZ*) and inserted this construct, in single copy, into the chromosome of PDO300 and PAO1. Using ß-galactosidase assays, we compared expression of the *algP* promoter in each strain. We found that, while the *algP* promoter was indeed expressed in both PAO1 and PDO300, there was a 40% decrease in PDO300 *algP* promoter activity compared to PAO1 (Fig. S2).

Over 80% of mutations leading to mucoid conversion occur in the *mucA* gene encoding an anti-sigma factor [9–13]. MucA is a critical negative regulator for maintaining control of alginate production. When MucA is inactivated, it no longer binds the sigma factor AlgT, which is then free to continuously direct transcription of the alginate biosynthesis operon as well as about 300 other genes [31, 32]. Since a large number of genes are differentially expressed between PAO1 and PDO300, and the *algP* promoter is expressed in both strains, albeit at different levels, we hypothesized that AlgP could regulate different genes in these strain backgrounds.

To determine the AlgP regulon, including any potential alginate biosynthesis genes, we used RNA sequencing to assess the effect of *algP* deletion on gene expression in PAO1 and PDO300. We first confirmed that there were no growth differences in either PAO1 or PDO300 when *algP* was deleted (Fig. S3) and then isolated mRNA from PAO1, PAO1 Δ*algP*, PDO300 and PDO300 Δ*algP* at exponential phase. We found that 26 genes were differentially regulated between PAO1 and PAO1 Δ*algP* (Tables S1 and S4). A similar number of genes were identified when comparing PDO300 and PDO300 Δ*algP* with 19 differentially regulated genes (Tables S2 and S5). In PAO1 and PDO300, AlgP acts as both a positive and negative regulator of gene expression. Of particular interest, none of the alginate biosynthetic genes were found to be differentially regulated due to the deletion of *algP*. As an internal control, the differences we found in gene expression between PAO1 and PDO300 were similar to those previously reported and included genes for alginate biosynthesis (Table S6) [33]. There was no overlap between the AlgP regulon of the nonmucoid strain compared to the mucoid strain. While we have not reconciled why this is the case, the mechanisms responsible could be (i) that different co-factors, which are only expressed in either a nonmucoid or mucoid background, may be required for AlgP function or (ii) that AlgP may be modified in one strain background compared to the other, resulting in different DNA-binding preferences.

Of note, the expression of genes for the operons *norCBD* and *nosRZ* controlling nitric oxide and nitrous oxide reductase

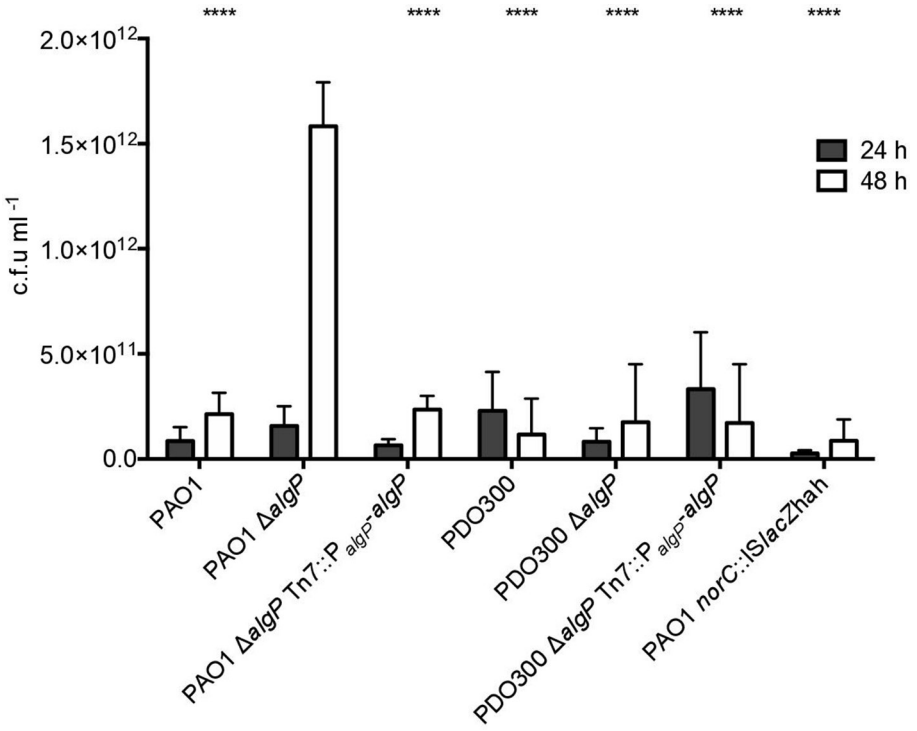

Fig. 2. Deletion of *algP* increases the survival of PAO1 during anaerobic growth. Strains were grown anaerobically in LB supplemented with potassium nitrate and c.f.u. were plated at 0, 24 and 48 h. Growth was calculated as final c.f.u. ml^{-1}–initial c.f.u. ml^{-1}. Significance was determined using a two-way analysis of variance (ANOVA) with Sidak's multiple comparisons analysis. Error bars represent the SD of three biological replicates. ****, $P<0.0001$.

