

Study on the Evolution and Expression of the Pyoverdine Receptor FpvA in
Pseudomonas aeruginosa

by
Amandip Singh

A THESIS

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Abstract approved: _____

Martin Schuster

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium that is capable of causing infections in people who are immune compromised. The bacterium can live in a variety of environments due to the variety of virulence factors it can produce. It is well adapted to living in iron-stringent conditions because it can acquire iron by secreting an iron-binding siderophore called pyoverdine. Pyoverdines are transported back into the cell through a ferripyoverdine receptor, FpvA. *P. aeruginosa* also secretes S-type pyocins, which degrade the DNA of sensitive cells. Interestingly, S-type pyocins gain entry through pyoverdine receptors and may play a role in the evolution of these receptors and their cognate biosynthesis pyoverdine. To study the interaction between pyocins and pyoverdines, we developed an *in vitro* selection scheme. We grew *P. aeruginosa* ATCC 27853 in the presence of pyocin S3 lysate in iron-stringent conditions. We utilized a spotting and passaging system to enrich for resistant cells. The passaging approach provides a foundation for future *fpvA* evolution studies. A pyocin S3-resistant mutant was analyzed using Sanger sequencing and found to have a 10-base pair deletion in *fpvAIIIb*.

Key Words: *Pseudomonas aeruginosa*, siderophore, pyoverdine, pyocin, evolution

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presented on May 23, 2017.

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

Amandip Singh, Author

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Study on the Evolution and Expression of the Pyoverdine Receptor FpvA in *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a ubiquitous gram-negative bacterium that thrives in diverse environments. Analysis of its 6.3 million base pair genome gives insight into how it adapts (32). A diverse array of metabolic genes allows *Pseudomonads* to display metabolic versatility and a range of different virulence genes allows it to infect different hosts (35). The organism's versatility is highlighted by its ability to grow both aerobically and anaerobically. Additionally, *P. aeruginosa* contains genes that confer intrinsic resistance to antibiotics, an adaptation that likely arose while competing with microorganisms that secrete antimicrobial compounds. The size and complexity of its genome explains why *P. aeruginosa* is able to survive in diverse ecological niches (32).

P. aeruginosa is an opportunistic human pathogen that infects damaged and compromised tissue. It is a common member of hospital bacterial communities and is notorious for causing nosocomial infections (1). Patients commonly become infected at burn and surgical sites, but immune-compromised cystic fibrosis patients are also at risk of acquiring a lung infection (3). Transmission of *P. aeruginosa* occurs through multiple routes, notably by exposure to strains present on the patient's skin, antibiotic-resistant strains on hospital workers, or medical devices contaminated with biofilms (1). Chemical cell-to-cell communication, termed quorum sensing, plays a major role in the expression of secreted virulence factors as well as in biofilm formation (10). Biofilms are highly organized bacterial structures that form on surfaces such as metals, plastics, medical implant devices, and human tissue (1). When biofilms form on host tissue, such as in the lungs of cystic fibrosis patients, infections become difficult to treat because biofilms provide a protective barrier

against antibiotics (31). Treatment is further complicated by a variety of intrinsic resistance mechanisms. *P. aeruginosa*'s outer membrane has low permeability to antibiotics and production of β -lactamase protects against multidrug efflux pumps (30). Together these evasion mechanisms allow *P. aeruginosa* to persist and cause declining lung function. Biofilms and intrinsic resistance mechanism are a major reason why *P. aeruginosa* is able to cause significant morbidity and mortality in cystic fibrosis patients (5, 11).

Pyoverdines

Iron acquisition is important for bacterial growth because many cellular mechanisms rely on iron cofactors (14). Iron uptake can be challenging for *P. aeruginosa* because it is often found in environments where useable iron is scarce. In aerobic environments iron exists in an insoluble Fe(III) form. Within hosts iron is tightly bound to proteins, such as transferrin and lactoferrin (17). To deal with the challenge of iron acquisition bacteria have developed uptake systems (29). *P. aeruginosa* utilizes an iron-uptake system that releases siderophores, iron-chelating molecules, into the environment to scavenge insoluble iron (9). Over 500 different siderophore structures have been characterized to date, but the source selecting for new siderophores is not known. Iron-chelated siderophores bind to cell surface receptors to transport iron into the cell (21).

Pyoverdine (PVD) is a yellow-green fluorescent molecule that serves as the primary siderophore produced by *Pseudomonas* species (22) (4,16). PVD production is under the global control of the ferric uptake regulator (Fur) protein (8). In iron-rich conditions Fur binds to the *pvdS* promoter repressing transcription of the PvdS sigma factor (33). In iron-limited conditions Fur does not bind to the *pvdS* promoter allowing gene transcription to occur. Once synthesized, the sigma factor PvdS binds to RNA Polymerase initiating transcription of PVD biosynthesis genes. Synthesized PVD is secreted into the environment where it binds to ferric iron. Iron-chelated PVD is transported into the cell through a PVD receptor (FpvA), and in the periplasm the

iron molecule is cleaved from PVD (7). PVD is released into the environment again and the iron molecule is transported into the cytoplasm after it is reduced (15).

Pyocins

Pyocins are bacteriocins produced and secreted by over 90% of *Pseudomonas aeruginosa* strains. Pyocins target and kill closely related strains (2). The abundance of bacteriocin genes in *Pseudomonas* species suggests they play an important role in competition among rival species (26). Pyocin synthesis is a community-regulated process where only a few cells within the population actively produce the compound (11). Currently, three types of pyocins have been distinguished: R-, F-, and S-type. S-type pyocins are soluble and protease sensitive. Each pyocin is a complex of two protein components transcribed as an operon (11). The larger protein contains a DNase activity domain that targets and degrades DNA non-specifically inside sensitive cells (26). The smaller protein provides immunity into the producing cell (23, 28). Inside the producing strain the immunity protein binds to the nuclease C-terminal domain of the killing protein, inhibiting its activity (23). The immunity protein and killing proteins are co-transcribed at a similar rate to prevent DNA degradation within the producing cell (23).

Pyocins only have a noticeable effect on cell growth and survival when taken up by sensitive cells. Killing by S-type pyocins is greatly increased when bacteria are grown under iron-stringent conditions (2). This finding led to the hypothesis that S-type pyocins use siderophore receptors to gain entry into cells (2). Studies have shown that strains susceptible to pyocin S3 produce type II PVD receptors. In contrast, pyocin S3-resistant strains produce either type I or type III PVD receptors (Figure 1) (2). The interactions between pyocins and PVD receptors could have implications for the evolution and diversification of the PVD gene locus, which is one of the most divergent loci in the *P. aeruginosa* genome (34). This process has been described as an example of “Darwinian positive selection” (35).

