Review

Targeting matrix exopolysaccharides to disrupt *Pseudomonas aeruginosa* biofilms in cystic fibrosis

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Abstract: In cystic fibrosis (CF), pulmonary infection with *Pseudomonas aeruginosa* is a cause of increased morbidity and mortality, especially in patients for whom infection becomes chronic and there is reliance on long-term suppressive therapies. Current antimicrobials, though varied mechanistically and by mode of delivery, are inadequate not only due to eradication failure in many cases, but also because they do not halt the progression of lung function decline over time. One of the reasons for this failure is thought to be the biofilm mode of growth of *P. aeruginosa*, wherein self-secreted exopolysaccharides (EPSs) provide physical protection against antibiotics and an array of niches with resulting metabolic and phenotypic heterogeneity. Targeting the three EPSs secreted by *P. aeruginosa* (alginate, Psl and Pel) is currently under investigation as a way of disrupting the biofilm extracellular matrix to potentiate the action of antibiotics. In this review, we look at each EPS as a potential therapeutic target for combatting pulmonary infection with *P. aeruginosa* in CF, with a particular focus on the current evidence for these emerging therapies and barriers to bringing these therapies into clinic.

Keywords: Cystic fibrosis, *Pseudomonas aeruginosa*, antibiotic adjunct, anti-biofilm, biofilm-degrading enzymes, matrix exopolysaccharides, alginate oligosaccharide

1. Introduction

Pseudomonas aeruginosa is a ubiquitous, Gram-negative bacteria that occupies environmental niches and is an opportunistic pathogen that poses risks to certain vulnerable groups of patients. Examples include people who are immunocompromised (including those with human immunodeficiency virus or neutropenia stemming from cancer chemotherapy), patients who are post-surgery, burn victims, and individuals with respiratory illnesses, including chronic obstructive pulmonary distress, primary ciliary dyskinesia, and most notably, cystic fibrosis (CF) [1–4]. CF is a genetic, multisystem disease that results in improper epithelial chloride ion transport. This leads to a dehydrated lung environment, impaired mucociliary escalator function, and the production of a sticky mucus that creates suitable grounds for colonization by a number of pathogens, including *P. aeruginosa* [5].

Within the lung, *P. aeruginosa* forms sputum-suspended aggregates (or biofilms) composed of exopolysaccharides (EPSs), proteins, and extracellular DNA (eDNA). This EPS matrix provides *P. aeruginosa* biofilm with a scaffolding framework which generates chemical and nutrient gradients that differentially affect cells, giving rise to the incredible metabolic and phenotypic diversity observed through the biofilm [6]. The EPS matrix

also provides physical protection against antimicrobials and host immune cells. *In vitro* work with antibiotics has consistently demonstrated that biofilm-grown *P. aeruginosa* possess minimum inhibitory concentrations (MICs) that are many orders of magnitude higher than their planktonic counterparts [7,8]. Neutrophils face tremendous difficult in eradicating biofilm infections, which results in the accumulation of immune-mediated debris and an aberrant inflammatory response that progressively damages the lung tissue [9]. Managing biofilm infections is an arms race, and as *P. aeruginosa* gradually accumulates adaptations that strengthens its defenses and survivability, our antibiotic arsenal dwindles. In cases where attempts at eradication fail, the infection becomes chronic, leading to further inflammation and scarring. This damage clinically manifests as progressive lung function decline, decreased quality of life, and increased mortality in patients infected with this organism [10,11].

Current strategies for eradicating and suppressing *P. aeruginosa* biofilms rely on combination high dose antibiotic therapy to overcome its naturally high tolerance. However, this approach has several drawbacks including the risk of accumulated side-effects (such as oto- or nephrotoxicity with aminoglycoside use) and the emergence of multidrug resistance (MDR) [12–15]. In 2021, the U.S. CF Foundation reported that amongst individuals who cultured *P. aeruginosa*, 12.3% were classified as having an MDR strain [16]. This percentage increases with patient age and is thought to represent the cumulative effect of mutagenic adaptation and antibiotic use in the form of chronic suppressive therapy with nebulized antibiotics and courses of antibiotics (nebulized, oral, and intravenous) prescribed in the setting of chronic suppressive therapy as well as acute pulmonary exacerbations. The emergence of MDR not only limits therapeutic options, but is associated with worse clinical outcomes in CF [17]. Given the scarcity of new antibiotics, there has been a surge of interest in compounds that aim to increase the efficacy of treatments currently in clinical use (antibiotic adjuncts), some of which target components specific to *P. aeruginosa*.

The use of biofilm degrading compounds to increase the efficacy of antibiotics is an approach with a well-established evidence base in CF. For instance, the use of the mucolytic human recombinant dornase alfa (rhDNase), an EPS-disruptor that degrades eDNA within biofilms, has been shown to be effective at reducing the rate of lung function decline and the number of pulmonary exacerbations in meta-analyses of clinical trials [18]. Targeting matrix EPSs specific to the biofilms of *P. aeruginosa* is a promising adjuvant strategy, as these polysaccharides plays a prominent role in substrate adhesion, cell aggregation, and community architecture, factors which confer survival benefits and lead to increased *P. aeruginosa* tolerance to antimicrobials and host defenses [19,20]. In this review, we focus on the disruption of the three *P. aeruginosa*-specific matrix EPSs as novel therapeutic targets for increasing the efficacy of antibiotics currently in clinical use. We summarize the current evidence base for EPS degrading enzymes and other strategies with a focus on the eventual clinical use alongside antibiotics for CF lung infection.

