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Nitrosative stress inhibits production of the virulence factor alginate in mucoid *Pseudomonas aeruginosa*

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Abstract

Alginate is a critical virulence factor contributing to the poor clinical prognosis associated with the conversion of *Pseudomonas aeruginosa* to mucoid phenotypes in cystic fibrosis (CF). An important mechanism of action is its ability to scavenge host innate-immune reactive species. We have previously analyzed the bacterial response to nitrosative stress by *S*-nitrosoglutathione (GSNO), a physiological NO· donor with diminished levels in the CF lung. GSNO substantially increased bacterial nitrosative and oxidative defenses and so we hypothesized a similar increase in alginate production would occur. However, in mucoid *P. aeruginosa*, there was decreased expression of the majority of alginate synthetic genes. This microarray data was confirmed both by RT-PCR and at the functional level by direct measurements of alginate production. Our data suggest that the lowered levels of innate-immune nitrosative mediators (such as GSNO) in the CF lung exacerbate the effects of mucoid *P. aeruginosa,* by failing to suppress alginate biosynthesis.

Keywords: *Nitrosative stress, virulence factor, alignate synthetic genes, GSNO*

Introduction

The progressive lung damage and deterioration of respiratory function in cystic fibrosis (CF) arises from a characteristic pattern of bacterial colonization of the lung, with chronic *Pseudomonas aeruginosa* infections being centrally important to the lung pathology and progressive tissue damage in CF patients [1,2]. More than 80% of CF patients over 26 years old are chronically infected with *P. aeruginosa* and these infections persist, despite aggressive antibiotic therapy [3]. These infections are only in the respiratory tract lumen, not systemic, so it appears that innate lung defense is specifically compromised by CF [4,5]. After initial infection with wild-type, non-mucoid strains, exemplified by strains such as PAO1, conversion of *P. aeruginosa* to the mucoid phenotype in the CF host occurs and increases bacterial resistance not only to host clearance and defense mechanisms, but also to antibiotic interventions [6,7]. The mucoid phenotype is characterized by production of large amounts of the exopolysaccharide alginate [6,8–10]. Mucoid conversion is concomitant with the establishment of chronic bacterial colonization [4,11,12] and increases inflammation and lung damage [13], while causing pulmonary function to decline [14,15]. Furthermore, alginate modulates host defenses to allow a persistent infection [16]. Accordingly, mucoid conversion results in a poor prognosis for CF patients [3,4,11] in which

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alginate production is an important and independent [11] virulence factor.

Aggressive antibacterial therapy, although beneficial, provides only a temporary delay in mucoid conversion [14] and could be a double-edged sword as it may be associated with increased alginate production for some antibiotics [17]. Mucoid conversion occurs as a result of mutations, primarily in *muc* genes [18] that render the stress response sigma factor AlgU constitutively active [7,19] and cause the characteristic activation of alginate biosynthesis genes [20]. This "runaway" mucoid phenotype is highly unstable, however, due to the toxic effects of uncontrolled AlgU on the bacterial cell and later phenotypic change is towards lowered alginate production through second-site mutations, e.g. in *algU* [21] exemplified by the strain PAO578II [22].

Alginate forms a viscous polysaccharide biofilm-like matrix around the bacteria, increasing resistance to both host defenses and antibiotics by a range of mechanisms [6,7]. The alginate matrix inhibits opsonization and phagocytosis [23–26] with *O*-acetylation by the *algFIJ* cluster [27] being important [23]. Alginate has also recently been shown to cause conformational changes in antimicrobial peptides [28]. The alginate matrix greatly restricts the diffusion of antibiotics and antibacterial innate-immune reactive oxygen and nitrogen species (ROS and RNS, respectively) to the bacteria [29–31] but also binds and scavenges these species before they can reach the microorganisms [26,31,32]. The combination of diffusion restriction [30] and consumption by scavenging will be particularly effective in limiting the path length of host ROS and RNS through the alginate matrix, to restrict their access to the bacteria. In light of the range of its deleterious effects, the hydrolysis of alginate by alginate lyases continues to be pursued as a potential therapeutic strategy [33–35] and there also appears considerable therapeutic promise in antibodies directed against alginate [36] but other pharmacological approaches to decrease alginate production may also prove to be clinically beneficial.

