Alginate-regulating genes are identified in the clinical cystic fibrosis isolate of *Pseudomonas aeruginosa* PA2192

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ABSTRACT

Cystic fibrosis (CF) is a genetic disorder that leads to a buildup of mucus in the lungs ideal for bacterial colonization. When *Pseudomonas aeruginosa* enters the CF lung, it undergoes a conversion from nonmucoid to mucoid; colonization by a mucoid strain of *P. aeruginosa* greatly increases mortality. The mucoid phenotype is due to the production of alginate. The regulator of alginate production is the AlgT/U sigma factor. The observed phenotypic conversion is due to a mutation in the *mucA* gene coding for an anti-sigma factor, MucA, which sequesters AlgT/U. This mucoid phenotype is unstable when the strains are removed from the lung as they acquire second-site mutations. This *in vitro* reversion phenomenon is utilized to identify novel genes regulating alginate production. Previously, second-site mutations were mapped to *algT/U*, *algO*, and *mucP*, demonstrating their role in alginate regulation. Most of these studies were performed using a non-CF isolate. It was hypothesized that second site mutations in a clinical strain would be mapped to the same genes. In this study, a clinical, hyper-mucoid *P. aeruginosa* strain PA2192 was used to study the reversion phenomenon. This study found that PA2192 has a novel *mucA* mutation which was named the *mucA180* allele. Twelve colonies were sub-cultured for two weeks without aeration at room temperature in order to obtain nonmucoid suppressors of alginate production (*sap*). Only 41 *sap* mutants were stable for more than 48 hours — a reversion frequency of 3.9% as compared to ~90% in laboratory strains showing that PA2192 has a stable mucoid phenotype. This

45 phenotype was restored in 28 of the 41 *sap* mutants when complemented with
 46 plasmids harboring *algT/U*. Four of the *sap* mutants are complemented
 47 by *algO*. Sequence analyses of the *algT/U* mutants have found no mutations in
 48 the coding region or promoter leading to the hypothesis that there is another, as
 49 yet unidentified mechanism of alginate regulation in this clinical strain.

INTRODUCTION

Cystic fibrosis (CF) is the most common cause of death due to genetic disorder (1). CF is an autosomal recessive disorder caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*cftr*) gene (2-4). The most common CF mutation (~90%) is the $\Delta F508$ which is the deletion of three nucleotides leading to the loss of a phenylalanine in the 508 position, and thus, a defective protein (3). A wide range of other mutations are possible that lead to either impaired function or total loss of activity (5).

Ordinarily, CFTR functions as anion transporter (6). When mutated, normal anion flow is restricted (7) and mucus accumulates in the CF lung; resulting pulmonary failure is the foremost killer of CF patients (8, 9) The mucus buildup provides a breeding ground for many pathogenic bacterial species especially *S. aureus*, *H. influenzae*, *Pseudomonas aeruginosa*, and *Burkholderia cenocepacia* (10); the relative population of each species fluctuates over the life of the individual (11). The pathogen that rises to prominence over the life of a CF patient and is the leading cause of mortality is *P. aeruginosa* (11, 12).

P. aeruginosa expresses a multitude of virulence factors (13). The major contributor to *P. aeruginosa* virulence in patients with CF is its ability to change from the standard, non-mucoid form to the mucoid form (14, 15). Mucoid *P. aeruginosa* is considered highly virulent because patients show poor clinical outcome despite having a heightened immune response (14, 15). The mucoid

phenotype is a result of the production of a complex polysaccharide called alginate (16).

Alginate protects *P. aeruginosa* from phagocytosis, antibiotics, oxygen radicals, and the host immune response (17-23) (Leid et al., 2005). The importance of alginate in the virulence of *P. aeruginosa* has also been demonstrated in mouse models (24, 25). In mice, an alginate-overproducing strain causes aggressive polymorphonuclear leukocyte (PMN) infiltration- similar to human infection- and causes inefficient pulmonary clearance. A protracted lung infection has the potential to spread to other organs such as the spleen. These properties suggest that alginate is an important virulence factor.

Alginate biosynthesis comes at a high metabolic cost, and thus is tightly regulated (Figure 1) by an intricate system of periplasmic and inner membrane proteins (26-29). The primary regulatory unit of alginate production is a five-gene operon containing *algT/U-mucA-mucB-mucC-mucD* (30). The first gene of this operon, *algT/U*, codes for a sigma factor able to bind to RNA polymerase (RNAP), guiding it to transcribe the genes necessary for alginate production (31, 32).

Under normal circumstances, *P. aeruginosa* is non-mucoid, as is the case with the prototypic reference strain, PAO1 (33). Upon colonizing the CF lung, *P. aeruginosa* must confront the host immune system and antibiotics. The typical response is to convert to a mucoid phenotype by producing alginate. This is commonly accomplished by mutating *mucA* which codes for the anti-sigma factor to AlgT/U

(34). Ordinarily, MucA sequesters AlgT/U to the inner membrane, preventing it from directing RNAP; however, when *mucA* is mutated, AlgT/U is left free to guide RNAP (Figure 1) to transcribe the genes needed for alginate biosynthesis (27, 35). The most common *mucA* mutation (~85%) found in clinical, mucoid strains of *P. aeruginosa* is the *mucA22* allele which is the deletion of a single G in a string of five Gs resulting in a frameshift mutation and premature stop codon (34, 35).

