# High prevalence of lipopolysaccharide mutants and R2-pyocin susceptible variants in *Pseudomonas aeruginosa* populations sourced from cystic fibrosis lung infections

Madeline Mei,[1,2,3] Preston Pheng,[1] Detriana Kurzeja-Edwards,[1] Stephen P. Diggle[1]

**AUTHOR AFFILIATIONS** See affiliation list on p. 10.

**ABSTRACT** Chronic, highly antibiotic-resistant infections in cystic fibrosis (CF) lungs contribute to increasing morbidity and mortality. *Pseudomonas aeruginosa*, a common CF pathogen, exhibits resistance to multiple antibiotics, contributing to antimicrobial resistance (AMR). These bacterial populations display genetic and phenotypic diversity, but it is unclear how this diversity affects susceptibility to bacteriocins. R-pyocins, i.e., bacteriocins produced by *P. aeruginosa*, are phage-tail-like antimicrobials. R-pyocins have potential as antimicrobials, however, recent research suggests the diversity of *P. aeruginosa* variants within CF lung infections leads to varying susceptibility to R-pyocins. This variation may be linked to changes in lipopolysaccharide (LPS), acting as the R-pyocin receptor. Currently, it is unknown how frequently R-pyocin-susceptible strains are in chronic CF lung infection, particularly when considering the heterogeneity within these strains. In this study, we tested the R2-pyocin susceptibility of 139 *P. aeruginosa* variants from 17 sputum samples of 7 CF patients and analyzed LPS phenotypes. We found that 83% of sputum samples did not have R2-pyocin-resistant variants, while nearly all samples contained susceptible variants. There was no correlation between LPS phenotype and R2-pyocin susceptibility, though we estimate that about 76% of sputum-derived variants lack an O-specific antigen, 40% lack a common antigen, and 24% have altered LPS cores. The absence of a correlation between LPS phenotype and R-pyocin susceptibility suggests that LPS packing density may play a significant role in R-pyocin susceptibility among CF variants. Our research supports the potential of R-pyocins as therapeutic agents, as many infectious CF variants are susceptible to R2-pyocins, even within diverse bacterial populations.

**IMPORTANCE** Cystic fibrosis (CF) patients often experience chronic, debilitating lung infections caused by antibiotic-resistant *Pseudomonas aeruginosa*, contributing to antimicrobial resistance (AMR). The genetic and phenotypic diversity of *P. aeruginosa* populations in CF lungs raises questions about their susceptibility to non-traditional antimicrobials, like bacteriocins. In this study, we focused on R-pyocins, a type of bacteriocin with high potency and a narrow killing spectrum. Our findings indicate that a large number of infectious CF variants are susceptible to R2-pyocins, even within diverse bacterial populations, supporting their potential use as therapeutic agents. The absence of a clear correlation between lipopolysaccharide (LPS) phenotypes and R-pyocin susceptibility suggests that LPS packing density may play a significant role in R-pyocin susceptibility among CF variants. Understanding the relationship between LPS phenotypes and R-pyocin susceptibility is crucial for developing effective treatments for these chronic infections.

**KEYWORDS** R-pyocin, bacteriocin, *Pseudomonas aeruginosa*, cystic fibrosis, lipopolysaccharide

Cystic fibrosis (CF) is a genetic disorder primarily characterized by chronic respiratory infections, leading to progressive loss of lung function and increased morbidity and mortality in affected individuals (1). The disease arises from a defective anion pump, known as the cystic fibrosis transmembrane regulator (CFTR), which results in an imbalance of chloride on epithelial cell surfaces (1). This imbalance causes mucus dehydration, making it too thick for proper clearing of airways along the respiratory tract in people with CF. Consequently, this creates an environment that promotes bacterial and fungal infections, which are notoriously challenging to treat.

*Pseudomonas aeruginosa*, a Gram-negative opportunistic bacterium, is the most prevalent bacterial pathogen that colonizes and persistently infects CF lungs (2–7). With a large genome, *P. aeruginosa* can regulate diverse metabolic processes, resist antibiotic treatment, and control various complex social behaviors that aid in its persistence in infections (8–16). Once established in the CF lung, *P. aeruginosa* is extremely difficult to eradicate and often becomes multi-drug resistant (MDR). Although *P. aeruginosa* inherently possesses many antibiotic-resistant mechanisms, strains sourced from chronic lung infections are known to become particularly problematic and resistant to multiple antibiotics over time (17–21). While recent data suggests that the approval and use of the new triple CF therapeutic TRIKAFTA (elexacaftor/tezacaftor/ivacaftor) have decreased the overall prevalence of *P. aeruginosa* infection, the 2019 CF Foundation Patient Registry Annual Report notes that the prevalence of MDR *P. aeruginosa* infection remains constant (22). In the era of antibiotic resistance, new treatment approaches are crucial against *P. aeruginosa* and other multi-drug resistant pathogens, especially in the case of CF chronic lung infections.

One of the challenges in eradicating *P. aeruginosa* from CF lungs is likely due to the high degree of phenotypic, and genomic diversity that evolves within *P. aeruginosa* populations over the course of infection (6, 18, 19, 23, 24). While this heterogeneity is known to affect antibiotic susceptibility, its impact on alternative therapeutic options, such as bacteriocins and bacteriophage, remains poorly understood (6, 18, 19). As pathogens like *P. aeruginosa* become increasingly difficult to eliminate, antimicrobials such as bacteriocins may serve as useful alternative therapies in combination with antibiotics and phage for more effective treatment.

Bacteriocins are narrow-spectrum, proteinaceous antimicrobials produced by one strain of bacteria and active against other strains of the same or closely related species (25, 26). *P. aeruginosa* produces various bacteriocins called pyocins, which are grouped based on similarities in their physical and chemical properties, export mechanisms, and mode of action (27–31). Pyocins produced by *P. aeruginosa* are classified as S-, R-, and F-pyocins, each with varying antimicrobial spectra (30–34). R-pyocins are narrow-spectrum, contractile, phage-tail-like bacteriocins, further categorized into subtypes R1–R5 based on specificity to different receptors on target cells, however, R-types 2–4 are often considered as a single, functional subtype (30, 31, 35–38). The proposed mode of action involves the R-pyocin tail fiber foot binding to a monosaccharide receptor on the lipopolysaccharide (LPS) (specific to each R-subtype), causing the sheath to contract and push the tail spike and core to puncture the outer membrane, resulting in membrane depolarization and eventual cell death (Fig. S1) (37, 39–44). R-pyocins have gained interest for their potential to combat *P. aeruginosa* due to their powerful ability to kill target cells, their inability to replicate without a DNA-containing head, and their well-documented mechanism of action (30, 31, 41, 43–45). In this study, we focus on R-pyocins because of their promising features as antimicrobial agents and the need for a better understanding of their effectiveness against clinical strains of *P. aeruginosa*.

The LPS of *P. aeruginosa* not only acts as the receptor for R-pyocins but importantly plays a significant role in virulence and immune evasion. LPS is composed of three major regions: the lipid A, the core oligosaccharide, and the O-specific antigen (OSA) (46). The core structure is relatively conserved across *P. aeruginosa* strains and acts as a platform for the attachment of various OSA side chains; both OSA and common polysaccharide antigen (CPA) can be found in *P. aeruginosa* LPS, but their presence and

heterogeneity vary among different isolates or variants even within the same strain (46–51). The core structure of *P. aeruginosa* LPS is essential for maintaining outer membrane integrity and plays a crucial role in bacterial survival, thus, it is costly for bacterial fitness to alter. LPS is composed of a conserved lipid A region, anchoring LPS to the outer membrane, and a core oligosaccharide region that extends from lipid A (47, 52–54). This core region serves as the attachment site for the OSA and CPA side chains, (as well as R-pyocins) contributing to the overall heterogeneity of *P. aeruginosa* LPS (38, 47, 54–58). OSA structures can differ in their length, sugar composition, and linkage patterns, which contributes to antigenic variation and immune evasion strategies employed by the bacterium during infection (59–61). The presence and composition of OSA and CPA side chains in *P. aeruginosa* LPS can vary significantly between strains, thus, the diverse repertoire of OSA structures can influence the bacterium's interactions with not only other pathogens in infection but also the host immune system, allowing for evasion of immune surveillance and facilitating chronic infection establishment in CF patients (59, 62–65). Moreover, certain *P. aeruginosa* isolates have been reported to downregulate or even lose OSA expression during chronic CF infections, contributing to increased adaptability and persistence in the host environment (51, 66–68)

