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Emerging strategies to target virulence in *Pseudomonas aeruginosa* respiratory infections

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen that is responsible for infections in people living with chronic respiratory conditions, such as cystic fibrosis (CF) and non-CF bronchiectasis (NCFB). Traditionally, in people with chronic respiratory disorders, *P. aeruginosa* infection has been managed with a combination of inhaled and intravenous antibiotic therapies. However, due in part to the prolonged use of antibiotics in these people, the emergence of multi-drug resistant *P. aeruginosa* strains is a growing concern. The development of anti-virulence therapeutics may provide a new means of treating *P. aeruginosa* lung infections whilst also combatting the AMR crisis, as these agents are presumed to exert reduced pressure for the emergence of drug resistance as compared to antibiotics. However, the pipeline for developing anti-virulence therapeutics is poorly defined, and it is currently unclear as to whether *in vivo* and *in vitro* models effectively replicate the complex pulmonary environment sufficiently to enable development and testing of such therapies for future clinical use. Here, we discuss potential targets for *P. aeruginosa* anti-virulence therapeutics and the effectiveness of the current models used to study them. Focus is given to the difficulty of replicating the virulence gene expression patterns of *P. aeruginosa* in the CF and NCFB lung under laboratory conditions and to the challenges this poses for anti-virulence therapeutic development.

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P. aeruginosa in CF and NCFB

Persistent respiratory diseases, such as chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis (CF) and non-CF bronchiectasis (NCFB) are responsible for approximately 4 million annual deaths worldwide, and thus, represent a major global health challenge (Soriano et al. 2020). (Maselli et al. 2017; Bierlaagh et al. 2021; Ghigo et al. 2021). Of the bacteria responsible for CF and NCFB pulmonary infections, *P. aeruginosa* is amongst the most commonly isolated (Mésinèle et al. 2022). Where antibiotic susceptibility allows, acute cases of *P. aeruginosa* infection in NCFB are treated with oral antibiotics, with those progressing to chronic infection often being managed with intravenous antibiotics during periodic acute exacerbations (García et al. 2011; Macfarlane et al. 2021). Intravenous antibiotics are also usually prescribed to people with CF (pwCF) experiencing periodic pulmonary exacerbations. However, ongoing inhaled antibiotic treatment is currently the standard of care for the management/suppression of chronic *P. aeruginosa* infection in pwCF, with such treatments acting to improve respiratory symptoms and decrease bacterial load within the lungs (Van de Kerkhove et al. 2016; Castellani et al. 2018; Haworth et al. 2019). Continuous antibiotic regimes put pwCF at severe risk of developing multi-drug resistant infections, and although they are used to manage chronic infection and alleviate exacerbations, antibiotics rarely resolve airway infections (Macfarlane et al. 2021). Anti-virulence therapeutics target bacterial virulence factors, without inducing bacteriostatic or bactericidal effects (Watson et al. 2020). The development of anti-virulence therapeutics may provide a clinically effective means of treating *P. aeruginosa* lung infections whilst also combatting

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the AMR crisis. Unlike antibiotics, anti-virulence therapies do not directly target essential bacterial processes and, therefore, it is expected that they will engender a weaker evolutionary pressure for AMR (Beceiro et al. 2013; Totsika 2016). Anti-virulence therapeutics may also act as host-targeted therapies by reducing inflammatory damage that occurs during bacterial infection and colonization (Watson et al. 2020). It may be possible to prescribe these therapeutics in conjunction with traditional agents, allowing reduced doses of antimicrobial to be used and thereby slowing the emergence of resistance (Rezzoagli et al. 2020).

An effective anti-virulence therapeutic for chronic respiratory infections must target a factor that is key for bacterial pathogenesis in the lung niche. However, due to the altered physical and chemical environment of the CF and NCFB lung, and the polymicrobial nature of many chronic lung infections, identifying such factors and developing *in vitro* models for their study can be a significant challenge (Reece et al. 2021). It is also difficult to determine whether the effects of virulence factors studied *in vitro* mimics the role they play within these disease syndromes (Ibberson et al. 2017; O'Brien and Welch 2019; Barton et al. 2022). This poses challenges when screening novel antimicrobial agents, as results obtained in simple growth media often do not reflect drug activity *in vivo*. Such challenges are exacerbated when screening anti-virulence drugs, as standard microbiological endpoints that measure bacterial density or viability, such as Minimum Inhibitory Concentration (MIC) assays do not provide an understanding of host-pathogen interactions, nor do they provide a full picture of drug-pathogen interactions (Totsika 2016). The pipeline for developing anti-virulence therapeutics is unclear, but for any chosen target, importance into the progression of the infection must be established and must also be implemented in the drug testing platforms.

Key virulence factors within respiratory syndromes

P. aeruginosa has an extensive repertoire of virulence factors (Figure 1) and they are expressed in an environment-dependent manner. Thus, virulence factor expression is influenced by co-infecting microbes, therapeutic administration and the physiochemical characteristics of the infection site (e.g. temperature, viscosity,