Since alginate production is metabolically expensive, mucoid strains revert to a non-mucoid phenotype when isolated from the lung and cultured *in vitro* (Figure 2), especially when grown at low oxygen levels (36). The isolates maintain the original *mucA* mutation but revert to Alg⁻ by mutating at another gene, a second site crucial to alginate biosynthesis (32, 37). This has proven to be a highly advantageous phenomenon when it comes to determining novel genes involved in alginate regulation. Several studies have utilized this *in vitro* reversion phenomenon to map second-site mutations to genes coding for the sigma factor AlgT/U (31, 32, 37), a putative periplasmic protease AlgO (37, 38), and an inner membrane protease MucP (Delgado *et al.*, submitted).

The study by DeVries *et al.*, (1994) used the mucoid CF isolate FRD1, but the great caveat tied to many of these other studies is that they were carried out in a laboratory-generated strain, PDO300 (22). This form is an isogenic derivative of PAO1 with the addition of the *mucA22* allele in order to imitate clinical isolates

(22). The potential issue with using PDO300 is that laboratory strains cannot faithfully mirror the real-world pathogenesis of a clinical isolate (39).

The present study was undertaken to map the location of second-site mutations in a clinical isolate. The strain utilized is *P. aeruginosa* 2192 which was isolated from a CF patient in Boston who passed away from the infection (40). *P. aeruginosa* 2192 produces about 60% more alginate than PDO300 (Delgado *et al.*, submitted) and is far more stable in its mucoid phenotype (this study). We hypothesized that the non-mucoid revertants of *P. aeruginosa* 2192 would harbor second-site mutations in *algT/U*, *algO*, and *mucP* while maintaining the original *mucA* mutation. This would demonstrate the role of these genes in *P. aeruginosa* 2192 alginate regulation.

MATERIALS AND METHODS

Bacterial Strains

The *P. aeruginosa* and *Escherichia coli* strains used in this study are listed in Table 1. The *E. coli* strains were grown on Luria-Bertani (LB) media supplemented with tetracycline (Tc) at 20 µg/ml and ampicillin (Ap) at 50 µg/ml. *Pseudomonas aeruginosa* strains were grown on LB or LB/PIA plates, which is a 1:1 mixture of LB and *Pseudomonas* isolation agar. These were supplemented with Tc at 100 µg/ml or carbenicillin (Cb) at 150 µg/ml when appropriate.

Isolation of *sap* mutants

The parent strain used to isolate suppressor of alginate production (*sap*) mutants was *P. aeruginosa* 2192, a hypermucoid, clinical CF isolate possessing a *mucA* mutation (Mathee *et al.*, 2008, Delgado *et al.*, Submitted). *P. aeruginosa* 2192 was plated and grown overnight on LB/agar plates. Twelve mucoid colonies were selected and inoculated into separate tubes containing 5 ml of LB nutrient broth. These were serially cultured at 25°C without aeration for two weeks. Dilutions of each of the 12 cultures were plated daily for single colonies. The *sap* mutant colonies were frozen in 1:1 culture/skim milk at -80°C for further analysis (Table 1). At the end of two weeks, all *sap* mutants were re-streaked on to LB/PIA plates and incubated at 37°C for 24 hours followed by 25°C for 24 more hours. Only the 41 *sap* mutants that maintained a non-mucoid phenotype after 48 hours during this secondary screening were used in subsequent analyses.

Complementation assays

Complementation of the *sap* mutants was accomplished by a modified tri-parental mating protocol (41) developed during this study. The donor *E. coli* strain containing the plasmid of interest was crossed on an LB plate with the two helper *E. coli* strains, pRK600 and pRK2013 (Table 1), and the recipient, in this case, the *sap* mutants. The following day, the mating conglomeration was homogenized in LB broth, diluted, and plated on selective media (LB/PIA supplemented by Tc 100 µg/ml or Cb 150 µg/ml). Colonies were checked for a mucoid or non-mucoid phenotype at 24 and 48 hours. Each of the *sap* mutants was complemented with pCD100 (Tc resistant) which contains the *algT/U-mucA22-mucB-mucC-mucD* operon (37). Those that saw a reversion to a mucoid phenotype were then complemented by pJG293 (Tc resistant) which contains *algT/U* alone (37). The remaining *sap* mutants that were not complemented by *algT/U* were conjugated with a plasmid containing *algO* (pAlgO), and another harboring *mucP* (pMucP).

Genomic DNA isolation

Genomic DNA was isolated from each of the *sap* mutants following a standard phenol-chloroform protocol (42). Briefly, 1 ml of an overnight culture was pelleted and mixed with lysozyme and proteinase K. After incubation at 37°C for 30 minutes, a 1:1 mixture of phenol:chloroform was added and vortexed to homogeneity. After centrifugation, the top layer containing the genomic DNA was removed and mixed with ethanol to precipitate the DNA and pelleted by

centrifugation at 16,000 xg for two minutes. The ethanol was decanted and the DNA was resuspended in water and stored at -20°C.

