R-PYOCIN REGULATION, RELEASE, AND SUSCEPTIBILITY IN *PSEUDOMONAS AERUGINOSA*

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R-PYOCIN REGULATION, RELEASE, AND SUSCEPTIBILITY IN *PSEUDOMONAS AERUGINOSA*

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“He who knows when to fight and when not to fight, will be victorious.”

- Sun Tzu, the Art of War, *circa* 500 BC [1]

I would like to dedicate this thesis to my husband, Blake, whose endless support, encouragement, and flexibility were paramount to achieving this work.

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LIST OF SYMBOLS AND ABBREVIATIONS

3O-C12-HSL	*N*-3-oxo-C12-homoserine lactone
AHLs	*N*-acyl homoserine lactones
ANR	anaerobic regulation of arginine deiminase and nitrate reduction
ANOVA	analysis of variance
AMR	antimicrobial resistance
APE	acute pulmonary exacerbation
AQs	alkyl-quinolones
bp	basepair
BLAST	Basic Local Alignment Search Tool
BRIG	BLAST Ring Image Generator
C	Celsius
C4-HSL	*N*-butanoyl-homoserine lactone
CDC	Centers for Disease Control and Prevention
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CFU	colony forming units
CLB	colicin-like bacteriocin
CPA	common antigen
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dpi	dots per inch
FDA	Food and Drug Administration
HHQ	3-hydroxy-4-quinolone
h	hours
IPCD	International Pseudomonas Consortium Database

IPTG	isopropyl β-D-1-thiogalacto-pyranoside
IRB	Institutional Review Board
kDa	kilodalton
L	liter
LAB	Lactic acid bacteria
LB	lysogeny broth
LPS	lipopolysaccharide
MRSA	Methicillin-resistant Staphylococcus aureus
M	molar
MDR	multi-drug resistant
min	minutes
µg	microgram
µL	microliter
µM	micromolar
mM	millimolar
mL	milliliter
MLST	multi-locus sequence type
mw	Molecular weight
nM	nanomolar
nm	nanometers
NCBI	National Center for Biotechnology Information
O6	O-serotype Type 6
OD	optical density
OSA	O-specific antigen
PAst	*Pseudomonas aeruginosa* serotyper
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PQS	Pseudomonas Quinolone Signal, 2-heptyl-3-hydroxy-4-quinolone
PTLB	Phage tail-like bacteriocin
QS	quorum sensing
R	resistant
Res.	resistant
rpm	revolutions per minute
S	susceptible
s	seconds
SNP	single nucleotide polymorphism
SNV	single nucleotide variant
SPAdes	St. Petersburg genome assembler
SRA	Sequence Read Archive
UT	untypeable
U.S.	United States
WGS	whole genome sequencing
WT	wild type
x g	times gravity

SUMMARY

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. Chronic infection with *P. aeruginosa* remains one of the main causes of mortality and morbidity in CF patients, thus new therapeutic interventions are 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 for *P. aeruginosa*, either in isolation, in combination with antibiotics, or as an alternative to phage therapy. There are five subtypes of R-pyocin (types R1-R5), and it is thought that each *P. aeruginosa* strain uniquely produces only one of these, suggesting a degree of strain-specificity. *P. aeruginosa* from CF lung infections develop increasing resistance to antibiotics, making new treatment approaches essential. It is known *P. aeruginosa* populations in CF chronic lung infection become phenotypically and genotypically diverse over time, however, little is known of the efficacy of R-pyocins against heterogeneous populations. Even less is known regarding the timing and regulation of R-pyocins in CF lung infections, or if *P. aeruginosa* utilizes R-pyocin production during infection for competition or otherwise – which may influence pressure towards R-pyocin resistance.

In this work, I evaluated R-pyocin type and susceptibility among *P. aeruginosa* isolates sourced from CF infections and found that (i) R1-pyocins are the most prevalent

R-type among respiratory infection and CF strains; (ii) a large proportion of *P. aeruginosa* strains lack R-pyocin genes entirely; (iii) isolates from *P. aeruginosa* populations collected from the same patient at a single time point have the same R-pyocin type; (iv) there is heterogeneity in susceptibility to R-pyocins within *P. aeruginosa* populations and (v) susceptibility is likely driven by diversity of LPS phenotypes within clinical populations. These findings suggest that there is likely heterogeneity in response to other types of LPS-binding antimicrobials, including phage, which is important for consideration of antimicrobials as therapeutics.

To investigate the prevalence of R2-pyocin susceptible strains in CF, I then utilized 110 isolates of *P. aeruginosa* collected from five individuals with CF to test for R2-pyocin susceptibility and identify LPS phenotypes. From our collection we i) estimated that approximately 83% of sputum samples contain heterogenous *P. aeruginosa* populations without R2-pyocin resistant isolates and all sputum samples contained susceptible isolates; ii) we found that there is no correlation between R2-pyocin susceptibility and LPS phenotypes, and iii) we estimate that approximately 76% of isolates sampled from sputum lack O-specific antigen, 42% lack common antigen, and 27% exhibit altered LPS cores. This finding highlights that perhaps LPS packing density may play a more influential role in mediating R-pyocin susceptibility in infection. Finding the majority of our sampled *P. aeruginosa* populations to be R2-pyocin susceptible further supports the potential of these narrow-spectrum antimicrobials despite facing heterogenous susceptibility among diverse populations.

In order to evaluate how R-pyocins may influence strain competition and growth in CF lung infection, I assessed R-pyocin activity in an infection-relevant environment

(Synthetic Cystic Fibrosis Sputum Medium; SCFM2) and found that (i) R-pyocins genes are transcribed more in the CF nutrient environment than in rich laboratory medium and (ii) in a structured, CF-like environment, R-pyocin induction is costly to producing strains in competition rather than beneficial. Our work suggests that R-pyocins may not be essential in CF lung infection and can be costly to producing cells in the presence of stress response-inducing stimuli, such as those commonly found in infection.

In this thesis I have studied R-pyocin susceptibility, regulation and release utilizing a biobank of whole populations of *P. aeruginosa* collected from 11 individuals with CF, as well as the CF infection model (SCFM) to understand the mechanisms of R-pyocin activity in an infection-relevant context and the role R-pyocins play in shaping *P. aeruginosa* populations during infection. The findings of this work have illuminated the impact of *P. aeruginosa* heterogeneity on R-pyocin susceptibility, furthered our understanding of R-pyocins as potential therapeutics, and built upon our knowledge of bacteriocin-mediated interactions.

CHAPTER 1: INTRODUCTION

Antimicrobial resistance (AMR) is a problem in many types of infectious disease, particularly in individuals with chronic infections, compromised immune systems, and people with cystic fibrosis (CF). In this chapter I (i) describe the opportunistic pathogen *Pseudomonas aeruginosa*, a major player in many different AMR chronic infections; (ii) highlight the importance of harnessing bacteriocins to combat widespread AMR and (iii) discuss R-type pyocins, the narrow-spectrum bacteriocins produced by this organism, for their potential as anti-pseudomonal agents against AMR infections.

1.1. The Global Issue of Antimicrobial Resistance

An estimated 4.95 million deaths were *associated* with bacterial AMR in 2019, including approximately 1.27 million deaths actually *attributable* to AMR bacteria [2]. Six leading pathogens for deaths associated with resistance in 2019 (*Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*) were responsible for between 660,000–1,270,000 deaths worldwide [2]. These estimates highlight that bacterial AMR is a health problem of a magnitude at least on a par with major diseases such as HIV and malaria, and potentially much larger [2, 3]. Bacterial AMR is responsible for a massive global burden in healthcare costs and resources globally and compromises the efficacy of treatment for other illnesses such as cancer chemotherapy and systemic autoimmune disorders [2-4].

The recent COVID-19 pandemic has not only lead to a delay or lack of available data on resistant infections for 2020-2022, but the data that is available show an alarming increase in AMR infections since 2019 [5]. During the first year of the pandemic, more

than 29,400 people died from AMR infections commonly associated with healthcare and of these, approximately 40% of the people acquired the infection from a hospital stay [5]. The Center for Disease Control and Prevention (CDC) reported a 78% increase in carbapenem-resistant *Acinetobacter* infection, a 32% increase in multi-drug resistant (MDR) *P. aeruginosa* infections, and a 13% increase in Methicillin-resistant *Staphylococcus aureus* (MRSA) infections, among others [5]. Even more concerning, is that these rates are estimated for the United States alone, and do not account for global increases, which are likely much higher. The CDC reports that 6 of the 18 most concerning antimicrobial resistance threats cost the U.S. more than $4.6 billion annually; these 6 threats include members of the notorious ESKAPE pathogens: Vancomycin-resistant *Enterococcus,* carbapenem-resistant *Acinetobacter,* MRSA, Carbapenem-resistant Enterobacterales, MDR *P. aeruginosa*, and extended-spectrum cephalosporin-resistant Enterobacterales [5, 6].

The acronym ESKAPE refers to six highly virulent pathogens with growing multi-drug resistance to current antibiotics; they include *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *P. aeruginosa*, and Enterobacter species [6]. ESKAPE pathogens are of key importance with respect to the development of new treatments, antibiotics, and antibacterials as they are associated with the highest risk of mortality, contributing to increased healthcare costs [7]. The mechanisms of resistance utilized by these pathogens can be grouped into three categories, which include enzymatic inactivation, target site modification, and accumulation reduction (either by altering membrane permeability or by increasing efflux) [6, 8]. Each year the number of antibiotics effective against ESKAPE pathogens is declining with few new options in the

developmental pipeline; it is therefore imperative to invest in the development of vaccines and alternative therapeutics for use to combat these pathogens.

1.2. *Pseudomonas aeruginosa*: a Gram negative Opportunistic Pathogen

P. aeruginosa is an ESKAPE pathogen listed as a serious threat in a report curated by the CDC. Known for possessing intrinsic antimicrobial resistance mechanisms, hospital-acquired strains of *P. aeruginosa* are of great concern because of their tendency to be MDR [9]. *P. aeruginosa* causes many types of infection including bacteremia, urinary tract infections, and pneumonia. However, it is especially difficult to eradicate for those with compromised immunity, chronic wounds, and particularly people with CF. With an estimated 36,000 patients in hospitals affected and approximately $767 million in attributable healthcare costs in 2017 alone, reducing cases of *P. aeruginosa* infection is imperative [10]. Continued monitoring of antibiotic use, infection control, and the development of additional therapeutics are currently the most promising avenues of addressing the concern of MDR *P. aeruginosa*.

P. aeruginosa is a Gram-negative, opportunistic pathogen that can be found ubiquitously in environments associated with human activity [9]. It is a heterotrophic, motile, facultative anaerobe capable of growing with aerobic respiration or with anaerobic respiration using nitrate as the terminal electron receptor [11,12]. This rod-shaped bacterium measures approximately 1-5 μM long and 0.5-1.0 μM wide [12]. A highly versatile survivor, *P. aeruginosa* possesses a circular genome encoding a plethora of intrinsic antibiotic resistance mechanisms, metabolic capabilities, virulence factors, and

social traits allowing adaptation to a number of environments, and plant, animal and human hosts [11, 12].

1.2.1. The Phylogeny of *P. aeruginosa*

The two most commonly used *P. aeruginosa* lab strains, PAO1 and PA14, have been studied extensively to develop genomic resources [11-13]. Early studies to classify *P. aeruginosa* strains used core genome single nucleotide polymorphism (SNP) phylogeny that organized strains into two major groups: group I included strains similar to PAO1 and group II included strains more similar to PA14 and unrelated clonal lineages [12]. Recent pan-genome studies have now outlined a five-group classification scheme, though little is known still regarding significant similarities between environmental and clinical strains [12, 14-16]. While multi-locus sequence typing (MLST) is the most widely accepted standard for building *P. aeruginosa* phylogenies and epidemiological surveillance, phylogenies have been modeled using highly conserved "housekeeping" genes such as *rpoB* (RNA polymerase beta subunit), *gyrB* (DNA gyrase subunit B), *rpoD* (RNA polymerase sigma factor), and *oprI* (outer membrane or peptidoglycan-associated lipoprotein) [17-21].

1.2.2. Molecular Biology of *P. aeruginosa*

The *P. aeruginosa* genome ranges in size from 5.5-7 Mbp depending on the strain; often, strains of environmental or clinical isolation have a larger genome than laboratory strains [11, 13, 14, 22-24]. The major part of the genome is referred to as the core genome and is highly conserved in all *P. aeruginosa* strains [12-14, 23, 25]. The extra-chromosomal elements including plasmids and inserted elements are referred to as the accessory genome

and account for most of the genomic diversity between *P. aeruginosa* strains. While genes in the accessory genome may be shared by multiple strains of *P. aeruginosa*, they may also occur in only a single strain where many 'variants' can exist within a population [26]. With approximately 5000-7000 genes encoded in the genome of many *P. aeruginosa* strains, several studies have found between 300 and 700 of these genes are core or essential genes (approximately 6.6% or more of the genome) and more than 50,000 genes flexible or unique [12, 14, 23, 24, 27, 28]. These flexible genes make up the accessory genome across the species [28]. Though horizontal gene transfer is responsible for some of the accessory genome, very often phages, transposons, or insertion elements are found, suggesting that the majority of the accessory genome originates from mobile DNA elements [11, 23, 28]. Together, the core genome and accessory genome are referred to as the pan-genome of the species.

1.2.2.1. Exoproducts of *P. aeruginosa*

P. aeruginosa produces many secondary metabolites and exports many products that aid in survival across a variety of niches. These exoproducts include antimicrobials (bacteriocins), toxins, rhamnolipids, pigments (pyocyanin, pyomelanin), proteases, hydrogen cyanide, iron-chelating siderophores (pyochelin, pyoverdine), polysaccharides and signaling molecules such as those used in cell-to-cell communication (quorum sensing, QS) [12, 29]. Many of these exoproducts are important for fitness in a number of environments, competition and biofilm formation.

In microbial populations, public goods can be defined as "compounds or functions that provide a collective benefit, generally through release into the extracellular environment" [29]. Secreted secondary metabolites may serve as public goods, supporting

microbial cooperation by facilitating interactions with other microbes, the host, and/or the environment [29-32]. Extracellular enzymes may degrade polymers (such as DNA or mucin) in the environment so that smaller fragments may be taken up and used by cells and may break down complex proteins into amino acids for uptake into cells [29, 33-35]. Siderophores are secreted to scavenge iron for microbes in iron-depleted environments, while toxins and virulence factors damage host tissues to release nutrients for metabolism [29, 36, 37]. Biosurfactants allow motility and movement of cells and exopolysaccharides like alginate, Psl and Pel promote biofilm formation and form a protective matrix around the cells which help to prevent desiccation an aid in survival against environmental stressors [29, 34, 35, 38].

To facilitate microbe-microbe interactions, *P. aeruginosa* may excrete antibiotics/bacteriocins, and toxins to harm competing bacteria and other microorganisms, while signaling molecules may coordinate concerted behaviors in a population via QS) [29, 32, 39-45]. With such a large toolbox of exoproducts to interact with the environment and other nearby organisms, *P. aeruginosa* is often chosen as the model of choice to study the impact of any of these mechanisms on community dynamics and survival.

1.2.2.2. Quorum Sensing (QS)

QS refers to the mechanism of cell-to-cell communication employed by many species of bacteria, including *P. aeruginosa* [12, 32, 46]. The QS system of *P. aeruginosa* involves a complex hierarchy of regulation, believed to control approximately 10% of genes in the genome [39-41, 47-50]. Signal molecules of this system include *N*-acyl homoserine lactones (AHLs) and alkyl-quinolones (AQs), which are small, diffusible molecules [39, 40, 47, 51]. As *P. aeruginosa* is also a favored model to study social

behaviors, this complex QS system has been used extensively to understand social evolution theory, cooperation, conflict, and virulence in microbial communities and infection [12, 30-32, 34, 43, 44, 46, 52-54]. QS in *P. aeruginosa* is density-dependent; the concentration of signal molecules are directly proportional to cell population density [12, 33, 41-43, 45, 46, 51, 55-57]. Upon detecting a threshold concentration of signal(s), gene expression and thus the behavior of the population is altered in a concerted fashion [32, 40, 46, 47, 58]. QS regulated pathways include those that control biofilm formation, motility, virulence factors, and antibiotic production [12, 29, 46].

As the first described autoinducing signals, acyl-homoserine lactones (AHLs) have since been described to regulate two circuits in *P. aeruginosa*; these are referred to as the Las and Rhl systems (Figure 1.1) [47, 51]. The Las system hierarchically coordinates the Rhl system, though recent studies have found evidence of de-coupling of this regulation leading to a more independently functioning Rhl system – especially in strains isolated from chronic infections [49, 59-65]. The AHLs secreted and detected by the Las and Rhl systems are *N*-3-oxo-C12-homoserine lactone (3O-C12-HSL) and *N*-butanoyl-homoserine lactone (C4-HSL) respectively (Figure 1.1), which then bind to the transcriptional regulators LasR and RhlR [47, 51, 60].

The second signaling system of the *P. aeruginosa* QS hierarchy produces the quinolone 3-hydroxy-4-quinolone (HHQ), which is then converted to 2-heptyl-3-hydroxy-4-quinolone, the Pseudomonas Quinolone Signal PQS (Figure 1.1) [59, 60, 66, 67]. The PQS system is closely linked to the AHL systems in that the production of HHQ and the transcriptional regulator *pqsR* are repressed by the Rhl system, while the expression of

pqsR and *pqsH* (which converts HHQ to PQS) are induced by the Las system (Figure 1.1) [59, 60]. In return, the PQS system activates the Rhl transcriptional regulator, RhlR [59].

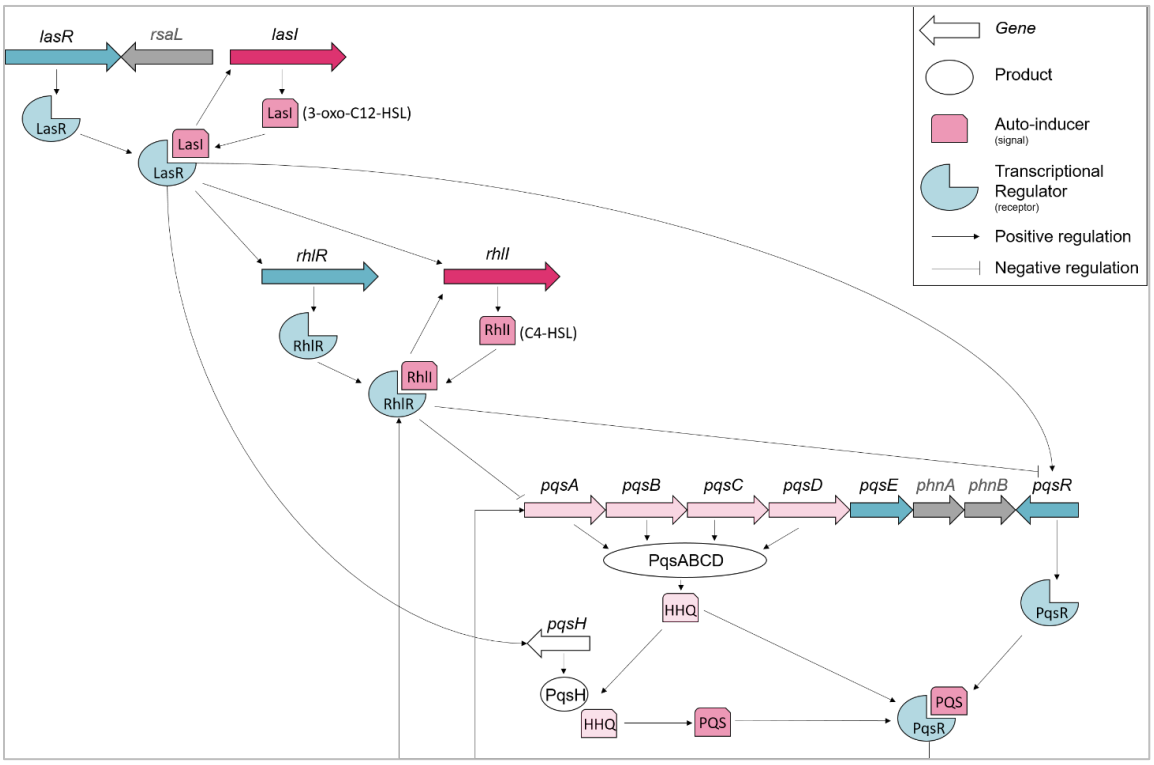

Figure 1.1: Simplified overview of interactions between three QS systems of *P. aeruginosa*. The Las system is canonically described at the top of the signaling hierarchy as it may regulate both the Rhl and PQS systems. The AHQ and AHL-dependent quorum sensing systems are closely linked in that the production of HHQ and the transcriptional regulator pqsR are repressed by the Rhl system, the expression of pqsR and pqsH are induced by the Las system, and the PQS system may activate the Rhl transcriptional regulator, RhlR.

QS mutants of *P. aeruginosa* have long been observed to exhibit autolytic plaquing, and the induction of pyocins (the bacteriocins of *P. aeruginosa*) had originally been considered the most probably cause of auto-plaquing [68]. In some cases, mutations that lead to an accumulation of PQS and HHQ can be attributed to autolysis [69, 70]. However, several studies examining the expression profiles of QS mutants and early-stationary-phase *P. aeruginosa* cultures do not provide evidence of pyocin production to be regulated by QS [48, 49, 68, 71]. The possible link between bacteriocin production and QS in *P. aeruginosa* remains unknown.

1.2.2.3. Secretion Systems

Protein secretion systems are molecular mechanisms used by Gram-negative bacteria to help adapt to their environment. These systems are nanomachines employed to release enzymes, scavenging molecules, and toxins [72, 73]. Five different types of secretion systems have been described in *P. aeruginosa* to date, and are referred to as types I , II, III, V, and VI (Figure 1.2) [12, 74]. Each of these secretion systems can be grouped into two categories determined by their secretion process. One-step secretion systems transport proteins directly from the cytoplasm to the cell surface; the two-step secretion mechanism requires a brief pit stop in the cytoplasm before the protein is transported outside of the cell; in principle, the secreted protein is able to reach the periplasm independently, before transported across the outer membrane [72, 73]. The most common examples of the general pathways that bring secreted proteins to the periplasm for export are the Sec and Tat pathways [75, 76]. The type II (T2SS) and type V secretion system (T5SS) are two-step mechanisms, while types I, III, and VI (T1SS, T3SS, T6SS) are one-step mechanisms (Figure 1.2) [72, 73]. Secretion systems allow *P. aeruginosa* to interact

with its environment by scavenging for resources, altering the environment for more suitable growth, and either interact with or evade host cell responses [73, 77]. While some bacterial species have been found to utilize protein secretion systems to release bacteriocins, in *P. aeruginosa*, bacteriocins are either released by cell lysis (phage-like bacteriocins) or the release is yet unknown (small molecule, soluble bacteriocins).

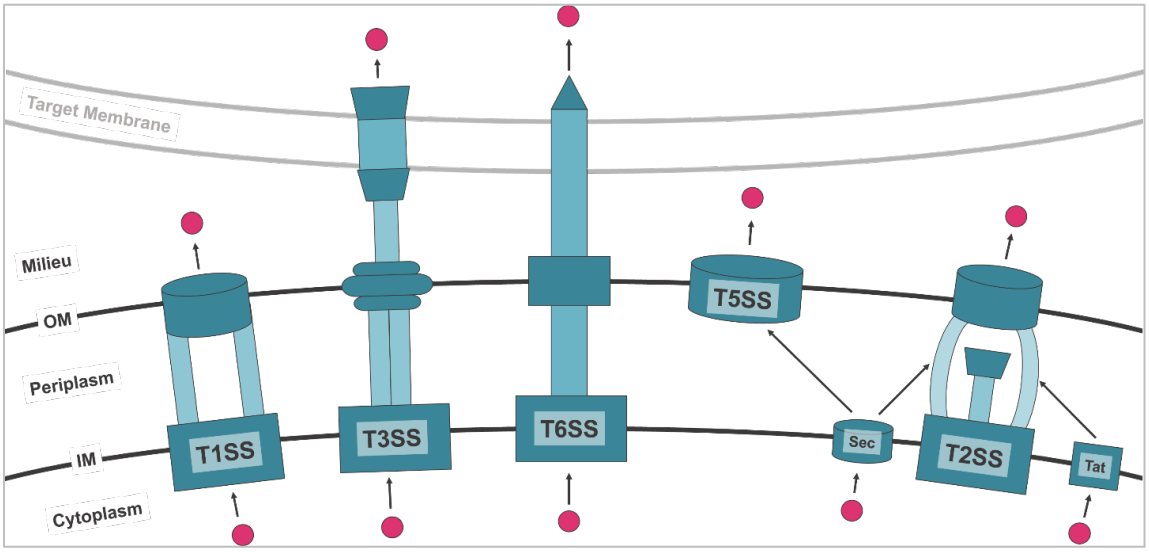

Figure 1.2 Secretion systems of *P. aeruginosa*. The types I, III, and VI (T1SS, T3SS, T6SS) are one-step mechanisms, while type II and type V secretion systems are two-step mechanisms utilizing the Sec and Tat systems. These systems allow *P. aeruginosa* to interact with its environment or nearby cells, release enzymes, scavenge molecules, and release toxins.

1.2.2.4. Outer Membrane Characteristics

Another mechanism of adaptation used by *P. aeruginosa* to adjust to different environments is the altering of its outer membrane. To facilitate movement and attachment to surfaces, *P. aeruginosa* may express "decorations" along the outer membrane such as lipopolysaccharide (LPS), pili, and flagella. Pili and flagella play important roles in movement for behaviors like swimming, twitching, and swarming, but are also involved in infection [12, 70, 78-80]. Pili are known to mediate attachment to epithelial cells in culture, and pili-mediated surface attachment is a critical first step in the formation of biofilms [78, 79]. Flagella also mediate attachment to host-related polymers such as mucin – a glycosylated protein excreted by host epithelial cells and commonly found in cystic fibrosis sputum [81].

The LPS of *P. aeruginosa* consists of a large polysaccharide chain anchored to a lipid (lipid A) within the outer membrane by a covalently bonded core [82-91]. Most strains of *P. aeruginosa* produce two forms of polysaccharide chain that is built into the LPS; one glycoform is a homopolymer of D-rhamnose and is referred to as the common polysaccharide antigen (CPA or A-band) [82-84, 89-92]. The second type of glycoform is a heteropolymer with three to five distinct sugars comprising its repeating units; this heteropolymer is referred to as O-specific antigen (OSA or B-band) and varies widely in composition by strain [82-84, 89, 91]. The differences in composition of the OSA allow for serotyping of *P. aeruginosa* strains, a method of classifying strains by their immunogenic surface characteristics. At least 20 major serotypes (O1 to O20) have been identified thus far [12, 91, 93]. The LPS of *P. aeruginosa* is notably heterogenous in its presentation due to the species' ability to modulate not only which polysaccharide antigen

will be “presented”, but also the chain length of either form, and the packing density of any combination of forms present on the surface in response to changes in the environment [90, 91].

The LPS plays a large role in interactions with the host in infection specifically through the immunogenicity of the OSA. Due to its immunogenic nature, OSA is often lost in strains isolated from chronic infection, and this loss is a one of many phenotypic changes *P. aeruginosa* undergoes to adapt from acute infection to chronic infection [90, 94, 95]. Another mechanism through which *P. aeruginosa* uses the LPS to interact with host cells is through a link with the type 3 secretion system; a strong association has been found between the absence of OSA and increased cytotoxicity through the type 3 secretion system, which punctures host cells and releases damaging toxins (ExoU, ExoS) [96, 97]. Though the complexity of the LPS in *P. aeruginosa* already adds to heterogenous behavior across strains, there is evidence to suggest even more diversity in LPS expression is likely due to chromosomal insertions in strains isolated from chronic infections [91, 98]. The LPS also serves as a receptor for a number of phage, including ϕPLS27, ϕCTX, KTN6 and KT28 among others [82, 91, 99-101].

1.2.2.5. Metabolism

As a facultative aerobe, *P. aeruginosa* is able to grow in a range of environments with varying oxygen. The ability to inhabit soil, water, animal-, human-, and plant-host associated infections is attributed to its versatile and robust metabolism. In the absence of oxygen, *P. aeruginosa* is able to grow by denitrification using nitrogen oxides as terminal electron acceptors; nitrate and nitrite are reduced and released as gaseous nitrous oxide and dinitrogen (N_2) – one of the few known mechanisms of generating atmospheric N_2 [12,

102, 103]. *P. aeruginosa* is also capable of anaerobic fermentation, though it grows very slowly [12, 103-105].

For aerobic respiration, *P. aeruginosa* possess five terminal oxidases, which vary in affinity for oxygen, efficiency in proton-translocation, and resistances to stresses (cyanide, reactive nitrogen species) [11, 103, 106]. These terminal oxidases are regulated by two redox-responsive transcriptional regulators known as ANR (anaerobic regulation of arginine deiminase and nitrate reduction) and RoxSR; ANR is a global regulator of aerobic respiration and a direct oxygen sensor while RoxSR is a two-component regulator with a membrane-bound sensor kinase and a response regulator [11, 103, 106, 107].

P. aeruginosa is unique in that the oxidases with high affinity to oxygen (cbb_3 oxidases) are dominantly expressed even under aerobic conditions with high oxygen [103, 108]. This suggests that the *P. aeruginosa* cells are maintained in a microaerobic state even under aerobic conditions. *P. aeruginosa* produces a microaerobic environment even at high aeration rates by reducing the transfer rate of oxygen from the gas phase into the liquid phase during growth [109]. *P. aeruginosa* strains isolated from the CF airway often overproduce the exopolysaccharide alginate, which is known to act as a physical barrier to oxygen entering the cell and likely facilitates a micro-anaerobic environment in the CF lung [110, 111]. As *P. aeruginosa* is exposed to reactive oxygen species from host immune responses during infection, the physiology of creating a polysaccharide layer to reduce oxygen diffusion could be a mechanism to improve fitness under oxidative stress [103, 110, 111].

1.2.3. Clinical Relevance of *P. aeruginosa*

Many of the described traits and exoproducts of *P. aeruginosa* play important roles in the microbe's adaptation to infectious environments. A versatile and virulent opportunist, *P. aeruginosa* is a pathogen of plants, animals, and humans and is known to contribute to chronic infections that are notoriously difficult to eradicate.

1.2.3.1. Zoonotic Infections

P. aeruginosa has been isolated from infections in a number of human-associated animals including dogs, horses, and reptiles [24]. With a metabolism capable of living in a wide range of hosts, *P. aeruginosa* strains may be picked up from plants, soil, or water before infecting animals, who may then serve as carriers or reservoirs to humans. Antibiotic treatment of pets with *P. aeruginosa* infections is yet another route evolving AMR in this intrinsically resistant pathogen.

1.2.3.2. Chronic Wounds

In the U.S. alone, chronic wounds affect approximately 6.5 million people and an estimated $25 billion is spent annually on the treatment of chronic wounds [112]. Soft tissue infections known to become chronic wounds including diabetic foot ulcers, burn wounds, venous leg ulcers and pressure sores, are a source of significant healthcare costs and burden worldwide [113]. Common features of each of these include prolonged and excessive inflammation, inability of host cells to respond to repair, and formation of drug-resistant microbial biofilms [114]. As chronic wounds commonly stall during the inflammation stage of the healing process, it is likely that bacterial infection contributes to this impediment [114-116]. While chronic wounds may host a number of different

pathogens, *P. aeruginosa* often becomes one of the most problematic and difficult to eradicate [115, 117].

Adaptive mechanisms employed by *P. aeruginosa* in both acute and chronic wounds include the downregulation of (and potential loss of) OSA of the LPS and *psl* polysaccharides, with the upregulation of siderophores, type II and type III secretion systems [113]. The type III and type VI secretion systems, *psl* polysaccharide genes, and the type IV pili have also been found to be essential for *P. aeruginosa* survival in both acute and chronic wounds suggesting the importance of the pathogen's ability to form biofilm, secrete toxins, and interact or attach to host cells [113]. These findings are consistent with what is known of the physiological changes to facilitate early biofilm formation (attachment, motility) [118, 119]. Common characteristics of bacterial biofilms include the ability to evade host immune responses as well as resist and tolerate antibiotic treatments, making chronic wounds infected with bacteria forming biofilms even more difficult to alleviate [115, 116]. The economic burden of wounds calls for more work to understand the microbiological mechanisms underlying chronic wound complications.

