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

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ABSTRACT

Cystic fibrosis (CF) lungs often suffer from chronic, debilitating infections that are highly resistant to antibiotics and can be fatal. *Pseudomonas aeruginosa* is a common pathogen in CF lungs and often displays strong resistance to multiple antibiotic classes, which contributes to antimicrobial resistance (AMR). These bacterial populations display a high degree of genetic and phenotypic diversity, but it is unclear how this diversity affects their susceptibility to non-traditional antimicrobials, such as bacteriocins. R-pyocins, a type of bacteriocin produced by *P. aeruginosa*, are phage tail-like protein complexes that are highly potent and have a narrow killing spectrum. They do not replicate and could potentially be used as antimicrobials. However, recent research suggests that the diversity of *P. aeruginosa* variants within CF lung infections leads to varying susceptibility to R-pyocins. This variation may be due to changes in the lipopolysaccharide (LPS) structure, which serves as the R-pyocin receptor. Currently, there is no estimation of how many CF strains may be susceptible to any of the five known R-pyocin subtypes (R1-R5), particularly when considering the diverse bacterial populations found in CF lungs. Additionally, the relationship between LPS phenotype and R-pyocin susceptibility is not well understood. In this study, we tested 139 *P. aeruginosa* variants from 17 sputum samples of seven CF patients for susceptibility to R2-pyocins and analyzed their LPS phenotypes. Our findings indicate that (i) approximately 83% of sputum samples contain diverse *P. aeruginosa* populations without R2-pyocin resistant variants, while all samples had some susceptible variants; (ii) there was no clear correlation between LPS phenotypes and R-pyocin susceptibility and (iii) 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 clear correlation between LPS phenotypes 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 use of R-pyocins as therapeutic agents, as a large number of infectious CF variants appear to be 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*. 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 *P. aeruginosa* 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 future effective treatments for these chronic infections.

INTRODUCTION

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 fosters 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 (8-15). 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 (16-20). While recent data suggests that the approval and use of the new triple therapeutic TRIKAFTA® (elexacaftor/tezacaftor/ivacaftor) have improved 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 (21). 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, 17, 18, 22, 23). While this heterogeneity is known to affect antibiotic susceptibility, its impact on alternative therapeutic options, such as bacteriocins and bacteriophage, remains poorly understood (16, 18, 20). As pathogens like *P. aeruginosa* become increasingly difficult to eliminate, non-traditional 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 (24, 25). *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 (26-30). Pyocins produced by *P. aeruginosa* are classified as S-, R-, and F-pyocins, each with varying antimicrobial spectra (29-34). 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 (29, 30, 35-37). 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**) (38-44). 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 (29, 30, 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*.

In previous research, we tested 20 individual *P. aeruginosa* variants from sputum samples collected from three different individuals with cystic fibrosis (CF) (60 variants total, from 3 *P. aeruginosa* populations). We found a variation in susceptibility to R2-pyocins within these populations (46). Additionally, we isolated and compared the lipopolysaccharide (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 (46). 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 (37, 46, 47). 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 (46) 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* 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), 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 (46), and (ii) nearly every *P. aeruginosa* population included variants with some degree of susceptibility to R2-pyocins. In our sample, we found that only 3 out of 7 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 years. 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 (48) and compared the LPS migration profiles of our clinical variants to those of isogenic LPS mutants of 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**) ($p > 0.05$).

169 We further evaluated the relationship between LPS phenotype and R2-pyocin susceptibility by
170 assessing the LPS as a whole. Using a numbered scale, we assigned an LPS Score of 0 when a
171 variant presented as CPA-, OSA-, and had an altered core; this variant was treated as having 0/3
172 WT LPS characteristics. A score of 1 was assigned when a variant exhibited one of the three LPS
173 glycoforms as WT (presence of OSA, CPA, or unaltered core), a score of 2 when two of three
174 glycoforms were WT, and a score of 3 when a variant presented CPA+, OSA+, with a WT core.
175 Using this classification system, we also found no correlation with R2-pyocin susceptibility (Fig.
176 4). However, we did find that three R2-pyocin resistant variants were all CPA- and OSA-. Only
177 20% of variants from all sputum samples exhibited CPA+, OSA+, and WT LPS cores.
178
179 **DISCUSSION**
180
181 Cystic fibrosis (CF) chronic lung infections present a significant challenge due to their high
182 antibiotic resistance, difficulty in eradication, and potentially lethal nature (1). *P. aeruginosa*, a
183 common pathogen found in CF lungs, contributes to infection-related morbidity and mortality due
184 to its significant antibiotic resistance and ability to diversify over time (2-7). As a result, it is crucial
185 to explore alternative antimicrobial and therapeutic options for treating these chronic infections.

186 Bacteriocins, particularly R-pyocins, have recently gained attention for their high potency, narrow
187 spectrum, and anti-pseudomonal properties. However, the efficacy of R-pyocins and other phage-
188 like bacteriocins remains uncertain due to the diverse phenotypic behavior of *P. aeruginosa* in
189 chronic CF lung infections. Previous work demonstrated that *P. aeruginosa* populations display
190 varying susceptibility to R-pyocins, emphasizing the need to better understand the implications of
191 this heterogeneity on treatment outcomes (46). This heterogeneity is particularly important when
192 considering resistant variants in infection, as the prevalence of resistant variants and the benefits
193 of resistance-related phenotypes to survival in chronic infection remain unclear.

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

202 The analysis of *P. aeruginosa* populations from CF lung infections revealed that (i) approximately
203 76% of sputum samples contained *P. aeruginosa* populations exhibiting heterogeneity among
204 LPS phenotypes; (ii) no correlation was found between R2-pyocin susceptibility and the presence
205 or absence of a specific LPS glycoform; and (iii) it was estimated that about 76% of the variants
206 sampled from sputum lacked O-specific antigen, 40% lacked common antigen, and 23% exhibited
207 altered LPS cores.

