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The influence of non-inhibitory concentrations of purified pyocin S2 on Pseudomonas aeruginosa isolates

Mehina, Nabiha

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The influence of non-inhibitory concentrations of purified pyocin S2 on *Pseudomonas aeruginosa* isolates

by

Nabiha Mehina

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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Abstract

*Pseudomonas aeruginosa* is an opportunistic pathogen whose impacts are primarily in clinical settings. It is possible for multiple *P. aeruginosa* strains to be involved in an infection and each strain can diverge into many morphotypes. Intraspecies interactions between strains and morphotypes leads to a complex environment involving both beneficial and antagonistic interactions. S-type pyocins are bacteriocin proteins produced by *P. aeruginosa* that can inhibit closely related strains. Previously, we observed a *P. aeruginosa* isolate using S-type pyocins to decrease the virulence of other isolates in a *Drosophila melanogaster* model. We have reason to believe that this decrease in virulence may not only be because of the direct inhibition of the target isolates but additionally from a reduction in their virulence factor production. As a result, we hypothesized that *P. aeruginosa* isolates use S-type pyocins to influence the overall virulence of microbial communities by decreasing the virulence factor production of target bacteria. In the current study, we purified a DNase S-type pyocin, S2, and used a variety of virulence assays to show that subinhibitory concentrations of this pyocin alter the virulence factor production of target *P. aeruginosa* isolates. In doing so, we also demonstrated that the effects of S2 on virulence factor production can vary for different strains. We further explored the interactions between S2 and target bacteria by demonstrating how one isolate was able to gain resistance towards S2. Using genetic sequencing, we identified mutations that treatment with S2 is selecting for which may account for this resistance and explored how these changes may explain the altered virulence factor production displayed by this isolate. From the results of this work, we shed new light upon the reactions of surrounding strains to S2 production and proposed a potential novel mechanism of resistance against this pyocin.
Acknowledgements

The successful completion of my graduate degree would not have been possible without my incredibly patient and supportive supervisor, Dr. Douglas Storey. I think every past member of the Storey lab can agree that you cannot find a more awesome supervisor than Doug. Going to lab was always something I looked forward to because of the wonderful lab environment created by Doug and my fellow lab mates. I enjoyed deep conversations with Jenny Nguyen, received random notes of encouragement and delicious home-made snacks from Camila Ching, and relied on Dr. Jessica Duong’s words of wisdom. Finally, I would like to thank my most important buddy whom I started out my master’s with and whom I am so glad to be finishing with – Nicole Jervis. Nicole, who I adventured with at conferences, who traded BTS videos with me throughout the COVID-19 lockdown, and who sat with me for hours when I was going through a hard time… I purple you!

I would also like to thank my examiner and my committee members: Dr. Rebekah DeVinney, Dr. Michael Parkins, and Dr. Joe Harrison. Your kind guidance and advice helped me to improve my project and I learnt so much from all of you.

Dr. Ray Turner and Dr. Joe Harrison were generous in allowing me to use their lab equipment throughout my project. Trevor Randall and Dr. Henrik Almblad from the Harrison lab never hesitated to share their extensive experience with me to help me to improve my work. Dr. Sui-Lam Wong, Dr. Gregory Moorhead, and Dr. Kenneth Ng provided me with useful insights as I explored techniques new to my lab.

It is key to acknowledge the funding and resources provided to me by the Department of Biological Sciences and the Faculty of Graduate Studies at the University of Calgary. I treasured the memorable experience of working as a teaching assistant for multiple semesters.
Additionally, day-to-day tasks ran smoother because of the support of so many department staff. These include Rebecca Lee and Karen Li, who manned the autoclave room and whose pleasant conversation brought a smile to the faces of graduate students. Additionally, Karen Barron was a huge support to graduate students, especially during the start of the COVID-19 pandemic.

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Dedication

To my mom – Sharon Clark
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<td>ABC</td>
<td>ATP-binding cassette</td>
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<td>ANOVA</td>
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<td>IQS</td>
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Chapter One: Introduction

1.1 Pseudomonas aeruginosa

*P. aeruginosa* is a Gram-negative bacterium that has an incredible ability to adapt, allowing it to survive in a multitude of different environments ranging from soil to water or associated with animals and plants (Monias 1928; Silby *et al*., 2011).

1.1.1 Virulence Factors

*P. aeruginosa* relies on an array of virulence factors to assist in competition and colonization, many of which have harmful impacts on human hosts. Acute infections are often associated with the planktonic lifestyle of this bacterium whereas chronic infections are correlated with a biofilm lifestyle consisting of communities of bacteria in an exopolysaccharide matrix (Eagon 1962; Costerton *et al*., 1978; Valentini *et al*., 2018). The expression of virulence factors can change depending on the needs of the bacterium. The Gac/Rsm global regulatory network has been suggested to be one of the contributing factors in the switch from acute infections to chronic infections. This network positively regulates virulence factors involved in acute infections (e.g., flagella, type IV pili, type III secretion system) and negatively regulates those involved in chronic infections (e.g., the biofilm exopolysaccharides *pel* and *psl*).

Quorum sensing (QS) is an important part of virulence factor regulation. Among the virulence genes controlled by QS are those involved in motility and the production of elastase, rhamnolipids, hydrogen cyanide, pyocyanin, lipase, and swarming (Latifi *et al*., 1996; Reimmann *et al*., 2002). QS is cell-density dependent system that uses diffusible molecules known as autoinducers to affect the transcription of target genes (Nealson 1977). These autoinducers are only expressed at basal levels until there is an increase in cell density (Nealson
As the concentration of cells increase, so does the concentration of autoinducer until the concentration is high enough that the autoinducer binds to a transcriptional regulator that can continue on to interact with the QS-regulated genes (Nealson 1977). To date, there are four QS systems known in *P. aeruginosa*: the *las* system, the *rhl* system, the *Pseudomonas* quinolone signal (PQS) system, and the integrated quorum sensing system (IQS) system (Passador et al., 1993; Latifi et al., 1995; Pesci et al., 1999; Lee et al., 2013).

Pyocyanin is a secreted virulence factor which leads to neutrophil apoptosis (Allen et al., 2005) and causes lung epithelial cells to experience cellular senescence that results in cell cycle arrest (Muller 2006), impaired ciliary movement which can further disrupt mucociliary clearance (Wilson et al., 1988), increased interleukin-8 (IL-8) expression that can cause increased neutrophil-mediated tissue injury (Denning et al., 1998a), and altered calcium homeostasis which disrupts proper epithelial cell functions (Denning et al., 1998b). Lipases are also secreted and can mediate the host immune response by, for example, inhibiting monocyte chemotaxis (Jaeger et al., 1991). *P. aeruginosa* can produce four types of proteases: alkaline proteases (AprA), elastase A (LasA) protease, elastase B (LasB) protease and protease IV (PIV) (Guzzo et al., 1991; Braun et al., 1998; Engel et al., 1998). Proteases can cause host tissue damage during infection including the cleavage of extracellular matrix (Heck et al., 1986a; Heck et al., 1986b; Azghani et al., 1990). This damage can lead to the formation of corneal ulcers and necrotic skin lesions (Johnson et al., 1966; Kreger and Gray 1978). The type III secretion system is a cell-surface attached apparatus that injects toxins, known as effector proteins, into host cells (Yahr et al, 1996; Roy-Burman et al., 2001). The effector proteins enhance pathogenesis with effects including hindering the ability of the host to phagocytose *P. aeruginosa* and preventing epithelial wound repair (Geiser et al., 2001; Sun and Barbieri 2003; Shaver and Hauser 2004).
Together, the type III secretion system and LasB protease can contribute to the disruption of the endothelial barrier, allowing for tissue invasion that may end in life-threatening septicaemia (Valentini et al., 2018).

Motility is another factor in virulence. *P. aeruginosa* has use of flagella, type IV pili, as well as rhamnolipid production, which allows it to be capable of swimming, swarming, and twitching motility (Drake and Montie 1988; O’Toole and Kolter 1998; Köhler et al., 2000). The flagellum is involved in the processes of swimming and swarming (Drake and Montie 1988; Köhler et al., 2000). By responding to chemical gradients, the flagellum allows the bacterium to move to areas of greatest nutrients or lowest toxicity (Moulton and Montie 1979; Masduki et al., 1993; Kato et al., 1999). To add to that, the flagella and type IV pili play a key role in the initial stages of infection and assists in adhesion – an important step for colonization (Arora et al., 1998; Feldman 1998; Valentini et al., 2018). Interestingly, the flagellum has been known to trigger the host immune response (Wolfgang et al., 2004). The triggering of the host immune system is known to further damage host tissue and various virulence factors can contribute to this. Pyocyanin increases IL-8 release in epithelial cells and macrophages through the MAPKs and NF-Kβ signalling pathway (Chai et al., 2014). Elastase regulates interleukin-1β (IL-1β) maturation, promoting its activation (Everett and Davies 2021). Both IL-8 and IL-1β are involved in attracting and activating neutrophils (Chai et al., 2014). Neutrophils are inflammatory cells that produce reactive oxygen species and secrete neutrophil elastase, which further damages lung tissue (Fischer and Voynow 2002; Winterbourn et al., 2016). They also secrete the proinflammatory protein calprotectin, worsening the cycle of inflammation (Stríz and Trebichavský 2004). Overall, the virulence factors produced by *P. aeruginosa* have complex implications for the bacterium and any host it infects.
1.1.2 Antibiotic Resistance

*P. aeruginosa* is an opportunistic pathogen that affects hospitalized or immunocompromised individuals (Wood 1976). It is most well known for being a prominent pathogen in infecting the lungs of cystic fibrosis (CF) patients, leading to increased rates of lung deterioration (Canadian Cystic Fibrosis Registry 2019). However, it can also infect wounds, burns, urinary tracts, ears causing otitis media, and eyes causing keratitis (Wood 1976). As *P. aeruginosa* has both intrinsic and acquired resistance mechanisms, treatment of its infections can be quite problematic. The low permeability of the outer membrane of *P. aeruginosa* contributes to its inherent antibiotic resistance, being 100 times less permeable than that of *E. coli* (Angus et al., 1982). Other intrinsic mechanisms of resistance include the expression of efflux pumps and antibiotic modifying enzymes such as extended-spectrum-beta lactamases that give resistance against a wide range of beta lactam antibiotics (Stover et al., 2000; Castanheira et al., 2004).

This bacterium can acquire resistance by horizontal gene transfer through conjugation, transduction, or transformation (Pang et al., 2019). Mutational changes can create resistant variants through, for example, homologous recombination or disruptions in the DNA repair process (Mandsberg et al., 2009; Hocquet et al., 2016). *P. aeruginosa* can form biofilms containing multidrug-tolerant persister cells (Mulcahi et al., 2010). These persister cells are metabolically inactive, slow-growing cells that worsen a patient’s prognosis by surviving antibiotic treatments and allowing for the repopulation of biofilms (Bigger 1944).

1.1.3 Strain Diversity and Intraspecies Interactions

A study of young children with CF found that single *P. aeruginosa* strains would enter the lungs and set up infections but would not remain over a long period of time (Burns 2001).
New strains would then infect the lungs and eventually a chronic infection would take place. These strains are often unique strains obtained from the environment, though in some areas epidemic strains transmitted from one patient to another, such as the prairie epidemic strain, are more common (Burns 2001; Parkins et al., 2014). Multiple strains can infect the lungs and each strain can later undergo clonal diversification from selective pressures, allowing a single strain to branch out into isolates with different characteristics (Smith et al., 2006; Sousa and Pereira 2014). This creates a dynamic and complex environment. The selective pressures that lead to clonal diversification can be from the host immune response, subinhibitory concentrations of antibiotic, or competition between strains or species of bacteria as they strive for advantages such as nutrients (Speert et al., 1990; Smith et al., 2006; Bakkal et al, 2010; Wright et al., 2013).

There is a great variety of virulence factor production between isolates of *P. aeruginosa*, as was determined by Lutter 2008 who screened the virulence factor production of 27 CF isolates (Figure 1.1). In the lungs, *P. aeruginosa* tends to form small colony variants, have increased antibiotic resistance and reduced virulence (Häußler et al., 1999; Burns et al., 2001). They frequently develop mucoid phenotypes despite this trait rarely being seen outside CF patients (Burns et al., 2001). Flagella are selected against in the CF lungs, which was initially thought to be to avoid triggering the host immune response or the metabolic benefit of not having to manufacture flagella, but recent research has pointed towards the reason being that flagellar mutants have an overproduction of the exopolysaccharides Pel and Psl (Harrison et al., 2020). An overproduction of Pel and Psl leads to increased antibiotic tolerances, helps prevent phagocytosis, and gives advantages in biofilms.

The isolate diversity that arises leads to complex intraspecies interactions as different morphotypes can influence the activity of other isolates. For example, when an isolate that has a
**Figure 1.1. Cluster plot of virulence factors detected in different CF isolates.**

A variety of virulence factor assays were carried out to determine the virulence factor production of each CF isolate. Fly feeding and fly nicking indicate the overall virulence determined in two different in-vivo *Drosophila melanogaster* models based on fly survival. 06HSL, C12HSL, C4HSL, C6HSL and C8HSL are QS molecules. The rows labelled ‘*’ indicate that the virulence factor production was compared to the average of the isolates with red indicating greater-than-average, black indicating average, and green indicating less-than-average virulence factor production. Rows labelled ‘+/−’ were not compared to an average and were categorized solely based on the production, as indicated by the red colour, or non-production, as indicated by the black colour, of the virulence factors. Image obtained from Lutter EI. 2008. *Pseudomonas aeruginosa* cooperativity. (Doctoral Dissertation). Biological Sciences. Calgary, University of Calgary.
mutated lasR, which encodes the transcriptional regulator for the las QS system, is co-cultured with an isolate that has a wildtype LasR, interactions ensue that lead to an increase in the production of virulence factors controlled by the rhl quorum sensing system, such as pyocyanin and rhamnolipids (Mould et al., 2020). The cause of this is believed to be that the lasR mutant produces the siderophore pyochelin which stimulates the wildtype isolate to produce citrate. This citrate stabilizes RhlI, which is involved in the production of the autoinducer for the rhl system. Additionally, different morphotypes may rely on one another for growth. An example of this would be where auxotrophic isolates require other isolates which are prototrophic to grow (Qin et al., 2012). Similarly, isolates that do not produce beneficial secreted factors like siderophores can benefit from using those factors secreted by other isolates without the cost of making them (Bisht et al., 2020).

Previous research in our laboratory looked at how interactions between CF isolates could affect virulence in a Drosophila melanogaster infection model (Lutter 2008). In this model, two isolates were infected together in D. melanogaster and the fly survival was measured. Three types of interactions became apparent. Firstly, some co-infections were able to increase the overall virulence of the bacteria in the fly model. One explanation for the increased overall virulence is that one isolate may provide a social cue that the other is lacking. For example, if one isolate is unable to produce QS molecules, the presence of QS molecules from the other isolate may lead to the activation of virulence factor genes which would otherwise be inactive. In the CF lungs, this interaction could possibly lead to an exacerbation due to a sudden increased virulence when one isolate’s QS system is activated by the other. The second possible outcome of a co-infection is that neither isolate affects the other’s virulence. Most interesting was the third interaction in which an isolate could decrease the overall virulence in the Drosophila model. It is
thought that this ability to attenuate the virulence of other isolates could be advantageous for establishing a chronic infection and evading the host immune response.

One isolate that was avirulent in the fly model, known as 14651, was able to attenuate the virulence of other more virulent isolates, one of which is named 14649 (Figure 1.2) (Lutter 2008). Similarly, in a rat prostate model, co-infection of 14651 with 14649 decreased rat tissue damage and edema (MacLean 2012). In vitro-studies found that this avirulent isolate also decreased the elastase and protease production of 14649 (Lutter 2008). These effects were further tested on two other virulent isolates, 14650 and 14673, and found to be similar (Lutter 2008, MacLean 2012). Upon further research, MacLean 2012 determined that 14651 was likely causing these affects by secreting a bacteriocin known as an S-type pyocin.

1.2 Pyocins: Bacteriocins Produced by P. aeruginosa

Bacteriocins are frequently used by bacteria to control interactions between and within species (Xavier and Russel 2006; Hawlena et al., 2012; Kommineni et al., 2015). Due to their inhibitory activity, bacteriocins provide their producer with an advantage over sensitive strains. Bacteriocins are proteins that can range in size from 2 kDa to 300 kDa and can vary in many respects (Ahmad et al., 2017). Bacteriocins are produced by both Gram-positive and Gram-negative bacteria, with Gram negative bacteria controlling bacteriocin production through the RecA DNA repair system whereas the regulation of Gram-positive bacteriocins is not yet fully known (Sano and Kageyama 1987; Ahmad et al., 2017). Pyocins are high molecular weight bacteriocins produced by P. aeruginosa (Jacob 1952). In the past, pyocins were widely used for
Figure 1.2. 14651 is able to attenuate the virulence of the more virulent isolate 14649 leading to an increase in fly survival.