1.2.4. *P. aeruginosa* in Cystic Fibrosis Infections

Cystic fibrosis (CF) is a life-limiting, autosomal recessive genetic disorder affecting epithelial cells of several body systems [120]. Disease is caused by a mutation in both copies of the cystic fibrosis transmembrane regulator (CFTR) gene, which encodes for an anion transporter responsible for managing chloride flow in and out of cells [121]. The dysfunctional CFTR transporter ultimately translates to dehydrated mucosal linings, causing a number of comorbidities including infertility, pancreatic insufficiency, cirrhosis

of the liver, and chronic respiratory infections [121]. Mortality is primarily caused by bronchiectasis and progressive loss of lung function [121].

P. aeruginosa is the most prominent bacterial pathogen that colonizes and chronically infects CF lungs [122-127]. The 2019 CF Foundation Patient Registry Annual Report noted that although the prevalence of *P. aeruginosa* continues to decrease, the prevalence of infection with MDR *P. aeruginosa* remains constant as *P. aeruginosa* often becomes the dominant CF lung pathogen over time [128-130]. CF patients are thought to be initially infected by an environmental or transmissible epidemic strain of *P. aeruginosa*, which evolves and acquires genetic mutations and genomic rearrangements within a host during a prolonged infection period, contributing to AMR, morbidity, and mortality [126, 131-134].

1.2.5. *P. aeruginosa* Population Heterogeneity and AMR

AMR is a key problem in individuals with CF infected with *P. aeruginosa* because the organism becomes resistant to many classes of antibiotic leaving few treatment options for many [135]. It has proven extremely challenging to eradicate *P. aeruginosa* from the CF lung, and this is likely due to the high degree of morphotypic, phenotypic and genomic diversity that evolves within *P. aeruginosa* populations during chronic CF infection [126, 130, 131, 136, 137]. The impact of this heterogeneity on acute pulmonary exacerbations (APEs), AMR, and disease progression is not well understood, and even less is known regarding the impact of this heterogeneity on alternative antimicrobials like bacteriocins and bacteriophage.

While significant progress has been made in understanding mechanisms of AMR in many bacterial species, including *P. aeruginosa*, most findings have been based on *in*

vitro experimentation, in conditions not necessarily relevant to chronic infection [138]. The availability of cheaper sequencing technologies has spurred a growing number of publications describing the genomic changes that occur as *P. aeruginosa* populations evolve naturally within the CF host, however, to better understand AMR in infection, it is imperative to also implement models in the laboratory that best recapitulate the nutritional and spatial characteristics of the infections of interest.

1.3. Bacteriocin Antimicrobials

1.3.1. Bacteriocins

As AMR continues to be a major concern and pathogens like *P. aeruginosa* are increasingly difficult to eradicate, non-traditional antimicrobials such as bacteriocins, may be useful alternative therapies in combination with antibiotics and phage treatment. Bacteriocins are a natural class of highly specific antibiotics, generally capable of killing other strains (sometimes other species) of the producing bacteria [139-141].

A number of bacterial species, both Gram-positive and Gram-negative produce bacteriocins, and as such each type of bacteriocin is named correspondingly to the producing species (ie. *Escherichia coli* produces *colicins*). Since 1925, bacteriocins have been studied and categorized by features such as morphology, mechanism of action, lytic capability, genetics (plasmids, chromosomal), molecular weight, and chemistry [140-142]. One common characteristic of all bacteriocins, including phage-like particles, is that they are incapable of replication [140]. While both Gram-positive and Gram-negative bacteria produce bacteriocins, they are classified and described very differently. It is of note that

bacteriocins of Gram-positive bacteria have been categorized into three, sometimes four classes, whereas Gram-negative bacteriocins are grouped into three groups [143, 144].

Bacteriocins produced by Gram-positive bacteria are often described to be grouped into three classes: class I bacteriocins are small (<5 kDa), thermostable peptides also referred to as lantibiotics [143, 144]. The most well characterized bacteriocin, Nisin, is produced by *Lactococcus lactis* and is an example of a class I lactibiotic bacteriocin [143, 144]. Class II bacteriocins are slight larger peptides (<10Da), also heat stable, but differ in that they possess an amphiphilic helical structure allowing insertion into the membrane of target cells and resulting in depolarization, then death [143, 144]. Pediocin PA-1 is an example of a class II bacteriocin produced by *Pediococcus acidilactici strain* PAC-1.0 [144, 145]. Class III (and when described, Class IV) bacteriocins are large, complex particles that can kill target cells by cell wall degradation or membrane depolarization [143, 144]. An example of a class III bacteriocin is megacin A-216, produced by *Bacillus megaterium* 216 [144, 146].

In contrast, the bacteriocins of Gram-negative bacteria are grouped into three major families: low molecular mass peptides (<10 kDa) known as microcins, high molecular mass peptides (30-80 kDa) known as colicins or colicin-like bacteriocins, and phage-tail-like tailocins [144, 147]. Low molecular mass peptides, often referred to as microcins, are more similar to the bacteriocins of Gram-positive bacteria in that they are thermostable, protease resistant, relatively hydrophobic, and pH resistant [148]. Microcins are the least studied of bacteriocins produced by Gram-negative bacteria, as only seven microcins have been isolated and properly characterized [148]. Colicin-like bacteriocins (CLBs), as implied by their description, primarily encompass colicins and other modular, protease-resistant

proteins that are found to be highly similar to colicins. CLBs generally target strains closely related to the producing strain [148]. Tailocins, also referred to as Phage Tail-Like Bacteriocins (PTLBs), are large particles that resemble headless phage tails [148-150]. PTLBs resemble contractile (R-type) or flexible (F-type) bacteriophage tails and utilized target binding mechanisms similar to their bacteriophage predecessors [151-153]. A fourth class is described by some to encompass Lectin-like (L-type) bacteriocins, though current knowledge of their mechanisms of action is limited [154, 155].

1.3.2. Therapeutic Potential of Bacteriocins

Bacteriocins are already commonly used in the agricultural and food industry for preservation and protection of goods, and are only recently gaining more interest as antimicrobials in clinical microbiology as well. Bacteriocins are most widely used in the preservation of dairy, vegetables, eggs, and meat products [141, 143, 144, 147, 148, 156-158]. Gram-positive bacteriocin-producing bacteria, Lactic acid bacteria (LAB) in particular, are the most well-known and frequently used bacteria in bio-preservation, though nisin is the only bacteriocin used worldwide and officially approved by the Food and Drug Administration (FDA) [144, 148, 156].

While useful in food preservation, LAB and other Gram-positive bacteriocins have been studied for their potential use against foodborne illnesses as well – so they may be useful not only in the protection of food against common foodborne pathogens, but in also in the treatment of disease [143, 144, 148, 156-159]. Initially bacteriocins have also been studied for their roles in competition and niche acquisition among Gram-negative bacteria, and somewhat recently gained traction specifically in the context of infection. Thus far, bacteriocins have been shown to play a role in influencing biodiversity, competition, and

biofilm behaviors in lab strains, as well as clinically derived strains in Gram-negative bacteria [147, 160-168].

In vitro studies of bacteriocin antimicrobial activity against relevant clinical pathogens have shown that a number of bacteriocins across a wide range of species exhibit broad spectrum killing capabilities across both Gram-negative and Gram-positive pathogens. Intra-species competition has been shown to be influenced by bacteriocins in various experiments studying *Streptococcus pneumoniae*, *P. aeruginosa, Pseudomonas chlororaphis,* and *E. coli* [166, 168-174]. Successful killing of these and other notoriously problematic pathogens, including *Klebsiella* species, *Salmonella* species, and *Neisseria gonorrhoeae* with bacteriocins highlight the variety of bacteriocin options already in existence to capitalize on for therapeutic purposes [175-178].

Promising examples of *in vivo* murine models supporting the therapeutic use of bacteriocins include one study showing Anti-*Helicobacter pylori* bacteriocin activity of probiotic strains *Lactobacillus johnsonii* LA1 and *Lactobacillus acidophilus* LB protected mice against *Helicobacter felis* and *Helicobacter pylori* gastroduodenal illness [179], Another group found the anti-pseudomonal bacteriocins of *P. aeruginosa* effective in treating murine models of *P. aeruginosa* sepsis, and acute lung infection [180, 181], and another study showed the successful reduction of *Listeria monocytogenes* load following treatment with the bacteriocin piscicolin 126 (P126) [182]. Even modified bacteriocins have been engineered and successful in targeting *Clostridium difficile,* protecting mice from colonization, treating *E. coli* O157:H7, as well as targeting multiple clinically relevant species at once (*E. coli, Yersinia pestis,* and *P. aeruginosa*) [183-185].

1.3.3. The Bacteriocins of *P. aeruginosa*

In 1945 Edwin Hays and colleagues first described antibiotic substances derived from *P. aeruginosa* and nine years later, François Jacob deemed these antibiotics as "pyocines" *P. aeruginosa* [186, 187]. Today, pyocins refer to the narrow-spectrum bacteriocins produced by *P. aeruginosa* (formerly referred to as *Pseudomonas pyocyanea*) [187, 188]. The pyocins produced by *P. aeruginosa* have since been described and categorized into three types referred to as S-, R-, and F-pyocins (Figure 1.3) [149-151, 189, 190]. Each subtype of pyocin differs in physical and chemical properties, export mechanisms, and mode of action though a strain may possess the genetic determinants to produce any combination of, or all three types of pyocin [139, 149, 150, 189].

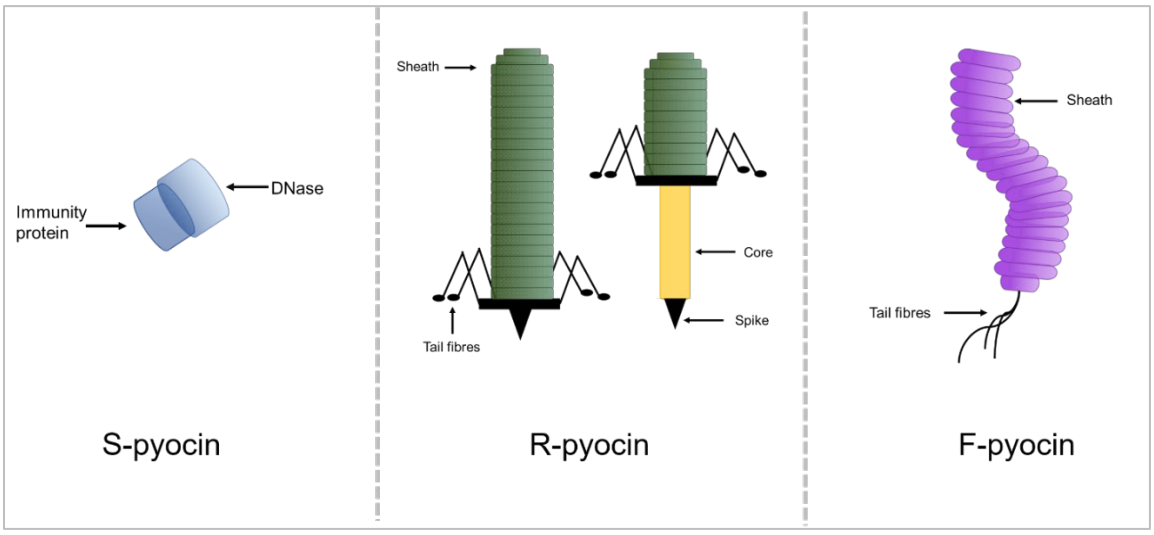

Figure 1.3: The S-, R-, and F-pyocins of *P. aeruginosa*. The bacteriocins of *P. aeruginosa* described as S-, R-, and F-pyocins. S-pyocins are small and soluble enzymatic-immunity protein complexes, while R- and F-pyocins resemble phage tails with sheaths or tubes, and tail fibers to recognize target cells. R-pyocins consist of a contractile sheath, with a spiked core to facilitate the puncture of a target cell membrane.

As the smallest of the pyocins, S- pyocins are membrane soluble, protease-sensitive antimicrobial enzymes produced by *P. aeruginosa* [180, 191-197]. S-pyocins consist of two protein components: a larger protein which exhibits killing activity and a smaller protein which provides the producing strain immunity, similar to colicin E2 of *E. coli* [150, 192, 194, 198, 199]. There have been a number subtypes of S-pyocin described based on killing specificity, which can then be grouped by mechanism of action. AP41, S1, S2, S3, S9, and S10 pyocins exhibit DNAse activity; pyocins S4, S11 and S12 are predicted to be tRNAses; and pyocin S5 is predicted to have pore-forming activity [150, 151, 191-194, 198, 200-202]. Pyocins S2-5 recognize TonB-dependent ferri-siderophore receptors of the outer membrane, and thus are particularly upregulated and uptaken in iron-limiting conditions [191, 203-205]. S-pyocin genetic determinants are located on the chromosome, and one strain may possess genes to produce multiple subtypes of S-pyocin [150, 192-194, 206-211]. While S-pyocins exhibit antimicrobial activity, they are the least potent relative to the other types of pyocin [171].

In contrast to S-pyocins, R-pyocins and F-pyocins are both PTLBs resembling phage tails without DNA-containing capsid heads [149, 212-214]. F-pyocin – named due to its flexuous structure resembling a filamentous, non-contractile bacteriophage are similar to *Siphoviridae* phage [150, 212-216]. Three subtypes of F-pyocin have been described and denoted as F1-3 as determined by their killing spectrum, however, little is known of their mechanism of action beyond that they are thought to form a channel through the membrane and disrupt respiration [149, 215]. Like S- and R-pyocins, F-pyocin genes also reside on the chromosome of *P. aeruginosa* and can be found in a single operon encoding for a single subtype of F-pyocin, immediately downstream of the R-pyocin genes

[150, 152, 161, 213-215]. R-pyocins and F-pyocins are thus controlled under the same regulatory conditions and are released by lysis of the producing cell [151, 190, 207, 213-215, 217-233]. Both R-pyocins and F-pyocins can kill a targed cell following the binding of only a single unit of either, however the mechanism of action and the genomic diversity of F-pyocins is generally unknown [149, 150, 214, 215].

R-pyocins have gained interest as anti-pseudomonal agents because of their potent ability to kill target cells, their inability to replicate without a DNA-containing head, and their thoroughly described mechanism of action against target cells [149, 190, 215, 234, 235]. In this thesis, I focus on R-pyocins due to their promising characteristics as antimicrobial agents along with the need for a better understanding of their activity in infection.

1.4. R-pyocins of *P. aeruginosa*

1.4.1. The Structure of R-pyocins

R-pyocins are contractile antimicrobial particles similar to *Myoviridae* phage tails, that lack the DNA-containing capsid of typical, replicating phages [150, 189, 190, 215, 217-219, 230, 234-247]. R-pyocin particles consist of a polymer tube core surrounded by a contractile sheath; a pointed tail-spike is attached to the core along with a baseplate and tail fibres [149, 217, 218, 234-236, 238, 242, 244, 245]. The tail fibres serve to recognize and bind to the receptor(s) on target cells, and are thus the determinant of killing spectra. As the C-terminal sequences of the tail fibers, corresponding to a “foot” of the fiber, differ among strains, there have been five subtypes of R-pyocin described to date denoted R1-5 [150, 184, 190, 215, 223, 230, 234, 238, 244, 248-259]. Types R2-4 have a similar amino

acid sequence and overlapping killing spectra and so are often categorized as a single, broad subtype under the R2 type [149, 185, 190, 228, 229, 256, 258, 260].

As narrow-spectrum, phage-like particles, R-pyocins also have specificity and the capability to target specific bacterial species in infection [140, 149, 176, 183-185, 211, 259, 261]. While they can be effectively used to kill target species without modification, they have also been successfully engineered to broaden their spectrum of killing activity against other *P. aeruginosa* strains and even other pathogenic bacterial species [149, 183-185, 261]. Given the similarities to phage and the promise of phage therapy, these PTLBs have been generally overlooked as novel therapeutic agents for use against *P. aeruginosa* in infections and yet, R-pyocins have a number of potential benefits as a therapeutic that are distinct from phage. R-pyocins tend to be highly specific to a particular species of bacteria, and therefore, in a clinical setting there is a low risk of undesirable, non-specific killing of other species [149, 150, 183-185, 258, 261]. As R-pyocins lack a DNA-containing capsid, they do not replicate like phage [149, 150, 190, 217, 218, 230, 234-237, 244, 255]. Hence, they may be used in doses, allowing for more precise and controlled treatment regimens than with self-replicating phage.

Since the 1960s R-pyocin typing has been used as an epidemiological method of strain-typing *P. aeruginosa* in infections in clinical settings, including tracing strains from CF lung infections [210, 262-279]. Rapid diagnostics of strain-type using R-pyocins and quickly determining which R-pyocin type (or combination of types) could be used for treatment, would likely be more efficient and specific than determining which phage to use.

1.4.2. Lipopolysaccharide (LPS) as the Receptor of R-pyocins

R-pyocins function as antimicrobials by recognizing a specific residue of the LPS on target bacteria, contracting, and puncturing the outer membrane [165, 184, 223, 230, 250, 251, 253, 256-259]. Various monosaccharide residues on the outer core of the LPS have been attributed to serving as the receptor for each of the subtypes of R-pyocin (Figure 1.4) [256, 257]. The proposed mode of action is that the foot of the tail fibre binds to a receptor on the LPS, and the sheath contracts, pushing the tail spike and core to puncture the outer membrane of a target cell, leading to inhibition of active transport and membrane depolarization before eventual death [149, 234, 235, 242, 256]. It is important to note that many bacteriocins, including S-pyocins, are accompanied by cognate immunity proteins while only LPS composition and packing density determine resistance to R-pyocin mediated killing.

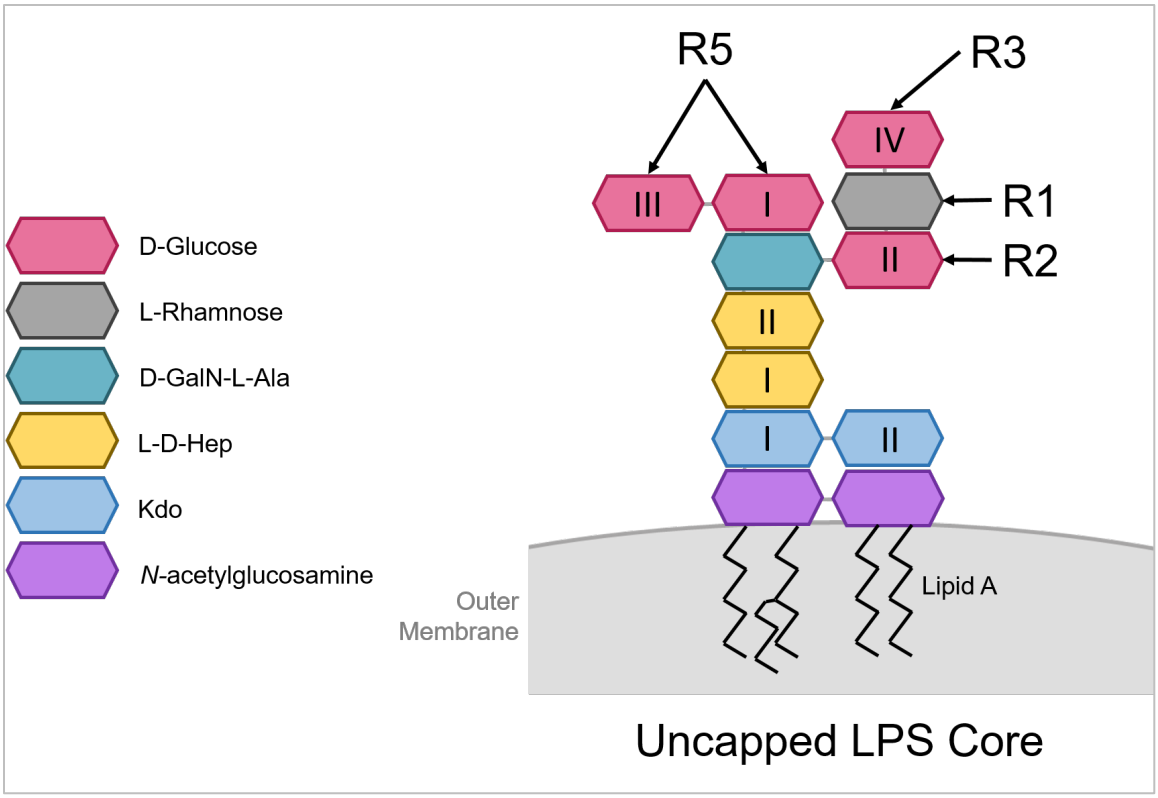

Figure 1.3: Lipopolysaccharide core residues are receptors for each of the subtypes of R-pyocin. The outer core of an uncapped LPS of *P. aeruginosa* exposing D-glucose (Glc^{I}-Glc^{IV}) residues, the receptors recognized by each of the five subtypes of R-pyocin. The inner core consists of two residues of 3-deoxy-α-D-manno-octulosonic acid (Kdo), and two residues of L-*glycero*-D-*manno*-heptose (L-D-Hep). The outer core consists of one residue of D-galactosamine (GalN) with alanyl (Ala) group (D-GalN-L-Ala), one L-Rhamnose, and three to four D-glucose residues.

1.4.3. R-pyocin Regulation

Similar to F-pyocins and prophages, R-pyocins are located on the *P. aeruginosa* chromosome and are induced by the SOS response via DNA-damaging agents [162, 165, 207, 231-233, 280-282]. These agents include UV radiation, mitomycin C, ciprofloxacin,

and oxidative stress [162, 165, 207, 231-233, 280-282]. Several transcription factors of *P. aeruginosa* have found to play a role in regulating R-pyocin production and assembly under SOS conditions, including PrtR, PrtN, and an unknown RecA*-induced factor. The current model predicts that following detection of DNA damage, activated RecA (RecA*) cleaves the negative repressor PrtR, allowing PrtN to activate expression of R-pyocin genes (Figure 1.5) [207, 233, 281, 282].

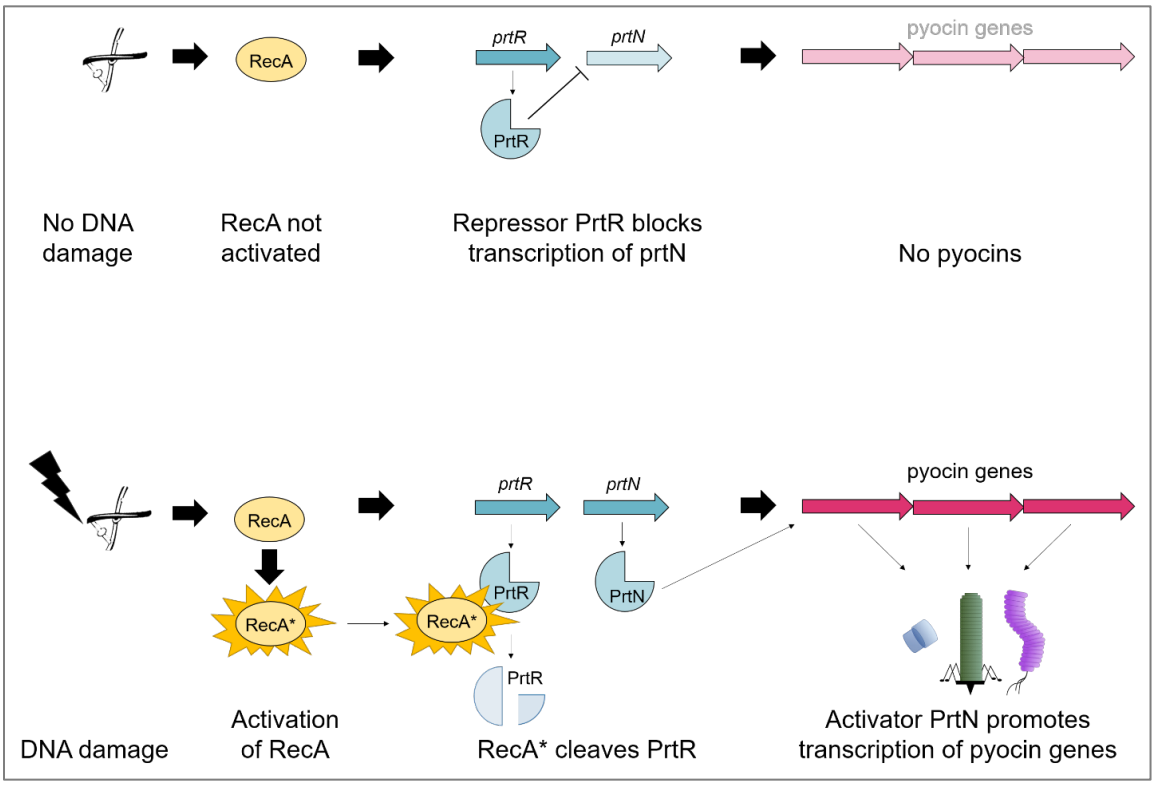

Figure 1.5: Schematic of RecA-dependent R-pyocin regulatory model. Following the detection of DNA damage, activated RecA becomes RecA* and cleaves the negative repressor PrtR. The cleavage of PrtR allows PrtN to activate expression of R-pyocin genes

As previously mentioned, R-pyocin genes are found in a single location on the chromosome as an operon and are directly upstream of F-pyocin genes [149, 150, 187, 207, 215, 220, 221, 225, 228, 229, 231, 232, 234, 235, 255]. The R-pyocin gene cassette consists of a set of genes encoding structural proteins encased by lytic genes, preceded by the regulatory genes PrtR and PrtN [220, 222, 225, 228, 233]. A second regulatory model has been proposed for the induction of R-pyocins that is RecA-independent, mediated by the deficiency of the tyrosine recombinase XerC, but the specific mechanisms of this regulation are not yet well understood (not shown) [283].

Similar to phages, the holin and lysin of the R-pyocin gene cassette are used to release the particles by cell lysis; however, as it does not benefit the producing cells for the entire population to lyse, it is observed that only a subpopulation of cells produce and lyse to release R-pyocins [149, 166, 167, 283, 284]. As within-population R-pyocin regulation and release has not been studied in detail, it is unknown when or how the lysing subpopulation is determined. There have been no signal sequences identified that would facilitate secretion of R-pyocins via any of the known secretion systems *P. aeruginosa* employs – thus lysis is currently the only known mechanism of R-pyocin release.

Temporal regulation of bacteriophages involves early stage (phage particle assembly) and late stage (host lysis and release) promoters that dictate the phage "life-history" [285, 286]. Similar to phage, the holin and lysin of the R-pyocin lysis cassette are used to release the particles by cell lysis [167]. Several promoter regions upstream of the R-pyocin structural genes have been putatively identified, and there is some evidence supporting their involvement in R-pyocin activity though there has been little work to further define these regulatory regions or their specific functions in R-pyocin activity [207,

215]. It is unknown if these promoters fire in synchrony, or if some are specifically responsible for directing R-pyocin assembly (early stage) and release (late stage) similar to the regulation of phage assembly. Although the lysis gene cassette associated with R-pyocins has been identified (PA0614; PA0629-PA0632), only the pore-forming endolysin (holin, PA0614) and murein hydrolase (lysin, PA0629) proteins have been proposed for their homology to known phage counterparts [167, 207, 215]. Several genes in this lysis cassette are homologous to late-stage regulatory phage genes but have yet to be characterized for their role in R-pyocin release. To understand the role R-pyocins play in shaping *P. aeruginosa* populations during infection, and assess their efficacy as a future therapeutic, it is imperative to understand their regulation, timing and release mechanisms.

1.4.4. R-pyocin Activity in Infection

Little is known regarding the activity of R-pyocins *in vivo*, and there have currently been no studies published with the intention to evaluate R-pyocin transcriptomic or proteomic behavior during infection. A survey of 15 published CF sputum transcriptomes found that 9 of these samples (60%) do not contain any detectable reads for R-pyocin structural genes (PA0615-PA0628), yet all exhibit some reads for the R-pyocin-associated lytic genes (PA0614, PA0629-PA032) and the adjacent regulatory genes (PA0612-PA0613) [287, 288]. In one proteomic study analyzing 35 CF sputum collections from 11 individuals, the R-pyocin sheath protein (PA0622) was identified with a Fold Change of 2-fold or greater in only 3 of these sputum samples [289]. These data suggest that few individuals in this cohort were infected with *P. aeruginosa* strains actively producing R-pyocins and that in the sputum of the individuals with *P. aeruginosa* that did produce R-pyocins, R-pyocin activity was induced [289].

When reviewing published transcriptomic reads from *in vitro* sampling in Synthetic Cystic Fibrosis Sputum Media (SCFM) with the wild type laboratory strain PA14, all samples produced reads for the R-pyocin-associated regulatory genes, lytic genes, and structural genes (PA0612-32) [287, 288]. Most of these genes show some degree of upregulation in the artifical CF sputum environment, suggesting that the nutritional environment of the CF lung may induce or upregulate R-pyocin production [287, 288, 290]. However, more sampling and genomic data is necessary to better understand whether or sputum samples lacking R-pyocin RNA are actually missing the R-pyocin genes, or if the strains have merely evolved regulatory changes to downregulate R-pyocins in the CF lung infection environment.

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SPECIFIC AIMS OF THESIS

P. aeruginosa is a Gram-negative opportunistic pathogen and a major determinant of declining lung function in individuals with CF. *P. aeruginosa* possesses many intrinsic antibiotic resistance mechanisms and isolates from chronic CF lung infections also tend to develop increasing resistance to multiple antibiotics over time - making infection with *P. aeruginosa* even more difficult to eradicate. As chronic infection with *P. aeruginosa* remains one of the main causes of morbidity and mortality in individuals with CF and other chronic infections, new therapeutic interventions are 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 for *P. aeruginosa* infection in combination with antibiotics, or as an alternative to phage therapy. R-pyocins vary by subtype (R1-R5), distinguished by binding to different residues on the lipopolysaccharide (LPS). Each type varies in killing spectrum, and each strain produces only one R-type, suggesting a degree of strain-specificity. *P. aeruginosa* populations in CF chronic lung infection become phenotypically and genotypically diverse over time, however, little is known of the efficacy of R-pyocins against heterogeneous populations. If R-pyocins are to be considered as a therapy, it is important to dive further into investigating the efficacy of R-pyocins against heterogenous clinical strains of *P. aeruginosa* as the frequency of R-pyocin-susceptible strains in CF chronic lung infections is also currently unknown.

Similar to prophages, R-pyocin genes are also located on the *P. aeruginosa* chromosome and are induced by the SOS response. It is likely that the chronic infection

environment, such as CF sputum, exposes the cells to a number of stress-inducing stimuli, including hydrogen peroxide (from host immune response) and ciprofloxacin (antibiotic), however the regulation of R-pyocins and the resulting lysis to facilitate their release is not well understood during infection. Furthermore, cells in aggregated biofilms exhibit higher tolerance to antimicrobials than in planktonic growth, yet it is not known how cells in a spatially structured, CF-relevant environment may impact the efficacy of R-pyocins, phage and other phage-like particles. Understanding this regulation and the resulting lysis is important for understanding how R-pyocins and other phage-like particles may already be affecting *P. aeruginosa* growth dynamics, strain competition, and biofilm development in infection, which could have a profound impact on the efficacy of their use as a therapeutic.

The scientific premise of this work is that R-pyocins have the potential to be useful antimicrobials in chronic infections and in CF therapy; however their regulation, release, and binding mechanisms in an infection-relevant context remain poorly understood. The goal of this work is to improve understanding of R-pyocins as novel, alternative therapies by i) assessing the impact of heterogeneity on R-pyocin susceptibility among CF isolates of *P. aeruginosa*, ii) estimating the frequency of CF strains that may be susceptible to R2-pyocins, and iii) determining how R-pyocins are regulated and influence strain competition in a nutritionally and spatially infection-relevant environment (SCFM). In this work, I tested the following hypotheses: that (i) in diverse populations of *P. aeruginosa* derived from CF chronic lung infections, there will be heterogeneity in susceptibility to R-pyocins; ii) there will be a high number of CF strains with LPS mutations conferring susceptibility to R2-pyocins, supporting R2-pyocin efficacy as an antimicrobial; and iii) R-pyocins are upregulated in the CF lung nutritional and spatially structured environment, and present a

greater fitness cost to producing strains due to spatial restraints and the required lysis to release them. I tested these hypotheses by the following aims:

Aim 1. Evaluate the intra-strain differential susceptibility to R2-pyocins. I will examine the diversity in R-pyocin susceptibility within single populations of *P. aeruginosa* isolated from chronic CF lung infections. Using up to 10 isolates from each CF sputum sample, I will R-pyocin genotype each population. I will then use 20 isolates from three heterogenous populations, isolated from three separate patients, test R2-pyocin susceptibility, and compare diversity among susceptibility profiles within each population and across all three populations.

