S-PYOCINS AS POTENTIAL ANTIMICROBIAL REAGENTS FOR

ERADICATING *Pseudomonas aeruginosa* BIOFILMS

by

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Submitted to the Graduate School of Engineering and Natural Sciences in partial

fulfillment of the requirements for the degree of Master of Science

Sabanci University

Spring 2014

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ABSTRACT

Pseudomonas aeruginosa (Pa) is a gram-negative opportunistic pathogen associated with life-threatening hospital-acquired and community-acquired infections affecting urinary tract, skin, eye, ear, and lungs. Antipseudomonal antibiotics are the most potent arsenals to treat Pa infections. However, Pa infections are still common and stubborn bacterial infections due to elevated resistance levels against most of the antibiotics used in clinics. Treating Pa infections becomes even harder especially if antibiotic resistant Pa strains form biofilms and cystic structures in human body. In such cases, drug doses for treating bacterial infections can be up to one thousand fold higher than the necessary dose for treating infections caused by planktonic bacteria. Thus, there is an obvious need for novel therapies for fighting against Pa infections and Pa biofilms. Recently, several studies performed on pyocins, proteinaceous bacterial toxins produced by Pa, to explore the potential of pyocins as novel antibiotics to treat Pa infections. Similar to other members of the bacteriocin family, pyocins mostly kill strains of the related species. There are three types of pyocins: S, R, and F pyocins. S-type pyocins are high molecular weight proteins and RF-type pyocins resemble bacteriophage tails. In this study, we applied four S-pyocins (S1, S2, S3, AP41) on Pa biofilms separately and in combination with six commonly used antibiotics (Tobramycin, Gentamicin, Colistin, Piperacillin, Ceftazidime, Ciprofloxacin) to determine the efficacy of S-pyocins in eradicating Pa biofilms and interactions between S-pyocins and antibiotics. We created Pa biofilms using Calgary Biofilm Device (CBD) to determine minimum biofilm eradication concentration (MBEC) of each antibiotics and S-pyocins separately and in

combination. This study aims to explore the potential use of S-pyocins as alternative drugs for eradicating biofilms where Pa biofilms show high resistance to antibiotics. Our preliminary results suggest wild type pyocins have a slight effect on Pa biofilms but there is an antagonism between pyocin AP41 and drugs Ciprofloxacin, and Colistin which can be used for selecting against drug resistant Pa strains.

Pseudomonas aeruginosa BİYOFİLMLERİNİ YOK ETMEK İÇİN POTANSİYEL ANTİMİKROBİYAL AJANLAR OLARAK S-PYOSİNLER

Enes Karaboğa

ÖZET

Pseudomonas aeruginosa (Pa) gram-negatif, oportünistik bir patojen olup genellikle hastanelerden bulaşarak ölümcül idrar yolları, deri, göz, kulak ve akciğer enfeksiyonlarına sebep olur. Antipseudomonal antibiyotikler günümüzde tedavi için kullanılan en yüksek potansiyelli tedavilerdir. Buna rağmen, Pa enfeksiyonları halen en çok görülen ve en inatçı enfeksiyonlardır. Bunun en temel sebebi ise Pa türlerinin antibiyotiklere karşı geliştirdiği yüksek direnç seviyeleridir. Hatta Pa biyofilm oluşturması durumunda antibiyotik direnci daha da artmakta ve tedavi daha da zorlaşmaktadır. Biyofilmler yapısal olarak antibiyotiklere planktonic fenotipe kıyasla bin kata kadar dirençli olabilmektedir. Dolayısıyla, yeni antibiyotik ajanlara ihtiyaç oldukça açıktır. Son yıllarda yapılan araştırmalarda pyosinlerin yani Pa tarafından üretilen toksik proteinlerin Pa tedavisinde kullanılmak üzere bir potansiyeli olduğu görülmüştür. Pyosinlerin özelliği sadece dar bir tür aralığını hedeflemesi ve sadece yakın türdeki Pa bakterilerini hedeflemesidir. Üç tip pyosin grubu vardır: S, R ve F pyosinler. S-pyosinler çözünebilen ve yüksek moleküler ağırlıkta proteinler olup, R ve F pyosinler bakteriyofaj şeklindedirler. Bu çalışmada, dört adet S-pyosinin (S1, S2, S3 ve AP41) Pa biyofilmleri üzerinde ayrı ayrı ve en çok kullanılan altı antibiyotik (Tobramisin, Gentamisin, Kolisin, Piperasillin, Ceftazidim ve Ciprofloxacin) ile antibiyotik çiftleri şeklinde denedik. Biyofilmleri Calgary Biyofilm Aleti kullanarak oluşturduk ve biyofilmlerin Minimum Biyofilm Eradikasyon Konsantrasyonlarını belirledik. Bu projenin hedefi Pa türleri üzerinde S-pyosinlerin ve antibiyotik-pyosin çiftlerinin Pa enfeksiyonları tedavisinde kullanılabilmesidir. İlk deney sonuçlar