The prevalence of *P. aeruginosa* in CF is likely to be multifactorial, but a significant component of this prevalence is thought to be due to a decrease in nitric oxide (NO·) mediated innate immunity [37]. In addition to its important signaling roles, NO· is a reactive, bactericidal and bacteriostatic, highly diffusible radical. Since NO· is a gas, in the context of the gas-exchanging environment of the lung, much of the innate-immune action of NO· is likely to be mediated by non-volatile nitrosothiol carriers of NO·, such as *S*-nitrosoglutathione (GSNO) [38]. GSNO is a physiologically relevant NO· donor in the lung [38] whose levels are decreased in the lower airways in CF [39]. However, the predominant chemistries of nitrosothiol action and detoxification are distinct from "free" NO· with the former being by nitrosonium equivalent transfer to target protein thiols and with the latter by specific GSNO metabolism [40]

We have shown that wild-type *P. aeruginosa* is able to mount inducible defenses against nitrosative stress and that upon mucoid conversion these defenses are constitutively up-regulated [13,20]. Furthermore, antipseudomonal therapy decreases this NO· consumption and restores nitrogen redox balance in the CF lung [41]. The combined deficiencies of lowered host NO·/GSNO levels and constitutive resistance against NO· stress by mucoid *P. aeruginosa,* may help explain the prevalence of the mucoid phenotype of this pathogen in CF. We hypothesized that gene expression responses of *P. aeruginosa* to mucoidy and nitrosative stress are broadly independent [20,42], nitrosative stress may modulate the mucoid phenotype by altering expression of important mucoidy-related genes. Since the metabolic costs of maintaining the mucoid phenotype are great [6], we reasoned that the additional nitrosative stress and the metabolic reprogramming required to deal with it [42] could negatively impact upon alginate production. Here, we show using microarrays and other assays that exposure to GSNO, a physiologically relevant NO· donor, inhibits alginate gene expression and lowers alginate production by mucoid *P. aeruginosa*.

Materials and methods

Bacterial strains and growth conditions

P. aeruginosa PAO578II (*mucA22 sup-2*) has been described previously [20,22]. For RNA isolation, strains were cultured shaking at 37°C overnight in Luria broth (LB). About 1 ml of the overnight culture was used to inoculate 100 ml of LB with 0.3 M NaCl (for a starting density equal to that of a saturated culture diluted 1:100 and grown for 4 h at 37°C to a mid-log optical density at 600 nm (OD_{600}) of 0.5 before GSNO treatments and use [20]. It is difficult to estimate the steady-state concentration of NO· that this will result in due to uncertainties in GSNO half-life and metabolism (about 10 min in CF lung as assessed by expired NO· release) [38] and also NO· consumption by both flavohemoglobin and nitric oxide reductase up-regulated in PAO578II [42]. One possible assumption (half-life ~ 10–15 min) leads to estimates of the steady-state concentration of NO· to which the bacteria are exposed to in this *in vitro* system is of the order of 1 μM, a value that is consistent with known pathophysiological levels of NO· [43]. Furthermore, as reported previously, this concentration produced only a mild and transitory growth arrest with no cell death [42], a situation analogous to the predominantly static biofilm-mode of growth in the CF lung [29,44,45], in which NO· is thought to play a major antibacterial role [46]. However, it is also clear that in this experiment, GSNO has the potential to act directly.

Cystic fibrosis lung cells and iNOS immunostaining

IB3-1 (CF) and S9 (CF-corrected) cells [47,48] were grown to 80% confluency. Cells were fixed with 1% paraformaldehyde followed by membrane permeabilization using 1% TritonX-100. The coverslips were washed three times with PBS prior to incubating with blocking solution (10% skim milk, 6% BSA fraction V and 2% goat serum in PBS). Airway epithelial cells were incubated with a polyclonal rabbit anti-inducible NO· synthase (iNOS) antibody (Transduction Labs, Lexington, KY) at 4°C overnight at a dilution of 1:500, followed by a secondary antibody. This antibody has previously been used in immunostaining and microscopic quantification of iNOS in CF cell lines [49]. Alexa 488-conjugated to goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) was diluted 1:1000 and incubated with samples for 5 h at 37°C in blocking solution. Coverslips were mounted on glass slides with PermaFluor (Immunon, Pittsburgh, PA) and analyzed using an Olympus IX70 microscope (Olympus, Melville, NY). Alexa 488 fluorescence was visualized using an excitation wavelength 490 ± 10 nm. All images were taken at a magnification of ×100. Confocal fluorescence microscopy images were collected and quantified using the Perkin-Elmer Life Sciences UltraView confocal microscopy system as previously [50].

