Heterogenous response to R-pyocin killing in populations of *Pseudomonas aeruginosa* sourced from cystic fibrosis lungs

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Abstract

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen and a major determinant of declining lung function in individuals with cystic fibrosis (CF). *P. aeruginosa* possesses many intrinsic antibiotic resistance mechanisms, and isolates from chronic CF lung infections develop increasing resistance to multiple antibiotics over time, making new treatment approaches necessary. R-type pyocins are narrow spectrum, phage tail-like bacteriocins, specifically produced by *P. aeruginosa* to kill other strains of *P. aeruginosa*. Due to their specific anti-pseudomonal activity and similarity to bacteriophage, R-pyocins have potential as additional therapeutics against *P. aeruginosa,* either in isolation, in combination with antibiotics, or as an alternative to phage therapy. While it is increasingly acknowledged that colonizing *P. aeruginosa* populations become both phenotypically and genetically diverse during chronic infection of the CF lung, little is known about the efficacy of R-pyocins against heterogenous populations of *P. aeruginosa*. To investigate this, we first evaluated the distribution of R-pyocin subtypes in different environments, by bioinformatically R-pyocin typing single strains from the International *Pseudomonas* Consortium Database (IPCD). We found that R1-type pyocins are the most prevalent across strains from all environments, including those sourced from CF. We corroborated these findings by R-pyocin typing whole populations of CF strains from our own biobank of isolates sourced from expectorated CF sputum and found that (i) R1-pyocins were the most prevalent R-type among our CF strains and (ii) isolates of *P. aeruginosa* from whole populations collected from the same patient have the same R-pyocin type. Moreover, we found heterogeneity in susceptibility to R-pyocins within populations of *P. aeruginosa,* which is likely due to differences in the lipopolysaccharide (LPS), supporting the idea that the core of the LPS is the receptor for R-pyocins. Our findings suggest there is likely heterogeneity in response to other types of LPS-binding antimicrobials, such as phage, and increases our understanding of the potential of bacteriophage and other phage-like, LPS-binding antimicrobial particles as novel alternative therapies in CF.

Importance

P. aeruginosa possesses many intrinsic antibiotic resistance mechanisms and isolates from chronic cystic fibrosis (CF) lung infections become resistant to multiple antibiotics over time, making new treatment approaches necessary. R-pyocins have potential as additional therapeutics against *P. aeruginosa,* however little is known about the efficacy and heterogeneity in resistance to R-pyocins in whole populations of *P. aeruginosa*, particularly diverse *P. aeruginosa* sourced from chronic CF lung infections. It is believed that in the CF lung, one strain of *P. aeruginosa* dominates and diversifies over the course of infection. Mechanistic explanations for this single strain domination are sparse, however, R-pyocins could play a key role given their strain-specificity and antimicrobial properties. In this study, we have found that *P. aeruginosa* populations of the same R-pyocin type exhibit heterogeneity in susceptibility to R-pyocins from other strains. Our findings suggest there is likely heterogeneity

in response to other types of LPS-binding antimicrobials, including phage, and highlights the necessity of further studying the potential of LPS-binding antimicrobial particles as alternative therapies in CF and chronic infection.

Keywords: R-pyocin, bacteriocin, *Pseudomonas aeruginosa*, cystic fibrosis, lipopolysaccharide

Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen found in a number of human infections and is the leading cause of morbidity and mortality in people with cystic fibrosis (CF). A large genome allows *P. aeruginosa* to regulate diverse metabolic processes, resist antibiotic treatment, and control a number of complex social behaviors (1-9). Once established in the CF lung, *P. aeruginosa* is extremely difficult to eradicate. *P. aeruginosa* possesses many intrinsic antibiotic resistance mechanisms, but *P. aeruginosa* isolates from these chronic lung infections are known to become increasingly resistant to multiple antibiotics over time (9-17). In the current era of antibiotic resistance, new treatment approaches are necessary for use in chronic infections against *P. aeruginosa* and other multi-drug resistant pathogens.

Pyocins are narrow spectrum antimicrobials, specifically produced by *P. aeruginosa* to kill other strains of *P. aeruginosa* (18-20). *P. aeruginosa* produces three types of pyocin, referred to as S-pyocins, F-pyocins, and R-pyocins (18-20). The focus of this work is R-pyocins, which are narrow spectrum, phage-tail-like bacteriocins produced by *P. aeruginosa* that vary by subtype (types R1-R5) (19-27). As types R2-R4 are approximately 98% similar in amino acid sequence, they are often grouped together under the R2 subtype (28-30). Each *P. aeruginosa* strain uniquely produces only one of these R-pyocin types, suggesting that there is strain specificity (19, 20). The R-pyocin subtype and specificity are conferred by differences in the "foot" of the tail fiber (Fig. 1), which is believed to bind to specific residues on the lipopolysaccharide (LPS) decorating the outer membrane (27, 30-41).

R-pyocins are similar to phage tails of the *Myoviridae* phage family, both in structure and in killing mechanism (19, 20, 30-32, 42, 43). The proposed mode of action is that the foot of the tail fiber binds to a receptor on the lipopolysaccharide (LPS) (18-20, 28, 31, 37-47); the sheath contracts, pushing the tail spike and core to puncture the outer membrane, leading to inhibition of active transport and membrane depolarization before eventual cell death (46-50). Several monosaccharide residues on the outer core of the LPS are proposed to confer susceptibility and specificity to some of the R-pyocin types (40), however, only the receptor of the R3-type pyocin has been clearly defined to be the Glc^{IV} terminal glucose on the outer core of the LPS (41). It remains unclear if the residues for each of the remaining subtypes reside on the common antigen (A-band), the variable O-antigen (B-band), or the uncapped core of the LPS. A strain may be susceptible or resistant to any variation of the different R types, but are generally thought to be resistant to the subtype they produce (19, 20, 42).

R-pyocins have previously been explored as potential therapeutics (28, 51, 52), but little is known about their binding to target cell receptors, or their killing efficacy in heterogenous populations of *P. aeruginosa*. It is known that multiple isolates sourced from populations of *P. aeruginosa* from CF lungs exhibit considerable variation in susceptibility to antibiotics (2, 15, 53-56), however heterogeneity in R-pyocin susceptibility, or susceptibility to other types of bacteriocins has not been explored. Here we observed that multiple isolates from a diverse population of *P. aeruginosa* evolved in a CF patient genetically encode the same R-pyocin type and that R-pyocin type does not change across populations collected over several years from a single patient. Moreover, we found

heterogeneity to R-pyocin susceptibility within a population of *P. aeruginosa* is likely due to differences in LPS composition among members, supporting evidence that the LPS is the receptor for R-pyocins in clinical strains of *P. aeruginosa*. Our findings highlight that diverse *P. aeruginosa* populations exhibit within-population heterogeneity in R-pyocin susceptibility, suggesting there is also likely heterogeneity in response to other types of LPS-binding antimicrobials, such as phage.

Results

Distribution of R-pyocin types in clinical *P. aeruginosa* strains. To determine the prevalence of the different R-pyocin subtypes in *P. aeruginosa* isolates sourced from CF infections, we conducted a screen to bioinformatically determine R-pyocin genotypes of *P. aeruginosa* isolates publicly available through the International Pseudomonas Consortium Database (IPCD) (57). By using the BLAST algorithm from NCBI, we searched 852 strains from the database for homology to R1-, R2-, or R5-pyocin tail fiber genes. As subtypes R2-R4 are highly similar, we considered these three subtypes as one larger R2-subtype for this analysis (28-30). Out of the 852 isolates in the entire database, we could not conclusively type 303 strains, and more analysis is necessary to determine if these strains are lacking R-pyocin genes entirely, or if the tail fiber genes are incomplete. Of the 549 typeable strains in the database, we categorized 263 strains as isolated from human "respiratory" sources; these include *P. aeruginosa* isolated from throat, CF, bronchiectasis, sputum, sinus, and nasopharynx. Among these 263 respiratory isolates, we typed 144 (32.14%) strains as R1-type, 76 (16.96%) as R2-type, and 43 (9.6%) as R5-type pyocin producers. The remaining 185 (41.29%) of strains were untypeable. Fig. S1 depicts the distribution of R-types for each source; more information regarding the categorization and typing of strains from the IPCD can be found in Supplementary Dataset 1. Due to the curation and labeling of the strain information in the database, we were not able to precisely distinguish CF strains from strains isolated from other respiratory infections, however is it likely that many of the samples labeled by anatomical respiratory sources are from CF patients, given the research areas of the curating research groups. The distribution of R-pyocin types among the typeable respiratory isolates shows that R1-pyocin producers are likely more prevalent in CF than the other R-pyocin subtypes (Fig. 2A).