Graph of *D. melanogaster* survival over 14 days following infection with isolates of *P. aeruginosa* using a fly feeding model. In the co-infection, 14651, which is believed to be producing an S- pyocin, is able to attenuate the virulence of the 14649, which is believed to be targeted by the S- pyocin. The black lines are where flies were fed a full dose of either 14651 or 14649 whereas the red line indicates a co-infection where the flies were fed half doses of 14651 and 14649 (combined they would equal a full dose). Each datum point is a mean of three or more replicates performed in triplicate. Analysis was done with the Log-rank test of Prism 5.0 using at least 90 flies per curve. Image obtained from: Lutter EI. 2008. *Pseudomonas aeruginosa* cooperativity. (Doctoral Dissertation). Biological Sciences. Calgary, University of Calgary (Lutter 2008).
typing *P. aeruginosa* strains (Farmer and Herman 1969; Fyfe *et al*., 1984). Although most other bacteria encode their bacteriocins in plasmids, pyocins are found in the chromosome (Sano and Kageyama 1984).

### 1.2.1 Pyocin Types

There are three types of pyocins: R-, F- and S- type. They are widely produced, with more than 90% of isolates producing at least one pyocin (France and Remold 2016; Bara *et al*., 2018; Snopkova *et al*., 2020). Although not well established, it has been proposed that lipid degrading pyocins should be a separate class from S-pyocins as they appear to have bacteriostatic effects on target cells not bacteriocidal and are even more narrow ranged than many S-pyocins (Ghequire and Kemland 2014). The genetic determinants of R- and F- pyocins are located between the *trpE* and *trpG* genes (Nakayama *et al*., 2000). There are several types of R- and F- pyocins but they are all structurally related except for their genes for tail fibre formation, which determine their killing specificity.

R- and F- pyocins resemble bacteriophage and lambda phage, respectively, and are nuclease- and protease- resistant (Figure 1.3) (Kageyama 1964; Ishii *et al*., 1965; Uratani and Hoshino 1984). These pyocins differ as R- pyocins are similar to an inflexible and contractile phage whereas F- pyocins bear resemblance to a flexible and non-contractile phage. They kill by puncturing the target cell, leading to membrane depolarization and cell death (Uratani and Hoshino 1984). S- pyocins, on the other hand, are similar to colicins, which are bacteriocins produced by *E. coli* (Ito *et al*., 1970). Unlike the other two pyocins, S- pyocins are soluble, protease-sensitive and have a range of killing methods such as DNAses, tRNAses, and pore
**Figure 1.3. Structures of the three types of pyocins.**

R- and F- pyocins are multiprotein structures that resemble bacteriophage and lambda phage, respectively. S- pyocins are multidomain proteins encoded by a single gene that is located upstream of their respective immunity proteins. Image created with BioRender.com.
forming proteins (Sano et al., 1993a; Parret et al., 2000). They are more narrow-spectrum as, unlike R- pyocins which can kill some other Gram-negative bacteria such as Neisseria gonorrhoeae and Haemophilus influenzae, S- pyocins are only known to target other P. aeruginosa strains (Andersen et al., 1995; Filiatrault et al., 2001). S- pyocins have multiple domains, which were stated to be organized from N-terminal to C-terminal: (1) Receptor binding domain, (2) unknown domain, (3) translocation domain, and (4) killing domain (Sano et al., 1993a; Sano et al., 1993b; Duport et al., 1995). However, Behrens et al., 2020 have recently suggested that the receptor binding and translocation domains should be switched, matching the domain organization of colicins. The purpose of the unknown domain has yet to be elucidated but it does not appear to be necessary for killing activity (Duport et al., 1995). Bacteria producing S- pyocins protect themselves against their toxicity by manufacturing an immunity protein to neutralize the toxic effects (Sano et al., 1993a). This immunity protein is encoded downstream of the killing protein (Figure 1.3) (Duport et al., 1995).

1.2.2 Pyocin Regulation

Pyocin production is regulated by the RecA DNA repair system, making it closely linked to the SOS response system that is similarly controlled by RecA (Figure 1.4) (Sano and Kageyama 1987). RecA is constitutively produced in small amounts and in an inactive conformation (Egelman and Stasiak 1993). During RecA’s inactivation, the repressor PrtR prevents the expression of prtN, which codes for the activator of pyocin genes (Matsui 1993). RecA becomes active when it binds to single stranded DNA (ssDNA), forming a filament structure (Egelman and Stasiak 1993). This activated RecA proceeds to induce the autocleavage of LexA, which is involved in repressing the SOS response system, PrtR, which is required for
Figure 1.4. Regulation of pyocin production.

RecA induces the autocleavage of three proteins, LexA (represses genes involved in the SOS response), PrtR (represses genes involved in pyocin production and release), and PA0906 (not shown in diagram - controls 6 genes of unknown function) (McPartland et al., 1980; Matsui et al., 1993; Cirz et al., 2006). Under normal conditions, the transcriptional activator for the pyocin genes (PrtN) is repressed by PrtR and thus there is minimal pyocin production (Matsui 1993). Similarly, the LexA blocks the expression of SOS regulon genes by binding to a section of the promoter known as the SOS box (Freudl et al., 1987). Under stressful conditions, such as when DNA is damaged or replication obstructed, RecA enters an active form (Egelman and Stasiak 1993). Once activated, RecA induces the autocleavage of LexA, allowing the transcription of SOS regulon genes that are involved in DNA repair, translesion DNA synthesis, and blocking cellular replication while DNA errors are being fixed (McPartland et al., 1980; Reuven et al., 1999). RecA additionally induces the autocleavage of PrtR, allowing for the expression of PrtN and subsequent activation of pyocin gene expression through its binding to the P-box sequence of pyocin promoters (Matsui 1993). Pyocin gene expression will lead to the production of pyocins (R-, F-, and S- type) as well as the proteins involved in lysing the producing cell to release the pyocins (holin and endolysin) (Nakayama et al., 2000). Image created with BioRender.com.
repressing pyocin production, and PA0906, which has unknown function (McPartland et al., 1980; Matsui et al., 1993; Cirz et al., 2006). The autocleavage of PrtR allows for the expression of prtN and the production of pyocins as a result (Matsui et al., 1993). PrtN activates transcription by binding to the regulatory sequence of pyocin genes known as the P-box (Matsui et al., 1993).

Pyocins are released through cell lysis, with S- pyocins being released in complex with their immunity protein (Nakayama et al., 2000; White et al., 2017). The genes involved in lysis are encoded in the R- and F- pyocin gene clusters (Nakayama et al., 2000). They consist of a holin protein, Hol and an endolysin protein, Lys. Hol produces a pore in the inner membrane, allowing Lys to access the peptidoglycan layer and degrade it. This degradation compromises the integrity of the cell wall and ultimately leads to the lysis of the producing cell, allowing the release of the pyocins into the surrounding environment. As there are many stressors that can lead to the formation of ssDNA and the subsequent activation of RecA, multiple factors are known to effect pyocin production. Environmental factors such as ultraviolet radiation and hydrogen peroxide upregulate pyocin production (Kageyama 1964; Chang et al., 2005). The potent DNA crosslinker, mitomycin C, and antibiotics that interfere with DNA replication such as fluoroquinolones increase pyocin expression (Kageyama 1964; Brazas and Hancock 2005). On the other hand, pyocin production is downregulated by the antibiotic ceftazidime (Blázquez et al., 2006).

As pyocin production ultimately leads to the lysis of the producing cell as a means of releasing the pyocins, pyocin expression must be under tight regulation. Indeed, the production of pyocins has been shown to increase susceptibility to antibiotics that trigger the SOS response system (Fan et al., 2019). Our knowledge of the mechanisms by which pyocin expression is
regulated is still growing. It remains unclear how the SOS response system, which is executed to save the producing cell, is balanced with pyocin production, which leads to the lysis of the producing cell. One suggested theory is that different rates of autocleavage could allow the SOS response system to be activated during acute stress and pyocin production to only be induced during chronic stress (Penterman et al., 2014). An additional level of regulation was recently determined by Fan et al., 2019 who found that the polynucleotide phosphorylase (PNPase) regulates PrtR production at the translational level. Mutants lacking a functional PNPase had increased PrtR expression and, as a result, increased resistance to ciprofloxacin (Fan et al., 2019).

There are approximately fifteen S-pyocins known or predicted to be produced by P. aeruginosa (Ghequire and de Mot 2014). The receptors that have been identified for S-pyocins are all hemin or iron siderophore receptors, including Hur, FpvAI, FpvAII, and FptA (Elfarash et al., 2012; Elfarash et al., 2014; White et al., 2017; Atanaskovic et al., 2020; Behrens et al., 2020). The uptake of the DNase S-type pyocin S2 (S2) and the pore-forming S-type pyocin S5 (S5), have been well studied (Figure 1.5) (White et al., 2017; Behrens et al., 2020). These pyocins are believed to aggregate on the target cell surface by binding the common polysaccharide antigen (CPA) (Behrens et al., 2020). They then hijack a nutrient receptor to cross the target cell’s outer membrane (White et al., 2017; Atanaskovic et al., 2020; Behrens et al., 2020). This leads to a force-dependent unfolding mediated by the proton motive force and the inner membrane protein TonB1 which causes the release of the immunity protein (Behrens et al., 2020; White et al., 2017). This way, the immunity protein, which would otherwise be bound to the C-terminal killing domain, is not imported into the target cell (White et al., 2017). Pore-forming pyocins can carry out their activity in the periplasm, but nuclease proteins are further
Figure 1.5. Import of S2 into a target cell.

(1) S2 binds to the common polysaccharide antigen (CPA) on the outer membrane of the target cell, assisting it in contacting the ferripyoverdine receptor FpvAI (White et al., 2017; Behrens et al., 2020). (2) The N-terminal (NT) domain binds FpvAI, leading to the force-dependent unfolding of the pyocin utilizing the proton motive force and TonB. (3) The pyocin refolds in the periplasm but the unfolding action has left the S2 immunity protein at the outer membrane. (4) The C-terminal (CT) nuclease domain is cleaved by FtsH and translocated into the cytoplasm where it carries out its killing activity.
translocated across the inner membrane with the help of the ATP-dependent protease FtsH (White et al., 2017; Atanaskovic et al., 2020; Behrens et al., 2020). Similar to colicins, FtsH cleaves the nuclease domain of these pyocins so that only the domain with killing activity enters the cytoplasm (Chauleau et al., 2011; White et al., 2017; Atanaskovic et al., 2020).

A major form of resistance would be the presence of the immunity protein to neutralize the toxicity of the C-terminal killing domain (Sano et al., 1993a). The genetic determinants of pyocins are all located in the chromosome of P. aeruginosa and the immunity gene for S-pyocins are located downstream of the gene for the killing protein (Sano and Kageyama 1984; Duport et al., 1995). Some strains have ‘orphan’ immunity proteins which are present without their corresponding killing proteins (Ghoul et al., 2015). Another obvious form of resistance would be the absence of primary or secondary target receptors.

1.2.3 Pyocins in Interstrain Interactions

Most of the research into the role of pyocins in interstrain competition has been published on R-pyocins. Multiple studies have shown that the production of R-pyocins can give a strain dominance in intraspecies competition (Yun-Jeong et al., 2005; Waite et al., 2009; Oluyombo et al., 2019). Oliveira et al., 2015 found that damage from R- and F- pyocins induces biofilm formation in target cells. Although there has been considerable research done into the importance of R-pyocins in strain competition, the extent to which S-pyocins are used in intraspecies competition remains largely unknown. We hope to shed light on the role of S-pyocins in interstrain competition by expanding on previous research done in our laboratory by MacLean 2012, which suggested that an S-pyocin may be decreasing the virulence of other target bacteria.
The reason why MacLean 2012 concluded the inhibitory factor leading to increased survival in the *Drosophila* co-infection model is likely an S-pyocin is that it is heat sensitive, pH sensitive, protease sensitive, and had a molecular weight greater than 5 kDa (MacLean 2012). These are all known characteristics of S-pyocins, with S-pyocins being known to be as large as 93 kDa (Elfarash *et al*., 2012). Furthermore, the spent medium of isolate 14651 was shown to inhibit the growth of other strains, which is characteristic of pyocins in general. These inhibitory effects were amplified with the addition of mitomycin C, an activator of pyocin expression.

S-pyocins are toxic to their target cells so it is believed that a reduction in the prevalence of the more virulent isolates is leading to overall reduced virulence (MacLean 2012). However, we have reason to believe that the S-pyocins released by 14651 may additionally be decreasing the virulence factor production of the more virulent isolates. The reasons for this include that during the 2-day acute rat prostate co-infection, there were equal percentages of 14651 and the more virulent isolates present but there was still decreased tissue damage. Furthermore, when the protease production of 14649 was tested in the presence of the spent medium of 14651, there was an absence of protease production observed despite there clearly still being bacterial growth (Figure 1.6) (Lutter 2008). This effect dissipated when the spent medium was heat-inactivated, suggesting it is caused by a protein which we believe is the secreted S-pyocin.

It is not unusual to find that subinhibitory levels of antimicrobials have a diverse range of effects on target cells. Fluoroquinolones, which are antibiotics that work by interacting with DNA gyrase and topoisomerase, are well known to have effects on the SOS-response system. The SOS-response system is believed to cause changes in gene regulation that involve general metabolism, DNA replication/repair, cell division, motility, QS, and cell permeability (Cirz *et al*., 2006). Sub-inhibitory concentrations of the fluoroquinolone, ciprofloxacin, have been shown
Figure 1.6. Protease production of 14649 is decreased in the presence of 14651 spent medium.

14649 was spotted on protease plates containing either no spent medium, 14651 spent medium, or 14651 spent medium that had been heat inactivated. Image modified from: Lutter EI. 2008. *Pseudomonas aeruginosa* cooperativity. (Doctoral Dissertation). Biological Sciences. Calgary, University of Calgary.
to reduce swimming and swarming in \textit{P. aeruginosa} while increasing biofilm formation (Linares \textit{et al.}, 2006). They have also been shown to cause an increase in pyocin gene expression (Brazas and Hancock 2005). If S2 affects the virulence factor production of target bacteria, this could have clinical importance as there is ongoing research into the possibility of using S- pyocins as novel antibiotics (Brown \textit{et al.}, 2012; Smith \textit{et al.}, 2012; Rasouliha \textit{et al.}, 2013; McCaughey \textit{et al.}, 2016a; McCaughey \textit{et al.}, 2016b; Turano \textit{et al.}, 2017; Turano \textit{et al.}, 2020).

1.3 Hypothesis

The manifold interactions that occur in the polymicrobial communities of CF lungs create a complex ecology that, overall, leads to a progressive decline in lung function. Most studies have focused on R- pyocins, with there being a lack of research done into the role S- pyocins play in influencing polymicrobial interactions. We believe 14651 secreted S- pyocins which, independent of their bactericidal actions, decreased virulence in co-infections by decreasing the virulence factors produced by their targets. This leads to our hypothesis that \textit{P. aeruginosa} CF isolates use S- pyocins to influence the overall virulence of microbial communities by decreasing the virulence factor production of target bacteria. If S- pyocins can decrease the virulence factor production of target bacteria, they could be playing a role in helping isolates establish a chronic infection by evading the host immune system. Our main aim is to characterize the virulence factor production of a population of sensitive bacteria treated with an S- pyocin compared to an untreated population of the same density. Additionally, we intend to investigate the origin and mechanism by which any observed differences occur.
1.4 Research Objectives

1. **Purify an S- pyocin and determine sensitive targets by surveying its activity against a spectrum of *P. aeruginosa* isolates that were previously found to respond to S-pyocins in general.** The S- pyocin was chosen based off which ones are available in our laboratory. Restriction cloning, Ni-NTA chromatography, and size exclusion chromatography were utilized for the purification of the pyocin. The inhibitory activity of the chosen S- pyocin was determined against 27 *P. aeruginosa* CF isolates available in our laboratory using an overlay inhibition assay.

2. **Examine the effects the S- pyocin has on the growth and virulence factor production of sensitive bacteria as compared to untreated populations of the same density.** Growth curves and preliminary experiments were performed to determine which concentrations of S- pyocin are subinhibitory and appear to have effects on the target population. Protease, elastase, pyocyanin, and lipase assays were carried out based on the known virulence traits of the sensitive isolates.