Microarray and RT-PCR analysis of gene expression

GSNO [51] was added to cultures of mucoid strain PAO578II (in LB containing 0.3 M NaCl) for 30 min at 5 mM final concentration, together with matched non-GSNO treated controls, then RNA extraction and microarray analysis performed as previously described [42]. These conditions were optimized to take into account the relatively short half-life of GSNO at 37°C (approximately 20–30 min). Results from three microarray chips, i.e. independent identical experiments, were obtained for each strain. Gene expression of alginate synthesis and mucoid associated genes in GSNO-treated PAO578II was compared to untreated controls and ratios of expression and statistical significance were calculated, using Students *t*-test. For RT-PCR confirmation of selected gene expression ratios, *P. aeruginosa* mucoid strain PAO578II was grown and treated with GSNO as described above (i.e. $n = 3$) and analyzed in triplicate as previously [42]. Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA): for *algD* these were GAGCACCGCGATCAAGGA and TTGTCCAGTTCGCCGATCA, for *algE* these were CGAGGCGCCGAAGAACT and CGCGATCGTTTTCCGATT, and for *algU* these were CGTGCGCTCGGCAATT and TTGATGGCGATCCGATACAG. Following PCR, relative expression ratios were calculated using $2^{\Delta CT}$ where ΔCT represents the difference in cycling times (CT) for the two samples being compared. CT is the point at which the PCR cycle crosses the preset logarithmic threshold. Statistical significance was calculated using Students *t*-test upon the ΔCT values. Statistical analyses was performed using SigmaStat 3.0, SPSS Inc., Chicago, IL.

Alginate biosynthesis assay

GSNO [51] was added to triplicate cultures of mucoid strain PAO578II (in LB containing 0.3 M NaCl) and non-mucoid strain PAO1 (in LB) for 30 min at 5 mM final concentration, the cultures were then briefly centrifuged, washed with LB containing 0.3 M NaCl and then resuspended in fresh (non-GSNO containing) medium and grown for 1 h. Removal of GSNO was necessary to prevent its interfering with alginate assay. After 1 h, culture fluid was collected and assayed for alginate production as previously [52].

Results and discussion

Lowered iNOS expression in CF cells

It has been documented that exhaled NO· in CF patients is lowered [53,54]. However, this may be an indirect result of infection with NO· consuming phenotypes of *P. aeruginosa* [41,42], rather than an actual decrease in host NO· synthesis. Since iNOS is the major source of NO· in the lung [49], we wanted to independently confirm that its expression was lower in the CF cells we have previously used [55–58]. Figure 1 shows that iNOS expression in the CF cell line IB3-1

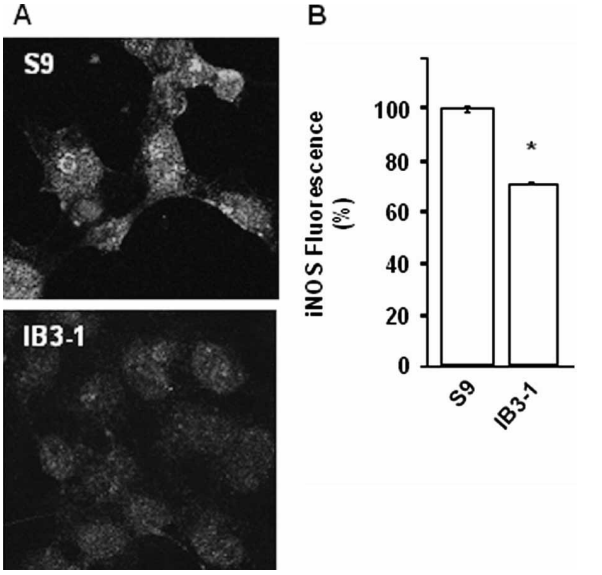

Figure 1. Diminished levels of iNOS in CF respiratory epithelial cells. Images show level of immunofluorescence observed with IB3-1 (CF) and S9 (CFTR-corrected) cells (A). Quantification (%) of iNOS expression visualized by immunofluorescence (B). Data, means of triplicate assays ± SEM (⋆, $p \leq 0.01$).