R-pyocin type does not vary within a clonal CF population of *P. aeruginosa*. Through a collaboration with Children's Healthcare of Atlanta and the CF Discovery Core at Emory University, we collected whole populations of *P. aeruginosa* from CF sputum samples from patients chronically infected with *P. aeruginosa*. Using multiplex PCR (Table S1), we R-pyocin typed isolates from 19 of these populations (183 isolates total) collected from 8 different patients over several years. In our biobank, R1 was also the most prevalent R-pyocin type found among CF patients in our cohort. We found that while R-pyocin type does not change within a population or over longitudinal collections from the same patient, different patients from the same clinic may be infected with *P. aeruginosa* containing different R-types. Out of the 8 patients we typed, 5 of these patients were chronically infected with an R1-pyocin producing strain, 2 patients were infected with untypeable populations, and 1 patient was infected with an R2-pyocin strain (Fig. 2B). We did not find any populations containing the R5-pyocin type in our biobank.

Effect of R-pyocin-mediated killing varies among *P. aeruginosa* isolates within a population. Our lab has previously shown that isolates from within the same population have diverse antibiotic resistance profiles (15), but diversity in R-pyocin susceptibility has yet to be evaluated within single populations of *P. aeruginosa*. We chose 3 *P. aeruginosa* populations from our biobank, each from a different CF patient, that exhibited diversity in

growth rate (Fig. 3A) and morphology (Fig. 3B). Populations from Patient 1 and Patient 2 were untypeable while the population from Patient 3 was determined to consist of R1- producers (Supplementary Dataset 2). To assess heterogeneity in susceptibility to R-pyocins within these populations, we used partially fractionated R2-pyocin cell lysate from PAO1. To show that other pyocin-associated lytic enzymes such as holin, lysin or other SOS-induced prophage are not responsible for the bactericidal activity against other strains, we included lysates extracted from an isogenic PAO1 R-pyocin mutant lacking the tail fiber gene and chaperone (PAO1ΔR), which produces nonfunctional partial pyocins (58). This strain has an intact lysis cassette, holin genes, and *Pf4* prophage. We found that each population as a whole, exhibited different levels of susceptibility, and that within each population, there was heterogeneity in susceptibility among the isolates (Fig. 3C). In *P. aeruginosa* populations sourced from Patient 1 and Patient 2, all 20 isolates were considered susceptible to R2-pyocins, however the response varied approximately 10-fold between individual isolates. In Patient 3, the response ranged from susceptible to completely resistant. This finding suggests that even within one population of *P. aeruginosa* producing the same R-pyocin type, there is diversity in susceptibility to R-pyocins of other types.

R-pyocin susceptibility levels are significantly different in *P. aeruginosa* isolates from the same population. To confirm differences in susceptibility between isolates, we chose three isolates from the R1-pyocin producing population of Patient 3 for further analysis. We first tested the three isolates for susceptibility to R-pyocins of other strains by visualizing killing activity with a standard, soft-agar overlay spot assay (Fig. 4A). We used R-pyocin containing lysates from the standard laboratory strain PAO1 (an R2-producer) and a PAO1 R-pyocin null mutant lacking a functional R-pyocin (PAO1ΔR), as well as previously described CF isolates A018 (an R2-producer), A026 (an R1-producer) and R-pyocin null mutants of each as controls (40, 58, 59). The spot assay showed that Isolate 1 from Patient 3 was resistant to the R2-pyocins of PAO1 and A018, while Isolates 2 and 3 were susceptible (depicted by the zones of inhibition where the R2-pyocin lysates were spotted) (Fig. 4A). All three R1-producing isolates of Patient 3 were resistant to the R1-pyocins of A026. We next tested pyocin susceptibility of these 3 isolates with R2-pyocin lysates from PAO1 in liquid culture, and measured Log_2 fold-change of colony forming units (CFU) after 4 hours of growth to compare cell density with and without R-pyocin-containing lysates. Through this assay, we confirmed that Isolates 2 and 3 are susceptible to R2-pyocins, while Isolate 1 is resistant (Fig. 4B). We confirmed in both planktonic growth and in an agar overlay, that two isolates were susceptible to R2-pyocins while one isolate was resistant, indicating that *P. aeruginosa* isolates of the same R-pyocin type and from the same population can exhibit different susceptibilities to other R-pyocins.

LPS characterization of isolates reveals differences in core and antigen presentation. As LPS is thought to be involved in the binding of R-pyocins to target cells (18-20, 28, 31, 37-47), we further characterized the three isolates by extracting and visualizing major LPS components by gel electrophoresis and staining. All three isolates showed differences in O-specific antigen (OSA, B-band) and common antigen (CPA, A-band) presentation, but a notable difference was that the R2-resistant Isolate 1 possessed a LPS core band that migrated differently from the susceptible isolates (Fig. 5). Along with an altered LPS core, Isolate 1 appeared to also lack both the A-band and B-band. We found that Isolate 2 appeared to lack A-band, presented some low molecular weight B-band, and had a normal core, while Isolate 3 also had a normal core and only presented A-band. This variation in LPS phenotypes among isolates within a population is in agreement with previous studies that have shown LPS mutations frequently arise in the CF environment, both *in vivo* and *in vitro* (60-68).

WGS sequencing of isolates reveals several possible SNVs responsible for R-pyocin resistance. We obtained whole genome sequences of each of the three isolates studied from Patient 3 to determine the strain type and serotype. We found that all three isolates are of the multi-locus sequence type (MLST) 2999, suggesting they are

derived from the same originating infecting strain. The serotype of each isolate was predicted to be O6, indicating that the isolates all carried the O6 gene cluster (encoding genes for synthesis of the variable O-specific antigen or B-band), and that serotype alone is likely not responsible for the differences in susceptibility. We found the clinical isolates shared 30,127 single nucleotide variants (SNVs) when mapped against a PAO1 reference genome (Fig. 6A). We found 232 SNVs unique to the R2-resistant Isolate 1 from Patient 3, when mapped to PAO1 and compared to SNVs of the two susceptible isolates from Patient 3 also mapped to PAO1 (Fig. 6B). Of the 232 SNVs unique to Isolate 1, 169 SNVs were nonsynonymous. Given the high number of SNVs and stark diversity between the isolates, through genetic analysis we were only able to identify several candidate SNVs potentially involved in the R-pyocin resistant phenotype of this isolate, though it could be any number of these mutations in combination that play a role in the R-pyocin resistant phenotype we observed. Noteable candidate SNVs unique to Isolate 1 included a C151T (Arg51Trp) missense variant at position 833604 in the *mucC* gene, a G710A (Arg237His) missense variant at position 1199237 of the *roeA* gene, and a T1169C (Leu390Pro) missense variant at position 3968193 in the *algK* gene.

Discussion

Chronic infections found in CF lungs are debilitating and often lethal. *P. aeruginosa* is a common pathogen found in CF lungs and is highly resistant to multiple classes of antibiotic which contributes hugely to antimicrobial resistance, making the development of new and alternative therapies critical. R-pyocins have potential as an alternative therapy, but little is known about the receptor specificity to target cells or their antimicrobial efficacy toward the heterogenous populations of *P. aeruginosa* found in individual CF patients. In this study we found that (i) R1-pyocins are the most prevalent R-type among CF strains of *P. aeruginosa*; (ii) isolates from *P. aeruginosa* populations collected from the same patient have the same R-pyocin type; (iii) there is heterogeneity in susceptibility to R-pyocins within *P. aeruginosa* populations and (iv) susceptibility is likely driven by the LPS composition in clinical strains.

We first assessed the prevalence of the different R-pyocin subtypes in *P. aeruginosa* isolates sourced from different environments by screening for R-pyocin genotypes of *P. aeruginosa* isolates through the International Pseudomonas Consortium Database (IPCD) and our own biobank of CF isolates (57). We found that R1-pyocin producers make up the majority of typeable CF strains from both sources. Our findings agree with previous work, as R1-producers have been shown to be the most prevalent subtype of CF isolates evaluated in a separate study which typed 24 *P. aeruginosa* CF isolates (62.5% of which were R1-producers) (58). We found a large number of strains from the IPCD that could not be typed by our initial screen. While we expect that some strains may in fact produce R-pyocins but were unable to be typed due to sequencing or contig errors, it is possible that many of these strains may not possess R-pyocins at all. Another group evaluating R-pyocin susceptibility among 34 CF isolates found that 23 (68%) of their isolates did not produce R-pyocins or could not be typed (69). This suggests that CF isolates of *P. aeruginosa* may lose the ability to produce R-pyocins later in chronic infection, but more work is necessary to confirm this phenotype and explore the potential fitness advantages of this evolutionary trajectory.