gösteriyor ki pyosinlerin biyofilmler üzerindeki etkisi düşük olmakla birlikte AP41 pyosin ve Ciprofloxacin ve Kolistin çiftleri antagonizme sebep olmakta. Bu antagonistik çiftler potansiyel olarak dirençli türlerin yok edilmesinde kullanılabilirler.

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INTRODUCTION

Identification of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (Pa) is a gram-negative, versatile bacteria that can be found in diverse environments ranging from soil to medical devises and human-polluted environments such as sinks, swimming pools and sewers, and prostheses [1, 2, 3]. Since Pa is able to metabolize wide-range of nutrients, it is very ubiquitous in nature and human-polluted environments with organic materials such as sinks, swimming pools, and sewers [4].

It is an opportunistic bacterium that can infect nematodes, plants, and animals [5, 6, 7]. In humans, Pa causes life-threatening hospital-acquired and community-acquired infections including sepsis, pneumonia, inflammation, gastrointestinal (GI) tract infections and skin infections, mostly affecting patients with cystic fibrosis and immunocompromised individuals such as cancer and AIDS patients, and burn victims[8].

Pathogenesis of Pa

First, Pa enters into the body through breathing; open wounds or skin abrasions and then exerts its pathogenesis after binding to the related tissue. Pa multiplies until reaching to a critical biomass before virulence factors show their effect. Pa virulence factors mostly include exotoxins and endotoxins. An example for Pa virulence factors is the secretion of ExoS and ExoT with Type III secretion system leading eukaryotic host cell cytoskeleton disruption and cell death [9]. Another example is Exotoxin A, aka Pseudomonas Exotoxin, which inhibits elongation factor-2 leading to inhibition of protein synthesis and eventually death of eukaryotic cells [10].

Pa is a great threat for Cystic Fibrosis (CF) patients. 80% of adult CF patients have chronic Pa infection in their lungs [11]. Pa infects CF patients through airway and

once it is in, it moves to low oxygen environment. Normally aerobic Pa switches anaerobic mode and starts forming biofilms that will cause long-term, persistent infections, which are resistant to many antibiotic classes.

Antibiotic Resistance of Pa

Pa infections are very challenging to treat because of antibiotic resistance mechanisms. Especially in clinical isolates, antibiotic resistance is much higher due to the intense usage of antimicrobial agents (Table 1).