PCR amplification

Primers for polymerase chain reaction (PCR) were designed for *algT/U*, *mucA*, *algO*, and *mucP* (Table 2; Integrated DNA Technologies Inc., Coralville, IA). Each set of primers was designed to fall about 100 bp up and downstream of the gene so that subsequent DNA sequencing would not cut off the beginning and end of each gene.

The *mucA* gene of each of the *sap* mutants as well as the gene identified in the complementation assay as containing a potential mutation were PCR amplified for sequencing. Two and a half microliters of genomic DNA was mixed with 1 µl of each primer (Table 2), 5 µl of bufferxII, 0.5 µl of HiFi Taq polymerase (Invitrogen, Carlsbad, CA) and the volume was made up to 50 µl with water. The mixture was amplified in the thermocycler with the following program: 95°C for 5 min; 95°C 30 sec, 61°C for 30 sec, 72°C for a time dependent on the amplicon size (1 min/kb) repeated for 30 cycles; 72°C for 10 min; hold at 4°C for further analysis. Products were run on a 2% agarose gel to verify amplification. PCR products were then cleaned according to a kit and standard protocol (Promega, Madison, WI).

185 **DNA sequencing**

186 Samples were sent to GeneWiz Inc. (Plainsfield, NJ) for sequencing. The *mucA*
 187 gene and the complementing genes of each *sap* mutants were sequenced.
 188 Samples were prepared according to the company's requirements. Each tube
 189 sent out was premixed with 5 µl of 5 µM primer (forward and reverse separately)
 190 and 40 ng of the PCR product.

191 **Sequence analysis**

192 Sequences were aligned using NCBI Blast, ClustalΩ, T-Coffee, LaserGene
 193 software, and Boxshade against the *P. aeruginosa* 2192 wildtype sequence (43,
 194 44) to check for mutations (45-48).

195

RESULTS

Isolation of *sap* mutants

The clinical isolate *P. aeruginosa* 2192 was grown for two weeks at 25°C without aeration. A total of 1058 colonies were analyzed during that period. Of these, 127 (12%) were nonmucoid and considered *sap* mutants. These were then plated on PIA media and allowed to grow for 48 hours to verify stability. Only 41 (3.8%) retained the Alg⁻ phenotype. Of these, 39 *sap* mutants produce alginate when the cells are at high density on a plate and they remain completely nonmucoid when they are single colonies. Two, *sap8* and *sap20*, remain completely nonmucoid indefinitely, whether in a dense community or single colony.

Complementation assays

Each of the *sap* mutants was complemented with genes previously identified as common second-site mutations (32, 37). Twenty-eight *sap* mutants (68%) were complemented by pCD100 containing the whole *algT/U* operon. These were also complemented by pJG293 containing *algT/U* alone (Table 1).

The mucoid phenotype was restored in 11 *sap* mutants (17%) when complemented by *mucP*. Similarly, *algO* successfully complemented four (10%) of the mutants that were also complemented by *mucP*.

Two *sap* mutants, *sap8* and *sap20*, were not complemented by any of the previously identified genes. When pCD100 (37) was introduced, the two mutants

failed to grow. In the presence of pJG293 (37), the strains grew and were nonmucoid.

Sequencing *mucA*

P. aeruginosa 2192 contains a mutation in the anti-sigma factor *mucA* which results in constitutive alginate production. To determine the exact mutation, the *mucA* of *P. aeruginosa* 2192 was aligned with PAO1, the common reference strain which has no mutation, and PDO300, the laboratory-generated strain containing a *mucA22* allele. *P. aeruginosa* 2192 was seen to have A343G resulting in a silent mutation, and G539T leading to a stop codon at the 180th position of the protein (Figure 4). The *mucA* genes of 10 of the *sap* mutants were aligned to *P. aeruginosa* 2192 and shown to possess the original mutations (Figure 4).

Analysis of *algT/U*, *algO*, *mucP* in *P. aeruginosa* 2192 and *sap* mutants

***algT/U*:** Alignment has shown that the *algT/U* ORF and promoter sequence is conserved between PAO1 and *P. aeruginosa* 2192 (data not shown). The *sap* mutants are also mutation free.

***algO*:** The *P. aeruginosa* 2192 *algO* sequence shows nine SNPs which all result in silent mutations. The *sap* mutants are yet to be sequenced.

***mucP*:** *mucP* of *P. aeruginosa* 2192 has two SNPs relative to PAO1; one relays a silent mutation, and the other a change of an alanine to a valine in the 313. This change is to an amino acid of similar functional group, and thus it is hypothesized

237 that the function is conserved. The *sap* mutants have not been sequenced as of
238 yet.

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DISCUSSION

Laboratory generated strains have been immensely useful in scientific research and have driven the depth of our knowledge to where it is today. However, laboratory strains, such as PDO300, will always fall short of perfectly mimicking the real-world pathogenesis of clinical isolates (39). This project has certainly confirmed the importance of utilizing a strain isolated directly from the lungs of a patient who passed away from the infection. It has also made the research eminently personal. The present study was designed to confirm the conclusions about alginate regulation drawn from studies using PDO300, as well as investigate the novelty and peculiarity of *P. aeruginosa* 2192.