2.   **Matrix Exopolysaccharides of *P. aeruginosa* biofilms**

To fully appreciate the potential *EPS* targets and strategies that will be discussed in this review, we begin with a summary of *P. aeruginosa* biofilm development. The life cycle of the *P. aeruginosa* biofilm is characterized by four stages, depicted in Figure 1: attachment, microcolony formation, biofilm maturation, and dispersal of biofilm bacteria to recolonize and propagate new biofilm development.
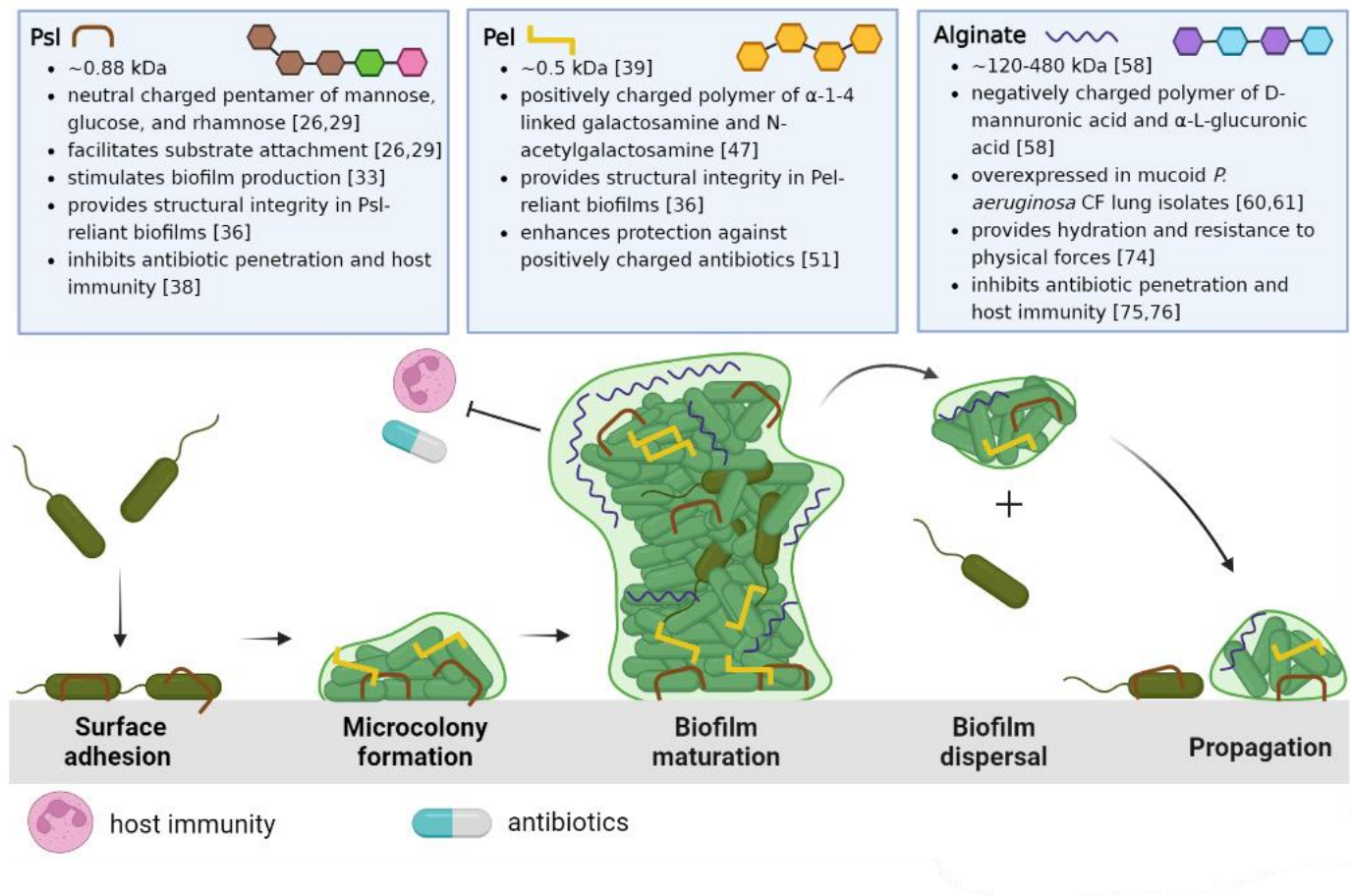

Figure 1: Schematic overview of the *P. aeruginosa* life cycle and the role of matrix exopolysaccharides Psl, Pel, and alginate. The biofilm life cycle begins with Psl-mediated surface/substrate attachment of *P. aeruginosa*. The association of *P. aeruginosa* cells results in microcolony formation as motile cells transition to a sessile phenotype. As the biofilm develops, Psl, Pel, or both EPSs are incorporated in the biofilm architecture to form large 3D macrostructures. The mature biofilm is characterized by the differential expression of these EPSs, the activation of quorum sensing, increasing population heterogeneity, and eventual dispersal to new areas of the lung. Created with Biorender.com

The life cycle described in Figure 1 begins when *P. aeruginosa* attaches to a substratum (which may include other cells) and shifts from a highly virulent, motile phenotype to a more sessile phenotype. This transition is characterized by the slowing of twitching motility and secretion of EPSs that anchor bacterial cells [21]. As *P. aeruginosa* proliferates, structured communities called microcolonies form and grow, eventually resulting in the development of mature biofilms. Maturing biofilms are identified by profound changes in the expression of genes that control twitching motility, EPS secretion, and

quorum sensing systems, resulting in an intricately structured, highly responsive, heterogeneous bacterial community [22]. Moreover, the biofilm structure is also influenced by the complex interactions between the bacteria and the host environment. Genomic studies have revealed that differing rates of up and down-regulation of genes encoding EPSs depend on the particular set of conditions in the bacterial microenvironment [23,24]. When appropriate, the final stage of the biofilm life cycle is characterized by the dispersal of the mature biofilm, releasing virulent cells and non-surface attached aggregates to colonize new areas in the CF lung [25].