208 In previous studies, R-pyocin susceptibility testing has typically focused on one variant per sputum
209 collection (37, 47). However, the current study expanded this approach by examining multiple
210 variants from a wide range of sputum samples from various individuals, including some
211 longitudinal samples. This comprehensive analysis revealed that nearly all bacterial populations
212 studied contained susceptible variants (16 out of 17 populations), with heterogeneity in
213 susceptibility observed among the susceptible variants (46). Only about 5% of the tested variants
214 were resistant to R2-pyocins, and these variants were obtained from only 4 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.

While previous studies have assessed correlations between serotype and R-pyocin susceptibility (37, 47), the relationship between LPS phenotype and R-pyocin susceptibility has remained unexplored. In this study, LPS phenotypes were analyzed based on the presence or absence of the common antigen (CPA), the O-specific antigen (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. Another group has proposed the importance of LPS packing density on R-pyocin susceptibility, though the mechanism or regulation of this behavior remains poorly understood (49).

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 previous work (46) 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). 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 isolate from CF sputum are listed and described in **Supplementary Dataset 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 construction of *P. aeruginosa* mutants. The construction of plasmids and generation of 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 PAO1*ΔrmlC* 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) (37). We then cloned these sequences

261 into the SphI-XbaI digested suicide vector pDM4 (50) by Gibson assembly using NEBuilder HiFi
262 Assembly master mix (New England Biolabs), transformed into *E. coli* S17-λpir by electroporation
263 and selected on LB agar plates supplemented with 30 µg/mL chloramphenicol. We verified cloned
264 inserts by colony PCR and Sanger sequencing (Eton Bioscience, NC). We introduced the deletion
265 construct into PAO1 wt by electroporation and strains carrying single crossover insertions of the
266 deletion constructs were selected on LB agar plates supplemented with 300 µg/mL
267 chloramphenicol (51,52). Resistant colonies were cultured in LB without antibiotic and plated on
268 LB agar plates with 0.25% NaCl and 5% sucrose to select for loss of the construct (53). We
269 screened sucrose-resistant colonies for antibiotic sensitivity to ensure loss of the vector sequence
270 and were assessed for presence of the gene deletion by colony PCR and Sanger sequencing of
271 the PCR product. We constructed the PAO1ΔPf4ΔR mutant by deleting the tail fiber and
272 chaperone genes of the R-pyocin gene cassette (PA0620-PA0621) from the PAO1ΔPf4 strain as
273 described previously (46).
274
275 **Expression and collection of R-pyocins.** We collected R-pyocins in lysates as previously
276 described (46). LB cultures of PAO1, PAO1ΔR (R-pyocin null mutant) (46), PAO1ΔPf4 (prophage
277 mutant) (54), and PAO1Δ*pf4* ΔR were inoculated at 1:100 from overnight planktonic cultures, and
278 grown to mid-logarithmic growth phase (approximately 4 h) in 10 mL of LB media. We then added
279 ciprofloxacin to each culture for a final concentration of 0.1 µg/mL to induce R-pyocin production
280 (46, 55, 56), and cultures were incubated for a further 3 h. Chloroform was used to lyse remaining
281 cells, before centrifuging lysates at ~3,300 *x g* for 10 min. The R-pyocin-containing supernatants
282 were separated and stored at 4°C.
283
284 **Spot assay for Relative R-pyocin Susceptibility.** Spot assays to assess R-pyocin activity were
285 conducted as previously described (46). We used overnight cultures of clinical *P. aeruginosa*
286 variants to inoculate 4 mL of cooled soft top (0.4%) agar at an optical density at 600 nm (OD_{600})
287 of 0.01. This mixture was poured onto LB agar plates for an overlay of the indicator strain. We
288 extracted R2-pyocin lysates from PAO1, PAO1ΔPf4 and respective R-pyocin null mutants were
289 vortexed, then serial diluted 10-fold in phosphate buffered saline (PBS) before spotting 5 µL of
290 each dilution onto the soft-top overlay indicator strains. Spots were allowed to dry before
291 incubating plates at 37°C overnight. Clear zones of growth inhibition from PAO1ΔPf4 lysates were
292 used to determine R-pyocin dependent activity against the indicator strains, and we determined
293 relative susceptibility by counting the number of these serial dilutions of lysate indicating inhibition.
294 PAO1 wild type was used as an R2-resistant control strain and PAO1Δ*rmlC* was used as an R2-
295 susceptible control strain to standardize and quality control each experiment (37). We conducted
296 spot assays for R-pyocin susceptibility in triplicate.
297
298 **LPS isolation and visualization.** We isolated LPS of bacterial cultures and visualized as
299 described by previous methods (48). We used overnight bacterial cultures of 5 mL LB grown at
300 37°C at 200 rpm shaking. We then diluted overnight cultures 1:10 with LB to measure OD_{600}
301 before making a 1 mL suspension of bacteria adjusted to an OD_{600} of 0.5 and pelleting by
302 centrifugation at 10,600 *x g* for 10 min. The cell pellet was resuspended in 200 µL of 1x SDS-
303 buffer. Suspended bacterial cells were boiled for 15 min before we prepared LPS by hot aqueous-
304 phenol extraction as previously described (48) but without the addition of DNase I and RNase
305 solutions (as deemed optional in the protocol). We visualized samples using 15 µL of LPS
306 preparation on a 4%-20% Tris-glycine mini gel using the Pro-Q Emerald 300 Lipopolysaccharide
307 Gel Stain Kit (ThermoFisher) (48).
308
309 **Statistical analysis.** We performed all data analysis using Prism (Graphpad software, version
310 9).
311

Data Availability. We have made raw data and code available in the Dryad Digital Repository: (URL in progress) (57).

Supplemental Material

Supplementary Dataset 1

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Figure legends

Table 1. Strains, primers and plasmids used in this study.

Figure 1. R2-pyocin inhibition zones for multiple CF variants from different patient sputum samples. The inhibition zones caused by R2-pyocin 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 as an R2-resistant control. Red boxes indicate patients with populations containing resistant variants. Up to ten variants per sputum sample were tested. An inhibition zone indicates R2-pyocin susceptibility, while the absence of a visible zone indicates resistance.