Antibiotic	Non-CF patients[2] (n=2067)	CF-patients[2] (n=127)	CF-patients[3] (n=282)
Amikacin	3.9	36	–
Gentamicin	9.1	43	47
Tobramycin	–	–	24
Ciprofloxacin	7.3	24	16
Ceftazidime	1.7	14	32
Imipenem	6.7	31	–
Meropenem	3.5	11	–
Piperacillin	3.4	11	24
Piperacillin/tazobactam	2.4	9	–
Colomycin	–	–	5

Table 1: Antibiotic resistance score differences between CF and non-CF patients [12]

Intrinsic Resistance Mechanisms

Increasing antibiotic resistance is achieved in several ways. The first way is general antibiotic resistance mechanisms (intrinsic) that Pa posses that are:

1) Efflux pumps
2) Inactivation of drugs
3) Target modifications

There are several sources of antibiotic resistance in Pa. Intrinsic mechanisms are one of them and include efflux pumps, drug inactivation, and target modification. Pa strains use efflux pumps located on membranes to pump the antibiotics outside of the cell. There are four types of efflux pumps found in Pa all targeting different class of antibiotics: mexAB-oprM, mexXY-oprM, mexCD-oprJand mexEF-oprN. MexAB-oprM pumps out mainly beta-lactams and quinolones [13], mexXY-oprM is responsible for aminoglycosides [14], and mexEF-oprN pumps carbapenems and quinolones [15]. Drug inactivation is another mechanism, which Pa uses to target to modify and inactivate antimicrobial molecules. AmpC gene is widely studied beta-lactamase targeting antipseudomonal penicillins [16]. Pa can also modify targets of antimicrobial agents in order to prevent binding antibiotic molecules to related sites. 16S rRNA modifications by methylation with 16S rRNA methylases such as RmtA and RmtD are shown to increase resistance against aminoglycoside group of antibiotics [17, 18].

Antibiotic Resistance of Biofilms

Pa`s ability to form biofilms is another cause of elevated antibiotic resistance. Biofilms are structures formed by bacterial aggregates attached on a surface and surround themselves with an extracellular polymeric substances (EPS), composed of polysaccharides, proteins, DNA, lipids, and other substances [19].

Biofilm formation starts with attachment of motile bacteria to an appropriate surface. Surface attachment is usually achieved by non-specific hydrophobic bonding if a surface is nonliving. If the surface to be attached is a living environment, several molecular mechanisms play role in adherence leading to a specific binding. Short after initial attachment, bacteria start to form irreversible attachments with surface and the other bacteria. These attachments induce the expression of genes that produce EPS and glue the cells together forming a mature biofilm (Figure 1).
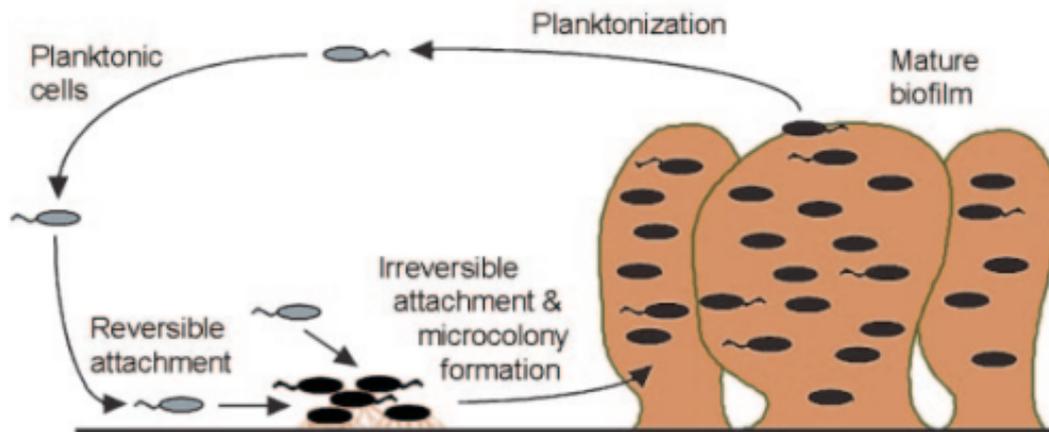
Figure 1: 4 steps of biofilm formation (1) Reversible attachment (2) Irreversible attachment (3) Maturation (4) Dispersion (Planktonization) [20]

After biofilm is formed, bacteria of biofilm community can communicate via system called quorum sensing. Using pheromones, Pa communicates with other members to achieve cell aggregation, virulence, and polysaccharide formation via signal molecules detected by other bacterial cells and activating target gene expression [21, 22].