3. **Explore whether genetic or physiological changes are occurring which could explain the observed alterations in virulence factor production.** The permanence of any observed differences was examined to discriminate between genetic and physiological changes, focussing in detail on one specific isolate. To examine genetic mutations, whole genome sequencing was carried out using short read sequencing and amino acid changes were noted.
Chapter Two: Materials and Methods

2.1 Bacterial Strains, Plasmids, and Growth Media Used

Table 2.1 lists all the strains and plasmids used in this study. A frozen stock of each strain was stored in 10% skim milk or 50% glycerol at -80°C. The following amounts of antibiotics were added when necessary: 34 μg/mL chloramphenicol and 50 μg/mL or 100 μg/mL ampicillin. Chloramphenicol was prepared to a working stock of 50,000 μg/mL in ultrapure ddH₂O. Ampicillin was prepared to a working stock of 100,000 μg/mL in ultrapure ddH₂O. During transformation experiments, *E. coli* were grown in Super Optimal Broth (SOB) (0.584 g NaCl, 0.186 g KCl, 0.952 g MgCl₂, 1.204 MgSO₄, 5 g yeast extract (EMD Chemicals) and 20 g tryptone (EMD Chemicals) per L) and rescued in Super Optimal Broth with Catabolite repression (SOC) (5 mL 1M glucose, 245 mL SOB). For protein expression, *E. coli* was grown in either Luria Bertani (LB) broth (10 g NaCl, 5 g yeast extract (EMD Chemicals) and 10 g tryptone (EMD Chemicals) per L) or on LB with 1.5% agar added (Select agar, Invitrogen, Canada). *P. aeruginosa* was grown in Tryptic Soy Broth (TSB) (VWR, Canada) or on Tryptic Soy Agar (TSA) (VWR, Canada).
<table>
<thead>
<tr>
<th>Isolate or plasmid</th>
<th>Description</th>
<th>Sequence type (Faria 2009)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>Laboratory strain</td>
<td>549</td>
<td>Holloway <em>et al.</em>, 1979</td>
</tr>
<tr>
<td>14651</td>
<td>Non-mucoid CF isolate, Patient 89, isolated on 04/29/98</td>
<td>179</td>
<td>Lutter 2008, MacLean 2012</td>
</tr>
<tr>
<td>14649</td>
<td>Non-mucoid CF isolate, Patient 89, isolated on 04/29/98</td>
<td>192</td>
<td>Lutter 2008</td>
</tr>
<tr>
<td>14650</td>
<td>Non-mucoid CF isolate, Patient 89, isolated on 04/29/98</td>
<td>192</td>
<td>Lutter 2008</td>
</tr>
<tr>
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<td>Non-mucoid CF isolate, Patient 91, isolated on 05/07/98</td>
<td>192</td>
<td>Lutter 2008</td>
</tr>
<tr>
<td>11 (also called 14716)</td>
<td>Non-mucoid CF isolate, Patient 38, isolated on 05/26/9</td>
<td>609</td>
<td>Lutter 2008</td>
</tr>
<tr>
<td>12 (also called 5166)</td>
<td>Non-mucoid CF isolate, Patient 35, isolated on 02/18/88</td>
<td>684</td>
<td>Raivio <em>et al.</em>, 1994</td>
</tr>
<tr>
<td>4384</td>
<td>Hypertoxigenic non-mucoid CF isolate, Patient 35, isolated on 02/02/87</td>
<td>685</td>
<td>Raivio <em>et al.</em>, 1994</td>
</tr>
<tr>
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<td>236</td>
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<td>236</td>
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<td>No.</td>
<td>Description</td>
<td>Year</td>
<td>Reference</td>
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<td>687</td>
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<td>Gallant et al., 2000</td>
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<td>Erickson et al., 2002</td>
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<td>298</td>
<td>Raivio et al., 1994</td>
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<td>---------------------------------</td>
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<td>-----------</td>
</tr>
<tr>
<td>Dh5α</td>
<td><em>E. coli</em> strain lacking T7 RNA polymerase used for maintaining vectors</td>
<td>N/A</td>
<td>Hanahan <em>et al.</em>, 1991</td>
</tr>
<tr>
<td><em>Nico21(DE3)</em></td>
<td><em>E. coli</em> derivative of BL21(DE3) used for protein expression. F– <em>glmS ompT gal dcm lon hsdSB</em> (rB− mB−) (DE3 [lacI lacUV5-T7 gene])</td>
<td>N/A</td>
<td>Robichon <em>et al.</em>, 2011</td>
</tr>
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<td>pLysS</td>
<td>Vector encoding T7 lysozyme, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>N/A</td>
<td>MilliporeSigma</td>
</tr>
<tr>
<td>pET15b</td>
<td>Protein expression vector, N-terminal his-tag, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>Novagen</td>
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<tr>
<td>pET15bS2</td>
<td>pET15b with coding sequences for PA1150 and PA1151</td>
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<td>This study</td>
</tr>
<tr>
<td>pET15bS4</td>
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<td>pET15bS5</td>
<td>pET15b with coding sequence for PA0985</td>
<td>N/A</td>
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</tr>
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</table>

<sup>†</sup> Resistance

* *Nico21(DE3) was kindly provided by Dr. Joe Harrison*
2.2 Cloning of Pyocins

Cloning was carried out based on a procedure used in literature (Elfarash \textit{et al.}, 2012; McCaughey \textit{et al.}, 2016a) (Figure 2.1). Primers containing NdeI and XhoI cut sites (S2fwd with S2rev for S2, S4fwd with S4rev for S4, and S5fwd with S5rev for S5) were used to PCR amplify (section 2.2.3) the S2 (PA1150), S4 (PA3866), and S5 (PA0985) genes from the PAO1 genome with an N-terminal His$_6$ (Table 2.2). The immunity proteins of S2 (PA1151) and S4 (PA3865.1) were cloned along with their respective killing proteins. S5 was cloned without its immunity gene because it is a pore-forming protein so it has activity when encountering a target cell from the outside, thus production of pyocins within \textit{E. coli} cells used for expression should be non-lethal. On the other hand, S2 and S4 were cloned with their immunity genes as they are both nucleases and could kill their \textit{E. coli} host if present without their corresponding immunity protein. To achieve the final construct illustrated in Figure 2.2, the genes were PCR amplified (section 2.2.3), gel extracted (section 2.2.5), restriction enzyme digested (section 2.2.6), and ligated into the expression vector pET-15b (section 2.2.7). Each final construct was confirmed through sequencing DNA sequencing (section 2.2.9) using the primers (S2Mid-Fwd, S2Mid-Rev, S4Mid-Fwd, S4Mid-Rev, T7 promoter, and T7 terminator) listed in Table 2.2.

The completed vector was transformed by electroporation (section 2.2.8) into the \textit{E. coli} strain Nico21(DE3) for S5 and NiCo21(DE3)pLysS for S2 and S4. Nico21(DE3) is a derivative of BL21(DE3), which is commonly used for protein expression as it is deficient in the proteases Lon and OmpT (Robichon \textit{et al.}, 2011). Nico21(DE3) was used as, unlike BL21(DE3), it lacks GlmS. GlmS is a contaminating protein that often binds to Ni-NTA resin. Pyocins with
Figure 2.1. Cloning of pyocin genes for protein expression.

The genome of PAO1 was extracted and used to PCR amplify the desired pyocin genes. The PCR primers used contained restriction sites for NdeI and XhoI. Desired pyocin genes included that of S2 along with its downstream immunity protein, S4 along with its downstream immunity protein, and S5 without its immunity protein. Gel extraction was used to purify and concentrate the PCR products before cloning. Restriction digestion was used on both the PCR products and the protein expression vector of interest (pET15b), which contains an N-terminal His$_6$-tag. Following ligation, the completed vector containing the desired pyocin gene was then transformed into the *E. coli* strain Nico21(DE3) through electroporation. Image created with BioRender.com.
Extract genome from PA01

Ndel

Pyocin Gene

Xhol

PCR amplify pyocin genes (S2 + S2imm, S4 + S4imm, or S5) with restriction sites

Concentrate and purify PCR products through gel extraction

pET15b + Pyocin Gene

Restriction digest plasmid and PCR product before ligating together

His6 Tag

Pyocin gene

pET15b

Amp

Transform protein expression vector into E. coli strain Nico21(DE3)
Table 2.2. Primers used to amplify pyocins from PAO1 genome and sequence plasmids following cloning to ensure correct gene insertion.

The underlined portion is the cut site for the restriction enzyme and the bolded part is complementary to the pyocin gene. The sequences for the S4fwd and S4rev primers were obtained from Elfarash et al., 2012 and the S5fwd and S5rev primers from McCaughey et al., 2016a.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence (5’ → 3’)</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2fwd</td>
<td>GGAATTCATATGGCTGTAATGATTACGAACCTG</td>
<td>NdeI</td>
</tr>
<tr>
<td>S2rev</td>
<td>CCGCTCGAGCTAAACCGGCTAAAGCC</td>
<td>XhoI</td>
</tr>
<tr>
<td>S4fwd</td>
<td>CCGCTCGAGTTATTTTCTGGAGCAATTGTTAC</td>
<td>XhoI</td>
</tr>
<tr>
<td>S4rev</td>
<td>GGAATTCATATGACAAATAATAGTGCACCCACCAC</td>
<td>NdeI</td>
</tr>
<tr>
<td>S5fwd</td>
<td>TTTGACGTCTCGAGTTAATGGATATTACAAGATTGTGTTCAC</td>
<td>XhoI</td>
</tr>
<tr>
<td>S5rev</td>
<td>GAGACATATGTCACACGAACGAAGTAC</td>
<td>NdeI</td>
</tr>
<tr>
<td>S2Mid-Fwd</td>
<td>GGATGAAATCGCCAGCCTG</td>
<td>N/A</td>
</tr>
<tr>
<td>S2Mid-Rev</td>
<td>GTACGACCAGAGTGTACTCG</td>
<td>N/A</td>
</tr>
<tr>
<td>S4Mid-Fwd</td>
<td>TACGACCAGAGTGTGCTTG</td>
<td>N/A</td>
</tr>
<tr>
<td>S4Mid-Rev</td>
<td>CAAGCAGATCGTAACGAAGGCC</td>
<td>N/A</td>
</tr>
<tr>
<td>T7 promoter</td>
<td>TAATACGACTCATAAGGG</td>
<td>N/A</td>
</tr>
<tr>
<td>T7 terminator</td>
<td>GCTAGTTATTGCTAGCG</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 2.2. Final construct of expression vector for S2.

The construct of a pET15b expression vector used for production of the S2 pyocin, S2, and its corresponding immunity protein. pET15b provides an N-terminal His6-tag. The expression of S2 and its immunity gene are controlled by a T7 promoter. An ampicillin resistance gene is present on the plasmid, allowing for positive selection. ‘pys2’ is the S2 killing gene, ‘imm2’ is the S2 immunity gene, ‘6xHis’ is the N-terminal His6-tag, and ‘AmpR’ is the ampicillin resistance gene. Image created by Dr. Henrik Almblad using SnapGene®.
nuclease activity additionally had the pLysS plasmid. pLysS is a plasmid that encodes a T7 lysozyme and was added as an extra safety measure to prevent the basal level expression of the nuclease genes controlled by the T7 promoter. This plasmid was additionally transformed into Nico21(DE3) for expression of S2 and S4 as an added protection against the nuclease activity of these proteins.

2.2.1 Genomic DNA Isolation

Genomic DNA isolation was carried out using a DNeasy® Tissue Kit (Qiagen, Mississauga, ON) according to manufacturer’s instructions. *P. aeruginosa* was streaked from frozen stocks onto TSA. A single colony was used to inoculate 20mL TSB, which was grown overnight shaking at 37 °C. One and a half mL of the overnight culture was spun down at 16,363 \( \times \) g (Eppendorf™ microcentrifuge model 5415D) for 2 mins and resuspended in buffer from the DNeasy® kit. Twenty μL proteinase K was added to the resuspension and the DNeasy® kit protocol for animal tissues was followed. The final product was eluted with 50 μL ultrapure ddH₂O.

2.2.2 Plasmid DNA Isolation

Plasmid isolation was carried out using QIAprep® Spin Miniprep Kit (Qiagen, Mississauga, ON). Strains carrying plasmids were streaked on LBA plates with required antibiotics. A single colony was used to inoculate LB similarly containing the appropriate antibiotics. Following overnight growth, 1.5mL of the overnight culture was spun down at 16,363 \( \times \) g (Eppendorf™ microcentrifuge model 5415D) for 2 min and resuspended in buffer from the QIAprep® kit. Instructions from the QIAprep® kit were followed to complete plasmid extraction. The final product was eluted with 50 μL ultrapure ddH₂O.
2.2.3 Polymerase Chain Reaction (PCR)

PCR reactions included 15 ng DNA template (or a small fraction of a colony if doing boiled colony PCR), 5 pmol of each primer, 1 × HF buffer (Invitrogen, Canada), 3 % dimethyl sulfoxide (DMSO), 0.2 mM dNTPs, and 2 U Phusion DNA polymerase High Fidelity Enzyme (Invitrogen, Canada) in a final volume of 20 μL. Primers used are listed in Table 2.2 and were purchased from the University Core DNA Services (University of Calgary). Primers were resuspended using IDTE and stored at room temperature. The PCR reactions were prepared in 0.2 mL thin-walled PCR tubes and the program was carried out using an Eppendorf® Mastercycler Gradient system. The PCR program included denaturation at 98 °C for 30 seconds using genomic DNA (5 minutes for boil colony PCR). This was followed by 34 cycles of denaturation at 98 °C for 10 seconds, annealing through gradient PCR (Temperature = 62 °C, gradient = 10 °C) for 15 seconds, and elongation at 72 °C for 20 seconds per kb of amplicon. A final elongation took place at 72 °C for 5 minutes. PCR products were stored at 4 °C and visualized using agarose gel electrophoresis as described in section 2.2.4.

2.2.4 Agarose Gel Electrophoresis

Gels of 1.0 % agarose (Invitrogen, Canada) were prepared using 1 × Tris-acetate-EDTA (TAE) buffer (4.84 g Tris, 1.14 mL glacial acetic acid, 0.675 g ethylenediaminetetraacetic acid (EDTA) per L). Five μL of the DNA sample was mixed with equal amounts of DNA loading dye (0.25 % bromophenol blue, 0.5 % xylene cyanol FF and 30 % glycerol) and loaded into wells alongside a 1Kb+ DNA ladder (Invitrogen, Canada). Gels were stained with RedSafe (iNtRON Biotechnology) and run in Mini-Sub Cell GT gel electrophoresis units (Bio-Rad) with 110 volts.
for 35 – 90 minutes. The Gel Doc 2000 (Bio-Rad Canada) with the Quantity One Software was used for band visualization.

2.2.5 Gel Extraction

Gel extractions were carried out using QIAquick® Gel Extraction Kit. PCR products were run on 1.0 % agarose gels as detailed in section 2.2.4. The bands were visualized with a UV light using a Transilluminator (Bio-Rad) and excised from the gel. Extraction was carried out as detailed in instructions for QIAquick® kit and the products were eluted with ultrapure ddH2O.

2.2.6 Restriction Digestion

Restriction enzymes were purchased from Invitrogen, Canada. Restriction digestions consisted of 1 µg of plasmid DNA or 0.2 µg of PCR product, 2 µL of 10 × Fast Digest buffer, and 1 uL of selected restriction enzymes in a final volume of 20 µL for plasmid digestions or 30 µL for PCR digestions. Restriction digestions were incubated for 4 hours at 37 °C before inactivation at 80 °C for 5 minutes. Products were stored at -20 °C and visualization was performed using agarose gel electrophoresis as described in section 2.2.4.

2.2.7 Ligation

Following the procedure detailed by McCaughey et al., 2016a, the pyocin genes were PCR amplified including the restriction sites for NdeI and XhoI using the primers in Table 2.2. Following gel extraction as detailed in section 2.2.5 and restriction digestion as detailed in section 2.2.6, the amplified pyocins were cloned into the expression vector pET-15b through a ligation reaction. Ligation reactions contained 50 ng of plasmid DNA, 0.035 ng of insert DNA per base pair (bp) of insert, and 1 µL of T4 DNA ligase (Invitrogen, Canada) in a final volume of
20 μL. The reaction was incubated overnight at 4 °C and inactivated by heating for 10 minutes at 70 °C. Products were stored at -20 °C.

2.2.8 Electroporation

The end products were transformed into the *E. coli* strain Dh5α for vector maintenance and Nico21(DE3) or Nico21(DE3)pLysS for protein expression. The *E. coli* were made competent using electroporation. The cells were grown overnight in SOB. Five mL SOB was inoculated with 50 μL of overnight culture and grown shaking at 37 °C to an OD$_{600}$ of 0.6. Two mL of cells were pelleted by centrifugation at 16,363 × g (Eppendorf™ microcentrifuge model 5415D) for 2 min and resuspended in 1 mL of chilled 10 % glycerol. Centrifugation and resuspension were repeated a second time, after which a final centrifugation at 16,363 × g (Eppendorf™ microcentrifuge model 5415D) for 2 min was carried out. The spent medium was discarded and the pellet was resuspended in 50 μL of chilled 10 % glycerol. DNA (200-400 ng) to be transformed was added to the pellet and the mixture was transferred to a chilled cuvette. The cuvette was electroporated with a Gene Pulser II electroporator (Bio-Rad) (2.5 kV, 200 ohms, 25 μF). Following electroporation, 1 mL of pre-warmed SOC recovery media was immediately added. The mixture was transferred to a separate tube and allowed to shake for 1 hr at 37 °C. The culture was then centrifuged at 16,363 × g (Eppendorf™ microcentrifuge model 5415D) for 2 min and resuspended in 100 μL of pre-warmed SOC. The 100 μL mixture would then be spread plated onto a pre-warmed LBA plate containing the required antibiotic and incubated at 37 °C for 24-48 hrs.
2.2.9 DNA Sequencing

Successful cloning was determined by visualizing inserts using plasmid preparation (section 2.2.2) and colony PCR (section 2.2.3) before a final confirmation through sequencing. For DNA sequencing, DNA samples were sent to University Core DNA Services (Calgary, Canada). The primer sets shown in Table 2.2 were used for sequencing. Sequence analysis was performed using Benchling (Benchling Inc., USA) and the Pseudomonas Genome DB found at Pseudomonas.com.