Figure 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 (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**.**

Figure 3. 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.

Figure 4. No correlation between combined LPS deficiency phenotypes and R2-pyocin susceptibility. An LPS Score of 0 to 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 ($p > 0.05$).

Figure S1. R-pyocin binding to LPS receptor and puncturing target cell membrane. When released into the environment by producing cells, R-pyocins bind to target cells by recognizing core residues of the lipopolysaccharide (LPS) on the outer cell membrane. After tail fiber binding, the baseplate dissociates, initiating sheath contraction and driving the iron-tipped tube through the cell surface, killing the target bacterium.

Figure S2. R2-pyocin lysate collection and susceptibility testing procedure. R-pyocins are isolated from *P. aeruginosa* by growing strains to early logarithmic growth, adding sub-minimal inhibitory concentration (MIC) ciprofloxacin, and lysing cells with chloroform after several hours for maximum R-pyocin production. Cultures are then centrifuged, separating into phases, allowing for the collection of cell-free R-pyocin-containing supernatant (lysates) to be stored. R-pyocin lysates are serially diluted and spotted onto soft agar overlays to test the susceptibility of strains of interest.

References

1. Elborn JS. 2016. Cystic fibrosis. The Lancet 388:2519-2531.
2. Hogardt M, Heesemann J. 2010. Adaptation of *Pseudomonas aeruginosa* during persistence in the cystic fibrosis lung. Int J Med Microbiol 300:557-62.
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 116:714-9.
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 65:3838-46.
5. Winstanley C, O'Brien S, Brockhurst MA. 2016. *Pseudomonas aeruginosa* Evolutionary Adaptation and Diversification in Cystic Fibrosis Chronic Lung Infections. Trends in Microbiology 24:327-337.
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 191:775-85.
7. Workentine M, Sibley C, Glezerson B, Purighalla S, Norgaard-Gron J, Parkins M, Rabin H, Surette M. 2013. Phenotypic Heterogeneity of *Pseudomonas aeruginosa* Populations in a Cystic Fibrosis Patient. PLoS One 8:e60225.
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.
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. Journal of Antimicrobial Chemotherapy 55:921-927.
10. Bradbury R, Tristram S, Roddam L, Reid D, Inglis T, Champion A. 2011. Antimicrobial susceptibility testing of cystic fibrosis and non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*: a comparison of three methods. British Journal of Biomedical Science 68:1-4.
11. Morita Y, Tomida J, Kawamura Y. 2014. Responses of *Pseudomonas aeruginosa* to antimicrobials. Frontiers in Microbiology 4.

414 12. Smith RS, Iglewski BH. 2003. *Pseudomonas aeruginosa* quorum-sensing systems and
415 virulence. Curr Opin Microbiol 6:56-60.
416 13. Diggle SP, Griffin A, S Campbell G, West S. 2007. Cooperation and conflict in quorum-
417 sensing bacterial populations, vol 450.
418 14. Pesci EC, Pearson JP, Seed PC, Iglewski BH. 1997. Regulation of las and rhl quorum
419 sensing in *Pseudomonas aeruginosa*. J Bacteriol 179:3127-32.
420 15. D'Costa VM, McGrann KM, Hughes DW, Wright GD. 2006. Sampling the Antibiotic
421 Resistome. Science 311:374-377.
422 16. Bhagirath AY, Li Y, Somayajula D, Dadashi M, Badr S, Duan K. 2016. Cystic fibrosis lung
423 environment and *Pseudomonas aeruginosa* infection. BMC Pulm Med 16:174.
424 17. Jorth P, Ehsan Z, Rezayat A, Caldwell E, Pope C, Brewington JJ, Goss CH, Benscoter D,
425 Clancy JP, Singh PK. 2019. Direct Lung Sampling Indicates That Established Pathogens
426 Dominate Early Infections in Children with Cystic Fibrosis. Cell Rep 27:1190-1204.e3.
427 18. Darch S, McNally A, Harrison F, Corander J, Barr H, Paszkiewicz K, Holden S, Fogarty A,
428 Crusz S, Diggle S. 2015. Recombination is a key driver of genomic and phenotypic
429 diversity in a *Pseudomonas aeruginosa* population during cystic fibrosis infection.
430 Scientific Reports 5:7649.
431 19. Williams D, Fothergill JL, Evans B, Caples J, Haldenby S, Walshaw MJ, Brockhurst MA,
432 Winstanley C, Paterson S. 2018. Transmission and lineage displacement drive rapid
433 population genomic flux in cystic fibrosis airway infections of a *Pseudomonas aeruginosa*
434 epidemic strain. Microbial Genomics 4:-.
435 20. Feliziani S, Luján AM, Moyano AJ, Sola C, Bocco JL, Montanaro P, Canigia LF, Argaraña
436 CE, Smania AM. 2010. Mucoidy, Quorum Sensing, Mismatch Repair and Antibiotic
437 Resistance in *Pseudomonas aeruginosa* from Cystic Fibrosis Chronic Airways Infections.
438 PLOS ONE 5:e12669.
439 21. Registry CFFP. 2019. Annual Data Report.
440 22. Clark S, Diaz Caballero J, Cheang M, Coburn B, Wang P, Donaldson S, Zhang Y, Liu M,
441 Keshavjee S, Yau Y, Waters V, Elizabeth Tullis D, Guttman D, Hwang D. 2015. Phenotypic
442 diversity within a *Pseudomonas aeruginosa* population infecting an adult with cystic
443 fibrosis. Scientific Reports 5:10932.
444 23. Roberts AE, Kragh KN, Bjarnsholt T, Diggle SP. 2015. The Limitations of In Vitro
445 Experimentation in Understanding Biofilms and Chronic Infection. J Mol Biol 427:3646-61.
446 24. Bradley DE. 1967. Ultrastructure of bacteriophage and bacteriocins. Bacteriological
447 reviews 31:230-314.
448 25. Reeves P. 1972. The bacteriocins (molecular biology, biochemistry and biophysics).
449 Spring-Verlag, New York.
450 26. Kageyama M, Egami F. 1962. On the purification and some properties of a pyocin, a
451 bacteriocin produced by *Pseudomonas aeruginosa*. Life Sciences 1:471-476.
452 27. Holloway BW. 1969. Genetics of Pseudomonas. Bacteriological reviews 33:419-443.
453 28. Kageyama M. 1975. Bacteriocins and bacteriophages in *Pseudomonas aeruginosa*.
454 Microbial drug resistance:291-305.
455 29. Michel-Briand Y, Baysse C. 2002. The pyocins of *Pseudomonas aeruginosa*. Biochimie
456 84:499-510.
457 30. Scholl D. 2017. Phage Tail-Like Bacteriocins. Annu Rev Virol 4:453-467.
458 31. Jacob F. 1954. Induced Biosynthesis and Mode of Action of a Pyocine, an Antibiotic
459 obtained from Ps. pyocyanea. Ann Inst Pasteur 86:149-60.
460 32. Ito S, Kageyama M. 1970. Relationship between pyocins and a bacteriophage in
461 *Pseudomonas aeruginosa*. The Journal of General and Applied Microbiology 16:231-240.
462 33. Govan JRW. 1974. Studies on the Pyocins of *Pseudomonas aeruginosa*: Morphology and
463 Mode of Action of Contractile Pyocins. Microbiology 80:1-15.