Table I. Microarray gene expression ratios of the alginate biosynthetic genes in mucoid *P. aeruginosa* PAO578II under nitrosative stress (GSNO treated) compared to control (no GSNO), Also shown are the expression ratios of the same genes that occurs upon loss of mucoid phenotype, by comparison of expression of mucoid strain PAO578II (*mucA22 sup-2 algU*$^{+}$) with that of its nonmucoid *algU* mutant derivative, PAO6865 (*algU*::Tc^{r}). [20].

Gene identity	PAO578II fold change GSNO vs. control	Fold change upon genetic loss of mucoidy [20]	Enzymatic activity or function
PA3540 *algD*	− 1.9 ns	7	GDP-mannose 6-dehydrogenase
PA3541 *alg8*	− 2.9 ns	2.9	Putative polymerase
PA3542 *alg44*	− 3.7⋆	3.4	Membrane protein
PA3543 *algK*	− 4.8⋆	5.8	Periplasmic protein
PA3544 *algE*	− 10⋆	3.2	Outer membrane protein for alginate export
PA3545 *algG*	− 4.8⋆	3.5	C5-mannuronan epimerase, protection from alginate lyase
PA3546 *algX*	− 6.3⋆	4.1	Periplasmic protein
PA3547 *algL*	− 4.6⋆	6.3	Periplasmic alginate lyase
PA3548 *algI*	− 3.7⋆	4.1	Alginate *O*-acetylation
PA3549 *algJ*	− 4.2⋆	7.6	Alginate *O*-acetylation
PA3550 *algF*	− 2.7⋆	16.0	Alginate *O*-acetylation
PA3551 *algA*	− 2.9⋆	4.0	Phosphomannose isomerase and GDP-mannose pyrophosphorylase
PA5322 *algC*	3.4⋆	–	Phosphomannomutase and phosphoglucomutase
Mean	− 3.8	+5.7	

⋆ $p \geq 0.05$ by Mann–Whitney rank sum test and ns, not significant.

is significantly lower than that of its CFTR-corrected and genetically-matched derivative S9. This, along with prior reports [37], indicates that host innate-immune nitrosative stress is lower in CF, regardless of consumption or detoxification of NO· by *P. aeruginosa*.

Effects of GSNO on alginate and mucoidy-associated gene expression

Microarray analysis of the expression of alginate-related genes has proven to be a useful approach in uncovering less overt aspects of the mucoid phenotype [13,20]. Although conventional biochemical alginate assays remain a gold standard, the complex nature of alginate structure and biosynthesis, as well as the large number of genes in its production and regulation [10], make in-depth studies of many steps difficult. The mucoid strain PAO578II was chosen as it has become the tester strain for complex gene expression patterns in mucoid cells [13,20,42]. Although wild-type non-mucoid strains such as PAO1 or PA14 can grow as biofilms, they do not appear to produce significant amounts of alginate, even in a biofilm [10,59] but in the light of a recent report that non-mucoid strains can produce alginate under certain conditions [60], alginate gene expression by PAO1 was also studied.

Nitrosative stress from exposure to GSNO in PAO578II caused a concerted down-regulation of many alginate-related genes. This was unexpected; as we had hypothesized that alginate synthesis would be increased to protect the bacteria from this stress. The expression of almost all genes whose products are involved in the biosynthesis and processing of alginate was significantly decreased (Table I), by values ranging from between − 1.9 and − 10-fold (Table I) with a mean of − 3.8. In contrast, the expression of the same genes was reported to be increased by mucoid conversion (i.e. PAO578II vs. its non-mucoid *algU* mutant derivative PAO6865) from between +2.9 and +16-fold, with a mean of +5.7-fold [20]. All the different classes of alginate biosynthetic genes: monomer synthesis, polymerization, acetylation and export, displayed down-regulation by GSNO. RT-PCR of selected genes was performed order to confirm the microarray data and reported in Table II. RT-PCR of *algD*, the key rate-limiting step in alginate biosynthesis [61] and *algE*, a critical porin for polymer export [62,63], showed expression decreases upon GSNO treatment of − 2.7-fold ($p < 0.01$) and − 9.8-fold ($p < 0.01$), supporting analysis of the microarray data. In comparison, no clear trend in effects on alginate regulatory genes was observed (Table III) and the unambiguous assignment of the mechanisms of alginate biosynthetic gene repression by GSNO was not possible given the complex nature of alginate gene control [6,10].