Biofilms are much more difficult to eradicate than free-floating bacteria. Compared to minimum inhibitory concentration (MIC), which is the minimum concentration of antibiotic agent needed to inhibit bacterial growth of planktonic bacteria, concentration needed to eradicate biofilms is hundreds and in some cases even thousand times more than MIC [23] (Table 2).

Antibiotic	MIC (μg/ml)		MBEC (μg/ml)	
	NCCLS assay[a]	Assay with CBD[a]	A_{650}[a]	0 CFU/peg[b]
Amikacin	2	4	16	16
Aztreonam	2	4	>1,024	>1,024
Ceftazidime	1	2	>1,024	>1,024
Ciprofloxacin	0.25	0.25	4	4
Gentamicin	2	4	128	128
Imipenem	1	4	>1,024	>1,024
Piperacillin	2	16	>1,024	>1,024
Tobramycin	0.5	1	2	2

[a] The values were obtained by measuring the turbidity at 650 nm (A_{650}) on a 96-well plate reader.
[b] The values were obtained by determination of plate counts.

Table 2: Comparison of MIC (Minimum Inhibitory Concentration and MBEC (Minimum Biofilm Eradication Concentration) values of *Pseudomonas Aeruginosa* ATCC 27853 [24]

Resistance mechanisms of biofilms differ from planktonic cells. Antibiotic resistance mechanisms usually seen in bacteria such as efflux pumps, inactivation of drugs and target modifications are not the reason of high resistance to antibiotics. When biofilms are disrupted and the cells of biofilm are suspended in a medium, antibiotic resistance of biofilms dramatically decrease and MIC values become same with free-floating bacteria [25].

There are several hypotheses on the high antibiotic resistance of biofilms to antibiotics. One hypothesis is incomplete penetrance of antibiotic agents into the biofilm [26]. Antibiotics may be degraded easier when penetrance is slower. Another hypothesis is the modified chemical environment in biofilm structure. Antibiotics may be degraded or disrupted in different environments such as low or high pH values, changes in oxygen concentrations etc. [27]. One another hypothesis suggests that increased antibiotic resistance may be caused by cell differentiation after forming biofilms [28]. It is also suggested that positioning of bacteria throughout the slime can create heterogeneity that is some bacteria are able to access nutrient easier while some are not. Therefore, different metabolic activity will lead to different levels of susceptibility of members of biofilm community [29].