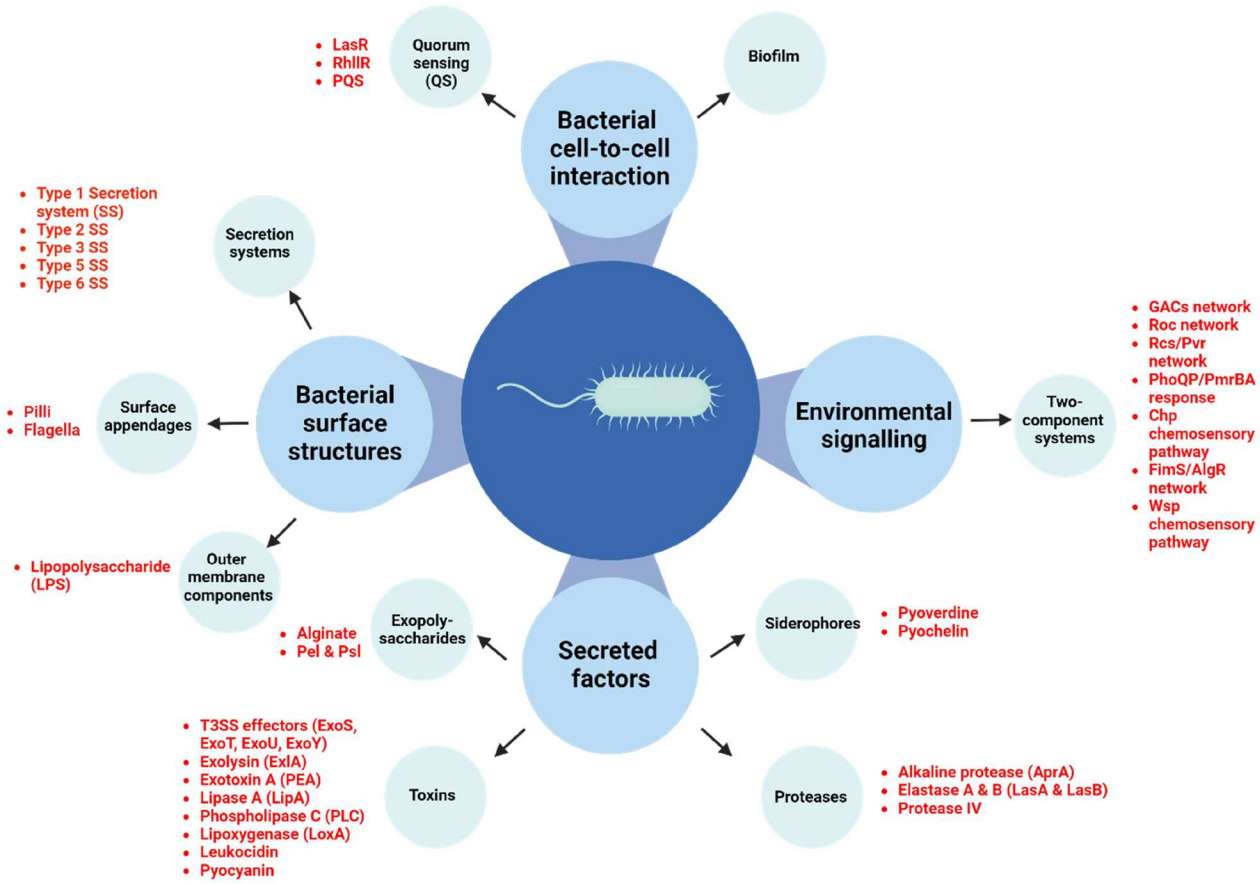

Figure 1. The virulence factors of P. aeruginosa (red).

nutrient and oxygen availability) (Winstanley et al. 2016). However, there are virulence factors which are described as "key" in initiating infection and in the development of disease (Table 1) and thus, such factors are increasingly becoming targets for the development of anti-virulence therapies. Those factors will be the subject of this review.

Adaption to the respiratory environment

The microbial landscape of the CF lung is patient specific and dynamic, particularly throughout childhood (Zemanick et al. 2017). However, subsequent to colonization with *P. aeruginosa*, microbial diversity is lost with age and disease progression, highlighting respiratory microbial diversity as a co-correlate of lung function (Françoise and Héry-Arnaud 2020). The NCFB lung is also a highly complex environment, and like in pwCF, *P. aeruginosa* is commonly isolated from NCFB sputum samples (Rogers et al. 2013; Woo et al. 2019; Dicker et al. 2021). Chronic colonization with *P. aeruginosa* is similarly associated with a decrease in microbial diversity, as well as an increase in disease progression and a decrease in lung function. This highlights *P. aeruginosa* as a cornerstone pathogen within CF and NCFB communities and therefore efforts into developing novel treatments to manage chronic infection is paramount. When developing and testing such treatments, it is important to consider the changes *P. aeruginosa* undergoes to establish chronic colonization within the pulmonary environment. Particularly the genetic changes which allow the bacterium to migrate from carriage in the non-sterile upper respiratory tract to chronic colonization within the lungs (Mahenthiralingam et al. 1994; Hansen et al. 2012; Markussen et al. 2014; Moore et al. 2021; Wardell et al. 2021; Eklöf et al. 2022). These include changes which allow the bacterium to cope with various stress factors that are typical of the CF lung environment, such as osmotic stress due to the presence of negatively charged, hydrophobic mucous, oxidative and nitrosative stresses owed to host responses, the presence of other microorganisms and, most notably in the CF lung, the exposure to sublethal concentrations of antibiotics (Winstanley et al. 2016). The effect of corrector and modifier drugs, such as CFTR modulators on the microenvironment of the CF lung must also be considered when developing preclinical models for the development of anti-virulence therapeutics.

The switch between a chronic and acute lifestyle is also an important manifestation of phenotypic flexibility in *P. aeruginosa,* and is significant in the initiation of pulmonary exacerbations. Cao et al. (2023) find that the deletion of *sicX,* which encodes a small RNA that is induced in low oxygen conditions, causes *P. aeruginosa* to transition back to an acute lifestyle in a range of *in vivo* models. Downregulation of *sicX* was also evident in acute infection (septicaemia) caused by dispersal of *P. aeruginosa* from chronic lung infection (Cao et al. 2023). The failure of studies to identify the expression of *sicX* in *in vitro* models to prior to this study – despite its centrality to a key pathogen lifestyle switch - highlights the challenges of capturing the *P. aeruginosa* virulome under laboratory conditions.

The upregulation of alginate production (referred to as mucoidy) by *P. aeruginosa* is often considered a marker for the transition to chronic infection in CF, due to its association with biofilm formation (Winstanley et al. 2016). Less research has been dedicated to study of the evolutionary changes *P. aeruginosa* undergoes to establish and maintain pulmonary infections in NCFB, but the presence of alginate-producing *P. aeruginosa* has also been described as a common feature in the NCFB lung. Hilliam et al. (2017) demonstrated that, amongst 99 *P. aeruginosa* genomes isolated from NCFB sputum, 13 independent mutations in *mucA*, a modulator of the sigma factor AlgU, were identified (Hilliam et al. 2017). These mutations can contribute to bacterial adaptation to the lung environment through the promotion of mucoidy. Woo et al. (2016) also identified *P. aeruginosa* presenting with a mucoid phenotype in sputum originating from the CF and NCFB lung; (Woo et al. 2016). The acquisition of a mucoid phenotype can increase tolerance to antibiotics, as alginate both protects the bacterium from host phagocytosis and can prevent penetration by antimicrobials (Davarzani et al. 2021; Gao et al. 2022; Liang et al. 2023). Activity against mucoid *P. aeruginosa* must also be considered when developing anti-virulence therapeutics which target *P. aeruginosa* colonization within CF and NCFB lungs.

Although *P. aeruginosa* chronic lung infections are common across both pwCF and pwNCFB, the importance of individual virulence factors in each condition is sometimes less clear. An ideal anti-virulence target would be produced in abundance within the respiratory environment; however, this is often challenging to demonstrate. For example, higher prevalence of elastase-null isolates in the NCFB compared with the CF lung has been reported by Woo et al. (2016), suggesting that elastase may not be necessary for lung survival and may have differing importance across respiratory conditions. However, limited data is available for the evolution of the *P. aeruginosa* virulome during NCFB lung infections making comparisons between pwCF and pwNCFB challenging. As elastase production is regulated by the LasR system, mutations

Table 1. *P. aeruginosa* virulence factors and their expression in laboratory models and CF sputum.