Previous studies have R-pyocin typed single isolates of *P. aeruginosa* from CF patients and compared R-types from longitudinal collections of single isolates (58, 69). The typing of single isolates does not consider the genotypic and phenotypic diversity of *P. aeruginosa* which evolves over the course of chronic infection (2-4, 10,

12, 14-16). To address this, we collected whole populations of *P. aeruginosa* from CF sputum samples of patients chronically infected with *P. aeruginosa,* and typed multiple isolates from each population, including longitudinal collections from the same patients up to 2 years. Out of the 8 patients we typed populations from, we found primarily R1-producing or R-pyocin-lacking strains. We found that all of the isolates sampled from each population, including longitudinal collections for some patients, consistently exhibited the same R-pyocin genotype. In addition, while R-pyocin type does not change within a population or over longitudinal sampling from the same patient, we found that different patients from the same clinic may be infected with *P. aeruginosa* of different R-pyocin genotypes. A previous study typed single isolates yearly for 8 years and found that R-pyocin type could change over the course of a patient's infection, but strain typing also showed that these patients were often infected with different strains during those events (69).

We are only just beginning to understand how heterogenous *P. aeruginosa* populations impact upon treatment and disease outcomes. Several studies have shown that diverse populations of *P. aeruginosa* from chronic infections show heterogeneity in susceptibility to a number of antibiotics, complicating susceptibility testing and treatments (2, 15, 53-56). We found that diverse populations of *P. aeruginosa* from chronic CF lung infections exhibit heterogeneity in susceptibility to R-pyocins. Specifically we found that (i) each population of *P.aeruginosa* as a whole exhibited different levels of susceptibility and (ii) within each population, there was heterogeneity in susceptibility among individual isolates. This means that similar to antibiotics, treatment with alternative therapies utilizing the LPS, such as R-pyocins and phage, may not eradicate strains within infections completely, potentially selecting for resistance in the infection. Our findings are in agreement with recent work demonstrating heterogeneous resistance mechanisms and persistence against tailocin killing by alterations of the LPS and associated glycosyltransferases in *Pseudomonas syringae* (70). To our knowledge, our work is the first to show heterogeneity in response to R-pyocins in *P. aeruginosa* clinical populations.

Previous studies have shown the effect of various LPS mutations on R-pyocin susceptibility in laboratory strains PAO1 and PAK (40), however our work is the first to examine LPS phenotypes associated with R-pyocin susceptibility and resistance in multiple clinical isolates of *P. aeruginosa* sourced from chronic CF lung infections. We found that differences in the core of the LPS are likely responsible for the differences in susceptibility and resistance to R-pyocins among the isolates from one patient; specifically, that a resistant CF isolate produces an altered LPS core. It is unknown however, if this altered LPS core phenotype is commonly found in CF, at which proportions it is found in *P. aeruginosa* populations, and the fitness benefit it confers during infection. This is important to understand because during early stages of infection in the initial colonization and strain establishment, the LPS (and therefore R-pyocin resistance) may play a role in antagonistic interactions between competing strains and ultimately determine the dominating strain. Over the course of infection, there may be a fitness tradeoff in altering or losing the LPS, which in turn could increase R-pyocin susceptibility.

When we probed the genomes of three isolates from a single clinical population exhibiting heterogeneous susceptibility to R2-pyocins, we identified several candidate SNVs unique to the resistant isolate that may be responsible for the R2-pyocin resistant LPS. We found a C151T (Arg51Trp) missense variant in the *mucC* gene in Isolate 1; as *mucC* is a positive regulator for alginate biosynthesis, as a part of an operon encoding a sigma factor *algT* involved in an expansive stress response (71-75), and it is known that alginate biosynthesis and LPS biosynthesis pathways are often entwined. It is possible that this defective transcriptional regulator hampers LPS presentation in the resistant isolate. We also found a G710A (Arg237His) missense variant in the *roeA* gene. As

roeA is a diguanylate cyclase involved in extracellular polysaccharide biosynthesis (76-78), it may also be involved in lipopolysaccharide biosynthesis. Another SNV found in an LPS biosynthesis gene was a T1169C (Leu390Pro) missense variant in the *algK* gene, another protein involved in LPS biosynthesis (61, 74, 75, 79-84). It has been shown that *algK* plays a role in alginate biosynthesis and is also the periplasmic component of an outer membrane secretin similar to other bacterial capsular polysaccharide secretion systems (61, 74, 75, 79-85), indicating it could also play a role in secretion of polysaccharide subunits of the LPS. All three variants may play a role in impeding presentation of various portions of the LPS or core residues. This in turn would result in R2-pyocin resistance, as the recognized residue would not be present on the core and the R2-pyocins could not bind to the target cells.

Overall our findings highlight that clinical populations of *P. aeruginosa* exhibit a heterogeneous response to R-pyocins and that this likely extends to any antimicrobial that utilizes the LPS. A number of LPS- and saccharide-binding phage have been identified and tested as antimicrobials against *P. aeruginosa* (39, 86-92) and other types of LPS-specific antimicrobials, including R-pyocins, are also being considered as treatments (28, 29, 51, 52, 93). Our work implies that treatment with alternative therapies utilizing the LPS may not eradicate strains within infections completely, potentially leading to highly resistant isolates taking over. This reiterates the importance of assessing multiple, diverse isolates from populations of *P. aeruginosa* rather than taking a single isolate from a population. To better understand how R-pyocins and other LPS-binding antimicrobial therapies can be utilized for alternative treatments, it is crucial to know more about the LPS and core phenotypes evolving in *P. aeruginosa* populations during infection, and the resulting impact on the potential efficacy of these antimicrobials.

Materials and Methods

Bacterial strains, media, and culture conditions. Expectorated sputum samples for this study were collected 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). *P. aeruginosa* populations were collected from each sputum sample using selective media (Pseudomonas Isolation Agar, Sigma-Aldrich) before isolating single colonies for further characterization. In total, 204 isolates of our biobank, sourced from CF chronic lung infections, were studied. All *P. aeruginosa* isolates, laboratory and indicator strains used in this work are listed and described in Supplementary Data 1. All bacterial cultures were grown in lysogeny broth (LB) medium at 37°C with 200rpm shaking agitation.

Assessing colony morphology diversity in clinical isolates. To evaluate the diversity in colony morphologies among *P. aeruginosa* clinical isolates, we used a Congo Red-based agar media (1% agar, 1×M63 salts (3 g of monobasic KHPO4, 7 g of K2PO4, and 2 g of NH4.2SO4, pH adjusted to 7.4), 2.5 mM magnesium chloride, 0.4 mM calcium chloride, 0.1% casamino acids, 0.1% yeast extracts, 40 mg/l Congo red solution, 100 µM ferrous ammonium sulfate, and 0.4% glycerol) (94). Isolates were grown overnight (16-18 hours) in LB medium at 37°C with 200rpm shaking agitation before spotting 10 µL onto the plates. We incubated the plates overnight at 37°C, and for a further 4 days at 22°C. The colonies were imaged with an Epson scanner at 800 dpi.

R-pyocin typing strains of the IPCD. Previously characterized *P. aeruginosa* strains were selected for query sequences for each R-type: PAK an R1, PAO1 an R2, and E429 an R5 (40). As the R-pyocin tail fiber sequences of PAO1 (NP_249311.1) (95-97) and PAK (Y880_RS29810; accession GCA_000568855.2) (98) are annotated, the corresponding nucleotide sequences were downloaded in FASTA format from NCBI. The E429 (R5) tail fiber

sequence was identified and downloaded from NCBI by using the PAO1 tail fiber sequence (PA0620) to BLAST against the E429 genome (57). NCBI's BLAST+ was also used to determine percent identity between the whole tail fiber sequences of each strain/R-type pair-wise, in order to determine the variable region of the tail fiber gene to use for typing. Homology information, percent identity, query coverage and parameters can be found in Supplementary Data 2 (99). The variable region to be used for typing was determined by identifying the query coverage between each pairwise alignment, subtracting the "uncovered" portion from the original base pair length of the query, which suggested approximated 800bp corresponding to the C-terminal end of the tail fiber gene that is most variable to the other types (R1) (Supplementary Data 2). Specifically the 800bp C-terminal sequence of each R-type of tail fiber sequence were used to further R-pyocin type the database. Genomes from the International Pseudomonas Consortium Database (IPCD; BioProject ID 325248) were downloaded from NCBI as nucleotide sequences and made into a "database" to use with blast+ locally (57, 99). Default BLAST parameters were used to align each R-type sequence with sequences in the IPCD database, to generate tables of IPCD strains for each R-pyocin type (99). Strains were considered to be of a R-pyocin genotype if they covered 99% or greater of the 800bp query sequence, and were determined to be of 96% or higher homology (identity) to the query sequence. A Genbank file of the IPCD was also retrieved from NCBI and used to extract information (source, strain, host, etc) for each strain, to analyze distribution of strains and sources of each R-type (57). Due to the curation and labeling of the strain information in the database, we were not able to precisely distinguish CF strains from strains isolated from other respiratory infections; CF isolates were considered with isolates of other respiratory sources.