During chronic CF infections, *P. aeruginosa* faces a challenging host environment characterized by immune responses and antibiotic exposure. In this context, certain variants of *P. aeruginosa* have been observed to downregulate or lose OSA expression (46, 51, 69). This phenomenon is thought to provide a survival advantage by reducing recognition and clearance by the host immune system. By modulating OSA expression, these isolates can persist and adapt more effectively within the CF lung environment, leading to prolonged and difficult-to-treat infections. The downregulation or loss of OSA expression by certain isolates during chronic CF infections further underscores the bacterium's adaptability and persistence in the host environment (46, 69). Understanding the structural and functional characteristics of *P. aeruginosa,* LPS is crucial for developing targeted therapeutic strategies to combat these challenging infections effectively, and particularly when considering LPS-binding antimicrobials such as bacteriophage and R-type bacteriocins. While there have been studies serotyping and R-pyocin typing panels of strains (38, 70, 71), there has not yet been a study examining LPS diversity among variants of the same strain or comparing variants within multiple different strains and the resulting impacts on R-pyocin susceptibility.

In previous research, we tested 20 individual *P. aeruginosa* variants from sputum samples collected from 3 different individuals with CF (60 variants total, from 3 *P. aeruginosa* populations). We found a variation in susceptibility to R2-pyocins within these populations (51). Additionally, we isolated and compared the LPS phenotypes of three of these variants from the same sputum sample and patient, discovering that each variant had different LPS glycoforms, which likely contributed to their varying susceptibility (51). If R-pyocins are to be considered as a therapy, it is essential to further investigate their efficacy against diverse clinical strains of *P. aeruginosa*, as the prevalence of R-pyocin-susceptible strains in CF chronic lung infections is currently not well understood.

We previously found that R1-pyocin producers make up the majority of R-pyocin-typeable CF *P. aeruginosa* strains, and other studies have shown that R1-type producers are often susceptible to R2-pyocins (38, 51, 70). As a result, we expected a high frequency of *P. aeruginosa* strains isolated from chronic lung infections with LPS mutations conferring R2-pyocin susceptibility. Although we discovered no correlation between LPS phenotype and R2-pyocin susceptibility, most *P. aeruginosa* strains isolated from CF lung infections were sensitive to R2-pyocins. Our findings support the potential use of R-pyocins as alternative anti-pseudomonal treatments in chronic infections.

## RESULTS

### The majority of *P. aeruginosa* populations in CF patients have variants susceptible to R2-pyocins

To determine the prevalence of R2-pyocin susceptible variants among *P. aeruginosa* populations from CF sputum samples, we analyzed up to 10 previously described variants (51) from longitudinal samples of 7 individuals with CF (139 total variants collected from 17 sputum samples or "populations") using the soft-agar overlay method (Fig. S2). For consistency, we used PAO1Δ*rmlC,* an LPS core mutant (38), as an R2-pyocin susceptible control and PAO1 wt as a resistant control in each experiment. Using R2-pyocins from PAO1 without the pf4 prophage (PAO1ΔPf4) (72), we assessed the relative susceptibility of each CF variant and found that (i) each population showed heterogeneity within the population, consistent with previous work using a microtiter plate method (51), and (ii) nearly every *P. aeruginosa* population included variants with some degree of susceptibility to R2-pyocins. In our sample, we found that only three out of seven individuals had sputum containing R2-pyocin resistant variants (Fig. 1). We also discovered that most populations contained R2-susceptible variants even across longitudinal sampling from the same patient, although the degree of relative susceptibility and sensitivity to R2-pyocins fluctuated over time (Fig. 2). Longitudinal sputum collections in our biobank showed no trend of variants becoming R2-pyocin resistant over a period of approximately 2 yr. To further confirm that these results were R2-pyocin dependent, we included lysates from a PAO1ΔPf4ΔR mutant (lacking both the prophage and a functional R2-pyocin) in each assay, ensuring no killing activity when R2-pyocins were absent.

### There is a high frequency of LPS mutants and diversity among LPS phenotypes in CF strains of *P. aeruginosa*

To characterize the LPS phenotypes of R2-pyocin susceptible and resistant variants in our biobank, we isolated LPS using a hot-aqueous phenol method (73) and compared the LPS migration profiles of our clinical variants to those of isogenic LPS mutants of
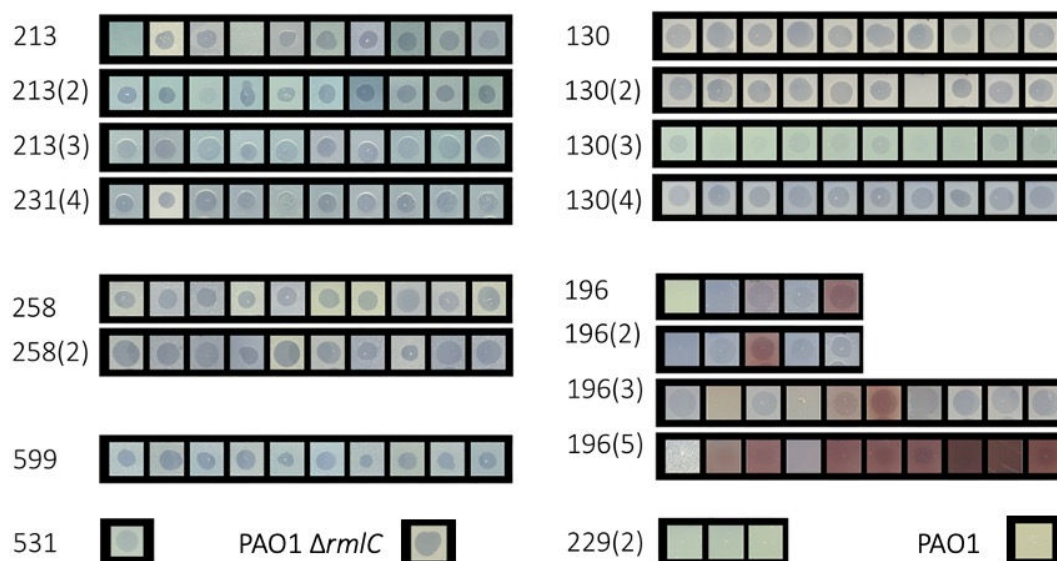