enzymes, respectively, was significantly increased in expression in PAO1 when *algP* was deleted. Previous studies have shown that expression of nitric oxide reductase and nitrous oxide reductase is necessary for the denitrification and growth of *P. aeruginosa* during anaerobic respiration and a PAO1 *norCB* mutant has a decreased growth rate under anaerobic conditions compared to wild-type PAO1 [34]. Based on this result, we hypothesized that PAO1 Δ*algP,* which has increased *norCB* and *nosRZ* expression*,* would grow better anaerobically compared to PAO1. To test this, we grew PAO1 and PAO1 Δ*algP* anaerobically with nitrate as a terminal electron acceptor and calculated colony forming units (c.f.u.) after 24 and 48 h of growth. In support of our hypothesis, deletion of *algP* significantly increased the survival of PAO1 after 48 h of anaerobic growth (Fig. 2). Complementation of *algP* in single copy under control of the native *algP* promoter (PAO1 Δ*algP* Tn7::P_{algP}-*algP*) returned growth yields to wild-type levels. We also monitored growth of PDO300 and PDO300 Δ*algP*; since there is no difference in *norCB* and *nosRZ* levels between these two strains, we hypothesized that deletion of *algP* would result in no growth difference under anaerobic conditions. Consistent with the RNA sequencing results, there was no difference in growth of PDO300, PDO300 Δ*algP*, and PDO300 Δ*algP* Tn7::P_{algP}-*algP* (Fig. 2). We used a PAO1 *norC* transposon mutant (PAO1 *norC*::IS*lacZ*hah) as a control for a strain with slow growth under anaerobic conditions. These results suggest that down regulation of *algP* in PAO1 may promote survival during an initial infection where *P. aeruginosa* must quickly adapt to growth in low-oxygen environments likely present in the CF lung [35, 36].

Recently published data implicate AlgP in the regulation of the small molecules pyoverdine and pyocyanin as well as biofilm formation [37, 38], but we did not find genes for the biosynthesis or transport of these molecules or any genes linked to biofilm formation in our RNA sequencing results. Because we only tested gene expression in MOPS succinate during exponential phase, it is still possible that other genes are regulated by AlgP at different growth phases and during different growth conditions. However, deletion of *algP* does not alter alginate production during stationary phase in MOPS succinate minimal media or in LB-rich media, or alginate gene expression during exponential growth in MOPS succinate minimal media (Figs 1 and S1, Tables S1, S2 and S4–S6).

Although AlgP was one of the first alginate regulators to be discovered, its role is still not fully understood. While it appears that the most accepted way for *P. aeruginosa* to rewire gene expression during chronic infection is through *mucA* mutation and overexpression of AlgT [31], this may not be the only way. Another way could be to alter the gene targets of a regulator that is already expressed, such as we see with AlgP. Our results show that there are few *algP*-regulated genes, that there is no significant overlap in the PAO1 and PDO300 AlgP

regulons, and, most surprisingly, that the alginate biosynthetic genes are not affected by deletion of *algP*. Altogether, these results suggest that AlgP is not a major regulatory factor controlling alginate production, helping clarify the existing literature surrounding AlgP. Still, since we only saw a small subset of genes significantly regulated by AlgP and most of the regulation we see is small, AlgP might be more involved in fine-tuning gene expression rather than regulating genes on or off. A thorough understanding of the multifaceted regulatory systems in *P. aeruginosa* will provide knowledge on how this complex opportunistic pathogen adapts to many environments, causes infections and persists.

SUPPLEMENTARY MATERIAL

Supplementary figures and tables (Tables S1–S3) and a detailed list of methods can be found in the Supplementary Material. Tables S4–S6 are Excel files. The RNA sequencing counts are available on GEO under accession number GSE148593.

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Author contributions
A.R.C. performed research and wrote the manuscript; A.R.C., E.E.C., F.L.D. and M.W. performed RNA sequencing; A.R.C. and V.R. analysed data; E.E.C., V.R., F.L.D., M.W., W.M.W. and J.B.G. helped design research and provided feedback.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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