Category	Virulence factor	Functions	Expression of gene in CF sputum (Y/N)	Expression compared to CF sputum			References
				LB	SCFM2	Mouse lung model	
Bacterial cell-to-cell interactions							
Quorum sensing	LasR	Regulates the maturation of biofilms, antimicrobial resistance, swarming motility and interactions with host cell signalling, as well as the production of elastases, pyocyanin and Type 3 secretion system (T3SS) effectors. The LasIR system has been described as the "master regulator," due to its roles in regulating the activities of the other QS system.	Y	*	*	*	(Venturi 2006)
	Pqs		Y	↑	*	*	
	RhlR		Y	*	↑	*	
Biofilm	–	Avoid host immune responses, survival of bacterial community during harsh conditions, confers resistance to antibiotics.	–	–	–	–	(Chen et al. 2015)
Bacterial surface structures							
Secretion systems	Type 3 Secretion system (T3SS)	Injection of virulence factors into host cell.	–	–	–	–	(Liao et al. 2022)
Secreted factors							
Proteases	Elastase B (LasB)	Degrades elastin, resulting in tissue damage and the liberation of nutrients that can be exploited by *P. aeruginosa*.	Y	*	*	*	(Cathcart et al. 2011)
Siderophores	Pyoverdine	Iron chelation, which can act to exert cytotoxicity, subsequently inducing mitochondrial damage, mitophagy and hypoxic crisis upon host cells.	Y	*	*	↑	(Kirienko et al. 2013; Kirienko et al. 2015
	Pyochelin	Iron chelation, which can act to exert cytotoxicity, subsequently inducing mitochondrial damage, mitophagy and hypoxic crisis upon host cells.	Y	*	*	↑	(Tyrrell and Callaghan 2016)
Toxins	T3SS ExoS	Disruption of host actin cytoskeleton, interference of cell junctions, inducing phagocytosis, host cell apoptosis and tissue injury.	Y	*	*	↑	(Liao et al. 2022; Jurado-Martín et al. 2021)
	T3SS ExoT		Y	*	*	↑	
	T3SS ExoU		Y	–	–	–	
	T3SS ExoY		Y	*	*	*	
	Pyocyanin	Cytotoxic, facilitates iron uptake through reducing iron bound to host proteins.	Y	*	*	*	(Liao et al. 2022; Ghssein and Ezzeddine 2022)

Expression in CF sputum was reported using data from pseudomonas.com (Winsor et al. 2016). The expression in laboratory models was based on data relative to CF sputum, reported as Z-scores (Cornforth et al. 2018). ↑ indicates higher expression than in CF sputum (>2-fold increase in Z-score). ↓ indicates lower expression than in CF sputum (<-2-fold decrease in Z-score). * indicates the expression in the model is comparable to CF sputum (-2< Z-score <2-fold). – indicates missing date for the expression in the models or CF sputum.

of the *lasR* quorum sensing (QS) transcription regulator gene are often responsible for the emergence of elastase-null isolates. Loss of function mutations in *lasR* leads to several clinically significant phenotypic changes, including growth advantages on carbon and nitrogen sources, and also increases in β-lactamase activity (D'Argenio et al. 2007; Woo et al. 2016). Clay et al. (2020) propose that QS mutations may benefit *P. aeruginosa* through increased fitness under low oxygen conditions, conferring a survival advantage in conditions relevant to both the CF and NCFB lung (Clay et al. 2020). The fact that QS mutants have survival advantages in hypoxic conditions is interesting, as such mutants have traditionally been described as being attenuated for virulence (Tang et al. 1996; Rumbaugh et al. 1999). These findings highlight the challenges in translating observations made using *in vivo* models to clinical benefit in the context of chronic infection, with individual virulence factors differing in importance across different infection contexts and with inhibition of one factor having often unpredictable impacts on co-regulated virulence pathways. A study by LaFayette et al. (2015) demonstrated that *P. aeruginosa lasR* mutants within the CF lung induced an exaggerated host inflammatory response in respiratory epithelial cells, coupled with an increased accumulation of

proinflammatory cytokines and neutrophil recruitment (LaFayette et al. 2015). Such findings suggest that, as well conferring a bacterial survival advantage, these mutants may worsen pulmonary inflammation, thus contributing to pathogenesis. However, the mechanisms behind the proinflammatory response are unclear.

P. aeruginosa rapidly develops resistance to antimicrobial agents, through spontaneous mutations or acquisition of mobile genetic elements harboring resistance genes. This, coupled with the exposure of pwCF to antibiotic therapy from infancy, means that the emergence of multidrug resistant (MDR) strains is alarmingly common. Two key mechanisms that *P. aeruginosa* uses to resist antibiotics are the AmpC β-lactamase, which confers resistance to β-lactam antibiotics, and upregulation of efflux pumps such as MexXY-OprM, which is associated with resistance to aminoglycosides and fluoroquinolones (Martin et al. 2018). López-Causapé et al. (2017) investigated the evolution of *P. aeruginosa* and discovered at least 3 mutational events associated with antibiotic resistance in blood and/or sputum isolates from 33 CF patients infected with the CC274 *P. aeruginosa* strain (López-Causapé et al. 2017). Particularly noteworthy amongst these were *mexB/mexY (*coding for efflux pump proteins) and *mexZ,* the main repressor of MexXY-OprM. High levels of expression of *mexY* and *mexX* was observed in CF sputum in a study by Cornforth et al. (2018), with *mexY* being highly discriminatory for human infection (Cornforth et al. 2018). Martin et al. (2018) also analyzed the expression of *mexXY* and reported that the expression of *mexX* was higher in CF sputum samples than when the corresponding bacteria were grown *in vitro* without the presence of antibiotics (Martin et al. 2018). Although not statistically significant*, mexX* expression in CF sputum was also higher than for bacteria grown on agar containing tobramycin, highlighting the variations in *P. aeruginosa* gene expression across different environments. As the expression of these genes is upregulated in CF sputum compared to in laboratory conditions, current laboratory models may provide an inaccurate representation of *P. aeruginosa* sensitivities to aminoglycosides (Cornforth et al. 2018). This is concerning as aminoglycosides, such as tobramycin are commonly used in the management of *P. aeruginosa* infections in pwCF (Shteinberg and Elborn 2015). It also further highlights the behavioral differences of *P. aeruginosa* in human infections compared to within laboratory models. However, the expression of *ampC* was similar in bacteria in sputum and in laboratory culture, highlighting that the effectiveness of laboratory models at reflecting gene expression varies depending on the gene in question (Martin et al. 2018). The expression of *ampC* has also been analyzed in *P. aeruginosa* strain PAHM4, a strain which is associated with NCFB (Varga et al. 2015). SYBR green qPCR assays determined that the basal expression of *ampC* was significantly higher in PAHM4 than in PAO1 (Varga et al. 2015). Although not regarded as a virulence factor, the emergence of antibiotic resistance can influence the virulence and survival of *P. aeruginosa* within the lung. This can be achieved through the upregulation of multi-drug efflux pumps that can extrude antimicrobials and the upregulation of alginate, which can increase antibiotic tolerance and result in resistance (Lorusso et al. 2022). Due to the increased antimicrobial tolerance of *P. aeruginosa* within the CF and NCFB lung, consideration should be taken when selecting bacterial strains for the screening of anti-virulence drugs. Although there is a shortage of studies exploring the similarities and differences in the routes to development of resistance in NCFB and CF isolates, it is clear that both airway environments select for AMR-conferring phenotypes. Whether those same phenotypes might also influence the efficacy of anti-virulence therapies remains to be determined.