Aim 2. Estimate the frequency of CF strains that may be susceptible to R2-pyocins. Using the same CF sputum samples, I will test up to 10 isolates per population for 12 populations (110 isolates total) for susceptibility to R2-pyocins. I will then isolate and characterize LPS phenotype profiles for each of the isolates to assess correlations between LPS phenotypes and R2-pyocin susceptibility, before providing an estimate of R2-susceptible strains based on my findings in our CF biobank of sputum collections.

Aim 3. Quantify the impact of R-pyocin regulation on strains in an infection-relevant environment (SCFM). I will generate transcriptional reporters of various R-pyocin putative promoter regions and compare R-pyocin regulatory activity in standard laboratory media to activity in CF-relevant media (SCFM without DNA and mucin). I will then grow *P. aeruginosa* in pairwise competition between an R2-producing, resistant strain (wt

PAO1) and a non-producing, R2-susceptible strain (PAO1 Δ*rmlC* ΔR) to assess the impact of R-pyocin activity and induction on strain fitness in the spatially structured, CF-relevant environment (SCFM with DNA).

The overall objective of this thesis is to further illuminate the mechanisms of R-pyocins regulation, release, and susceptibility in an infection-relevant context to better understand the role R-pyocins play in shaping *P. aeruginosa* populations during infection and assess their efficacy as a therapeutic.

CHAPTER 2: HETEROGENOUS SUSCEPTIBILITY TO R-PYOCINS IN POPULATIONS OF *PSEUDOMONAS AERUGINOSA* SOURCED FROM CYSTIC FIBROSIS LUNGS

Reproduced in part from Mei, M., J. Thomas, and S.P. Diggle, *Heterogenous Susceptibility to R-Pyocins in Populations of Pseudomonas aeruginosa Sourced from Cystic Fibrosis Lungs.* mBio, 2021. **12**(3).

2.1. Abstract

Bacteriocins are proteinaceous antimicrobials produced by bacteria, which are active against other strains of the same species. R-type pyocins are phage tail-like bacteriocins produced by *P. aeruginosa*. Due to their anti-pseudomonal activity, R-pyocins have potential as therapeutics in infection. *P. aeruginosa* is a Gram-negative opportunistic pathogen and is particularly problematic for individuals with CF. *P. aeruginosa* from CF lung infections develop increasing resistance to antibiotics, making new treatment approaches essential. *P. aeruginosa* populations become phenotypically and genotypically diverse during infection, however, little is known of the efficacy of R-pyocins against heterogeneous populations. R-pyocins vary by subtype (R1-R5), distinguished by binding to different residues on the LPS. Each type varies in killing spectrum, and each strain produces only one R-type. To evaluate the prevalence of different R-types, we screened *P. aeruginosa* strains from the International Pseudomonas Consortium Database (IPCD) and from our biobank of CF strains. We found that (i) R1-types were the most prevalent R-type among strains from respiratory sources; (ii) there is a large number of strains lacking R-pyocin genes, and (iii) isolates collected from the same patient have the same R-type. We then assessed the impact of intra-strain diversity on R-pyocin susceptibility and found a heterogenous response to R-pyocins within populations, likely due to differences in the LPS core. Our work reveals that heterogeneous populations of microbes exhibit variable susceptibility to R-pyocins and highlights that there is likely heterogeneity in response to other types of LPS-binding antimicrobials, including phage.

2.2. Introduction

Pyocins are narrow-spectrum antimicrobials, specifically produced by *Pseudomonas aeruginosa*, that have antimicrobial activity against members of the same species [1-4]. *P. aeruginosa* produces three types of pyocin, referred to as S-pyocins, F-pyocins, and R-pyocins [1-3]. The focus of this chapter is R-pyocins, which are narrow-spectrum, phage-tail-like bacteriocins that vary by subtype (types R1-R5) [2, 3, 5-11]. Each *P. aeruginosa* strain uniquely produces only one of these R-pyocin types [2, 3]. As the variable C-terminus region of the tail fibres of sub-types R2-R4 are approximately 98% similar in amino acid sequence, they are often grouped together under the R2 subtype [12-14]. R-pyocin subtype and specificity are conferred by differences in the "foot" of the tail fibre (Figure 2.1), which is believed to bind to specific residues on the LPS decorating the outer membrane of Gram-negative bacteria [12-26]. A strain may be susceptible or resistant to any variation of the different R-types [2, 3, 14, 25, 27-29].

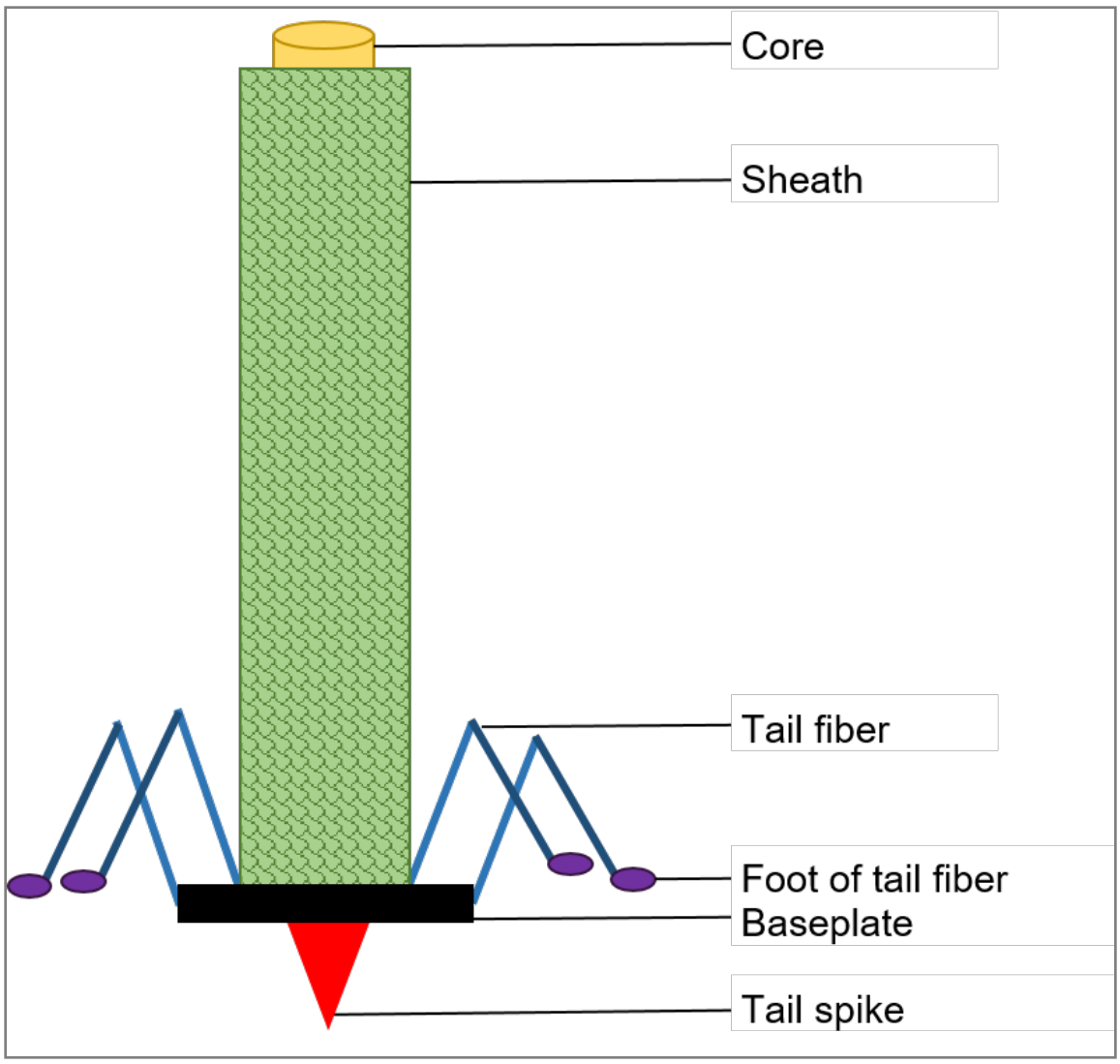

Figure 2.1: R-pyocin structure. R-type specificity is determined by the sequence on the foot of the tail fibre, which is believed to recognize glycosylated structures on the outer membrane (such as the LPS). Upon binding, the sheath contracts to push the core into the cell membrane. Puncture by the tail spike depolarizes the membrane potential, leading to cell death [1-3, 14, 15, 22-27, 29-36]. R-pyocin structure is depicted as described in previous studies [2, 3, 11, 14-21, 27, 29-31, 36, 37].

2.2.1. R-pyocin Structure and Mode of Action

R-pyocins are similar to phage tails of the Myoviridae phage family, both in structure and in killing mechanism [2, 3, 14-17, 24, 29]. The proposed mode of action is through the foot of the R-pyocin tail fibre recognizing a receptor on the LPS; binding to the target cell triggers the sheath to contract, pushing the tail spike and core through the cell envelope [1-3, 14, 15, 22-27, 29-36]. The puncture causes cytoplasmic membrane depolarization, inhibiting active transport and eventually leading to cell death [32, 33, 36-38]. Several monosaccharide residues on the outer core of the LPS have been proposed to confer susceptibility and specificity to some of the R-pyocin types [24, 25], however, only the receptor of the R3-type pyocin has been clearly defined to be the GlcIV terminal glucose on the outer core of the LPS [26]. R-pyocins have previously been explored as potential therapeutics [12, 13, 39, 40], but little is known about their binding to target cells, or their killing efficacy in heterogeneous *P. aeruginosa* populations.

2.2.2. Heterogeneity and AMR of *P. aeruginosa*

P. aeruginosa is found in a number of human infections and is the leading cause of morbidity and mortality in individuals with CF. Once established in the CF lung, single-strain populations become heterogeneous - evolving genotypic and phenotypic diversity during the course of infection [41, 42]. *P. aeruginosa* possesses many intrinsic antibiotic resistance mechanisms, and isolates from CF lung infections are known to become increasingly resistant to multiple antibiotics over time [4, 43-50]. It is also known that isolates sourced from clinical populations of *P. aeruginosa* can exhibit considerable variation in susceptibility to antibiotics [48, 51-55], however heterogeneity in R-pyocin susceptibility, or heterogeneity in susceptibility to other types of bacteriocins have not been explored at the population level.

2.3. Results

2.3.1. R1 Genotype is the Most Prevalent R-pyocin Type in Clinical *P. aeruginosa* Strains

To determine the prevalence of different R-pyocin subtypes in *P. aeruginosa* strains sourced from CF infections, we conducted an *in silico* screen to determine R-pyocin genotypes of *P. aeruginosa* strains publicly available through the International *Pseudomonas* Consortium Database (IPCD) [56]. The IPCD is a repository of *P. aeruginosa* strains (single isolates) collected from a variety of sources (with an emphasis on CF), created to facilitate metadata analyses specifically to improve prognostic approaches for CF treatment [56]. By using the BLAST algorithm from NCBI, we screened 852 strains from the database for nucleotide sequence homology to R1-, R2-, or R5-pyocin tail fibre genes. As subtypes R2-R4 are highly similar, we considered these three subtypes as one larger R2-subtype for this analysis [12-15]. Out of the 852 strains in the database, we could not conclusively type 303 strains. We then further screened the 303 untypeable strains for the presence or absence of genes flanking the R-pyocin gene cassette; these include *trpE* (PA0609), regulatory genes and holin (PA0610-PA0614), lytic genes (PA0629-PA0631), F-pyocin structural genes (PA0633-PA0648) and TrpG (PA0649). While all 303 strains contained *trpE* and *trpG*, regulatory genes (PA0610-PA0613), holin, and lytic genes, 297 strains were found to lack R-pyocin structural genes entirely. The six strains with intact and complete R-pyocin structural genes were confirmed to possess a full R-pyocin tail fibre gene, though it was not homologous to the known types. In addition, 300 of the untypeable strains still maintained some degree of F-pyocin structural genes. It

is important to note that typing alone does not have implications on whether a strain produces functional R-pyocins or not.

Of the 852 strains in the database, we categorized 448 strains as isolated from human "respiratory" sources; these include *P. aeruginosa* isolated from throat, CF, bronchiectasis, sputum, sinus, and nasopharynx. Among the 448 respiratory strains, 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, with only two of these strains possessing R-pyocin structural genes. Supplementary Figure 2.1 depicts the distribution of R-types for each source. Due to the curation and labelling of the strain information in the database, we were not able to precisely distinguish all CF strains from strains isolated from other respiratory infections; however, it is likely that many of the samples labelled 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 strains shows that (i) R1-pyocin producers are likely more prevalent in CF than the other R-pyocin subtypes (Figure 2.2A) and (ii) a large proportion of strains lack R-pyocin structural genes entirely, though they maintain the flanking regulatory elements.

2.3.2. R-Pyocin Type Does Not Vary within Clonal CF Populations 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 fresh CF sputum samples of patients with chronic lung infections. In this work, we use the term "population" to describe a collection of bacteria (*P. aeruginosa*) isolated from

the expectorated sputum of a CF patient at a specific time point. Up to 10 isolates were chosen at random to R-pyocin type for each population, from each patient. Using multiplex PCR (Supplementary Table 2.1), we R-pyocin typed isolates from 19 of these populations (183 isolates total) collected from 8 different patients over a 2 year period. In our cohort, the R1 genotype was the most prevalent R-pyocin type found among CF patients. 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 (Figure 2.2B). It appeared that in the period sampled (over 2 years) there were no strain switching events within these patients. We did not find any populations of the R5-pyocin type in our biobank.

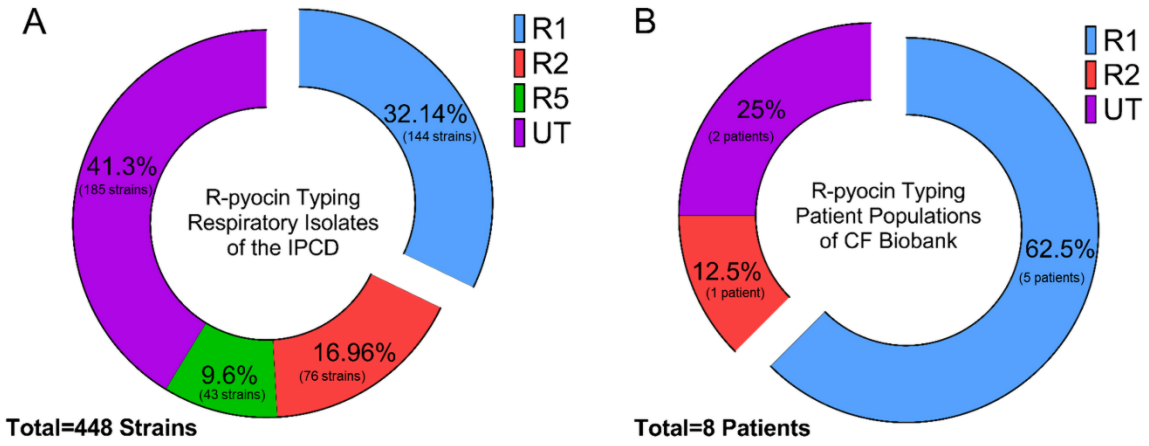

Figure 2.2: R-pyocin typing of clinical *P. aeruginosa* strains and whole populations isolated from CF patients. (A) Using BLAST homology-based alignment, we R-pyocin-typed publicly available *P. aeruginosa* sequences from respiratory sources of the IPCD to determine the prevalence of each pyocin type. 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 R-pyocin genotype).

2.3.3. R-Pyocin-Mediated Killing Varies among Heterogeneous *P. aeruginosa* Populations

Previous work has shown that isolates from within the same population have diverse antibiotic resistance profiles [48], but diversity in R-pyocin susceptibility has yet to be evaluated within whole populations of *P. aeruginosa*. We chose 20 random isolates from three *P. aeruginosa* populations from our biobank (each from a different CF patient), that exhibited diversity in growth rate (Figure 2.3A) and morphology (Figure 2.3B). Populations from Patient 1 and Patient 2 were untypeable (UT) and did not exhibit R-

pyocin killing activity (Supplementary Figure 2.3), while the population from Patient 3 was determined to consist of R1-pyocin producers, and did exhibit R-pyocin activity. Preliminary experimentation suggested that these populations each exhibited some susceptibility to R2-pyocins, regardless of their own R-pyocin genotype. 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 (required for R-pyocin release by forming pores in the host cell's membrane) or lysin (murein hydrolase that degrades peptidoglycan through pores created by holin; found in an operon of several lytic genes) are not responsible for the bactericidal activity against other strains, we included lysates extracted from an isogenic PAO1 R-pyocin null mutant lacking the tail fibre gene and chaperone (PAO1 ΔR). This strain has an intact lysis cassette, holin genes, and Pf4 prophage, however filamentous prophage has previously been shown to be deactivated by chloroform [57-59], which was used during the R-pyocin isolation process to lyse the cells.

By comparing growth (optical density) of *P. aeruginosa* isolates with and without R2-pyocins added to liquid culture, we found that across patients, each population as a whole exhibited different levels of susceptibility. We also found that within each population, there was heterogeneity in susceptibility among the isolates (Figure 2.3C). In *P. aeruginosa* populations sourced from Patient 1 and Patient 2, all 20 isolates were 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 demonstrates 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.

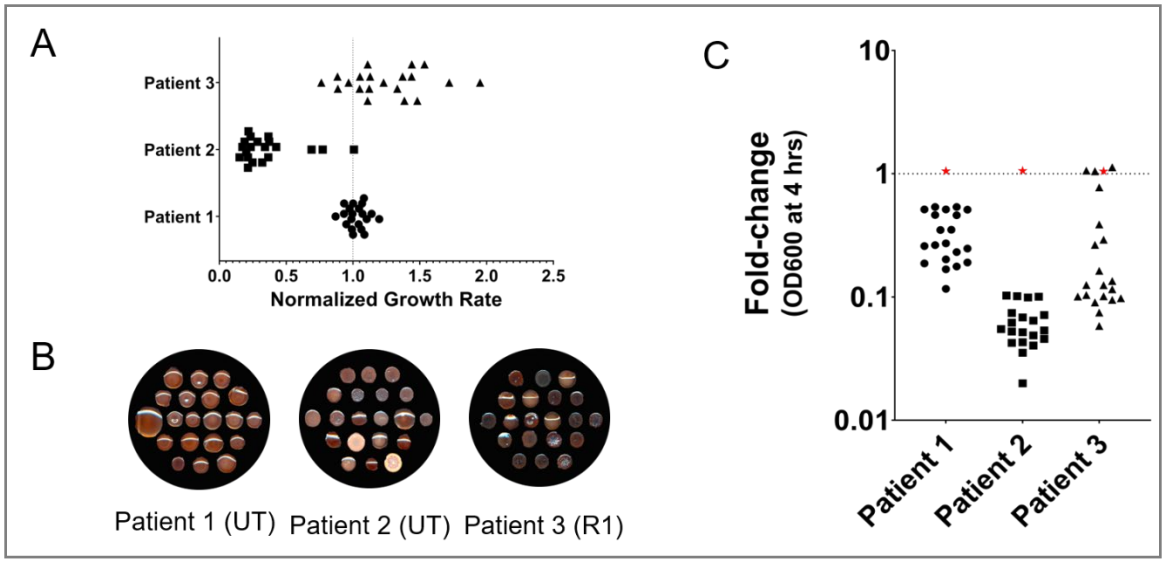

Figure 2.3: Diverse *P. aeruginosa* populations from CF exhibit heterogeneity in growth, morphology, and R2-pyocin susceptibility. (A) Growth rates vary between 20 isolates each from 3 populations collected from expectorated CF sputum. Growth rate was calculated from optical density (at 600nm) after 16 h 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. The populations of Patients 1 and 2 were untypeable (UT) by classical R-pyocin typing methods, however the population of Patient 3 were typed as R1-producers. (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. PAO1 is denoted as a red star, and was measured with each population as a control for R2-pyocin resistance. A fold-change of 1 indicates resistance to R2-pyocin whereas a fold-change less than 1 indicates R2-pyocin susceptibility. UT = Untyped R-pyocin genotype.

2.3.4. There is Significant Heterogeneity in R-Pyocin Susceptibility within a *P. aeruginosa* Population.

To further distinguish 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 from several R2-pyocin producing strains by visualizing killing activity with a standard, soft-agar overlay spot assay (Figure 2.4A). We used R2-pyocin containing lysates from the standard laboratory strain PAO1 and a PAO1 R-pyocin null mutant lacking a functional R-pyocin (PAO1 ΔR), as well as a previously described CF isolate A018 (also a R2-pyocin producer) and a corresponding R-pyocin null mutant [25, 60, 61]. The spot assay indicated 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) (Figure 2.4A).

We next measured R2-pyocin susceptibility of the three isolates with lysates from PAO1 in liquid culture, and measured Log2 fold-change of colony forming units (CFU) after 4 h of growth to compare cell density with and without R-pyocin-containing lysates. Through this assay, we confirmed that Isolates 2 and 3 from Patient 3 are susceptible to R2-pyocins, while Isolate 1 is resistant (Figure 2.4B). We confirmed in both planktonic growth and in an agar overlay that within a single population, 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.

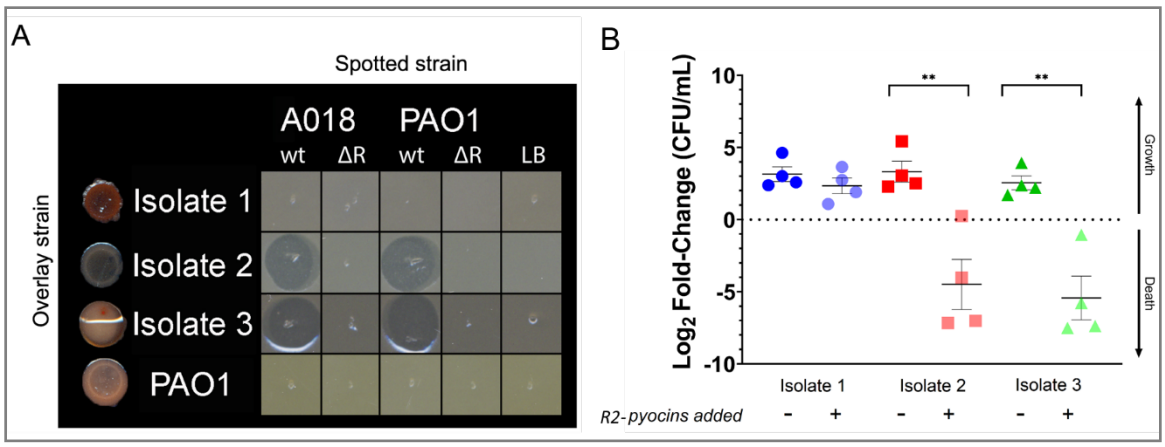

Figure 2.4: Clinical CF isolates of *P. aeruginosa* from the same population exhibit differential susceptibility to R2-pyocins. (A) Three R1-type pyocin producing clinical CF isolates from the same population (Patient 3) were tested for susceptibility to spotted lysates with a standard spot assay, and show differences in susceptibility to R2-pyocin lysates. Isolates 2 and 3 show zones of inhibition, indicating susceptibility. Spotted strains include the previously described CF strain A018 (R2), the laboratory strain PAO1 (R2), and a cell-free media preparation. (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 h for each isolate to depict growth or death in the presence or absence of R2-pyocins. 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).

2.3.5. R-Pyocin Susceptibility of Isolates is Dependent on the LPS Core Structure and Not Alginate Production

Alginate is a type of exopolysaccharide that some *P. aeruginosa* cells secrete, giving them a characteristic mucoid morphology during growth; it contributes to a more robust biofilm and has been shown to be associated with strains that are more tolerant to antibiotics [62-64]. We tested alginate production to determine whether alginate may

provide resistance to R-pyocin killing or influence R-pyocin susceptibility in any way among isolates. As there was diversity in colony morphology and mucoidy among the *P. aeruginosa* population of Patient 3 (Figure 2.5B), we quantified the alginate production of Isolates 1-3 from Patient 3 to determine if alginate production may influence the susceptibility differences among these isolates. We found that only Isolate 3 (R2-susceptible) produced alginate while Isolate 2 (R2-susceptible) and Isolate 1 (R2-resistant) did not produce alginate (Figure 2.5A), suggesting that alginate production did not influence R2-pyocin susceptibility between the three isolates.

As LPS has been shown to be involved in the binding of R-pyocins to target cells [1-3, 12, 14, 15, 22-27, 29-33, 65], we further characterized Isolates 1-3 from Patient 3 by extracting and visualizing major LPS components via 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 (Figure 2.5B). Along with an altered LPS core, Isolate 1 lacks both the A-band and B-band. We found that Isolate 2 lacks 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* [66-75].

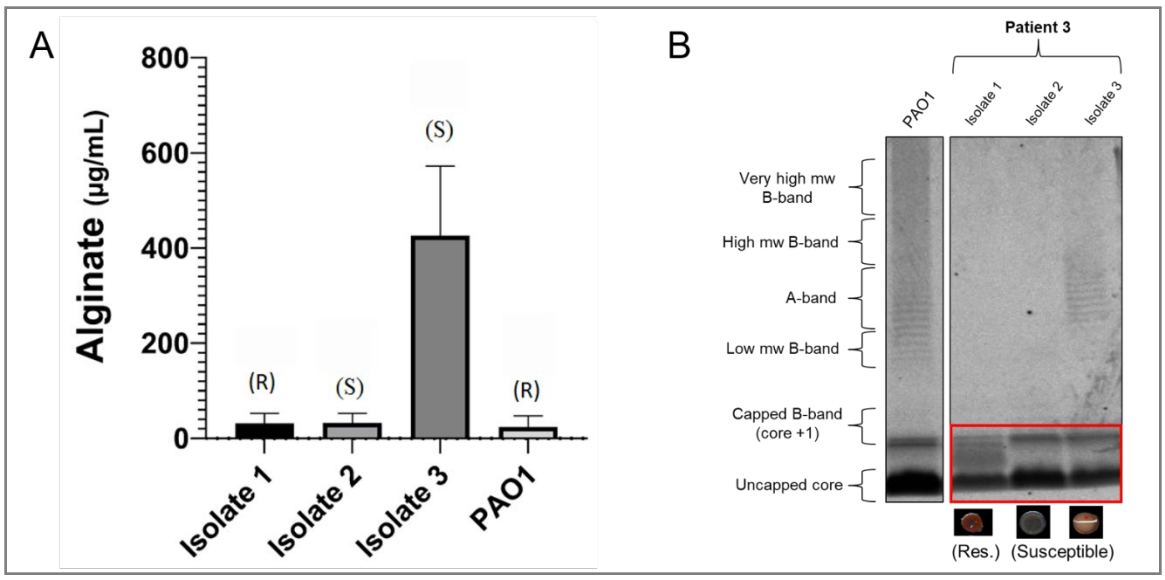

Figure 2.5: Alginate production and LPS characterization of three CF isolates of *P. aeruginosa* from the same population. (A) Alginate was isolated and quantified from clinical Isolates 1-3 (of Patient 3). Susceptibility to R2-pyocins is denoted by R (resistance) or S (susceptible). Alginate concentrations measured from three biological replicates are represented by the mean value and error bars depict standard error of the mean (SEM). The means of alginate concentration from each isolate were determined by comparison to a standard curve generated and analyzed with a one-way ANOVA and Tukey-Kramer post-hoc test. Isolate 3 exhibited a mucoid morphology and produces more alginate in comparison ($P<0.025$) though it is still susceptible to R2-pyocins, while Isolates 1 and 2 did not produce detectable levels of alginate (<50 µg/mL) and differ in R2-pyocin susceptibility. (B) The three isolates each exhibit different LPS phenotypes; Isolate 1 presents neither A-band nor B-band, and has a different core from PAO1. Isolate 2 also does not present A-band or B-band, but has a similar core to Isolate 3 and PAO1. Isolate 3 presents a normal core, and presents A-band antigen.

2.3.6. Whole Genome Sequencing of Isolates Reveals Possible Single Nucleotide Variants Responsible for R-Pyocin Resistance

We obtained whole genome sequences of Isolates 1-3 from Patient 3 to determine the strain type and serotype. We found that all three isolates are of the 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 R-pyocin susceptibility. We found that all three isolates shared 30,127 single nucleotide variants (SNVs) when mapped against a PAO1 reference genome (Figure 2.6A). 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 (Figure 2.6B). Of the 232 SNVs unique to Isolate 1, 169 SNVs were non-synonymous.

Given the high number of SNVs and stark diversity between the isolates, through genetic analysis we were 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. Notable candidate SNVs unique to Isolate 1 included a C151T (Arg51Trp) missense variant at position 833604 in the *mucC* gene (PA0765), a G710A (Arg237His) missense variant at position 1199237 of the *roeA* gene (PA1107), and a T1169C (Leu390Pro) missense variant at position 3968193 in the *algK* gene (PA3543). Each of these genes are involved in alginate production or polysaccharide biosynthesis but may indirectly influence LPS biosynthesis or presentation. However, when we complemented each of these genes (using the gene sequences from PAO1) into Isolate 1, there were no differences to R2-pyocin susceptibility; the complemented strains of Isolate 1 remained resistant to R2-pyocins (Supplementary Figure 2.5) and did not alter LPS presentation (Supplementary Figure 2.5).

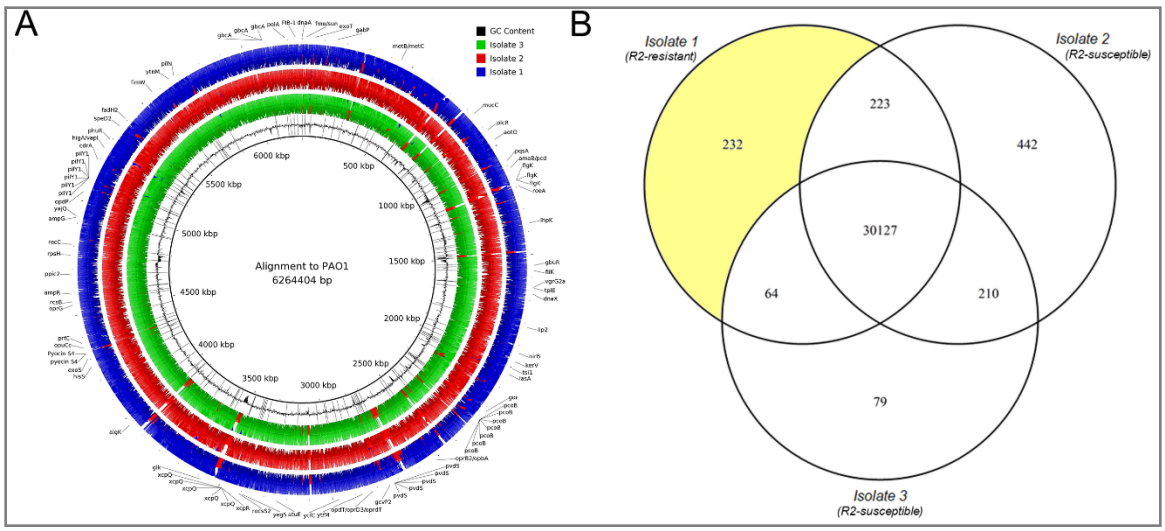

Figure 2.6: Whole-genome analysis of three CF isolates of *P. aeruginosa* from the same population. (A) Three R1-type pyocin producing clinical CF isolates from the same population were sequenced and aligned to a PAO1 reference genome for comparison. Only annotated single nucleotide variants (SNVs) unique 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, 232 were unique to Isolate 1 when compared to Isolates 2 and 3 from the same population.

2.4. 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 [4, 41-52, 76-81], making the development of new and alternative therapies critical. Bacteriocins have long been considered in food preservatives as bio-controlling agents and also as therapeutics [82-88]. R-pyocins have potential as an anti-pseudomonal therapeutic, but little is known about their antimicrobial efficacy against the

heterogeneous *P. aeruginosa* populations found in CF lungs. We evaluated R-pyocin type and susceptibility among *P. aeruginosa* isolates sourced from CF infections and found that (i) R1-pyocins are the most prevalent R-type among respiratory infection and CF strains; (ii) a large proportion of *P. aeruginosa* strains lack R-pyocin genes entirely; (iii) isolates from *P. aeruginosa* populations collected from the same patient at a single time point have the same R-pyocin type; (iv) there is heterogeneity in susceptibility to R-pyocins within *P. aeruginosa* populations and (v) susceptibility is likely driven by diversity of LPS phenotypes within clinical populations.