2.3 Protein Purification

2.3.1 Protein Expression and Ni-NTA Chromatography

Pyocin purification was carried out as detailed in McCaughey et al., 2016a. The pyocins were overexpressed in either Nico21(DE3) (for pyocin S5) or Nico21(DE3)pLysS (for pyocins S2 and S4). The transformed bacteria were grown in LB broth with 100 μg/mL of ampicillin in a 37 °C shaking incubator to an OD₆₀₀ of 0.6. As the T7 RNA polymerase is under the control of a lacUV5 promoter in Nico(DE3), 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce pyocin expression. After IPTG addition, the cultures were grown for 3.5 h at 37 °C. Following centrifugation (3316 g, 20 min), cells were resuspended in 20 mM Tris/HCl (pH 8), 500 mM NaCl and 10 mM imidazole. 1:100 phenylmethanesulfonyl fluoride (PMFS) was added to resuspended cells, which were then lysed by sonication (Misonix microson™ Ultrasonic Cell Disruptor XI). After lysis and centrifugation (36000 g, 30 min), the spent medium was added to a column containing Ni-NTA resin equilibrated in 20 mM Tris/HCl (pH 8), 500 mM NaCl and 10 mM imidazole and eluted with 20 mM Tris/HCl (pH 8), 5 % glycerol,
and 500 mM Imidazole. The Ni-NTA chromatography was carried out using a gravity flow column (Bio-Rad, Canada) and 1-2 mL of HisPur™ Ni-NTA Resin (Invitrogen, Canada). Visualization was accomplished with sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) as detailed in section 2.3.3.

2.3.2 Size Exclusion Chromatography

Size exclusion chromatography was carried out with a size exclusion machine (GE AKTApurifier) and with the UNICORN™ 5.20 software. Ni-NTA purified pyocins were run through a Superose 12 10/300GL column (bed volume of 24 mL, mol wt of 1-300 kDa) using a buffer of 50 mM Tris/HCl (pH 7.5) and 200 mM NaCl. Prior to running the sample, 40 mL of buffer was used to wash the system at a flow rate of 0.5 mL/min. If the absorbance line was not completely flat, another 40 mL of buffer was run through to remove residue. Samples were concentrated to a volume of 500 μL using a MilliporeSigma™ Amicon™ Ultra Centrifugal Filter Units (Millipore, Canada) and spun down (4000 g, 15 min) at 4 °C to remove aggregates. The 500 μL sample was injected into a 1 mL loop and buffer was pumped through the system at 0.5 mL/min. The flow through was collected in 1 mL aliquots. Once all samples had been run, the system was washed with 40 mL of 20 % ethanol at a flow rate of 0.7 mL/min. The column was similarly stored in 20 % ethanol but at 4 °C. Aliquots of interest were determined using a chromatogram (Figure 2.3) produced by the UNICORN™ 5.20 software and visualized with SDS-PAGE (section 2.3.3). The aliquots containing the desired pyocin were stored at -20 °C. When required for use, an aliquot was removed, centrifuged at 9,391 × g (Eppendorf™ microcentrifuge model 5415D) for 10 min at 4 °C to remove aggregates. Following this, the aggregates were measured using a Bradford Assay as detailed in section 2.3.4. If higher protein
**Figure 2.3. The chromatogram of S2 size exclusion shows elution around 10 mL.**

The separation of the components of a S2 purification. Fragments run through a Superose 12 10/300GL column, which separates 1 – 300 kDa proteins based on their size. The x-axis represents the progression of one column volume of buffer (24 mL) through the column, with each 1 mL being collected as an aliquot. The y-axis shows the signal intensity, representing the abundance, in milli-Absorbance Units for each of the separated components.
concentrations were required, the purified pyocins were concentrated using MilliporeSigma™ Amicon™ Ultra Centrifugal Filter Units (Millipore, Canada). The pyocins would then be stored at 4 °C while in use.

2.3.3 SDS-PAGE

A discontinuous buffer system (Neville, 1971) was used for SDS-PAGE. Samples were mixed with Laemmli Solubilization Buffer (100 mM dithiothreitol (DTT), 50 mM Tris pH 6.8, 2 % SDS, 20 % Glycerol, 0.1 % bromophenol blue) and boiled for 10 minutes prior to electrophoresis. Samples were electrophoresed in a polyacrylamide gel consisting of a 5 % stacking and 12 % separating portion. Gels were run at 100 V for 10 min then 180 V for 70 min. The running buffer consisted of 25 mM Tris base, 200 mM glycine (electrophoresis grade), and 0.1 % SDS. The gels were stained in Coomassie blue dye (Sigma Aldrich, Canada) for 30 minutes and then destained with Coomassie destain (14 % ethanol, 10 % glacial acetic acid). A PageRuler™ Prestained Protein Ladder (Invitrogen, Canada) was run for the approximation of molecular weights and the Gel Doc 2000 (Bio-Rad Canada) with the Quantity One Software was used for band visualization.

2.3.4 Bradford Assay

Protein samples (10 μL) were mixed with 200 μL of Bradford reagent (Bio-Rad, Canada) in a 96 well plate (VWR, Canada). The Bradford reagent was diluted 1 in 5 prior to using. Following 15 minutes of incubation at room temperature, the OD_{600} was measured with a Perkin Elmer Victor X4 model 2030 multilabel counter. Bovine serum albumin (BSA) (Invitrogen, Canada) was used to create a standard curve for protein quantification.
2.4 Overlay Inhibition Assay

PAO1 and indicator isolates were grown overnight in 10 mL of TSB (VWR, Canada) shaking at 37 °C. Indicator isolates are *P. aeruginosa* isolates used to form the bacterial lawn of the overlay plates. The supernatant of PAO1 was collected for use as a positive control because it is known to produce pyocins, which should have inhibitory ability when spotted on the lawns of indicator isolates. PAO1 was standardized to an OD$_{600}$ of 0.1 in 10 mL of fresh TSB and grown until mid-log phase. PAO1 was pelleted at 4,602 × g (Eppendorf™ microcentrifuge model 5415D) for 10 min and its supernatant filter sterilized with a 0.2 μm filter (VWR, Canada). For pyocin induction, 1.5 μg/mL of mitomycin C (Sigma-Aldrich, Canada) was added and the cells were grown for an additional 2 hours prior to supernatant collection. Cell-free supernatant was stored at 4 °C for up to two weeks.

Indicator isolates were standardized to an OD$_{600}$ of 0.1. Standardized culture (500 μL) was added to 4500 μL soft agar overlay (1 % peptone (Difco), 0.5 % select agar (Invitrogen, Canada)) for a final concentration of 0.01 and poured over a 20 mL TSA plate. The plates were allowed to dry before spotting 5 μL of each treatment in triplicate onto the plates. The plates were incubated overnight at 37 °C before measuring the zones of inhibition.

2.5 Minimum Inhibitory Concentration (MIC)

The MIC procedure is based off that described by EUCAST and ESCMID (EUCAST 2000). Four or five isolate colonies were used to inoculate Mueller Hinton Broth II (MHBII) (VWR, Canada) and subsequently adjusted to match a 0.5 McFarland standard. Samples were diluted to a final concentration of approximately 1 × 10$^6$ CFU/mL and added to 96 well plates in
a 1:1 ratio with S2. The final concentrations of S2 ranged in doubling dilutions from 64 μg/mL to 0.125 μg/mL. Ten μL of a positive control well (the isolate with 0 μg/mL of S2) was diluted in 10 mL of MHBII and 100 μL was plated on TSA and incubated (overnight at 37 °C) to confirm the isolate was diluted correctly. An optimal dilution would yield around 50 colonies/plate. The 96 well plates were sealed to keep from drying and left for 18 hours. Naked eye observations of the wells were used to determine the MICs (i.e., the first well that has no visible bacterial growth).

2.6 Growth Curve

Isolates were grown overnight at 37 °C in TSB and standardized to an OD600 of 0.01 in the presence or absence of S2. OD600 measurements were taken periodically over the interval of 24 hours using a spectrophotometer (Thermo Scientific GENESYS 20).

Colony counts were performed by removing 20 μL of culture and diluting it in 180 μL of phosphate-buffered saline (PBS). The mixture was further diluted 5 times and then 5 μL of each dilution was spotted on TSA plates in duplicates. The plates were incubated overnight at 37 °C before the colonies were counted.

2.6.1 Growth Curve – Genetic or Physiological Effects

A growth curve was carried out as described in section 2.6 for the isolate I1. At the 24-hour time point, 100 μL of the culture treated with 0 or 8 μg/mL S2 was pelleted and resuspended in 100 μL of fresh TSB. Note that the following procedure was carried out for both I1 treated with buffer and I1 treated with S2. The resulting resuspension was streaked onto TSA twice to produce a secondary streak plate. For ease of communication, single colonies
of I1 treated with S2 on this streak plate were referred to as ‘R1’. A single colony was taken from the secondary streak plate and grown overnight in TSB. New growth curves were carried out as detailed in section 2.6. This procedure is visualized in Figure 2.4.

2.7 Virulence Factor Assays

2.7.1 Lipase Assay

Lipase activity was measured as described by Lonon et al., 1988. Isolates were grown at 37 °C overnight or to a specific OD$_{600}$ in TSB in the presence or absence of S2. Following this, the cultures were pelleted by centrifugation at 4,355 x g (Beckman™ centrifuge model JA-20) for 10 min and 0.2 μm filter sterilized (VWR, Canada). A mixture of 500 μL cell-free spent medium, 0.15 mL 10 % Tween 20, 0.1 mL 1M CaCl$_2$, and 2.3 mL 50 mM TrisHCl (pH 7.6) was incubated for 2 hours at 37 °C. The increase in turbidity of the mixture was measured at OD$_{400}$. One unit of lipase activity was defined as an increase in OD$_{400}$ of 0.01 per 2 hours of incubation. Lipase activity per mL was calculated using the following equation:

$$\frac{OD_{400}^{Final} - OD_{400}^{Initial}}{OD_{600} \times Volume\ in\ mL \times 0.01}$$

2.7.2 Protease Assays

2.7.2.1 Skim Milk Protease Assay

Isolates were grown at 37 °C overnight or to a specific OD$_{600}$ in TSB in the presence or absence of S2. Following this, the cultures were pelleted by centrifugation at 4,355 x g.
Figure 2.4. Visualization of the Growth Curve – Genetic or Physiological Effects procedure.

A visualization of the procedure used to determine whether the extended lag phase of I1 is genetic or physiological in origin. I1 was cultured from a secondary streak plate and subsequently standardized to perform a growth curve. The growth curve treatments were 0 and 8 μg/mL S2. A sample was taken from each growth curve at 24 hours (both I1 treated with buffer and I1 treated with S2), resuspended in fresh broth, and then streaked out. When I1 treated with S2 was resuspended and streaked out, it became known as R1. R1 was cultured and standardized to perform growth curves again with the treatments of 0 and 8 μg/mL S2. As a negative control, the culture of I1 treated with buffer was put through the same steps as R1. Image created with BioRender.com.
(Beckman™ centrifuge model JA-20) for 10 min and filter sterilized (0.2 μm filter from VWR, Canada). Three μL of each cell-free spent medium was spotted in triplicate on skim milk plates. The plates were incubated overnight at 37 °C and the zones of clearance measured.

Dialyzed skim milk plates were made for assessing protease production (Sokol et al., 1979). The plates were produced by first dialyzing BHI broth (9.25 g BHI powder from EMD chemicals in 25 mL ddH2O) at 4 °C overnight in 500mL of ddH2O using Spectrum Labs® 15,000 MWCO dialysis tubing. Noble agar (Difco) at 3 % was added to the 500 mL dialysate and autoclaved. At the same time, a mixture of 3 % skim milk (Difco) in 500 mL ddH2O was produced and autoclaved. The two mixtures were added together and poured to produce the protease plates (15 mL per plate). The plates were air-dried overnight and stored at 4 °C before use.

2.7.2.2 Elastin Congo Red Assay

The procedure for elastase activity was modified from Ohman et al., 1980. Isolates were grown at 37 °C overnight or to a specific OD600 in TSB in the presence or absence of S2. Following this, the cultures were pelleted by centrifugation at 4,355 × g (Beckman™ centrifuge model JA-20) for 10 min and filter sterilized (0.2 μm filter from VWR, Canada). Half a mL of cell-free spent medium was added to a 1 mL mixture of 1mM TrisHCl (pH 7.2) and 5 mg Elastin Congo Red (Sigma Aldrich, Canada). The mixture was incubated for 3 hours at 37 °C with mixing each hour. The change in OD495 was relative to the elastase activity and the following equation was used to determine elastase activity normalized for growth:

\[
\frac{OD495_{Final} - OD495_{Initial}}{OD600 \times Volume \ in \ mL}
\]
2.7.3 Pyocyanin Assay

A pyocyanin extraction was carried out based on Essar et al., 1990. Isolates were grown overnight in TSB in the presence or absence of S2. Following this, the cultures were pelleted by centrifugation at 4,355 × g (Beckman™ centrifuge model JA-20) for 10 min and filter sterilized (0.2 μm filter from VWR, Canada). Chloroform (1.8 mL) was added to 3 mL of cell-free spent medium. The mixture was vortexed (10 × 2 s) and then centrifuged at 9,391 × g (Eppendorf™ microcentrifuge model 5415D) for 10 min to separate the aqueous and organic layer before the green-blue organic layer was transferred to a new tube. The organic layer (1.2 mL) was combined with 600 μL 0.2 M HCl. This new mixture was vortexed (10 × 2 sec) and then centrifuged at 9,391 × g (Eppendorf™ microcentrifuge model 5415D) for 2 min to produce a pink top layer. The OD₅₂₀ of the pink layer was measured and the resulting absorbance correlated with the quantity of pyocyanin. The μg/mL of pyocyanin normalized for growth was calculated using the following equation:

\[
\frac{OD_{520} \times 17.072}{OD_{600} \times Volume \ in \ mL}
\]

2.8 Genome Sequencing

Isolates were grown overnight in TSB in the presence or absence of 8 μg/mL S2. The resulting cultures were streaked onto TSA twice to produce a secondary streak plate. A single colony was taken from the secondary streak plate and grown overnight in TSB. Following this overnight growth, a genomic DNA isolation was carried out as detailed in section 2.2.1. The isolated genomes were sent to the Microbial Genome Sequencing Center (MiGS) under a 300 Mb sequencing offering. The resulting reads were assembled and analyzed using Geneious
Prime ® 2020.2.4 (https://www.geneious.com). This procedure is visualized in Figure 2.5 and was carried out for both I1 and PAO1.

Draft reference genomes (i.e., I1 and PAO1) were assembled using the SPAdes plugin. SPAdes builds a de Bruijn graph for de novo genome assembly. Short reads from the treated genomes (i.e., R1 and PAO1 treated with 8 μg/mL S2) were mapped to the assembled draft reference genomes. As detailed in the Geneious de novo assembly and alignment instructions, reads were trimmed using BBDuk before use and low coverage regions removed during SNP analysis. The Geneious variant finder was used to locate SNPs between the treated genomes and the draft reference genomes.

Annotations from RAST (Aziz et al., 2008) and the completed PAO1 (NC_002516) genome were used to annotate each draft reference genomes. The functions of hypothetical proteins were examined using the Geneious InterProScan plugin. InterPro assigns functions to proteins by searching 13 databases for protein signatures that match the protein sequence of interest. The MLST of each draft reference genome was checked using PubMLST.

2.9 Data Analysis and Statistics

All data was analyzed and graphed using GraphPad Prism 5 or 8 (Graphpad Software, Inc). A Student’s t-test was used in comparing 2 groups together and an analysis of variance (ANOVA) followed by a Dunnett’s or Tukey’s test was used in situations when there were more than 2 groups.
**Figure 2.5. Visualization of procedure for genome sequencing.**

Steps carried out to obtain and analyze sequences. I1 from a secondary streak plate was cultured and grown in TSB before standardization and exposure to 8 μg/mL S2. During this exposure, I1 treated with S2 may form multiple genotypes as a result of the selective pressures from S2. Then, the resuspended cells were streaked on a TSA plate. Each of the individual colonies that grow on this streak plate should only be of one genotype and will be referred to as R1. These colonies were cultured in TSB and their genomes extracted. These genomes, as well as a genome from the untreated isolate, were then sent for sequencing at the Microbial Genome Sequencing Center (MiGS). It should be emphasized that this whole procedure was repeated for each replicate. Assembly and analysis of the resulting sequences was carried out using Geneious Prime ®. Image created with BioRender.com.
Chapter Three: Purifying S-type Pyocins and Determining Their Spectrum of Activity Against a Variety of P. aeruginosa Isolates

3.1 Introduction

Lutter 2008 showed that the spent medium of isolate 14651 (from a CF patient) was able to decrease the protease production of isolates 14649, 14650, and 14673. This decrease in protease production is believed to be from a protein as this effect dissipated when the spent medium was heat inactivated (Lutter 2008). When co-infected with its more virulent target strains in a fly infection model, 14651 increased fly survival (Lutter 2008; MacLean 2012). Subsequently, MacLean 2012 found that the P. aeruginosa isolate 14651 was likely producing S-pyocins, which may be causing the inhibition that 14651 exhibited on the target strains 14649, 14650, and 14673. These observations led us to wonder if we could determine whether S-pyocins could be causing the decrease in protease production that Maclean 2012 witnessed. This would mean that, in addition to having bacteriocidal effects, S-pyocins could decrease the virulence factor production of target cells when present at subinhibitory concentrations.