The developing anti-virulence therapeutics space

Quorum sensing

QS systems are probably the most extensively explored targets for anti-virulence therapies. QS systems are widely distributed bacterial communication systems and, due to their role in regulating the expression of an array of other virulence factors, QS inhibitors have been considered an attractive candidate for novel therapeutic development for the treatment of P. aeruginosa infections. P. aeruginosa has three well-characterised QS systems referred to as the las, rhl and pqs systems (Warrier et al. 2021). Chloroacetamide and maleimide analogues are examples of non-natural ligands that have been demonstrated by O'Brien et al. (2015) to possess potent LasR antagonist activity and to inhibit pyocyanin expression by *P. aeruginosa* PAO1 and PA14 strains (O'Brien et al. 2015). Due to the ability of pyocyanin to generate reactive oxygen species (ROS), such therapies may act to reduce pulmonary damage induced by ROS production (Hall et al. 2016). Synthetic QS inhibitors that exhibit antagonist activity against the LasR receptor, and the RhlR receptor have been developed, including a series of halogenated furanone derivatives identified by Chang et al. (2019). Amongst these derivatives, compound 29 (3,4-Dibromo-5-oxo-2,5-dihyd rofuran-2-yl 2-(3,4-dimethoxyphenyl)acetate) showed

promise as an antibiofilm agent, along with antagonist activity against pyocyanin production and *lasB* expression (Chang et al. 2019). However, the compound did generate an MIC against three *P. aeruginosa* strains, suggesting it has direct antimicrobial activity and therefore may exert pressure for the development of resistance (Chang et al. 2019). Indeed, acquired resistance to QS-targeting agents has been recorded. Resistance to the brominated furanone C-30, which targets both the LasR and RhlR QS pathways through interference with acyl homoserine lactone (AHL) receptors, has been demonstrated against *P. aeruginosa* laboratory strains (PAO1 and PA14) as well as several clinical isolates (García-Contreras et al. 2013; 2015; Bové et al. 2023). Interestingly, the mechanisms of resistance differed amongst the clinical isolates. García-Contreras et al. (2013) state that CI-7 produces low levels of *N* -(3-oxo-dodecanoyl), which suggests a disruption in AHL-mediated QS, rendering C-30 treatment ineffective. This would mean that for *P. aeruginosa* isolates that have disrupted AHL-mediated QS systems, treatment with QSI's like C-30 would be redundant. However, CI-6 produce normal *N* -(3-oxo-dodecanoyl) levels (García-Contreras et al. 2013). The authors propose CI-6 resistance may be due to efflux via the the MexAB-OprM pump, which is the only known C-30 resistance mechanism. Isolates with this mutation are also likely to demonstrate increased resistance to existing antibiotics, due to MexAB-OprM's role as a multidrug efflux pump (Maeda et al. 2012; García-Contreras et al. 2013; 2015; Bové et al. 2023). The differences in resistance mechanisms observed across the clinical isolates, including the identification of previously unknown resistance mechanisms, challenges the notion that anti-virulence therapies are inherently resistance-proof and suggests that the application of QS inhibitors to treat *P. aeruginosa* may require a more nuanced approach. How identified QS inhibitor resistance mechanisms influence *in vivo* fitness will be an important consideration for their further development.

The role of *P. aeruginosa* QS systems within the CF lung compared to in laboratory models must also be considered when assessing the appropriateness of QS inhibitors for use in pwCF. For example, a study published by Cornforth et al. (2018) comparing the transcriptomes of *P. aeruginosa* grown in *in vitro* conditions and those directly isolated from human infections (CF expectorated sputum and soft-tissue infections) found that levels of mRNA for genes controlled by the Las QS system were considerably lower under *in vivo* conditions (Cornforth et al. 2018). Several studies have reported inactivating mutations in the *las* system within *P. aeruginosa* CF isolates (Asfahl et al. 2022; Collalto et al. 2022). However, the frequency of inactivating mutations in *rhlR* and *pqs* genes are reported to be low, with Collalto et al. (2022) reporting that 85% of *P. aeruginosa* strains isolated from CF sputum were functional in *pqs* (Collalto et al. 2022).

Another potential challenge for QS inhibition lies in the overlapping regulons controlled by the various QS systems. Asfahl et al. (2022) reported RhlR control of several virulence factors *(lasB* elastase, hydrogen cyanide biosynthesis (*hcnABC*), and phenazine biosynthesis (*phzA1B1))* that are also under the control of Las (Asfahl et al. 2022). The study also reinforced that, unlike *lasR,* mutations of *rhlR* are uncommon within CF isolates, which is in agreement with earlier studies (Smith et al. 2006; Marvig et al. 2015). Collectively, work to date suggests that developing anti-virulence therapies that target the RhlR or Pqs, rather than the LasR system, may serve as more effective therapeutic agents for treating *P. aeruginosa* infections within the CF or NCFB lung. Even so, challenges remain, with a need for more in depth assessment of the possibility of resistance emergence and consideration given to redundancy within QS systems.