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 biobank of CF isolates [56]. We found that R1-pyocin producers make up the majority of typeable CF strains from both sources. This suggests that many CF infections may initially be colonized with R1-producing ancestor strains, or that there is a possible benefit to producing R1-type pyocins during strain competition in early stages of infection. 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) [60]. We found a large number of strains from the IPCD that could not be typed by our screen using classical R-pyocin typing methods, the majority of which (98%) were missing the R-pyocin structural gene cassette. Another group evaluating R-pyocin susceptibility among 34 CF isolates found that 23 (68%) of the tested isolates did not produce R-pyocins or could not be typed [28]. Other work has also shown that CF isolates of *P. aeruginosa* exhibited loss of pyocin

production when compared to environmental strains [89]. This suggests that CF isolates of *P. aeruginosa* initially possessing R-pyocin genes may lose the ability to produce R-pyocins later in chronic infection, however further study is necessary to explore the potential fitness advantages of this evolutionary trajectory. This hypothesis is explored further in Chapter 4.

We are only just beginning to understand how heterogeneous *P. aeruginosa* populations impact upon treatment and disease outcomes. Previous studies have R-pyocin typed *P. aeruginosa* from CF patients and compared R-types from longitudinal collections, only evaluating single isolates from each patient [28, 60, 89]. The typing of single isolates, however, does not consider the genotypic and phenotypic diversity of *P. aeruginosa* populations, which evolve over the course of chronic infection [41-43, 51, 52, 76-81]. Out of the *P. aeruginosa* populations collected from eight CF patients we found primarily R1-producing or untypeable strains. We also found that all of the isolates in each population consistently exhibited the same R-pyocin genotype over a 2-year period. 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, suggesting that the observed changes in R-pyocin type were likely due to transient strains [28]. 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 [41, 48, 51, 53-55]. Though heterogeneity did not influence R-pyocin genotypes within our CF populations, 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* exhibited differential levels of susceptibility and (ii) within each population, there was heterogeneity in susceptibility among individual isolates.

Previous studies have shown the effect of various LPS mutations on R-pyocin susceptibility in laboratory strains PAO1 and PAK [24-26], however, to our knowledge, our work is the first to examine LPS phenotypes associated with R-pyocin susceptibility and resistance among clinical isolates. We found that differences in the core of the LPS appear to be correlated with the differences in susceptibility and resistance to R-pyocins among three isolates from one patient; specifically, that a resistant CF isolate produces an altered LPS core. Our findings are in agreement with recent work demonstrating heterogenous resistance mechanisms and persistence against tailocins killing by alterations of the LPS and associated glycosyltransferases in *Pseudomonas syringae* [90]. It is unknown if the altered LPS core phenotype we have found is commonly identified in CF *P. aeruginosa* populations, or if there is a fitness benefit it confers during infection. During initial colonization and strain establishment in the early stages of infection, the LPS (and therefore R-pyocin susceptibility) may play a role in antagonistic interactions between competing strains and ultimately determine the dominating strain.

While investigating possible mechanisms for the variable R2-pyocin susceptibility of Isolates 1-3 from Patient 3, we showed that serotype, strain type, and alginate production were not responsible for the resistance in Isolate 1. When we probed the genomes of three isolates from a single clinical population exhibiting heterogenous susceptibility to R2-pyocins, we identified several candidate SNVs in *mucC* (PA0765), *roeA* (PA1107), and *algK* (PA3543), unique to the resistant isolate; however ultimately these genes did not effect R2-pyocin resistance or LPS presentation when complemented. All three genes are

involved with the alginate production pathway or other extracellular polysaccharides [76, 91-93], known to be closely intertwined with various LPS synthesis and secretory pathways; thus, any combination or all three variants combined may play a role in indirectly impeding presentation of various portions of the LPS or core residues. As there are many regulatory mechanisms and biosynthetic genes still yet to be understood that are involved in LPS synthesis and presentation, it is also possible that there is a mechanism at play that has yet to be described.

Overall, our findings highlight that clinical populations of *P. aeruginosa* exhibit a heterogenous 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* [24, 94-100] and other types of LPS-specific antimicrobials, including R-pyocins, are being considered as treatments [12, 13, 39, 40, 101, 102]. 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, core phenotypes, and how they are evolving in *P. aeruginosa* populations during infection, to better understand the resulting impact on the potential efficacy of these antimicrobials.

2.5. Methods

2.5.1. 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 from our biobank, sourced from CF chronic lung infections, were studied for R-pyocin typing, and 60 of these isolates were chosen at random for further study (20 isolates from three different patients). All *P. aeruginosa* isolates, laboratory and indicator strains used in this work are listed and described in Supplementary Dataset 2 of the published work. All bacterial cultures were grown in lysogeny broth (LB) medium at 37°C with 200 rpm shaking agitation.

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 [103] 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 [104] 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 [105] 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.

Complemented strains of Isolate 1 from Patient 3 were constructed as follows: DNA sequences of each gene from PAO1 Nottingham strain (*mucC* PA0765; *algK* PA3543; *roeA* PA1107) were PCR amplified using Q5 DNA polymerase (New England Biolabs, Ipswich, MA). These sequences were cloned into the EcoRI-KpnI digested expression vector pME6032 [106] to be under inducible control of the *tac* promoter 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 10 µg/mL tetracycline. Cloned inserts were verified by colony PCR and Sanger sequencing (Eurofins Genomics, Louisville, KY). Expression constructs were introduced into Isolate 1 by electroporation [104] and strains carrying the complementation constructs were selected on LB agar plates supplemented with 200 µg/mL tetracycline. All plasmids and primers used for mutant generation can be found in Supplementary Table 2.1.

2.5.2. Assessing Colony Morphology Diversity in Clinical Isolates

To evaluate the diversity in colony morphologies between *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) [107]. Isolates were grown overnight (16-18 h) in LB medium at 37°C with 200 rpm 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.

2.5.3. R-Pyocin Typing Strains of the International *Pseudomonas* Consortium Database

Previously characterized *P. aeruginosa* strains were selected for query sequences for each R-type: PAK an R1, PAO1 an R2, and E429 an R5 [25]. As the R-pyocin tail fibre sequences of PAO1 (NP_249311.1) (112-114) and PAK (Y880_RS29810; accession GCA_000568855.2) [108, 109] are annotated, the corresponding nucleotide sequences were downloaded in FASTA format from NCBI. The E429 (R5) tail fibre sequence was identified from previous work [25] and downloaded from NCBI by using the PAO1 tail fibre sequence (PA0620) to BLAST against the E429 genome [56, 109, 110]. NCBI's BLAST+ was also used to determine percent identity between the whole tail fibre sequences of each strain/R-type pair-wise, in order to determine the variable region of the tail fibre gene to use for typing.

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 fibre gene that is most variable to the other types (R1). Specifically, the 800bp C-terminal sequence of each R-type of tail fibre 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 [56, 110]. 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 [110]. 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 [56]. 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.

2.5.4. 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) [111] in a previous study [60]. 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 [56]; this strain has been reported as an R5-type pyocin producer by other groups [25]. The R5 primers were input into the Sequence Manipulation Suite (https://www.bioinformatics.org/sms2/pcr_products.html) [112] to verify specificity to R5-pyocin producers and product size. R5-pyocin primers were designed using Benchling (www.benchling.com) [113] and ordered from Eurofins Genomics. R-pyocin typing primer details and product sizes are listed in Supplementary

Table 2.1. 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 [25, 56, 60, 114, 115]. 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°C 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 min. R-pyocin typing primer details and product sizes are listed in Supplementary Table 2.1

2.5.5. 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 [12]. LB cultures of PAO1 and PAO1 ΔR (R-pyocin null mutant) were inoculated at 1:100 from overnight planktonic cultures, and grown to mid-logarithmic growth phase (approximately 3 h) 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 [12], and cultures were incubated for a further 3 h. Chloroform was used to lyse remaining cells and inactivate filamentous prophage [57-59], and lysates were centrifuged at ~3,300 *x g* for 10 min. The R-pyocin-containing supernatant was separated and stored at 4°C. R-pyocin lysates were extracted on three separate occasions for biological replication.

2.5.6. 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_{600}) of 0.01. For complemented strains, 4μL of Chromomax isopropyl β-D-1-thiogalacto-pyranoside (IPTG)/X-Gal solution (Fisher Scientific) was added per 1 mL of soft agar for induction of gene expression. This mixture was poured onto LB agar plates for an overlay of the indicator strain. For Figure 2.4A, R2-pyocin lysates extracted from PAO1, A018, and respective R-pyocin null mutants and were vortexed before spotting 5 μL of each lysate onto the soft-top overlay indicator strains. For Supplementary Figure 2.3 lysates were collected from clinical isolates, were vortexed and serially diluted in LB 10-fold before spotting 5 μL of each dilution onto the soft-top overlay of previously described indicator strain A026 [60]. Spots were allowed to dry before 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.

2.5.7. Microtiter Plate Method for R-Pyocin Activity

R2-pyocin susceptibility was measured by changes optical density under conditions with and without added R2-pyocins, using a 96-well microtiter plate. Susceptibility to R2-pyocins was measured by fold-change of the optical density (OD_{600}) 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 h 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_{600} of 0.01, before 10 µL of R-pyocin lysates were added for a total volume of 200 µL 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_{600} was measured for each well every 20 min over a total of 16 h while incubating at 37°C with 200 rpm orbital shaking. Growth rates of isolates not treated with R-pyocins were calculated using Growthcurver in R [116, 117]. Microtiter plate assays for R-pyocin susceptibility were conducted in triplicate. CFUs were quantified at t=0 h and t=4 h by sampling 10 µL of each culture, serially diluting in phosphate buffered saline (PBS), and spotting on LB plates. Colonies were counted after 18 h of growth at 37°C. CFUs were determined for three biological replicates of each isolate.

2.5.8. Alginate Isolation and Quantification

Alginate was isolated and quantified by carbazole methods described previously [118, 119]. Single colonies of each isolate were used to inoculate 3 mL of LB and grown to mid-logarithmic phase at 37°C, 200 rpm. Using a BioTek Synergy H1, OD_{600} was measured and used to inoculate fresh overnight cultures of 20 mL of LB at an OD_{600} of 0.01. 10 mL of 0.85% sodium chloride (Fisher Scientific) was combined with 10 mL of overnight culture, vortexed, and centrifuged at ~3,300 *x g* for 30 min. Supernatant was removed and combined with 20 mL of 2% cetylpyridinium chloride (Sigma), inverted to precipitate the alginate (10 times), and centrifuged at ~3,300 *x g* for 10 min. The supernatant was poured off and discarded, preserving the pellet. 10 mL of 1 M sodium

chloride was used to resuspend the alginate pellet and vortexed before incubating at room temperature for 30 min. Then, 10 mL of isopropanol (Fisher Scientific) was added to the alginate solution, inverted 10 times, vortexed, and centrifuged at ~3,300 *x g* for 10 min. The supernatant was poured off and discarded. The alginate pellet was then resuspended in 10 mL of 0.85% sodium chloride and incubated at 4°C overnight.

Following the incubation of the isolated alginate samples in a borate-carbazole solution at 55°C [118, 119], a BioTek Synergy H1 was used to measure the OD_{530}. All values were compared to a standard curve generated by diluting laboratory grade alginic acid (Sigma) in 0.85% sodium chloride to concentrations of 50-1000 µg/ml. Alginate production was analyzed and compared for three biological replicates for Isolates 1-3 of Patient 3 by comparing plate reader values to the standard curve generated.

2.5.9. Statistical Analysis

Data analysis was performed using Prism (Graphpad software, version 9). Population heterogeneity experiments were normalized to a corresponding R-pyocin null mutant lysate control culture for each isolate (to account for heterogeneity in growth rates) for fold-change, and analyzed with Prism. CFU data was analyzed for four isolates by normalizing CFU measurements at t=0 h and t=4 h for cultures with and without R-pyocin lysates to compare log2 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 and alginate production were analyzed using a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons analysis in Prism. Figure 2.3C and Figure 2.5A show mean values of three biological replicates for each individual isolate, while Figure 2.7B shows

four biological replicates (averaged from technical replicates) as individual data points. Error bars represent standard error and significance is denoted as follows: **, P = 0.005.

2.5.10. LPS Extraction and Characterization

LPS of bacterial cultures were isolated and visualized as described by previous methods [120]. Overnight bacterial cultures of 5 mL LB were grown at 37°C at 200 rpm shaking. Complemented Isolate 1 strains were grown in 5mL LB supplemented with 200 µg/mL tetracycline to maintain the construct, and 20 µL Chromomax IPTG/X-Gal solution (Fisher Scientific) for induction of gene expression. The overnight cultures were diluted 1:10 with LB for an OD_{600} measurement and diluted to make a 1.5 mL suspension of bacteria adjusted to an OD_{600} of 0.5 before pelleting by centrifugation at 10,600 *x g* for 10 min. The cell pellet was resuspended in 200 µL of 1x SDS-buffer. Suspended bacterial cells were boiled for 15 min and LPS were prepared by hot aqueous-phenol extraction as previously described [120] 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) [120].

2.5.11. 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 [121], before mapping with default parameters to the PAO1 reference genome (NC_002516) using Bowtie2 (version 2.1.0) [122, 123]. 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 [124, 125]. To annotate SNV lists, the same PAO1 reference files used for mapping were built into a database to annotate SNV lists through SnpEff version 4.3 [126]. SNV lists were compared across all three isolates for shared SNVs and visualized for comparison with R (version 3.5.1) [117]. Sam files generated from mapping each isolate to PAO1 were used to visualize coverage with BRIG (version 0.95) [127] and CGView [128]. Sequences were compiled into de novo assemblies using SPAdes [129] through KBASE [130] for serotype prediction with PAst 1.0 [131] and strain typing with PubMLST [132].

2.5.12. Data Availability

All sequences were deposited in the National Center for Biotechnology Information's SRA database under the accession number PRJNA67996 [133]. Raw data and code have been made available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.573n5tb67 [134].

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2.7. Acknowledgements

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CHAPTER 3: HIGH FREQUENCY OF LPS MUTANTS AND R2-PYOCIN-SUSCEPTIBLE ISOLATES OBSERVED IN POPULATIONS OF *PSEUDOMONAS AERUGINOSA* FROM CF LUNG INFECTION

3.1. Abstract

Chronic infections found in cystic fibrosis (CF) lungs are debilitating, highly antibiotic resistant and often lethal. *Pseudomonas aeruginosa* is a common pathogen found in CF lungs and is highly resistant to multiple classes of antibiotic, contributing to antimicrobial resistance (AMR). *P. aeruginosa* populations in the CF lung are known to be phenotypically and genotypically diverse, but little is understood regarding how this heterogeneity influences susceptibility to non-traditional antimicrobials, such as bacteriocins (protein complexes produced by a species of bacteria with efficacy against members of the same species). *P. aeruginosa* produces phage tail-like bacteriocins, called R-pyocins, which cannot replicate, are highly potent and have a narrow killing spectrum. R-pyocins have potential for use as antimicrobials, however, the heterogeneity of *P. aeruginosa* evolved in CF lung infection has recently been found to contribute to heterogeneity in susceptibility to R-pyocins. This heterogeneity is likely caused by alterations of the lipopolysaccharide, which is the receptor of R-pyocins. To date, there is currently no estimation of CF strains that may exhibit some degree of susceptibility to any of the 5 subtypes of R-pyocins, particularly considering multiple isolates from diverse populations. There is also a limited understanding of correlation between LPS phenotype and R-pyocin susceptibility in CF lung infection. In this work we tested 110 isolates of *P. aeruginosa* collected from 12 longitudinal samples of sputum from 5 individuals with CF for susceptibility to R2-pyocins, and characterized the LPS phenotypes. Based on findings

from our biobank we i) estimated that approximately 83% of our sputum samples contain heterogenous *P. aeruginosa* populations without R2-pyocin resistant isolates and all sputum samples contained susceptible isolates; ii) there is no correlation to LPS phenotypes, and iii) we estimate that approximately 76% of isolates sampled from sputum may lack O-specific antigen, 42% lack common antigen, and 27% exhibit altered LPS cores. The lack of correlation between LPS phenotypes and R-pyocin susceptibly, suggests LPS packing density may play a more important role in R-pyocin susceptibility in CF. Our work contributes to the evaluation of R-pyocins as potential therapeutics as we estimate a large number of infectious strains to be susceptible to R2-pyocins – supporting its efficacy in chronic infections even with heterogeneous evolved pathogens.

3.2. Introduction

Cystic fibrosis (CF) is a genetic disorder predominantly characterized by chronic respiratory infections resulting in a progressive loss of lung function and an increased rate of morbidity and mortality in CF individuals [1]. CF disease results from a defective anion pump, referred to as the cystic fibrosis transmembrane regulator (CFTR), which leads to an imbalance in chloride on epithelial cell surfaces [1]. This imbalance leads to dehydrated mucous, ultimately too thick to allow proper clearing of airways along the respiratory tract of people with CF; this inability to clear the airways creates an environment conducive to bacterial and fungal infections, which are notoriously difficult to treat.

The Gram-negative opportunistic bacterium *Pseudomonas aeruginosa*, is the most prominent bacterial pathogen that colonizes and chronically infects CF lungs [2-7]. Equipped with a large genome, *P. aeruginosa* is able to regulate diverse metabolic processes, resist antibiotic treatment, and control a number of 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). In general, *P. aeruginosa* already possesses many intrinsic antibiotic resistant mechanisms, but *P. aeruginosa* sourced from these chronic lung infections are known to become particularly problematic and resistant to multiple antibiotics over time [16-20]. While recent data suggests that with the approval and use of the new triple therapeutic TRIKAFTA® (elexacaftor/tezacaftor/ivacaftor), the overall prevalence of *P. aeruginosa* infection is improving, the 2019 CF Foundation Patient Registry Annual Report has noted that the prevalence of infection with MDR *P. aeruginosa* remains constant [21]. In the era of antibiotic resistance, new treatment approaches are crucial for use in chronic infections against *P. aeruginosa* and other multi-drug resistant pathogens – especially in the case of CF chronic lung infections.

Part of the challenge of eradicating *P. aeruginosa* from the CF lung is likely due to the high degree of morphological, phenotypic and genomic diversity that evolves within *P. aeruginosa* populations over the course of infection [6, 17, 18, 22, 23]. While it is known that this heterogeneity affects antibiotic susceptibility, the impact of this heterogeneity on other therapeutic alternatives, such as bacteriocins and bacteriophage are poorly understood [16, 18, 20]. As pathogens like *P. aeruginosa* are increasingly difficult to eliminate, non-traditional antimicrobials such as bacteriocins, may be 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 multiple types of bacteriocins called pyocins, which be

grouped by similarities in their physical and chemical properties, export mechanisms, and mode of action [26-30]. The pyocins produced by *P. aeruginosa* are referred to as S-, R-, and F-pyocins and vary in their antimicrobial spectra [29-34]. R-pyocins are narrow spectrum, contractile, phage tail-like bacteriocins which can further be categorized by the subtypes R1-R5, differentiated by specificity to different receptors on target cells [29, 30, 35-37]. The proposed mode of action is that the foot of the tail fibre of the R-pyocin binds to a receptor – a monosaccharide- on the lipopolysaccharide (LPS) (specific to each R-subtype), the sheath contracts, pushing the tail spike and core to puncture the outer membrane; and the membrane depolarizes before eventual death (Figure 3.1) [38-44]. R-pyocins are gaining interest in their anti-pseudomonal potential for their potent ability to kill target cells, their inability to replicate without a DNA-containing head, and their thoroughly described mechanism of action against target cells [29, 30, 41, 43-45]. In this work, we focus on R-pyocins due to their promising characteristics as antimicrobial agents along with the need for a better understanding of their activity among clinical strains of *P. aeruginosa*.

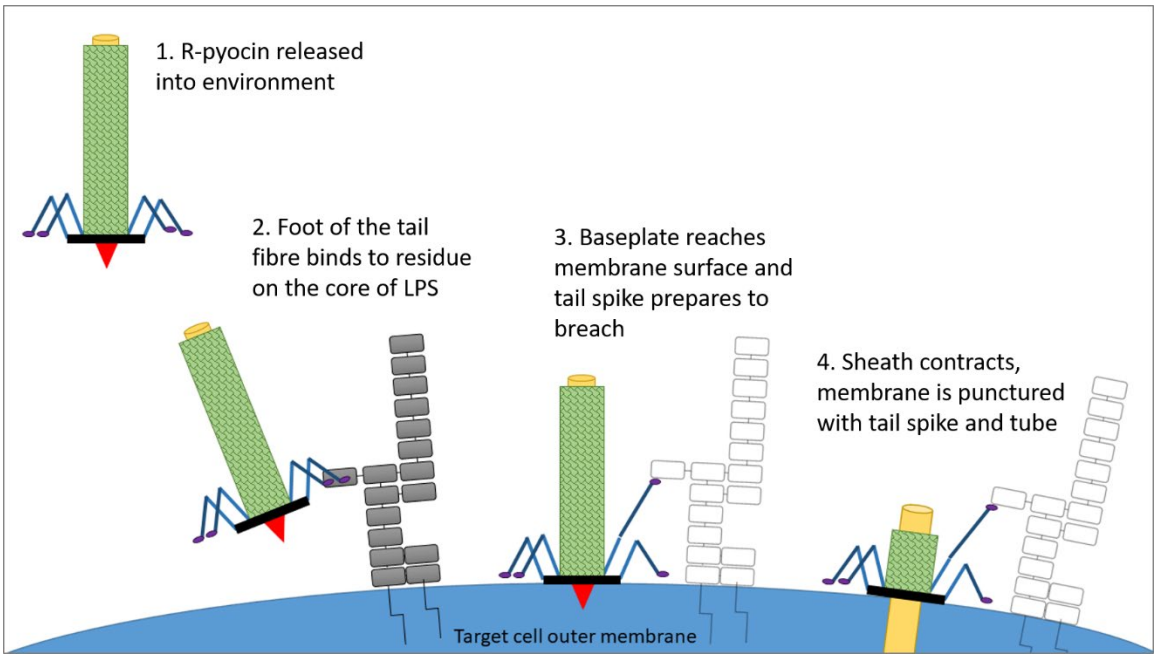

Figure 3.1: R-pyocin unit binding to LPS receptor and puncturing target cell membrane. Upon release into the environment by producing cells, R-pyocins bind to target cells by the recognition of core residues of the lipopolysaccharide (LPS) decorating the outer cell membrane. Following the binding of the tail fibres, the baseplate is dissociated thus initiating sheath contraction and driving the iron-tipped tube through the cell surface killing the target bacterium.

In previous work, we tested up to 20 individual isolates of *P. aeruginosa* from 3 expectorated sputum samples collected from different individuals with CF (60 isolates total, from 3 *P. aeruginosa* populations); we showed heterogeneity in susceptibility to R2-pyocins within these populations of *P. aeruginosa* [46]. We also isolated and compared lipopolysaccharide (LPS) phenotypes among 3 of these isolates, derived from the same sputum collection and patient, and found that each isolate presented different LPS glycoforms likely contributing to the differences in susceptibility [46]. If R-pyocins are to

be considered as a therapy, it is important to dive further into investigating the efficacy of R-pyocins against heterogenous clinical strains of *P. aeruginosa* as the frequency of R-pyocin-susceptible strains in CF chronic lung infections is currently poorly understood. We found in prevous work that R1-pyocin producers are the majority of R-pyocin-typeable CF *P. aeruginosa* strains, and other groups have shown that R1-type producers are often susceptible to R2-pyocins [37, 46, 47]. Thus, we expect to find a high frequency of *P. aeruginosa* strains isolated from chronic lung infection with LPS mutations conferring R2-pyocin susceptbility. In this work, we utilized our biobank of whole *P. aeruginosa* populations isolated from CF sputum, to describe R2-pyocin susceptibility profiles and characterize LPS phenotypes to better understant the potential for R-pyocin use as an alternative anti-pseudomonal in chronic infection.

3.3. Results

3.3.1 Majority of CF Populations of *P. aeruginosa* Contain Isolates Susceptible to R2-Pyocins.

To determine the frequency of R2-pyocin susceptible isolates among *P. aeruginosa* populations from CF sputum samples, we screened up to 10 isolates previously described [46] from longitudinal samples of 5 individuals with CF (110 isolates total collected from 12 sputum samples or "populations") utilizing the soft-agar overlay method (Figure 3.2). We assessed relative susceptibility of each isolate to R2-pyocins and found that i) each population showed heterogeneity within the population by this method (in agreement with previous work using a microtiter plate method) [46] and ii) nearly every population of *P. aeruginosa* included isolates with some degree of susceptibility to R2-pyocins. In this

sampling of strains, we found that even across longitudinal sampling from the same patient, most populations contained R2-susceptible isolates, though the degree of relative susceptibility and sensitivity to the R2-pyocins fluctuated over time (Figure 3.2). We found that only 3 out of 110 total isolates were R2-pyocin resistant (2.7%), with all 5 individuals producing sputum containing R2-susceptible isolates (Figure S3.1). Longitudinal sputum collections in our biobank did not show trends of isolates becoming R2-pyocin resistant over a period of approximately 2 years.

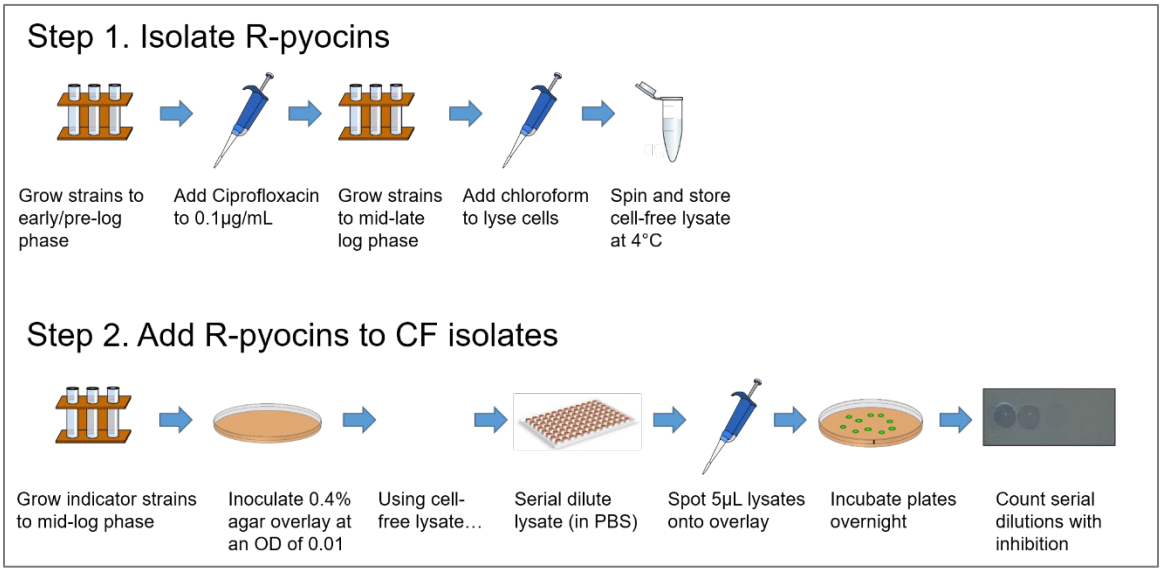

Figure 3.2: Collection of R2-pyocin lysate and susceptibility testing protocol. R-pyocins are isolated from *P. aeruginosa* by growing strains to early logarithmic growth, adding sub-minimal inhibitory concentration (MIC) ciprofloxacin, then lysing the cells with chloroform following several hours to allow time for maximum R-pyocin production. Cultures are then centrifuged, separating into phases, so that cell-free R-pyocin-containing supernatant (lysates) can be stored for use. R-pyocin lysates are then serially diluted and dilutions are spotted onto soft agar overlays to test susceptibility of strains of interest.

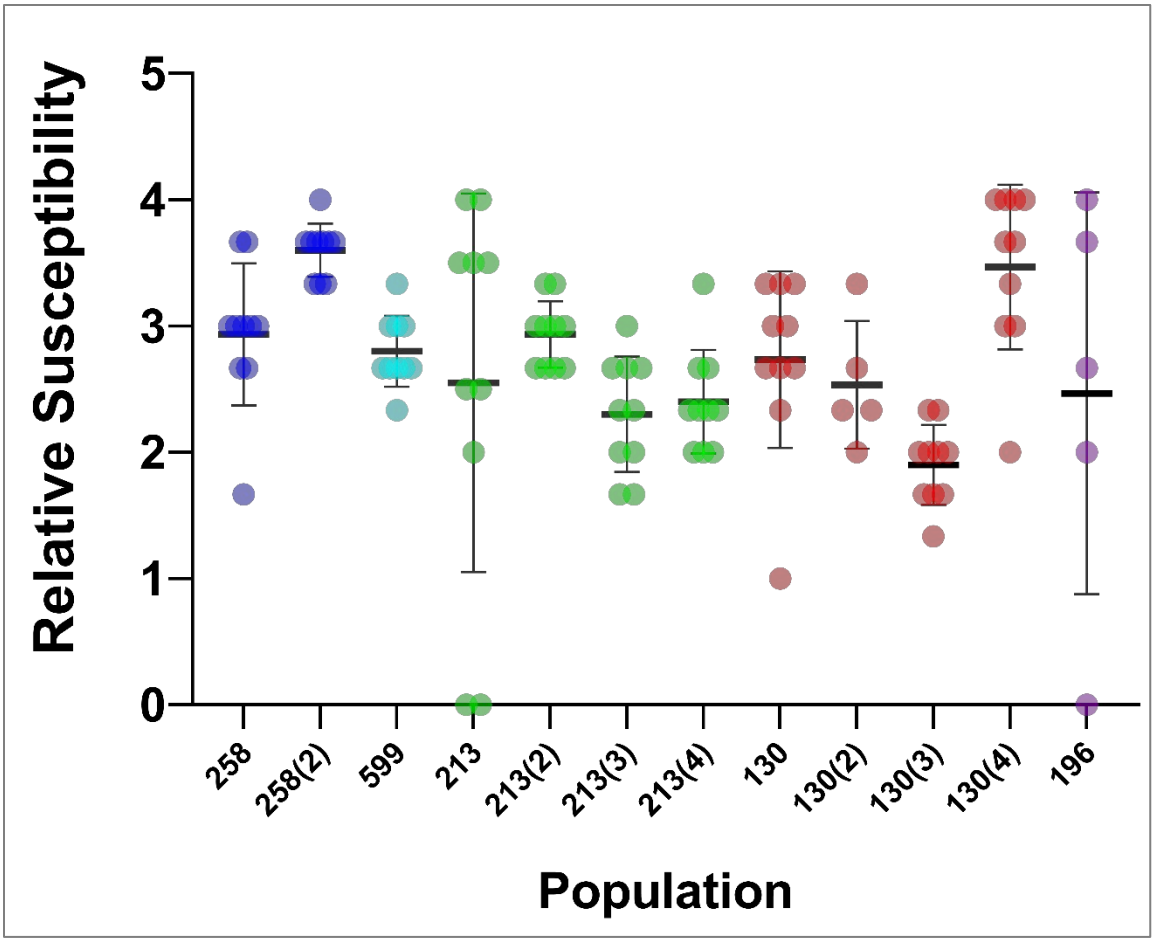

Figure 3.3: Clinical *P. aeruginosa* populations contain R2-susceptible isolates and heterogeneity in relative susceptibility fluctuates over time. Relative susceptibility to R2-pyocins vary by isolates in each populaiton, and across populations. Heterogeneity in susceptibility ranges can be seen both across all five patients, and within longitudinal samplings from the same patient. Of the longitudinally samples (consecutively numbered populations with collection number in parentheses), there is not trend of strains becoming more resistant to R2-pyocins. Two sputum samples contained at least one isolate that was resistant to R2-pyocins (Relative Susceptibility = 0). Relative Susceptibility was determined by counting the number of serial dilutions (including neat) that which zones of inhibition can be seen where R2-pyocins were spotted onto agar overlays. Means are shown with error bars of 95% confidence intervals.