***P. aeruginosa* 2912 shows a hyperstable mucoid phenotype**

Non-mucoid variants of *P. aeruginosa* 2192 were isolated in the same way as previous studies that utilized PDO300 as the parent strain (37). Studies using PDO300 saw a 90% reversion to *sap* mutants after just 48 hours of culturing at 25°C without aeration (37). In contrast, PA2192 took two weeks under the same conditions to yield even a 3.8% reversion that could be utilized in further analyses. This extended time needed to isolate non-mucoid variants is unique to *P. aeruginosa* 2192 when compared with another clinical strain as well. One study obtained mutants from FRD1, a CF isolate, in 24 hours under the same conditions (32). When compared with the PDO300 and FRD1 studies, *P. aeruginosa* 2192 has a hyperstable mucoid phenotype since it took seven and fourteen times longer

before any non-mucoid colony was isolated. It remains to be seen what the contributing factors to this hyperstability are, including the chemical makeup of *P. aeruginosa* 2192 alginate when compared with PDO300. It is interesting to speculate that the hyperstability is directly related to the clinical virulence of *P. aeruginosa* 2192.

***P. aeruginosa* 2192 possesses a novel *mucA* mutation**

Sequence analysis of *mucA* shows that PA2192 does not have the common *mucA22* allele (22) (Figure 4). Instead, it has a previously undocumented *mucA* mutation, which has been named the *mucA180* allele. Alginate production is frequently accomplished in clinical isolates by mutating the *mucA* anti-sigma factor (34). The most common mutation is the *mucA22* allele possessed by ~85% of mucoid *P. aeruginosa* strains (34, 35). It was in this light that PDO300 was constructed from PAO1 with the *mucA22* allele to imitate clinical, mucoid strains (22). The majority of *mucA* mutations are toward the 3' end of the sequence (24, 49, 50). This results in an altered or truncated C-terminus of MucA that is the end that protrudes from the inner membrane into the periplasm (Figure 4) and interacts with MucB; as a result, MucB binding is reduced or inhibited altogether (50-52). Without MucB binding, MucA is destabilized, and the AlgT/U sigma factor is released resulting in alginate production (50).

It is hypothesized that MucA180 also has a reduced interaction with MucB due to protein truncation. Further experimentation with a yeast two-hybrid system is

required to demonstrate this. It could be accomplished by designing a MucA180 bait protein and a MucB fish protein. When compared to wildtype MucA, the MucA180 two-hybrid system should show reduced transcription of the reporter gene.

***P. aeruginosa* 2192 *algT/U* shows no mutation**

The *mucA* sequence alignment of the non-mucoid variants revealed that they maintain the *mucA180* allele. Thus, the loss of alginate production is not the result of a true reversion that has restored the function of MucA, but is due to a second-site mutation, as was hypothesized.

Complementation assays have shown that a majority (68%) of the second-site mutations may be mapped to *algT/U*. Interestingly, no mutation was detected in the *algT/U* open reading frame (ORF) or in the promoter region upstream of the ORF.

This suggests that there may be a novel mechanism of alginate regulation in *P. aeruginosa* 2192 that is bypassed when AlgT/U is overexpressed. Further studies will investigate this possibility. This does not rule out the possibility that this set of revertants may harbor an entirely novel mutation.

***mucP* and *algO* are involved in *P. aeruginosa* 2192 alginate regulation**

This study also found that *mucP* was able to restore the Alg⁺ phenotype of 17% of the non-mucoid variants, indicating that second-site mutations in *mucP* were the

second most common mode of alginate repression in *P. aeruginosa* 2192. The third most common second-site mutation in alginate suppression is in *algO* (10%). Moreover, all the non-mucoid variants that were complemented by *algO* were also complemented by *mucP*, suggesting that *algO* mutations can be bypassed in *P. aeruginosa* 2192 by increasing the *mucP* copy number as previously demonstrated (Delgado *et al.*, 2018, Submitted). The exact function of AlgO has not been elaborated as yet but presumed to be a periplasmic protease (37). The *mucP* and *algO* genes in the *sap* mutants are being sequenced to confirm the mutations and find the exact region of the proteins that is mutated.

Uncomplemented non-mucoid variants possess novel second-site mutations

Two of the non-mucoid variants of *P. aeruginosa* 2192 (*sap2192-8* and *sap2192-20*; Table 1) were not complemented by any of the genes previously identified as liable to second-site mutations. Interestingly, these two also remain completely nonmucoid indefinitely while the others begin producing alginate after 48 hours. This indicates that these possess mutations in one or more novel genes that previously have not been identified as involved in alginate regulation. The *mucA* of these mutants did not possess true reversions and restoration of function. The mutants will be complemented with a previously constructed *P. aeruginosa* cosmid library (37) to identify any novel mutations elsewhere on the chromosome. These two mutants prove fatal when the *algT/U* operon was introduced on pCD100. It is hypothesized that either these two do not take up the plasmid for

some reason, and thus are killed on the selective media, or the novel mutation, which is yet to be identified, will be able to explain this highly unusual phenomenon.