Elastase

The differing prevalence's of LasB within respiratory conditions as described by Woo et al. (2016) may mean that targeting this factor for anti-virulence therapeutic development is not appropriate for respiratory conditions. However, LasB has been described in other studies to be an important virulence factor of *P. aeruginosa* within the lung in initiation pulmonary damage (Everett and Davies 2021). Furthermore, LasB is an extracellular enzyme, so direct inhibitors are not faced with the difficult task of penetrating the *P. aeruginosa* outer membrane in order to interact with the target (Everett and Davies 2021). As a metalloprotease, LasB belongs to a validated drug target class, aiding novel therapeutic design (Croston 2017; Everett and Davies 2021). The abolition of LasB production can be achieved through inhibiting QS-mediated activities, either through targeting the QS regulators (LasR, RhlR, PQS) directly, or through inhibiting the production of QS autoinducers (O'Loughlin et al. 2013). QS inhibitors aimed at targeting *lasB* expression have been developed, with a recent example being the natural product derivatives psammaplin A and bisaprasin (Oluwabusola et al. 2022). Synthetic LasB inhibitors are a possible alternative to QS inhibitors. Cathcart et al. (2011) evaluated the ability of an array of synthetically derived dipeptide compounds to block *in vitro* activity of LasB on its *in vivo* targets, namely immunoglobin G (IgG), a human host

defence effector, and nucleoside diphosphate kinase (NDK), a key component of the *P. aeruginosa* biofilm pathway (Cathcart et al. 2011). Amongst these compounds, HS-CH_2-CO-Phe-Tyr-NH_2 ("Phe") demonstrated particularly high antagonist activity against LasB (K_I = 41 nM) (Cathcart et al. 2011). "Phe" was able to achieve a 99% decrease in biofilm cell numbers and, also acted to protect IgG from LasB-catalysed degradation (Cathcart et al. 2011). Like synthetic therapies, human-derived components have also shown anti-LasB activity coupled with IgG protection. α1- antitrypsin (AAT) is an example of such and has been demonstrated to achieve significant reductions in airway inflammation amongst pwCF (Griese et al. 2007). AAT has since been FDA approved for use in the treatment of pulmonary emphysema owed to AAT deficiency, and has also exhibited promising results in several clinical trials related to protease-antiprotease imbalances in coronavirus disease 2019 (COVID-19) (NCT04799873; NCT04495101; NCT04385836; NCT04495101) (Vianello and Braccioni 2020; McEvoy et al. 2021; Strassmair and Stangl 2021; O'Brien et al. 2022).

Pyocyanin

Pyocyanin has been found at concentrations up to 100 µmol/L within CF airways (Wilson et al. 1988). This, coupled with the effects pyocyanin has on the host immune system and ROS production, makes the toxin an attractive target for anti-virulence drug development (Hall et al. 2016). As with LasB, many therapeutics that target pyocyanin act to inhibit its production *via* obstructing the QS systems. Benzoxazolone derivatives are examples of QS inhibitors that mimic the Acyl-homomserine lactone (AHL) autoinducers utilized by the Gram-negative QS systems (Chen et al. 2023). B20, a benzoxazolone derivative designed by Chen et al. (2023) inhibited the production of pyocyanin in PAO1 with no influence on bacterial growth and division (Chen et al. 2023). The expression levels of QS promoting genes (*lasB, rhlA* and *pqsA*) were decreased in a dose-dependent manner upon exposure to B20, suggesting influence on QS may be responsible for the observed reductions in pyocyanin production (Chen et al. 2023). The study did not, however, explore the action of B20 on other genes related to pyocyanin synthesis, such as *phzH, phzM* and *phzS,* and thus its actions on pyocyanin are not fully understood. Due to the reduced expression of QS genes in the CF lung, as described by Cornforth et al. (2018), targeting genes directly involved in pyocyanin synthesis, such as *phzH, phzM* and *phzS* may be a more appropriate direction for developing anti-virulence therapeutics (Cornforth et al. 2018). Thees et al. (2021) advocated targeting the cysteine-rich protein PmtA for therapeutic intervention (Thees et al. 2021). PmtA is a member of the metallothionein protein family which has been well-characterised in eukaryotes as essential for zinc and copper homeostasis. Thees et al. (2021) demonstrated that PmtA is essential for pyocyanin production and a *pmtA* deletion mutant was shown to be defective in biofilm formation, resulting in increased sensitivity to cefepime and ciprofloxacin (Thees et al. 2021). Targeting these genes for anti-virulence therapeutic development appears to be a relatively unexplored area, however they represent potential future directions for inhibiting pyocyanin-induced virulence.

Pyoverdine and pyochelin

A novel approach to combat the harmful activities of *P. aeruginosa* is to exploit stresses already imposed on the organism by the environment or by host immune responses (Kaneko et al. 2007). Iron metabolism is a major vulnerability for *P. aeruginosa* because, as well as being vital for survival, free iron levels are low within *in vivo* environments. Siderophores such as pyoverdine are thus vital contributors to *P. aeruginosa* success in infectious settings (Kaneko et al. 2007). Gallium nitrate ($Ga(NO_3)_3$) is FDA approved for the treatment of several conditions, including intravenous treatment of cancer-related hypercalcaemia, and is increasingly gaining attention as a potential antipseudomonal agent (Guo et al. 2019; Kang et al. 2021). Ga^{3+} possesses an ionic radius almost identical to that of Fe^{3+} and thus, *P. aeruginosa* is unable to distinguish between the two, likening Ga^{3+} to a "Trojan horse" in the disruption of Fe metabolism (Kaneko et al. 2007). *P. aeruginosa* cannot reduce and subsequently oxidize Ga^{3+}, meaning the redox reactions which are critical for the virulence-promoting activities of iron cannot occur (Kaneko et al. 2007). The effects of $Ga(NO_3)_3$ on *P. aeruginosa* PAO1 were explored by Kaneko et al. (2007) who found the compound repressed *pvdS* (a transcriptional regulator of pyoverdine synthesis and the pyoverdine receptor), decreasing iron uptake *via* this mechanism (Kaneko et al. 2007). Several recently published studies have also observed anti-virulence effects of gallium (Richter et al. 2017; Scott et al. 2022; Mosina et al. 2023). A study by Kang et al. (2021) found that treatment with $Ga(NO_3)_3$ under iron-limiting conditions induces the expression of genes related to pyochelin biosynthesis, and that pyochelin then binds Ga^{3+} and deposits it intracellularly, where subsequent interference with bacterial cell function occurs (Kang et al. 2021). Consequently, pyochelin synthesis mutants, such as *P.*