Mutant generation. Standard genetic techniques were used for construction of *P. aeruginosa* mutants. Pyocin tail mutants were constructed as follows: 600 bp DNA sequences flanking the open reading frames of PA0620-PA0621 were PCR amplified using Q5 DNA polymerase (New England Biolabs, Ipswich, MA). These sequences were cloned into the SphI-XbaI digested suicide vector pDM4 (100) by Gibson assembly using NEBuilder HiFi Assembly master mix (New England Biolabs), transformed into *Escherichia coli* S17-λpir by electroporation and selected on LB agar plates supplemented with 30 µg/mL chloramphenicol. Cloned inserts were verified by colony PCR and Sanger sequencing (Eurofins Genomics, Louisville, KY). Deletion constructs were introduced into PAO1 and other strains by electroporation (101) and strains carrying single crossover insertions of the deletion constructs were selected on LB agar plates supplemented with 300 µg/mL chloramphenicol. Chloramphenicol resistant colonies were cultured in LB without antibiotic and plated on LB agar plates with 0.25% NaCl and 5% sucrose (102) to select for loss of the deletion construct. Sucrose resistant colonies were screened for chloramphenicol sensitivity to ensure loss of the vector sequence and were assessed for presence of the gene deletion by colony PCR and Sanger sequencing of the PCR product.

PCR conditions and R-pyocin typing. Primers for typing R1- and R2-pyocins were designed using the *P. aeruginosa* Genome Database as a reference (http://www.pseudomonas.com) (101) in a previous study (58). Primers for typing R5-pyocins were designed using the publicly available genome sequence of strain E429, isolate 15108-1 (NZ_MCME00000000) of the International Pseudomonas Consortium Database (55) ; this strain has been reported as an R5-type pyocin producer by other groups (40). The R5 primers were inputted into the Sequence Manipulation Suite (https://www.bioinformatics.org/sms2/pcr_products.html) (104) to verify specificity to R5-pyocin producers and product size. R5-pyocin primers were designed using Benchling (www.benchling.com) (105) and ordered from Eurofins Genomics. R-pyocin typing primer details and product sizes are listed in Table S1. Clinical isolates were R-pyocin-typed with multiplex PCR comprising of R1, R2, and R5 primers, with laboratory strains already available PAO1 (R2), PAK (R1) and TuD199 (R5) used as positive

controls for each PCR (39, 55, 104-106). The PCR volume of 15 µL contained 3 µL of One *Taq* Standard Reaction Buffer, 0.3 µL of each primer (10 µM) and deoxynucleoside triphosphates (dNTPs [10nM]), 0.075 µL of One *Taq* DNA polymerase and 10.225 µL of nuclease-free deionized water. Template DNA was obtained by using 1 colony per reaction. The PCR conditions used included 94° for 30 s during initial denaturation, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 68°C for 30 s, followed by final extension at 68°C for 5 minutes.

Expression and extraction of R-pyocins. Expression of R2-pyocins used in the susceptibility assays were induced and pyocins extracted from PAO1 with a modified method from published work (28). LB cultures of PAO1 and PAO1ΔR (R-pyocin null mutant) were inoculated at 1:100 from overnight planktonic cultures, and grown to mid-logarithmic growth phase (approximately 3 hours) in 10mL of LB media. Ciprofloxacin was then added to each culture for a final concentration of 0.1µg/mL to induce R-pyocin production (28), and cultures were incubated for a further 3 hours. Chloroform was used to lyse remaining cells, and lysates were centrifuged at ~3,300 x *g* for 10 minutes. The R-pyocin-containing supernatant was separated and stored at 4°C. R-pyocin lysates were extracted on three separate occasions for biological replication.

Spot assay for R-pyocin activity. Overnight cultures of clinical *P. aeruginosa* isolates were used to inoculate 4 mL of cooled soft top (0.7%) agar at an optical density at 600 nm (OD_{600nm}) of 0.01. This mixture was poured onto LB agar plates for an overlay of the indicator strain. R2-pyocin lysates extracted from PAO1 and control cultures were vortexed before spotting 5 µL of each lysate onto the soft-top overlay indicator strains. Spots were allowed to dry, then plates were incubated at 37°C overnight. Clear zones of growth inhibition indicated R-pyocin dependent activity against the indicator strains. Spot assays for R-pyocin susceptibility were conducted in triplicate.

Microtiter plate method for R-pyocin activity. Susceptibility to R2-pyocins was measured by fold-change of the optical density (OD_{600nm}) of each culture treated with R-pyocin-containing lysates, normalized by the optical density of the culture treated with PAOΔR-pyocin mutant lysates after 4 hours of growth. This normalization allows for the consideration of growth heterogeneity and response to any non-R-pyocin particles in the lysates, confirming that the variation in response seen among isolates is R-pyocin-dependent. A standard 96-well plate was used to assess R-pyocin susceptibility of *P. aeruginosa* clinical isolates. Using mid-log phase cultures of the clinical isolates (grown separately), each well of the plate contained LB broth and was inoculated to an adjusted OD_{600nm} of 0.01, before 10µL of R-pyocin lysates were added for a total volume of 200µL in each well. Cultures measured included normal growth cultures of each isolate with no R-pyocin lysates added, cultures with R-pyocin lysates, cultures with R-pyocin null mutant lysates, and cultures with blank media "lysates" added. Using a BioTek Synergy H1, OD_{600nm} was measured for each well every 20 minutes over a total of 16 hours while incubating at 37°C with 200rpm orbital shaking. Growth rates of isolates not treated with R-pyocins were calculated using Growthcurver in R (109, 110). Microtiter plate assays for R-pyocin susceptibility were conducted in triplicate. Colony forming units (CFUs) were quantified at t=0 hours and t=4 hours by sampling 10µL of each culture, serially diluting in phosphate buffered saline (PBS), and spotting on LB plates. Colonies were counted after 18 hours of growth at 37°C. CFUs were determined for three biological replicates of each isolate.

Statistical analysis. Data analysis was performed using Prism (Graphpad software, version 8). Population heterogeneity experiments were normalized to R-pyocin null mutant lysate controls for fold-change, and analyzed with Prism. CFU data was analyzed for three isolates by normalizing CFU measurements at t=0 hours and t=4 hours for cultures with and without R-pyocin lysates to compare $\log_2$ fold-change between treatments for each isolate. Statistical significance between treatments for each isolate was measured with a paired, parametric

Student's T-test. Differences across all three isolates with the R-pyocin treatments were analyzed using a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons analysis in Prism. Fig. 3C shows mean values of three biological replicates for each individual isolate, while Fig. 4B shows four biological replicates (averaged from technical replicates) as individual data points. Error bars represent standard error (SEM) and significance is denoted as follows: **, $P = 0.005$.

LPS extraction and characterization. Overnight bacterial cultures of 5 mL LB were grown at 37°C at 200 rpm shaking. The overnight cultures were diluted 1:10 with LB for an OD_{600nm} measurement and diluted to make a 1.5 mL suspension of bacteria adjusted to an OD_{600nm} of 0.5 before pelleting by centrifugation at 10,600x *g* for 10 minutes. The cell pellet was resuspended in 200 µL of 1x SDS-buffer. Suspended bacterial cells were boiled for 15 minutes and LPS were prepared by hot aqueous-phenol extraction as previously described (111) but without the addition of DNase I and RNase solutions (as deemed optional in the protocol). Samples were visualized using 15 µL of LPS preparation on a 4%-12% gradient Tris-glycine gel using the Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (ThermoFisher) (111).

Whole genome sequencing analysis. Genomic DNA was prepared from overnight cultures using the DNeasy UltraClean Microbial Kit (Qiagen). Library prep and sequencing was performed by the Georgia Institute of Technology Molecular Evolution Core Facility. We obtained 250 bp, paired-end reads sequenced on the Illumina MiSeq platform for an average of at least 60X coverage. Adapters were trimmed following sequencing before receipt for analysis. Reads were checked for quality with FastQC (112), before mapping with default parameters to the PAO1 reference genome (NC_002516) using BowTie2 (version 2.1.0) (111, 112). SNV lists between each clinical isolate and PAO1 were generated using Samtools (version 0.1.19) and filtered for a minimum quality score of 20 (113, 114). To annotate SNV lists, the same PAO1 reference files used for mapping were built into a database to annotate SNV lists through SnpEff version 4.3t (117). SNV lists were compared across all three isolates for shared SNVs and visualized for comparison with R (version 3.5.1) (110). The full list of SNVs unique to each isolate can be found in Supplementary Data 3. Sam files generated from mapping each isolate to PAO1 were used to visualize coverage with BRIG (version 0.95) (118) and CGView (119). Sequences were compiled into de novo assemblies using SPAdes (120) through KBASE (119) for serotype prediction with PAst 1.0 (122) and strain typing with PubMLST (123).

Supplemental Material

Table S1

Supplementary Data 1

Supplementary Data 2

Supplementary Data 3

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Figure legends

Figure 1. R-pyocin structure. R-type specificity is determined by the sequence on the foot of the tail fiber, which is believed to recognize glycosylated structures on the outer membrane (such as the LPS). Upon binding, the sheath contracts to push the core into the cell membrane. Puncture by the tail spike depolarizes the membrane potential, leading to cell death (45-49). R-pyocin structure is depicted as described in previous studies (17-19, 26, 32-35, 41-44, 48, 49, 122).