As previously noted, co-infections with 14651 seemed to lessen the virulence of other CF isolates in a co-infection model, likely through a secreted factor (Lutter 2008). Further, MacLean 2012 determined that 14651 most likely produces S-pyocins that were having this effect. Previously, I had purified three S-pyocins: S2 with DNase activity, S4 with tRNase, and the pore-forming protein S5. This chapter will go into detail on how the three pyocins were purified and their activity confirmed. Additionally, this work will determine which purified pyocin to use for our studies.
3.2 Results

3.2.1 Cloning and Expressing Pyocins from PAO1 in *E. coli*

The purpose of this section is to obtain a purified S-pyocin to determine whether S-pyocins could potentially affect the virulence factor production of target bacteria. For a prior project, I had cloned (section 2.2) and purified through Ni-NTA chromatography (section 2.3.1) three S-pyocins (S2, S4, S5). These three S-pyocins had originated from the genome of PAO1.

The SDS-PAGE results verified the presence of the three pyocins following Ni-NTA chromatography. S2 (which is 74 kDa) showed a band between the 72 and 95 kDa ladder bands (Figure 3.1 A) (Sano *et al.*, 1993a). S4 (which is 93kDa) showed a band close to the 95kDa ladder band (Figure 3.1 B) (Elfarash *et al.*, 2012). S5 (which is 57 kDa) showed a band between the 55 and 72 kDa ladder bands (Figure 3.1 C) (Saeidi *et al.*, 2014).

The presence of the pyocin proteins does not mean they are biologically active. To confirm the activity of the purified pyocins, an overlay assay (section 2.4) was carried out where the purified pyocins were spotted on lawns of CF isolates to observe the presence of inhibition. To find sensitive isolates, a random selection of isolates was tested from the 27 available in our laboratory. They were spotted on a lawn of PAO1 as a negative control as PAO1 has the immunity proteins for S2, S4, and S5 so it should not be inhibited by any of these pyocins. Each test was repeated three times. For S2 and S5, very clear zones of inhibition were observed when spotted on a sensitive isolate (Figure 3.2 A, Figure 3.2 C). A faint zone of inhibition was observed when S4 was spotted on a sensitive isolate (Figure 3.2 B). Likely the zone of inhibition
Figure 3.1. Pyocins S2, S4, and S5 are visualized on SDS-PAGE following Ni-NTA chromatography.

SDS-PAGE gel of aliquots taken following the Ni-NTA chromatography of the pyocins: (A) S2, (B) S4, and (C) S5. Lane 1 of each gel is a PageRuler™ Prestained Protein Ladder whereas lane 2 contains 1.5mL aliquots taken following Ni-NTA chromatography. The His\textsubscript{6}-tagged pyocin sizes are expected to be: 74 kDa for S2, 93 kDa for S4, and 57 kDa for S5 (Sano et al., 1993a; Elfarash et al., 2012; Saeidi et al., 2014). The gel in (C) was cropped to remove lanes between the ladder and the lane of interest, the full uncropped image can be found in the Appendix under Figure A.1.
Figure 3.2. Purified S2, S4, and S5 have activity against sensitive isolates.

Overlay plates of an isolate sensitive to (A) S2, (B) S4, or (C) S5 treated with the respective purified pyocins. An overlay of PAO1 was used as a negative control because it expresses the immunity protein for pyocins S2, S4, and S5.
was so much fainter for S4 because there was a much lower amount of S4 collected as elute from Ni-NTA (Figure 3.1 B). These lower amounts of S4 might be due to the larger size of the S4 protein (93 kDa) as compared to S2 (74 kDa) and S5 (57 kDa) (Sano et al., 1993a; Elfarash et al., 2012; Saeidi et al., 2014).

3.2.2 The DNase S2 Was Chosen for Purification

If S- pyocins are triggering a change in virulence factor production in target bacteria, we do not know the exact mechanism of this change. However, both pyocins and colicins have been found to trigger a reciprocal bacteriocin attack in their target cells (Sano and Kageyama 1981; Majeed et al., 2013). This attack is RecA-dependent and occurs along with the induction of the SOS-response system (Sano and Kageyama 1981; Majeed et al., 2013). DNase colicins have been reported to trigger the SOS response system in target cells (Vankemmelbeke et al., 2005; Toshima et al., 2007). In the E. coli serotype O157:H7 this was noted to lead to a significant increase in Shiga toxin production (Toshima et al., 2007). Majeed et al., 2011 found that subinhibitory levels of colicins trigger a reciprocal colicin production by target strains through the induction of the SOS-response system, with DNases causing the strongest response. The DNase S- pyocin, AP41, has also been shown to induce reciprocal pyocin expression (Sano and Kageyama 1981). Consequently, it stands to reason that S2 may similarly induce the SOS-response system and perhaps through this mechanism it can cause an alteration in virulence factor production. Therefore, S2 was chosen to be used in later experiments and was further purified with size exclusion chromatography (section 2.3.2). The purity of the final product was inspected using SDS-PAGE (section 2.3.3) and protein concentration measured with a Bradford assay (section 2.3.4). Eluted S2 showed a clean band in the SDS-PAGE gel (Figure 3.3).
Figure 3.3. S2 was purified from size exclusion chromatography.

SDS-PAGE gel of S2 aliquot taken following size exclusion chromatography. Lane 1 is a PageRuler™ Prestained Protein Ladder whereas lane 2 contains a 1 mL aliquot taken following size exclusion chromatography. The expected size of S2 is 74 kDa (Sano et al., 1993a). The gel was cropped to remove lanes between the ladder and the lane of interest, the full uncropped image can be found in the Appendix under Figure A.2.
3.2.3 Inhibitory Effects of S2 on a Collection of *P. aeruginosa* CF Isolates

DNase pyocins have a catalytic core with a conserved HNH motif (Joshi *et al.*, 2015). This motif binds to the minor groove of the target cell’s genomic DNA and randomly cleaves their DNA. Death of the target cell likely occurs when there are too many cleavages for its DNA repair machinery to handle. If the target cell has an immunity protein it can block the cytotoxic activity by binding beside the nuclease domain.

The spent medium of 14651 had inhibitory activity against 14649, 14650 and 14673, suggesting the attenuation in virulence is related to the S- pyocin’s ability to enter or exhibit cytotoxicity on the target bacterium (MacLean 2012). In section 3.2.2, it was decided that S2 should be used in further testing. This section will discuss the sensitivity of an array of isolates to S2. Twenty-seven CF isolates were available in our laboratory. Based on the work of Bakkal *et al.*, 2010 who found that about 18% of a set of *P. aeruginosa* strains were inhibited by S2, we postulated that S2 would only inhibit a small subset of CF isolates. An overlay inhibition assay (section 2.4) was carried out on these isolates to determine which ones were sensitive to S2. PAO1 was used as a negative control as S2 was purified from PAO1 so this isolate should be immune against inhibition.

A Bradford assay (section 2.3.4) was used to measure the concentration of S2 and a range of concentrations (doubling dilutions from 256 – 0.125 μg/L) were tested to approximate how sensitive the isolates were to S2. The supernatant of PAO1 treated with and without mitomycin C was used as a positive control. All 27 of the CF isolates available in our laboratory have been found to be susceptible to the pyocins of both PAO1 and 14651 (MacLean 2012). The presence of mitomycin C, which is a potent DNA crosslinker, increases the expression of pyocins and
makes it more likely to see inhibition on target strains (MacLean 2012). As S2 is one of the pyocins produced by PAO1, we would expect that isolates inhibited by the purified S2 would similarly show zones from the supernatant of PAO1 treated with mitomycin C. The buffer used to suspend S2 was also spotted on the overlays as a negative control.

Full pictures of all the overlay plates can be found in the appendix (Figures A.3-A.10). As expected, none of the treatments showed any obvious inhibition against PAO1 (Table 3.1). Out of the 27 isolates, 7 of them were inhibited in some degree by S2: 14715, 14716, 14717, 5552, 4384, 5166, and 5585 (Table 3.1). The strongest effects of S2 were seen on isolates 14715, 14716, and 14717 with there being clear inhibition around 8 μg/mL (Figure 3.4). All three of these isolates are from the same patient and are of the same sequence type, so it is not surprising that they are all similarly affected by S2 (Table 3.1). The sequence type of all the isolates had been determined previously with multilocus sequence typing (MLST) (Faria 2009). MLST is a technique used to identify isolates with a common genetic background by categorizing unique sequences in their housekeeping genes (Maiden et al., 1998). Isolates with the same sequence type have a shared genetic background.

Isolates 4384 and 5166 both had cloudy zones of inhibition beginning at about 8 μg/mL (Figure 3.4). Unlike the isolates of patient 38, isolates 4384 and 5166 never obtained completely clear zones. Both these isolates are from patient 35 but are of different sequence types (Table 3.1). Isolates 5552 and 5585 were from different patients and only showed cloudy inhibition beginning at around 64 μg/mL (Figure 3.4; Table 3.1).

The sequence type, virulence factor production, and sensitivity to S2 were all taken into account in determining which isolates to use in further testing. 14716 was chosen for initial
Table 3.1. Seven out of 27 isolates were inhibited by S2.

Inhibition was determined following an overlay inhibition assay where S2 was spotted on lawns of isolates. The concentrations of S2 used ranged in doubling dilutions from 256 – 0.125 μg/mL. X correlates to no observable inhibition at any concentration, +++ correlates to clear zones of inhibition observed at greater than about 8 μg/mL, ++ correlates to cloudy zones of inhibition observed at concentrations greater than about 8 μg/mL, and + correlates to cloudy zones of inhibition observed at concentrations greater than about 64 μg/mL. The sequence type (ST) of each isolate determined by multilocus sequence typing is noted (Faria 2019). ‘Source’ is the patient where each of the isolates were taken from, excepting PAO1 which is a laboratory strain.

The results are representative of three independent replicates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Inhibition</th>
<th>Sequence type</th>
<th>Source</th>
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<td>X</td>
<td>236</td>
<td>Patient 92</td>
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<td>179</td>
<td>Patient 90</td>
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<tr>
<td>14671</td>
<td>X</td>
<td>179</td>
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<td>X</td>
<td>192</td>
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<tr>
<td>14673</td>
<td>X</td>
<td>192</td>
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</tr>
<tr>
<td>14715</td>
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Figure 3.4. S2 inhibited 7 isolates on overlay plates.

Overlay plates of isolates 14715, 14716, 14717, 4384, 5166, 5552, and 5585 treated with purified S2 at concentrations around 8 or 64 µg/mL.
examination as it was among the most strongly inhibited by S2, along with 14715 and 14717 (Table 3.1). It was not as practical to use 14715 as it is an extremely slow grower. 14716 and 14717 have almost the same virulence profile except that 14716 produces more lipase than 14717, so it was chosen for that reason.

For the next isolate, we wanted to choose one from a different patient and sequence type. Following the isolates from patient 38, the isolates from patient 35 were the most inhibited by S2 so they were the next choice for examination. 5166 was preferred over 4383 because, unlike 4384, it has above average lipase production (Lutter 2008). In summary, the effects of subinhibitory concentrations of S2 will be tested in further experiments on 14716 and 5166.

3.3 Summary

The S- pyocins S2, S4, and S5 were PCR amplified and cloned into an expression vector. The expressed proteins were then purified by selection on an Ni-NTA column and by size exclusion chromatography. All three purified pyocins displayed inhibitory activity on sensitive isolates.

The spectrum of activity of S2 against 27 CF isolates available in our laboratory was examined using an overlay inhibition assay. It was found that 7 of the 27 isolates were inhibited to some degree by S2. This number (26 %) is close to the percentage of isolates we predicted would be sensitive to S2 based off the work of Bakkal et al., 2010, which was 18 %. The inhibited isolates fell into three clusters. There were those that showed clear inhibition at concentrations of greater than about 8 μg/mL which included 14715, 14716, and 14717. All three of these isolates were of the same sequence type and from the same patient. Then there were those that showed cloudy inhibition at concentrations greater than about 8 μg/mL such as 5166.
and 4384. These isolates were similarly from the same patient but were of different sequence types. Lastly, there were those that showed cloudy inhibition only at concentrations greater than about 64 μg/mL, such as 5552 and 5585. Both these isolates were from different patients and sequence types.

This data provides basis for the selection of an S-pyocin and target isolates for use in further testing. The chosen target isolates include 14716 and 5166. For the sake of simplicity from here on out 14716 will be referred to as I1 and 5166 will be referred to as I2. The next chapter will delve into what effects subinhibitory S2 has on the virulence factor production of these isolates.
Chapter Four: Examining the Effect of Subinhibitory Concentrations of S-type Pyocin S2 on *P. aeruginosa* Isolates

4.1 Introduction

Repeated cycles of bacterial infection and inflammation wear down the lungs of CF patients, ultimately leading to respiratory failure for many individuals (Heeckeren *et al*., 1997; Canadian Cystic Fibrosis Registry 2019). *P. aeruginosa* infection exacerbates the inflammatory response and worsens patient prognosis (Henry *et al*., 1992; Heeckeren *et al*., 1997). Importantly, *P. aeruginosa* produces an array of virulence factors which can further damage the host.

Among its secreted virulence factors are proteases, lipases, and pyocyanin. Proteases, for instance, can affect the host immune system by cleaving vital components like immunoglobulins (Döring *et al*., 1981). They can cause further damage by destroying tissues, leading to the formation of ulcers and other lesions (Johnson *et al*., 1966; Kreger and Gray 1978). Pyocyanin plays an important part in pathogenesis (Mahajan-Miklos *et al*., 1999; Lau *et al*., 2003; Lau *et al*., 2004). The effects of this phenazine are wide ranging from the obstruction of ciliary motion and increasing reactive oxygen species to causing neutrophil apoptosis and disrupting protease inhibitors (Hassan and Fridovich 1980; Wilson *et al*., 1988; Britigan *et al*., 1999; Allen *et al*., 2005). Lipase is an extracellular protein which can affect the movement of some host immune cells (Jaeger *et al*., 1991). Previous work in our laboratory established that some *P. aeruginosa* isolates can modulate the virulence of other isolates during co-infection (Lutter 2008). When one isolate increases the virulence of another it may lead to an exacerbation in the patient’s lungs, worsening patient outcome. Conversely, if an isolate can attenuate the virulence of other isolates
this could assist the population as a whole in evading the immune system, contributing to chronic infection.

One isolate that our laboratory looked at, 14651, was able to decrease the virulence of other isolates in both a Drosophila model and a rat prostate model (Figure 4.1) (Lutter 2008; MacLean 2012). It was believed to be doing so by secreting S-pyocins (MacLean 2012). There was evidence that the S-pyocins of 14651 may not only be decreasing the virulence of target isolates through their bactericidal activity but, additionally, they may be able to decrease their virulence factor production. In the previous chapter, we purified the S-pyocin S2 and examined its ability to inhibit a collection of isolates. Isolates were chosen for further investigation based on their sensitivity to S2 and virulence factor production (Table 4.1). This chapter will examine the effects of subinhibitory concentrations of S2 on the growth and virulence factor production of isolates I1 (previously known as 14716) and I2 (previously known as 5166).

4.2 Results

4.2.1 Effect of S2 on Growth of I1 and I2

In this chapter, the effects of S2 will be tested against two isolates: I1 and I2. The purpose of this section is to determine which concentrations of S2 to use and examine the effects of S2 on the growth of these isolates utilizing growth curves.

A minimum inhibitory concentration (MIC) assay (section 2.5) was carried out with the intention of gaining a better idea as to which concentrations of S2 would be subinhibitory for the chosen isolates. Three replicates of the MIC assay were carried out for both isolates and the MICs ranged between 1-8 μg/mL for I1 and >64 μg/mL for I2 (data not shown). Unexpectedly, the presence of trailing endpoints, where the growth gradually fades over a couple wells, made it difficult to determine an exact MIC (EUCAST2000). These trailing endpoints could be due to
In the *D. melanogaster* and rat prostate models, the *P. aeruginosa* isolate 14651 decreased overall virulence when co-infected with the more virulent *P. aeruginosa* isolates 14649, 14650, and 14673. Its supernatant is believed to contain DNase S-pyocins. Treatment with the supernatant was shown to decrease the virulence and protease production of the target bacteria 14649, 14650, and 14673. The DNase S2 was purified in this study and used on the target bacteria I1 and I2. For all experiments, the buffer used to purify S2 was used as a negative control. Image created with BioRender.com.
Table 4.1. Virulence factor production of I1 and I2 as determined by Lutter 2008.