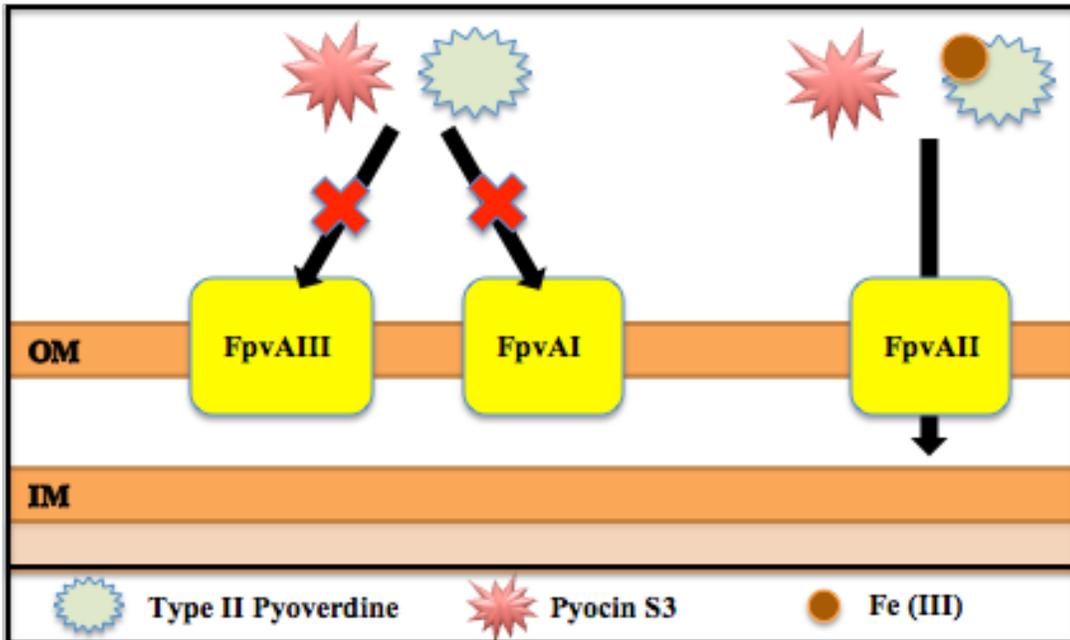


Figure 1. Pyoverdine and pyocins are receptor-specific. Type II pyoverdine and Pyocin S3 enter the cell through FpvAII located on the outer membrane of the cell.

Significance of Study

Antibiotic resistance is an emerging concern in *P. aeruginosa* species (18). Currently, different types of bacteriocins are being evaluated as alternatives to antibiotics (6, 12). Certain bacteriocins have narrower strain specificity than antibiotics, which has the advantage of leaving commensals unharmed (27). In trials, pyocins have shown excellent antimicrobial activity *in vivo* when used to treat murine lung infections (19); however, one potential concern is pyocin-resistance could generate in a short period of time given that the PVD gene locus is the most divergent gene locus in *Pseudomonas* species (34). Pyocins might apply a selective pressure on *fpvA* conferring a fitness advantage to mutant strains. Cells with mutated PVD receptors could prevent pyocin from gaining entry, negating their antimicrobial activity. Therefore it is important to understand if pyocins are driving the evolution of the PVD gene locus when exploring the use of pyocins as therapeutic agents.

Introduction to Research

The goal of this study has been to investigate the evolutionary impact of pyocins on the PVD gene locus of *P. aeruginosa* strain ATCC 27853. We hypothesize that pyocins apply a selective pressure on the PVD gene locus under iron-limiting conditions. We predict mutations to the PVD receptor will provide a selective fitness advantage to cells that are able to keep pyocins out. This advantage may come at the cost of PVD having a weaker binding affinity for its cognate PVD receptor.

For our studies, we have used *P. aeruginosa* strain ATCC 27853 because it only produces one type of PVD receptor, FpvAIIIb. Additionally, this PVD receptor gene is expressed at both low and high concentrations (13). We will use pyocin S3 because it target cells through type II PVD receptors (Baysee). We therefore anticipate pyocin S3 will apply a selective pressure that results in mutations in *fpvAIIIb*. In our experimental design, *P. aeruginosa* strain ATCC 27853 will be grown in iron-stringent media in the presence of pyocin S3. Cells will need to transcribe PVD genes in these conditions to obtain iron. We anticipate that only cells with mutated PVD receptors will be able to survive in the presence of pyocin S3. We expect surviving cells to grow slowly because their cognate PVD might not bind the mutated PVD receptor as efficiently.

Surviving cells will have their PVD receptor gene sequenced and mutations will be mapped on a three-dimensional model. This will allow us to determine if there is a specific location that is a hot spot for mutations on the PVD receptor. Additionally, we will note if any mutants grow more quickly than other mutants. These fast growing mutants might be producing a novel PVD that binds more tightly to the mutated PVD receptor. The evolution of a novel PVD in the presence of pyocin S3 would provide evidence that pyocins apply a selective pressure on the PVD gene locus.

Prior work on this project has shown that a working PVD-uptake system is required to grow in culture medium supplemented with a strong synthetic chelator. The

amount of pyocin S3 needed to produce killing in this system has also been determined. Finally, pyocin S3 appears to have greater killing ability at 30°C but not at 37°C.

In this study we will sequence this mutant and address the limitations of the prior experimental design. The main limitation of the prior study was that the mutant was isolated in iron-replete conditions on LB medium. This is significant because any mutant obtained on iron-replete LB medium does not need a functional PVD receptor to survive. We hope to build on previous work by conducting testing in iron-stringent conditions in CAA medium. We will employ isolation techniques using both solid and liquid culture experiments to obtain PVD receptor mutants grown in the presence of pyocin S3 in iron limiting conditions.

Materials and Methods

Bacterial strains used

The primary strain used in this study was *P. aeruginosa* ATCC 27853. The strain was originally isolated from a blood culture and selected for this study because it only produces one PVD receptor. Table 1 describes the important characteristics of all the bacterial strains used for this thesis.

Table 1: Bacterial Strains

Strain	Relevant Property	Reference
<i>P. aeruginosa</i> ATCC 27853	Type II PVD receptor producer	(20)
<i>P. aeruginosa</i> PyoS3 ^R 1	Pyocin S3-resistant mutant	(13)
<i>P. aeruginosa</i> PAO1	Well-characterized wild-type strain	
<i>P. aeruginosa</i> PAO1 Δ <i>pvdS</i>	Unable to produce PvdS σ -factor	(24)
<i>E. coli</i> DH5 α pPYS3-3	Contains plasmid conferring pyocin S3 production genes	(11)

Growth Conditions

Growth experiments with *P. aeruginosa* were carried out in a medium containing 0.50% (w/v) casamino acids (CAA), 25 mM 3-morpholinopropane-1-sulfonic acid (MOPS), 0.25 g L⁻¹ MgSO₄ • 7 H₂O, 1.18 g L⁻¹ K₂PO₄ • 3 H₂O. *E. coli* growth experiments were carried out in LB medium. *P. aeruginosa* ATCC 27853 was grown at two temperatures (30°C and 37°C) as indicated throughout the thesis. *P. aeruginosa* PAO1 and *E. coli* strains were grown at 37°C. A salt solution consisting of 25 μ M MOPS, 0.25 g L⁻¹ MgSO₄ • 7 H₂O, and 1.18 g L⁻¹ K₂PO₄ • 3 H₂O was used to wash overnight cultures. All liquid cultures and plates were grown for 18 hours.