Figure 2. R-pyocin typing of clinical *P. aeruginosa* strains and whole populations isolated from CF patients. (A) Using BLAST homology-based alignment, we R-pyocin typed publicly available *P. aeruginosa* sequences from respiratory sources of the IPCD (55) to determine the prevalence of each pyocin type. Out of the 448 respiratory isolates in the entire database, 185 strains were not able to be typed. (B) Using multiplex PCR, we R-pyocin typed our own biobank of whole populations of *P. aeruginosa* isolated from expectorated sputum of CF patients to compare the prevalence of R-types found among CF patients in our cohort. We used 19 longitudinal samples collected from 8 patients and R-pyocin-typed 183 isolates total. We found that in our biobank, R1 was also the most prevalent R-pyocin type found among CF patients in our cohort (UT = untyped).

Figure 3. Diverse *P. aeruginosa* populations from CF are heterogeneous in growth, morphology, and R2-pyocin susceptibility. (A) Growth rates vary between 20 isolates each from 3 populations collected from expectorated CF sputum. Growth rate was calculated from optical density (at 600nm) after 16 hours of growth in LB medium and normalized to PAO1. (B) The same isolates from each population are also morphologically diverse as shown on Congo Red Agar medium. Both mucoid and non-mucoid isolates are found within the same populations. (C) The diverse isolates vary in susceptibility to the R2-pyocins of PAO1, when R2-pyocins are added to the medium during growth. Each data point represents the mean of three independent biological replicates for an individual isolate. Fold-change in optical density (OD) at 600nm was calculated by normalizing OD of cultures with pyocins by OD of cultures without R-pyocins. Each isolate was normalized to a R-pyocin-free control culture of the same isolate, thus heterogeneity in growth rates were accounted for. UT = Untyped R-pyocin genotype.

Figure 4. Clinical CF isolates of *P. aeruginosa* from the same population exhibit differential susceptibility to R2-pyocins. (A) Three R1-type pyocin producing clinical CF isolates from the same population (Patient 3) were tested for susceptibility to spotted strains with a standard spot assay, and show differences in susceptibility to R2-pyocin lysates. Isolates 2 and 3 show zones of inhibition, indicating susceptibility. Spotted strains include previously described CF strains A018 (R2), A026 (R1) and the laboratory strain PAO1 (R2). (B) The same three isolates were grown in Lysogeny broth (LB), and treated with R2-pyocins extracted from PAO1. Each data point depicts a biological replicate (averaged from technical replicates). Log2 fold-change in CFU was measured at t=0 and t=4 hours for each isolate to depict growth or death in the presence or absence of R2-pyocins. There is heterogeneity in susceptibility to R2-pyocins within each population, and a significant difference in susceptibility levels to R2-pyocins between clinical isolates of Pa. Two-tailed, paired Student's T-test between treatments confirm that Isolates 2 and 3 are susceptible to R2-pyocins (**P=0.005), while Isolate 1 is resistant (ns). A one-way ANOVA and Tukey-Kramer post-hoc test determined that Isolates 2 and 3 significantly decrease in CFU from Isolate 1 when treated with R2-pyocins (not shown, $P<0.05$).

Figure 5. LPS characterization of three CF isolates of P. aeruginosa from the same population. Three R1-type pyocin producing clinical CF isolates from the same population were analyzed for LPS phenotype by extraction with a hot aqueous phenol and visualization by SDS-page (111). The three isolates each exhibit

different LPS phenotypes; Isolate 1 presents neither A-band nor B-band, and appears to have a different core from PAO1 wt. Isolate 2 also does not present A-band or B-band, but appears to have a similar core to Isolate 3 and PAO1 wt. Isolate 3 presents a wild type core, and presents A-band antigen.

Figure 6. Whole-genome analysis of three CF isolates of *P. aeruginosa* from the same population. (A) Three R1-type pyocin producing clinical CF isolates from the same population were sequenced and aligned to a PAO1 reference genome for comparison. Only annotated single nucleotide variants (SNVs) unique to the R2-pyocin resistant Isolate 1 are depicted for simplicity. (B) Single nucleotide variant lists between each of the isolates when mapped to PAO1 were compared, to reveal that approximately 30,127 SNVs distinguished this population from the laboratory strain PAO1. Out of the 30,646 SNVs between Isolate 1 and PAO1, only 232 were unique to Isolate 1 when compared to Isolates 2 and 3 from the same population.

Figure S1. Distribution of R-pyocin types across *P. aeruginosa* strains isolated from various sources as a part of the IPCD. Using BLAST+ (99), 852 publically available strains through the International Pseudomonas Consortium Database (IPCD) were assessed for R-pyocin genotype (55). Of the 852 strains, 549 strains were typeable (assigned to either R1- R2- or R5-pyocin types). The distribution of R-pyocin types among typeable respiratory isolates suggest that R1-pyocin producers are the most prevalent subtype found, including in CF and in other types of respiratory infections.