The effects of GSNO upon PAO578II at the gene expression level were also borne out when its impaction upon alginate synthesis was tested. The assay was normalized to c.f.u. in order to correct for

Table II. RT-PCR of selected alginate genes in mucoid *P. aeruginosa* PAO578II under GSNO-imposed nitrosative stress compared with untreated controls.

Gene identity	Fold change GSNO vs. control by RT-PCR
PA0762 algU	− 1.02⋆
PA3540 algD	− 2.7†
PA3544 algE	− 9.8†

⋆ p values: ≤ 0.01; † ≥ 0.05.

Table III. Micorarray expression ratios of alginate regulatory genes in mucoid *P. aeruginosa* PAO578II under nitrosative stress with GSNO compared non-GSNO treated controls, *p* values, as in Table I. Also shown are the expression ratios of the same genes that occurs upon genetic loss of mucoid phenotype, by comparison of expression in mucoid strain PAO578II (*mucA22 sup-2 algU*$^+$) with that of its nonmucoid *algU* mutant derivative, PAO6865 (*algU*::Tc^r). [20].

Gene identity	PAO578II fold change GSNO vs. control	Fold change upon genetic loss of mucoidy	Alginate expression regulatory function
PA0762 *algU*	−2.7 ns	49.2	Alternative sigma factor responsible for transcription of algD, algR, algT, algZ and rpoH
PA0763 *mucA*	1.1 ns	21	Anti-sigma factor: negatively regulates alginate production
PA0764 *mucB*	1.4 ns	7	Negatively regulates AlgU
PA0765 *mucC*	1.0*	4.1	Postulated to act with MucA and MucB in negative regulation of AlgU
PA0766 *mucD*	3.3*	2.2	Putative HtrA-like protease, negatively regulates AlgU
PA1754 *cysB*	3.8*	–	Activator of algD transcription
PA4446 *algW*	−1.4*	–	Putative HtrA-like protease, inhibits alginate synthesis
PA4462 *rpoN*	3.2*	–	Negative regulator of algD transcription
PA5253 *algP*	2.9*	−1.2	Histone-like protein; activates algD transcription
PA5255 *algQ*	1.3*	−1.4	Activator of algD transcription;
PA5261 *algR*	−1.3 ns	3.1	Activator of algC and algD transcription
PA5262 *algZ*	−2*	2.2	Hypothesized cognate sensor kinase for AlgR; negative regulator of alginate production
PA5483 *algB*	1.4*	–	Positive activator of algD transcription
Regulatory gene mean	−1.2	+9.6*	

*$p \geq 0.05$ by Mann–Whitney rank sum test and ns, not significant.

the bacteriostatic effects of the GSNO treatment. We assayed alginate production directly and observed a significant decrease in alginate biosynthesis upon GSNO treatment (Figure 2). Although repression of alginate synthesis was not complete, it is in accord with the gene expression data and the difference most likely reflects the microarrays assessing change in new gene product synthesis, while alginate production at the polymer level assesses steady state levels of enzymes and their activity.

The non-mucoid strain PAO1 exhibited no coordinated or significant effects of GSNO upon alginate biosynthetic gene activity (Table IV). Alginate synthesis was not detected by carbazole assay (i.e. below the levels of sensitivity) in either the absence or presence of GSNO. Thus although under some conditions [60], this non-mucoid strain does produce some alginate, there was no effect of nitrosative stress under the culture conditions used here. We also note that expression of *adhC*, which codes for a GSNO-metabolizing aldehyde dehydrogenase, was not significantly up-regulated (+1.5-fold in PAO578II, −1.3-fold in PAO1 respectively, not significant) although others have noted a lack of *adhC* up-regulation in GSNO-treated *Escherechia coli* [64].