Pyocin S3 Extraction

Pyocin S3 was obtained from the lysates of *E. coli* DH5 α pPYS3-3, a strain that constitutively expresses a pyocin S3 plasmid. Cells were grown at 37°C in liquid LB medium containing 50 mM MOPS and 50 $\mu\text{g mL}^{-1}$ ampicillin. Cells were centrifuged for 30 minute at 4°C and 3400rpm. Afterwards, cells were washed with 25 mM Tris-HCL pH 7.5 and centrifuged for 30 minute at 4°C and 3400 rpm. Cells were resuspended in 25 mM Tris-HCL pH 7.5 and 1 mM EDTA. Cells were then incubated with 120 $\mu\text{g/mL}$ lysozyme for 30 minutes at 37°C. Then, cells were centrifuged for 15 minutes at 4°C and 3,400 rpm. The pyocin S3 lysate was filtered and stored at 4°C.

Iron-stringent conditions

The chelator ethylenediamine-N,N'-bis (2-hydroxyphenylacetic acid), referred to as EDDHA, was used to obtain iron-stringent conditions. For liquid cultures iron-stringent conditions were defined as CAA medium with 1 μM FeCl₃ and 100 μM EDDHA. This combination of medium will be referred to as “Chelated CAA”. Iron-stringent conditions were achieved in solid media by adding 1 μM FeCl₃ and 100 μM EDDHA after autoclaved sterilized CAA medium (1.5% agar) reached 65°C (13).

Chelator comparison

A new EDDHA (Complete Green Co.) preparation was deferrated to pH 9.00 and compared to an old EDDHA preparation in the laboratory. Single colonies of strain PAO1 and PAO1 ΔpvdS grown on LB plates were inoculated in LB liquid medium and incubated for 18 hours at 30°C and 250 rpm. Cells were washed in low iron salts containing 100 μM of either the old or new EDDHA and centrifuged for 5 minutes at 10,000 rpm. Each well condition was inoculated to an OD₆₀₀ of 0.02. Samples were placed in a 96-wel plate according to Figure 2. Cells were grown for 19 hours at 30°C in a microplate reader, which measured the OD₆₀₀ and fluorescence (excitation 400 nm, emission 460 nm) of each sample every 15 minutes.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 μ M Fe old EDDHA, PAO1			0 μ M Fe old EDDHA, <i>ApvdS</i>			0 μ M Fe new EDDHA, PAO1			0 μ M Fe new EDDHA, <i>ApvdS</i>		
B	1 μ M Fe old EDDHA, PAO1			1 μ M Fe old EDDHA, <i>ApvdS</i>			1 μ M Fe new EDDHA, PAO1			1 μ M Fe new EDDHA, <i>ApvdS</i>		
C	25 μ M Fe old EDDHA, PAO1			25 μ M Fe old EDDHA, <i>ApvdS</i>			25 μ M Fe new EDDHA, PAO1			25 μ M Fe new EDDHA, <i>ApvdS</i>		
D	50 μ M Fe old EDDHA, PAO1			50 μ M Fe old EDDHA, <i>ApvdS</i>			50 μ M Fe new EDDHA, PAO1			50 μ M Fe new EDDHA, <i>ApvdS</i>		
E	100 μ M Fe old EDDHA, PAO1			100 μ M Fe old EDDHA, <i>ApvdS</i>			100 μ M Fe new EDDHA, PAO1			100 μ M Fe new EDDHA, <i>ApvdS</i>		
F	200 μ M Fe old EDDHA, PAO1			200 μ M Fe old EDDHA, <i>ApvdS</i>			200 μ M Fe new EDDHA, PAO1			200 μ M Fe new EDDHA, <i>ApvdS</i>		
G	Old 0 μ M Fe	Old 1 μ M Fe	Old 25 μ M Fe	Old 50 μ M Fe	Old 100 μ M Fe	Old 200 μ M Fe	New 0 μ M Fe	New 1 μ M Fe	New 25 μ M Fe	New 50 μ M Fe	New 100 μ M Fe	New 200 μ M Fe
H	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank	blank

Figure 2. 96-well plate design to compare the growth and PVD production of PAO1 and PAO1 Δ *pvdS* in old and new EDDHA chelator preparations.

Spotting to Isolate Pyocin S3-Resistant Mutants

A pyocin spotting method adopted from Baysse et al. (2) was used to evolve pyocin-resistant colonies. *P. aeruginosa* ATCC 27853 was grown on solid LB agar for 18 hours at 37°C. Colonies were suspended in LB liquid medium containing 200 μ M MOPS and placed in the incubator for 18 hours at 30°C and 250rpm. 1mL of the liquid culture suspension was washed with salts and diluted to an OD₆₀₀ of 2.0. Then, 20 μ L of cells were plated onto Chelated CAA medium. After the cells had settled into the agar, 10 μ L, 30 μ L, 50 μ L, 100 μ L, or 300 μ L of pyocin S3 was spotted onto the surface of the plate to obtain a sufficient zone of clearing.

The pyocin S3 lysate produced a zone of clearing where it was applied. Bacterial colonies that grew in the zone of clearing were selected as pyocin S3-resistant candidates. These candidates were inoculated into LB overnight cultures for screening. Cells cultures were washed, resuspended in Chelated CAA, and diluted to an optical density (OD) at 600 nm of 2.0. Each large Chelated CAA agar plate received 200 μ L of cell suspension. Then, 10 μ L of pyocin S3 lysate was spotted on

to the surface of the agar. 20 μL of each suspension was spread onto small Chelated CAA agar plates and allowed to settle. Then, 50 μL of pyocin S3 lysate was spotted on to the surface of the agar. Plates were incubated at 30°C for 18 hours.

Passaging to Isolate Pyocin S3-Resistant Mutants

It has been observed that a 1:10 pyocin S3 lysate dilution conditions in liquid CAA medium containing 100 μM EDDHA produces sufficient killing (13). *P. aeruginosa* ATCC 27853 was grown on LB agar for 18 hours at 37°C. Colonies were suspended in CAA liquid medium containing 200 μM MOPS and grown for 18 hours at 37°C and 250 rpm. Cells were washed and resuspended in Chelated CAA medium.