3.3.2 High Frequency of LPS Mutants and Heterogeneity in LPS Phenotypes among CF Strains of *P. aeruginosa*.

To characterize the LPS phenotypes of the R2-pyocin susceptible and resistant isolates in our biobank, we isolated LPS using a hot-aqueous phenol method [48] and compared the LPS migration profiles of our clinical isolates to those of isogenic LPS mutants of our Nottingham PAO1 wild type strain (Figure 3.4). We observed that out of 149 isolates across all populations 42.7% lacked common antigen (CPA, or A-band) (Figure 3.5B), 76.3% lacked o-antigen (or B-band) (Figure 3.5D), and 27.2% presented altered LPS cores (Figure 3.5F), as determined by comparing to the wild type (WT) PAO1. We found that 10 of the 12 sputum collections sampled contained isolates with heterogeneity in LPS profiles; at least 83.3% of our *P. aeruginosa* populations contained a mixture of isolates with varying LPS phenotypes.

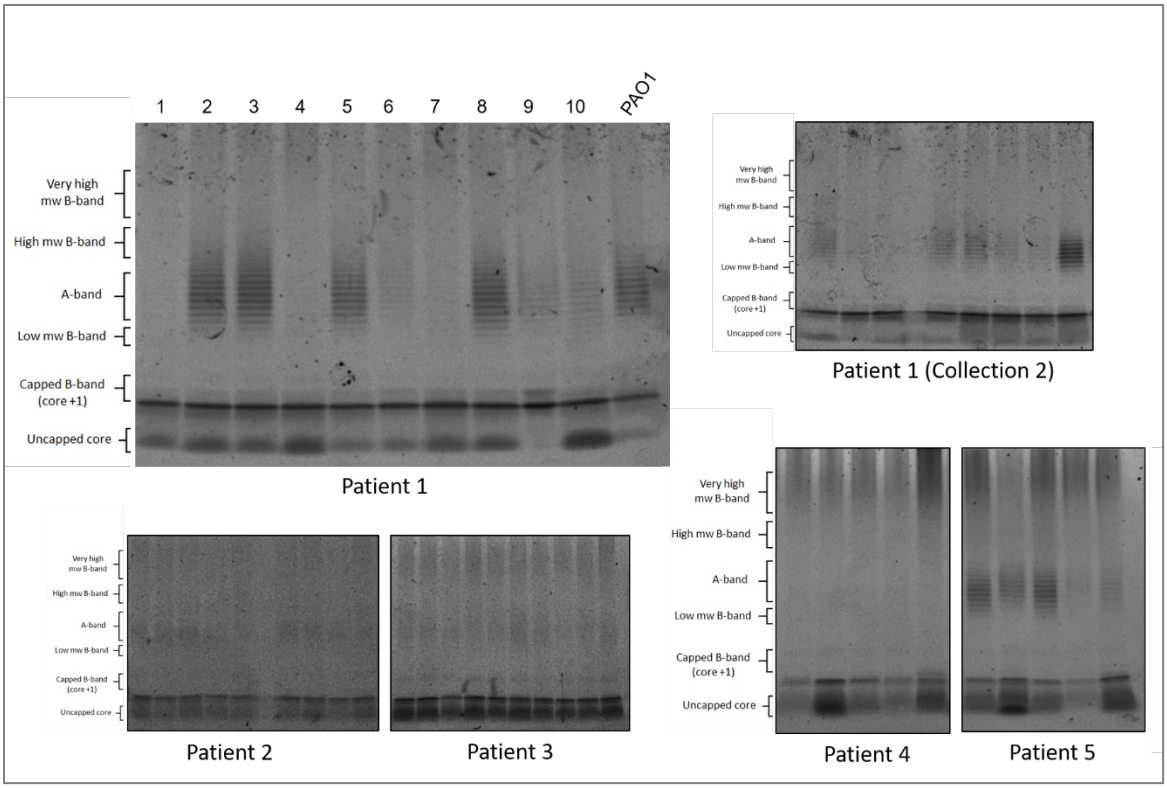

Figure 3.4: SDS-Page visualization of clinical *P. aeruginosa* isolates from a representative sputum sample from 5 patients. Up to ten isolates were visualized for lipopolysaccharide (LPS) phenotypes from each of 12 populations of *P. aeruginosa* sampled from five CF patients. Isolates from two different sputum collections from a single patient are also shown for comparison of heterogeneity across longitudinal samples. Some sputum samples contained isolates with similar LPS phenotypes, while others exhibited heterogeneity in LPS. Most frequently found were strains lacking O-specific antigen (OSA), or B-band. LPS were isolated with a hot-aqueous phenol method and stained with the Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit.

3.3.3 Correlation Between R2-Pyocin Susceptibility and LPS Phenotypes Observed in CF Strains.

To assess correlation between R2-pyocin relative susceptibility and a particular LPS glycoform phenotype we ran a one-tailed, Spearman correlation analysis for each phenotype individually. For these analyses we assumed independence between the three glycoform phenotypes (CPA^+ or CPA^-, OSA^+ or OSA^-, WT or altered core), though it is understood that there are some dependencies between presence or absences of each glycoform given overlaps in biosynthetic pathways and the essentiality of the LPS core. We found that individually, there were no correlations specifically between the presence of absence of the CPA (Figure 3.5A), the OSA (Figure 3.5C) nor whether the core appeared altered (Figure 3.5E) ($p > 0.05$).

We further evaluated the relationship between LPS phenotype and R2-pyocin susceptibility by assessing the LPS as a whole. Using a numbered scale, we assigned an LPS Score of 0 when an isolate presented as CPA^-, OSA^- and had an altered core; this isolate was treated as presenting 0/3 WT LPS characteristics. A score of 1 was assigned when an isolate exhibited one of the three LPS glycoforms as WT (presence of OSA, CPA, *or* unaltered core), a score of 2 when two of three glycoforms were WT, and a score of 3 when an isolate appeared CPA^+, OSA^+ with a WT core. Using this LPS Score, we also found no correlation with R2-pyocin susceptibility (Figure S3.2) however we did find that three isolates resistant to R2-pyocins were all CPA^- and OSA^-. Only 20.9% of isolates from all sputum collections exhibited CPA^+, OSA^+, and WT LPS cores.

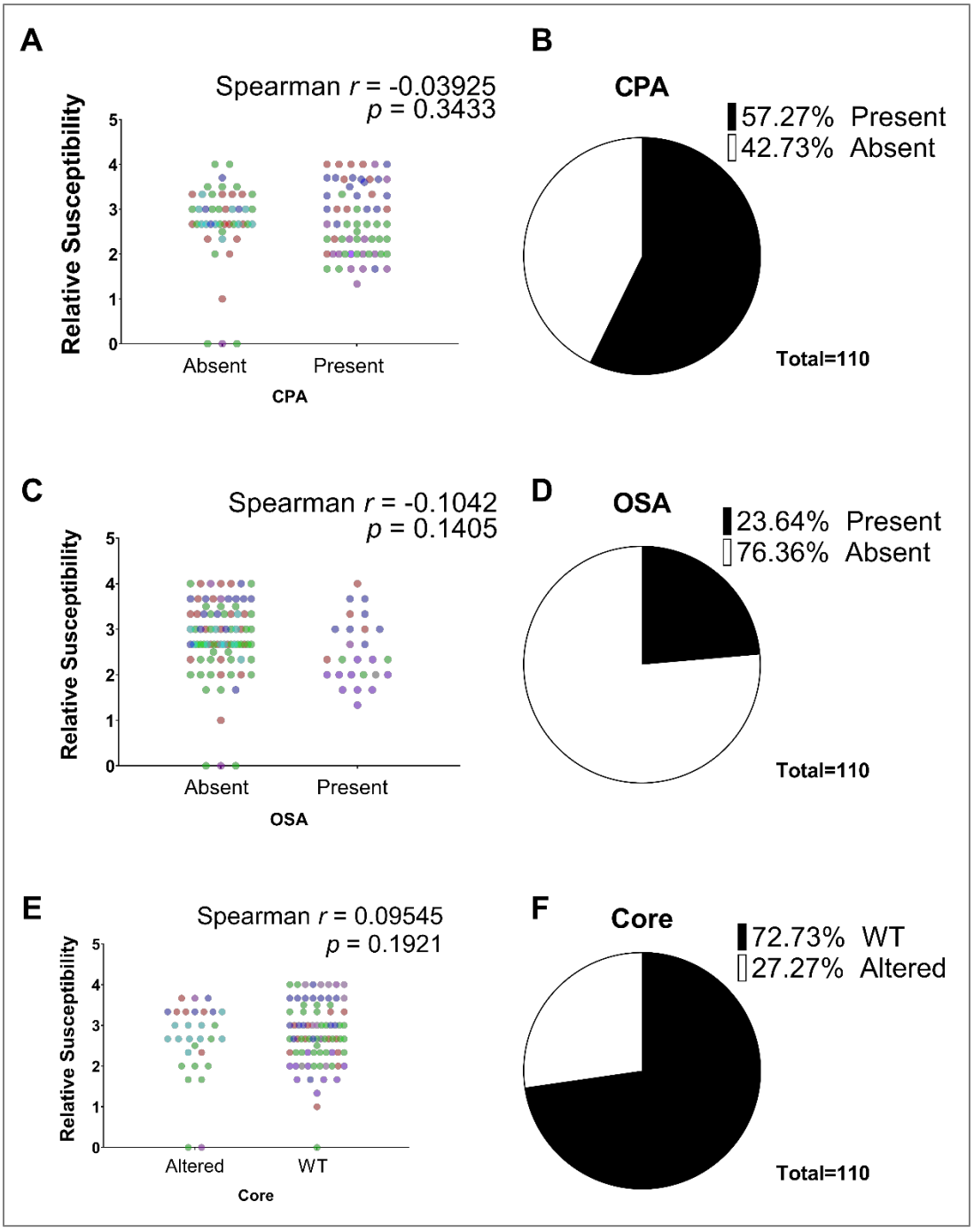

Figure 3.5: Heterogeneity among LPS phenotypes but no correlation with R2-susceptibility. Using a one-tailed, Spearman correlation analysis for each phenotype, we found no correlations between R2-pyocin susceptibility and absence of (A) common antigen (CPA), (C) O-specific antigen (OSA), or (E) an altered core ($p > 0.05$). We found CPA absent in 42.73% of isolates (B), OSA absent in 76.36%, and (F) 27.27% of isolates with altered LPS cores. Each data point represents the average Relative Susceptibility of an isolate, and isolates are colored by their longitudinal collection.

3.4. Discussion

Cystic fibrosis chronic lung infections are highly antibiotic resistant, difficult to eradicate and often-lethal [1]. *P. aeruginosa* is a pathogen commonly found in CF lungs and is a highly antibiotic-resistant contributor to infection-related morbidity and mortality [2-7]. In addition to intrinsic and evolved mechanisms of antibiotic resistance, *P. aeruginosa* diversifies over the course of long-term infection, adding to the difficulty in eliminating the bacteria. As one of the most challenging pathogens to treat in chronic infection, it is critical to consider alternative antimicrobials and therapeutics.

Bacteriocins, and specifically R-pyocins have gained interest recently for their high potency, narrow spectrum, anti-pseudomonal use. However, given the heterogeneity in phenotypic behavior of *P. aeruginosa* found in chronic CF lung infection, the efficacy of R-pyocins and other phage-like bacteriocins is poorly understood. In previous work, we showed that diverse populations of *P. aeruginosa* exhibit heterogeneity in susceptibility to R-pyocins, highlighting the importance of understanding the implications of heterogeneity on treatments [46]. This heterogeneity is especially important to consider in regards to resistant isolates in the infection - if the phenotypes associated with this resistance are selected for or beneficial to survival in chronic infection, and if resistant isolates are highly prevalent. In this work, we utilized 110 isolates of *P. aeruginosa* collected from 12 expectorated sputum samples (including longitudinal collections) of five individuals with CF to test for R2-pyocin susceptibility and identify LPS phenotypes. From our collection of *P. aeruginosa* populations from CF lung infection we i) estimated that approximately 83% of our sputum samples contain heterogenous P. aeruginosa populations without R2-pyocin resistant isolates and all sputum samples contained susceptible isolates; ii) we found

that there is no correlation between R2-pyocin susceptibility and LPS phenotypes, and iii) we estimate that approximately 76% of isolates sampled from sputum lack O-specific antigen, 42% lack common antigen, and 27% exhibit altered LPS cores.

As there have been no estimates of prevalence regarding *P. aeruginosa* strains in CF lung infections that may be susceptible to R2-pyocins, we first tested for R2-susceptiblity among multiple diverse isolates collected from the same sputum samples. Whereas previously the majority of R-pyocin susceptibility testing has been limited to 1 isolate per sputum collection [37, 47], this work is unique in collecting information on susceptibility from within multiple bacterial populations as well as across patients.

In previous work we assessed the R2-pyocin susceptibility of 20 isolates from 3 sputum collections (3 CF individuals), however in this work we sample a broader number of sputum collections from more individuals, including longitudinal samples for some patients. From these experiments we found that all of the bacterial populations studied included susceptible isolates – and we again found heterogeneity in susceptibility within the susceptible isolates as previously described [46]. Less than 3% of isolates tested were resistant to R2-pyocins, and these isolates were derived from only two sputum collection. We also did not find a trend of increasing nor decreasing R2-pyocin susceptibility across longitudinal samples. We did not find that R2-pyocin susceptibility clustered by patient, but by longitudinal collection (Figure 3.3). A high frequency of R2-pyocin susceptible strains in CF lung infections suggests that i) there is not strong selective pressure for *P. aeruginosa* to alter LPS in a fashion that leads to R2-pyocin resistance and ii) R2-pyocins would be effective for at least approximately 83% of patients, who in our collections did not produce any isolates with R2-pyocin resistance.

While there have been studies assessing correlation between serotype and R-pyocin susceptibility [37, 47], it is currently unknown if there is a correlation between LPS phenotype and R-pyocin susceptibility. In this study we examined LPS phenotypes by considering presence or absence of the common antigen (CPA) and the O-specific antigen (OSA) as well as the altered or PAO1-like (WT) LPS core. We did not find a correlation between any of the phenotypes, nor did we find a correlation by assessing various combinations of LPS phenotypes with R2-pyocin susceptibility. We did find that in some sputum collections, all or most isolates studied expressed similar LPS phenotypes; for example, in one collection of sputum, all isolates examined exhibited the phenotype CPA^+, OSA^+, and present a WT core while another collection contained isolates that all exhibited CPA^-, OSA^-, and altered cores. We found a high percentage of isolates lacked OSA (76.3%), while more than half maintained the CPA (57.2%). The lack of correlation between the absence or presence of any of the specific LPS glycoform phenotypes suggests that perhaps another LPS-modifying behavior, such as packing density, may be more influential in determining R2-pyocin susceptibility. Another group has suggested the importance of LPS packing density on R-pyocin susceptibility, though little is understood of the mechanism or regulation of this behavior [49]. It is unknown how the CF lung environment impacts LPS packing density.

Overall our findings suggest that the majority of *P. aeruginosa* strains isolated from CF lung infections are likely susceptible to R2-pyocins, supporting the potential for R-pyocins as effective antimicrobials. Our work also reveals that there is no correlation between LPS phenotype and R2-susceptiblity in strains evolved in chronic infection, suggesting more work is necessary to evaluate other LPS-modifying behaviors and packing

density in the CF lung environment – and the implications of these behaviors for the use of LPS-binding phage and bacteriocins.

3.5. Methods

3.5.1 Bacterial Strains, Media, and Culture Conditions.

Expectorated sputum samples for this study were collected 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). *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, 110 isolates from our biobank, sourced from CF chronic lung infections, were studied for R2-pyocin susceptibility and LPS phenotype. All *P. aeruginosa* lab strains used in this work are listed and described in Supplementary Table 3.1. All bacterial cultures were grown in lysogeny broth (LB) medium at 37°C with 200 rpm shaking agitation. Standard genetic techniques were used 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 Supplementary Table 3.1.

3.5.2 Generation of Mutants.

PAO1 *ΔrmlC* mutant was constructed 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]. These sequences were cloned into the SphI-

XbaI digested suicide vector pDM4 [50] by Gibson assembly using NEBuilder HiFi Assembly master mix (New England Biolabs), transformed into *E. coli* S17-λpir by electroporation and selected on LB agar plates supplemented with 30 µg/mL chloramphenicol. Cloned inserts were verified by colony PCR and Sanger sequencing (Eton Bioscience, NC). Deletion constructs were introduced into PAO1 by electroporation and strains carrying single crossover insertions of the deletion constructs were selected on LB agar plates supplemented with 300 µg/mL chloramphenicol [51]. Resistant colonies were cultured in LB without antibiotic and plated on LB agar plates with 0.25% NaCl and 5% sucrose to select for loss of the construct [52]. Sucrose-resistant colonies were screened for antibiotic 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.

The PAO1 ΔPf4 ΔR mutant was constructed by deleting the tail fibre and chaperone genes of the R-pyocin gene cassette (PA0620-PA0621) from the PAO1 ΔPf4 strain as described previously [46].

3.5.3 Expression and Collection of R-Pyocins.

R-pyocins were extracted in lysates as previously described [46]. LB cultures of PAO1, PAO1 ΔR (R-pyocin null mutant) [46], PAO1 ΔPf4 (prophage mutant) [53] , and PAO1 Δpf4 ΔR were inoculated at 1:100 from overnight planktonic cultures, and grown to mid-logarithmic growth phase (approximately 4 h) in 10 mL of LB media. Ciprofloxacin was then added to each culture for a final concentration of 0.1 µg/mL to induce R-pyocin production [46, 54, 55], and cultures were incubated for a further 3 h. Chloroform was used to lyse remaining cells, and lysates were centrifuged at ~3,300 *x g* for 10 min. The R-pyocin-containing supernatants were separated and stored at 4°C.

3.5.4 Spot Assay for Relative R-Pyocin Susceptibility.

Spot assays to assess R-pyocin activity were conducted as previously described [46]. Overnight cultures of clinical *P. aeruginosa* isolates were used to inoculate 4 mL of cooled soft top (0.4%) agar at an optical density at 600 nm (OD_{600}) of 0.01. This mixture was poured onto LB agar plates for an overlay of the indicator strain. R2-pyocin lysates extracted from PAO1, PAO1 ΔPf4 and respective R-pyocin null mutants and were vortexed, then serial diluted 10-fold in phosphate buffered saline (PBS) before spotting 5 µL of each dilution onto the soft-top overlay indicator strains. Spots were allowed to dry before plates were incubated at 37°C overnight. Clear zones of growth inhibition indicated R-pyocin dependent activity against the indicator strains, and relative susceptibility was determined by counting the number of serial dilutions of lysate indicating inhibition. PAO1 wild type was used as an R2-resistant control strain and PAO1 Δ*rmlC* was used as an R2-susceptible control strain for each experiment [37]. Spot assays for R-pyocin susceptibility were conducted in triplicate.

3.5.5 LPS Isolation and Visualization.

LPS of bacterial cultures were isolated and visualized as described by previous methods [48]. 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_{600} measurement and diluted to make a 1 mL suspension of bacteria adjusted to an OD_{600} of 0.5 before pelleting by centrifugation at 10,600 *x g* for 10 min. The cell pellet was resuspended in 200 µL of 1x SDS-buffer. Suspended bacterial cells were boiled for 15 min and LPS were prepared by hot aqueous-phenol extraction as previously described [48] 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%-20% Tris-glycine mini gel using the Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (ThermoFisher) [48].

3.5.6 Statistical Analysis.

Data analysis was performed using Prism (Graphpad software, version 9).

3.6. References

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CHAPTER 4: SPATIAL DYNAMICS OF *PSEUDOMONAS AERUGINOSA* R-PYOCIN REGULATION AND RELEASE IN AN INFECTION-RELEVANT ENVIRONMENT

4.1. Abstract

Bacteriocins are antimicrobial particles produced by bacteria that have long been studied for their role in microbial competition and more recently as therapeutics for treating infection. The opportunistic pathogen *Pseudomonas aeruginosa* is often multi-drug resistant and problematic in a number of infections, including chronic cystic fibrosis (CF) lung infections. *P. aeruginosa* produces bacteriocins called R-pyocins, which resemble headless phage-tails and are highly potent anti-pseudomonals. While it is thought that R-pyocins, like phage, reach target cells by diffusion, there has been little experimental evidence showing R-pyocins can diffuse between strains in CF sputum to mediate competition between strains, and even less is known regarding the timing and regulation of R-pyocins in CF lung infections. Further, cells in aggregated biofilms exhibit higher tolerance to antimicrobials than in planktonic growth, yet it is not known how cells in a spatially structured, CF-relevant environment may impact the efficacy of R-pyocins, phage and other phage-like particles. Here we evaluated R-pyocin activity in an infection-relevant environment (Synthetic Cystic Fibrosis Sputum Medium; SCFM2) and found that (i) R-pyocins genes are transcribed more highly in the CF nutrient environment than in rich laboratory medium and (ii) in a structured, CF-like environment, R-pyocin induction is costly to producing strains in competition rather than beneficial. Our work suggests that R-pyocins may not be essential to CF lung infection and can be costly to producing cells in the presence of stress response-inducing stimuli.

4.2. Introduction

Bacteriocins are antimicrobials produced by bacteria, which are capable of killing other closely-related strains or other species of bacteria [1]. Bacteriocins have generally been studied in microbiology for their roles in intra-strain and intra-species competition, but recently there has been a resurgence of interest in the use of bacteriocins to combat infection, because they present great potential as adjunctive therapeutic treatments. It is well known that bacteria in infections spatially structure in the form of aggregates [2-10]. However, it is unclear how this spatial organization impacts diffusive competitive mechanisms like R-pyocins, as the ability of R-pyocins to diffuse in a structured environment is not well understood. Thus the efficacy of R-pyocins as antimicrobials is unknown in infection, particularly those conducive to structured or aggregate growth.

Cystic fibrosis chronic lung infections are one type of chronic infection known to facilitate pathogenic growth in dehydrated sputum, creating a spatially structured environment. These chronic infections are also known for being dominated by primarily one strain of multi-drug resistant and difficult to eradicate *Pseudomonas aeruginosa* [11-15]. The mechanisms behind this single-strain takeover are still poorly understood. R-pyocins, a potent and strain-specific bacteriocin produced by *P. aeruginosa* have been proposed to be one type of mechanism of intra-strain competition that could play a role in determining the primary infectious strain [16-19], however the *in vivo* regulatory behavior and timing of R-pyocin induction in the CF lung environment are unknown. In order to fully appreciate the efficacy of R-pyocins for their potential use in medicine, it is crucial to understand their regulatory activity and timing during infection to better understand their efficacy and the potential risks of selective pressures evolving resistance.

Similar to other types of bacteriocins, R-pyocins can be produced sponateously at sub-basal levels or upregulated by SOS-inducing agents and stimuli such as ciprofloxacin or mitomycin C [20, 21]. Following SOS activation, R-pyocins are produced and then released into the environment by cell lysis. While several SOS-inducing agents have been described to induce R-pyocins, it is unknown if each of these agents induce R-pyocin production with differences in timing or response and in general. The agent of choice varies by study. The CF lung environment includes many stressful and SOS-inducing cues, including antibiotics, nutrient limitation, iron starvation, anoxic microenvironments, and reactive oxygen species from the host immune response [2, 15, 22-29]. Thus, it remains unclear how different stimuli may influence R-pyocin activity in infection.

In a previous study, we screened 849 *P. aeruginosa* genomes publicly available as a part of the International *Pseudomonas* Consortium Database (IPCD) and found that, 40.8% of strains isolated from respiratory infection sources (including cystic fibrosis) lacked R-pyocin genes (PA0615-28) entirely [30, 31]. This absence of R-pyocin genes in infectious strains, suggests that even if R-pyocins are involved in strain competition early in acute infection, R-pyocins may not be essential for survival in chronic infection, and specifically in chronic CF lung infection. Despite many transcriptomic studies to analyze *P. aeruginosa* behavior in the CF lung or in sputum, little data is available regarding R-pyocin transcriptional or proteomic activity and thus the induction and timing of R-pyocin production in infection is not yet known. As R-pyocin genes are not discussed in many transcriptomic and proteomic studies, it is possible that this may be because the infecting strains have lost R-pyocin genes or evolved to lose activation of R-pyocin genes, rather than a lack of R-pyocin induction in the infection. We predicted that the minimal CF

nutritional environment induces R-pyocin activity, but the spatial biogeography of CF sputum renders R-pyocin production and lysis more costly than beneficial in competition with a non-producing strain. In this work, we evaluated R-pyocin transcriptional activity in SCFM and assessed the fitness of R-pyocin production in a spatially structured, CF-relevant environment to understand R-pyocin activity and efficacy in chronic CF lung infection.

4.3. Results

4.3.1. Timing and Induction of R-Pyocin Associated Genes.

To evaluate R-pyocin transcriptional activity, we evaluated the R-pyocin gene cassette (Figure 4.1A) to identify potential promoter regions upstream of the R-pyocin lytic and structural genes. We generated transcriptional reporters for the R-pyocin-associated holin (PA0614) (Figure 4.1B), lysin (PA0629) (Figure 4.1C), and an R-pyocin structural gene (PA0622) (Figure 4.1D) in the wild-type (WT) laboratory strain PAO1. Using the SAPPHIRE regulatory element prediction analyzer, we identified three putative promoter regions upstream of each gene of interest (Figure 4.1E). Using a CTX-*lux* reporter, we tested each putative promoter for activity with and without ciprofloxacin at 0.1 μg/mL to induce the SOS response [21]. We found that the holin is most active gene of the R-pyocin cassette during SOS induction, while the lysin putative promoter sequence was active but not as responsive to induction as the holin (Figure 4.2). We tested the activity of the holin reporter in several isogenic mutants lacking R-pyocin lytic genes and genes encoding for the filamentous prophage pf4 of PAO1 (Supplementary Figure 4.1), and found that the PAO1 Δlysin (Supplementary Figure 4.1A) showed the greatest difference in R-pyocin

associated activity as holin activity decreased under each agent relative to the activity seen in the PAO1 Δholin (Supplementary Figure 4.1B), PAO1 ΔPf4 (Supplementary Figure 4.1C), and PAO1 ΔPf4 ΔR (Supplementary Figure 4.1D) strains. We were unable to generate an active and SOS-responsive reporter of an R-pyocin sheath, possibly due to the R-pyocin structural genes acting as a single transcriptional unit under the regulation of a single promoter.

To evaluate differences in response to various agents known to induce R-pyocins, we tested our reporters under the induction of ciprofloxacin at 0.1 μg/mL, mitomycin C at 3 μg/mL, and R2-pyocins. We found that each agent varies in the magnitude (Figure 4.3A) and the timing of regulatory influence (Figure 4.3B). Mitomycin C induced the strongest response in holin activity, while the addition of R2-pyocins (produced by the wild type PAO1) did not elicit detectable activity. The lysin putative regulatory region was active under all conditions; however, it was not significantly responsive to SOS induction by any of the agents used.

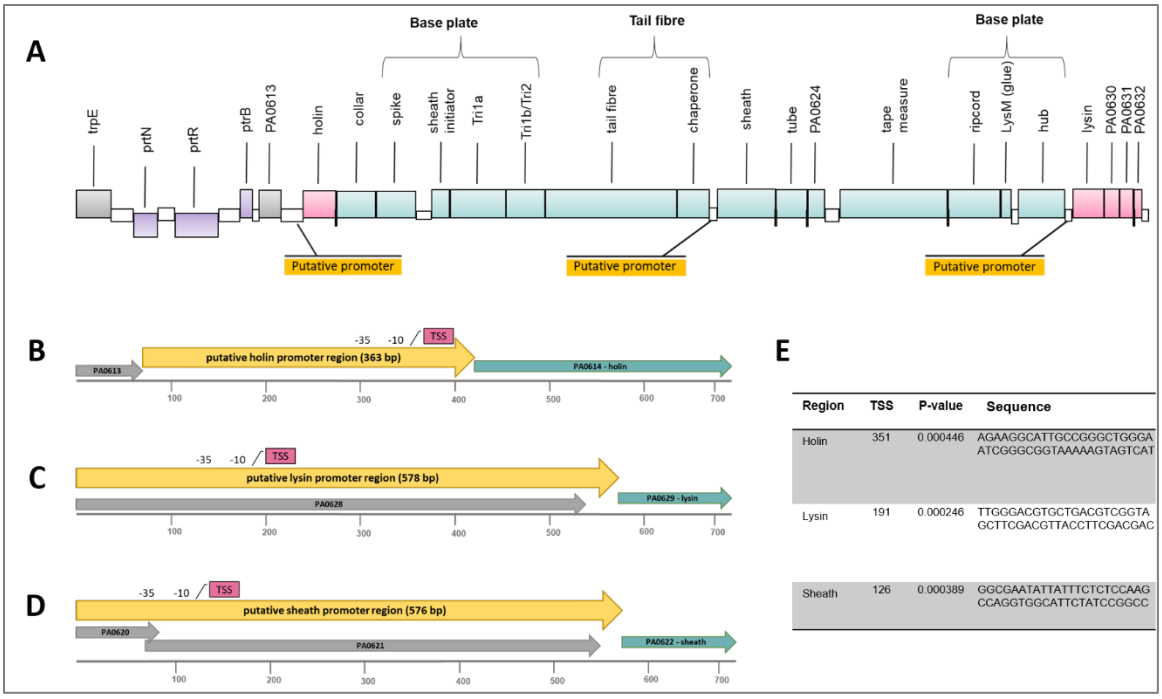


Figure 4.1: R-pyocin genes & reporter composition. The R-pyocin gene cassette and putative regulatory regions. The R-pyocin genes (A) are located between trpE (PA0609) and the F-pyocin genes (PA0633-PA0648) on the chromosome of *P. aeruginosa*. Structural genes are indicated in cyan, lytic genes are indicated in magenta, and known transcription factors are indicated in purple. Using SAPPHIRE to predict promoters and regulatory regions of interest, we generated and tested (B) a 363 bp region upstream of the holin gene (PA0614), (C) a 546 bp region upstream of the lysin (PA0629) gene, and (D) a 576bp region upstream of the sheath (PA0622) gene. The SAPPHIRE predicted promotor features including -35 box, -10 box, and predicted transcription start site (TSS) are included for reference (E).