**FIG 1** R2-pyocin inhibition zones for multiple cystic fibrosis (CF) variants from different patient sputum samples. The inhibition zones caused by R2-pyocin were applied to soft agar overlays containing clinical variants from the CF sputum samples listed on the left. PAO1Δ*rmlC* serves as an R2-susceptible control and PAO1 wt serves as an R2-resistant control. Red boxes indicate patients with populations containing resistant variants. Up to 10 variants per sputum sample were tested. An inhibition zone indicates R2-pyocin susceptibility, while the absence of a visible zone indicates resistance.
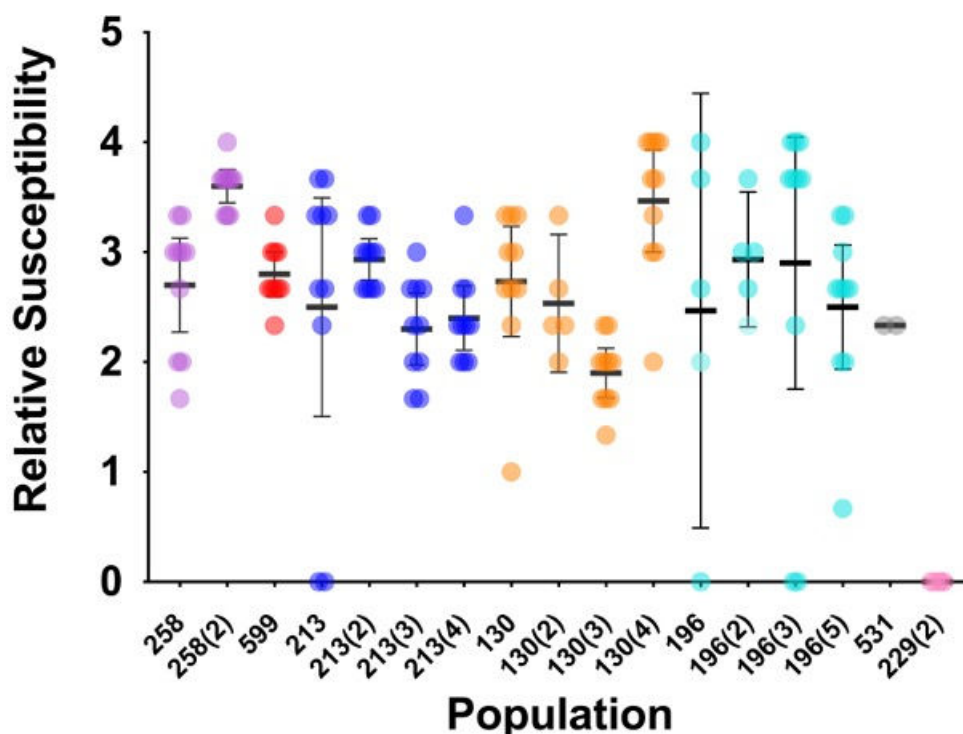
**FIG 2** R2-pyocin susceptibility varies among clinical *P. aeruginosa* populations and changes over time. Relative susceptibility to R2-pyocins differs among variants in each population and across populations. Susceptibility heterogeneity is observed across all five patients and within the same patient's longitudinal samples. There is no trend of strains becoming more resistant to R2-pyocins in longitudinally sampled populations, up to 2 yr (consecutive numbers with collection number in parentheses). Two sputum samples contained at least one variant resistant to R2-pyocins (Relative Susceptibility = 0). Relative Susceptibility was determined by counting the number of serial dilutions (including neat) where inhibition zones appeared after R2-pyocins were applied to agar overlays. Means are shown with 95% confidence interval error bars.

our Nottingham PAO1 wild-type strain. Out of 139 variants across all populations, we observed that 40% lacked common antigen (CPA or A-band) (Fig. 3A) and 76% lacked O-specific antigen (OSA or B-band) (Fig. 3B). Additionally, 24% presented altered LPS cores (Fig. 3C), as determined by comparing to PAO1. We found that 13 out of the 17 sputum collections sampled contained variants with diverse LPS profiles; approximately 76% of our *P. aeruginosa* populations contained a mixture of variants with varying LPS phenotypes.
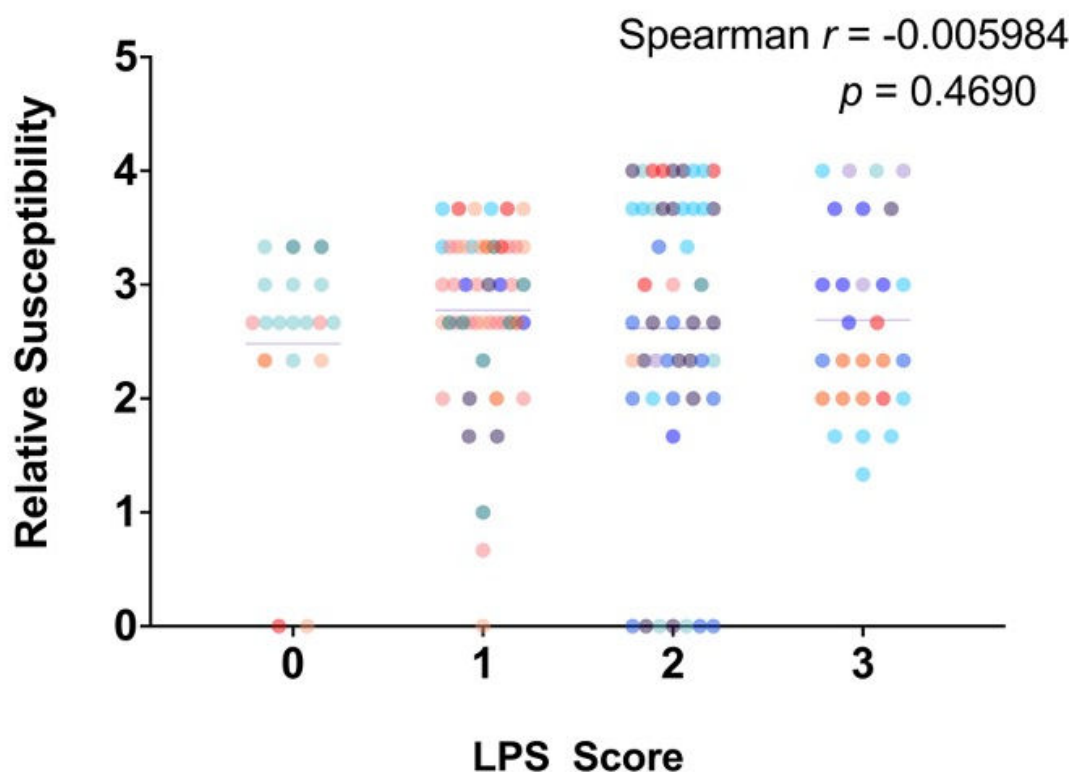
To assess the correlation between R2-pyocin susceptibility and specific LPS glycoform phenotypes in CF strains, we conducted a one-tailed Spearman correlation analysis for each phenotype individually. We assumed independence between the three glycoform phenotypes (CPA +or CPA-, OSA +or OSA-, WT, or altered core) for these analyses, even though some dependencies may exist due to overlapping biosynthetic pathways and the essentiality of the LPS core. We found no significant correlations specifically between the presence or absence of CPA (Fig. 3D), OSA (Fig. 3E), or an altered core (Fig. 3F) ($P >$ 0.05).

We further evaluated the relationship between the LPS phenotype and R2-pyocin susceptibility by assessing the LPS as a whole. Using a numbered scale, we assigned an LPS score of 0 when a variant presented as CPA−, OSA−, and had an altered core; this variant was treated as having 0/3 WT LPS characteristics. A score of 1 was assigned when a variant exhibited one of the three LPS glycoforms as WT (presence of OSA, CPA, or unaltered core), a score of 2 when two of three glycoforms were WT, and a score of 3 when a variant presented CPA+, OSA+, with a WT core. Using this classification system, we also found no correlation with R2-pyocin susceptibility (Fig. 4). However, we did find
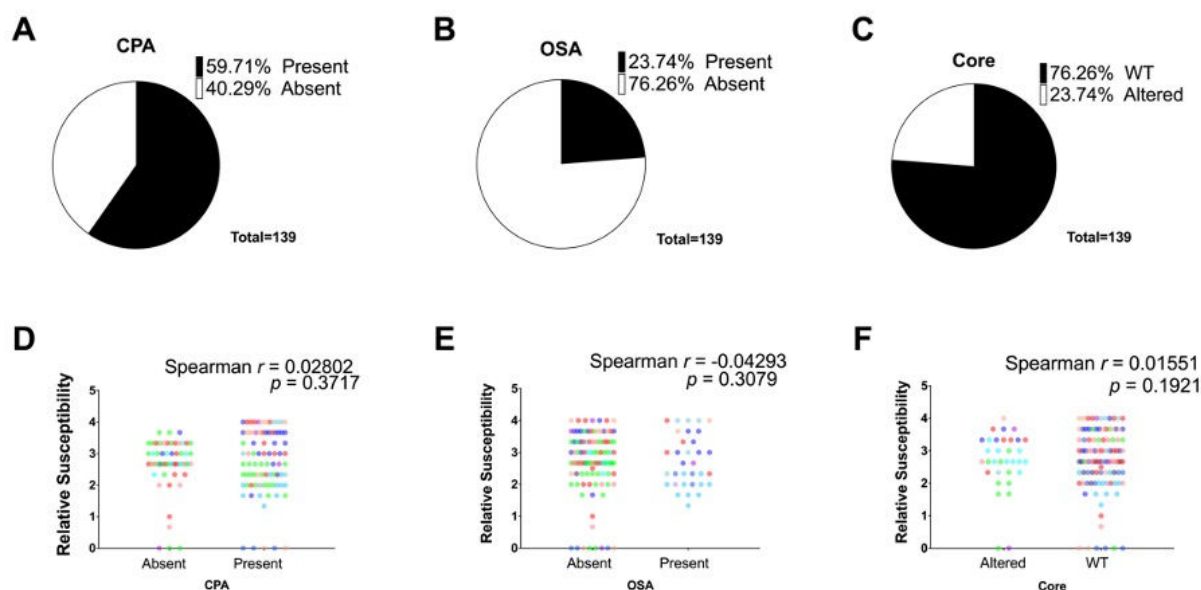
FIG 3  Lipopolysaccharide (LPS) phenotype heterogeneity and lack of correlation with R2-susceptibility. A one-tailed Spearman correlation analysis for each phenotype revealed no correlations between R2-pyocin susceptibility and the absence of (A) common antigen (CPA), (C) O-specific antigen (OSA), or (E) an altered core ($P > 0.05$). CPA was absent in 42.73% of variants (B), OSA was absent in 76.36%, and (F) 27.27% of variants had altered LPS cores. Each data point represents the average relative susceptibility of a variant, and variants are colored based on their longitudinal collection.