The three EPSs of *P. aeruginosa* biofilms, Psl, Pel, and alginate, are all distinct in structure and function within the biofilm, from enabling surface attachment or bacterial aggregation, to maintaining architecture and nourishment in the biofilm, to offering physical protection from antibiotics and host defenses. Their specific properties and physiological significance in the CF lung will be elaborated upon below.

2.1. *Psl and Pel*

Psl is a branched neutral pentasaccharide composed of repeating d-mannose, d-glucose and l-rhamnose subunits and is involved in all stages of *P. aeruginosa* biofilm development [26]. Psl synthesis requires proteins encoded in the *psl* operon, which consists of 15 co-transcribed genes (*pslA – O*) [27,28]. Upon encountering a surface or substrate, Psl anchors *P. aeruginosa* and connects neighboring cells [26,29]. As *P. aeruginosa* swims, it deposits Psl trails that enhance the surface motility of subsequent cells, aiding in the formation of microcolonies [30,31. Sensing Psl further stimulates biofilm development by increasing levels of an important intracellular secondary messenger, cyclic di-guanosine monophosphate (cGMP) [32,33,34]. As the biofilm matures, Psl provides structural integrity by cross-linking CdrA, an extracellular adhesin, and eDNA [21,35–37]. Psl limits the penetration of antibiotics by sequestration, and weakens host immunity by preventing efficient complement deposition, thereby inhibiting the production of neutrophil reactive oxygen species (ROS) and efficient phagocytosis of *P. aeruginosa* [38]. Clinically, Psl is associated with increased *P. aeruginosa* tolerance to tobramycin and is associated with aggregation in the sputum of children with CF, and this has been put forth as a reason for failure of eradication therapy with this antibiotic [39–41]. Although Psl is primarily produced by non-mucoid bacteria, studies have shown it remains utilized in mucoid strains, contributing to biofilm development, maintaining architecture and stability, and evading host immune effectors [42–44].

Pel is a cationic polymer important for biofilm formation, specifically pellicle development at the air-liquid interface, and like Psl, it is primarily associated with non-mucoid *P. aeruginosa* biofilms [45,46]. Pel is synthesized by proteins expressed by the *pel* operon (*pelA – G*), and structurally, it is composed of galactosamine and N-acetyl galactosamine residues linked by partially de-N-acetylated alpha 1-4 glycosidic linkages [47]. In strains that rely on Pel for biofilm formation, such as PA14, the EPS acts as a primary scaffold, maintaining cell-to-cell interactions between bacterial cells in a similar fashion as Psl in PAO1 [36,48–50].The positively charged Pel has been observed to cross-link the negatively charged eDNA through ionic interactions, conferring tolerance against positively

charged antibiotics like tobramycin, but not neutrally charged antibiotics, such as ciprofloxacin [39,51]. Moreover, Pel-eDNA interactions protect eDNA from digestion by DNase I, and thus, (in theory) rendering nuclease-based therapies less effective [39,51].

Although *P. aeruginosa* can produce both Psl and Pel, their relative contribution to biofilm formation and structural integrity is strain dependent. In certain strains understood to be more reliant on Pel such as PA14, knockout Pel mutants are unable to form viable biofilms [52]. However, in strains that rely more on Psl, such as PAO1, knockout Psl mutants inhibit biofilm formation [52]. The localization of Psl and Pel within the biofilm is also strain dependent. In PAO1, Psl is localized to the periphery of mature microcolonies and bacterial aggregates while Pel is localized to the base of mature microcolonies [39]. However, in PA14, it is Pel that localizes to the periphery of mature microcolonies [21,39]. The ability to synthesize both Psl and Pel allows for redundancy in which EPS is incorporated into the matrix, highlighting the adaptive capacity of *P. aeruginosa*. For example, it has been observed that Psl-deficient PAO1 mutants upregulate Pel expression in the peripheral region after several repeat cultures [39,52]. Based on these findings, four classes of *P. aeruginosa* strains have been proposed by Colvin et al.: I) strains that rely on Pel as the dominant matrix polysaccharide (e.g., PA14), II) strains that rely on Psl as the dominant matrix polysaccharide (e.g., PAO1), III) strains that produce an EPS redundant matrix where single *psl* or *pel* mutations lead to impaired biofilm formation, and IV) strains that produce significantly elevated matrix levels such that a single *psl* or *pel* mutation has minimal impact on biofilm biomass [52–54].

2.2. *Alginate*

Alginate is the most studied and extensively characterized EPS secreted by *P. aeruginosa*. In contrast to Psl and Pel, both of which are specific to *P. aeruginosa*, it must be noted that alginate genes can be found in many different bacterial species [55–57]. Alginate is an anionic linear polymer with a high molecular weight (MW: 120 – 480 kDa), and is composed of β-1,4-linked D-mannuronic and α-L-guluronic acids [20,58,59]. The overproduction of alginate results in a mucoid phenotype and is a significant virulence factor in *P. aeruginosa* CF lung infections. [20,60,61]. Clinically, there has been a noted correlation between infection with mucoid *P. aeruginosa* and faster rates of lung function decline than with non-mucoid strains [62–64]. Alginate overproduction is often attributed to an inactivating mutation in the *mucA* gene, which encodes an anti-sigma factor that sequesters AlgT, an activator of alginate biosynthesis. Thus, *mucA* mutations result in constitutive alginate synthesis and the appearance of the mucoid phenotype [61,65–67].