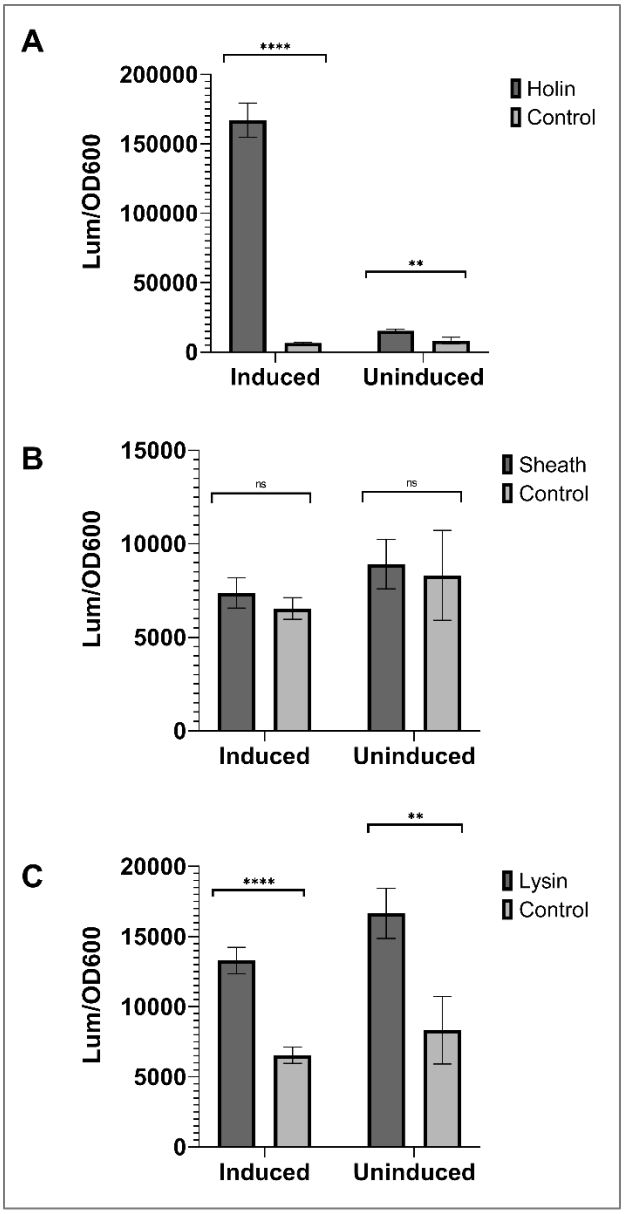


Figure 4.2: The holin is the most active gene of R-pyocin cassette during SOS induction. Using PAO1, we constructed transcriptional-lux reporters under the control of the putative promoter regions preceding the (A) holin (PA0614), the (B) sheath (PA0622) and the (C) lysin (PA0629) in the insertion vector pMini-CTX-lux and inserted each into the attCTX site by electroporation to observe the timing of transcriptional activity of each following SOS induction. We measured luminescence and optical density (at 600nm) simultaneously for up to 10 hours to assess transcriptional activity both under induction with ciprofloxacin (0.1 µg/mL) and without induction. We compared peak activity between each reporter strain with an empty insertion vector control to assess transcriptional activity of each sequence. Significance between each condition was determined with a Welch's t test (**p<0.005, ****p <0.0001).*

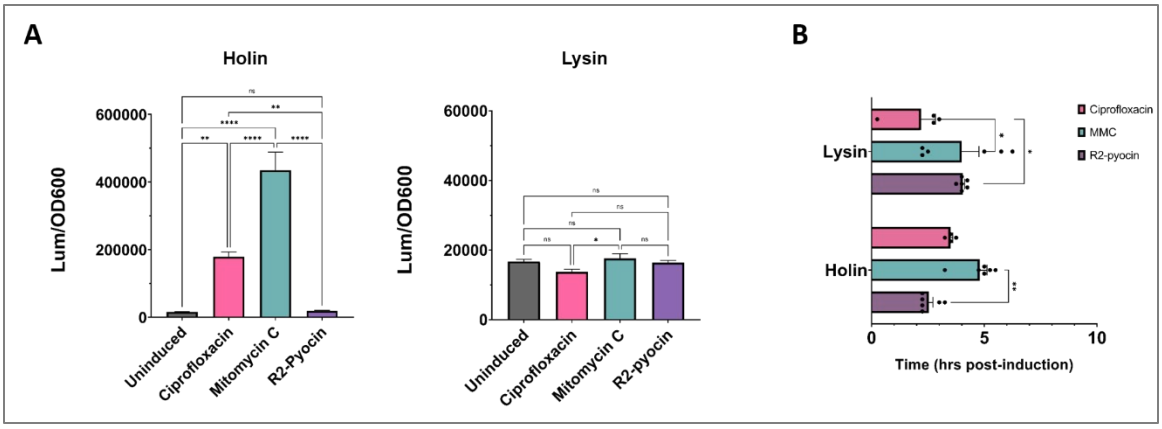


Figure 4.3: R-pyocin induction agents vary in magnitude and timing of regulatory influence. We measured luminescence and optical density at 600nm simultaneously for up to 10 hours to assess transcriptional activity of the holin (PA0614) and the lysin (PA0629) under induction with ciprofloxacin (0.1 µg/mL), mitomycin C (3 µg/mL) and R2-pyocins. (A) Mitomycin C was determined to be the most effective agent for inducing R-pyocin activity, though the lysin is not as influenced by SOS-inducing agents as the holin putative promoter sequence. (B) The holin and the lysin putative promoters peak in transcriptional activity peak between 2.5-5 hours post-induction, and each SOS-induction agent varies in the timing of peak activity. Significance was evaluated between agents for each reporter with a one-way ANOVA and differences in peak activity timing was analyzed with a two-way ANOVA (*$p<0.05$, **$p<0.005$, ****$p<0.0001$).

4.3.2. Nutritional Environment Influences Regulatory Activity of R-Pyocins.

To evaluate R-pyocin transcriptional activity in an environment relevant to infection, we tested transcriptional reporters using SCFM – a defined medium developed to mimic the nutritional composition of CF sputum [32]. We found that the R-pyocin-associated holin is more transcriptionally active in CF infection-relevant media (SCFM) than in nutrient-rich lysogeny broth (LB) medium (Figure 4.4A). Even when un-induced

the putative holin promoter region is approximately 3-fold more active in SCFM than in LB. We also found that the holin takes longer to reach peak transcriptional activity in SCFM (5 h) when un-induced compared to when in LB medium (3.5 h) (Figure 4.4B).

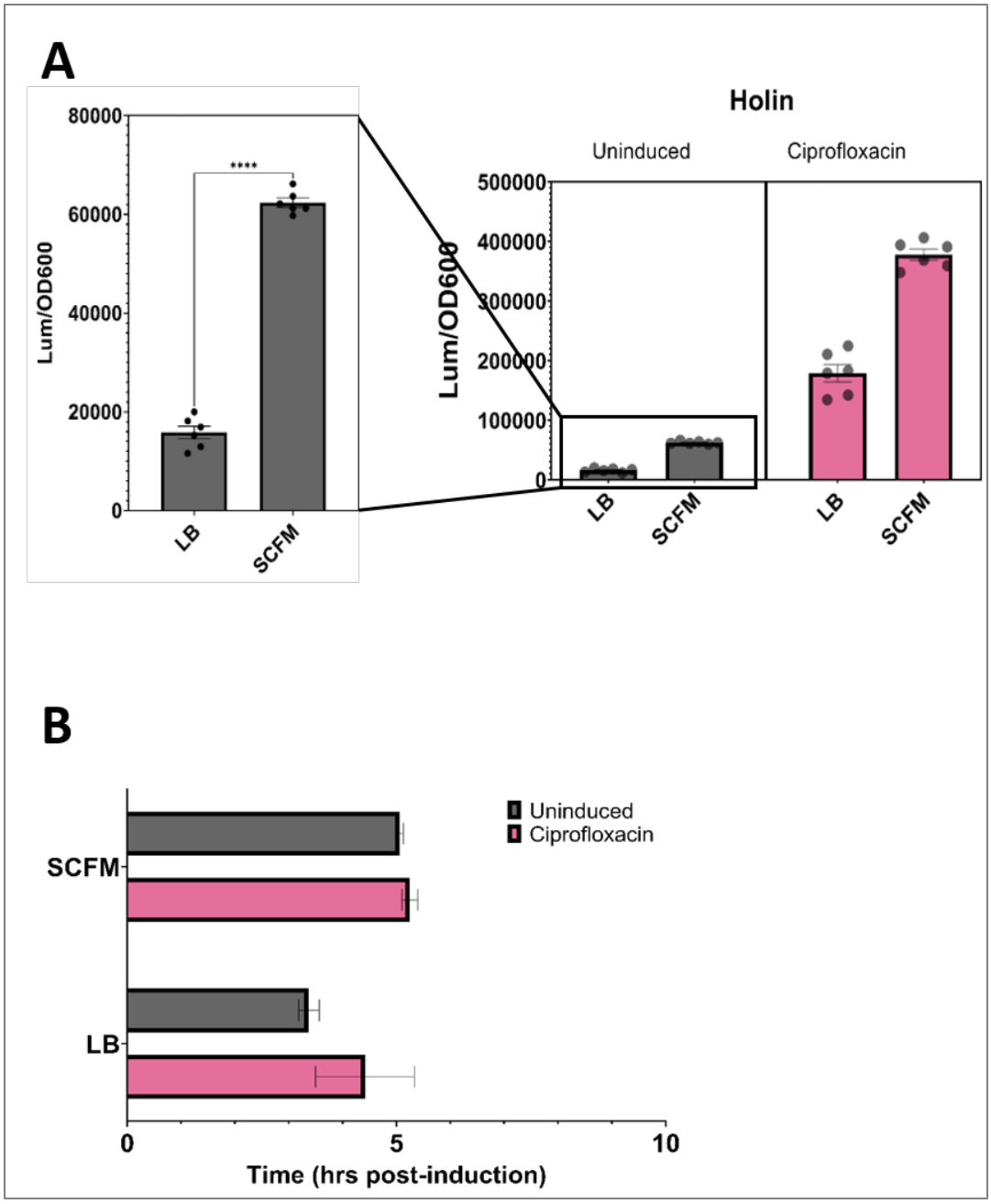
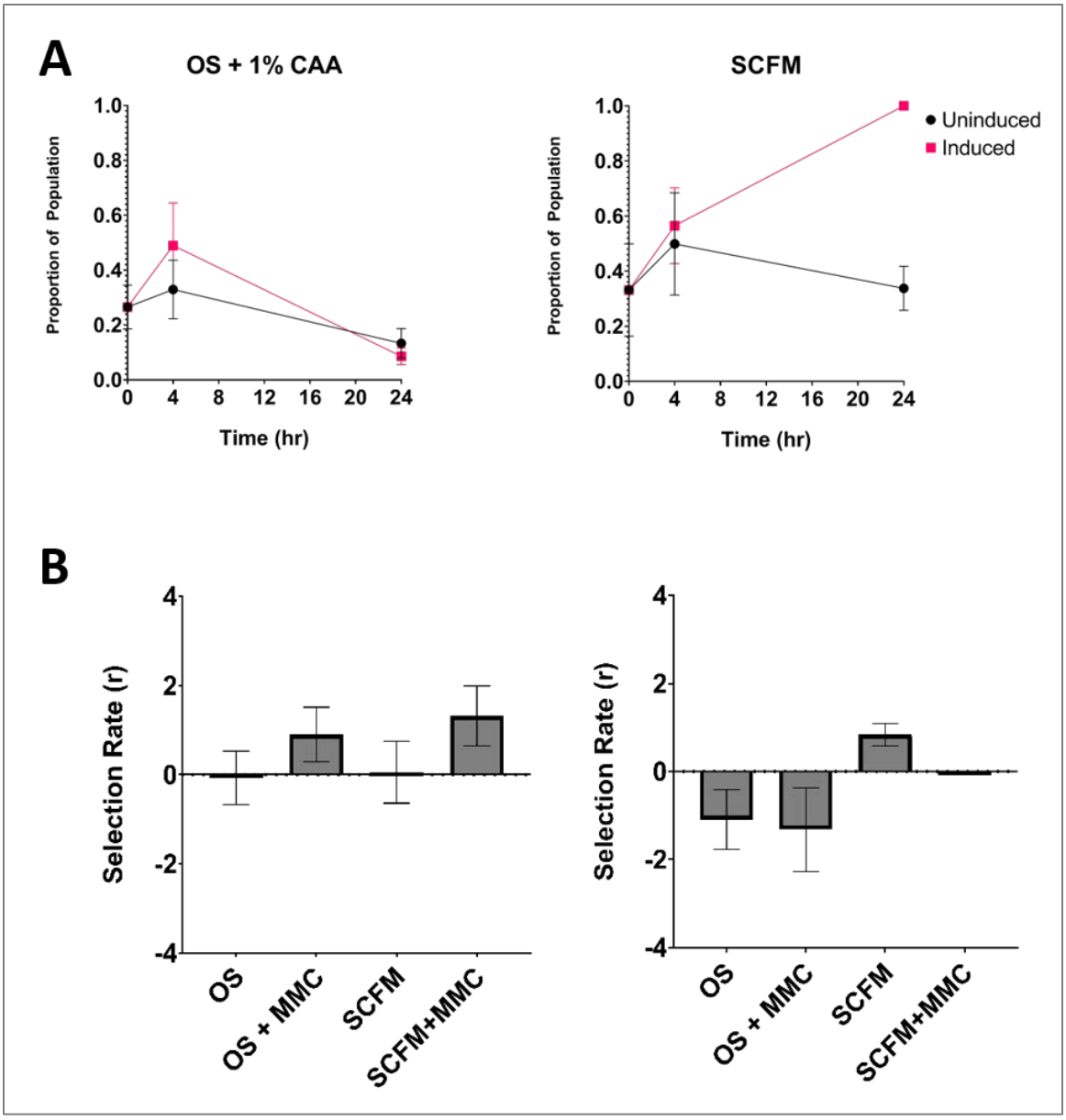


Figure 4.4: R-pyocin-associated holin (PA0614) is more transcriptionally active in infection-relevant media (SCFM) than in nutrient-rich LB medium. We measured transcriptional activity of the holin putative promoter region with and without induction by ciprofloxacin (0.1 µg/mL) in LB and Synthetic Cystic Fibrosis Medium (SCFM) for up to 6 hours. The R-pyocin-associated holin (PA0614) is (A) more transcriptionally active though (B) takes longer to reach peak transcriptional activity in infection-relevant media (SCFM) than in nutrient-rich LB medium. We determined significance with a paired, two-tailed Student's T-test (**** $p<0.0001$).

4.3.3. R-Pyocin Induction Mediates Competition Between Strains During Planktonic Growth in Infection Relevant Media.

After finding R-pyocin genes to be more transcriptionally active in SCFM than LB, we sought to investigate the influence of R-pyocins on competition in SCFM planktonic growth (without mucin or DNA). We grew equal inoculations of PAO1 WT (R2-pyocin producer, R2-pyocin resistant) and a PAO1 *ΔrmlC ΔR* strain (susceptible to R2-pyocins and cannot produce its own functional R-pyocins) in planktonic competition. We used two types of minimal media to determine if the increase in R-pyocin induction could be due to nutrient limitation in general or if the difference is CF-nutrient specific. We used OS media supplemented with 1% casamino acids [33] and SCFM without polymer. As we found mitomycin C to be the most effective induction agent, we grew strains both with and without mitomycin C at 3 µg/mL [34]. We found that when R-pyocins are induced and around peak R-pyocin activity (approximately 4 h), the R-pyocin producing PAO1 WT increased in population proportion (Figure 4.5A) and was more fit than the R-pyocin susceptible strain PAO1 *ΔrmlC ΔR* (Figure 4.5B). Under the same conditions, a R-pyocin null strain, PAO1 ΔR [30], decreased in the co-culture (Figure 4.5C) and was less fit than the susceptible strain (Figure 4.5D). This fitness effect ultimately determined the fate of the co-culture in SCFM, as after 24 h, the WT PAO1 took over the co-culture, whereas the PAO1 ΔR strain was outcompeted.


A
OS + 1% CAA
SCFM
Proportion of Population
Time (hr)
Uninduced
Induced
B
Selection Rate (r)
OS
OS + MMC
SCFM
SCFM+MMC

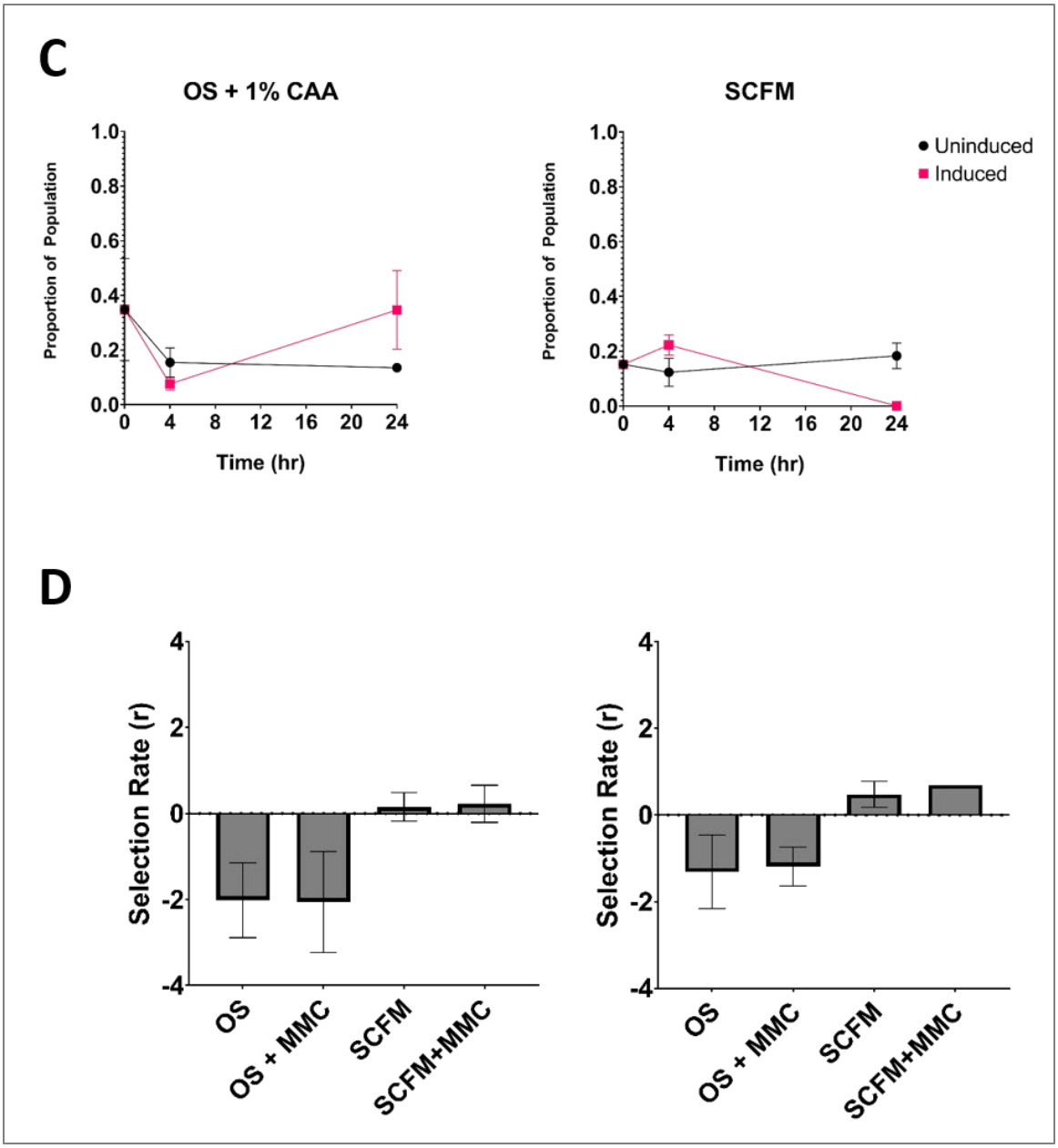


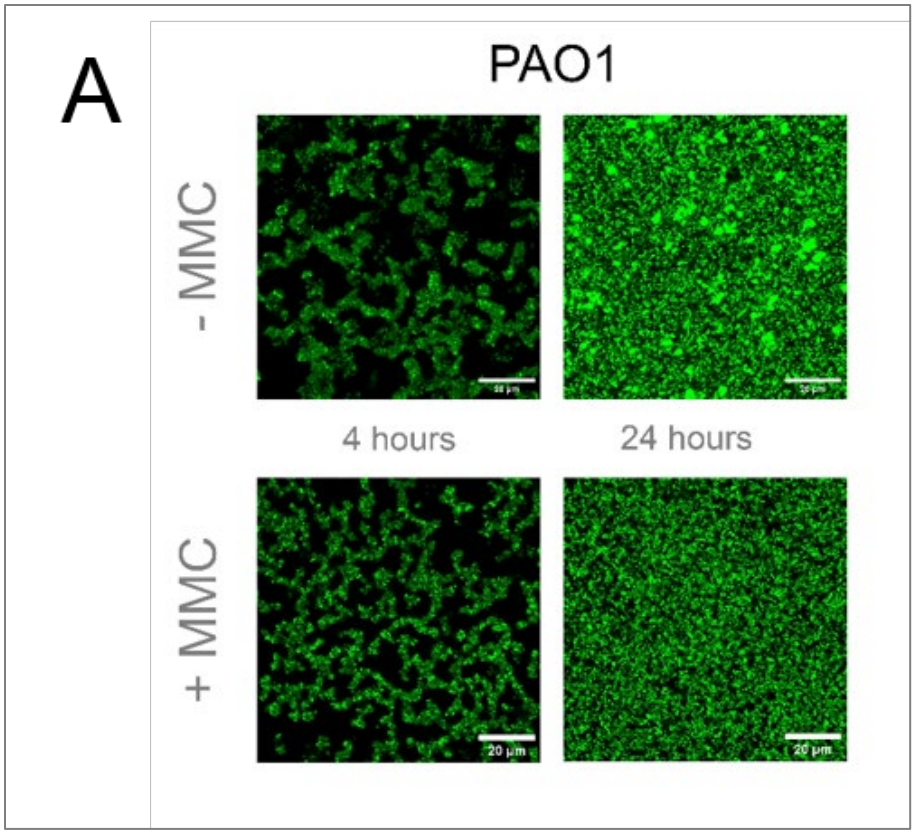
Figure 4.5: R-pyocin induction mediates competition between strains in planktonic growth. (A) Proportion of PAO1::PAO1 *ΔrmlC ΔR* and (C) PAO1 ΔR::PAO1 *ΔrmlC ΔR* in planktonic growth, with and without mitomycin C (3 μg/mL) for R-pyocin induction. (B) The selection rate constant (*r*) as a metric of fitness of PAO1 and (D) PAO1 ΔR in OS + 1% CAA or SCFM media, with and without mitomycin C for R-pyocin induction measured by counting CFUs at 4 hours (left) and 24 hours (right) post-induction.

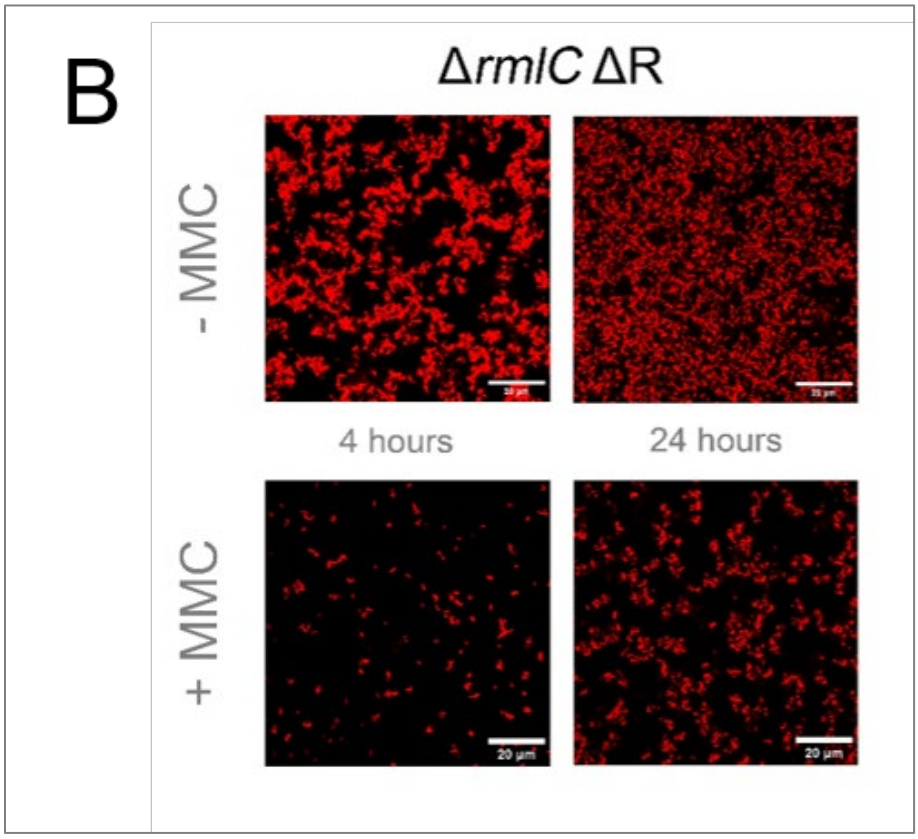
4.3.4. R-Pyocin Induction Does Not Benefit Producing Strain in Infection-Like Biofilm.

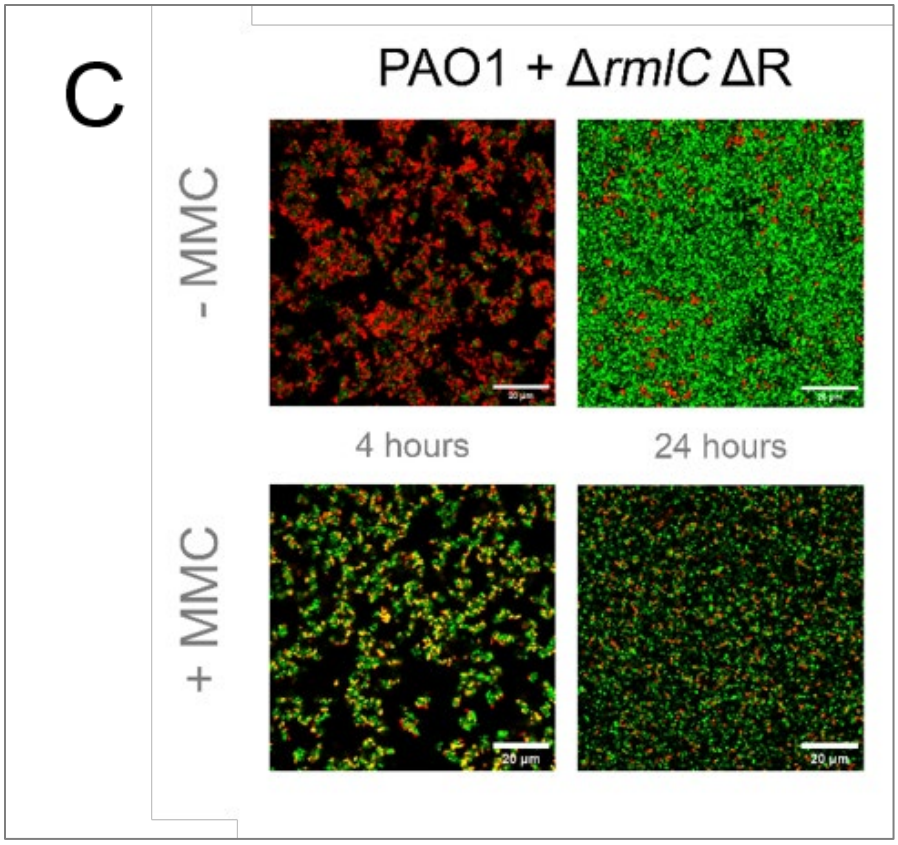
Continual movement in planktonic growth aids in distribution of particles, including R-pyocins, however bacteria grow in structured biofilms as aggregates in infection [2-10]. To evaluate the fitness impact of R-pyocin induction in structured competition similar to an infection-relevant environment, we then grew equal inoculations of PAO1 WT (R-pyocin producer, resistant) and PAO1 *ΔrmlC ΔR* R-pyocin (non-producer, susceptible) in SCFM with 0.6 mg/mL of DNA as a structuring polymer, both with and without mitomycin C at 3 µg/mL as an induction agent [23, 32, 34]. We imaged and sampled these mono-cultures (Figure 4.6A and Figure 4.6B) and co-cultures (Figure 4.6C) at 4 h and 24 h post-inoculation/induction. We repeated these experiments with the R-pyocin null strain PAO1 ΔR and imaged mono-cultures (Figure 4.6D and Figure 4.6E) and the co-culture (Figure 4.6F) at 4 h and 24 h post-inoculation/induction as well.

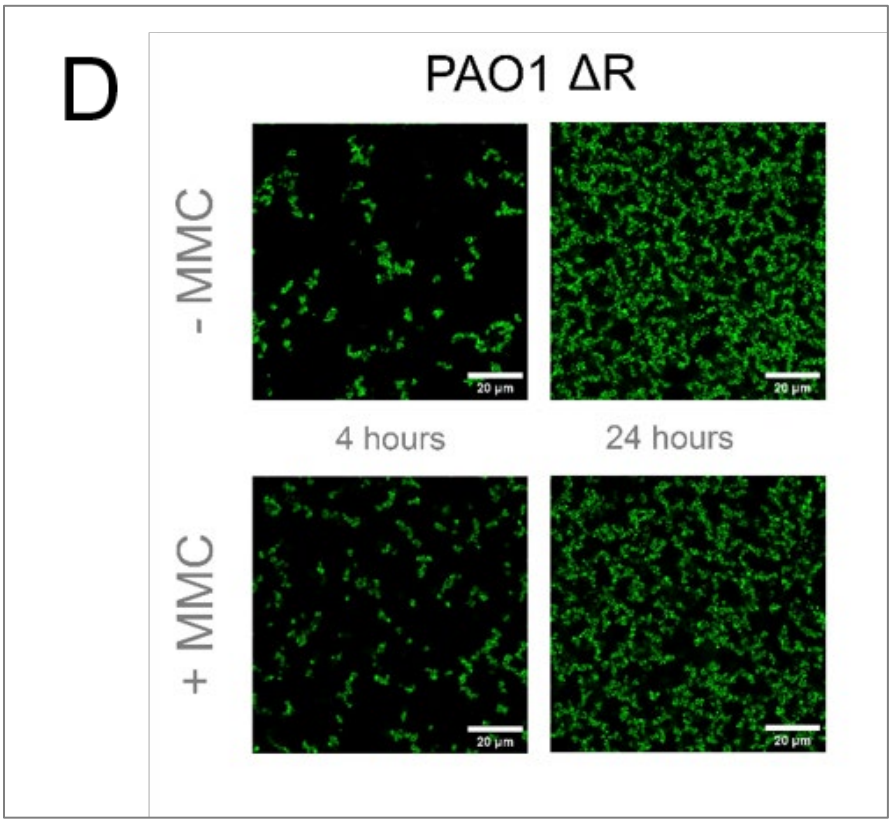
Image analysis showed the WT R-pyocin producer outcompeted the susceptible strain under control conditions, however, it was not able to significantly outcompete the susceptible strain when R-pyocins were induced with mitomycin C (Figure 4.7A). The R-pyocin null strain was unable to significantly outcompete the susceptible strain under either condition (Figure 4.7B). Cultures were sampled for colony forming units (CFUs) immediately following imaging at 4 h (Figure 4.7C) and 24 h (Figure 4.7D) post-inoculation/induction and the selection rate constant (*r*) was calculated as a metric for relative fitness. CFU data suggested that at both time points, R-pyocin induction with mitomycin C negated growth differences between strains. Although image analysis suggested that the WT strain was able to outcompete the R-pyocin susceptible strain PAO1

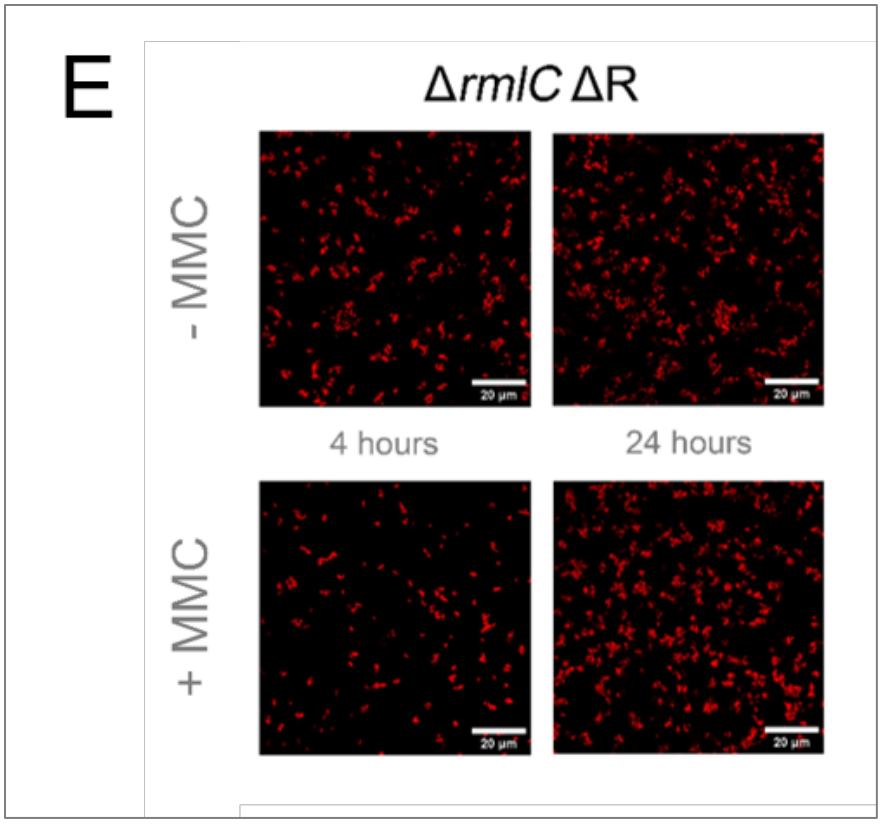
Δ*rmlC* ΔR without induction, CFU data suggested that the WT strain was not necessarily more fit; after 4 hr post-inoculation, the WT strain grew approximately 1-2 natural logs worse than PAO1 Δ*rmlC* ΔR, and both strains grew at a similar rate by 24 h. Monoculture experimental images of PAO1 (Supplementary Figure 4.2A), PAO1 ΔR (Supplementary Figure 4.2B) and PAO1 Δ*rmlC* ΔR (Supplementary Figure 4.2C) were analyzed for percentage of fluorescent area (% Area) when grown with and without mitomycin C.