Eppendorf tubes prepared with 1:10 pyocin S3 lysate Chelated CAA medium were inoculated to an OD_{600} of 0.02 and placed in a shaking incubator for 24 hours at 30°C and 250 rpm. After 24 hours a freezer stock (20% glycerol) was prepared for each tube. Additionally, a new set of tubes containing 1:10 pyocin S3 lysate Chelated CAA medium were inoculated to an OD_{600} of 0.02 and placed in a shaking incubator for 24 hours at 30°C. This process was repeated for five days. After the fifth day we compared the PVD fluorescence of the liquid cultures grown with and without pyocin S3 lysate. Then, ancestral cells and evolved cells grown in the presence of pyocin S3 lysate from days two and five were screened for resistance to pyocin S3. Cells were plated onto LB medium and incubated for 18 hours at 37°C. Isolated colonies were patched onto four different growth media and incubated for 18 hours at 30°C: CAA + 50 μM Fe, CAA + 50 μM Fe + 1:10 Pyocin S3 lysate, Chelated CAA, and Chelated CAA + 1:10 Pyocins S3 lysate.

Characterization of Pyocin Resistant Mutants

Candidates that grew in the presence of pyocin S3 lysate during screening were prepped for genomic sequencing. Polymerase chain reaction (PCR) reaction mixtures were prepared according to Table 2. A touchdown PCR method was used to increase binding specificity and accuracy. The full program is shown in Table 3. PCR products were analyzed using gel electrophoresis on a 1.0% agarose gel in 1x TBE salt buffer (containing ethidium bromide) at 80V. Each well received 10 μL of amplicon and two

wells received 5 μL of 100 bp DNA ladder. Gels were analyzed under UV light after 60 minutes.

Table 2. PCR Reaction Mixture

Reagent	Volume (μL)
10x Buffer #3	2.5
dNTPs (10 mM)	1.25
ELT Polymerase	0.35
Template DNA (conc. not determined)	0.5
Forward Primer (150 pM)	0.5
Reverse Primer (150 pM)	0.5
Water	19.4 ^a
Total	25

^a 20.4 μL of water was added in ‘No Primer’ control samples.

Table 3. PCR Program

Temperature	Time
95°C	5 minutes
94°C	30 seconds
58°C	40 seconds
68°C	60 seconds
94°C – 68°C steps repeated 30 times	-
68°C	10 minutes
4°C	Hold

PCR samples were purified with the using a PCR clean up kit to remove nucleotides and primers. A spectrophotometer was used to read the DNA concentrations after clean up. Samples were diluted to 25 ng/ μL and sent for sequencing at the Center for Genome Research and Biocomputing. The *fpvAIIb* sequences of the ancestral ATCC 27853 strain and PyoS3^{R1} were compared using sequence alignment program.

Results

Characterization of PyoS3^{R1}

In a prior study a pyocin-S3 mutant candidate named PyoS3^{R1} (derived from the ATCC 27853 ancestor) was isolated from a LB plate that had been spotted with pyocin S3 lysate (13). In the previous study the candidate was studied in depth and potential mutation of the *fpvAIIIb* receptor gene was analyzed by using PCR and gel electrophoresis. PCR was conducted using primers designed for *P. aeruginosa* strain PAO1. The PAO1 primers had been tested for compatibility using the wildtype ATCC 27853 strain. The gel results showed an amplicon present for the wild-type ATCC 27853 but no amplicon for PyoS3^{R1} (Figure 3) (13). It was hypothesized that PyoS3^{R1} had a complete *fpvAIIIb* deletion based on these findings.

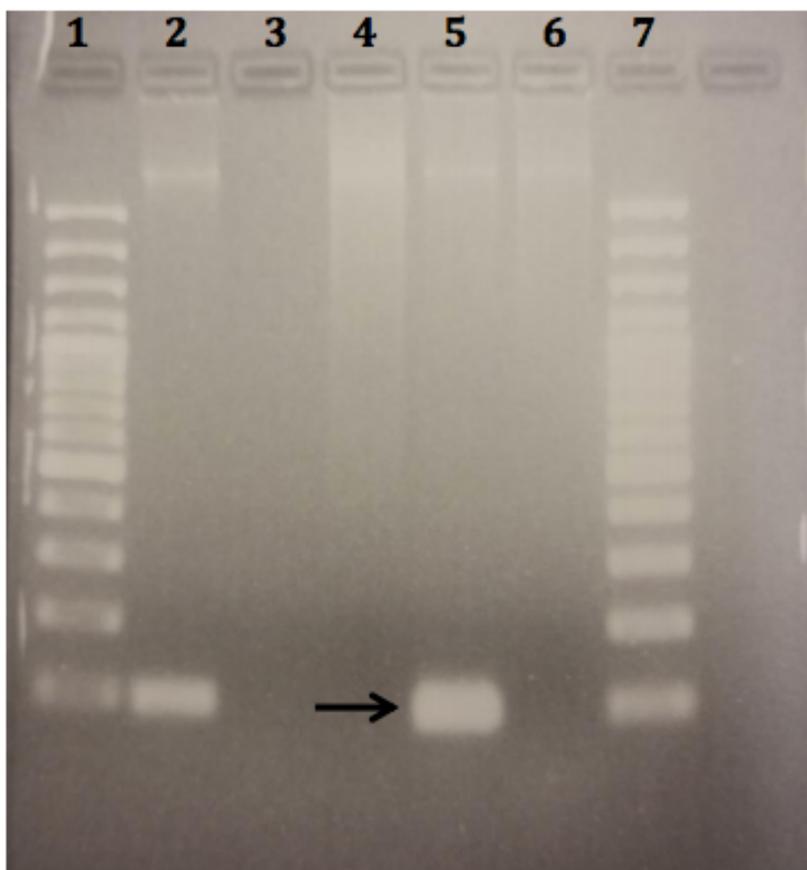


Figure 3. Gel electrophoresis of *fpvA* amplicon using PAO1 primer pairs. The arrow indicates a band corresponding to *fpvA* in ATCC 27853. Lane designations are as follows: 1) DNA ladder; 2) PAO1 wild-type; 3) no template; 4) PAO1 template with no primers; 5) ATCC 27853 wild-type; 6) PyoS3^{R1} template; DNA ladder

To determine if there was a total gene deletion in PyoS3^R1's *fpvAIIb*, primers were specifically designed for ATCC 27853. Four primer sets with overlap were constructed and used to amplify *fpvAIIb* (Figure 4). PCR gel results showed amplicon in all lanes corresponding to the new primers. This indicated that *fpvAIIb* was still present in PyoS3^R1 (Figure 5). The results obtained using ATCC27853 primers contradicted results obtained using PAO1 primer (Figure 3).