<table>
<thead>
<tr>
<th></th>
<th>I1</th>
<th>I2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pyocyanin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pyoverdine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total siderophore</td>
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<td>+</td>
</tr>
<tr>
<td>Protease</td>
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<td>+</td>
</tr>
<tr>
<td>Elastase</td>
<td>Below average</td>
<td>Above average</td>
</tr>
<tr>
<td>Lipase</td>
<td>Above average</td>
<td>Above average</td>
</tr>
</tbody>
</table>
contamination, resistant variants, or regrowth of the organism if the pyocin is deteriorating (EUCAST 2000). The formation of variants resistant to pyocins has been reported in literature and we examined this further in Chapter Five (Holloway et al., 1973; Hocquet et al., 2016; Inglis et al., 2016).

Preliminary experiments on lipase activity, pyocyanin production, and growth curves were carried out using a range of S2 concentrations (0.5 – 32 μg/mL). Effects of S2 on the pyocyanin production of I2 were significantly changed at concentrations as low as 2 μg/mL (data not shown). Initial testing found lipase activity appeared to be altered for I2 at concentrations between 8 and 32 μg/mL and at all tested concentrations for I1 (data not shown). As there did not appear to be a difference between the effects of 32 μg/mL and 16 μg/mL, it was decided not to use concentrations of higher than 16 μg/mL. As a result, the main concentrations this research focused on are 4, 8 and 16 μg/mL as this range demonstrated the strongest influence on both strains.

Growth curves were carried out on PAO1, I1, and I2 as detailed in section 2.6 using the concentrations 0, 4, 8, and 16 μg/mL of S2. PAO1 was used as a negative control and S2 did not appear to have any significant effect on the growth of PAO1 (Figure 4.2 A). I1 treated with S2 showed a clear difference from I1 treated with buffer where growth at all the time points between 2 and 24 hours were significantly different (p ≤ 0.0001) (Figure 4.2 B). Across all three concentrations, there was an increase in the lag phase from around 2 hours to around 13 hours. To address if this effect was only found in I1 we tested isolate 14715, an isolate taken from the same patient as I1 with the same MLST but different morphology (Figure 4.2 D). This lag phase was also observed in isolate 14715 where there was a significant difference between 14715
**Figure 4.2. Effects of S2 on the growth curves of PAO1, I1, I2, and 14715.**

Growth curve of the *P. aeruginosa* clinical isolate (A) PAO1, (B) I1, (C) I2, or (D) 14715 grown in the presence or absence of different concentrations of S2. All cultures were grown in TSB and were incubated in round bottom tubes shaking at 37°C. ‘0’ was treated with the buffer that was used to suspend S2. ‘4’ – ‘16’ were treated with 4 -16 μg/mL S2, respectively. Data was visualized with GraphPad Prism 8 and fitted with Gompertz non-linear regression. Each datum point is a mean of three biological replicates with one technical replicate each and error bars represent standard deviation. A Student’s t-test compared each group with the ‘0’ group. * denotes P value ≤ 0.05, ** denotes P value ≤ 0.01 and **** denotes P value ≤ 0.0001. Note that the significant difference in the growth between cultures treated with and without S2 is observed for all time points between 2 and 24 hours for I1 and for all the time points between 2 and 34 hours for 14715.
treated with S2 and 14715 treated with buffer for all the time points between 2 and 34 hours (p ≤ 0.0001). The extended lag phase for 14715 was around 28 hours, likely due to this isolate growing much slower than I1. Though not nearly as prominent as that of I1 treated with S2, I2 treated with S2 had a slightly significant difference in growth that showed at 6 and 7 hours, possibly due to a small increase in the lag phase (Figure 4.2 C). For all the strains, the stationary phase did not significantly change with S2 treatment (Figure 4.2).

The above-mentioned growth curves were carried out with small volumes (3 mL) in round bottom tubes. When testing virulence factor production, larger volumes (7.5 mL) in flasks were used to collect spent medium for use in experiments. To ensure the results observed in Figure 4.2 are reflective of the experimental conditions used, the procedure in section 2.6 was carried out with a larger volume in flasks and the results are shown in Figure 4.3. 4384 was used later, in comparison to I2, so it was included in the growth curves. CFU counts were checked to further support the concentration of cells collected between the treated and untreated samples being equal. It should be pointed out that the x-axis of Figure 4.3 B is OD<sub>600</sub>, not time. The reason for this is because there was such a huge discrepancy between the growth of I1 treated with S2 and I1 treated with buffer, the spent mediums were collected for use in experiments not at a specific time but at a specific OD<sub>600</sub> (OD<sub>600</sub> = 7.5). As such, we only tested the CFU/mL of cultures stopped at an OD<sub>600</sub> of 7.5. On the other hand, both the treated and untreated I2 and 4384 grew at similar rates, so we could grow them overnight and collect the spent medium from all the treatments at the same time point. To make sure that the growth rates were similar for I2 and 4384, we took CFU counts at multiple time points (6, 12, and 18 hours) (Figure 4.3 D, F). Similar to the previous growth curves, I1 treated with S2 showed an increased lag phase compared to I1 treated with buffer (Figure 4.3 A). I2 treated with S2 and 4384 treated with S2
Figure 4.3. Effects of S2 on the growth of I1, I2, and 4384 in flasks.

Growth curves of the *P. aeruginosa* clinical isolates (A) I1, (C) I2, or (E) 4384 in the presence or absence of different concentrations of S2. On the right are CFU counts taken during the growth curves of (B) I1, (D) I2, or (F) 4384. Due to the large difference in the lag phase of I1 treated with and without S2, CFU counts were taken for I1 at the same OD$_{600}$ (OD$_{600}$ = 7.5) not the same time as was done for I2 and 4384. All cultures were grown in TSB and were incubated in flasks shaking at 37°C. ‘0’ was treated with the buffer that was used to suspend S2 and ‘8’ was treated with 8 μg/mL S2. Data was visualized with GraphPad Prism 8 and fitted with Gompertz non-linear regression. Each datum point is a mean of three biological replicates with one technical replicate each and error bars represent standard deviation. A Student’s t-test compared each group with the ‘0’ group. * denotes P value ≤ 0.05 and ** denotes P value ≤ 0.01.
showed a slight difference in growth at some of the time points but not to the same extent as I1 treated with S2 (Figure 4.3 C, E). At the 18-hour time point, which is around when the spent mediums were collected following overnight growth, I2 treated with S2 and 4384 treated with S2 did not show a significant difference compared to the respective cultures treated with buffer for either OD$_{600}$ (Figure 4.3 C, E) or CFU/mL (Figure 4.3 D, F). The spent medium of I1 treated with buffer and I1 treated with S2 were collected at an OD$_{600}$ of 7.5 which is, again, due to the difference in lag phase. A CFU count collected at this OD$_{600}$ showed no significant difference between I1 treated with buffer and I1 treated with S2 (Figure 4.3 B).

**4.2.2 Effect of Subinhibitory Concentrations of S2 on the Secreted Virulence Factor Production of Target Isolates**

In the previous section, it was observed that subinhibitory concentrations of S2 significantly increased the lag phase time of I1 and potentially I2; but by the end of the growth curve the bacteria had acquired a stable ability to grow in the presence of the pyocin. This outcome suggested that subinhibitory concentrations of S2 can affect its target cells to some degree while not preventing the growth of the cells. This next section will delve deeper into the effects of subinhibitory S2 on virulence factor production. Our initial hypothesis was that subinhibitory S- pyocin would decrease the virulence factor production of target isolates. Based on this, we expect that subinhibitory S2 will decrease the production of secreted virulence factors in I1 and I2. For each experiment, PAO1 was used as a negative control as it has the immunity protein for S2 so we would not expect S2 to have any effects on it. Treatments of 4, 8 and 16 μg/mL of S2 were used and results compared to that of a 0 μg/mL treatment consisting of the buffer used to suspend the pyocin.
4.2.2.1 Protease Production

I2 has been previously reported to produce proteases, including elastase (Lutter 2008). Protease production was of particular interest as MacLean 2012 found that the spent medium of 14651 was able to decrease the protease production of target isolates. To address this, a skim milk protease assay was carried out as described in section 2.7.2.1 and an elastin-Congo red assay as described in section 2.7.2.2. These assays examined the amount of protease present in the spent medium following overnight growth of PAO1 and I2 in the presence or absence of S2.

As expected, there was no change in the protease production of PAO1 following treatment with S2 (Figure 4.4 A, 4.5 A). Some replicates from I2 treated with S2 had a complete absence of protease production whereas there was no obvious change in the protease production of others (Figures 4.4 B, 4.5 B). To uncover possible explanations for this confounding result, we investigated previous research done into the mutation rates of our laboratory’s *P. aeruginosa* collection. The mutation rates of I1, I2, and 4384 had been determined previously based on their frequency of developing resistance to rifampicin (Faria 2009). Neither I1 nor 4384 were considered mutators and had lower mutation frequencies than PAO1. On the other hand, I2 was determined to be a mutator with over a 20-fold higher mutation frequency than PAO1 due to a mutation in the mismatch DNA repair genes *mutS* and *uvrD*. Being inadequately equipped to handle DNA damage, the presence of the S2 may induce more mutations that I2 is not able to properly correct, some of which may result in the decrease in protease. To determine if this result would be seen in a non-hypermutant, we examined the protease production of 4384, which is an isolate from the same patient as I2. A skim milk protease assay showed 4384 consistently having the same level of protease production as the untreated samples (Figure 4.6).
Figure 4.4. The effect of S2 on the protease production of PAO1 and I2.

(A) PAO1 and (B) I2 were grown overnight in different treatments of S2 before their protease production was measured by the zones of clearance on skim milk protease plates. ‘0’ is the negative control and was treated with the buffer that was used to purify S2. ‘4-16’ were treated with 4-16 μg/mL S2, respectively. n consists of three biological replicates with three technical replicates each. An ANOVA followed by a Dunnett’s test was used to compare each group with the ‘0’ group. *** denotes P value ≤ 0.001, **** denotes P value ≤ 0.0001.
Figure 4.5. The effect of S2 on elastase production of PAO1 and I2.

(A) PAO1 and (B) I2 were grown overnight at different concentrations of S2 before their elastase activity was measured by the $\Delta\text{OD}_{495}/\text{OD}_{600}$ following an elastin-Congo red assay. ‘0’ is the negative control and was treated with the buffer that was used to purify S2. ‘4-16’ were treated with 4-16 μg/mL S2, respectively. n consists of three biological replicates with three technical replicates each. An ANOVA followed by a Dunnett’s test was used to compare each group with the ‘0’ group. Error bars represent standard deviation.
Figure 4.6. The effect of S2 on protease production of the non-hypermutant 4384 compared to the hypermutant I2.

(A) The hypermutant I2 and (B) the non-hypermutant 4384 were grown overnight in different treatments of S2 before their protease production was measured by the zones of clearance on skim milk protease plates. ‘0’ was treated with the buffer that was used to purify S2 and ‘8’ was treated with 8 μg/mL S2. n consists of three biological replicates with three technical replicates each for I2 and six biological replicates with three technical replicates each for 4384. A Student’s t-test compared each group with the ‘0’ group. Error bars represent standard deviation.
As there was a slight difference in the growth curves of I2, we wanted to do an extra check to make sure that our results are not because of that small difference in growth. To do this, we checked the protease production of I2 in the presence or absence of S2 at the same OD\textsubscript{600} (OD\textsubscript{600} = of 3.5) and found no significant difference in the protease production between the two treatments (Figure 4.7). Overall, these results do not support the hypothesis that S2 is able to affect the protease production of target isolates and the alteration in protease production observed for I2 treated with S2 may be linked to this isolate being a hypermutant rather than a direct effect from S2.

4.2.2.2 Pyocyanin Production

Interestingly, it was observed that I2 cultures grown in the presence of S2 were noticeably more blue-green than those grown in the absence of the pyocin. As I2 was determined to produce pyocyanin but not pyoverdine, an increase in pyocyanin was the suspected candidate for the change in colour (Lutter 2008). To verify if more pyocyanin was being produced, a pyocyanin extraction was carried out on the spent medium of the overnight cultures as described in section 2.7.3. No significant change was observed in the pyocyanin activity of PAO1 (Figure 4.8 A). As predicted, the assay showed an increase in the pyocyanin production of I2 after it was grown in the presence of S2, though this was only statistically significant for the 4 μg/mL treatment (p ≤ 0.01) (Figure 4.8 B).

Interestingly, when examining mixed infection of \textit{P. aeruginosa} isolates in a \textit{Drosophila} model, Lutter 2008 found that isolates 14670 and 14671 had increased pigment production when mixed together. The pigment could be seen in the sucrose media where the bacteria were inoculated for fly feeding assays (Lutter 2008). It was stated that the pigment initially had a bluish tinge but evolved to a reddish hue after six days post-infection. It is known that pyocyanin
Figure 4.7. S2 showed no effect on the protease or elastase production of I2 when tested at an OD\textsubscript{600} of 3.5.

I2 was grown to an OD\textsubscript{600} of 3.5 before its protease production was measured by the zones of clearance on (A) skim milk protease plates as well as by the $\Delta$OD\textsubscript{495}/OD\textsubscript{600} following a (B) elastin-Congo red assay. ‘0’ was treated with the buffer that was used to purify S2 and ‘8’ was treated with 8 μg/mL S2. n consists of three biological replicates with three technical replicates. A Student’s t-test compared each group with the ‘0’ group. Error bars represent standard deviation.
Figure 4.8. The presence of S2 increases pyocyanin production in I2.

(A) PAO1 and (B) I2 were grown overnight in different treatments of S2 before a pyocyanin extraction was carried out. ‘0’ is the negative control and was treated with the buffer used to purify S2. ‘4-16’ were treated with 4-16 μg/mL S2, respectively. n consists of three biological replicates with one technical replicate each. An ANOVA followed by a Dunnett’s test was used to compare each group with the ‘0’ group. ** denotes P value ≤ 0.01. Error bars represent standard deviation.
produces a reddish colour when acidified and pyocyanin plates will turn brown when left out for a while (Ogunnariwo and Hamilton-Miller 1974). This is made more interesting by the fact that previous work in our laboratory found that 14670 had inhibitory effects on 14671 (MacLean 2012). These inhibitory effects are seen only in the presence of mitomycin C, an inducer of pyocin expression, suggesting that 14670 is producing pyocins that can inhibit 14671. To add to that, 14671 is known to produce pyocyanin, which means the pyocins of 14670 could be upregulating its pyocyanin production (Lutter 2008). Unlike how 14651 decreased virulence in a Drosophila infection model, the co-infection of these two isolates did not show an alteration in virulence. However, it is evident these isolates are interacting in some way and these findings may support our results that pyocins can increase pyocyanin production in their target cells.

4.2.2.3 Lipase Production

The effects of subinhibitory S2 on lipase production were examined using a lipase assay as described in section 2.7.1. S2 did not have any effects on PAO1 lipase production (Figure 4.9 A). Likewise, I2 did not have a significant change in lipase production at any of the concentrations of S2 (Figure 4.9 B). Lipase activity was also tested at an OD\textsubscript{600} of 3.5 and, similarly, there was no significant change in production (Figure 4.10). Lipase production was tested for I1 and contrary to the results for I2, lipase was significantly higher in the treated cultures at all three concentrations than the untreated (Figure 4.11).

4.3 Summary

Previous work in our laboratory revealed that spent medium containing S- pyocins decreases the virulence of target strains but it was unclear whether this was occurring from a decrease in target cell number only or whether S- pyocins could additionally influence virulence factor production (MacLean 2012). In this study, the effects of subinhibitory concentrations of
Figure 4.9. The presence of S2 does not affect lipase production in PAO1 or I2.

Lipase activity/mL of (A) PAO1 or (B) I2 after they were allowed to grow overnight in different treatments of S2. ‘0’ is the negative control and was treated with the buffer that was used to purify S2. ‘4-16’ were treated with 4-16 µg/mL S2, respectively. n consists of three biological replicates with three technical replicates each. An ANOVA followed by a Dunnett’s test was used to compare each group with the ‘0’ group.
Figure 4.10. S2 showed no effect on the lipase production of I2 at an OD$_{600}$ of 3.5.

Lipase activity/mL of I2 after it was allowed to grow to an OD$_{600}$ of 3.5 in different treatments of S2. All cultures were grown in TSB. I2 was treated with either the buffer used to purify S2 or 8 μg/mL S2. n consists of three biological replicates with three technical replicates each. A Student’s t-test compared each group with the ‘0’ group. ** denotes P value ≤ 0.01. Error bars represent standard deviation.
Figure 4.11. Cultures of I1 treated with S2 showed a significantly higher lipase production than the cultures grown in the absence of S2.

Lipase activity/mL of I2 after it was allowed to grow to an OD$_{600}$ of 7.5 in different treatments of S2. ‘0’ is the negative control and was treated with the buffer that was used to purify S2. ‘4-16’ were treated with 4-16 µg/mL S2, respectively. n consists of three biological replicates with three technical replicates each. An ANOVA followed by a Dunnett’s test was used to compare each group with the ‘0’ group. ** denotes P value ≤ 0.01.
the S- pyocin, S2, on the growth and virulence of three isolates were examined. Two scenarios were observed in terms of virulence factor production. Firstly, the production could be increased as in the lipase production of I1, as well as the pyocyanin production of I2. Secondly, the production could be unaffected, as was observed for the lipase, protease, and elastase production of I2 and the protease production of 4384. PAO1, as a strain that has the immunity protein for S2, showed no changes in either growth or virulence factor production when treated with S2. This suggests that the observed effects are linked to the toxic activity of S2, which is blocked by the S2 immunity protein. It is important to note that some isolates respond to S- pyocins differently. This can be seen in terms of lipase production where I1 showed an increase in production whereas I2 had unchanged production.