Treatment of Pa infections is mostly achieved by antibiotic therapies. However, the more we use the antibiotics, the more antibiotic resistance is emerging. In addition, the number of novel antibiotics introduced by pharmaceutical companies is declining. It is obvious that novel therapies for antibiotic resistance bacteria are needed [30] (Figure 2).
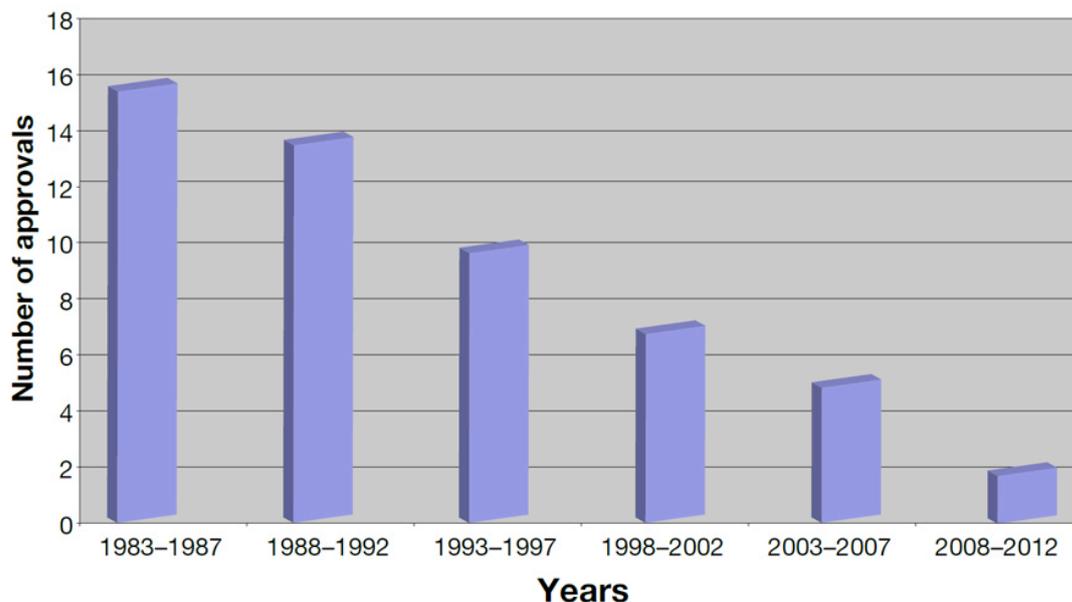
Figure 2: Number of antibiotics approved by FDA as 4-year periods starting from 1983 [31]

Bacteriocins

Bacteriocins, discovered by A. Gratia in 1925, are small toxic proteins produced by wide variety of Bacteria and Archaea to inhibit or inhibit the growth of the closely related species [32]. In other words, bacteriocins are narrow spectrum antibiotics produced by bacteria to gain advantage on other bacteria. The first bacteriocin discovered killed *E. coli* therefore it is called "colicin".

Colicins

Colicins are produced by *E. Coli* and all colicin-producing genes are carried by plasmids. These plasmids are called pCol plasmid and colicin-producing bacteria are called colicinogenic bacteria. There are two main groups of colicin; group A and group B colicins that are classified according to the membrane proteins which translocate these toxins into target cell cytoplasm [33, 34]. Group A colicins are small proteins that are secreted to medium, while group B colicins are bigger and do not secreted to medium. Both groups of colicins show nuclease and pore-forming activities.

As it has shown on the figure below, colicin operon has two genes: the first gene (toxin gene) codes for nuclease or pore-forming protein. Toxin gene is followed by the second gene (immunity gene) which codes for immunity protein. Immunity protein prevents colicin protein to attack and kill bacteriocin producing bacteria [35].
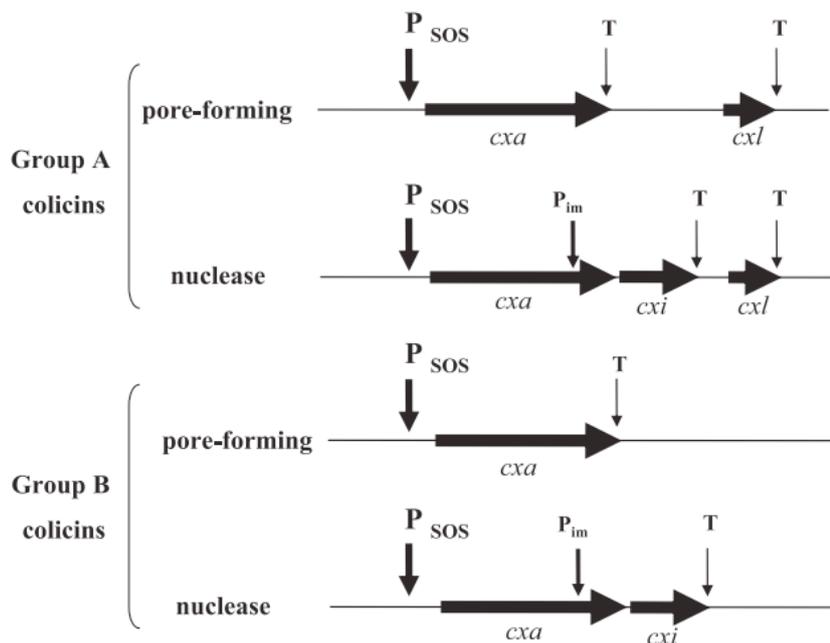
Figure 3: Organization of colicin operons. PSOS: SOS promoters, Pim: Immunity Protein, T: Transcription Terminator, cxa: Colicin Killer Gene, cxi: Colicin Immunity Gene, cxl: Lysis Protein Gene [35]