aeruginosa Δ*pchBA* are more resistant to $Ga(NO_3)_3$, unlike pyoverdine synthesis mutants (Δ*pvdF*), which appear to sequester Ga^{3+} extracellularly or within the bacterial periplasmic space and thus demonstrate enhanced susceptibility to $Ga(NO_3)_3$ (Kang et al. 2021) Similar trends in resistance were observed by Scott et al. (2022) when exposing mice infected with *P. aeruginosa* PA103 ATCC to $Ga(NO_3)_3$, and Gallium protoporphyrin (GaPP) combination therapy (Scott et al. 2022). $Ga(NO_3)_3$ uptake *via* the high affinity iron ABC transporter system (HitABC) has also been demonstrated suggesting a further possible route of cell entry for Ga^{3+} as an anti-virulence therapy (Guo et al. 2019). However, several studies have reported bacteriostatic effects following treatment, both in *in vitro* (Choi et al. 2019) and *in vivo* (Scott et al. 2022) studies of exposure of *P. aeruginosa* PA103 ATCC to $Ga(NO_3)_3$/GaPP. This is a concern due to the association between the use of therapeutics that target bacterial metabolism and the development of resistance. Iron uptake mechanisms are under strong selective pressure, due to the limited unbound iron *in vivo*, a factor which could affect the long term effectiveness of $Ga(NO_3)_3$ (Kaneko et al. 2007). However, Kang et al. (2021) states that the compound demonstrates low selective pressure for *P. aeruginosa* resistance even at concentrations that significantly inhibit bacterial growth (Kang et al. 2021).

Biofilms

P. aeruginosa is notoriously inclined to aggregate and form surface-attached biofilms (Lu et al. 2021). The presence of these biofilms results in a much higher tolerance to antibiotics than is observed with planktonic cells, driven by protective extracellular polymeric substances (EPS) produced by bacteria and scavenged from the local host environment (Ciofu and Tolker-Nielsen 2019). Because bacteria residing in biofilms are up to 1000-fold more resistant to antibiotics, developing anti-biofilm therapeutics has potential for managing *P. aeruginosa* infection in pwCF and pwNCFB (Sommer et al. 2013).

Treatment with nitric oxide (NO) donors has been proposed as a potential treatment to disperse *P. aeruginosa* biofilms, through reducing cyclic-di-GMP (c-di-GMP), a messenger involved in the establishment of biofilms (Cai and Webb 2020; Andersen et al. 2021). A decrease in intracellular c-di-GMP levels promotes bacterial motility and initiates biofilm dispersal (Römling et al. 2013). The NO donors, sodium nitroprusside (SNP) and spermine NONOate (S150) successfully reduced >60% bacterial biomass within 24 and 2 h respectively in a study by Cai and Webb (2020) using 24 h pre-established *P. aeruginosa* PAO1 biofilms grown in M9 minimal media (Cai and Webb 2020). A 2 h incubation time with S150, successfully disrupted 72 h biofilms of several *P. aeruginosa* clinical CF isolates at low doses, without inducing cytotoxic effects against *P. aeruginosa* (Cai and Webb 2020). The authors propose S150 as a potential treatment for preventing *P. aeruginosa* biofilm development or disrupting existing biofilms, whilst also acknowledging that the mechanisms at play are unclear (Cai and Webb 2020). Studies have been performed to determine safe doses of inhaled NO donors, such as that published by Safaee Fakhr et al. (2021) which determined dosage of NO treatment in patients with COVID-19, demonstrating safe treatment with NO donors is clinically feasible (Safaee Fakhr et al. 2021). Determining effective dosage must also be considered, particularly how effective dosage may differ between healthy individuals and individuals with airway inflammation, as seen in pwCF and NCFB. There are also concerns that repeated exposure of *P. aeruginosa* to NO donors may lead to mutations, resulting in higher tolerance of biofilms to NO, particularly as mutations in c-di GMP are common in NCFB isolates (Hilliam et al. 2017; Cai and Webb 2020). The efficacy of anti-biofilm agents in polymicrobial biofilms is also an important consideration for their use within the CF and NCFB lung. The effectiveness of NO at dispersing polymicrobial biofilms has been investigated (Slomberg et al. 2013; Sorbo et al. 2022), as well as the effectiveness of other anti-biofilm agents, such as glycoside hydrolases in dual-species *P. aeruginosa* and *Staphylococcus aureus* biofilms (Redman et al. 2020). There are also concerns that the disruption of biofilms and the subsequent release of planktonic *P. aeruginosa* into the pulmonary environment could initiate an exacerbation of respiratory symptoms and a possible immune reaction, resulting in host-induced pulmonary damage. A possible method to combat this would be to co-administer antibiotics during the NO donor treatment to reduce planktonic bacterial load within the lungs (Daboor et al. 2021).

Exotoxins

As the most damaging of the *P. aeruginosa* exotoxins, ExoU is a prime target for anti-virulence therapeutics (Jurado-Martín et al. 2021). Although the mechanisms of ExoU activation are yet to be fully explored, it is understood that the toxin relies on non-covalent binding of host ubiquitin and phosphatidylinositol 4,5-bisphosphate (PIP_2) in order to become fully activated (Foulkes et al. 2019; 2021). The conformational changes ExoU undergoes subsequent to activation

may be targeted by small molecules, such as pseudolipasin A as a means to attenuate virulence. Pseudolipasin A exhibited inhibitory effects against ExoU within a study by Foulkes et al. (2021). The study utilized *P. aeruginosa* PA103 ΔUT: ExoU (a strain which solely employs ExoU as the T3SS cytotoxic effector) in a human corneal cell (HCE-T) scratch and infection assay in which HCE-T cells were infected with the bacteria and subsequent cell lysis induced by ExoU production was observed (Foulkes et al. 2021). Upon addition of pseudolipasin A, significant decreases in wound sizes were observed. There was also a decrease in lactate dehydrogenase production – an indicator of protection from cell lysis – upon exposure of the HCE-T cells to pseudolipasin A when compared with DMSO controls (Foulkes et al. 2021). According to Jurado-Martín et al. (2021) *P. aeruginosa* isolated from chronically infected CF sputum often presents with the *exoS*$^{+}$/*exoU*$^{-}$ virulotype and therefore an ExoU-specific therapeutic may have limited impact in this indication. There are currently no novel therapeutics that target ExoS inhibition.