In this work, we have shown that nitrosative stress significantly down-regulates the vast majority of genes involved in alginate synthesis and maintenance of the mucoid phenotype in *P. aeruginosa*. The concerted decrease in alginate gene expression assessed by microarrays occurred against the backdrop of active and increased mRNA and protein synthesis nitrosative defenses [42] and thus was not merely the result of a generalized metabolic or transcriptional machinery depression. The microarray findings are supported by RT-PCR analyses and direct measurement of alginate production. All of these assays confirm a significant decrease in alginate synthesis by GSNO treatment. We are led to conclude that an inability of the CF host to suppress alginate production by nitrosative stress, due to the derangement in NO·-mediated innate immunity in the CF host [37–39,53,54] (see also Figure 1), is likely to play a role in the distinct pathogenesis of colonization with mucoid phenotypes. Future studies planned include assessing the magnitude of GSNO

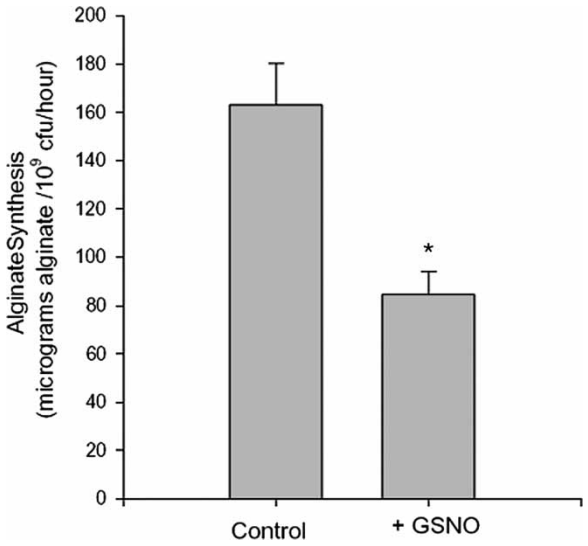

Figure 2. GSNO Inhibition of alginate synthesis. Triplicate cultures of mucoid *P. aeruginosa* PAO578II was treated for 30 min with 5 mM GSNO, washed and then alginate synthesis measured after 1 h incubation as in the text. Data, means ± SD (*, $p \leq 0.05$).

Table IV. Micorarray expression ratios of alginate biosynthetic genes in non-mucoid *P. aeruginosa* PAO1 under nitrosative stress with GSNO compared non-GSNO treated controls.

Gene identity	PAO1 fold Change GSNO vs. control	Enzymatic activity or function
PA3540 algD	−1.7 ns	GDP-mannose 6-dehydrogenase
PA3541 alg8	−1.1 ns	Putative polymerase
PA3542 alg44	−1.4 ns	Membrane protein
PA3543 algK	−2.0 ns	Periplasmic protein
PA3544 *algE*	−1.7 ns	Outer membrane protein for alginate export
PA3545 algG	−2.3 ns	C5-mannuronan epimerase, protection from alginate lyase
PA3546 algX	−1.5 ns	Periplasmic protein
PA3547 algL	+1.1 ns	Periplasmic alginate lyase
PA3548 algI	−1.1 ns	Alginate *O*-acetylation
PA3549 algJ	+1.8*	Alginate *O*-acetylation
PA3550 algF	1 ns	Alginate *O*-acetylation
PA3551 algA	−1.5 ns	Phosphomannose isomerase and GDP-mannose pyrophosphorylase
PA5322 algC	1.6 ns	Phosphomannomutase and phosphoglucomutase
Mean	−1.5	

* $p \geq 0.05$ by Mann–Whitney rank sum test and ns, not significant.

effects upon alginate synthesis in a range of CF isolates, as although PAO578II represents a suitable model strain for CF isolates, there may be differences in how mutations in other CF isolates interact.

We propose that pharmacological correction of the NO· defect in CF, or direct boosting of NO· levels by administration of GSNO or other NO· donors, may provide viable strategies to suppress alginate synthesis in patients colonized by mucoid *P. aeruginosa*. Such interventions may synergize with other treatments that alter airway surface fluid composition and properties, such as alginate lyases [34,65] and DNAses [66,67]. Since mucoid phenotypes are notorious for their recalcitrance and resistance to eradication by antibiotics with conventional treatments once mucoid conversion occurs [6,14], there is a need for less conventional strategies to deal with mucoid *P. aeruginosa* in the CF host.

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