Conclusion

Care for CF patients over the last thirty years has dramatically improved. Life expectancy has risen from 18 years in the 1980's to nearly 50 today (9). *P. aeruginosa*, a ubiquitous bacterium, is devastatingly efficient as a CF pathogen (53). The most indicative factor pointing towards poor patient outcome is the production of alginate by the colonizing strain (54). As of yet, there is no effective anti-alginate therapy. Understanding all variables of *P. aeruginosa* alginate regulation and synthesis is inseparable from combating this deadly pathogen. The present study sought to contribute to this end by investigating regulatory genes involved in the mucoid to non-mucoid reversion of the clinical strain *P. aeruginosa* 2192

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345 **Conflicts of interest**

346 There are no conflicts of interest.

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348 **Ethical Statement**

349 Not applicable.

Bibliography

1. **FitzSimmons SC**. The changing epidemiology of cystic fibrosis. The Journal of pediatrics. 1993;122(1):1-9.
2. **Rommens JM, Iannuzzi MC, Kerem B, Drumm ML, Melmer G, Dean M, et al.** Identification of the cystic fibrosis gene: chromosome walking and jumping. Science. 1989;245(4922):1059-65.
3. **Kerem B**. Identification of the cystic fibrosis gene: genetic analysis. Trends in Genetics. 1989;5:363.
4. **Riordan JR, Rommens JM, Kerem B-s, Alon N, Rozmahel R**. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science. 1989;245(4922):1066-1072.
5. **Tsui LC**. Mutations and sequence variations detected in the cystic fibrosis transmembrane conductance regulator (CFTR) gene: a report from the Cystic Fibrosis Genetic Analysis Consortium. Human Mutation. 1992;1(3):197-203.
6. **Anderson MP, Gregory RJ**. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. Science. 1991;253(5016):202.
7. **Gadsby DC, Vergani P, Csanády L**. The ABC protein turned chloride channel whose failure causes cystic fibrosis. Nature. 2006;440(7083):477-83.
8. **Boat TF, Cheng PW, Iyer RN, Carlson DM, Polony I**. Human respiratory tract secretions: mucous glycoproteins of nonpurulent tracheobronchial secretions, and sputum of patients with bronchitis and cystic fibrosis. Archives of Biochemistry and Biophysics. 1976;177(1):95-104.
9. **CFFoundation**. Cystic Fibrosis Foundation Patient Registry. 2015:1-92.
10. **Govan J, Harris G**. *Pseudomonas aeruginosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. Microbiological Sciences. 1986;3(10):302-6.

- 375 11. **Sibley CD, Rabin H, Surette MG.** Cystic fibrosis: a polymicrobial infectious disease. Future
376 Microbiology. 2006;1(1):53-61.
- 377 12. **Pedersen SS.** Lung infection with alginate-producing, mucoid *Pseudomonas aeruginosa* in
378 cystic fibrosis. Acta Pathologica, Microbiologica et Immunologica Scandinavica Supplementum.
379 1991;28:1-79.
- 380 13. **Moradali MF, Ghods S, Rehm BH.** *Pseudomonas aeruginosa* lifestyle: A paradigm for
381 adaptation, survival, and persistence. Frontiers in Cellular and Infection Microbiology.
382 2017(7):39-45.
- 383 14. **Pedersen S, Højby N, Espersen F, Koch C.** Role of alginate in infection with mucoid
384 *Pseudomonas aeruginosa* in cystic fibrosis. Thorax. 1992;47(1):6-13.
- 385 15. **Højby N.** *Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid
386 strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta Pathologica,
387 Microbiologica, et Immunologica Scandinavica. 1974;82(4):551-8.
- 388 16. **Evans LR, Linker A.** Production and characterization of the slime polysaccharide of
389 *Pseudomonas aeruginosa*. Journal of Bacteriology. 1973;116(2):915-24.
- 390 17. **Schwarzmann S, Boring JR.** Antiphagocytic effect of slime from a mucoid strain of
391 *Pseudomonas aeruginosa*. Infection and immunity. 1971;3(6):762-7.
- 392 18. **Govan J, Fyfe JA.** Mucoid *Pseudomonas aeruginosa* and cystic fibrosis: resistance of the
393 mucoid form to carbenicillin, flucloxacillin and tobramycin and the isolation of mucoid variants
394 *in vitro*. Journal of Antimicrobial Chemotherapy. 1978;4(3):233-40.
- 395 19. **Kulezkycki LL, Murphy T, Bellanti JA.** *Pseudomonas* colonization in cystic fibrosis. Jama.
396 1978;240:30-4.
- 397 20. **Oliver A, Weir D.** Inhibition of bacterial binding to mouse macrophages by *Pseudomonas*
398 alginate. Journal of clinical & laboratory immunology. 1983;10(4):221-4.