FIG 4  No correlation between combined lipopolysaccharide (LPS) deficiency phenotypes and R2-pyocin susceptibility. An LPS Score of 0–3 was assigned to each variant from each sputum collection to assess the correlation between overall LPS phenotypes and R2-pyocin susceptibility. Variants from each population are shown as data points of the same color. No correlation was found between LPS score and R2-pyocin susceptibility ($P > 0.05$).

that three R2-pyocin resistant variants were all CPA− and OSA−. Only 20% of variants from all sputum samples exhibited CPA+, OSA+, and WT LPS cores.

## DISCUSSION

CF chronic lung infections present a significant challenge due to their high antibiotic resistance, difficulty in eradication, and potentially lethal nature (1). *P. aeruginosa*, a common pathogen found in CF lungs, contributes to infection-related morbidity and mortality due to its significant antibiotic resistance and ability to diversify over time (2–7). As a result, it is crucial to explore alternative antimicrobial and therapeutic options for treating these chronic infections.

Bacteriocins, particularly R-pyocins, have recently gained attention for their high potency, narrow spectrum, and anti-pseudomonal properties. However, the efficacy of R-pyocins and other phage-like bacteriocins remains uncertain due to the diverse phenotypic behavior of *P. aeruginosa* in chronic CF lung infections. Previous work demonstrated that *P. aeruginosa* populations display varying susceptibility to R-pyocins, emphasizing the need to better understand the implications of this heterogeneity on treatment outcomes (51). This heterogeneity is particularly important when considering resistant variants in infection, as the prevalence of resistant variants and the benefits of resistance-related phenotypes to survival in chronic infection remain unclear.

In a previous study, we tested the R2-pyocin susceptibility of 60 isolates from 3 patients' expectorated sputum samples, but the limited sample size did not provide a comprehensive understanding of R-pyocin sensitivity and resistance behaviors over time or across more CF patients. In the current study, a larger number of variants were tested, collected from multiple longitudinal samples and more individuals with chronic CF lung infections. A total of 139 *P. aeruginosa* variants were collected from 17 expectorated sputum samples (including longitudinal collections) of 7 individuals with CF and were tested for R2-pyocin susceptibility and LPS phenotypes.

The analysis of *P. aeruginosa* populations from CF lung infections revealed that (i) approximately 76% of sputum samples contained *P. aeruginosa* populations exhibiting heterogeneity among LPS phenotypes; (ii) no correlation was found between R2-pyocin susceptibility and the presence or absence of a specific LPS glycoform; and (iii) it was estimated that about 76% of the variants sampled from sputum lacked O-specific antigen, 40% lacked common antigen, and 23% exhibited altered LPS cores.

In previous work, R-pyocin susceptibility testing has typically focused on one variant per sputum collection (38, 70). However, the current study expanded this approach by examining multiple variants from a wide range of sputum samples from various individuals, including some longitudinal samples. This comprehensive analysis revealed that nearly all bacterial populations studied contained susceptible variants (16 out of 17 populations), with heterogeneity in susceptibility observed among the susceptible variants (51). Only about 5% of the tested variants were resistant to R2-pyocins, and these variants were obtained from only four sputum samples from 3 patients. No trends in R2-pyocin susceptibility were identified across longitudinal samples, and susceptibility did not cluster by patient, but by longitudinal collection.

The high frequency of R2-pyocin susceptible strains in CF lung infections suggests that (i) there is no strong selective pressure for *P. aeruginosa* to alter LPS in a way that leads to R2-pyocin resistance, and (ii) R2-pyocins could be effective against *P. aeruginosa* for the majority of individuals with CF lung infections, as 94% of individuals in the cohort were infected with R2-pyocin susceptible variants.

Previous studies have shown that there is some correlation between OSA serotype and R-pyocin genotype, and though susceptibility to R1, R2, and R5 pyocins was tested for representative strains of varying serotypes, no statistic was provided linking O-antigen serotype with R-pyocin susceptibility (38, 70). Several specific residues on the outer core have been identified as binding factors for each of the three functional subtypes of R-pyocins (38) and this implies that O-serotype alone is not the sole determinant of R-pyocin susceptibility as the residues recognized are present among

all naturally occurring glycoforms of LPS regardless of serotype. It has not been shown that any of the serotypes have differently structured outer cores, thus, in this work, we sought to first look at the presence or absence of the LPS "decorations" (OSA and CPA) and the impacts of variations in these phenotypes rather than correlate serotypes. This study is novel in examining LPS glycoform diversity (i.e., the presence or absence of OSA and CPA) among variants of the same strain or comparing variants within multiple different strains and the resulting impacts on R-pyocin susceptibility.

In this study, LPS phenotypes were analyzed based on the presence or absence of the CPA, the OSA, and the altered or PAO1-like (WT) LPS core. No correlations were found between any of the phenotypes or between various combinations of LPS phenotypes and R2-pyocin susceptibility. However, some sputum samples contained variants with similar LPS phenotypes. For example, one sputum collection had all variants exhibiting the phenotype CPA+, OSA+, and presenting a WT core. A high percentage of variants lacked OSA (76%), while more than half maintained the CPA (40%).

The absence of correlation between specific LPS glycoform phenotypes suggests that other LPS-modifying behaviors, such as "packing density", may be more influential in determining R2-pyocin susceptibility. LPS packing density refers to the arrangement and density of LPS molecules on the outer membrane of Gram-negative bacteria, which is also essential for the stability and integrity of the bacterial outer membrane (38, 74–76). Packing density determines how closely the LPS molecules are arranged on the surface of the membrane, which can influence several aspects of bacterial physiology, including the permeability of the outer membrane to antimicrobial agents, the accessibility of OSA side chains, affecting the recognition of bacterial pathogens by immune cells and the production of antibodies, biofilm formation, and the exposure and accessibility of residues targeted by bacteriophage and bacteriocins (including R-pyocins) (74–76). Other groups have proposed the importance of LPS packing density and the impact on R-pyocin susceptibility (38, 50). One group found several genes responsible for outer membrane lipid homeostasis and LPS packing via TnSeq, including the *mla* and *lpt* transport pathways (50). Though there is some identification of pathways involved in maintaining outer membrane integrity, lipid homeostasis, and LPS arrangement, the regulation of these behaviors remains poorly understood.

In summary, the majority of *P. aeruginosa* strains isolated from CF lung infections appear to be susceptible to R2-pyocins, supporting the potential for R-pyocins as effective antimicrobials. This study also revealed no correlation between LPS phenotype and R2-susceptibility in strains evolved in chronic infection, indicating that further research is necessary to evaluate other LPS-modifying behaviors and packing density in the CF lung environment, as well as the implications of these behaviors for the use of LPS-binding phage and bacteriocins.