Although wild-type *P. aeruginosa* has the genetic ability to over-produce alginate, it is rarely seen outside the CF lung and arises due to selection pressures in this specific niche. The mucoid phenotype has drastic effects on biofilm structure and the perseverance of infection within the lung, a fact that is corroborated by the observation that clinical isolates from CF patients often possess alginate-overproducing mucoid phenotypes [68–71]. Using whole genome sequencing, Marvig et al. followed the evolution of 474 *P. aeruginosa* isolates collected longitudinally from 34 patients with CF, beginning at initial

infection and continuing for a mean timespan of 4.8 years. They found significant convergent, within-host evolution of 52 pathoadaptive genes, especially those responsible for alginate overproduction [72].

This observed survival advantage in mucoid *P. aeruginosa* has been attributed to several factors, specifically the thick and viscous structure of alginate-overproducing biofilms opposed to the flat and uniform colonies observed in their non-mucoid counterparts [61,73]. In the dehydrated lung mucus of patients with CF, alginate helps maintain the hydration of the bacterial cells, a vital function for cell survival and maintenance [68,74]. Furthermore, alginate serves as a protective barrier against antimicrobial therapy and host-mediated immune responses like macrophage phagocytosis [75,76]. This evidence supports the hypothesis that alginate overproduction reduces the clinical efficacy of antibiotic therapy in patients with CF, and for this reason alginate-specific therapeutics will be explored further in this review [77–80].

3. **Therapies**

3.1. *Matrix degrading enzymes*

Proposed anti-biofilm therapies employing matrix degrading enzymes include PslG glycoside hydrolase (PslG$_h$), PelA glycoside hydrolase (PelA$_h$), and alginate lyase (AgLase). While PslG$_h$ and PelA$_h$ are both synthesized by and specific to *P. aeruginosa* matrix EPSs, AgLase can be isolated from a variety of sources including algae, mollusks, bacteria, viruses, and fungi [81]. The known properties, effects, and toxicities of these compounds (including OligoG discussed later in this review) are summarized in Figure 2.
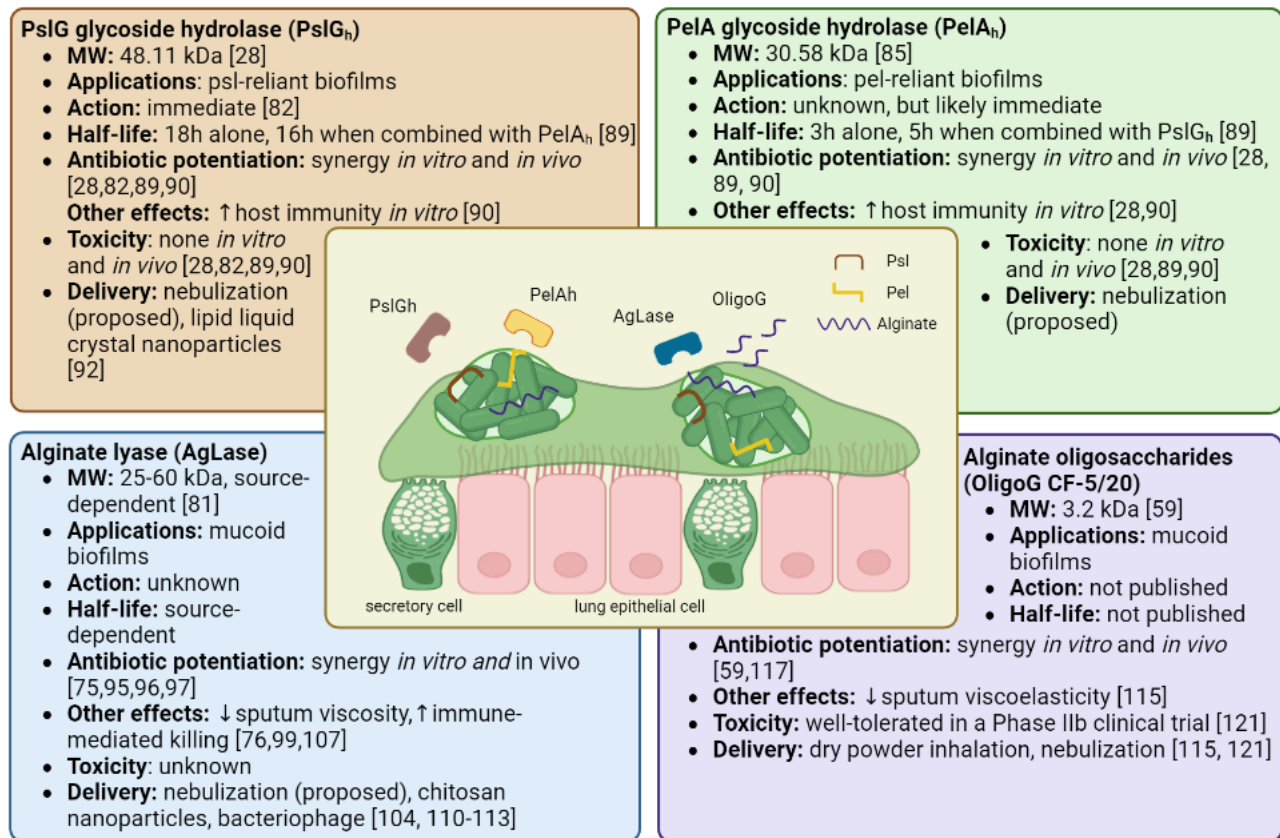
Figure 2: Overview of therapeutics targeting matrix EPSs of *P. aeruginosa* biofilms suspended in CF airway sputum. Outlined are potential therapeutic applications, drug mechanisms, half-life, synergistic antibiotics, immune effects, safety, and methods of delivery for PslG$_h$ (top left), PelA$_h$ (top right), AgLase (bottom left), and OligoG (bottom right).