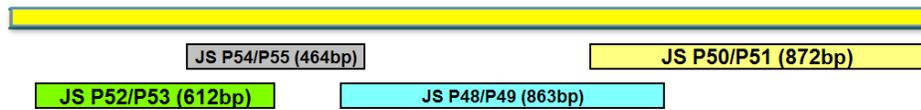


Figure 4. ATCC 27853 specific primer set. The four blocks represent the primer sets for *fpvAIIb* in *P. aeruginosa* ATCC 27853. Each block corresponds to the region a primer set covers.

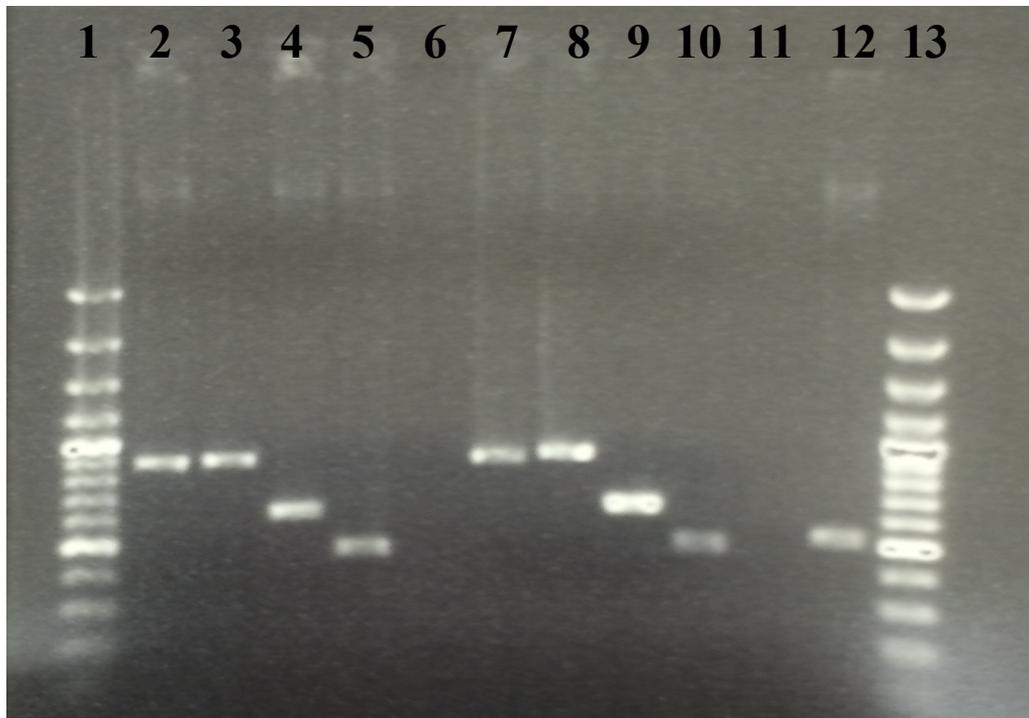
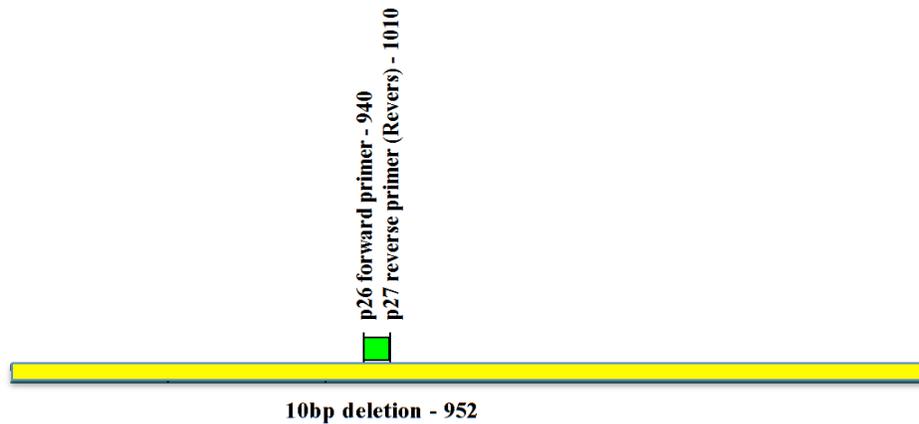


Figure 5. Gel electrophoresis of *fpvA* amplicon using four primer pairs designed for ATCC 27853. Lane designations are as follows: 1) DNA Ladder; 2) ATCC 27853 P48/49; 3) ATCC 27853 P50/51; 4) ATCC 27853 P52/53; 5) ATCC 27853 P54/55; 6) No template; 7) PyoS3^R1 P48/49; 8) PyoS3^R1 P50/51; 9) PyoS3^R1 P52/53; 10) PyoS3^R1 P54/55; 11) No template; 12) PAO1 using PAO1 primer pair; 13) DNA Ladder

To resolve the inconsistent results we sequenced PyoS3^{R1}'s *fpvAIIb*. Using Sanger Sequencing we determined there was a 10 base pair deletion in the PVD receptor gene. The deletion occurred 12 base pairs upstream from the start of the annealing site of the forward PAO1 primer (Figure 6) and caused poor annealing of the forward PAO1 primers during amplification. The 10 base pair deletion caused a frameshift resulting in a null receptor, thereby limiting the potential for future evolution of *fpvAIIb* in PyoS3^{R1} (Figure 6). We adopted a new approach to isolate a comparable mutant in Chelated CAA, where a functional PVD receptor would be necessary.

A.



B.

950
|

Wildtype: TTCATGCTGGCGCCGAGCTTTACCTGGAACCCCAACGAGCAGACCAGCCTGA
 F M L A P S F T W N P N E Q T S L

Mutant: TTCATGCTGGCG _____ CCTGGAACCCCAACGAGCAGACCAGCCTGA
 F M L A P G T P T S R P A Stop

Figure 6. The *fpvAIIb* gene marked with PAO1 primer locations and deletion site. A. Overview of *fpvAIIb* gene labeled with the location of the deletion and the binding sites for PAO1 forward and reverse primers. B. Comparison of the amino acid and DNA sequences between wildtype ATCC 27853 and PyoS3^{R1}, surrounding the deletion in *fpvAIIb*. The number above the sequence indicates the nucleotide distance from the start of the gene.

EDDHA Comparison

While conducting this thesis another lab member was unable to replicate an experiment he had conducted in the past. He suspected that the EDDHA chelator preparation had lost some of its chelating ability. To test this hypothesis we prepared a new EDDHA chelator preparation and compared it to the old preparation. In CAA medium with 100 μM of old EDDHA and 0 μM Fe, PAO1 $\Delta pvdS$ was able to grow to a similar OD_{600} as PAO1. In CAA medium with 100 μM of new EDDHA and 0 μM Fe, PAO1 $\Delta pvdS$ only reached an OD_{600} of 0.025 in comparison to an OD_{600} of 0.200 for PAO1 (Figure 7).