## MATERIALS AND METHODS

### Bacterial strains, media, and culture conditions

We collected expectorated sputum samples for this study in the previous work from adult CF patients through Emory–Children's Center for Cystic Fibrosis and Airways Disease Research by the Cystic Fibrosis Biospecimen Laboratory with IRB approval (Georgia Tech approval H18220) (51). We isolated *P. aeruginosa* populations from each sputum sample using selective media (Pseudomonas Isolation Agar, Sigma-Aldrich) before isolating single colonies for further characterization. In total, we studied 139 variants sourced from CF chronic lung infections for R2-pyocin susceptibility and LPS phenotype. All *P. aeruginosa* lab strains used in this work are listed and described in Table 1. All *P. aeruginosa* strains isolated from CF sputum are listed and described in Supplementary Data set 1. We grew all bacterial cultures in lysogeny broth (LB) medium at 37°C with 200 rpm shaking agitation and used standard genetic techniques for the construction of *P. aeruginosa* mutants. The construction of plasmids and generation of

**TABLE 1** Strains, primers, and plasmids used in this study

| Strain | Description | Source |
|---|---|---|
| PAO1N | Wildtype Nottingham laboratory strain | (52) |
| *E. coli* S17-1 λ*pir* | galU galK rpsL(Strr) endA1 nupG thi pro hsdR hsdM +recA (RP4-2 Tc::Mu Km::Tn7) λ*pir* | (51) |
| PAO1N ΔR | R-pyocin null mutant; lacking tail fiber protein (PA0620) and chaperone protein (PA0621) | (46) |
| PAO1N Δ*rmlC* | R2-pyocin susceptible; lacking *rmlC* (PA5164) | This study |
| PAO1 ΔPf4 | Nottingham PAO1 lacking filamentous prophage pf4 | (54) |
| PAO1 ΔPf4ΔR | Nottingham PAO1 lacking filamentous prophage pf4, tail fiber protein (PA0620) and chaperone protein (PA0621) of R-pyocin | This study |

| Primer (bp)[a] | Sequence (5′ to 3′) | Description | Reference or Source |
|---|---|---|---|
| rmlC-del upstream-Forward (600) | GAGCTCAGGTTACCCGCATGCGAGTCGTTCAT-CGGCAACG | Δ*rmlC* mutant generation; To clone PA5164 flanking regions into pDM4 SphI and XbaI sites for deletion | This study |
| rmlC-del upstream-Reverse | GCTGCGAGTATCCGGGGCATCATCAGTACACG-GTCTCGGTCA | Δ*rmlC* mutant generation; To clone PA5164 flanking regions into pDM4 SphI and XbaI sites for deletion | This study |
| rmlC-del downstream-Forward (600) | ACCGAGACCGTGTACTGATGATGCCCCGGATA-CTCGCAGCCACGGC | Δ*rmlC* mutant generation; To clone PA5164 flanking regions into pDM4 SphI and XbaI sites for deletion | This study |
| rmlC-del downstream-Reverse | CACTAGTGGGGCCCTTCTAGGCGCCGGTGCG-TCGGCGGAT | Δ*rmlC* mutant generation; To clone PA5164 flanking regions into pDM4 SphI and XbaI sites for deletion | This study |
| TF-del upstream-Forward (600) | GAGCTCAGGTTACCCGCATGGGTCCTGGATGA-AACCCAGG | R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct | (46) |
| TF-del upstream-Reverse | GTAACGCCGTGGAAGAAACTCATGTCACTGTT-TCCAGGGGA | R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct | (46) |
| TF-del downstream-Forward (600) | CCCCTGGAAACAGTGACATGAGTTTCTTCCAC-GGCGTTACGGTAACCAAC | R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct | (46) |
| TF-del downstream-Reverse | CACTAGTGGGGCCCTTCTAGCGAAGCCGGGG-CGTTGC | R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct | (46) |

| Plasmids | Description | Source |
|---|---|---|
| pDM4 | Suicide vector, Cm$^r$ | Nottingham Collection |
| pJT95 | pDM4 bearing construct gene with deletion of PA0620 and PA0621; Cm$^r$ | (46) |
| pME6032 | Expression vector, Tc$^r$ | Nottingham Collection |
| pMM08 | pDM4 bearing construct gene with deletion of PA5164; Cm$^r$ | This study |

[a]The size of the product of each primer pair is given as base pairs in parentheses.

mutants are described below and all plasmids and primers used for mutant generation can be found in Table 1.

## Generation of mutants

We constructed the PAO1Δ*rmlC* mutant as follows: 600 bp DNA sequences flanking the open reading frames of *rmlC* (PA5164) were PCR amplified using Q5 DNA polymerase (New England Biolabs, Ipswich, MA) (38). We then cloned these sequences into the SphI-XbaI digested suicide vector pDM4 (50) by Gibson assembly using the NEBuilder HiFi Assembly master mix (New England Biolabs), transformed into *E. coli* S17-λpir by electroporation and selected on LB agar plates supplemented with 30 µg/mL chloramphenicol. We verified cloned inserts by the colony PCR and Sanger sequencing (Eton Bioscience, NC). We introduced the deletion construct into PAO1 wt by electroporation and strains carrying single crossover insertions of the deletion constructs were selected on LB agar plates supplemented with 300 µg/mL chloramphenicol (77, 78). Resistant colonies were cultured in LB without antibiotics and plated on LB agar plates with 0.25% NaCl and 5% sucrose to select for loss of the construct (79). We screened sucrose-resistant colonies for antibiotic sensitivity to ensure loss of the vector sequence and were assessed for the presence of the gene deletion by colony PCR and Sanger sequencing of the PCR product. We constructed the PAO1ΔPf4ΔR mutant by deleting the tail fiber and chaperone genes of the R-pyocin gene cassette (PA0620-PA0621) from the PAO1ΔPf4 strain as described previously (51, 72).

## Expression and collection of R-pyocins

We collected R-pyocins in lysates as previously described (51). LB cultures of PAO1, PAO1ΔR (R-pyocin null mutant) (46), PAO1ΔPf4 (prophage mutant) (72), and PAO1Δ*pf4* ΔR were inoculated at 1:100 from overnight planktonic cultures, and grown to mid-logarithmic growth phase (approximately 4 h) in 10 mL of LB media. We then added ciprofloxacin to each culture for a final concentration of 0.1 µg/mL to induce R-pyocin production (51, 80, 81), and cultures were incubated for a further 3 h. Chloroform was used to lyse remaining cells, before centrifuging lysates at ~3,300 × $g$ for 10 min. The R-pyocin-containing supernatants were separated and stored at 4°C.

## Spot assay for relative R-pyocin susceptibility

Spot assays to assess R-pyocin activity were conducted as previously described (51). We used overnight cultures of clinical *P. aeruginosa* variants to inoculate 4 mL of cooled soft top (0.4%) agar at an optical density at 600 nm ($OD_{600}$) of 0.01. This mixture was poured onto LB agar plates for an overlay of the indicator strain. We extracted R2-pyocin lysates from PAO1, PAO1ΔPf4, and respective R-pyocin null mutants were vortexed, then serially diluted 10-fold in phosphate-buffered saline (PBS) before spotting 5 µL of each dilution onto the soft-top overlay indicator strains. Spots were allowed to dry before incubating plates at 37°C overnight. Clear zones of growth inhibition from PAO1ΔPf4 lysates were used to determine R-pyocin-dependent activity against the indicator strains, and we determined relative susceptibility by counting the number of these serial dilutions of lysate indicating inhibition. PAO1 wild type was used as an R2-resistant control strain and PAO1Δ*rmlC* was used as an R2-susceptible control strain to standardize and quality control each experiment (38). We conducted spot assays for R-pyocin susceptibility in triplicate.

## LPS isolation and visualization

We isolated LPS of bacterial cultures and visualized as described by previous methods (73). We used overnight bacterial cultures of 5 mL LB grown at 37°C at 200 rpm shaking. We then diluted overnight cultures 1:10 with LB to measure $OD_{600}$ before making a 1 mL suspension of bacteria adjusted to an $OD_{600}$ of 0.5 and pelleting by centrifugation at 10,600 × $g$ for 10 min. The cell pellet was resuspended in 200 µL of 1 × SDS buffer. Suspended bacterial cells were boiled for 15 min before we prepared LPS by hot aqueous-phenol extraction as previously described but without the addition of DNase I and RNase solutions (as deemed optional in the protocol) (73). We visualized samples using 15 µL of LPS preparation on a 4%–20% Tris-glycine mini gel using the Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (ThermoFisher) (73).