PslG is a 48.11 kDa molecular weight (MW) periplasmic protein involved in Psl synthesis, and is co-transcribed with 15 other genes in the *psl* operon [28,82]. PslG contains an electronegative glycoside hydrolase domain (PslG$_h$) that cleaves Psl chains by targeting adjacent mannose residues [28,82]. PelA is a 101.3 kDa multidomain, periplasmic protein encoded by the first gene of the *pel* operon and contains a deacetylase domain and a glycoside hydrolase domain with distinct functions [52,83]. Its deacetylase domain is essential to Pel synthesis as it gives Pel a positive charge, allowing it to associate with other charged elements in the biofilm matrix such as eDNA [39,83,84]. Its glycoside hydrolase domain (MW: 30.58 kDa), herein PelA$_h$, cleaves cationic, partially deacetylated residues within Pel residues and is involved in biofilm dispersal [85–87]. Given the highly selective action of PslG$_h$ and PelA$_h$ against their substrates Psl and Pel, which have critical roles in biofilm development and functioning, PslG$_h$ and PelA$_h$ have been studied as adjuvant therapies for eradicating *P. aeruginosa* biofilms.

Several lines of evidence from *in vitro* studies support the potential of PslG$_h$ and PelA$_h$ for the treatment of *P. aeruginosa* biofilms. When supplied exogenously, PslG$_h$ and PelA$_h$ can disrupt *P. aeruginosa* biofilms at nanomolar concentrations with extremely high specificity [82,86]. In developing biofilms, PslG$_h$ impairs the ability of *P. aeruginosa* to associate with surfaces and other bacteria, leading to more rapid, scattered movement of *P.*

aeruginosa, delaying microcolony formation and inhibiting biofilm development [88]. The half maximal effective concentration (EC50) values of PslG$_h$ and PelA$_h$ to disrupt mature PAO1 biofilms after 1 hour were 12.9 ± 1.1 and 35.7 ± 1.1 nM, respectively [86]. When combined, PslG$_h$ - PelA$_h$ improved the penetration and efficacy of ciprofloxacin, tobramycin, colistin, neomycin, and polymyxin, allowing for smaller antibiotic dosages [89,90]. PslG$_h$ also works remarkably quickly, completely disrupting all Psl fibers within 5 minutes of administration and triggering sudden biofilm dispersal [82]. Moreover, PslG$_h$ -dispersed bacteria has a lower MIC for tobramycin and ciprofloxacin compared to planktonic bacteria [82]. PslG$_h$ further sensitizes the host immune system towards *P. aeruginosa* biofilms, increasing the deposition of C3 complement proteins which stimulates macrophage and neutrophil phagocytosis and neutrophil ROS production [28,82,90]. When tested *in vitro*, both PslG$_h$ and PelA$_h$ were observed to be non-toxic to neutrophils, lung fibroblasts, and red blood cells, and PslG$_h$ was additionally non-toxic to colonic epithelial cells and macrophages. [28,82,90]. Experiments using animal models of *P. aeruginosa* have validated *in vitro* findings of the efficacy and safety of PslG$_h$ and PelA$_h$. When injected locally, PslG$_h$ potentiated tobramycin killing of mature *P. aeruginosa* biofilms on mouse peritoneum implants [82]. In a wound infection model, prophylactic treatment with PslG$_h$ showed an additive killing effect when tobramycin was added 24 hours later and was not toxic to the wound [90]. PelAh embedded in a bacterial cellulose membrane effectively destabilized *P. aeruginosa* biofilms when topically applied to infected murine chronic wounds [91]. Although a majority of studies have utilized wound and implant models, the results demonstrate the proof of concept for topical (i.e. nebulized) application in pulmonary infection in CF.

In the most comprehensive pre-clinical evaluation of PslG$_h$ and PelA$_h$ to date, Ostapska et al. prophylactically administered PslG$_h$ and PelA$_h$ intratracheally to mice with *P. aeruginosa* lung infections with or without antibiotics [89]. They found that the antimicrobial effects of ciprofloxacin, but not ceftazidime, were potentiated when administered every 8 hours following initial treatment with PslG$_h$ - PelA$_h$. In healthy mice, a single intratracheal dose of up to 250/250 ug of PslG$_h$ - PelA$_h$ was well tolerated, with no changes in weight, temperature, mortality, makers of pulmonary injury, or numbers of macrophage, eosinophils, and neutrophils cells compared to buffer-treated mice. However, there was an increase of pulmonary lymphocytes following PslG$_h$ - PelA$_h$ treatment, highlighting the need to further investigate the adaptive immune response to this therapy. When administered alone, PslG$_h$ has a half-life of 18 hours and PelA$_h$ has a half-life of 3 hours. However, PelA$_h$ stability increased to 5 hours when co-administered with PslG$_h$ and catalytic activity was retained >24 hours. However, PslG$_h$ - PelA$_h$ treatment of *P. aeruginosa* infected mice in the *absence of antibiotics* triggered pulmonary inflammation and lethal septicemia, underscoring the necessity of antibiotic co-treatment [89].