- 399 21. **Hodges NA, Gordon CA.** Protection of *Pseudomonas aeruginosa* against ciprofloxacin and
400 beta-lactams by homologous alginate. Antimicrobial Agents and Chemotherapy.
401 1991;35(11):2450-2.
- 402 22. **Mathee K, Ciofu O, Sternberg C, Lindum PW, Campbell JI, Jensen P, et al.** Mucoid
403 conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence
404 activation in the cystic fibrosis lung. Microbiology. 1999;145(6):1349-57.
- 405 23. **Leid JG, Willson CJ, Shirtliff ME, Hassett DJ, Parsek MR, Jeffers AK.** The
406 exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN- γ -
407 mediated macrophage killing. The Journal of Immunology. 2005;175(11):7512-8.
- 408 24. **Boucher J, Yu H, Mudd M, Deretic V.** Mucoid *Pseudomonas aeruginosa* in cystic fibrosis:
409 characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model
410 of respiratory infection. Infection and Immunity. 1997;65(9):3838-46.
- 411 25. **Bragonzi A, Worlitzsch D, Pier GB, Timpert P, Ulrich M, Hentzer M, et al.** Nonmucoid
412 *Pseudomonas aeruginosa* expresses alginate in the lungs of patients with cystic fibrosis and in a
413 mouse model. The Journal of Infectious Diseases. 2005;192(3):410-9.
- 414 26. **Mathee K, Kharazmi A, Høiby N.** Role of exopolysaccharide in biofilm matrix formation:
415 the alginate paradigm. Molecular Ecology of Biofilms. 2002:1-34.
- 416 27. **Pandey S, Martins KL, Mathee K.** Posttranslational regulation of antisigma factors of RpoE:
417 a comparison between the *Escherichia coli* and *Pseudomonas aeruginosa* systems. Stress and
418 Environmental Regulation of Gene Expression and Adaptation in Bacteria. 2016:361-7.
- 419 28. **Franklin MJ, Nivens DE, Weadge JT, Howell PL.** Biosynthesis of the *Pseudomonas*
420 *aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. *Pseudomonas aeruginosa*,
421 Biology, Genetics, and Host-pathogen Interactions. 2011(1):49-60.
- 422 29. **Damron FH, Goldberg JB.** Proteolytic regulation of alginate overproduction in *Pseudomonas*
423 *aeruginosa*. Molecular Microbiology. 2012;84(4):595-607.

30. **Chitnis CE, Ohman DE.** Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. *Molecular Microbiology*. 1993;8(3):583-90.
31. **Deretic V, Schurr M, Boucher J, Martin D.** Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. *Journal of Bacteriology*. 1994;176(10):2773-8.
32. **DeVries CA, Ohman DE.** Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternate sigma factor, and shows evidence for autoregulation. *Journal of Bacteriology*. 1994;176(21):6677-87.
33. **Holloway B, Morgan A.** Genome organization in *Pseudomonas*. *Annual Reviews in Microbiology*. 1986;40(1):79-105.
34. **Martin D, Schurr M, Mudd M, Govan J, Holloway B, Deretic V.** Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proceedings of the National Academy of Sciences*. 1993;90(18):8377-81.
35. **Schurr M, Yu H, Martinez-Salazar J, Boucher J, Deretic V.** Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *Journal of Bacteriology*. 1996;178(16):4997-5004.
36. **Ohman DE, Chakrabarty AM.** Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. *Infection and Immunity*. 1981;33(1):142-8.
37. **Sautter R, Ramos D, Schneper L, Ciofu O, Wassermann T, Koh C-L, et al.** A complex multilevel attack on *Pseudomonas aeruginosa* *algT/U* expression and *algT/U* activity results in the loss of alginate production. *Gene*. 2012;498(2):242-53.
38. **Reiling S, Jansen J, Henley B, Singh S, Chattin C, Chandler M, et al.** Prc protease promotes mucoidy in *mucA* mutants of *Pseudomonas aeruginosa*. *Microbiology*. 2005;151(7):2251-61.

- 451 39. **Fux C, Shirliff M, Stoodley P, Costerton JW.** Can laboratory reference strains mirror ‘real-
452 world’ pathogenesis? Trends in Microbiology. 2005;13(2):58-63.
- 453 40. **Mathee K, Narasimhan G, Valdes C, Qiu X, Matewish JM, Koehrsen M, et al.** Dynamics
454 of *Pseudomonas aeruginosa* genome evolution. Proceedings of the National Academy of Sciences.
455 2008;105(8):3100-5.
- 456 41. **Walkerpeach CR, Velten J.** Agrobacterium-mediated gene transfer to plant cells: cointegrate
457 and binary vector systems. Plant Molecular Biology Manual. 1994(1):33-51.
- 458 42. **Wilson K.** Preparation of genomic DNA from bacteria. Current Protocols in Molecular
459 Biology. 1987(24):1-5.
- 460 43. Database TPG. The Pseudomonas Genome Database - Genome annotation and comparative
461 genome analysis 2017 [Available from: <http://pseudomonas.com/>].
- 462 44. **Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS.** Enhanced annotations
463 and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome
464 database. Nucleic Acids Research. 2016;44(D1):D646-D53.
- 465 45. NCBI. BLAST: Basic Local Alignment Search Tool 2017 [Available from:
466 <https://www.ncbi.nlm.nih.gov/pubmed/>].
- 467 46. Clustal_Ω. Clustal Omega 2017 [Available from: <http://www.ebi.ac.uk/Tools/msa/clustalo/>].
- 468 47. T-Coffee. T-Coffee Server 2017 [Available from:
469 <http://tcoffee.crg.cat/apps/tcoffee/do:mcffee>].
- 470 48. BoxShade. BoxShade Server 2017 [Available from:
471 http://www.ch.embnet.org/software/BOX_form.html].
- 472 49. **Pulcrano G, Iula DV, Raia V, Rossano F, Catania MR.** Different mutations in *mucA* gene
473 of *Pseudomonas aeruginosa* mucoid strains in cystic fibrosis patients and their effect on *algU* gene
474 expression. New Microbiologica. 2012;35(3):295-305.