## Statistical analysis

We performed all data analysis using Prism (GraphPad software, version 9).

## AUTHOR AFFILIATIONS

[1]School of Biological Sciences, Center for Microbial Dynamics and Infection, Georgia Institute of Technology, Atlanta, Georgia, USA
[2]Department of Pediatrics, Division of Pulmonary, Allergy and Immunology, Cystic Fibrosis, and Sleep, Emory University School of Medicine, Atlanta, Georgia, USA
[3]Emory+Children's Center for Cystic Fibrosis and Airway Disease Research, Emory University School of Medicine, Atlanta, Georgia, USA

## AUTHOR ORCIDs

Stephen P. Diggle  http://orcid.org/0000-0003-3093-0412

## AUTHOR CONTRIBUTIONS

Madeline Mei, Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review and editing | Preston Pheng, Investigation, Methodology | Detriana Kurzeja-Edwards, Investigation, Methodology | Stephen P. Diggle, Conceptualization, Writing – original draft, Writing – review and editing

## DATA AVAILABILITY

We have made raw data and code available in the Dryad Digital Repository (82).

## ADDITIONAL FILES

The following material is available online.

### Supplemental Material

**Data Set S1 (Spectrum01773-23-s0001.xlsx).** Detailed description of bacterial strains, average susceptibility, and LPS scoring used in this study.
**Fig. S1 (Spectrum01773-23-s0002.tif).** R-pyocin binding to LPS receptor and puncturing target cell membrane.
**Fig. S2 (Spectrum01773-23-s0003.tif).** R2-pyocin lysate collection and susceptibility testing procedure.
**Supplemental legends (Spectrum01773-23-s0004.docx).** Legends for Fig. S1 and S2 and Data Set S1.

## REFERENCES

1. Elborn JS. 2016. Cystic fibrosis. Lancet 388:2519–2531. https://doi.org/10.1016/S0140-6736(16)00576-6
2. Hogardt M, Heesemann J. 2010. Adaptation of *Pseudomonas aeruginosa* during persistence in the cystic fibrosis lung. Int J Med Microbiol 300:557–562. https://doi.org/10.1016/j.ijmm.2010.08.008
3. Kerem E, Corey M, Gold R, Levison H. 1990. Pulmonary function and clinical course in patients with cystic fibrosis after pulmonary colonization with *Pseudomonas aeruginosa*. J Pediatr 116:714–719. https://doi.org/10.1016/s0022-3476(05)82653-8
4. Boucher JC, Yu H, Mudd MH, Deretic V. 1997. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. Infect Immun 65:3838–3846. https://doi.org/10.1128/iai.65.9.3838-3846.1997
5. Winstanley C, O'Brien S, Brockhurst MA. 2016. *Pseudomonas aeruginosa* evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. Trends Microbiol. 24:327–337. https://doi.org/10.1016/j.tim.2016.01.008
6. Williams D, Evans B, Haldenby S, Walshaw MJ, Brockhurst MA, Winstanley C, Paterson S. 2015. Divergent, coexisting *Pseudomonas aeruginosa* lineages in chronic cystic fibrosis lung infections. Am J Respir Crit Care Med 191:775–785. https://doi.org/10.1164/rccm.201409-1646OC
7. Workentine ML, Sibley CD, Glezerson B, Purighalla S, Norgaard-Gron JC, Parkins MD, Rabin HR, Surette MG, Battista JR. 2013. Phenotypic heterogeneity of *Pseudomonas aeruginosa* populations in a cystic fibrosis patient. PLoS ONE 8:e60225. https://doi.org/10.1371/journal.pone.0060225
8. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FSL, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GKS, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock REW, Lory S, Olson MV. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 406:959–964. https://doi.org/10.1038/35023079

9. Foweraker JE, Laughton CR, Brown DFJ, Bilton D. 2005. Phenotypic variability of *Pseudomonas aeruginosa* in sputa from patients with acute infective exacerbation of cystic fibrosis and its impact on the validity of antimicrobial susceptibility testing. J Antimicrob Chemother 55:921–927. https://doi.org/10.1093/jac/dki146

10. Bradbury RS, Tristram SG, Roddam LF, Reid DW, Inglis TJJ, Champion AC. 2011. Antimicrobial susceptibility testing of cystic fibrosis and non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*: a comparison of three methods. Br J Biomed Sci 68:1–4. https://doi.org/10.1080/09674845.2011.11732833

11. Morita Y, Tomida J, Kawamura Y. 2014. Responses of *Pseudomonas aeruginosa* to antimicrobials. Front Microbiol 4:422. https://doi.org/10.3389/fmicb.2013.00422

12. Smith RS, Iglewski BH. 2003. *P. aeruginosa* quorum-sensing systems and virulence. Curr Opin Microbiol 6:56–60. https://doi.org/10.1016/s1369-5274(03)00008-0

13. Diggle SP, Griffin AS, Campbell GS, West SA. 2007. Cooperation and conflict in quorum-sensing bacterial populations. Nature 450:411–414. https://doi.org/10.1038/nature06279

14. Pesci EC, Pearson JP, Seed PC, Iglewski BH. 1997. Regulation of las and rhl quorum sensing in *Pseudomonas aeruginosa*. J Bacteriol 179:3127–3132. https://doi.org/10.1128/jb.179.10.3127-3132.1997

15. D'Costa VM, McGrann KM, Hughes DW, Wright GD. 2006. Sampling the antibiotic resistome. Science 311:374–377. https://doi.org/10.1126/science.1120800

16. Cao P, Fleming D, Moustafa DA, Dolan SK, Szymanik KH, Redman WK, Ramos A, Diggle FL, Sullivan CS, Goldberg JB, Rumbaugh KP, Whiteley M. 2023. A *Pseudomonas aeruginosa* small RNA regulates chronic and acute infection. Nature 618:358–364. https://doi.org/10.1038/s41586-023-06111-7

17. Bhagirath AY, Li Y, Somayajula D, Dadashi M, Badr S, Duan K. 2016. Cystic fibrosis lung environment and *Pseudomonas aeruginosa* infection. BMC Pulm Med 16:174. https://doi.org/10.1186/s12890-016-0339-5

18. Jorth P, Ehsan Z, Rezayat A, Caldwell E, Pope C, Brewington JJ, Goss CH, Benscoter D, Clancy JP, Singh PK. 2019. Direct lung sampling indicates that established pathogens dominate early infections in children with cystic fibrosis. Cell Rep 27:1190–1204. https://doi.org/10.1016/j.celrep.2019.03.086

19. Darch SE, McNally A, Harrison F, Corander J, Barr HL, Paszkiewicz K, Holden S, Fogarty A, Crusz SA, Diggle SP. 2015. Recombination is a key driver of genomic and phenotypic diversity in a *Pseudomonas aeruginosa* population during cystic fibrosis infection. Sci Rep 5:7649. https://doi.org/10.1038/srep07649

20. Williams D, Fothergill JL, Evans B, Caples J, Haldenby S, Walshaw MJ, Brockhurst MA, Winstanley C, Paterson S. 2018. Transmission and lineage displacement drive rapid population genomic flux in cystic fibrosis airway infections of a *Pseudomonas aeruginosa* epidemic strain. Microb Genom 4:e000167. https://doi.org/10.1099/mgen.0.000167

21. Feliziani S, Luján AM, Moyano AJ, Sola C, Bocco JL, Montanaro P, Canigia LF, Argaraña CE, Smania AM. 2010. Mucoidy, quorum sensing, mismatch repair and antibiotic resistance in *Pseudomonas aeruginosa* from cystic fibrosis chronic airways infections. PLoS One 5:e12669. https://doi.org/10.1371/journal.pone.0012669

22. Cystic Fibrosis Foundation Patient Registry. 2019. Annual data report. Cystic Fibrosis Foundation.

23. Clark ST, Diaz Caballero J, Cheang M, Coburn B, Wang PW, Donaldson SL, Zhang Y, Liu M, Keshavjee S, Yau YCW, Waters VJ, Elizabeth Tullis D, Guttman DS, Hwang DM. 2015. Phenotypic diversity within a *Pseudomonas aeruginosa* population infecting an adult with cystic fibrosis. Sci Rep 5. https://doi.org/10.1038/srep10932