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Pyocins

Pa is also known to produce bacteriocins called pyocins. 90% of Pa strains are known to produce pyocins. There are three types of pyocins classified according to their mechanisms of action and biological properties: F type, R type, and S type pyocins. F type (Flexuous) pyocins are group of pyocins have a structure and mechanism of action like contractile bacteriophages, such as bacteriophage λ. R type pyocins are also phage-like proteins, yet their structure is similar to bacteriophage T4 (Figure 4). The main difference between R and F type pyocins is R type pyocins are contractile while F type pyocins are non-contractile [36].
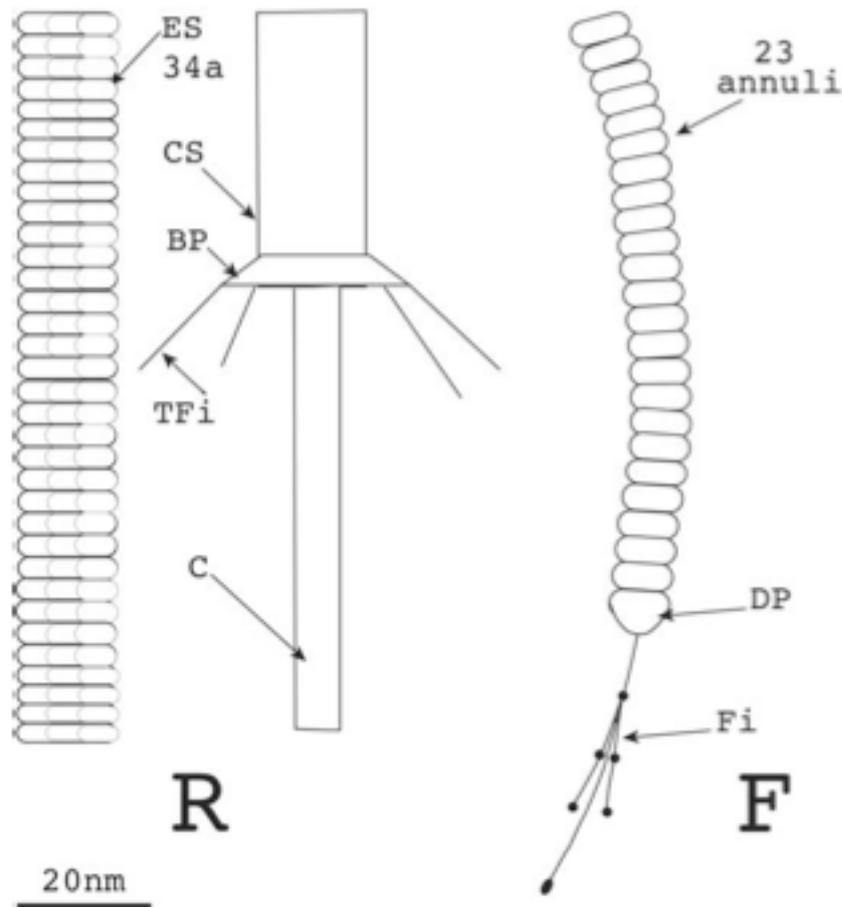
Figure 4: Schematic representation of R and F type pyocins (CS = Contracted Sheath, BP = Base Plate, TFi = Tail Fibers, C = Core, DP = Distal Part, Fi = Fiber Part) [36]