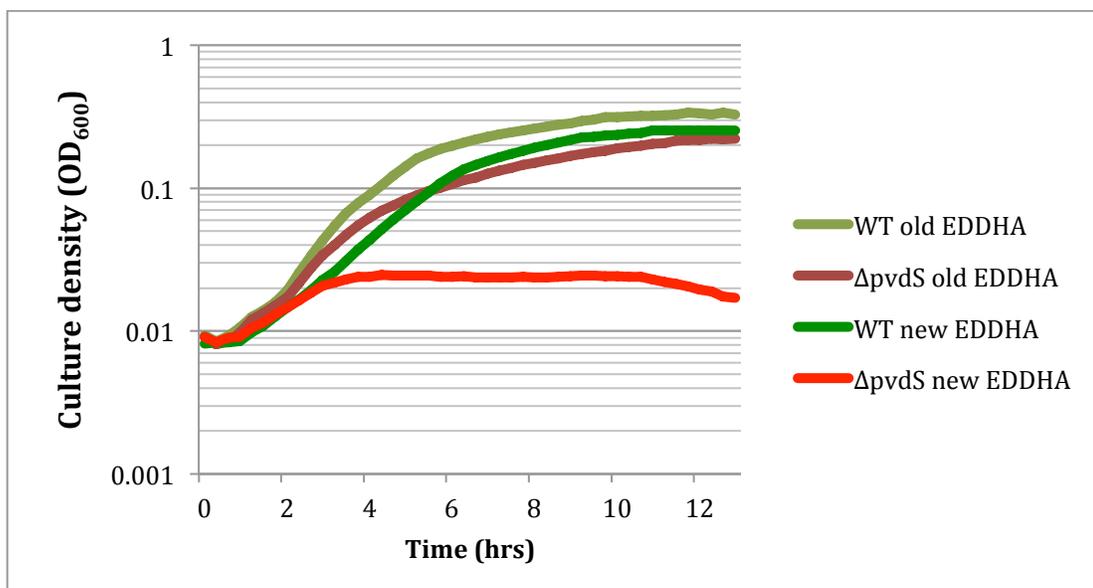


Figure 7. Culture density (OD_{600}) of $\Delta pvdS$ and PAO1 grown in CAA medium with 100 μM old and new EDDHA and no Fe added.

Isolation of Mutants via spotting

The spotting method adopted from Baysse *et al.* was used to put cells grown on Chelated CAA under the selective pressure of pyocin S3. A few approaches were used to expose cells to pyocin S3 lysate. The first method involved spotting 10 μL of pyocin S3 lysate directly onto the surface of the plate after a lawn of cells was plated. Isolated colonies grew in the zone of clearing and were suspected to be pyocin S3-resistant. These candidates were grown in liquid overnight cultures and plated onto chelated CAA plates and spotted with pyocin S3. After 18 hours a zone of clearing was present indicating the candidates were not resistant to pyocin S3 (Figure 8).

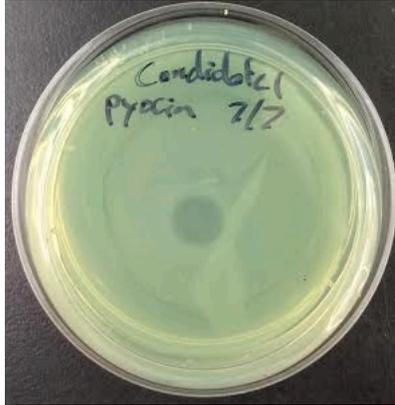


Figure 8. Pyocin-resistance screen plate Candidate that previously grew along the edge of the zone of clearing did not grow in the presence of pyocin S3 the second time.

Different volume sizes were explored after 10 μ L of pyocin S3 produced a small zone of clearing. We spotted 30, 50, 100, and 300 μ L of pyocin S3 onto Chelated CAA medium. The 300 μ L plate did not dry even after 24 hours. Plates with volumes below 100 μ L took between 60 and 120 minutes to dry; however, they produced a sufficiently large zone of clearing. No colonies were isolated from the zone clearing.

Another approach involved spotting pyocin S3 on to the agar 24 hours before spreading cells. The protocol modification reduced the time plates sat at room temperature to dry allowing us to place the plates in the incubator within 30 minutes. Reducing the time plates spend at room temperature is important given the temperature dependent killing of pyocin S3. This method produced the same zone of clearing found on plates spotted with pyocin S3 after plating cells (Figure 9).

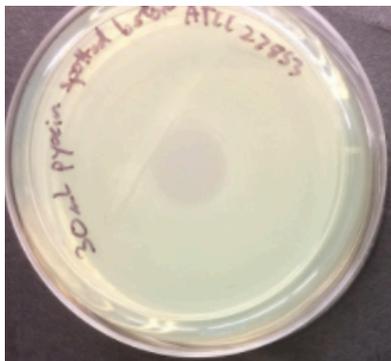


Figure 9. Plate spotted with pyocin S3 before plating lawn of cells. Zone of clearing present after 18 hours at 30°C

We were unable to isolate a pyocin S3-resistant candidate because no colonies emerged within the zone of clearing. The spotting method was still not producing reliable results after adding the new EDDHA. We decided to move to liquid culture experiments to increase the odds of isolating a mutant. We hypothesized not enough cells were growing in the presence of pyocin S3 lysate on the plates. However, even with liquid culture experiments we would eventually need a consistent solid medium screen. We decided to construct 1:10 pyocin S3 lysate CAA-EDDHA plates to see if they would produce more consistent results. Pyocin S3 lysate was added to the media after cooling to 65°C after autoclaving to prevent pyocin S3 from degrading. A lawn of cells were plated and incubated at 30°C for 18 hours. No growth was present indicating that the pyocin S3 retained its killing ability (Figure 10).

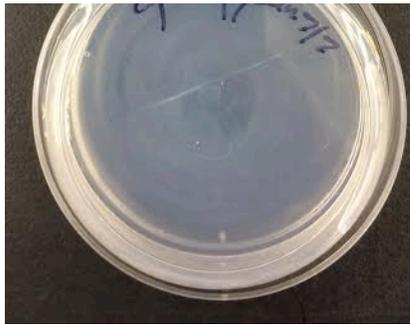


Figure 10. 1:10 pyocin S3 lysate Chelated CAA plates. Total zone of clearing present after 18 hours at 30°C.