34. Govan JRW. 1974. Studies on the Pyocins of *Pseudomonas aeruginosa*: Production of Contractile and Flexuous Pyocins in *Pseudomonas aeruginosa*. Microbiology 80:17-30.
35. Ikeda K, Egami F. 1969. Receptor Substance for Pyocin R I. Partial Purification and Chemical Properties. The Journal of Biochemistry 65:603-609.
36. Ikeda K, Egami F. 1973. Lipopolysaccharide of *Pseudomonas aeruginosa* with special reference to pyocin R receptor activity. The Journal of General and Applied Microbiology 19:115-128.
37. Köhler T, Donner V, van Delden C. 2010. Lipopolysaccharide as Shield and Receptor for R-Pyocin-Mediated Killing in Pseudomonas aeruginosa. Journal of Bacteriology 192:1921-1928.
38. Higerd TB, Baechler CA, Berk RS. 1969. Morphological Studies on Relaxed and Contracted Forms of Purified Pyocin Particles. Journal of Bacteriology 98:1378-1389.
39. Yui C. 1971. Structure of Pyocin R I. Isolation of Sheath from Pyocin R by Alkali Treatment and Its Properties. The Journal of Biochemistry 69:101-110.
40. Ikeda K, Nishi Y. 1973. Interaction between pyocin R and pyocin R receptor. The Journal of General and Applied Microbiology 19:209-219.
41. Ohsumi M, Shinomiya T, Kageyama M. 1980. Comparative Study on R-type Pyocins of Pseudomonas aeruginosa. The Journal of Biochemistry 87:1119-1126.
42. Uratani Y, Hoshino T. 1984. Pyocin R1 inhibits active transport in Pseudomonas aeruginosa and depolarizes membrane potential. J Bacteriol 157:632-6.
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. Nature Structural & Molecular Biology 22:377-382.
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.
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 38:213-31.
46. Mei M, Thomas J, Diggle SP. 2021. Heterogenous Susceptibility to R-Pyocins in Populations of *Pseudomonas aeruginosa* Sourced from Cystic Fibrosis Lungs. mBio 12.
47. Redero M, López-Causapé C, Aznar J, Oliver A, Blazquez J, I Prieto A. 2018. Susceptibility to R-pyocins of *Pseudomonas aeruginosa* clinical isolates from cystic fibrosis patients, vol 73.
48. Davis MR, Jr., Goldberg JB. 2012. Purification and visualization of lipopolysaccharide from Gram-negative bacteria by hot aqueous-phenol extraction. J Vis Exp doi:10.3791/3916.
49. 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.
50. Milton DL, O'Toole R, Horstedt P, Wolf-Watz H. 1996. Flagellin A is essential for the virulence of Vibrio anguillarum. J Bacteriol 178:1310-9.
51. Choi K-H, Schweizer HP. 2006. mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. Nature Protocols 1:153.
52. Holloway BW. 1955. Genetic recombination in *Pseudomonas aeruginosa*. J Gen Microbiol 13:572-81.
53. 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 10:1820-41.
54. Schmidt AK, Fitzpatrick AD, Schwartzkopf CM, Faith DR, Jennings LK, Coluccio A, Hunt DJ, Michaels LA, Hargil A, Chen Q, Bollyky PL, Dorward DW, Wachter J, Rosa PA,

515 Maxwell KL, Secor PR. 2022. A Filamentous Bacteriophage Protein Inhibits Type IV Pili
516 To Prevent Superinfection of *Pseudomonas aeruginosa*. mBio 13:e0244121.
517 55. Brazas MD, Hancock REW. 2005. Ciprofloxacin Induction of a Susceptibility Determinant
518 in Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy 49:3222-3227.
519 56. Penterman J, Singh PK, Walker GC. 2014. Biological cost of pyocin production during the
520 SOS response in *Pseudomonas aeruginosa*. J Bacteriol 196:3351-9.
521 57. [Dryad dataset.]
522