50. **Rowen D, Deretic V.** Membrane to cytosol redistribution of ECF sigma factor AlgU and conversion to mucoidy in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Molecular Microbiology*. 2000;36(2):314-27.

51. **Goldberg JB, Gorman W, Flynn J, Ohman D.** A mutation in *algN* permits trans activation of alginate production by *algT* in *Pseudomonas* species. *Journal of Bacteriology*. 1993;175(5):1303-8.

52. **Mathee K, McPherson CJ, Ohman DE.** Posttranslational control of the *algT* (*algU*)-encoded sigma²² for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its antagonist proteins MucA and MucB (AlgN). *Journal of Bacteriology*. 1997;179(11):3711-20.

53. **Lyczak JB, Cannon CL, Pier GB.** Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes and Infection*. 2000;2(9):1051-60.

54. **Henry RL, Mellis CM, Petrovic L.** Mucoid *Pseudomonas aeruginosa* is a marker of poor survival in cystic fibrosis. *Pediatric Pulmonology*. 1992;12(3):158-61.

Tables

Table 1: Strains and primers generated and used in this study.

Strain	Genotype	Phenotype	Source
<i>P. aeruginosa</i>			
sap2192-1	<i>mucA180</i>	Alg ⁻	BC01; This study
sap2192-2	<i>mucA180</i>	Alg ⁻	BC02; This study
sap2192-3	<i>mucA180</i>	Alg ⁻	BC03; This study
sap2192-4	<i>mucA180</i>	Alg ⁻	BC04; This study
sap2192-5	<i>mucA180</i>	Alg ⁻	BC05; This study
sap2192-6	<i>mucA180</i>	Alg ⁻	BC06; This study
sap2192-7	<i>mucA180</i>	Alg ⁻	BC07; This study
sap2192-8	<i>mucA180</i>	Alg ⁻	BC08; This study
sap2192-9	<i>mucA180</i>	Alg ⁻	BC09; This study
sap2192-10	<i>mucA180</i>	Alg ⁻	BC10; This study
sap2192-11	<i>mucA180</i>	Alg ⁻	BC11; This study
sap2192-12	<i>mucA180</i>	Alg ⁻	BC12; This study
sap2192-13	<i>mucA180</i>	Alg ⁻	BC13; This study
sap2192-14	<i>mucA180</i>	Alg ⁻	BC14; This study
sap2192-15	<i>mucA180</i>	Alg ⁻	BC15; This study
sap2192-16	<i>mucA180</i>	Alg ⁻	BC16; This study
sap2192-17	<i>mucA180</i>	Alg ⁻	BC17; This study
sap2192-18	<i>mucA180</i>	Alg ⁻	BC18; This study
sap2192-19	<i>mucA180</i>	Alg ⁻	BC19; This study
sap2192-20	<i>mucA180</i>	Alg ⁻	BC20; This study
sap2192-21	<i>mucA180</i>	Alg ⁻	BC21; This study
sap2192-22	<i>mucA180</i>	Alg ⁻	BC22; This study
sap2192-23	<i>mucA180</i>	Alg ⁻	BC23; This study
sap2192-24	<i>mucA180</i>	Alg ⁻	BC24; This study
sap2192-25	<i>mucA180</i>	Alg ⁻	BC25; This study