It is worth mentioning several technological developments that may enhance the feasibility of PslG$_h$ and PelA$_h$ in clinical applications. Lipid liquid crystal nanoparticles encapsulating PslG$_h$ and tobramycin offer protection against proteolytic degradation and are releasde upon encountering *P. aeruginosa*. This product was seen to be 10-100 fold

more effective at eradicating *P. aeruginosa* infections *in vivo* using a Caenorhabditis elegans infection model [92]. PslG$_h$ constructs with significantly enhanced trypsin resistance have also been developed, which may extend the half-life of PslG$_h$, allowing for smaller and less frequent doses [93]. PslG$_h$ can also be immobilized on the lumen surface of medical-grade polyethylene, polyurethane, and polydimethylsiloxane (silicone) catheter tubing, reducing initial attachment of *P. aeruginosa*. The bacterial burden of PslG$_h$ -modified catheters decreased by 3 logs for up to 11 days under dynamic flow culture conditions and 1.5 logs when used in *in vivo* rat infection models [94].

Published research has reported that mucoid, alginate-rich *P. aeruginosa* isolates produce heterogenous biofilms with a high tolerance to antibiotics [61]. Many anti-biofilm strategies have been designed and examined for increasing the susceptibility of antibiotics through disrupting the alginate EPS. One such promising therapeutic technique is using the enzyme AgLase, which can be derived from various sources, and weigh anywhere between 25-60 kDa [81]. Depending on its source, AgLase can degrade alginate at a variety of cleavage points, disrupting established mucoid *P. aeruginosa* biofilms, and enhancing antibiotic penetration and host immune functions [81]. AgLase has been reported *in vitro* and *in vivo* to disrupt biofilms, enhancing the efficacy of amikacin, tobramycin, ciprofloxacin, and gentamicin [75,95,96,97]. AgLase co-administration with DNase has been found to potentiate antimicrobial biofilm eradication to a greater effect than seen with either agent on its own [97,98]. AgLase enhances neutrophil killing, macrophage phagocytosis, and alveolar macrophage efferocytosis [76,99]. It is worth noting, however, the conflicting evidence for AgLase as an antibiotic adjunct. One study reported that AgLase did not have any effect on pure or mixed cultures of *P. aeruginosa* biofilms, a fact which authors attributed in certain instances to alginate not being the main contributing component of the biofilm and in others to protection from enzymatic degradation due to the presence of other molecules [100]. Another study compared two AgLases to proteins similar in structure, but without the capacity to enzymatically degrade alginate, and observed equal rates of bacterial biofilm disruption and antibiotic synergy between these compounds, suggesting that the potentiation effects of AgLases are uncoupled to their catalytic activity [101]. AgLase was also unable to degrade mucoid *P. aeruginosa* biofilms embedded in sputum [102].

Though the evidence is unclear if and how AgLases combat bacterial biofilms, AgLase products with therapeutic implications are under development. AgLase-polyethylene glycol conjugates have been developed that significantly reduce its immunoreactivity [103]. AgLase functionalized chitosan nanoparticles of ciprofloxacin are novel delivery carriers, which has demonstrated enhanced biofilm degradation in *in vitro* experiments with a mucoid clinical *P. aeruginosa* strain, without toxicity to human lung epithelial cells or rat lung tissues [104]. In another distinct approach, it has long been known that bacteriophage (viruses that infect and lyse bacterial cells) employ polysaccharide depolymerases to migrate through polysaccharide-rich biofilms and infect embedded bacterial cells [105,106]. One study using a mucoid *P. aeruginosa* isolate revealed that *P. aeruginosa*-specific bacteriophage could reduce the viscosity of purified CF alginate by up

to 40% and penetrate through the mucoid alginate matrix to lyse biofilm-associated bacteria [107]. In a subsequent study, Glonti et al. isolated an alginate lyase responsible for the alginate-degrading properties of a *P. aeruginosa* bacteriophage, and reported activity against alginate purified from many sources, including from clinical CF *P. aeruginosa* isolates [108]. Finally, a *P. aeruginosa* phage isolated from hospital sewage was able to disrupt mature *P. aeruginosa* biofilms and potentiate serum bactericidal activity [109]. A number of clinical trials involving the use of bacteriophage for *P. aeruginosa* lung infection in CF are currently underway, and although the contribution of specific depolymerases to the antibiofilm properties of *P. aeruginosa* bacteriophages are to be determined, it will be interesting to see whether such enzymes will play a role in the success or failure of these therapies (NCT04596319; NCT04684641; NCT05010577; NCT05453578) [110–113].

3.2. *Other novel strategies*

Alongside the development of matrix degrading enzymes as adjunctive therapies for patients CF, other novel therapeutics that may enhance airway clearance and lung function are being investigated. One such example is the 3.2 kDa alginate oligosaccharide, OligoG CF-5/20 (OligoG), which is sourced from the brown seaweed, *Laminaria hyperborean* and can reduce the viscoelasticity of CF sputum [59,114,115]. Being relatively small, OligoG can also readily diffuse through mucoid biofilms and disrupt EPS within biofilm matrices [59,116]. OligoG disrupts biofilms in a time and dose dependent manner and can acts as an antibiotic potentiator, increasing the penetration and efficacy of tobramycin, erythromycin, colistin, and ciprofloxacin [59,116–118]. Mice intratracheally infected with a mucoid clinical *P. aeruginosa* isolate showed a 2.5 log reduction in bacterial colony-forming units (CFUs) following treatment with 5% OligoG [117]. Like the aforementioned matrix disruptors, as OligoG is not bactericidal, it is unlikely to create an adaptive pressure, a theory supported by studies showing prolonged treatment with OligoG does not induce resistance [114]. Furthermore, OligoG-colistin conjugates have been developed that can produce a sustained biofilm inhibitory effect, while limiting the toxicity of colistin [119]. There are also novel compounds under development that combine the mucoactive properties of alginate oligomers with nitric oxide release as an added antibiofilm agent to improve the efficacy of antibiotics [120].