Figure 1

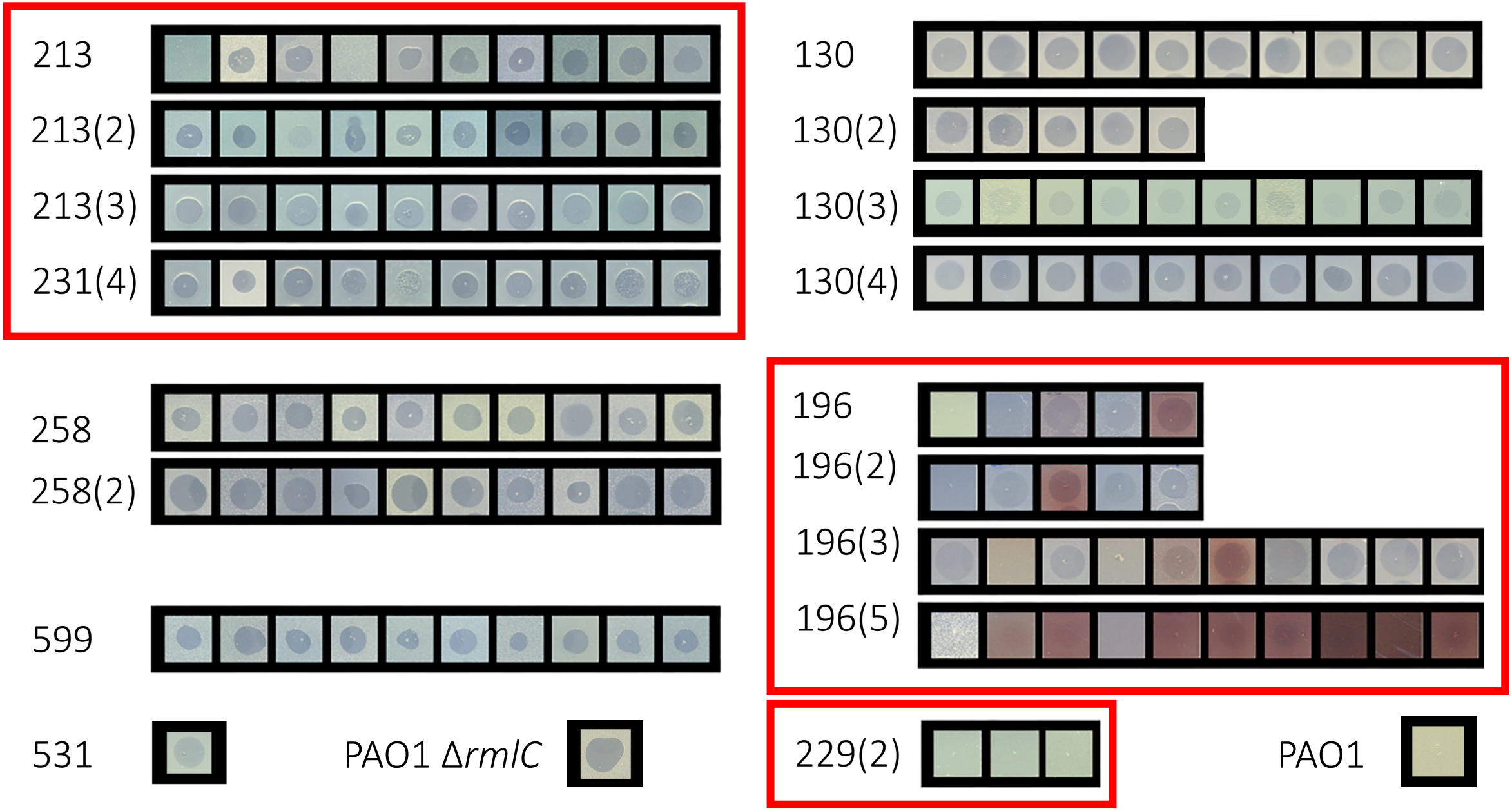

213
213(2)
213(3)
231(4)
130
130(2)
130(3)
130(4)
258
258(2)
196
196(2)
196(3)
196(5)
599
531
PAO1 Δ*rmlC*
229(2)
PAO1