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Figure 1

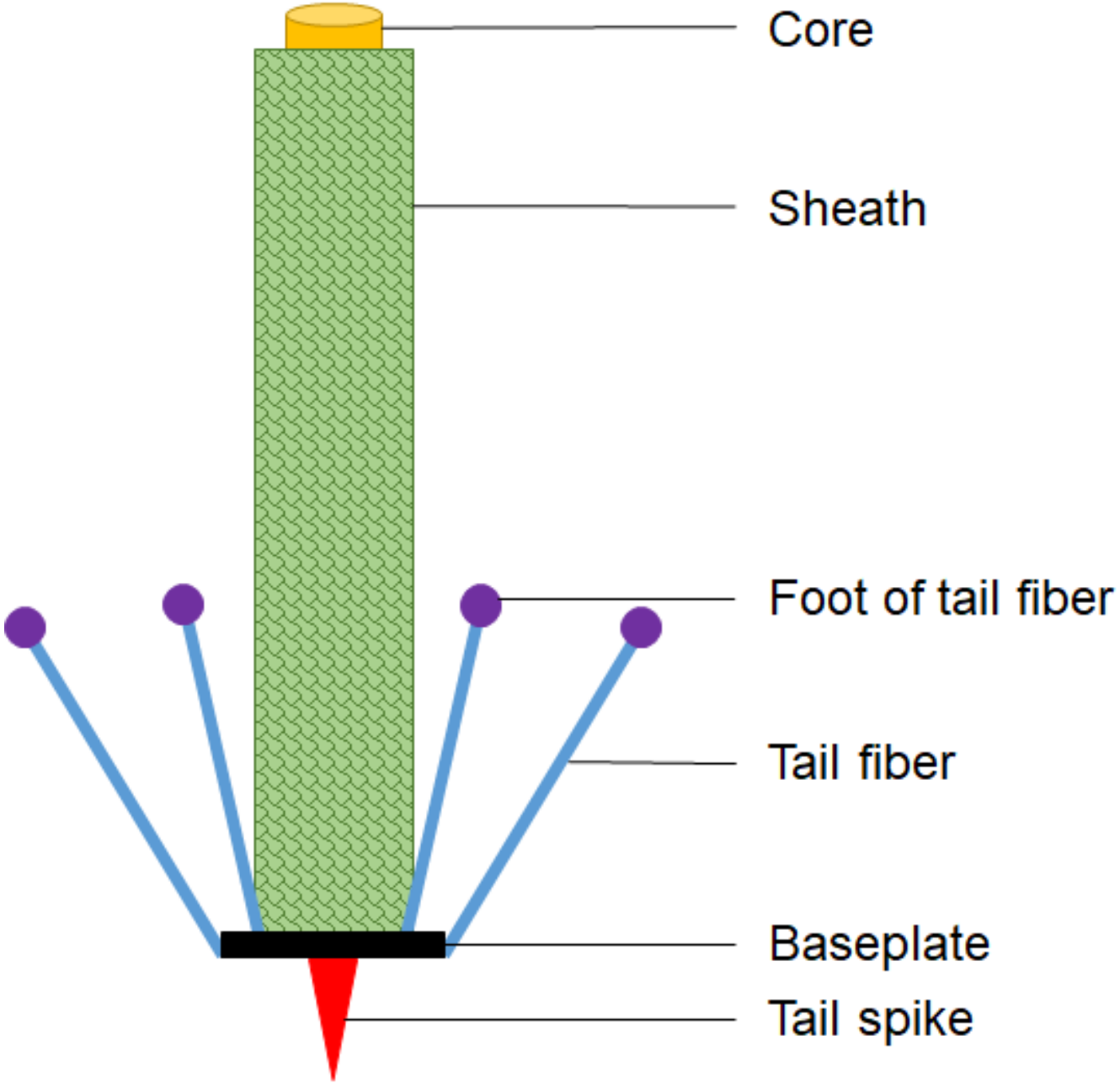

Core
Sheath
Foot of tail fiber
Tail fiber
Baseplate
Tail spike

Figure 2

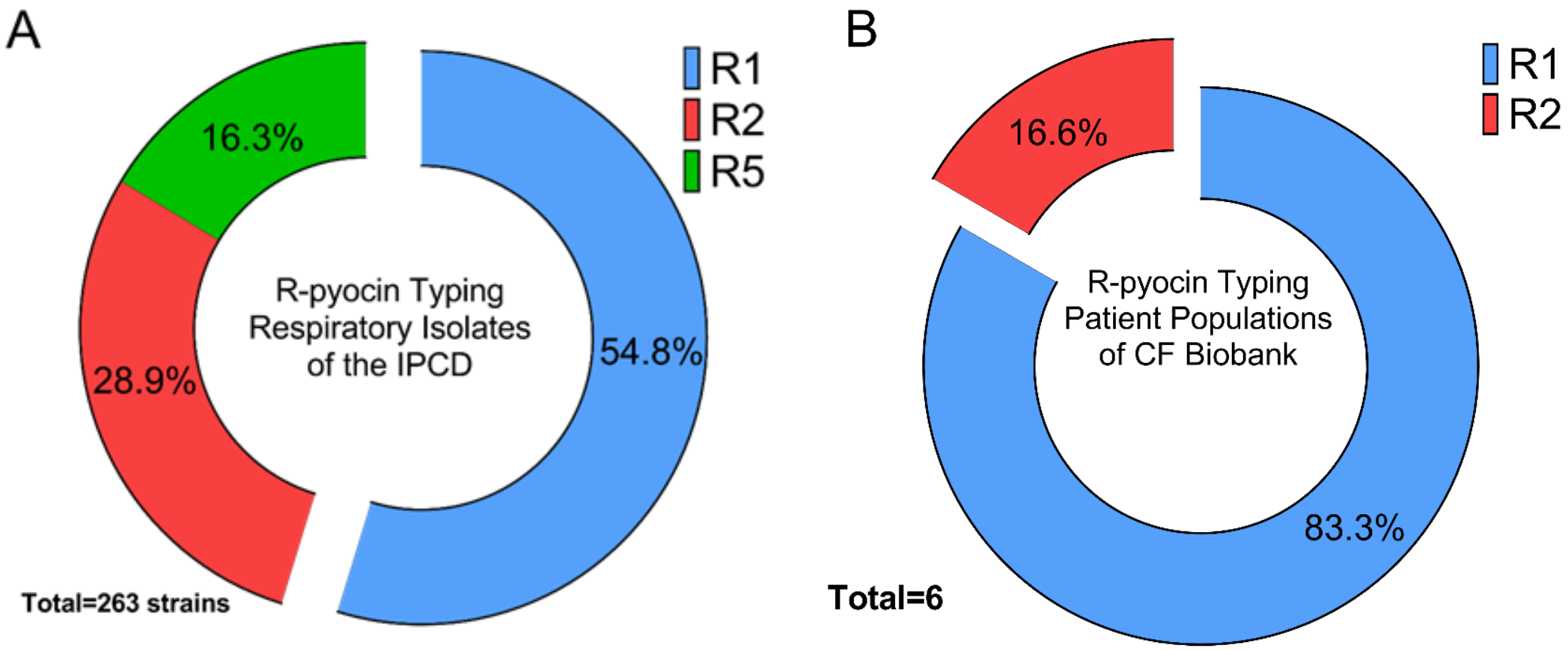
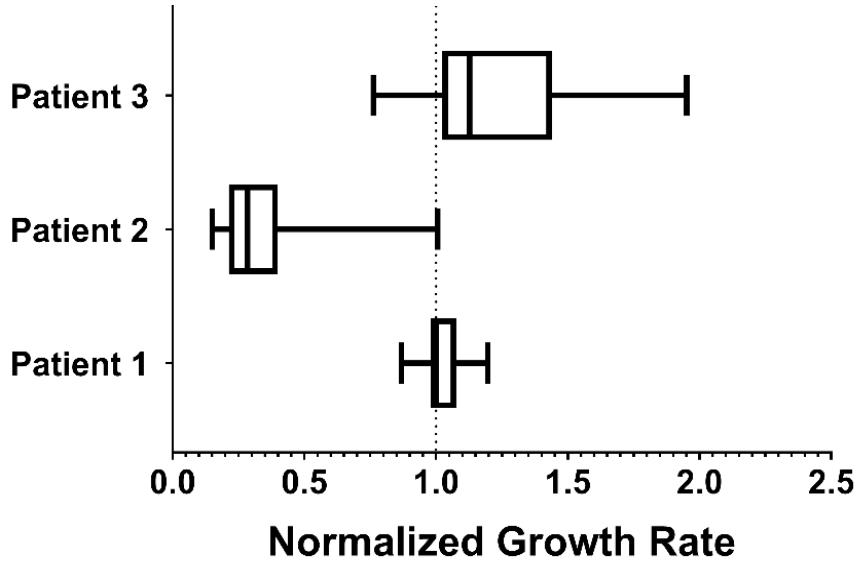
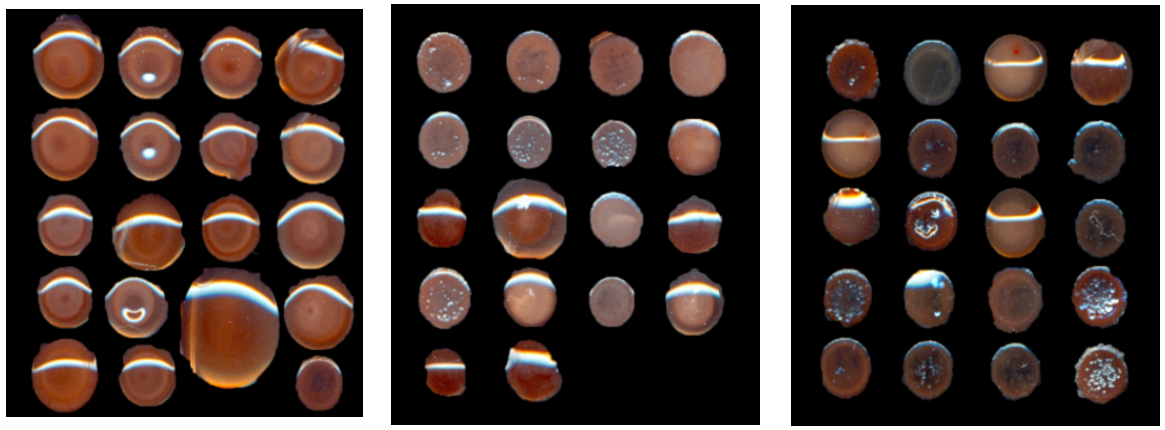

A
R1
R2
R5
16.3%
28.9%
54.8%
R-pyocin Typing
Respiratory Isolates
of the IPCD
Total=263 strains
B
R1
R2
16.6%
83.3%
R-pyocin Typing
Patient Populations
of CF Biobank
Total=6

Figure 3

A



B



Patient 1 (UT) Patient 2 (UT) Patient 3 (R1)