Challenges associated with anti-virulence therapeutic development

The development of antimicrobials that target respiratory infections in diseases such as CF and NCFB is a challenging venture. There are difficulties associated with determining the pharmacokinetic/pharmacodynamic (PK/PD) relationship of a therapeutic, which include ascertaining host-drug relationships and defining plasma drug concentrations. Furthermore, the sputum composition, polymicrobial community interactions, biofilm mode of bacterial life and host factors associated with certain chronic respiratory conditions can be difficult to replicate within *in vivo* and *in vitro* models (Bulitta Jürgen et al. 2019; Barton et al. 2022; Kolpen et al. 2022). As already discussed, these difficulties are exacerbated further when developing anti-virulence therapies rather than direct-acting antimicrobials. This raises the question: *Do we have the models required to develop anti-virulence therapeutics against P. aeruginosa in chronic respiratory conditions?*

Methods used in early anti-virulence drug development are often dependent on the targeted virulence factor. For example, fluorescence spectrometry assays are employed to assess the anti-QS activities of compounds (Chang et al. 2019; Soukarieh et al. 2021; Oluwabusola et al. 2022). These assays are appropriate to measure the inhibitory action of therapeutics which directly target QS genes, however they are not appropriate when testing therapeutics that target virulence protein production (Oluwabusola et al. 2022). Bespoke assays such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) and thin-layer chromatography have been used to assess the detection of QS signals within bodily fluids and could be used to assess the effectiveness of QS-specific anti-virulence therapeutics (Fletcher et al. 2018; Webb et al. 2022). Webb et al. (2022) used LC-MS/MS to assess the presence of 2-alkyl-4-quinolones (AQs) in saliva and sputum from pwCF with known *P. aeruginosa* infection (Webb et al. 2022). Using saliva to determine the effectiveness of a therapeutic in place of sputum is simpler and easier, as well as being beneficial for patients that cannot expectorate, such as infants or those on highly-effective CF modulator therapy (Caverly et al. 2022; Webb et al. 2022). Employing virulence assays to assess the effects of a therapeutic has potential, however, the development of such assays is likely to be costly, as the assays would need to be bespoke to the therapeutic.

There is currently no gold standard model to assess the effectiveness of anti-virulence agents in conditions that recapitulate the CF and NCFB lung. A factor contributing to the lack of a gold standard model is that sampling of the microbial environment in order to facilitate study of these virulence factors under relevant conditions is difficult (Caverly et al. 2022). There are several sampling options to study CF pathogens: bronchoscopy can be used to acquire bronchoalveolar lavage (BAL) samples. This is considered the gold standard sampling method in pwCF, as these samples do not have contact with the non-sterile upper respiratory tract during sampling, therefore avoiding contamination with commensal microorganisms from the URT and oral cavity (Eyns et al. 2018; Caverly et al. 2022). However, this procedure is invasive, not without risk and not feasible for studies which require analysis of serial samples across a large patient cohort. Sputum is the preferred specimen type for CF and NCFB, and has been used to recover and study the CF lung microbiome for several years (Lu et al. 2020). As these samples are highly reflective of the CF lung environment, sputum may provide the most effective model to develop and test anti-virulence therapeutics. However, there are a variety of factors which make using CF sputum in anti-virulence therapeutic development challenging. As already stated, obtaining sputum samples may not be possible for certain individuals, and in those individuals that can expectorate, it can be an unpleasant experience (Caverly et al. 2022). It is also likely that the demand for sputum in anti-virulence therapy development will outweigh the amount of sputum available, and these samples may not be accessible for all researchers. Due to its viscous and adhesive nature, CF sputum is also very challenging to work with, and must

be diluted prior to the commencement of laboratory work (Chatham et al. 2004). Therefore, using CF sputum in therapeutic development is likely to be time consuming, suffer from low reproducibility of findings and will prove in-appropriate for high-throughput testing.

Due to the issues with using patient sputum in anti-virulence therapeutic development, alternative models are required. Various CF sputum mimics have been developed to recapitulate the conditions of the CF lung, including CF lung media (CFLM), CF sinus media (CFSM), Synthetic CF Media 2 (SCFM2) and SCFM2-Calprotectin as well as various *in vitro* models (Ruhluel et al. 2022; Lewin et al. 2023). These models may provide an effective alternative to CF sputum in the development and testing of anti-virulence therapies. However, as can be seen in Figure 2 and Table 1, the effectiveness of such media varies dependent on the virulence gene and *P. aeruginosa* strain in question, and no one media completely recapitulates the pattern of expression of these genes observed within CF sputum.

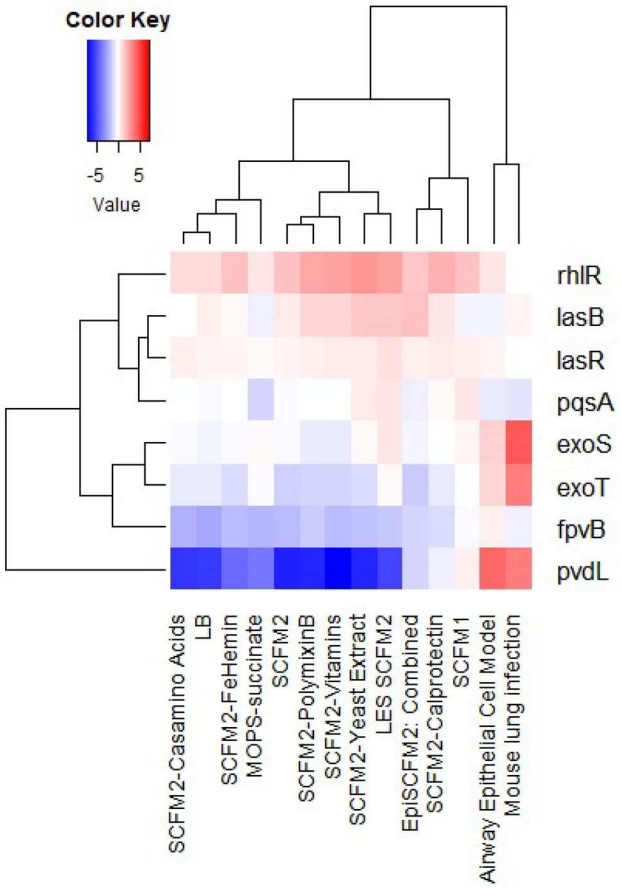

Figure 2. The expression of QS and virulence genes (*rhlR, lasB, lasR, pqsA, exoS, exoT, fpvB, pvdL)* across various models within *P.* aeruginosa PAO1 or LES (LES SCFM2). the gene expression is in comparison to that of CF sputum samples isolated from patients chronically infected with *P. aeruginosa.* Values at 0 represent no difference in expression between human CF sputum. Values >0 indicate higher expression in the model than in human sputum. Values <0 indicate less expression in the model than in human sputum. Created with rstudio (Lewin et al. 2023).

This variation in virulence gene expression means that, although CF sputum mimics and other models such as the mouse long model may recapitulate certain characteristics of the CF lung, as well as provide insights into bacterial behavior, these models do not fully recapitulate the *P. aeruginosa* virulome as it is seen in the CF lung. This variation in *P. aeruginosa* virulence gene expression may, in part, be due to the difficulties in replicating the microenvironment of the CF lung in laboratory models (Bjarnsholt et al. 2022).