Figure 2

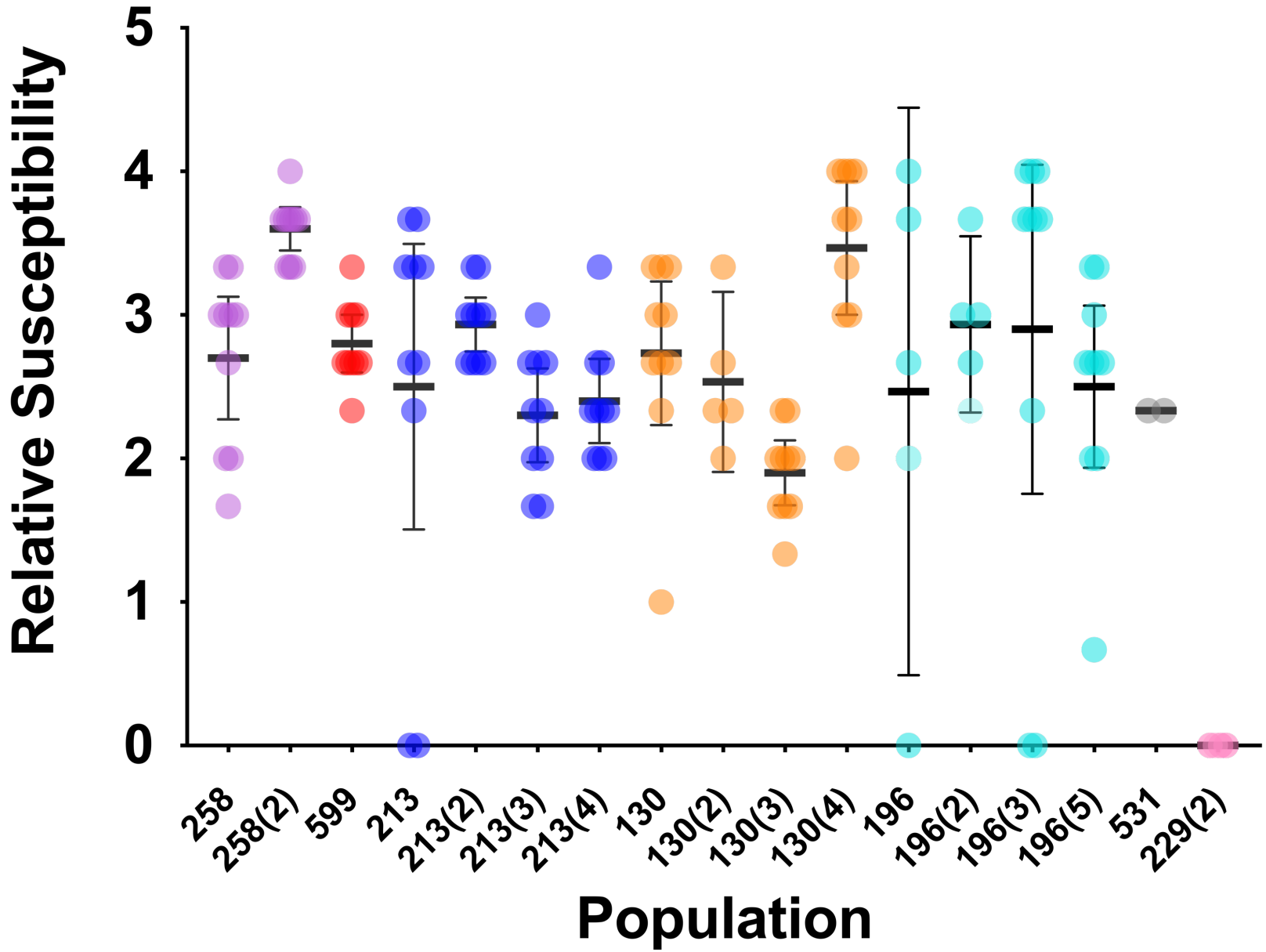

Relative Susceptibility
5
4
3
2
1
0
258
258(2)
599
213
213(2)
213(3)
213(4)
130
130(2)
130(3)
130(4)
196
196(2)
196(3)
196(5)
531
229(2)
Population

Figure 3

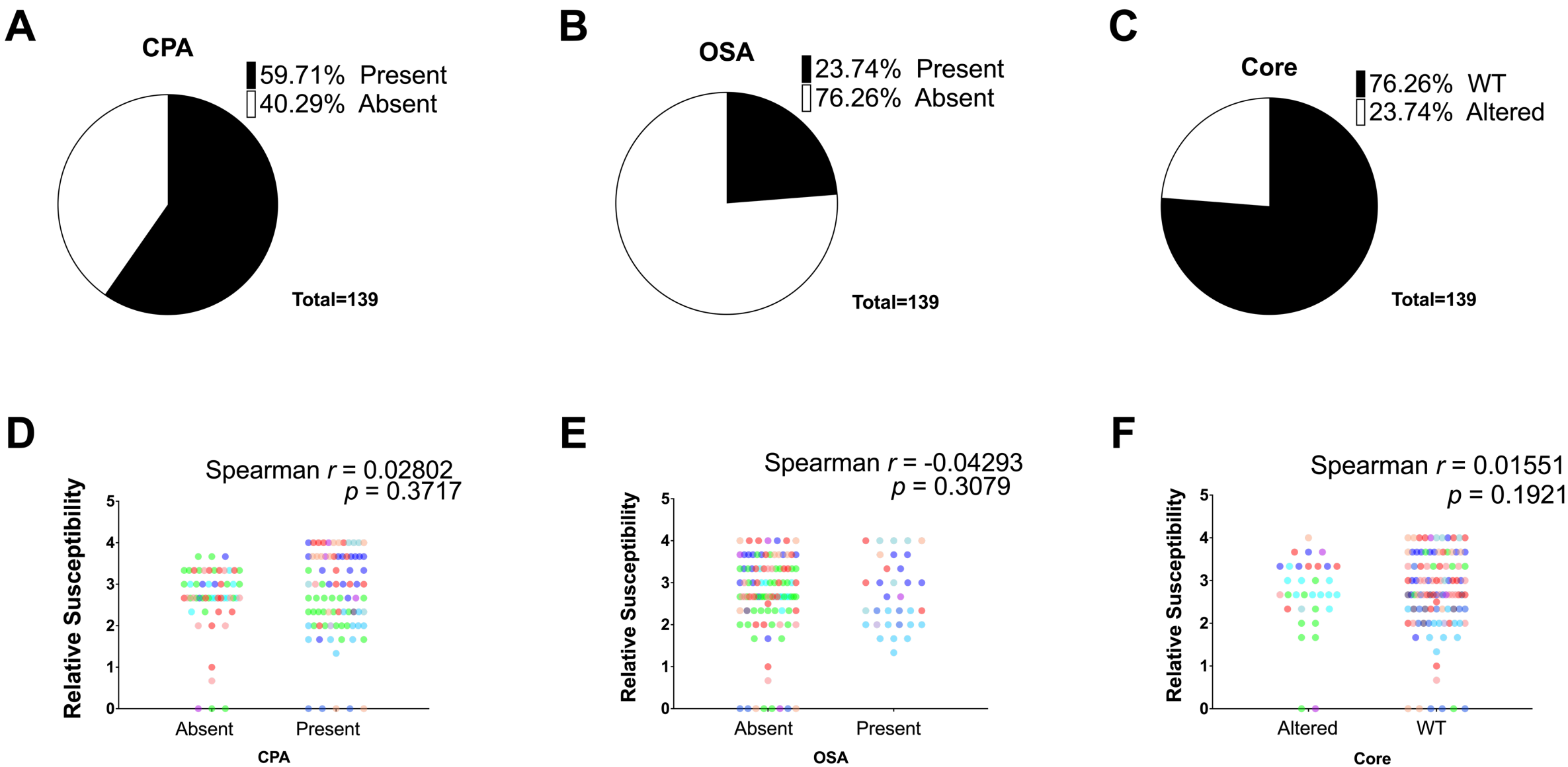

A
CPA
59.71% Present
40.29% Absent
Total=139
B
OSA
23.74% Present
76.26% Absent
Total=139
C
Core
76.26% WT
23.74% Altered
Total=139
D
Spearman r = 0.02802
p = 0.3717
Relative Susceptibility
Absent
Present
CPA
E
Spearman r = -0.04293
p = 0.3079
Relative Susceptibility
Absent
Present
OSA
F
Spearman r = 0.01551
p = 0.1921
Relative Susceptibility
Altered
WT
Core