The results of this chapter found that, apart from having bactericidal activity, S2 is able to affect the virulence factor production of target isolates, albeit not in the way we had initially expected which would have been a decrease in protease production and overall virulence. The findings of this chapter led to the question of what is the mechanism by which these virulence factor changes are occurring. In the next chapter, we will examine this question by investigating whether the observed changes are occurring through genetic or physiological mechanisms.
Chapter Five: Genetic Changes are Occurring in I1 When Exposed to S2

5.1 Introduction

Initially, we had hypothesized that S2 may be able to decrease the virulence factor production of target strains. Contrarily, we observed that treatment of S2 increased the pyocyanin production of I2. Although an increase in lipase was not observed for I2 it was observed for I1. These changes in virulence factor production could result from either physiological or genetic alterations.

An example of a physiological response is the activation of the SOS-response system. Damage to DNA is known to trigger the SOS-response system, which can have a range of effects on the cell. The SOS-response system can cause changes in gene regulation that, among other things, involve general metabolism, DNA replication/repair, motility, and quorum sensing (Cirz et al., 2006). There have been reports of both antibiotics and bacteriocins causing changes to virulence through the activation of the SOS-response system. Sub-inhibitory concentrations of the fluoroquinolone ciprofloxacin decrease swimming and swarming in P. aeruginosa but increase biofilm formation and pyocin gene expression (Brazas and Hancock 2005; Linares et al., 2006).

Genetic changes can occur through the selection of mutations and resistant mutants are selected for when grown in the presence of an antimicrobial. Over 18 % of CF isolates have increased rates of mutation (Oliver et al., 2000; Montanari et al., 2007). An induction of the SOS response system can further increase the rates of mutation (Faria 2009). Prolonged stress detected by the SOS response leads to the production of error-prone polymerases, which cause the cell to temporarily be a hypermutator (Cirz et al., 2006; Mo et al., 2016). This may increase the chances of resistance occurring and survival of the target cell. Depending on where the
mutation(s) occur in the genome, a change in virulence factor production can accompany the genetic changes. Hocquet et al., 2016 studied a large chromosomal deletion including hmgA that they believed led to resistance to AP41 and S1. This deletion is known to lead to lower clearance in a murine model (Rodríguez-Rojas et al., 2009). Furthermore, it causes hypersecretion of pyomelanin, which gives it slightly more resistance to oxidative stress and may assist against neutrophil attack as neutrophil levels are 1500x higher in CF lungs (Rodríguez-Rojas et al., 2009; Hocquet et al., 2016). Additionally, these resistant mutants were more susceptible to osmolarity and aminoglycosides (Hocquet et al., 2016). Whereas physiological changes are temporary, genetic changes are permanent even after the removal of a stimuli.

5.2 Results

5.2.1 Genetic Resistance is Occurring in I1 Following Exposure to S2

We examined whether the change in the lag phase observed in I1 treated with S2 was from a temporary (physiological) or permanent (genetic) change. This was done following the procedure described in section 2.6.1. Note that, as described in section 2.6.1, single colonies taken from a streak plate of I1 that had undergone treatment with S2 will be referred to as ‘R1’. As expected, both I1 and the I1 buffer control showed an extended lag phase when treated with S2 (Figure 5.1 A, C). However, R1 re-treated with S2 does not show any discernable lag phase extension (Figure 5.1 B). The absence of the extended lag phase in R1 suggests the formation of resistance against S2 from a genetic change. This is backed up by our results in Chapter Four where the MIC test for I1 clearly showed a trailing endpoint, which might stem from the formation of resistant variants.
**Figure 5.1. The extended lag phase is absent in R1 treated with S2.**

Growth curves of (A) I1, (B) R1, and (C) negative control of I1 previously grown in buffer. All cultures were grown in TSB and were incubated in round bottom tubes shaking at 37°C. ‘0’ was treated with the buffer that was used to suspend S2 and ‘8’ was treated with 8 μg/mL S2. Data was visualized with GraphPad Prism 8 and fitted with Gompertz non-linear regression. Each datum is a mean of three biological replicates with one technical replicate each. Error bars represent standard deviation.
Further experiments were carried out testing the effect of quadrupling dilutions of S2 concentrations ranging from 4 to 0.0039 µg/mL on the growth of I1. As concentrations of S2 decrease, the length of the lag phase decreases (Figure 5.2). These results further emphasize the effects S2 has on I1.

To confirm R1 has gained genetic resistance against S2, I1 was treated with 8 µg/mL of S2 and streaked on a TSA plate to create single colonies of R1. These single colonies of R1 were used to carry out an overlay assay (section 2.4). Even at concentrations of S2 as high as 256 µg/mL, there were no visible zones of inhibition for R1 (Figure 5.3). The absence of zones of inhibitions at high concentrations of S2 suggest that R1 has obtained a genetic change that provides it with resistance against S2. It should be noted that for each replicate of R1, I1 was re-exposed to 8 µg/mL of S2 and a new streak plate carried out.

5.2.2 Sequencing

In the previous sections, we established that there are likely genetic changes occurring in R1 as a result of treatment with subinhibitory concentrations of S2. Such genetic changes may explain the alterations in growth and virulence factor production observed in Chapter Four. To expand on these findings, we set up a parallel evolution experiment as described in section 2.8. A parallel evolution experiment tests if certain genotypic traits are repeatedly selected for in different populations. In this experiment, we grew I1 in the presence of 8 µg/mL of S2 and isolated a colony from the treated culture. We repeated this 10 times so that we had 10 separate colonies isolated from separate treated cultures. These colonies should each represent a single genotype and, again, are designated as R1. As a negative control, a colony was picked from PAO1 treated with 8 µg/mL S2 as well. To look for changes arising from S2 treatment, the genomes of these isolates were sequenced and compared to draft reference genomes we
Figure 5.2. Effects of quadrupling dilutions of S2 on the growth of I1.

Growth curve of the *P. aeruginosa* clinical isolate I1 grown in the presence or absence of different concentrations of S2. All cultures were grown in TSB and were incubated in round bottom tubes shaking at 37°C. ‘0’ was treated with the buffer that was used to suspend S2. ‘0.0039’ – ‘4’ were treated with 0.0039 - 4 μg/mL S2, respectively. Data was visualized with GraphPad Prism 8 and fitted with Gompertz non-linear regression. Each datum is a mean of three biological replicates with one technical replicate each. Error bars represent standard deviation.
Figure 5.3. Overlay plates demonstrate I1 gains resistance to S2.
Overlay assay plates with 5 μL of 64, 128, and 256 μg/mL of S2 spotted on in triplicate. The lawns of bacteria are either (A) I1 or (B) R1. Each lawn was produced bacteria grown from single colonies so the lawn should have originally stemmed from bacteria of a single genotype. These images are representative of 10 biological replicates.
assembled of untreated PAO1 and I1. Metrics for the draft genomes assembled can be found in Table 5.1. We observed the nucleotide changes resulting in amino acid alterations that occurred in the 10 genomes of R1 as compared to draft reference genome of I1. PAO1 treated with and without S2 was used as a negative control as we would not expect S2 to select for mutations in it.

As expected, the results showed that PAO1 had no nucleotide modifications that resulted in amino acid changes from treatment with S2. For R1, sequence changes that resulted in amino acid changes were listed for each replicate in Tables 5.2 and 5.3. Mutations that appear in multiple replicates would signify convergent evolution being selected for by the treatment with S2 (Table 5.2). Interestingly, all 10 replicates had the same single point mutation in phzA1 leading to an amino acid change from glutamine to glutamic acid. phzA1 produces a protein involved in phenazine biosynthesis. Fifty percent of replicates had a mutation in PA2149 and, similarly, 50% had a mutation in PA1414. Forty percent of replicates had a mutation in phaJ3, which is a 3-Hydroxyacyl-thioester dehydratase HTDZ-related protein. Two replicates had a mutation in only phzA with no detected amino acid changes in other genes. R1-4 was the only replicate to have mutations in all four of these genes (phzA, PA2149, PA1414, phaJ3) as well as the two additional genes (PA4970 and PA2151) (Tables 5.2 and 5.3). Mutations that appear in only one replicate of an isolate, such as those in PA4970 and PA2151, are more likely to be random mutations and not necessarily linked to the presence of S2 (Table 5.3).

Both PA2149 and PA1414 encode hypothetical proteins and were examined using InterProScan. InterProScan did not reveal any information for PA2149. For PA1414, it showed a disorder prediction. Protein disorder often means there are non-globular units in a protein that may allow for more interaction sites (Linding et al., 2003). In the literature, PA1414 has been shown to have a potential link with the oxidative stress response as it was repressed upon
Table 5.1. Metrics from genome assembly and annotation.

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Table 5.2. Amino acid changes were present in R1 compared to I1.

1Nomenclature adapted from Dunnen and Antonarkakis 2000. Comparisons were carried out with the parental strain I1.

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<th>Replicate</th>
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<th>$PA2149$: Hypothetical protein</th>
<th>$PA1414$ (Hypothetical protein)</th>
<th>$phaJ3$ (<em>PA4788</em>): 3-Hydroxyacyl-thioester dehydratase HTDZ-related</th>
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</tr>
<tr>
<td>R1-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1-8</td>
<td></td>
<td></td>
<td>PA1414\textsubscript{62C&gt;G} (G21A)</td>
<td>phaJ\textsubscript{325C&gt;G} (P9A)</td>
</tr>
<tr>
<td>R1-9</td>
<td></td>
<td>PA2149\textsubscript{232G&gt;C} (G78R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1-10</td>
<td></td>
<td></td>
<td></td>
<td>phaJ\textsubscript{325C&gt;G} (P9A)</td>
</tr>
<tr>
<td>Total Replicates</td>
<td>10/10</td>
<td>5/10</td>
<td>5/10</td>
<td>4/10</td>
</tr>
</tbody>
</table>
Table 5.3. Some amino acid changes were present in only one replicate of R1.

Nomenclature adapted from Dunnen and Antonarkakis 2000. Comparisons were carried out with the parental strain I1.

<table>
<thead>
<tr>
<th></th>
<th>PA4970: Conserved hypothetical protein</th>
<th>PA2151: Alpha-1,4-glucan:maltose-1-phosphate maltosyltransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1-4</td>
<td>PA4970_{125C&gt;G} (R42P)</td>
<td>PA2151_{392C&gt;G} (A131G)</td>
</tr>
</tbody>
</table>
treatment with hydrogen peroxide (Chang et al., 2005). PA2149 has been suggested to interact with polycyclic aromatic hydrocarbon degradation genes (Yan and Wu 2017).

5.3 Summary

The origin of changes induced by subinhibitory concentrations of the S-type pyocin, S2, on a *P. aeruginosa* isolate was examined. It was determined that a genetic change causes R1 to gain resistance against S2. To further examine these possibilities, we carried out a parallel evolution experiment where we looked for mutations occurring in multiple replicates of R1. We found evidence of convergent evolution acting on an array of genes in R1 following exposure to S2, suggesting that these genes give survival advantages against S2. These genes were *PA2149*, *PA1414*, *phzA*, and *phaJ3*. Interestingly, mutations in *phzA* occurred in each R1 replicate, suggesting this mutation may be involved in some or all of the phenotypic differences between I1 and R1. Overall, this chapter sheds light on the genetic side of the response to S-type pyocins and which genes are important in this response.
Chapter Six: Discussion

6.1.1 Effects of S2 on Virulence Factor Production

Lutter 2008 found that the spent medium of a CF isolate known as 14651 attenuated the virulence of other isolates. It was believed to be doing so by secreting S-pyocins that inhibit the target isolates (MacLean 2012). The spent medium of 14651 was found to inhibit the protease production of the target isolates while still allowing for growth (Figure 1.6). This was an effect which disappeared when the spent medium was heat-inactivated, suggesting the responsible factor was a protein – such as an S-pyocin. This led to our hypothesis that *P. aeruginosa* CF isolates use S-pyocins to influence the overall virulence of microbial communities by decreasing the virulence factor production of target bacteria. We purified a DNase S-pyocin, S2, and tested its effect on the virulence of two target isolates (I1 and I2) at concentrations that still permitted growth. The virulence factor production of the isolates treated with S2 were compared to those treated with buffer when they were at the same cell numbers, ruling out any observed effects as being a result of a reduced cell number. The results suggested that S2 does not select for a decrease in protease production (Figures 4.6 B, 4.7). According to these results, the decrease in protease production that MacLean 2012 observed in response to the spent medium of 14651 is likely due to a different protein that is not an S-pyocin. That being said, we did observe that isolates can respond differently to S-pyocins, with I1 showing an increase in lipase production (Figure 4.11) and I2 (Figures 4.9 B, 4.10) having unaffected production. We saw an increase in the lipase production of I1 (Figure 4.11) and the pyocyanin production of I2 (Figure 4.8 B). Overall, our results do not support our initial hypothesis that CF isolates use S-pyocins to influence the overall virulence of microbial communities by decreasing the virulence factor production of target bacteria. However, although the changes to the virulence factor production
of target isolates are not the decrease we had initially expected, there are definitely changes that are occurring. The fact that changes do occur in response to an S-pyocin lines up with research done into DNase colicins which found that they are able to increase the production of Shiga toxin 64-fold (Toshima et al., 2007). Therefore, CF isolates could still be using S-pyocins to influence the overall virulence of microbial communities but in a way more complicated than we had initially thought. This can be seen as some virulence factors are left unchanged (lipase/protease/elastase for I2 and protease for 4384) and some are increased (lipase for I1 and pyocyanin for I2).

From cultures of I1 that underwent selective pressure from treatment with S2, we isolated single colonies representing only one R1 genotype and used them to examine which changes were genetic or physiological in nature. We found that R1 is gaining resistance to S2 though whether the changes to virulence factors were contributing to resistance or were unintended collateral by a change in, for example, a global regulatory protein, is unknown but will be expanded upon in the following section. Having proof of phenotypic changes, we used sequencing to determine what amino acid alterations are being selected for. In the following section, we will discuss possible pathways explaining the results.

6.1.2 A Proposed Pathway Explaining the Observed Results

We noticed a clear development of resistance towards S2 when I1 was treated with the pyocin. There has been previous research into the cause of resistance and tolerance towards S-pyocins. Holloway et al., 1973 looked at spontaneous mutants that were tolerant towards the DNase AP41 and found they had altered membrane composition. Hocquet et al., 2016 described a large chromosomal deletion that led to resistance against pyocins AP41 and S1. Inglis et al., 2016 co-cultured an S2 producing strain and an S2 sensitive strain to examine the appearance of
pyocin resistance. Upon genetic analysis of fpvAI, the S2 pyocin receptor gene, they found that the resistant mutation did not appear to be in the receptor gene and the source of the resistance was left unknown (Inglis et al., 2016). Interestingly, 30% of isolates have been found to contain a killing gene without a corresponding immunity gene, suggesting that if these killing genes are active there are mechanisms of resistance that we are not yet aware of (Ghoul et al., 2015; Bara et al., 2018).

In Chapter Five, it was observed that 100% of R1 had developed a mutation in phzA1, a gene involved in phenazine biosynthesis (Table 5.2). Dietrich et al., 2006 found that pyocyanin regulates the production of various genes. It upregulates the efflux pump system mexGHI-OpmD and redox control genes (Dietrich et al., 2006). It is possible that the 20-fold upregulation in the efflux pump system could contribute to pyocin resistance. The multidrug efflux system CmeABC has been known to increase resistance against bacteriocins in Campylobacter jejuni (Hoang et al., 2011). On the other hand, pyocyanin downregulates genes for ferric iron acquisition (Dietrich et al., 2006). Importantly, it was shown that fpvA is downregulated approximately 7-fold and tonB 3-fold. If the mutation in phzA1 restores pyocyanin production in R1 or if it affects the production of other phenazines which have similar effects as pyocyanin, then the downregulation of two proteins involved in the import of S2 (the target receptor FpvAI and the TonB transporter) may be what is leading to the genetic resistance of R1 against S2. If there is a decreased expression of fpvAI and tonB then it could be that S2 is binding to the CPA and then being unable to get into the cell due to the decreased number of FpvAI and TonB transporters. If pyocyanin increases resistance against S2, this may further explain why I2 showed an increase in pyocyanin production upon exposure to S2 (Figure 4.8).
Two other prominent mutations were in *PA1414* and *PA2149* (Table 5.2). Unfortunately, we were unable to determine the exact function of these genes. However, *PA1414* is likely linked to an oxidative stress response (Chang *et al.*, 2005). Pyocyanin has been known to lead to $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ formation (Hassan and Fridovich 1980). For that reason, if pyocyanin production is being selected for this could be leading to increased oxidative stress. Hydroxyl radicals are dangerous to cells because they can damage DNA, proteins, and membrane lipids (Kohanski *et al.*, 2007).