A
PAO1
- MMC
4 hours
24 hours
+ MMC
20 µm
20 µm


B
ΔrmlC ΔR
- MMC
4 hours
24 hours
+ MMC
20 µm
20 µm


C
PAO1 + ΔrmlC ΔR
- MMC
+ MMC
4 hours
24 hours
20 µm


D
PAO1 ΔR
- MMC
+ MMC
4 hours
24 hours
20 µm


E
ΔrmlC ΔR
- MMC
+ MMC
4 hours
24 hours
20 µm

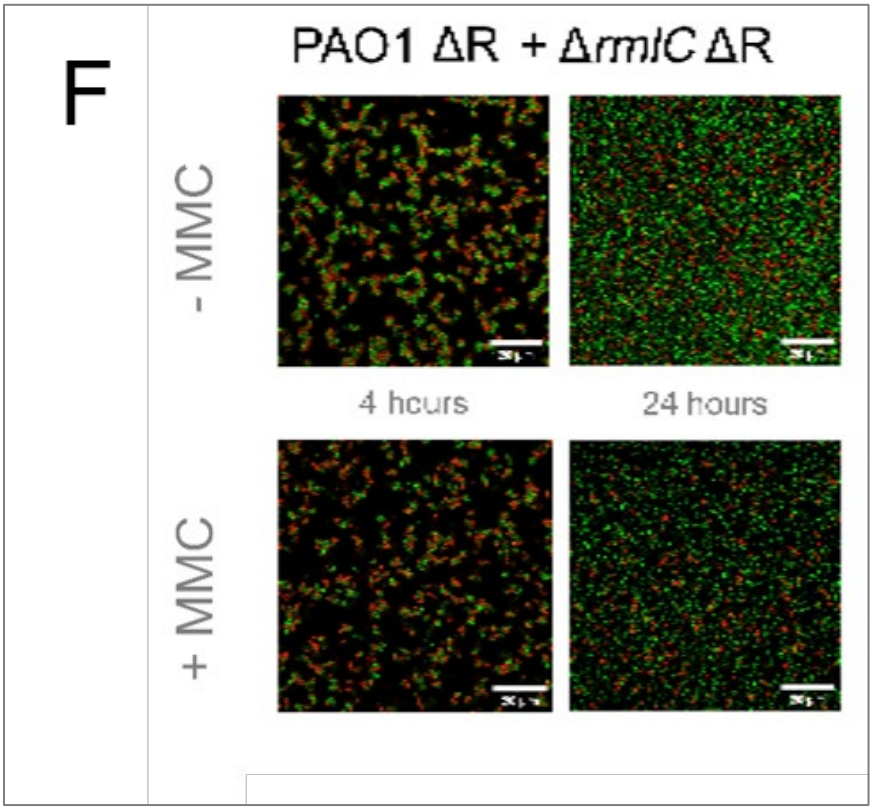

F
PAO1 ΔR + ΔrmlC ΔR
- MMC
+ MMC
4 hours
24 hours

Figure 4.6: R-pyocin producing and R-pyocin susceptible *P. aeruginosa* strain growth in SCFM2 with and without mitomycin C. With confocal microscopy, we imaged monocultures of the R-pyocin producing strain (PAO1) (A) and the R-pyocin-susceptible strain (PAO1 *ΔrmlC ΔR*) (B) without induction (top row) and with R-pyocin induction (bottom row), and co-cultures with and without induction with mitomycin C. Scale bars depict 20 μm.

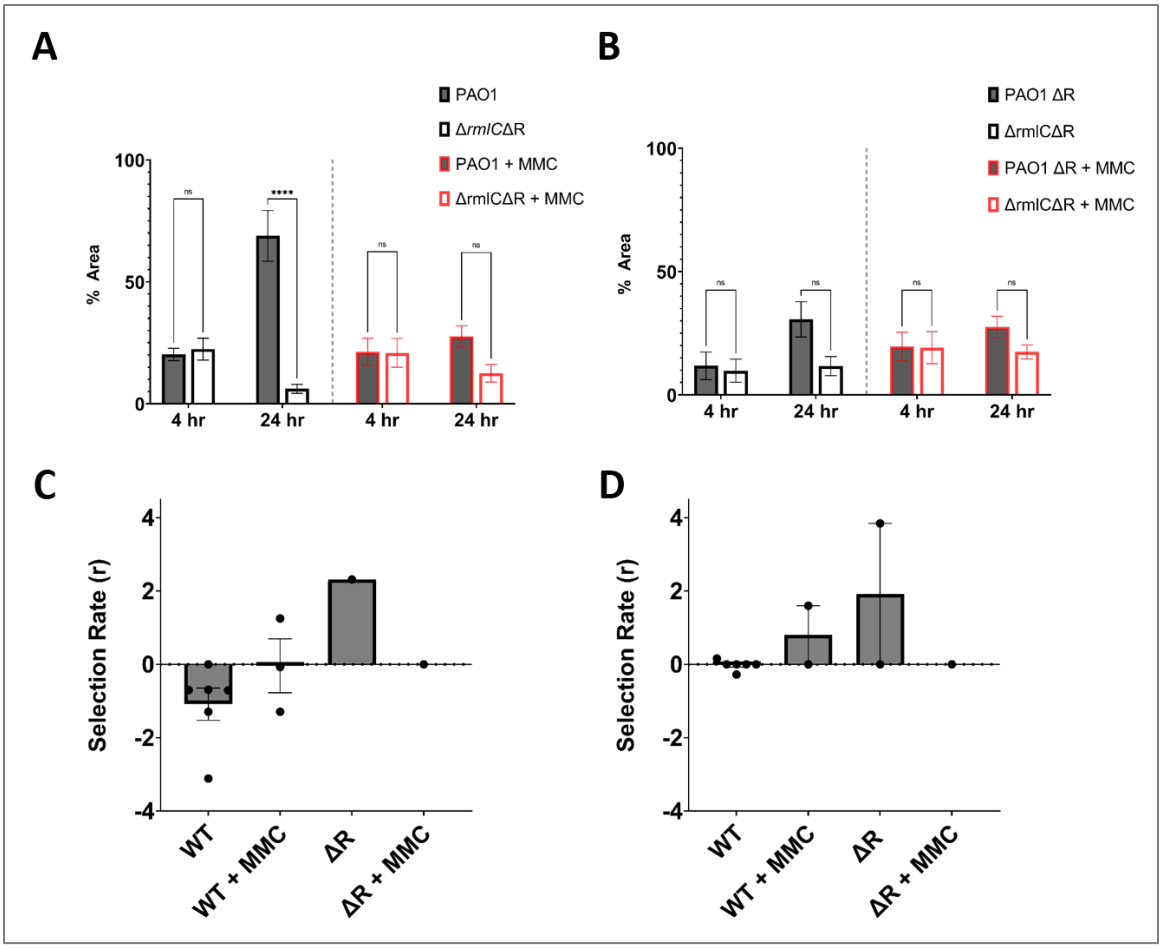


Figure 4.7: R-pyocin induction does not benefit the producing strain in a structured, infection-like environment. We analyzed confocal microscopy images and compared Percentage of fluorescent area (% Area) was measured from images taken at 4 hours (A) and 24 hours (B) post inoculation and induction. Significance was analyzed with a two-way ANOVA. At 0 hours, 4 hours, and 24 hours post inoculation and following imaging, cultures were sampled for colony forming units (CFUs) to assess fitness by selection rate (*r*) of the R-pyocin producing or null strain relative to the R-pyocin susceptible PAO1 *ΔrmlC ΔR* in a spatially structured, infection-like environment (SCFM) at 4 h (C) and 24 h (D). (*****p<0.0001*).

4.4. Discussion

Bacteriocins antimicrobials have long been studied for their role in microbial competition however the role of bacteriocins in infection is still poorly understood. Cystic fibrosis chronic lung infections are one type of chronic infection known to be difficult to eradicate and harbor multiple species and strains. These chronic infections are also known for being dominated by primarily one strain of *P. aeruginosa,* though the mechanisms behind this single-strain takeover are still poorly understood [11-15]. R-pyocins, a phage tail-like bacteriocin produced by *P. aeruginosa* have been proposed to be one type of mechanism of intra-strain competition that could play a role in determining the primary infectious strain [16-19], however the *in vivo* regulatory behavior and timing of R-pyocin induction in the CF lung environment are still unknown.

While it is thought that R-pyocins reach target cells by diffusion, there has been little work done to evaluate how R-pyocins may influence strain competition and growth in CF sputum. Even less is known regarding the timing and regulation of R-pyocins in CF lung infections. In this work, we assessed R-pyocin activity in an infection-relevant environment (Synthetic Cystic Fibrosis Sputum Medium; SCFM2) and found that (i) R-pyocins genes are transcribed more in the CF nutrient environment than in rich laboratory medium and (ii) in a structured, CF-like environment, R-pyocin induction is costly to producing strains in competition rather than beneficial. Our work suggests that R-pyocins may not be essential to CF lung infection and can be costly to producing cells in the presence of stress response-inducing stimuli, such as those commonly found in infection.

While R-pyocin regulatory behavior has been studied before, this behavior has not yet been characterized in nutrient-limited, infection-relevant conditions yet is crucial for

understanding the ecological pressures shaping chronic infection [24, 35-41]. To evaluate R-pyocin activity in an infection-relevant environment, we generated transcriptional reporters of three R-pyocin associated genes (holin PA0614; lysin PA0629; sheath PA0622) and tested them in SCFM, a media replicating the nutritional composition of CF sputum. We found that R-pyocin related genes were approximately three times more active in SCFM than in LB, even when no R-pyocin induction agent was added to the medium (Figure 4.4). As pathogens likely encounter a number of SOS-response inducing stimuli over the course of infection, we also tested our holin reporter under the induction of several different agents, found that mitomycin C induced higher R-pyocin related activity than ciprofloxacin, while the addition of R2-pyocins did not influence R-pyocin activity (Figure 4.3). It is important to note that R2-pyocins were added, and the strains used were R2-pyocin resistant (as the same strain background was used for the isolation of added pyocins). This data suggests that i) R-pyocins do not induce the SOS response nor R-pyocin induction in cells that are resistant, ii) mitomycin C is a much stronger and more effective R-pyocin induction agent than ciprofloxacin, and iii) the CF sputum nutritional environment induces higher R-pyocin activity than nutrient-rich conditions. This finding implies that the CF nutritional environment alone induces a stress response that upregulates R-pyocin activity, even without the addition of a DNA-damaging stimulus.

R-pyocins have been shown to play a role in strain competition in flow cells or biofilms [19, 38, 42-46], however it is unknown how well R-pyocins can diffuse in a spatially structured and nutrient-limited environment, such as CF sputum, with polymers (DNA) creating a matrix capable of inhibiting the diffusion of particles. To test the influence of R-pyocin induction on strain competition in an infection-relevant context, we

first showed that R-pyocin induction does contribute to competition in well-mixed, planktonic cultures in SCFM (Figure 4.5).

We then grew the same strains in SCFM with DNA to determine how R-pyocin induction influences strain growth in the structured, nutrient-limited environment. Through this work we found that while the R-pyocin producer was able to easily outcompete the R-pyocin susceptible strain when no induction agent was present, when mitomycin C was added to induce R-pyocin production the response and resulting lysis reduced competition so that neither strain could take over. R-pyocin induction was not beneficial in structured, SCFM, but was actually a cost to the producing strain so that it could no longer take over (Figure 4.7). By 24 hours both strains were left with few replicating cells (as per CFU data), though we were able to visualize strain growth prior to the effects of mitomycin C via imaging as the non-replicating cells still fluoresced until lysis (Figure 4.6). This suggests that while it may be beneficial in competition for *P. aeruginosa* strains to be able to produce R-pyocins in infection, however if an SOS-inducing stimulus is present, the resulting induction is too costly for the cells to succeed in total niche acquisition. The costs of R-pyocin production has been examined before, primarily in the context of small, culture-impaired colonies being formed by strains developing mutations facilitating resistance and protection from R-pyocins [38, 45]. However, this is the first work to our knowledge that has shown the cost of R-pyocin induction from an ecological perspective, on strain competition in a CF-like environment.

In previous work, we genotyped 849 *P. aeruginosa* genomes publicly available as a part of the International *Pseudomonas* Consortium Database (IPCD) and found that, 40.8% of strains isolated from respiratory infection sources (including cystic fibrosis)

lacked R-pyocin genes (PA0615-28) entirely [30, 31]. This absence of R-pyocin genes in infectious strains, implies that perhaps R-pyocins may not be essential for survival in chronic infection, and specifically in chronic CF lung infection. As we also identified a few strains possessing only partial R-pyocin genes, as if in a state of decay [30]. As R-pyocin genes are not mentioned in many transcriptomic and proteomic studies, this may be because the infecting strains have lost R-pyocin genes, are evolving to lost R-pyocin genes, or have evolved to lose activation of R-pyocin genes, rather meaning there is a lack of R-pyocin activity in the infection.

Further evidence suggesting the loss of R-pyocin genes in the CF lung environment can be found in transcriptomes sampling CF sputum [47, 48]. Out of a total 15 published CF sputum transcriptomes, 9 of these samples (60%) do not contain any detectable reads for R-pyocin structural genes (PA0615-PA0628), yet all exhibit some reads for the R-pyocin-associated lytic genes (PA0614, PA0629-PA032) and the adjacent regulatory genes (PA0612-PA0613) [47, 48]. With all structural genes of the R-pyocin operon lacking reads, it is likely that the strain(s) in these samples have lost these genes. Another 5 (20%) of these transcriptomes include reads for some R-pyocins structural genes, but do not give reads for others, suggesting they may be in a state of decay [47, 48]. In proteomic sampling of 35 sputum collections from 11 individuals with CF, the R-pyocin sheath protein (PA0622) was identified with a Fold Change of 2-fold or greater in only 3 of these sputum samples [49]. This suggests that i) few individuals in this cohort were infected with *P. aerguginosa* strains producing R-pyocins and ii) in the sputum of the individual(s) with *P. aeruginosa* that did produce R-pyocins, R-pyocin activity was induced [49].

When evaluating transcriptomic reads from *in vitro* sampling in Synthetic Cystic Fibrosis Sputum Media (SCFM) with the wild type laboratory strain PA14, all samples produce reads for the R-pyocin-associated regulatory genes, lytic genes, and structural genes (PA0612-32) [47, 48]. Most of these genes show some degree of upregulation in the artifical CF sputum environment, suggesting that the nutritional environment of the CF lung does in fact induce R-pyocin production in strains with an intact R-pyocin cassette [22, 47, 48].

Albeit limited, genomic, transcriptomic, and proteomic data of R-pyocin genes and regulatory activity related to CF strains and chronic lung infection seem to tell a story of a stressful enviornment in which R-pyocin production is too costly for survival. It is possible that *P. aeruginosa* in chronic infections like CF may be under selective pressure to be rid of R-pyocin production, however more work is necessary to test this hypothesis. Our work shows that in a structured, CF-like environment, R-pyocin induction is costly to producing strains in competition rather than beneficial, suggesting that R-pyocins may not be essential to CF lung infection and providing ecological evidence as to why a significant portion of *P. aeruginosa* strains from chronic infection lack R-pyocin genes. This work contributes to our understanding of the role of bacteriocins in chronic infection and illuminates another facet of how the infection environment influences competitive measures of pathogens.

4.5. Methods

4.5.1. Bacterial Strains, Media, and Culture Conditions.

All *P. aeruginosa* strains used in this work are listed and described in Supplementary Table 4.1. All bacterial cultures were grown in lysogeny broth (LB)

medium, OS medium with 1% Casamino acids [33], or Synthetic Cystic Fibrosis Sputum Media (SCFM) [32] at 37°C with 200 rpm shaking agitation or at static growth. Standard genetic techniques were used 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 Supplementary Table 4.2.

4.5.2. Generation of Mutants and Reporter Strains.

Reporter strains were constructed as follows: DNA sequences 363 bp, 578 bp, and 576 bp upstream of loci PA0614, PA0620, and PA0629 respectively were PCR amplified using Q5 DNA polymerase (New England Biolabs, Ipswich, MA). Upstream sequences were input into SAPPHIRE [50] to evaluate potential as a promoter region and identify likely regulatory elements. These sequences were then cloned into the EcoRI-HindIII digested insertion vector pMini-CTX-lux [51] 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 10 µg/mL tetracycline. Cloned inserts were verified by colony PCR and Sanger sequencing (Eton Bioscience Inc., NC). Once verified, reporter constructs were inserted into PAO1 by electroporation and strains carrying insertions were selected on LB agar plates supplemented with 200 µg/mL tetracycline [52]. Reporter candidates were assessed for presence of the inserted sequence by colony PCR and Sanger sequencing of the PCR product.

For competition assays, PAO1 WT [53] and PAO1 ΔR were electroporated with pME6032 [54] with constitutively expressed green fluorescent protein (GFP), then selected for tetracycline resistance. Both strains were then given gentamycin resistance by

electroporating the Tn7 helper plasmid pTNS3 [55] with the empty pUC18-miniTn7T-Gm insertion vector [56], then selecting for resistant colonies on LB plates supplemented with 100 µg/mL of gentamycin and 200 µg/mL tetracycline to ensure maintenance of fluorescent reporter plasmids.

PAO1 *ΔrmlC ΔR* mutant was constructed 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)[46]. These sequences were cloned into the SphI-XbaI digested suicide vector pDM4 [57] by Gibson assembly using NEBuilder HiFi Assembly master mix (New England Biolabs), transformed into *E. coli* S17-λpir by electroporation and selected on LB agar plates supplemented with 30 µg/mL chloramphenicol. Cloned inserts were verified by colony PCR and Sanger sequencing (Eton Bioscience, NC). Deletion constructs were introduced into PAO1 by electroporation and strains carrying single crossover insertions of the deletion constructs were selected on LB agar plates supplemented with 300 µg/mL chloramphenicol [52]. Resistant colonies were cultured in LB without antibiotic and plated on LB agar plates with 0.25% NaCl and 5% sucrose to select for loss of the construct [58]. Sucrose-resistant colonies were screened for antibiotic 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. After generating the PAO1 Δ*rmlC* mutant, the R-pyocin tail fibre gene and chaperone (PA0620-1) were deleted using the same method [30]. The PAO1 ΔPf4 strain was generously gifted by Patrick Secor [59], and the PAO1 ΔPf4 ΔR strain was generated by deleting the R-pyocin tail fibre gene and chaperone (PA0620-1) as previously described [30].

PAO1 Δholin and Δlysin mutants were constructed as follows: 750 bp DNA sequences flanking the open reading frames of PA0614 and PA0629 respectively, were PCR amplified using Q5 DNA polymerase (New England Biolabs, Ipswich, MA)[46]. These sequences were cloned into the KpnI-XbaI digested suicide vector pEXG2 [60] by Gibson assembly using NEBuilder HiFi Assembly master mix (New England Biolabs), transformed into *E. coli* S17-λpir by electroporation and selected on LB agar plates supplemented with 15 μg/mL gentamycin. Cloned inserts were verified by colony PCR and Sanger sequencing (Eton Bioscience, NC). Deletion constructs were introduced into PAO1 by electroporation and strains carrying single crossover insertions of the deletion constructs were selected on LB agar plates supplemented with 150 μg/mL gentamycin [52]. Resistant colonies were cultured in LB without antibiotic and plated on LB agar plates with 0.25% NaCl and 5% sucrose to select for loss of the construct [58]. Sucrose-resistant colonies were screened for antibiotic 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.

4.5.3. Expression and Collection of R-pyocins

R-pyocins were extracted in lysates as previously described [30]. LB cultures of PAO1 ΔPf4 and PAO1 ΔPf4 ΔR (R-pyocin null) were used to ensure no killing activity would be attributed to pf4 prophage activity. Cultures for both strains were inoculated at 1:100 from overnight planktonic cultures, and grown to mid-logarithmic growth phase (approximately 4 h) in 10 mL of LB media. Ciprofloxacin was then added to each culture for a final concentration of 0.1 μg/mL to induce R-pyocin production [21, 30, 38], and cultures were incubated for a further 3 h. Chloroform was used to lyse remaining cells, and

lysates were centrifuged at ~3,300 *x g* for 10 min. The R-pyocin-containing supernatant was separated and stored at 4°C.

4.5.4. Microtiter Plate Method for Promoter Activity.

Promoter activity was measured using a 96-well microtiter plate, by normalizing changes in luminescence to changes in growth (optical density at 600nm). Using mid-logarithmic growth phase cultures of the strains (grown separately), each well of the plate contained LB broth or SCFM (without mucin or DNA) and was inoculated to an adjusted OD_{600} of 0.1. Then 10 µL of either R2-pyocin lysates (isolated from PAO1 ΔPf4), 2 µg/mL ciprofloxacin (for final concentration of 0.1 µg/mL), 60 µg/mL mitomycin C (final concentration of 3 µg/mL) as added for a total volume of 200µL in each well. Cultures measured also included wells of each strain with no inducing agent added, cultures with PAO1 ΔPf4 ΔR lysates, and cultures with media and induction agents only as controls. Using a BioTek Synergy H1, luminescence and OD_{600} were measured for each well every 20 min over a total of 10 h while incubating at 37°C with 200 rpm orbital shaking.

4.5.5. Planktonic Competition Assay

Relative fitness of R-pyocin-producing strains in planktonic competition was measured using 24-well cell culture plates for up to 24 h following inoculation and R-pyocin induction. We grew each strain in Luria Broth (LB) supplemented with 200 µg/ml of tetracycline and 100 µg/ml of gentamycin (when appropriate) overnight in flasks. We washed overnight cultures of each strain twice in phosphate buffered saline (PBS) before concentrating 100,000-fold, and inoculating 1.5 mL of either OS media supplemented with 1% CAA or SCFM (without DNA or mucin) with 20 µL of concentrated cells

(approximately 10^8 CFU/mL). Monocultures of each strain were grown alongside co-cultures and sampled for colony forming units (CFU/mL) at 0 h, 4 h, and 24 h post inoculum. For R-pyocin-induced cultures, mitomycin C was added to a final concentration of 3 μg/mL immediately following inoculation. Planktonic assays were conducted in biological triplicates.

4.5.6. Image Acquisition and Analysis of Structured Competition Assay

We evaluated the fitness of R-pyocin-producing strains in a structured environment by growing aggregates of competing strains in SCFM with 0.6 mg/mL of DNA (without mucin). Inoculum was prepared as described prior, by growing each strain overnight in Luria Broth (LB) supplemented with 200 μg/ml of tetracycline and 100 μg/ml of gentamycin (when appropriate), washed, then concentrated 100,000-fold before inoculating. Approximately 10^8 CFU/mL of cells were inoculated into 400 μL of freshly made SCFM containing 0.6 mg/ml of DNA [32]. Cultures were incubated static at 37°C for 4 h and 24 h in chamber slides (Lab-Tek I) before image acquisition. An LSM880 confocal microscope was used to image, equipped with a 63× oil immersion lens. Aggregates were viewed with a diode laser at 488 nm, and fluorescence emission between 480 and 530 nm was used for imaging cells with green fluorescent protein (GFP) or mCherry. For image analysis, we used FIJI (ImageJ) [61] software to analyze the percentage of fluorescent area (% Area) of 12 images acquired for each strain and co-culture in six independent experiments; a total of 260 images were analyzed. Following imaging, each culture was collected and serial diluted for quantifying CFUs.

4.5.7. Statistical Analysis

Data analysis was performed using Prism (Graphpad software, version 9). Fitness was evaluated using the selection rate constant (r) as a measure of relative performance, calculated as

$$r_{ij} = ln\left[\frac{N_i(1)}{N_i(0)}\right] - ln\left[\frac{N_j(1)}{N_j(0)}\right]$$

where N_i (0) and N_j(0) are the initial densities of the R-pyocin producer (PAO1) or null (PAO1 ΔR) and the R-pyocin susceptible mutant (PAO1 Δ*rmlC* ΔR) respectively and N_i (1) and N_j(1) are the corresponding densities at either 4 h or 24 h post inoculation [62].

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4.7. Acknowledgements

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CHAPTER 5. CONCLUDING REMARKS

Antimicrobial resistance (AMR) is a problem in many types of infectious disease, particularly in individuals with chronic infections, compromised immune systems, and people with cystic fibrosis (CF). *P. aeruginosa* is a Gram-negative opportunistic pathogen known for possessing intrinsic antimicrobial resistance mechanisms and is notoriously difficult to eradicate. *P. aeruginosa* causes many types of infection including bacteremia, urinary tract infections, and pneumonia. However, it is especially difficult to eradicate for those with compromised immunity, chronic wounds, and particularly people with CF. *P. aeruginosa* infection is a major determinant of declining lung function in individuals with CF. As chronic infection with *P. aeruginosa* remains one of the main causes of morbidity and mortality in individuals with CF and a number of other chronic infections, new therapeutic interventions are 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 for *P. aeruginosa* infection however our understanding of R-pyocin efficacy in chronic infection environments, including the CF lung, is still poorly understood. It is known *P. aeruginosa* populations in CF chronic lung infection become phenotypically and genotypically diverse over time, however, little is known of the efficacy of R-pyocins against heterogeneous populations, nor if *P. aeruginosa* utilizes R-pyocin production during infection for competition or otherwise – which may influence pressure towards R-pyocin resistance.

Similar to prophages, R-pyocin genes are also located on the *P. aeruginosa* chromosome and are induced by the SOS response. It is likely that the chronic infection environment, such as CF sputum, exposes the cells to a number of stress-inducing stimuli, including hydrogen peroxide (from host immune response) and ciprofloxacin (antibiotic), however the regulation of R-pyocins and the resulting lysis to facilitate their release has not been well understood during infection. It is not known how cells in a spatially structured, CF-relevant environment may impact the efficacy of R-pyocins, phage and other phage-like particles. Understanding this regulation and the resulting lysis is important for understanding how R-pyocins and other phage-like particles may already be affecting *P. aeruginosa* growth dynamics, strain competition, and biofilm development in infection, which could have a profound impact on the efficacy of their use as a therapeutic.

In this work I have studied R-pyocin susceptibility, regulation and release utilizing a biobank of whole populations of *P. aeruginosa* collected from 11 individuals with CF, as well as the most relevant CF infection model to date (SCFM) to understand the mechanisms of R-pyocin activity in an infection-relevant context. The scientific contributions of this thesis include the following major findings:

- Utilizing an *in silico* screen to determine R-pyocin genotypes of *P. aeruginosa* strains publicly available through the International Pseudomonas Consortium Database (IPCD), I have found that R1-type pyocin producing strains are the most prevalent R-pyocin genotype among respiratory infection and CF strains.

- A significant proportion of *P. aeruginosa* strains (approximately 40%) isolated from infections lack R-pyocin genes entirely, but maintain the flanking regulatory genes (PrtN, PrtR), as well as the lytic cassette (holin, lysin).

- By testing 20 isolates each from 3 diverse populations of *P. aeruginosa* from expectorated sputum samples, I found heterogeneity in susceptibility to R-pyocins. This suggests there is likely heterogeneity in susceptibility to other phage-tai like bacteriocins and LPS-binding phage as well, which is an important consideration for alternative therapies.

- I further tested more populations of *P. aeruginosa* and found that the majority of sputum samples consist of isolates with some degree of susceptibility to R2-pyocins, suggesting most individuals with CF likely carry strains that would be sensitive to R2-pyocins as a potential additional therapy.

- Upon surveying LPS profiles of the 12 *P. aeruginosa* populations tested for R2-pyocin susceptibility, I found a high degree of heterogeneity among LPS profiles within each population. There were no correlations between the presence and absence of CPA, OSA, or an "unaltered" LPS core and R2-pyocin susceptibility.

- In characterizing the LPS phenotypes among the *P. aeruginosa* populations, I determined that 42.7% lacked common antigen (CPA, or A-band), 76.3% lacked o-specific antigen (OSA, or B-band), and 27.2% presented altered

LPS cores. Based on finding from our biobank, we estimate more than 75% of CF strains of *P. aeruginosa* lack OSA.

- Using transcriptional reports and a CF-relevant media, SCFM, I found that the nutritional environment influences regulatory activity of R-pyocins even when the SOS response is not induced. Specifically, I showed that infection-like media upregulates R-pyocin associated genes.

- By growing pairwise combinations of an R-pyocin producing strain and an R-pyocin susceptible strain in a structured, infection-like media (SCFM2), I showed that the nutritional and physical limitations of the CF lung environment ultimately create a niche in which R-pyocin induction is not beneficial but is instead costly for the producing strain. This finding also suggests that R-pyocins are not able to diffuse across aggregates in a spatially structured environment such as infection, so that the cost of R-pyocin release and lysis is greater than the competitive benefit of using R-pyocins to kill the competition.

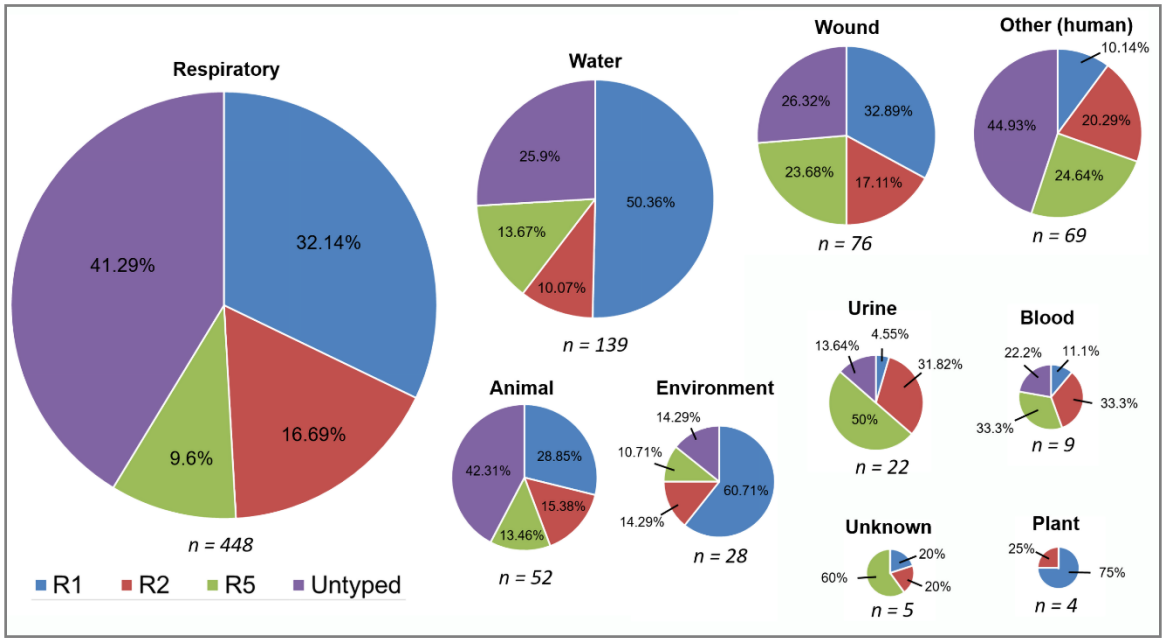
To improve upon our knowledge and understanding of how R-pyocins can be utilized as an effective antimicrobial therapy in infection, there are several areas that which require further study. The first is the matter of understanding how using R-pyocins as an antimicrobial treatment influences the strain composition and thus, patient outcomes. While I have shown that there is heterogeneity in R2 susceptibility among diverse populations of *P. aeruginosa*, it is still unknown how well R-pyocins can truly penetrate through the physical limitations of infections (ie. dehydrated mucous of CF sputum). It is

also unknown how eradicating R-pyocin susceptible isolate but potentially leaving behind susceptible isolates will influence the infection and thus the patient. Regarding the efficacy of R-pyocins in infection, the next obvious step is to test their antimicrobial capabilities more thoroughly in animal infection models, with host factors and a number of additional influences on pathogenic behavior to better assess their potential.