Figure 4

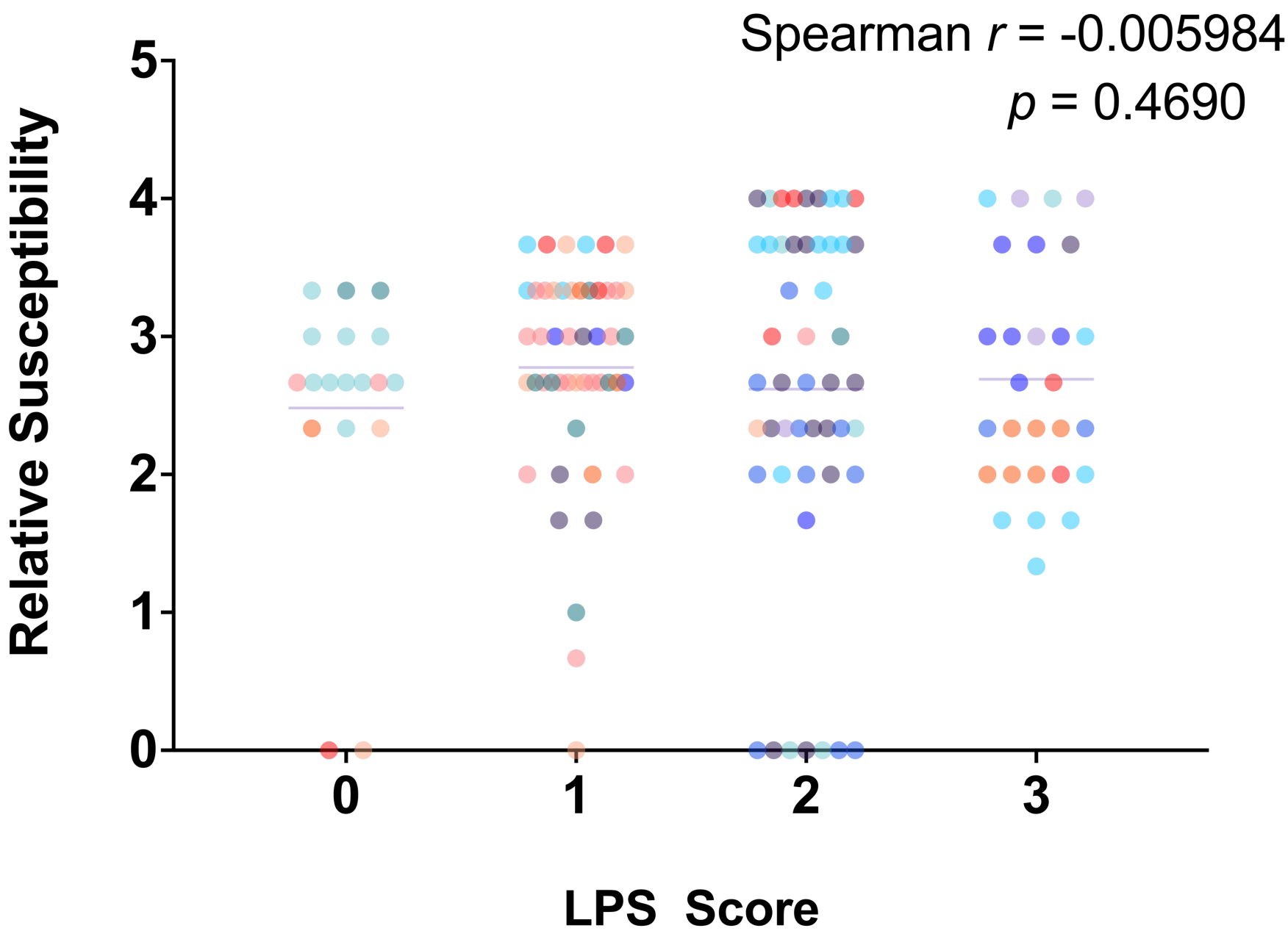

Spearman r = -0.005984
p = 0.4690
Relative Susceptibility
5
4
3
2
1
0
0
1
2
3
LPS Score