It may also be argued that certain virulence factors might not be produced within *in vitro* conditions without the presence of a host factor to act on. The *P. aeruginosa* genome encodes an arsenal of proteins that enable success in diverse environments, and whilst those that contribute to disease are often termed virulence factors, this terminology can be misleading. As an organism with a predominantly environmental lifestyle, it is likely that many of these factors first arose and have been maintained due to advantages they confer in extra-host environments. We should not assume, therefore, that a host is necessarily required for induction of their expression. Rather, an unbiased assessment of the factors that drive expression of virulence-promoting phenotypes is required. Figure 2 also shows that models which consider host factors, such as the *in vitro* airway epithelial cell model and the *in vivo* mouse lung infection model do not fully recapitulate the CF virulome, particularly in reference to the expression of the exotoxins *exoS* and *exoT*.

pvdL is a major gene in the pyoverdine synthesis pathway and is expressed, alongside *fpvB* (a gene involved in ferripyoverdine synthesis) during iron-limiting conditions (Ghysels et al. 2004). Figure 2 shows that the expression of these genes was underrepresented within all the sputum mimicking media, with the exception of SCFM1, compared to CF sputum. As these media types contain an iron source it is likely that siderophores for iron scavenging would not be require, therefore *pvdL* and *fpvB* would not be expressed. The employment of an iron-limiting media such as M9 minimal media may be more appropriate for the *in vitro* study of these virulence genes. The airway epithelial cell model and the mouse lung model also do not recapitulate expression of *pvdL* and *fpvB* obsereved within CF sputum, with overexpression recorded in these models. The variation in expression across the models further highlights that for many virulence genes there is no model which completely recapitulates their expression within the CF lung.

The use of CF mimicking media allows for the incorporation of a nutrient environment similar to that of the CF lung, which traditional *P. aeruginosa* culture media, such as LB and MOPS succinate does not (Ruhluel et al. 2022). Most of these model's, like those shown in Figure 2, study *P. aeruginosa* in mono-culture, but CF lung infections are polymicrobial. Microbial co-colonisers may affect *P. aeruginosa* virulence, through influencing *P. aeruginosa* gene expression or affecting the physical characteristics of a community, such as increasing biofilm formation (Bisht et al. 2020; Al-Wrafy et al. 2023). Monoculture models do not consider the influence of co-colonising microorganisms on the virulence gene expression of *P. aeruginosa*. They also do not consider how the presence of other microorganisms can impact the host response against *P. aeruginosa*. Thus, there is significant effort toward developing polymicrobial models which consider the heterogenous environments of the CF and NCFB lung. However, these models are challenging to develop as the interactions between microbes are complex, impacted by both the environment and biogeography (Ruhluel et al. 2022). One approach could be to test therapeutics directly in sputum. Sputum however, is highly variable and with the introduction of effective modulator therapy, less abundant. Polymicrobial *in vivo* models, primarily murine models, are increasingly employed in an effort to observe the mechanisms of interspecies interactions, as employed by Lindgren et al. (2023) utilizing CFTR knockout mice co-infected with *P. aeruginosa* and *Stenotrophomonas maltophilia*/or with *P. aeruginosa* and *H. influenzae* (Lindgren et al. 2023). Unlike many *in vitro* models, utilizing animal models provides observations regarding the influence of host immune components on novel therapeutics and vice versa. Another key advantage of *in vivo* models is the determination of how host pathology is influenced by a therapeutic (O'Toole et al. 2021). However, an important caveat to this is that the microbiome of mice is very different to that of humans and many CF pathogens do not naturally colonize the murine airways. CF-mutant zebrafish models are also becoming an increasingly popular choice for research, as zebrafish CFTR is structurally similar to human CFTR (Galdino et al. 2023). Furthermore, zebrafish larval innate immunity is homologous to the human innate response, and their optical transparency allows for the real-time-monitoring of pathogenesis in a noninvasive capacity (Bernut et al. 2019). However, this model is yet to be exploited to study the effects of anti-virulence therapies in polymicrobial infections.

As well as the challenges associated with replicating *P. aeruginosa* virulome within current models relevant to the pulmonary environment, there is also the possibility that some virulence factors are naturally difficult to study *in vitro*. Crystallized ExoU, for example, has only been studied whilst bound to its chaperone, SpcU, as unbound ExoU is thought to be too flexible to crystallize alone (Foulkes et al. 2021). As crystallography plays an important role in determining drug targets, the study of ExoU as a target may be challenging.

It must be noted that disease phenotype has a significant bearing on the pulmonary microbial community in chronic lung infections, and a "one size fits all approach" is not appropriate for developing a suitable model for the study of anti-virulence therapeutics in these patients (O'Toole et al. 2021). Particularly for pwCF, the development of several models may need to be employed for the development of anti-virulence therapeutics for use on a large scale, considering patient age (paediatric versus adult), disease severity (mild phenotype versus severe), antibiotic regime and anti-virulence therapeutic target (O'Toole et al. 2021). The use of polymicrobial models should also be implemented within anti-virulence therapeutic development for pwCF, as many bacterial factors are equally involved in intra-species competition such as the Type VI secretion system, meaning anti-virulence therapies may have unintended impacts on the balance of species in the airways. This highlights that different models may be required to answer different questions in relation to the action of anti-virulence therapies.

The introduction of CFTR modulators has revolutionized the treatment regime of pwCF, and some may argue that their introduction renders anti-virulence therapeutic research redundant. However, not all pwCF can benefit from modulators, particularly individuals with premature termination codons or nonsense mutations, as no protein is produced as a target for such therapies (King et al. 2022). Furthermore, such therapies are not effective in individuals suffering from chronic pulmonary infections that are not owed to CF and infection remains an ongoing issue for those pwCF who were already chronically infected at onset of modulator therapy. This highlights that there is still a need for effective anti-virulence therapies (Saluzzo et al. 2022). It is also unclear what effects CFTR modulators have on the pulmonary microenvironment, and numerous questions remain regarding the impact these therapies place on new and stable lung infections.

Outlook

P. aeruginosa is a significant threat for individuals with chronic pulmonary disorders and it is apparent that managing this bacterium with antibiotics is becoming

increasingly challenging, with the threat of the AMR crisis ever looming. Anti-virulence therapeutics represent a novel approach that acts to combat the current resistance crisis and improve the quality of life for patients with chronic pulmonary disorders, including CF and NCFB. Despite the growing evidence of the need for novel approaches such as anti-virulence therapies, it is clear that research into appropriate models to accelerate their development is desperately required. An increased understanding of how best to develop *in vitro* and *in vivo* polymicrobial biofilm models that represent the harsh pulmonary environment is needed, with consideration given to the everchanging microbial landscape observed within these conditions.

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