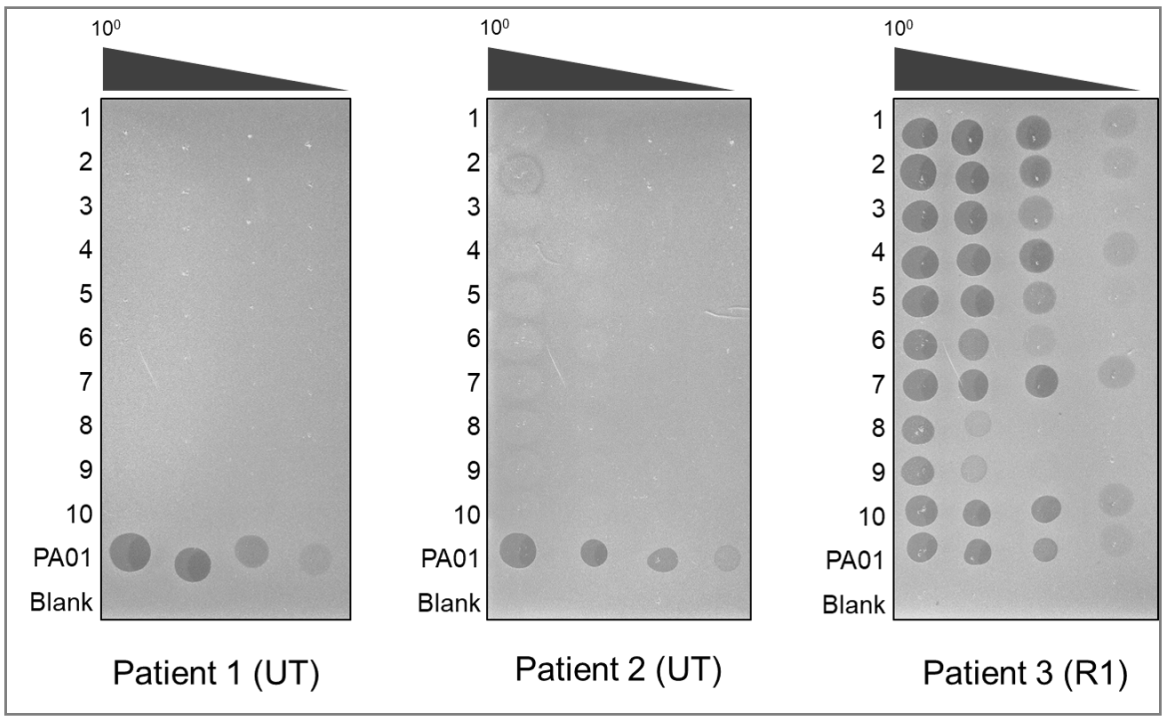
Another area of interest that this work brings to light is the need to understand why many *P. aeruginosa* strains from infections lack R-pyocin structural genes but have the regulatory and lytic genes. It is unclear if they once possessed the entire R-pyocin gene cassette and are losing the R-pyocin genes through selected pressures in the infection environment (as I hypothesize in Chapter 4), or if perhaps there are multiple ancient lineages of infectious strains each with differences in the R-pyocin or phage-like genes they carry. Understanding the evolution of R-pyocin genes in infections may contribute to understanding of their efficacy, as selective pressures towards evolving resistance is always a concern when considering antimicrobials.

The work presented in this thesis has advanced our understanding of R-pyocin activity in infection, the impact of *P. aeruginosa* heterogeneity on R-pyocin susceptibility, and built upon our knowledge of bacteriocin-mediated interactions.

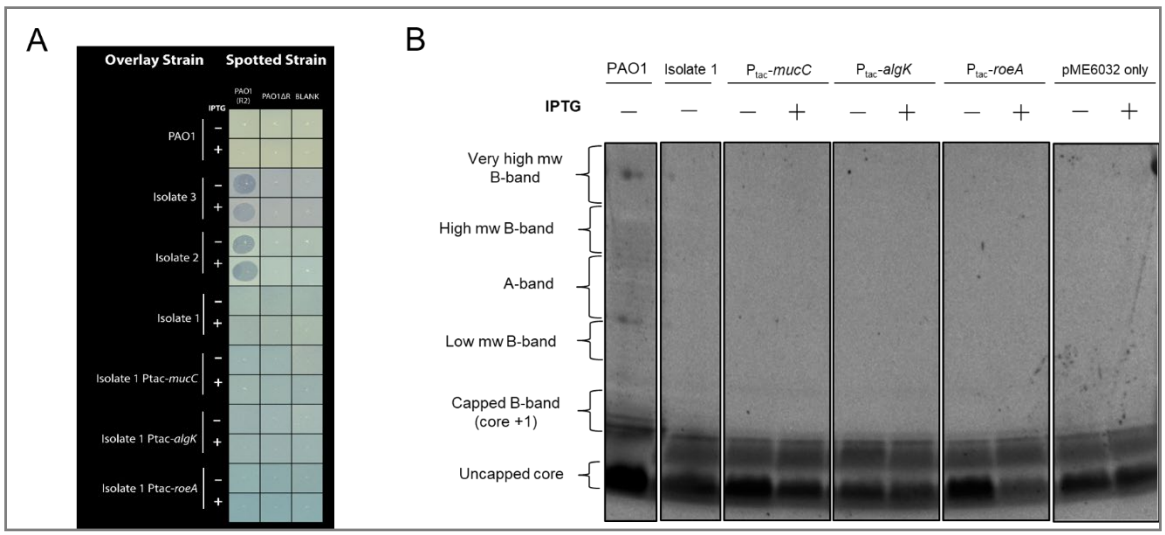
APPENDIX A. CHAPTER 2 SUPPLEMENTARY FIGURES AND TABLES



Supplementary Figure 2.1: Distribution of R-pyocin types across *P. aeruginosa* strains isolated from various sources as a part of the IPCD. Using BLAST+, 852 publicly available strains through the International *Pseudomonas* Consortium Database (IPCD) were assessed for R-pyocin genotype. 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.



Supplementary Figure 2.2: Untypeable *P. aeruginosa* isolates from two CF patients do not exhibit R-pyocin activity. From each population of Patient 1, Patient 2 and Patient 3, isolates 1-10 were tested for R-pyocin activity. Partially fractionated lysates were serially diluted 10-fold in lysogeny broth (LB) and each dilution was spotted (5 µL) onto an agar overlay inoculated with a previously described CF strain, A026. Untypeable (UT) isolates from Patients 1 and 2 do not exhibit R-pyocin activity, while isolates from Patient 3 (R1-pyocin genotype) does show killing activity against the indicator strain. PAO1 (an R2-pyocin producer) is included as a control for killing activity. Zones of inhibition indicate R-pyocin killing.



Supplementary Figure 2.3: R2-pyocin resistance and LPS presentation in complemented strains of clinical Isolate 1 from Patient 3. (A) The clinical Isolates 1-3 from Patient 3 depicted in a spot assay with strains of Isolate 1 complemented with mucC, algK, and roeA of PAO1 inducible under Ptac. Isolates 2 and 3 show susceptibility to the R2-pyocins of PAO1 (denoted by the zones of inhibition), whereas Isolate 1 and all complemented strains of Isolate 1 are resistant. Each isolate or strain was tested with and without Chromomax IPTG/X-Gal for induction. (B) The complemented strains of Isolate 1 were extracted and visualized by SDS-Page, confirming no alterations to the LPS presentation resulting from complementation. LPS extractions without the Chromomax induction agent were included.

Supplementary Table 2.1: Primers and Plasmids used in Chapter 2.

Primer (bp)*	Sequence (5' to 3')	Description	Reference or Source
R1-Forward (441)	ATGATTTTTTTCC ATGCCGCCACG	R-pyocin typing; gives fragment for R1 tail fibre	[60]
R1-Reverse	TCAGGGGGTGAT GAGCGATTGG	R-pyocin typing; gives fragment for R1 tail fibre	[60]
R2-Forward (257)	ATGCCGATGCTT CGATTAC	R-pyocin typing; gives fragment for R2 tail fibre	[60]
R2-Reverse	AAACCTCTCGCA AGGAGG	R-pyocin typing; gives fragment for R2 tail fibre	[60]
R5-Forward (601)	AATGCACAGGCC GAAAGTGGGG	R-pyocin typing; gives fragment for R5 tail fibre	This study
R5-Reverse	TGACATCTGCGA GGGTGACGGT	R-pyocin typing; gives fragment for R5 tail fibre	This study
TF-del upstream-Forward (600)	GAGCTCAGGTTA CCCGCATGGGTC CTGGATGAAACC CAGG	R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct	This study
TF-del upstream-Reverse	GTAACGCCGTGG AAGAAACTCATG TCACTGTTTCCAG GGGA	R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct	This study
TF-del downstream-Forward	CCCCTGGAAACA GTGACATGAGTT TCTTCCACGGCGT TACGGTAACCAA C	R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct	This study

Supplementary Table 2.1 (continued)

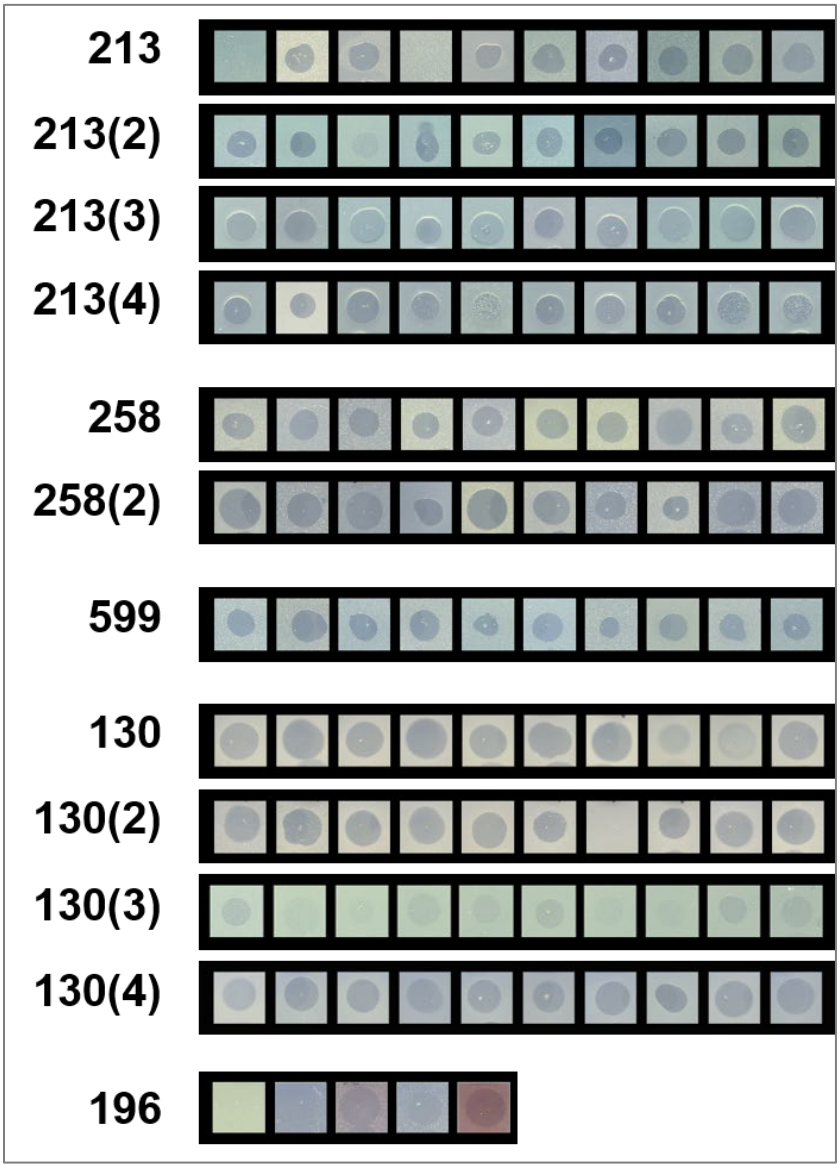
TF-del downstream-Reverse	CACTAGTGGGGCCCTTCTAGCGAAGCCGGGGCGTTGC	R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct	This study
mucC-Forward (521)	ATCGATGCATGCCATGGTACTCAGGCCGAATCGGACGGCCCGGAAATCAGCG	Complementation; To clone PA0756 *mucC* into pME6032 (under P_{tac}) in Isolate1	This study
mucC-Reverse	TTCACACAGGAAACAGAATTTCCGGCCCGAGGCCGCCGCC	Complementation; To clone PA0756 *mucC* into pME6032 (under P_{tac}) in Isolate1	This study
algK-Forward (1495)	ATCGATGCATGCCATGGTACTCATAGGCTTTCTGGCTCTTCTTCGTTGATCG	Complementation; To clone PA3543 *algK* into pME6032 (under P_{tac}) in Isolate1	This study
algK-Reverse	TTCACACAGGAAACAGAATTCCACCGCTCGCTGACCGGTA	Complementation; To clone PA3543 *algK* into pME6032 (under P_{tac}) in Isolate1	This study
roeA-Forward	ATCGATGCATGCCATGGTACTTACCGCAGGCTTTCCGCGAGGC	Complementation; To clone PA1107 *roeA* into pME6032 (under P_{tac}) in Isolate1	This study
roeA-Reverse	TTCACACAGGAAACAGAATTGCCGGATAGGGCGGCCGTCG	Complementation; To clone PA1107 *roeA* into pME6032 (under P_{tac}) in Isolate1	This study

Supplementary Table 2.1 (continued)

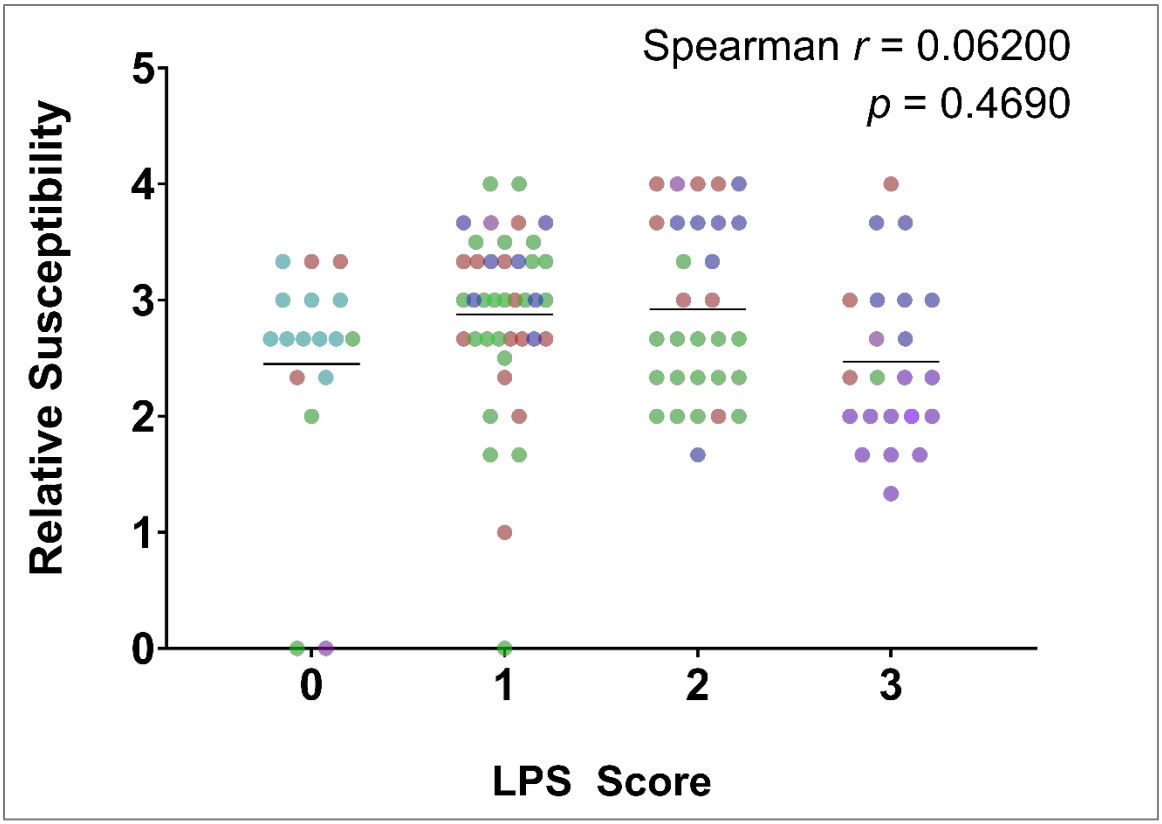
Plasmids	**Description**	**Source**
pDM4	Suicide vector, Cm^r	Nottingham Collection
pJT95	pDM4 bearing construct gene with deletion of PA0620 and PA0621; Cm^r	This study
pME6032	Expression vector, Tc^r	Nottingham Collection
pMM01	pME6032 containing PA0756 gene under inducible P_{tac} (pME6032::*mucC*), Tc^r	This study
pMM02	pME6032 containing PA3543 gene under inducible P_{tac} (pME6032::*algK*), Tc^r	This study
pMM03	pME6032 containing PA1107 gene under inducible P_{tac} (pME6032::*roeA*), Tc^r	This study

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

APPENDIX B: CHAPTER 3 SUPPLEMENTARY FIGURES AND TABLES



Supplementary Figure 3.1: Zones of R2-pyocin inhibition of multiple CF isolates from different CF patient sputum samples. The zones of inhibition created by the neat spots of R2-pyocin onto soft agar overlays of clinical isolates from the CF sputum sample numbers listed on the left. Up to ten isolates per sputum sample were tested. A zone of inhibition indicates susceptibility to R2-pyocin, whereas no visible zone indicates resistance to R2-pyocin.



Supplementary Figure 3.2: Combinatorial LPS deficient phenotypes are not correlated with R2-pyocin susceptibility. Using a numbered scale, we assigned an LPS Score of 0 to 3 for each isolate from each sputum collection to evaluate correlation between LPS phenotypes as a whole and R2-pyocin susceptibility. Isolates from each population are depicted as data points of the same color. There was not a correlation between LPS Score and R2-pyocin susceptibility ($p > 0.05$).

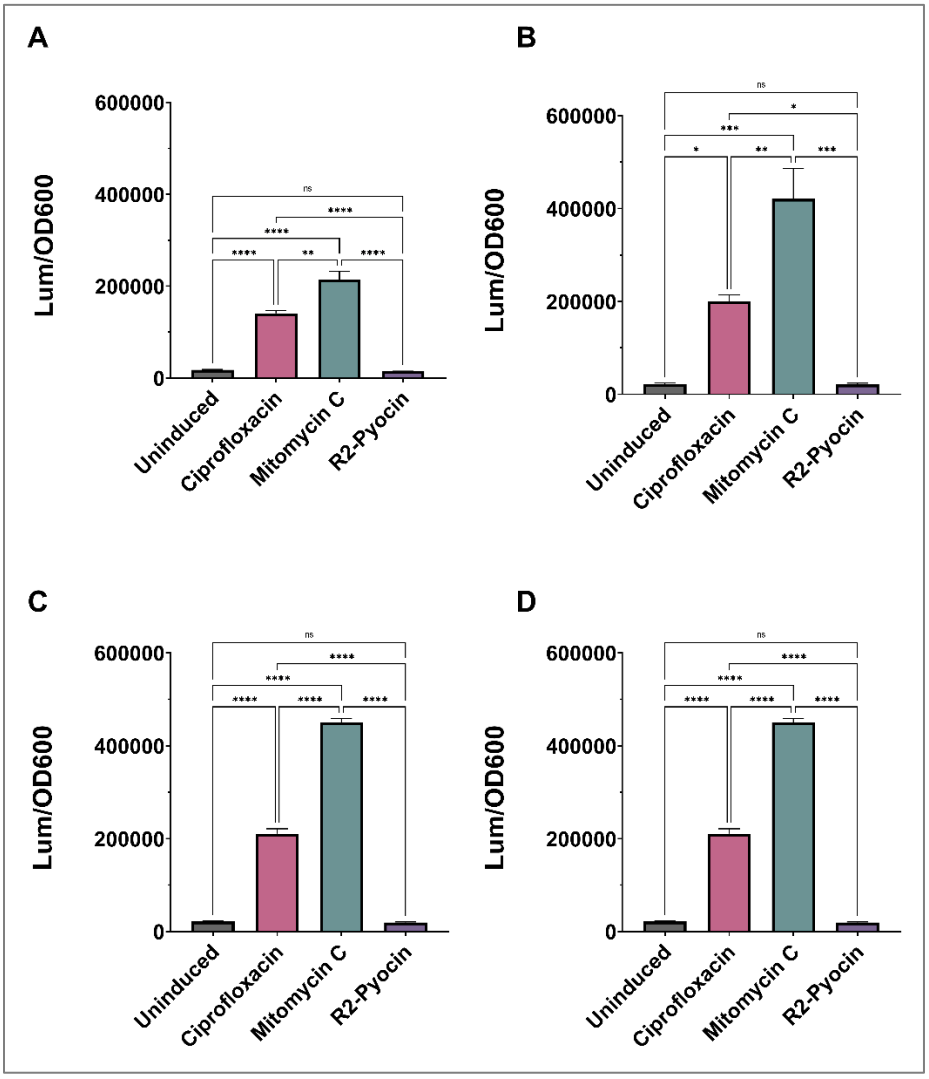
Supplementary Table 3.1: Strains, primers and plasmids used in Chapter 3.

Strain		Description	Source
PAO1N		wildtype Nottingham laboratory strain	[56]
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*	[57]
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	[52]
PAO1 ΔPf4 ΔR		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**	**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]

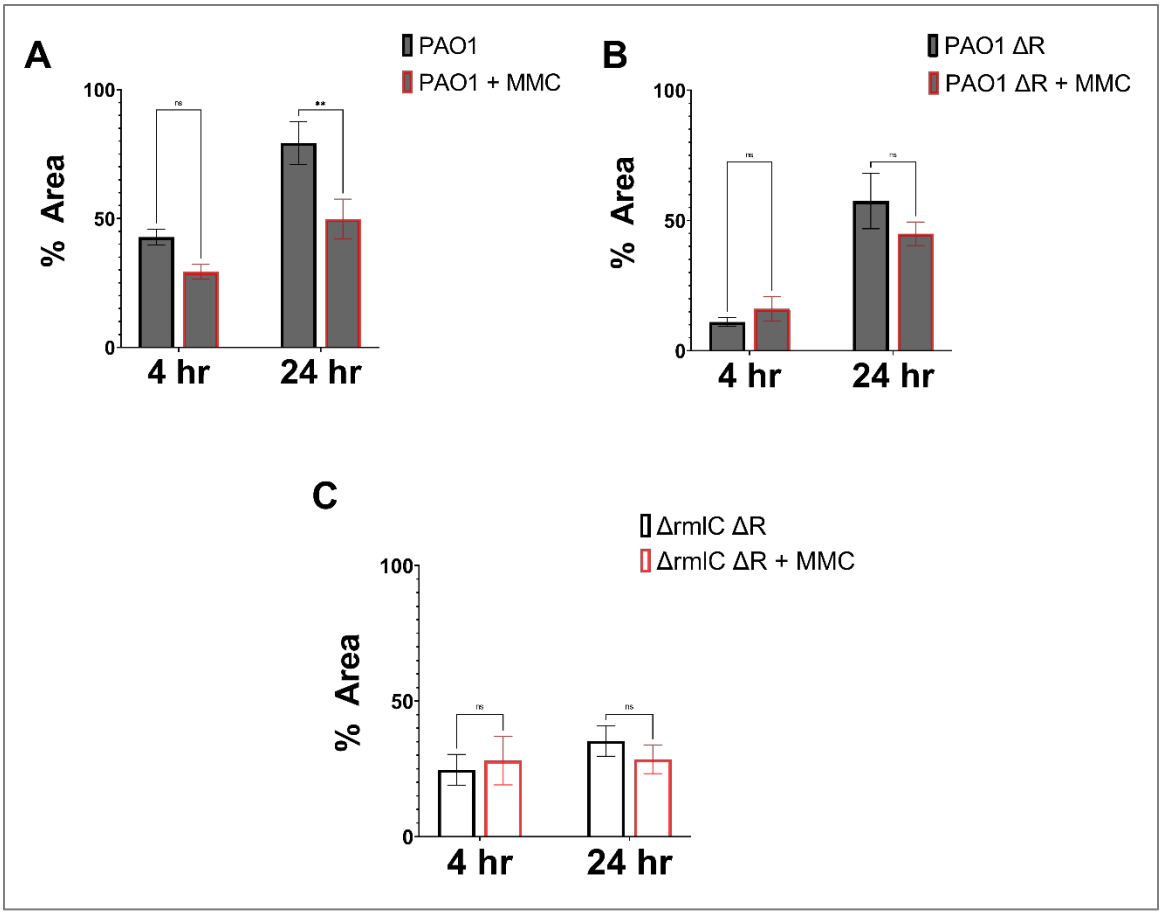
Supplementary Table 3.1: Strains, primers and plasmids used in Chapter 3.Supplementary Table 3.1 (continued)

Primer (bp)*	**Sequence (5' to 3')**	**Description**	**Source**
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

APPENDIX C: CHAPTER 4 SUPPLEMENTARY FIGURES AND TABLES



Supplementary Figure 4.1: R-pyocin associated holin activity in various strains lacking R-pyocin genes. We measured luminescence and optical density at 600nm simultaneously for up to 10 hours to assess transcriptional activity of the holin (PA0614) under induction with ciprofloxacin (0.1 µg/mL), mitomycin C (3 µg/mL) and R2-pyocins. While each SOS-induction agent varies in activity for each strain, the PAO1 Δ*lysin* (A) showed the greatest difference in R-pyocin associated activity as holin activity decreased under each agent relative to the activity seen in the PAO1 Δ*holin* (B), PAO1 ΔPf4 (C), and PAO1 ΔPf4 ΔR (D) strains. Significance was evaluated between agents for each reporter with a one-way ANOVA and differences in peak activity timing was analyzed with a two-way ANOVA (*$p<0.05$, **$p<0.005$, ***$p=0.0001$).



Supplementary Figure 4.2: Percent Area of monocultures in structured SCFM. Percentage fluorescent area (% Area) was measured from images of (A) PAO1, (B) PAO1 ΔR, and (C) PAO1 *ΔrmlC ΔR* monocultures taken at 4 h (left) and 24 h (right) post inoculation and induction with mitomycin C (MMC). Significance was analyzed with two-way ANOVA. (**p=0.005).

Supplementary Table 4.1: Bacterial strains used in Chapter 4.

Strain	Description	Source
PAO1N	wildtype Nottingham laboratory strain	[53]
PAO1N::Tn7-Gm/pME6032 *ptac*-GFP	wildtype Nottingham laboratory strain; Tn7 insertion for Gent resistance; pME6032 for constitutive GFP; Tc^r Gm^r	This study
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*	Nottinham Collection
PAO1N ΔR::Tn7-Gm/pME6032 *ptac*-GFP	R-pyocin null mutant; lacking tail fiber protein (PA0620) and chaperone protein (PA0621); Tn7 insertion for Gent resistance; pME6032 for constitutive GFP; Tc^r Gm^r	This study
PAO1N Δ*rmlC* ΔR/pME6032 *ptac*-mCherry	R-pyocin null; R2-susceptible; lacking *rmlC* PA5164, tail fiber protein (PA0620) and chaperone protein (PA0621); pME6032 for constitutive mCherry; Tc^r	This study
PAO1 attCTX::*p*Holin-lux	Transcriptional reporter; Mini-CTX-lux insertion into the attCTX site; Tc^r	This study
PAO1 attCTX::*p*Lysin-lux	Transcriptional reporter; Mini-CTX-lux insertion into the attCTX site; Tc^r	This study
PAO1 attCTX::*p*Sheath-lux	Transcriptional reporter; Mini-CTX-lux insertion into the attCTX site; Tc^r	This study
PAO1N Δ*rmlC*	R2-pyocin susceptible; lacking *rmlC* (PA5164)	This study
PAO1 ΔPf4	Nottingham PAO1 lacking filamentous prophage pf4	[59]
PAO1 ΔPf4 ΔR	Nottingham PAO1 lacking filamentous prophage pf4, tail fiber protein (PA0620) and chaperone protein (PA0621) of R-pyocin	This study
PAO1 Δholin	Nottingham PAO1 lacking R-pyocin associated holin (PA0614)	This study
PAO1 Δlysin	Nottingham PAO1 lacking R-pyocin associated lysin (PA0629)	This study

Supplementary Table 4.2: Primers and plasmids used in Chapter 4.

Primer (bp)*	Sequence (5' to 3')	Description	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	[30]
TF-del upstream-Reverse	GTAACGCCGTGGAAGAAACTCATGTCACTGTTTCCAGGGGA	R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct	[30]
TF-del downstream-Forward (600)	CCCCTGGAAACAGTGACATGAGTTTCTTCCACGGCGTTACGGTAACCAAC	R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct	[30]
TF-del downstream-Reverse	CACTAGTGGGGCCCTTCTAGCGAAGCCGGGGCGTTGC	R-pyocin null mutant generation; To clone PA0620-1 600 bp flanking regions into pDM4 for deletion construct	[30]
pHolin-Forward (363)	CTATAGGGCGAATTGGGTACGTTCCTGGACCGGATACCGTG	To clone the R-pyocin holin promoter in into pmini-CTXlux	This study

Supplementary Table 4.2 (continued)

pHolin-Reverse	CAGGAATTCGATATCAAGCTGAGTGCCTCCCTGGGGACGC	To clone the R-pyocin holin promoter in into pmini-CTXlux	This study
pLysin-Forward (578)	CTATAGGGCGAATTGGGTACTACCACTTCGTCACCCGTCT	To clone the R-pyocin lysin putative promoter into pmini-CTXlux	This study
pLysin-Reverse	CAGGAATTCGATATCAAGCTCGATCCTCCTGCACTCCGAT	To clone the R-pyocin lysin putative promoter into pmini-CTXlux	This study
pSheath-Forward (576)	GTCGACGGTATCGATAAGCTAGCGACTACCACTACCGCGG	To clone the R-pyocin sheath putative promoter into pmini-CTXlux	This study
pSheath-Reverse	TCCCCCGGGCTGCAGGAATTAGGTAGATCTCCATTAATGAAAAACCCCGC	To clone the R-pyocin sheath putative promoter into pmini-CTXlux	This study
PA0614-del upstream-Forward (750)	CTTCTGCAGGTCGACTCTAGCGGGCAGTGAAAGGAGACAC	To clone pA0614 750 bp flanking regions into pEXG2 KpnI and XbaI sites for deletion construct	This study
PA0614- del upstream-Reverse	GACTCCTTCGATCAGTTTCACACGAGTGCCTCCCTGGGGA	To clone pA0614 750 bp flanking regions into pEXG2 KpnI and XbaI sites for deletion construct	This study
PA0614-del downstream -Forward (750)	TCCCCAGGGAGGCACTCGTGTGAAACTGATCGAAGGAGTCAACC	To clone pA0614 750 bp flanking regions into pEXG2 KpnI and XbaI sites for deletion construct	This study
PA0614- del upstream-Reverse	GTGGAAATTAATTAAGGTACCCGACGGGCTGAGCAGTACC	To clone pA0614 750 bp flanking regions into pEXG2 KpnI and XbaI sites for deletion construct	This study
PA0629-del upstream-Forward (750)	CTTCTGCAGGTCGACTCTAGTGCCAAGGCCGGCGACATGC	To clone pA0629 750 bp flanking regions into pEXG2 KpnI and XbaI sites for deletion construct	This study
PA0629- del upstream-Reverse	AGGAGCAGAGCGAGCCGGCTCATCGATCCTCCTGCACTCCGATG	To clone pA0629 750 bp flanking regions into pEXG2 KpnI and XbaI sites for deletion construct	This study
PA0629-del downstream -Forward (750)	GGAGTGCAGGAGGATCGATGAGCCGGCTCGCTCTGCTCCT	To clone pA0629 750 bp flanking regions into pEXG2 KpnI and XbaI sites for deletion construct	This study

Supplementary Table 4.2 (continued)

PA0629- del upstream-Reverse	GTGGAAATTAATTA AGGTACTCAGCGGC AGGCACGCAATG	To clone pA0629 750 bp flanking regions into pEXG2 KpnI and XbaI sites for deletion construct	This study
Plasmids	**Description**		**Source**
Mini-CTX-lux	self-proficient integration vector, V-FRT-attP-MCS, ori, int, and oriT; lux insertion; Tc^r		[51]
pTNS3	plasmid expressing *tnsABCD* from *P1* and *Plac*; Ap^r		[55]
pUC18-miniTn7T-Gm	mini-Tn7 insertion vector with MCS; Gm^r		[56]
pDM4	Suicide vector, Cm^r		[57]
pEXG2	Suicide vector, Gm^r		[60]
pJT95	pDM4 bearing construct gene with deletion of PA0620 and PA0621; Cm^r		[30]
pME6032	Expression vector, Tc^r		Nottingham Collection
pMM08	pDM4 bearing construct gene with deletion of PA5164; Cm^r		This study
pJT47	Mini-CTX-lux insertion construct bearing 363 bp upstream of PA0614 (holin) coding sequence; Tc^r		This study
pMM18	Mini-CTX-lux insertion construct bearing 576 bp upstream of PA0622 (sheath) coding sequence; Tc^r		This study
pMM21	Mini-CTX-lux insertion construct bearing 578 bp upstream of PA0629 (lysin) coding sequence; Tc^r		This study

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