Isolation of Mutants via Liquid Culture Enrichment

To increase the probability of isolating a pyoverdine receptor mutant we adopted an enrichment scheme that exposed a greater quantity of cells to pyocin S3 lysate using a liquid culture. We inoculated liquid cultures at a low density in both chelated CAA and unchelated CAA. Cultures were grown to high density. The cycle was repeated five times to enrich for resistant mutants. After 24 hours the average OD₆₀₀ of the liquid cultures with pyocin S3 lysate was lower than the control liquid cultures. After 48 hours both cultures had similar OD₆₀₀ readings (Figure 11). We interpreted the rise in OD₆₀₀ reading between days one and two as an indication that pyocin S3-resistant cells had been enriched. The relative fluorescence of the cultures grown in the presence of pyocin S3 lysate was approximately half of the fluorescence of the cultures grown in the absence of pyocin S3 (Figure 12).

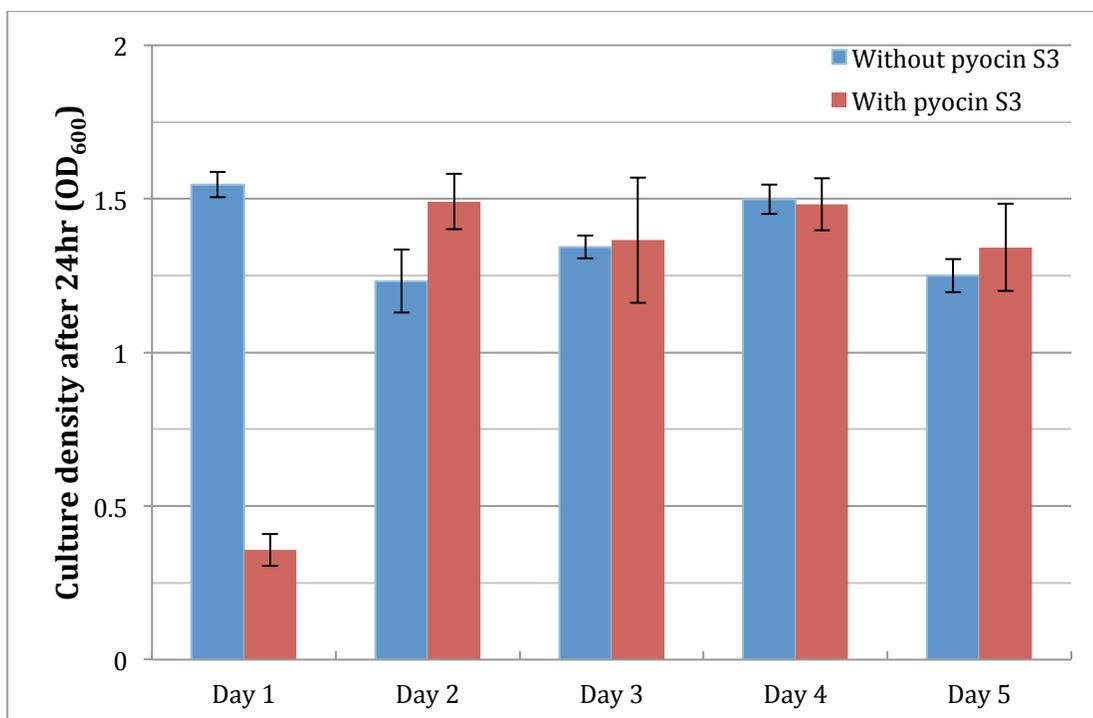


Figure 11. Growth as a function of pyocin S3. OD₆₀₀ Sample OD was measured after 24 hours of growth. Three replicates were performed; error bars indicate standard deviation.

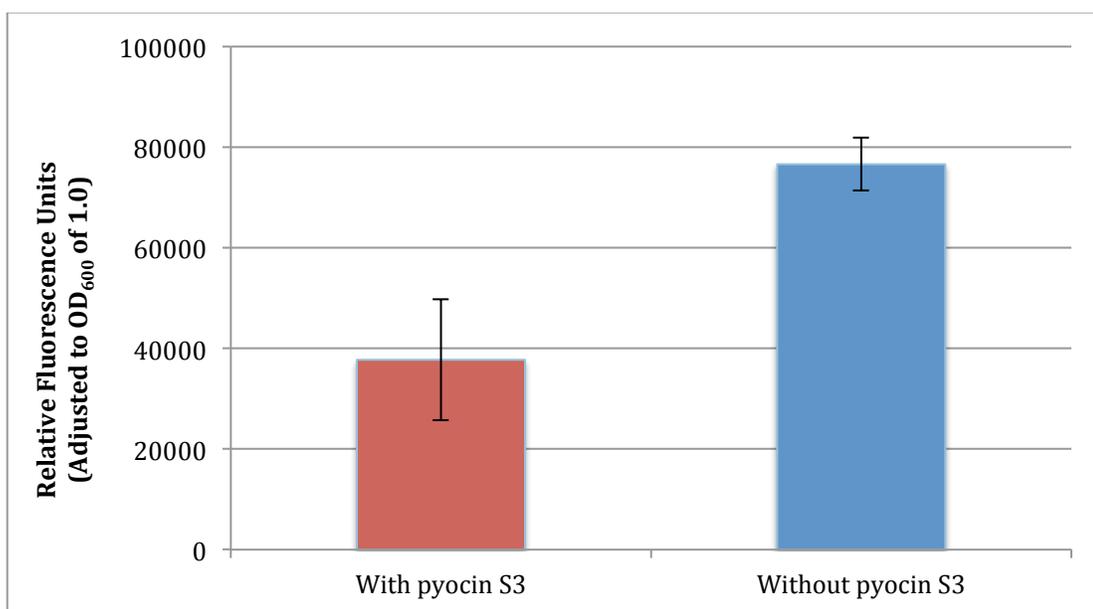


Figure 12. Pyoverdine Production as a function of pyocin S3. Sample relative fluorescence units measured after 24 hours of growth on day 5. Three replicates were performed; error bars indicate standard deviation.

Screening Liquid Culture Enrichment Strains

Ancestral ATCC 27853 and ATCC 27853 enriched for two and five days in the presence of pyocin S3 were screened for pyocin S3 resistance. This was accomplished by patching colonies onto selection plates. The screen consisted of four conditions. The first condition was iron-replete (CAA + 50 μ M Fe); we expected no PVD production would be needed to grow. The second condition was iron-replete with pyocin S3 (CAA + 50 μ M Fe + 1:10 Pyocin S3 lysate); we expected no PVD production would be needed to grow. The third condition was iron-stringent (Chelated-CAA); we expected PVD production would be needed to grow. The fourth condition was iron-stringent with pyocin S3 (Chelated-CAA + 1:10 pyocin S3 lysate); we only cells expected cells with mutated functional PVD receptors to grow.