24. Roberts AEL, Kragh KN, Bjarnsholt T, Diggle SP. 2015. The limitations of *in vitro* experimentation in understanding biofilms and chronic infection. J Mol Biol 427:3646–3661. https://doi.org/10.1016/j.jmb.2015.09.002

25. Bradley DE. 1967. Ultrastructure of bacteriophage and bacteriocins. Bacteriol Rev 31:230–314. https://doi.org/10.1128/br.31.4.230-314.1967

26. Reeves P. 1972. The Bacteriocins. In The Bacteriocins (molecular biology, biochemistry and biophysics). Spring-Verlag, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-46289-4

27. Kageyama M, Egami F. 1962. On the purification and some properties of a pyocin, a bacteriocin produced by *Pseudomonas aeruginosa*. Life Sciences 1:471–476. https://doi.org/10.1016/0024-3205(62)90055-3

28. Holloway BW. 1969. Genetics of pseudomonas. Bacteriol Rev 33:419–443. https://doi.org/10.1128/br.33.3.419-443.1969

29. Kageyama M. 1975. Bacteriocins and bacteriophages in *Pseudomonas aeruginosa*. Microbial drug resistance:291–305.

30. Michel-Briand Y, Baysse C. 2002. The pyocins of *Pseudomonas aeruginosa*. Biochimie 84:499–510. https://doi.org/10.1016/s0300-9084(02)01422-0

31. Scholl D. 2017. Phage tail–like bacteriocins. Annu Rev Virol 4:453–467. https://doi.org/10.1146/annurev-virology-101416-041632

32. Jacob F. 1954. Induced biosynthesis and mode of action of a pyocine, an antibiotic obtained from PS. pyocyanea. Ann Inst Pasteur 86:149–160.

33. Ito S, Kageyama M. 1970. Relationship between pyocins and a bacteriophage in *Pseudomonas aeruginosa*. J Gen Appl Microbiol 16:231–240. https://doi.org/10.2323/jgam.16.3_231

34. Govan JRW. 1974. Studies on the pyocins of *Pseudomonas aeruginosa*: production of contractile and flexuous pyocins in *Pseudomonas aeruginosa*. J Gen Microbiol 80:17–30. https://doi.org/10.1099/00221287-80-1-17

35. Ikeda K, Egami F. 1969. Receptor substance for Pyocin R I. J Biochem 65:603–609. https://doi.org/10.1093/oxfordjournals.jbchem.a129053

36. Ikeda K, Egami F. 1973. Lipopolysaccharide of *Pseudomonas aeruginosa* with special reference to Pyocin R receptor activity. J Gen Appl Microbiol 19:115–128. https://doi.org/10.2323/jgam.19.115

37. Ikeda K, Nishi Y. 1973. Interaction between Pyocin R and Pyocin R receptor. J Gen Appl Microbiol 19:209–219. https://doi.org/10.2323/jgam.19.209

38. Köhler T, Donner V, van Delden C. 2010. Lipopolysaccharide as shield and receptor for R-Pyocin-mediated killing in *Pseudomonas aeruginosa*. J Bacteriol 192:1921–1928. https://doi.org/10.1128/JB.01459-09

39. Higerd TB, Baechler CA, Berk RS. 1969. Morphological studies on relaxed and contracted forms of purified pyocin particles. J Bacteriol 98:1378–1389. https://doi.org/10.1128/jb.98.3.1378-1389.1969

40. Yui C. 1971. Structure of Pyocin R I. isolation of sheath from Pyocin R by alkali treatment and its properties. J Biochem 69:101–110. https://doi.org/10.1093/oxfordjournals.jbchem.a129437

41. Ohsumi M, Shinomiya T, Kageyama M. 1980. Comparative study on R-type pyocins of *Pseudomonas aeruginosa*. J Biochem 87:1119–1125.

42. Uratani Y, Hoshino T. 1984. Pyocin R1 inhibits active transport in *Pseudomonas aeruginosa* and depolarizes membrane potential. J Bacteriol 157:632–636. https://doi.org/10.1128/jb.157.2.632-636.1984

43. Ge P, Scholl D, Leiman PG, Yu X, Miller JF, Zhou ZH. 2015. Atomic structures of a bactericidal contractile nanotube in its pre- and postcontraction states. Nat Struct Mol Biol 22:377–382. https://doi.org/10.1038/nsmb.2995

44. Ge P, Scholl D, Prokhorov NS, Avaylon J, Shneider MM, Browning C, Buth SA, Plattner M, Chakraborty U, Ding K, Leiman PG, Miller JF, Zhou ZH. 2020. Action of a minimal contractile bactericidal nanomachine. Nature 580:658–662. https://doi.org/10.1038/s41586-020-2186-z

45. Nakayama K, Takashima K, Ishihara H, Shinomiya T, Kageyama M, Kanaya S, Ohnishi M, Murata T, Mori H, Hayashi T. 2000. The R-type pyocin of *Pseudomonas aeruginosa* is related to P2 phage, and the F-type is related to lambda phage. Mol Microbiol 38:213–231. https://doi.org/10.1046/j.1365-2958.2000.02135.x

46. Huszczynski SM, Lam JS, Khursigara CM. 2019. The role of *Pseudomonas aeruginosa* lipopolysaccharide in bacterial pathogenesis and physiology. Pathogens 9:6. https://doi.org/10.3390/pathogens9010006

47. Lam JS, Taylor VL, Islam ST, Hao Y, Kocíncová D. 2011. Genetic and functional diversity of *Pseudomonas aeruginosa* lipopolysaccharide. Front Microbiol 2:118. https://doi.org/10.3389/fmicb.2011.00118

48. Hao Y, King JD, Huszczynski S, Kocíncová D, Lam JS, Davies JE. 2013. Five new genes are important for common polysaccharide antigen biosynthesis in *Pseudomonas aeruginosa*. mBio 4. https://doi.org/10.1128/mBio.00631-12

49. Anbo M, Jelsbak L. 2023. A bittersweet fate: detection of serotype switching in *Pseudomonas aeruginosa* Microb Genom 9:mgen000919. https://doi.org/10.1099/mgen.0.000919

50. Carim S, Azadeh AL, Kazakov AE, Price MN, Walian PJ, Lui LM, Nielsen TN, Chakraborty R, Deutschbauer AM, Mutalik VK, Arkin AP. 2021. Systematic discovery of pseudomonad genetic factors involved in sensitivity to tailocins. ISME J 15:2289–2305. https://doi.org/10.1038/s41396-021-00921-1

51. Mei M, Thomas J, Diggle SP, Hogan DA. 2021. Heterogenous susceptibility to R-pyocins in populations of *Pseudomonas aeruginosa* Sourced from cystic fibrosis lungs. mBio 12:mBio https://doi.org/10.1128/mBio.00458-21

52. Lerouge I, Vanderleyden J. 2002. O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. FEMS Microbiol Rev 26:17–47. https://doi.org/10.1111/j.1574-6976.2002.tb00597.x

53. Byrd MS, Sadovskaya I, Vinogradov E, Lu H, Sprinkle AB, Richardson SH, Ma L, Ralston B, Parsek MR, Anderson EM, Lam JS, Wozniak DJ. 2009. Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. Mol Microbiol 73:622–638. https://doi.org/10.1111/j.1365-2958.2009.06795.x

54. King JD, Kocíncová D, Westman EL, Lam JS. 2009. Review: lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa*. Innate Immun 15:261–312. https://doi.org/10.1177/1753425909106436

55. de Kievit TR, Lam JS. 1994. Monoclonal antibodies that distinguish inner core, outer core, and lipid A regions of *Pseudomonas aeruginosa* Lipopolysaccharide. J Bacteriol 176:7129–7139. https://doi.org/10.1128/jb.176.23.7129-7139.1994

56. Kintz E, Goldberg JB. 2008. Regulation of Lipopolysaccharide O antigen expression in *Pseudomonas aeruginosa*. Future Microbiol 3:191–203. https://doi.org/10.2217/17460913.3.2.191