Jin *et al.*, 2016 suggested that changes in genes that cause a flux towards the TCA cycle help in the increased production of pyocyanin by providing necessary energy to sustain growth. Forty percent of R1 had a mutation in the (R)-specific enoyl-CoA hydratase *phaJ3* (Table 5.2) (Tsuge *et al.*, 2003). *phaJ3* is involved in the production of polyhydroxyalkanoates (PHA), which are a source of stored energy, from fatty acids. Fatty acids are formed from acetyl-CoA, the starting molecule used in the TCA cycle, so a mutation in *phaJ3* could increase the flux towards the TCA cycle (Bodner 1986; Jin *et al.*, 2016). The increase in lipase activity for I1 treated with S2 may be explained by the *phaJ3* mutation. PHA is made from short- to medium-chain fatty acids (Tsuge *et al.*, 2002). Short chain fatty acids are known to stimulate the production of lipase (Chander *et al.*, 1979; Lee *et al.*, 2012). Therefore, a mutation in *phaJ3* could lead to more short-chain fatty acids, which would then increase lipase activity, as was observed for I1 treated with S2.

A distinct phenotypic characteristic that I1 treated with S2 exhibited was an increased lag phase (Figure 4.2 B). Some bacteria show an extended lag phase in response to antibiotic treatment (Li *et al.*, 2016). It is suggested that the extended lag phase allows the bacteria to gain tolerance to the antibiotics and it was found that bacteria that have an extended lag phase are
more likely to be able to survive in concentrations of antibiotic higher than what MIC assays may suggest. This is what we observed in Chapter Three, where according to MIC tests, I1 had an MIC of $\leq 8 \, \mu g/mL$ but we found that this isolate could survive in concentrations of at least $32 \, \mu g/mL$ in flasks. The lag phase is characterized by two sub-phases: (1) after inoculation and before biomass growth and (2) between when the cell biomass starts increasing and the start of cell division (Madar et al., 2013). During the first sub-phase, there is minimal gene expression except for those genes involved in utilizing the specific carbon source present in the media. In the second sub-phase, other genes, such as those involved in amino acid production, are turned on. An extended lag phase allows cells to gain transient tolerance by remaining metabolically inert for the duration of the antibiotic treatment (Fridman et al., 2014). If key receptors, such as FpvA, are not expressed during the lag phase then this would allow the survival of I1 throughout the lag phase period. This tolerance may increase the chance of resistant variants forming by increasing the number of viable cells that are available for mutation and potentially by increasing the mutation rates of those cells (Windels et al., 2019).

Naturally, the above is all speculation as we do not know for sure what the effect of each mutation is on each protein let alone its effect on the broader cell function. Future experiments need to be carried out to determine the exact effects of each mutation and how they relate to both the resistance against S2 and the observed changes in virulence factor production. The pathway proposed in this section potentially explains some of the observations of this study, but it still leaves many questions open. Firstly, I1 was not recorded as producing pyocyanin (Lutter 2008). Unless the mutation in PhzA restores pyocyanin production or that of another phenazine that has similar regulatory actions as pyocyanin, the downregulation of FpvA and TonB by pyocyanin leading to resistance does not provide answers for the resistance observed in R1. Additionally,
what role does PA2149 play? This hypothetical protein was present in half of the replicates, yet we do not know what effects it has on the bacteria and why it is being selected for. Undoubtedly, there are mechanisms of regulation occurring that we have not yet uncovered.

6.1.3 Implications for Clinical and Environmental Settings

As our repertoire of antibiotics that are effective against *P. aeruginosa* are dwindling due to antibiotic resistance, much research on S-type pyocins relates to the possibility of eventually using them to treat *P. aeruginosa* infections (Brown *et al.*, 2012; Smith *et al.*, 2012; Rasouliha *et al.*, 2013; McCaughey *et al.*, 2016a; McCaughey *et al.*, 2016b; Turano *et al.*, 2017; Turano *et al.*, 2020). Although pyocins are large proteins, which according to Lipinski’s rule of five is unfavourable for use as an oral antibiotic, there are a number of reasons why their prospective use as antibiotics is attractive (Lipinski *et al.*, 1997). Firstly, pyocins are narrow spectrum, meaning they would have a lower impact on host microbiota (Smith *et al.*, 2012). Furthermore, S- pyocins are generally stable in the lung, non-inflammatory, not very immunogenic, and still show protection following repeated administration in a murine infection model (McCaughey *et al.*, 2016b). The S- pyocin focussed on in this study, S2, is one of the most researched S- pyocins for use as a potential antibiotic. S2 has been shown to be effective in killing certain *P. aeruginosa* biofilms, even more so than tobramycin or aztreonam, which are two antibiotics commonly used to treat CF bacterial infections (Smith *et al.*, 2012). This pyocin has demonstrated protection in a *Galleria mellonella* caterpillar model against *P. aeruginosa* infection and has not been found to have any significant inhibition against normal human cells (Abdi-Ali *et al.*, 2004; Smith *et al.*, 2012).

In this study, we observed I1 to have a spontaneous formation of resistance against S2. To prevent the development of resistance, there has been the idea of using a ‘pyocin cocktail’
where multiple pyocins are administered together (McCaughey et al., 2016b). McCaughey et al. 2016b used the pyocins L1, S2, and AP41 together to increase efficacy in protecting mice against *P. aeruginosa* infection compared to using them alone. Additionally, we noted the phenomenon of an extended lag phase for two *P. aeruginosa* isolates in response to S2 (Figure 4.2 B, D). 14715, for example, had a lag phase extended to around 28 hours which means its growth would not be detected in a typical MIC assay in which measurements are taken following about 18 hours of incubation (EUCAST 2000). Li et al., 2016 suggested taking the lag time extension (measured as the lag time at X µg/mL divided by the lag time at 0 µg/mL) into account in addition to the MIC when predicting the effectiveness of an antibiotic.

One of the most striking findings of this research was the upregulation of pyocyanin in I2 and the genetic changes in R1 potentially leading to resistance to S2 by influencing phenazine production. Pyocyanin is a metabolite that is toxic to a wide range of organisms, including plants and humans (Wilson et al., 1988; Mahajan-Miklos et al., 1999). Mutants deficient in pyocyanin are less virulent in *Caenorhabditis elegans, Arabidopsis, D. melanogaster*, and a murine model (Mahajan-Miklos et al., 1999; Lau et al., 2003; Lau et al., 2004). In humans, pyocyanin has a range of negative effects in the CF lungs, including inhibition of ciliary motion and causing neutrophil apoptosis (Wilson et al., 1988; Allen et al., 2005). If a patient were to be treated with S2 and there were to be a significant increase in pyocyanin production, this may have adverse effects on the patient’s health. As S- pyocins are being considered for use as antibiotics, these potential side-effects must be looked into as this study has only scratched the surface of the possible effects S- pyocins could have on their target cells.

*P. aeruginosa* is a bacterium that produces many virulence factors and plays a role in diverse aspects of life. Apart from a clinical setting, it is important to understand the intricacies
of inter- and intra-species competition for environmental applications. For instance, microbial communities are being turned to for use in the biodegradation of hydrocarbon contamination and \textit{P. aeruginosa} can frequently be found in these contaminated sites (Frontera-Suau et al., 2002; Norman et al., 2004; Yan and Wu 2017). To maximize the benefit of these microbial communities, we need to understand how to optimize their hydrocarbon degrading capabilities and it was found that pyocyanin decreased hydrocarbon degradation in crude oil-degrading cultures by lowering the overall microbial diversity (Norman et al., 2004). Mutations in \textit{PA2149} may additionally be relevant to bioremediation as \textit{PA2149} likely interacts with polycyclic aromatic hydrocarbon degradation genes (Yan and Wu 2017). Polycyclic aromatic hydrocarbons are of a particular concern for bioremediation because of their harmful, carcinogenic nature (Petry et al., 1996).

6.1.4 Limitations and Future Directions

Although a potential pathway to explain the detected resistance and alterations in virulence factor production was elucidated above, a direct link has not yet been established between the observed mutations and any of the altered phenotypes. An experiment which would establish this would be to carry out a two-step allelic exchange to produce single or multiple nucleotide changes that mimic those observed in this study (Hmelo et al., 2015). The resistance towards S2 and virulence factor production of each mutant can be determined, linking the mutants back to the observed characteristics. The wild type allele can then be complemented back into the mutant isolate to confirm the association. It is possible that multiple mutations are working together to produce the altered characteristics, in which case the two-step allelic exchange method can be further used to create these multiple mutations together. Particular
attention should be made to the point mutation \( phzA1_{340C>G} \) (Q114E) observed in R1, as it would be of interest to see how this mutation affects the resistance level of I1.

Transcriptomic studies could be carried out utilizing microarrays to determine the broader effects of each of the mutations on gene expression. To investigate the possible link between pyocyanin production and S2 resistance, one could make a pyocyanin knockout in I2 to see if that would make it more inhibited by S2. An overlay assay can then be carried out to compare the inhibition of the mutated isolate to the wildtype. It would also be of interest to carry out overlay assays on I2 treated with S2 to determine if, similar to R1, it is gaining genetic resistance against S2. Additionally, gas chromatography-mass spectrometry could be used to determine if there are changes in the phenazine production of R1 compared to I1.

Limitations of this study include that only one S-pyocin was tested in detail and there are many more types of S-pyocins with a range of different killing activities and target receptors. Therefore, the results of this study may not be reflective of all S-pyocins. In future studies, the effects of the tRNase S-pyocin, S4, and pore-forming S-pyocin, S5, purified in Chapter Three could be examined to determine if they have similar effects to the DNase S-pyocin, S2. Furthermore, the effects of S2 were only tested on two isolates. Thus, we cannot say that the results of this study are applicable to all isolates as we even showed here that there can be a variance in response to S2 between isolates (e.g., I1 having increased lipase production and I2 having unchanged lipase production). As well, the effect of S2 on a greater number of virulence factors, such as biofilm formation, should be looked into to gain a better idea of the effect S2 is having on target cells.

Another limitation is that indels and rearrangements could not be fully determined. The reason for this is that the reference genome for I1 was a draft genome. Given the time constraint
of this project they were assembled using short read sequencing only. Draft genomes created from short read sequencing are known to have trouble properly sequencing repeated areas (Alkan et al., 2011). To carry out an accurate investigation of indels and rearrangements, the draft genome should be completed by carrying out a hybrid assembly using long reads (e.g., PacBio or Nanopore) combined with the short reads obtained in this study.

6.1.5 Conclusion

The initial aim of this work was to determine if, apart from having bactericidal activity, S-pyocins are capable of affecting virulence factor production at subinhibitory concentrations. We successfully purified three S-pyocins, a tRNase, a pore-forming protein, and a DNase, for this purpose. The tRNase and pore-forming protein were not studied in depth here but are now readily accessible for future work. Using the purified DNase, S2, we showed that S-pyocins can indeed alter the virulence factor production of their targets at subinhibitory concentrations. Through genome sequencing, we expanded upon the possible origin of these altered phenotypes and how they might relate to the formation of resistance against S2. In conclusion, this research has led to an enhanced understanding of the effects S-pyocins can have on target cells and has potentially uncovered a new mechanism of resistance towards S-pyocins that has not hitherto been investigated.
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Appendix

Figure A.1. Full SDS-PAGE gel for S5 following Ni-NTA chromatography.

SDS-PAGE gel of aliquots taken following Ni-NTA chromatography of S5. Lane 1 is a PageRuler™ Prestained Protein Ladder. Lanes 2-8 are 1.5mL aliquots taken following Ni-NTA chromatography. The His<sub>6</sub>-tagged S5 protein is expected to be 57 kDa (Saeidi et al., 2011).
Figure A.2. Full SDS-PAGE gel for S2 following size exclusion chromatography.

SDS-PAGE gel of different aliquots taken following size exclusion chromatography of S2. Lane 1 is a PageRuler™ Prestained Protein Ladder. Lanes 2-6 are 1 mL aliquots taken following size exclusion chromatography. The His$_6$-tagged S2 protein is expected to be 74 kDa (Sano et al., 1993a).
Figure A.3. Overlay assay plates of the isolate PAO1 treated with different concentrations of S2.

‘P’ indicates treatment with the supernatant of PAO1 (which contains S2). ‘PM’ indicates treatment with the supernatant of PAO1 grown in the presence of mitomycin C (which increases pyocin production). ‘B’ indicates treatment with the buffer used to suspend the purified pyocin following purification. ‘0.125-256’ indicates treatment with concentrations of S2 ranging in doubling dilutions from 256 μg/mL to 0.125 μg/mL.
Figure A.4. Overlay assay plates of the isolate 14715 treated with different concentrations of S2.

‘P’ indicates treatment with the supernatant of PAO1 (which contains S2). ‘PM’ indicates treatment with the supernatant of PAO1 grown in the presence of mitomycin C (which increases pyocin production). ‘B’ indicates treatment with the buffer used to suspend the purified pyocin following purification. ‘0.125-256’ indicates treatment with concentrations of S2 ranging in doubling dilutions from 256 μg/mL to 0.125 μg/mL.
Figure A.5. Overlay assay plates of the isolate 14716 treated with different concentrations of S2.

‘P’ indicates treatment with the supernatant of PAO1 (which contains S2). ‘PM’ indicates treatment with the supernatant of PAO1 grown in the presence of mitomycin C (which increases pyocin production). ‘B’ indicates treatment with the buffer used to suspend the purified pyocin following purification. ‘0.125-256’ indicates treatment with concentrations of S2 ranging in doubling dilutions from 256 μg/mL to 0.125 μg/mL.
Figure A.6. Overlay assay plates of the isolate 14717 treated with different concentrations of S2.

‘P’ indicates treatment with the supernatant of PAO1 (which contains S2). ‘PM’ indicates treatment with the supernatant of PAO1 grown in the presence of mitomycin C (which increases pyocin production). ‘B’ indicates treatment with the buffer used to suspend the purified pyocin following purification. ‘0.125-256’ indicates treatment with concentrations of S2 ranging in doubling dilutions from 256 μg/mL to 0.125 μg/mL.
Figure A.7. Overlay assay plates of the isolate 5166 treated with different concentrations of S2.

‘P’ indicates treatment with the supernatant of PAO1 (which contains S2). ‘PM’ indicates treatment with the supernatant of PAO1 grown in the presence of mitomycin C (which increases pyocin production). ‘B’ indicates treatment with the buffer used to suspend the purified pyocin following purification. ‘0.125-256’ indicates treatment with concentrations of S2 ranging in doubling dilutions from 256 μg/mL to 0.125 μg/mL.
Figure A.8. Overlay assay plates of the isolate 4384 treated with different concentrations of S2.

‘P’ indicates treatment with the supernatant of PAO1 (which contains S2). ‘PM’ indicates treatment with the supernatant of PAO1 grown in the presence of mitomycin C (which increases pyocin production). ‘B’ indicates treatment with the buffer used to suspend the purified pyocin following purification. ‘0.125-256’ indicates treatment with concentrations of S2 ranging in doubling dilutions from 256 μg/mL to 0.125 μg/mL.
Figure A.9. Overlay assay plates of the isolate 5552 treated with different concentrations of S2.

‘P’ indicates treatment with the supernatant of PAO1 (which contains S2). ‘PM’ indicates treatment with the supernatant of PAO1 grown in the presence of mitomycin C (which increases pyocin production). ‘B’ indicates treatment with the buffer used to suspend the purified pyocin following purification. ‘0.125-256’ indicates treatment with concentrations of S2 ranging in doubling dilutions from 256 μg/mL to 0.125 μg/mL.
Figure A.10. Overlay assay plates of the isolate 5585 treated with different concentrations of S2.

‘P’ indicates treatment with the supernatant of PAO1 (which contains S2). ‘PM’ indicates treatment with the supernatant of PAO1 grown in the presence of mitomycin C (which increases pyocin production). ‘B’ indicates treatment with the buffer used to suspend the purified pyocin following purification. ‘0.125-256’ indicates treatment with concentrations of S2 ranging in doubling dilutions from 256 μg/mL to 0.125 μg/mL.
Figure A.11. Permission from Erika Lutter for use of her figures.

The figures that were used in this thesis from Lutter 2008 were figures 1.1, 1.2, and 1.6.

Permission to use Figures

Lutter, Erika
Fri 2021-04-23 2:03 PM
To: Nabiha Mehina
[Δ EXTERNAL]

To whom it may concern,

I, Erika Lutter, hereby grant Nabiha Mehina permission to use multiple figures from my PhD thesis titled 'Lethality of Pseudomonas aeruginosa Cystic Fibrosis Isolates in Drosophila melanogaster Suggests Cooperativity among Isolates'. These figures include: Figure 23 from p. 154 (complete cluster analysis of virulence determinants produced by P. aeruginosa CF isolates), Figure 34 from p. 195 (mixed infections involving P. aeruginosa CF isolate 14651 resulted in decreased lethality), and Figure 44 from p. 225 (heat inactivated supernatant from CF isolate 14651 does not affect protease production of other CF isolates).

Sincerely,
Erika Lutter

Dr. Erika Lutter, PhD