sap2192-26	<i>mucA180</i>	Alg ⁻	BC26; This study
sap2192-27	<i>mucA180</i>	Alg ⁻	BC27; This study
sap2192-28	<i>mucA180</i>	Alg ⁻	BC28; This study
sap2192-29	<i>mucA180</i>	Alg ⁻	BC29; This study
sap2192-30	<i>mucA180</i>	Alg ⁻	BC30; This study
sap2192-31	<i>mucA180</i>	Alg ⁻	BC31; This study
sap2192-32	<i>mucA180</i>	Alg ⁻	BC32; This study
sap2192-33	<i>mucA180</i>	Alg ⁻	BC33; This study
sap2192-34	<i>mucA180</i>	Alg ⁻	BC34; This study
sap2192-35	<i>mucA180</i>	Alg ⁻	BC35; This study
sap2192-36	<i>mucA180</i>	Alg ⁻	BC36; This study
sap2192-37	<i>mucA180</i>	Alg ⁻	BC37; This study
sap2192-38	<i>mucA180</i>	Alg ⁻	BC38; This study
sap2192-39	<i>mucA180</i>	Alg ⁻	BC39; This study
sap2192-40	<i>mucA180</i>	Alg ⁻	BC40; This study
sap2192-41	<i>mucA180</i>	Alg ⁻	BC41; This study
PAO1	Prototypic strain	Alg ⁻	BC42; Holloway & Morgan 1986
PA2192	Clinical isolate; <i>mucA180</i>	Alg ⁺	BC43; Mathee et al. 2008
Plasmids			
pRK600	pRK600	Cm ^R (mating helper)	BC44; Heeb et al. 2000
pRK2013	pRK2013	Km ^R (mating helper)	BC45; Figurski & Helinski 1979
pCD100	pRK404; <i>algTmucAmucBmucCmucD</i>	Tc ^R	BC46; Sautter et al. 2012
pJG293	pRK404; <i>algT/U</i>	Tc ^R	BC47; Sautter et al. 2012
pAlgO	pMF54; <i>algO</i>	Ap ^R	BC48; Caballos submitted
pMucP	pMF54; <i>mucP</i>	Ap ^R	BC49; Caballos submitted

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Primer	Sequence (5' to 3')	Amplicon (bp)
AlgT Fw	TGTTGATAATGTTGGCTCATGCCCCGCATTTC	1183
AlgT Rv	AGCGATATCCAGCTTCGGCAGGGTAG	
MucA Fw	AGGACGTAGCGCAGGAAGCCTTCATC	1221
MucA Rv	AAGCTGCCATTGCGCTCGTAGACGAAG	
AlgO Fw	TTCTGCAACAGGTCGGCGCGGTTGAG	2738
AlgO Rv	ACTCCGGGGAGACGTTGAGGAACAGCATG	
MucP Fw	AGCGTGATCCACTCGATGGTGGAC	2212
MucP Rv	GAGCAGGTCTTCCTTGGAATCGCCTTG	
AlgT Seq Fw	ATATCAGAAAGACTCGTGA	Seq. only
AlgT Seq Rv	CATCCGCTTCGTTATCCAT	

493 N.B.: Colored boxes group the *sap* mutants that came from the same parent
494 colony during the isolation process.

495

496

Figure Legends

Figure 1: The *Pseudomonas aeruginosa* alginate regulation pathway. Alginate production is controlled by the sigma factor AlgT/U which is ordinarily bound to the inner membrane by the anti-sigma factor MucA to prevent interaction with RNAP. AlgT/U must be freed from MucA to begin alginate production. When stress is sensed MucE misfolds (1) and induces periplasmic cleavage of MucA by AlgW (2). MucA is also cleaved by MucP (3) on the cytoplasmic end to release AlgT/U. AlgT/U is now free to interact with RNAP and initiate alginate biosynthesis by transcribing the *algD* operon. When MucA is mutated, it is unable to sequester AlgT/U and a mucoid phenotype ensues. Proteins marked with a red star are under investigation in this study. Adapted from Pandey *et al.*, 2016 (27).

Figure 2: The *P. aeruginosa in vitro* reversion phenomenon. Many clinical isolates are mucoid when removed from the lung due to *mucA* mutations. When cultured *in vitro*, they cease producing alginate and become non-mucoid. *mucA* has been seen to remain mutated, and so the reversion is due to second-site mutations in other alginate-regulating genes.

Figure 4: The alignment of MucA. **A.** *mucA* DNA alignment between PAO1, PDO300 (*mucA22*), PA2192, and a sap mutants displaying the differences in *mucA* mutations. Ten *sap* mutants all had the same sequence. PAO1 contains no

mutations and is presented as a reference. **B.** Alignment of the respective MucA proteins. **C.** Model of the differences in the MucA protein. MucA22 and MucA180 both have a truncated C-terminus in the periplasm. As a result, MucB cannot bind and the protein loses stability, releasing AlgT/U and bestowing the mucoid phenotype.

Figures

Figure 2

Figure 4

A.

	1	337	426	534
PA01_mucA	ATGAGTC.....	CCGCAAATGGC.....	GGGGGCGCCGCAGG.....	TACAGAG...
PDO300_mucA	ATGAGTC.....	CCGCAAATGGC.....	GGGG-CGCCGCAGG.....	TACAGAG...
PA2192_mucA	ATGAGTC.....	CCGCAGATGGC.....	GGGGGCGCCGCAGG.....	TACATAG...
sap1_mucA	ATGAGTC.....	CCGCAGATGGC.....	GGGGGCGCCGCAGG.....	TACATAG...
		Silent	mucA22	Stop

B.

	1	139	180
PA01_MucA	MSREA.....	SEEQGAPQVITNSSSSDTRWHEQRLPIYLRQHVQOSAVSGTESALPYARAASLENR	
PDO300_MucA22	MSREA.....	SEEQGRRR-----	
PA2192_MucA	MSREA.....	SEEQGAPQVITNSSSSDTRWHEQRLPIYLRQHVQOSAVSGT-----	
sap1_MucA	MSREA.....	SEEQGAPQVITNSSSSDTRWHEQRLPIYLRQHVQOSAVSGT-----