Importantly, OligoG is the first inhaled polymer therapy that has been investigated in humans as a potential novel therapeutic approach for airway clearance in CF patients. AlgiPharma, the lead developer of OligoG, has been spearheading efforts to develop OligoG as a novel CF therapeutic and have completed several clinical trials, although the results of only one Phase 2b trial has been published so far. In this randomized, double-blind, placebo-controlled, multi-centered, crossover study, 90 adult CF patients were screened, and 65 patients were randomly allocated to receive either OligoG via dry powder inhalation (1050 mg per day) or a placebo. The primary endpoint was forced expiratory volume in 1 second (FEV1), measured at the end of the 28-day treatment regimen [121]. This study revealed that OligoG administered thrice daily via dry powder inhalation was well tolerated, with no significant differences in serious adverse events between treatment and placebo groups. Furthermore, concentration in plasma was in the range

of 0.5-8.98 µg·mL^{-1}, with no detectable OligoG in plasma after 28 days of washout (day 56 of the study). Despite this reassuring safety data, however, no significant improvement in FEV1 was observed in the treatment group. Interestingly, post hoc exploratory analyses indicated that patients on inhaled tobramycin and patients <25 years of age showed positive trends in lung function, highlighting that further studies of OligoG are essential [121]. A prospective clinical study is currently being planned under the framework of HORIZON2020 by the European Clinical Trial Network [122]. Whatever the outcome, these trials will shed new light on the therapeutic potential of disrupting alginate biofilms in *P. aeruginosa* pulmonary infection.

4. **Discussion**

    Successful treatment of *P. aeruginosa* CF pulmonary infections is difficult to achieve, in part due to the heterogeneity of bacterial populations within the biofilm (cells with differing metabolism, mutation rate, phenotypic expression of virulence factors, etc.) that limits the effectiveness of broad-spectrum antibiotics at our disposal. The agents described in this review all have the potential to disrupt the biofilm structure. Although not bactericidal by themselves, by disrupting the extracellular matrix, the hope is that an adjuvant effect will result from exposing bacterial cells to both effectors of the immune system (like alveolar macrophages and neutrophils), as well as to antibiotics. Additionally, their selectivity for targeting *P. aeruginosa* infections make them appealing adjuncts in an era of personalized medicine, while limiting off-target effects. These agents are summarized in Table 1, which focuses on the challenges and considerations that exist as these compounds transition from laboratory bench to CF patient, or from clinical trial to effective therapy.

Table 1: EPS-Targeted Therapies of *P. aeruginosa* Biofilms in CF

Product	Therapeutic Challenges and Considerations
PslG glycoside hydrolase (PslG$_h$)	• Unclear relevance of Psl to biofilm infections across clinical isolates • Uncertain stability of PslG$_h$ in CF sputum • Optimal dosing concentration and regimen unknown • Risk of disseminated infection if not given with antimicrobials [89]
PelA glycoside hydrolase (PelA$_h$)	• Unclear relevance of Pel to biofilm infections across clinical isolates • Uncertain stability of PelA$_h$ in CF sputum • Optimal dosing concentration and regimen unknown • Risk of disseminated infection if not given with antimicrobials [89]

Alginate Lyase (AgLase)	• Unclear stability of compound in CF sputum • Different AgLases have been isolated from diverse sources, each with distinct enzymatic profiles [81] • Data conflicting on antibiotic potentiation effects (e.g. not as effective when alginate not main component of biofilm) [123] • Biofilm degrading capacity of AgLase may be independent of its catalytic activity [101] • AgLase degradation of alginate in CF mucoid *P. aeruginosa* biofilm was ineffective *in vitro*, when incubated with sputum [102]
Alginate Oligosaccharide (OligoG, CF-5/20)	• Primary endpoint (improved FEV1) not met in Phase 2b clinical trial [121] • 45% of patients did not adhere to three times daily dosing regimen • Younger patients and those on continuous or inhaled antibiotics showed trend towards improved efficacy • Larger Phase 3 clinical trials needed

One should note that some of the compounds mentioned in Table 1 are further along the development pipeline than others, with OligoG being the only compound administered to humans in clinical trial. Although this trial failed to meet its primary endpoint (improvement in FEV1), there were several trends in post-hoc analyses that should be taken into consideration for clinical trials in development, including the use of a placebo inhaler unexpectedly reduced bacterial burden in sputum, and some promising trends in certain patient groups.

The matrix degrading enzymes $PslG_h$, $PelA_h$, and AgLase all have shown efficacy *in vitro* and *in vivo* as potent biofilm-disruptors and antibiotic potentiators in their own right, although it is clear from Table 1 that if these compounds are to be developed into viable antibiotic adjuncts in CF patients, more research is needed. Specifically, investigations across many different clinical strains are required to demonstrate whether there is the potential for widespread applicability or whether the promising effects that have been observed are specific to particular bacterial strains. To overcome this high level of specificity, studies bringing these therapies to animal models have focused on co-administration of certain compounds, whether it is a DNase or another glycoside hydrolase [97,124]. Combining therapy of different matrix-degrading enzymes may have many benefits, from targeting multiple EPSs at once and achieving broader therapeutic coverage, to extending the half-life of the compounds, as observed when combining $PelA_h$ with $PslG_h$ [124]. Moreover, there is no reason that antibiotics with different mechanisms of action should all act in additive or synergistic ways with these compounds, and this is illustrated by some of the preliminary *in vitro* work showing additional benefit when $PslG_h$ was administered with the fluoroquinolone ciprofloxacin, but not with the third generation cephalosporin ceftazidime – both commonly used agents in *P. aeruginosa* infection. [124]

Additional information is also needed for optimal dose concentrations for safety and efficacy, as well as whether specific antibiotic combinations have more synergistic potential than others. Although PslG$_h$ and PelA$_h$ have been demonstrated to be non-cytotoxic to mammalian cells in all the studies included in this review, Ostapska et al. showed an increase in the number of pulmonary lymphocytes following glycoside hydrolase administration, warranting further study, as these compounds may trigger specific adaptive immune responses over-time. Of critical importance is the hematological dissemination and lethal septicemia that has been seen in animal models if glycoside hydrolases are used in the absence of antibiotics [124]. Additionally, most of the *in vivo* studies use wound or implant infection models, leaving a gap in knowledge concerning the efficacy of these compounds in pulmonary infection models, which better represent CF lung infection.