C

$$\text{Fold-change} = \frac{OD\ with\ pyocins}{OD\ without\ pyocins}$$

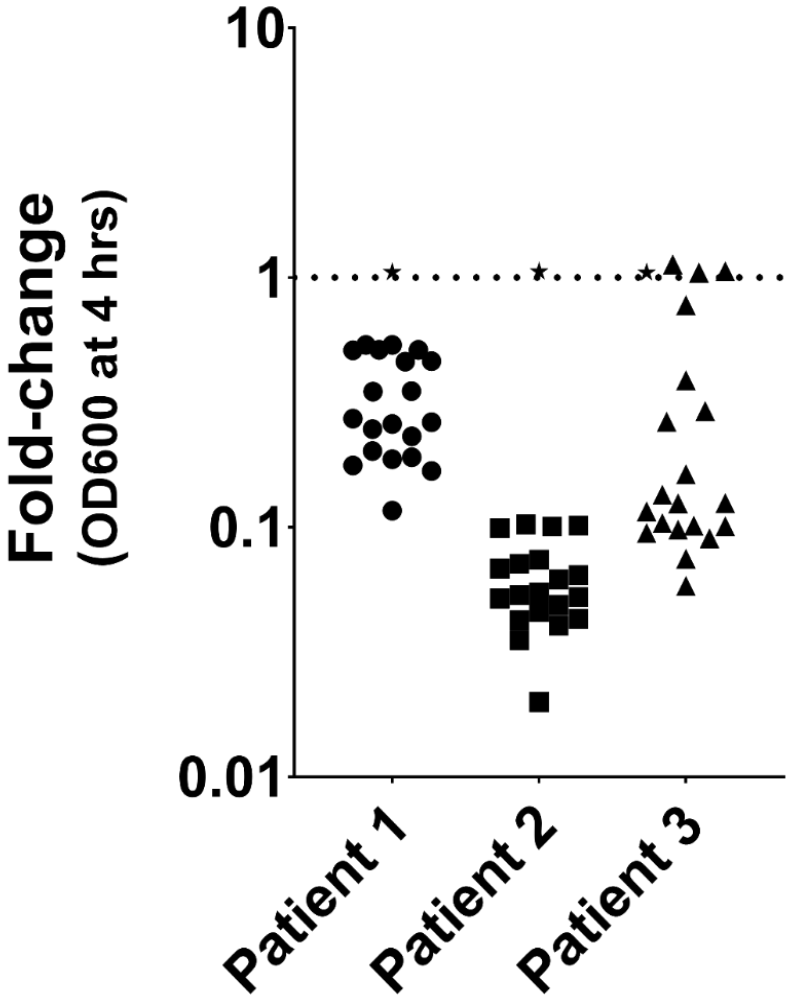


Figure 4

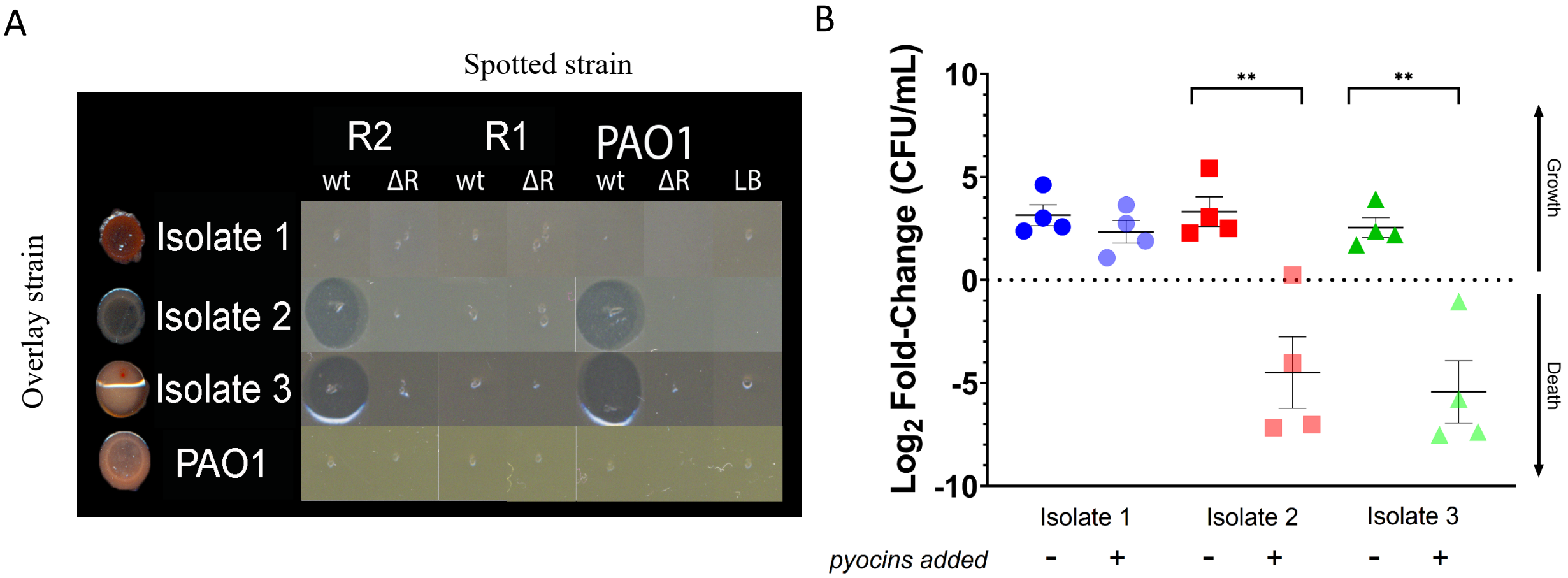

A
Spotted strain
R2
R1
PAO1
wt
ΔR
wt
ΔR
wt
ΔR
LB
Overlay strain
Isolate 1
Isolate 2
Isolate 3
PAO1
B
Log2 Fold-Change (CFU/mL)
10
5
0
-5
-10
**
**
Growth
Death
Isolate 1
Isolate 2
Isolate 3
pyocins added
-
+
-
+
-
+

Figure 5

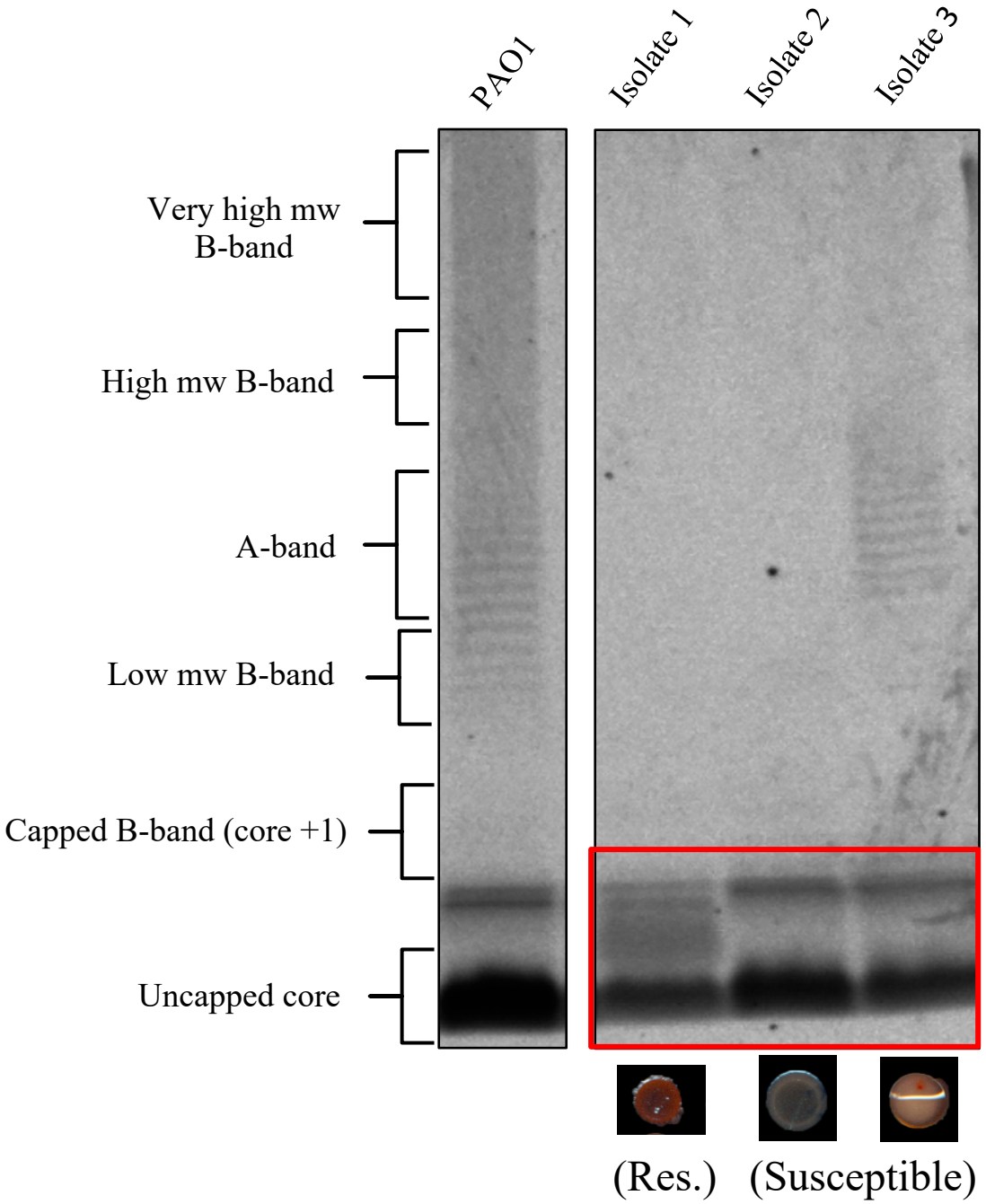
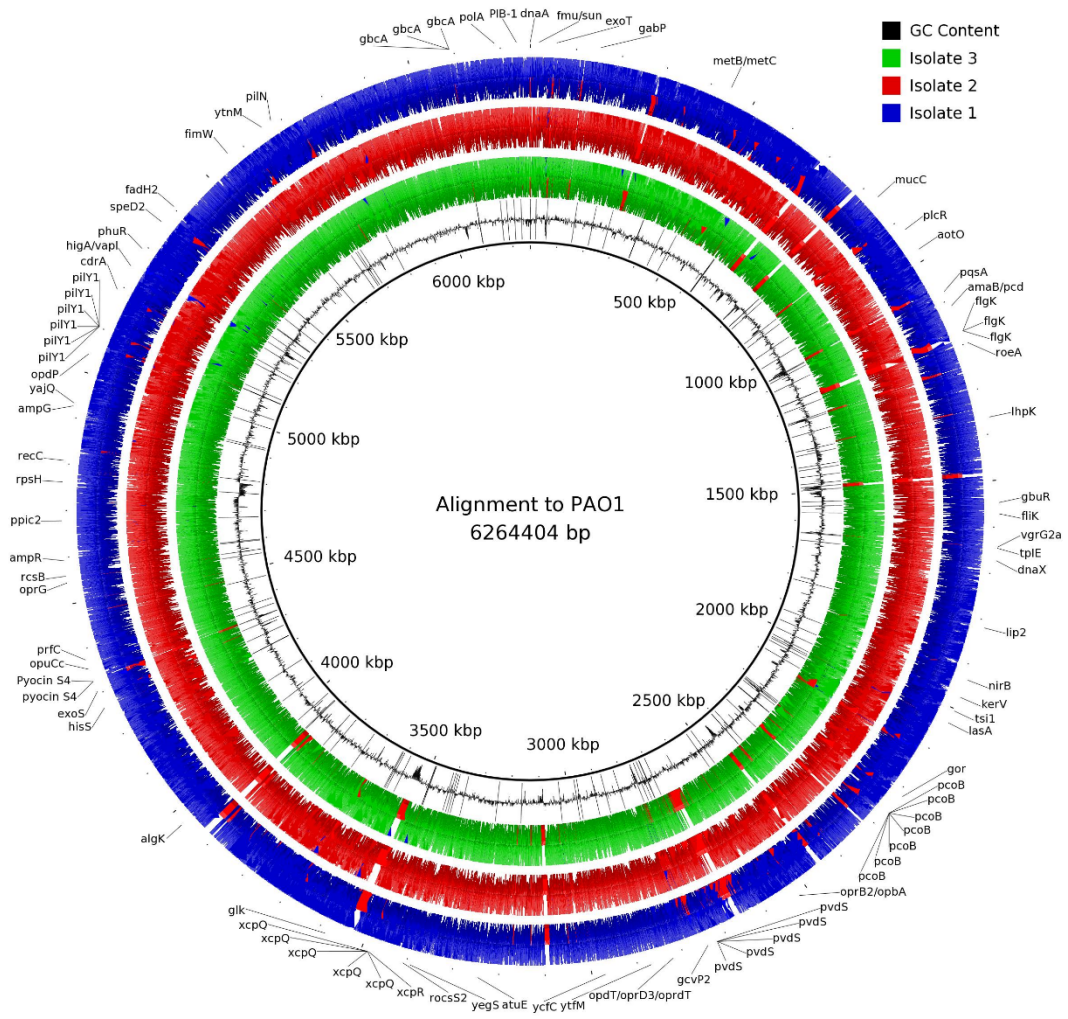