The results obtained from conditions one, two, and three mirrored our expectations. In condition one all colonies patched from the three strains grew without producing PVD. In condition two there was higher survival for the evolved strains compared to the ancestral strain and the patched colonies grew without producing PVD. In condition three all three strain grew but not all produced PVD. Interestingly, PVD production decreased the longer cells were enriched in the presence of pyocin S3 lysate. The results from condition four did not match our expectations because the 46% of the colonies derived from the ancestral culture were able to survive in the presence of pyocin S3 lysate (Table 4).

Table 4. Liquid Culture Screen

	Ancestral*		Pyocin Evolved Day 2**		Pyocin Evolved Day 5**	
	Survival (%)	PVD (%)	Survival (%)	PVD (%)	Survival (%)	PVD (%)
CAA + 50 μ M Fe	100	0	100	0	100	0
CAA + 50 μ M Fe + 1:10 Pyocin S3 lysate	84	0	100	0	99.3	0
CAA + 1 μ M + 100 μ M EDDHA	100	100	100	90	100	81.3
CAA + 1 μ M + 100 μ M EDDHA + 1:10 Pyocins S3 lysate	46	0	58.7	0	29.3	0

* 50 colonies

** 150 colonies

Discussion

Background

This study explored one potential explanation for the diversity of siderophores, more specifically PVDs. We attempted to create a model system to observe the influence of pyocins on the evolution of the PVD gene locus *in vitro*. We explored many methods to obtain *P. aeruginosa* ATCC 27853 PVD receptor mutants

Analyzing the alteration of PyoS3^{R1}'s *fpvAIIb*

In a previous study a mutant that could grow in the presence of pyocin S3 lysate was isolated from a zone of clearing on LB medium. Although, LB medium is iron-replete *P. aeruginosa* ATCC 27853 expresses *fpvAIIb* at all iron concentrations (13).

Therefore, all cells grown on LB were susceptible to pyocin S3 killing. PyoS3^{R1}'s *fpvAIIb* was sequenced and alignment with the wildtype gene showed a 10 base pair deletion (Figure 6). The deletion resulted in a null-receptor that prevented both PVD and pyocin S3 from entering the cell. PyoS3^{R1} was able to grow because there is sufficient iron present in the LB medium, so a functional PVD receptor is not needed to survive. *P. aeruginosa* can acquire iron without the use of siderophores in iron-replete conditions.

In vitro Evolution Spotting System

We are interested in isolating mutants with a functional PVD receptor. Therefore, we constructed a medium that was iron-stringent and would require a functional PVD receptor for cells to grow. We adapted a spotting method to isolate PVD receptor mutant candidates. Initially, this method proved inconsistent as non-resistant colonies grew in the zone of clearing. One possible explanation for this result is that the pyocin S3 lysate was distributed in a bell-shaped manner on the agar surface. Therefore, at the edge of the spotted region there could have been less pyocin S3 present compared to the middle of the spotted region. This would allow colonies to grow inside the zone of clearing in locations where a lower concentration of pyocin S3 present. To overcome the theoretically bell-shaped distribution of pyocin S3 a greater volume of

pyocin S3 lysate was spotted to increase the zone where there was sufficient pyocin S3.

The spotting method did not produce a pyocin S3 resistant colony. In antibiotic resistance studies one in 10^9 cells has a mutation conferring resistance. The spotting method was putting orders of magnitudes less cells under selective pressure compared to what is required in antibiotic studies. To address this we took a new approach to isolate PVD receptor mutants by adopting a liquid culture enrichment scheme.

***In vitro* Evolution Liquid Culture Passaging System**

Ancestral ATCC 27853 and ATCC 27853 enriched for two and five days in the presence of pyocin S3 were screened for pyocin S3 resistance. Colonies were patched onto four different mediums and survival and PVD production were measured. The results obtained from conditions one, two, and three matched our expectations. The results from condition four did not match our expectations. Ancestral colonies were able to survive and grow in the presence of pyocin S3 lysate in iron-stringent conditions (Table 4). One explanation for this result is that pyocin S3 lysate is unpurified and contains nutrients and components originally inside the *E. coli* cells. Therefore, it is possible that the pyocin S3 lysate is contaminated with high amounts of iron. If that is the case, then true iron-stringent conditions might not have been produced during the liquid passaging experiment and in the screening plates.

When ATCC 27853 is grown in iron-replete conditions it does not produce PVD. When ATCC 27853 is grown in iron-stringent conditions it does produce PVD. Binding of PVD to the PVD receptor creates a positive feedback loop that increases PVD and PVD receptor production. This feedback loop would not be present in iron-replete conditions where ATCC 27853 does not produce PVD. Therefore, there would be a lower number of PVD receptors on a cell grown in iron-replete compared to a cell grown in iron-stringent conditions. This would limit pyocin S3's ability to kill cells.

An alternative explanation is that pyocin S3 only retains its killing activity long enough to kill a certain amount of cells. It is currently unknown how many cells a single pyocin S3 can kill. It is possible that after killing a cell pyocin S3 becomes stuck inside the cell.

EDDHA Activity Appears to Degrade Over Time

The data we obtained indicates that the iron-chelator EDDHA appears to lose activity over time. PAO1 $\Delta pvdS$, a strain that does not produce a PVD receptor, was able to grow in CAA medium chelated with the old EDDHA. The same strain was not able to grow in CAA medium chelated with EDDHA prepared a few days earlier (Figure 7). It is known that EDDHA's cleaving structure deteriorate in the presence of light (25). Perhaps it is also possible that EDDHA's cleaving structure deteriorate over time.

Significance and Future Work

Antibiotic resistance among pathogens is an increasing problem. Bacteriocins are being investigated as an alternative to antibiotics. Pyocins have shown signs of antimicrobial activity *in vivo* but there is still much not known about these compounds (19). A potential concern with the use of pyocins is that resistance will develop. Resistance could develop if pyocins select for PVD receptor mutants. Therefore, it is imperative to increase our understanding of pyocins and their effects on cellular structures.

Future work should analyze the concentration of EDDHA chelator required to produce sufficient iron-stringency in CAA medium supplemented with pyocin S3 lysate. Then, a passaging experiment should be conducted until the OD_{600} of the culture grown in the presence of pyocin S3 lysate increases. I would recommend using the same plate screening system because each of the four conditions will provide valuable information, when sufficient iron-stringency is achieved. Results from these experiments could provide evidence towards the theory that pyocins are driving the evolution of the PVD gene locus.

Additionally, *P. aeruginosa* ATCC 27853's PVD receptor production should be further analyzed. In a prior study it was noted that the strain expresses the same amount of *fpvAIIIb* mRNA in both iron-replete and iron-stringent conditions; however, it is unknown if the mRNA is translated into protein.

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