Concerning the mode of administration for these compounds, with the exception of OligoG, it is unknown whether (i) they are stable when nebulized or inhaled and (ii) can be administered in sufficient quantity to exert clinically significant antibiotic potentiation. Given that the glycoside hydrolases PslG$_h$ and PelA$_h$ disperse biofilms almost immediately, it seems reasonable to develop these compounds either for administration prior to or for co-administration with antibiotics. Furthermore, there is lacking information on the stability of these compounds in CF sputum, where it is possible that the presence of proteases and other specifics of the environmental niche (e.g. temperature, pH, etc.) could denature these enzymes before they have the ability to degrade the biofilm. Some of these concerns may be addressed by technological modifications briefly introduced in this review, such as novel lipid liquid crystal and chitosan nanoparticles to enhance delivery, protease-resistant hydrolase/lyases that may increase enzymatic longevity, and polyethylene glycol-conjugation that may decrease immunoreactivity and improve safety profiles.

Lastly, we must address the chasm that exists between clinical outcomes in patients with CF lung infection and *in vitro* laboratory methods for testing antibiotic sensitivity [125]. This is likely due to the simplicity of *in vitro* assays and their inadequacy of simulating a biofilm mode of growth and the myriad of conditions encountered in the human lung. However, even when clinical trials have assessed choice of antibiotic based on assays that have used bacterial biofilms, there has been no link with patient outcome [126,127]. There are a variety of assays that have been developed/are in development that aim to rectify this disconnect, including the use of artificial or CF sputum, animal models of pulmonary infection, as well as the polymicrobial nature of the lung environment.

Concerning the latter, *P. aeruginosa* matrix EPSs have also been noted to play important roles in mixed-species communities, conferring both communal benefits and competitive advantages. For instance, Psl is required to integrate *P. aeruginosa* into mixed-species biofilms with *Pseudomonas protegens* and *Klebsiella pneumoniae* and also confers communal stress resistance [128]. Psl and Pel promote the development of mixed-species biofilms with *Staphylococcus aureus* and can further grant antibiotic-tolerance to Psl non-producers such as *Escherichia coli* and to *Staphylococcus aureus* by binding secreted staphylococcal protein A [129–131]. The alginate overproduction of mucoid *P. aeruginosa*

isolates from the CF lung not only render them more permissive to the growth of *S. aureus* [132], but have been seen to protect *Burkholderia cenocepacia* from inflammatory responses and host immune effectors [133]. Thus, disruption of *P. aeruginosa* matrix polysaccharides may yield a greater therapeutic effect beyond the scope of the primary infection [134].

5. Conclusion

Despite tremendous progress in our understanding of bacterial biofilms in recent years, *P. aeruginosa* lung infection remains a significant cause of morbidity and mortality in the CF population [16]. The recent introduction of highly effective modulator therapy (HEMT) in CF has shown great promise in restoring some CFTR protein function and thus impacting mucus and the CF lung environment in clinically meaningful ways, improving pulmonary function, BMI, patient-reported quality of life, and decreasing rates of pulmonary exacerbations [135]. Additionally, there have been reports that the modulator ivacaftor (used in most of the currently available HEMT) might possess some antimicrobial activity both directly [136], and when combined with common antibiotics [137,138]. Though it is not clear yet what impact these therapies will have on lung infection generally, by "normalizing" the lung environment, the hope is that younger patients starting on these therapies will not be affected by common CF pathogens. However, current evidence suggests that even with HEMT, CF lung disease still progresses, albeit slower, and organisms like *P. aeruginosa* will continue to be problematic for the majority of individuals with well-established lung disease [139–141]. Additionally, the high cost of HEMT and lack of uniform provision across different health systems means that many people in developing countries with CF will not be able to benefit from these compounds in the short term [135]. There also remains a population of CF patients that possess CFTR mutations not amenable to treatment by current HEMT, and patients have withdrawn from HEMT due to adverse hepatic, somatic, or psychiatric symptoms [139]. It is thus important to continue searching for novel antimicrobial strategies and compounds to improve outcomes in patients.

The three EPSs produced by *P. aeruginosa* (Psl, Pel and alginate) each represent novel targets for antibiotic adjuvant therapy. As we enter an era of more personalized medicine, tailored therapies may hold the key to antibiotic potentiation that we have been searching for, whether the target is an alginate over-producing mucus isolate or a Psl/Pel-rich one. Despite this promise, the only compound discussed in this review that has been given in clinical trials (OligoG) failed to meet the primary endpoint of increased lung function at the end of the trial period. Many of the other compounds, including matrix-degrading enzymes, have only been administered in animals and data are lacking on applicability across clinical strains found in the CF lung. Hence, there are several steps before such compounds come to human clinical trials. Further *in vitro* work will need to establish efficacy of these compounds across a variety of clinical strains, in addition to considering other clinically relevant questions, including optimizing concentrations, and assessing mode and timing of delivery in relation to other therapies.

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