PAO1
Isolate 1
Isolate 2
Isolate 3
Very high mw B-band
High mw B-band
A-band
Low mw B-band
Capped B-band (core +1)
Uncapped core
(Res.)
(Susceptible)

Figure 6

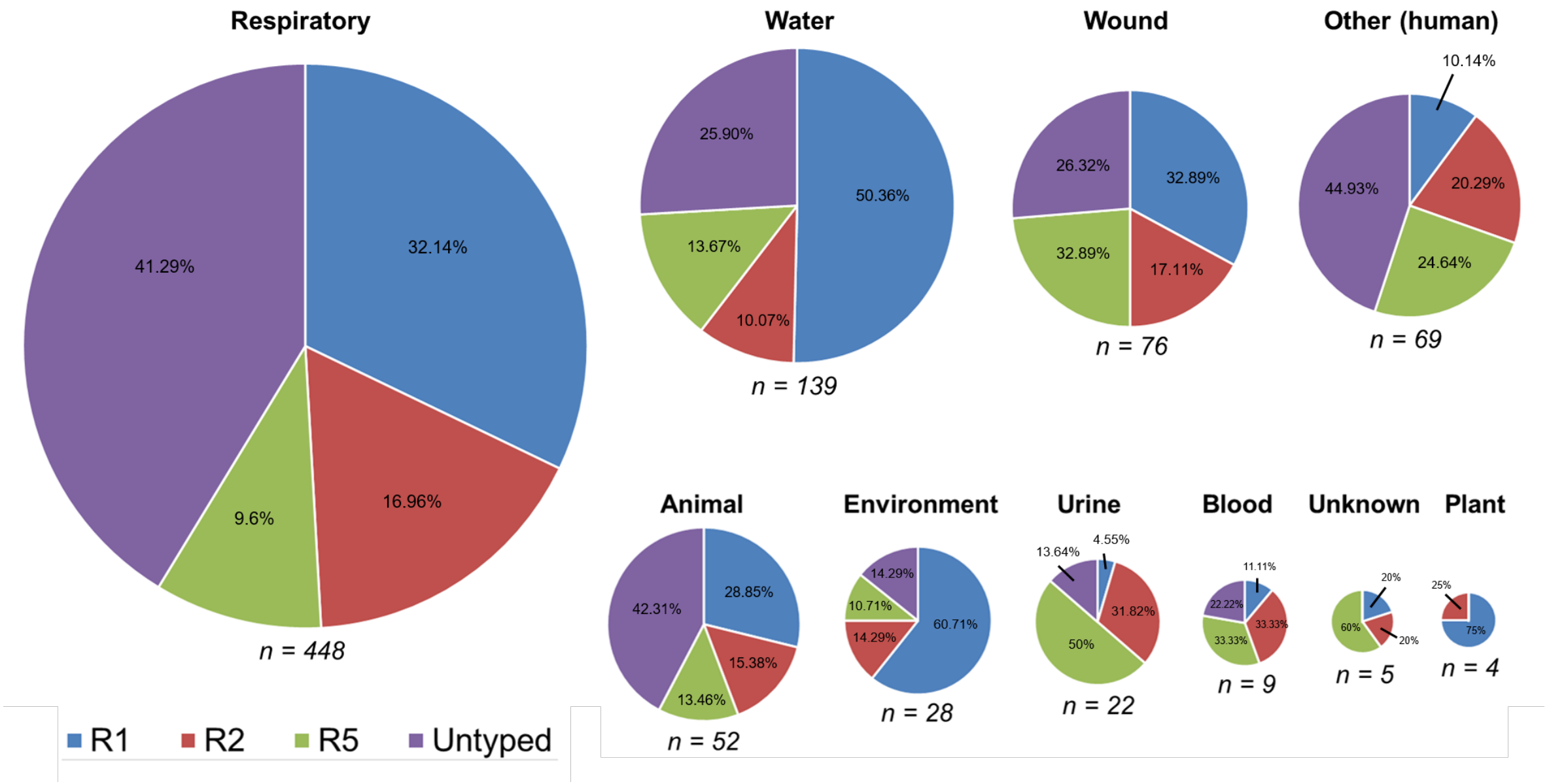
A


GC Content
Isolate 3
Isolate 2
Isolate 1
Alignment to PAO1
6264404 bp
500 kbp
1000 kbp
1500 kbp
2000 kbp
2500 kbp
3000 kbp
3500 kbp
4000 kbp
4500 kbp
5000 kbp
5500 kbp
6000 kbp
gbcA
gbcA
gbcA
polA
PIB-1
dnaA
fmu/sun
exoT
gabP
metB/metC
mucC
plcR
aotO
pqsA
amaB/pcd
flgK
flgK
flgK
roeA
lhpK
gbuR
fliK
vgrG2a
tplE
dnaX
lip2
nirB
kerV
tsi1
lasA
gor
pcoB
pcoB
pcoB
pcoB
pcoB
pcoB
pcoB
oprB2/opbA
pvdS
pvdS
pvdS
pvdS
pvdS
gcvP2
opdT/oprD3/oprdT
ycfC ytfM
yegS atuE
rocsS2
xcpR
xcpQ
xcpQ
xcpQ
xcpQ
xcpQ
glk
algK
hisS
exoS
pyocin S4
Pyocin S4
opuCc
prfC
rcsB
oprG
ampR
ppic2
rpsH
recC
ampG
yajQ
opdP
pilY1
pilY1
pilY1
pilY1
pilY1
pilY1
pilY1
cdrA
higA/vapI
phuR
speD2
fadH2
fimW
ytnM
pilN

B

Isolate 1
Isolate 2
Isolate 3
232
223
442
30127
64
210
79

Figure S1


Respiratory
32.14%
16.96%
9.6%
41.29%
n = 448
Water
50.36%
10.07%
13.67%
25.90%
n = 139
Wound
32.89%
17.11%
32.89%
26.32%
n = 76
Other (human)
10.14%
20.29%
24.64%
44.93%
n = 69
Animal
28.85%
15.38%
13.46%
42.31%
n = 52
Environment
60.71%
14.29%
14.29%
10.71%
n = 28
Urine
4.55%
31.82%
50%
13.64%
n = 22
Blood
11.11%
33.33%
33.33%
22.22%
n = 9
Unknown
20%
20%
60%
n = 5
Plant
25%
75%
n = 4
R1
R2
R5
Untyped