Figure S1

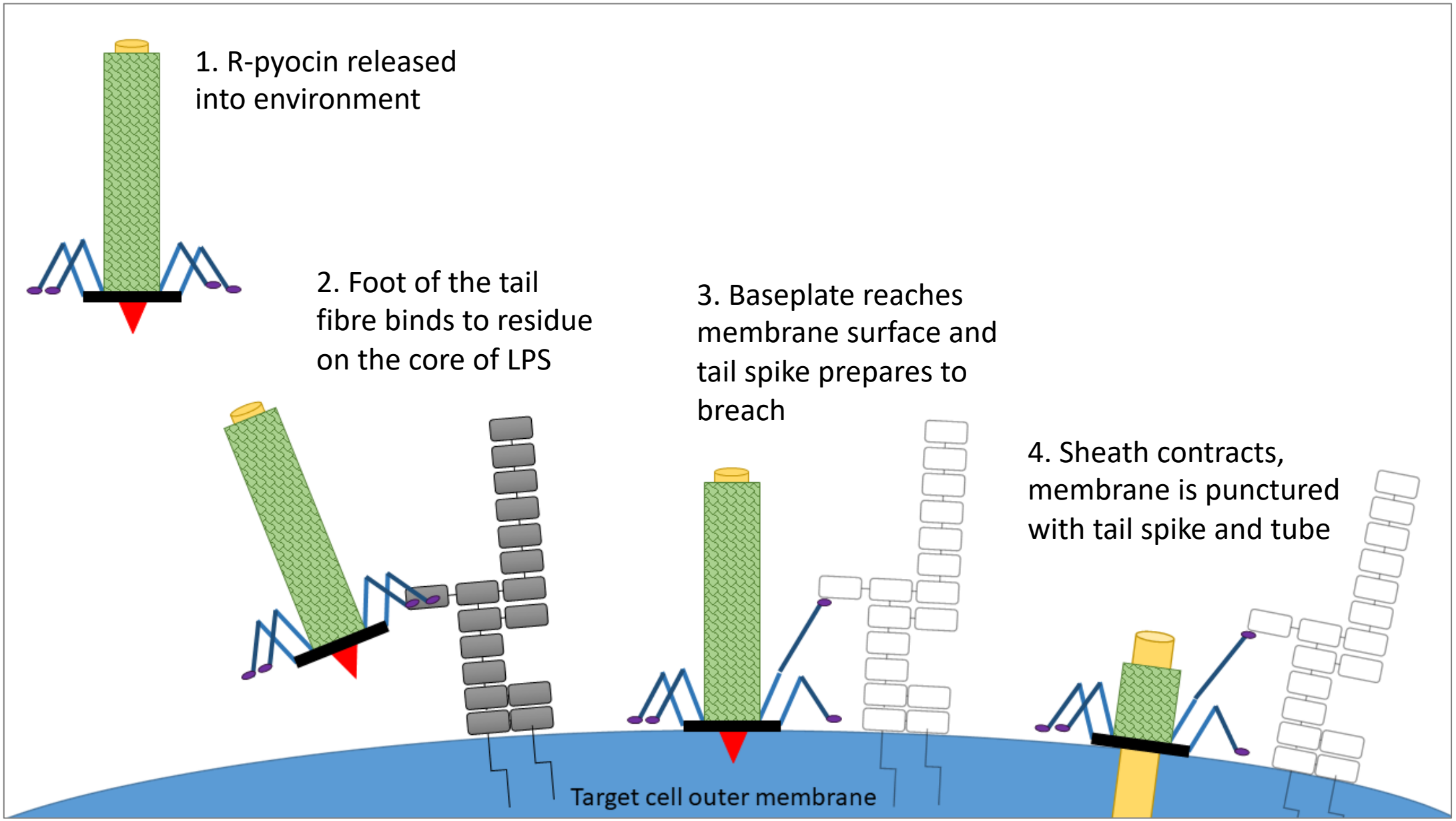
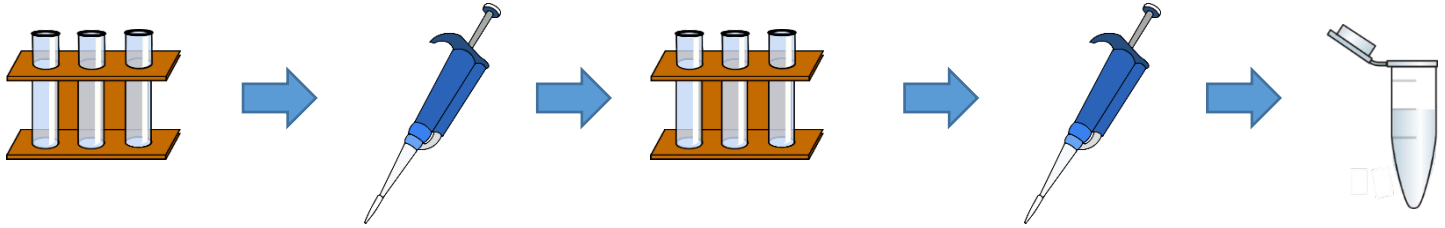

1. R-pyocin released into environment
2. Foot of the tail fibre binds to residue on the core of LPS
3. Baseplate reaches membrane surface and tail spike prepares to breach
4. Sheath contracts, membrane is punctured with tail spike and tube
Target cell outer membrane

Figure S2

Step 1. Isolate R-pyocins



Grow strains to early/pre-log phase

Add Ciprofloxacin to 0.1µg/mL

Grow strains to mid-late log phase

Add chloroform to lyse cells

Spin and store cell-free lysate at 4°C

Step 2. Add R-pyocins to CF isolates

Grow indicator strains to mid-log phase

Inoculate 0.4% agar overlay at an OD of 0.01

Using cell-free lysate…

Serial dilute lysate (in PBS)

Spot 5µL lysates onto overlay

Incubate plates overnight

Count serial dilutions with inhibition

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)*	**Sequence (5' to 3')**	**Description**	**Reference or Source**
rmlC-del upstream-Forward (600)	GAGCTCAGGTTACCCGCATGCGAGTCGTTCATCGGCAACG	Δ*rmlC* mutant generation; To clone PA5164 flanking regions into pDM4 SphI and XbaI sites for deletion	This study
rmlC-del upstream-Reverse	GCTGCGAGTATCCGGGGCATCATCAGTACACGGTCTCGGTCA	Δ*rmlC* mutant generation; To clone PA5164 flanking regions into pDM4 SphI and XbaI sites for deletion	This study
rmlC-del downstream-Forward (600)	ACCGAGACCGTGTACTGATGATGCCCCGGATACTCGCAGCCACGGC	Δ*rmlC* mutant generation; To clone PA5164 flanking regions into pDM4 SphI and XbaI sites for deletion	This study
rmlC-del downstream-Reverse	CACTAGTGGGGCCCTTCTAGGCGCCGGTGCGTCGGCGGAT	Δ*rmlC* mutant generation; To clone PA5164 flanking regions into pDM4 SphI and XbaI sites for deletion	This study
TF-del upstream-Forward (600)	GAGCTCAGGTTACCCGCATGGGTCCTGGATGAAACCCAGG	R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct	(46)

TF-del upstream-Reverse	GTAACGCCGTGGAAGAAACTCATGTCACTGTTTCCAGGGGA	R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct	(46)
TF-del downstream-Forward (600)	CCCCTGGAAACAGTGACATGAGTTTCTTCCACGGCGTTACGGTAACCAAC	R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct	(46)
TF-del downstream-Reverse	CACTAGTGGGGCCCTTCTAGCGAAGCCGGGGCGTTGC	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

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