57. Poon KKH, Westman EL, Vinogradov E, Jin S, Lam JS. 2008. Functional characterization of MigA and WapR: putative rhamnosyltransferases involved in outer core oligosaccharide biosynthesis of *Pseudomonas aeruginosa*. J Bacteriol 190:1857–1865. https://doi.org/10.1128/JB.01546-07

58. Lau PCY, Lindhout T, Beveridge TJ, Dutcher JR, Lam JS. 2009. Differential lipopolysaccharide core capping leads to quantitative and correlated modifications of mechanical and structural properties in *Pseudomonas aeruginosa* Biofilms . J Bacteriol 191:6618–6631. https://doi.org/10.1128/JB.00698-09

59. Maldonado RF, Sá-Correia I, Valvano MA. 2016. Lipopolysaccharide modification in gram-negative bacteria during chronic infection. FEMS Microbiol Rev 40:480–493. https://doi.org/10.1093/femsre/fuw007

60. Pier GB. 2007. *Pseudomonas aeruginosa* lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. Int J Med Microbiol 297:277–295. https://doi.org/10.1016/j.ijmm.2007.03.012

61. Kocíncová D, Lam JS. 2011. Structural diversity of the core oligosaccharide domain of *Pseudomonas aeruginosa* Lipopolysaccharide. Biochemistry (Mosc) 76:755–760. https://doi.org/10.1134/S0006297911070054

62. Wang L, Wang Q, Reeves PR. 2010. The variation of O antigens in gram-negative bacteria. Subcell Biochem 53:123–152. https://doi.org/10.1007/978-90-481-9078-2_6

63. Qin S, Xiao W, Zhou C, Pu Q, Deng X, Lan L, Liang H, Song X, Wu M. 2022. *Pseudomonas aeruginosa*: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. Signal Transduct Target Ther 7:199. https://doi.org/10.1038/s41392-022-01056-1

64. Di Lorenzo F, Silipo A, Bianconi I, Lore' NI, Scamporrino A, Sturiale L, Garozzo D, Lanzetta R, Parrilli M, Bragonzi A, Molinaro A. 2015. Persistent cystic fibrosis isolate *Pseudomonas aeruginosa* strain RP73 exhibits an under-acylated LPS structure responsible of its low inflammatory activity. Mol Immunol 63:166–175. https://doi.org/10.1016/j.molimm.2014.04.004

65. del Campo R, Martínez E, del Fresno C, Alenda R, Gómez-Piña V, Fernández-Ruíz I, Siliceo M, Jurado T, Toledano V, Arnalich F, García-Río F, López-Collazo E. 2011. Translocated LPS might cause endotoxin tolerance in circulating monocytes of cystic fibrosis patients. PLoS One 6:e29577. https://doi.org/10.1371/journal.pone.0029577

66. Cigana C, Curcurù L, Leone MR, Ieranò T, Lorè NI, Bianconi I, Silipo A, Cozzolino F, Lanzetta R, Molinaro A, Bernardini ML, Bragonzi A. 2009.

67. *Pseudomonas aeruginosa* exploits lipid A and muropeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection. PLoS One 4:e8439. https://doi.org/10.1371/journal.pone.0008439

68. Ciofu O, Mandsberg LF, Bjarnsholt T, Wassermann T, Høiby N. 2010. Genetic adaptation of *Pseudomonas aeruginosa* during chronic lung infection of patients with cystic fibrosis: strong and weak mutators with heterogeneous genetic backgrounds emerge in mucA and/or lasR Mutants. Microbiology (Reading) 156:1108–1119. https://doi.org/10.1099/mic.0.033993-0

69. Giordano NP, Cian MB, Dalebroux ZD. 2020. Outer membrane lipid secretion and the innate immune response to gram-negative bacteria. Infect Immun 88:e00920-19. https://doi.org/10.1128/IAI.00920-19

70. Tognon M, Köhler T, Gdaniec BG, Hao Y, Lam JS, Beaume M, Luscher A, Buckling A, van Delden C. 2017. Co-evolution with *Staphylococcus aureus* leads to lipopolysaccharide alterations in *Pseudomonas aeruginosa*. ISME J 11:2233–2243. https://doi.org/10.1038/ismej.2017.83

71. Redero M, López-Causapé C, Aznar J, Oliver A, Blázquez J, Prieto AI. 2018. Susceptibility to R-pyocins of *Pseudomonas aeruginosa* clinical isolates from cystic fibrosis patients. J Antimicrob Chemother 73:2770–2776. https://doi.org/10.1093/jac/dky261

72. Redero M, Aznar J, Prieto AI. 2020. Antibacterial efficacy of R-type pyocins against *Pseudomonas aeruginosa* on biofilms and in a murine model of acute lung infection. J Antimicrob Chemother:dkaa121. https://doi.org/10.1093/jac/dkaa121

73. Secor PR, Sweere JM, Michaels LA, Malkovskiy AV, Lazzareschi D, Katznelson E, Rajadas J, Birnbaum ME, Arrigoni A, Braun KR, Evanko SP, Stevens DA, Kaminsky W, Singh PK, Parks WC, Bollyky PL. 2015. Filamentous bacteriophage promote biofilm assembly and function. Cell Host & Microbe 18:549–559. https://doi.org/10.1016/j.chom.2015.10.013

74. Davis MR, Goldberg JB. 2012. Purification and visualization of lipopolysaccharide from gram-negative bacteria by hot aqueous-phenol extraction. J Vis Exp, no. 63 :3916.:3916. https://doi.org/10.3791/3916

75. Jacobson KH, Gunsolus IL, Kuech TR, Troiano JM, Melby ES, Lohse SE, Hu D, Chrisler WB, Murphy CJ, Orr G, Geiger FM, Haynes CL, Pedersen JA. 2015. Lipopolysaccharide density and structure govern the extent and distance of nanoparticle interaction with actual and model bacterial outer membranes. Environ Sci Technol 49:10642–10650. https://doi.org/10.1021/acs.est.5b01841

76. Snyder S, Kim D, McIntosh TJ. 1999. Lipopolysaccharide bilayer structure: effect of chemotype, core mutations, divalent cations, and temperature. Biochemistry 38:10758–10767. https://doi.org/10.1021/bi990867d

77. Clifton LA, Ciesielski F, Skoda MWA, Paracini N, Holt SA, Lakey JH. 2016. The effect of lipopolysaccharide core oligosaccharide size on the electrostatic binding of antimicrobial proteins to models of the gram negative bacterial outer membrane. Langmuir 32:3485–3494. https://doi.org/10.1021/acs.langmuir.6b00240

78. Choi K-H, Schweizer HP. 2006. mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. Nat Protoc 1:153–161. https://doi.org/10.1038/nprot.2006.24

79. Holloway BW. 1955. Genetic recombination in *Pseudomonas aeruginosa*. Microbiology 13:572–581. https://doi.org/10.1099/00221287-13-3-572

80. Hmelo LR, Borlee BR, Almblad H, Love ME, Randall TE, Tseng BS, Lin C, Irie Y, Storek KM, Yang JJ, Siehnel RJ, Howell PL, Singh PK, Tolker-Nielsen T, Parsek MR, Schweizer HP, Harrison JJ. 2015. Precision-engineering the *Pseudomonas aeruginosa* genome with two-step allelic exchange. Nat Protoc 10:1820–1841. https://doi.org/10.1038/nprot.2015.115

81. Brazas MD, Haaerugaerugcock REW. 2005. Ciprofloxacin induction of a susceptibility determinant in *Pseudomonas aeruginosa* . Antimicrob Agents Chemother 49:3222–3227. https://doi.org/10.1128/AAC.49.8.3222-3227.2005

82. Penterman J, Singh PK, Walker GC. 2014. Biological cost of pyocin production during the SOS response in *Pseudomonas aeruginosa*. J Bacteriol 196:3351–3359. https://doi.org/10.1128/JB.01889-14

83. Mei M, Pheng P, Kurzeja-Edwards D, Diggle SP. 2023. High prevalence of lipopolysaccharide mutants and R2-Pyocin susceptible variants in *Pseudomonas aeruginosa* populations sourced from cystic fibrosis lung infections . Microbiology. https://doi.org/10.1101/2023.04.26.538445