S-type pyocins (S1, S2, S3, and AP41) are small nucleases that cause target cell DNA breakdown. Similar to colicins, pyocins protein consist of two components; the large component which is located to C-terminal of protein that exerts its nuclease activity, and the small component that do not show nuclease activity by itself and acts as an immunity protein [37] (Figure 5).
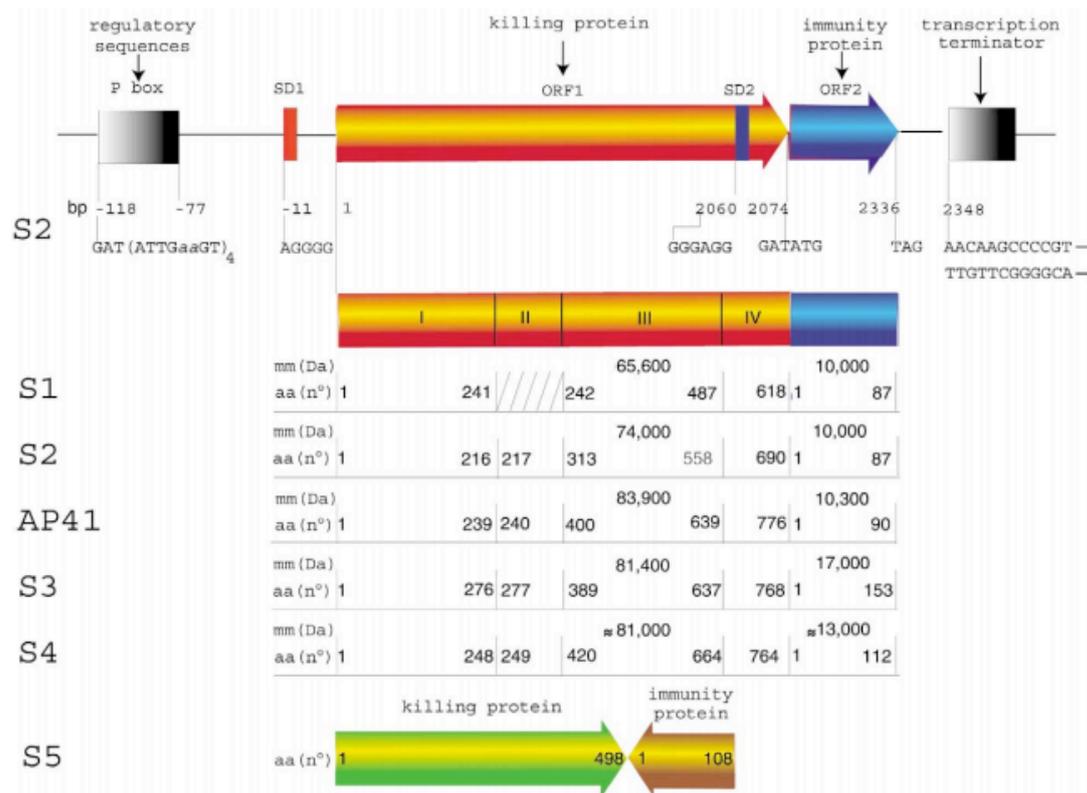
Figure 5: Genetic organization of pyocins. ORF1: Killing Protein, ORF2: Immunity Protein. [36]

There is also a remarkable similarity between E2 group of colicins and the killing domains of S1, S2, S3 and AP41 pyocins. S1, S2, and AP41 nuclease domains show 41% homology with E2 nuclease domain and 53% of their amino acids of immunity proteins are identical [38]. This situation also suggests that there is an evolutionary relationship between colicins and pyocins. They are possibly originated from a common ancestor.

It is shown that S1, S2, S3, and AP41 are able to effectively kill sensitive strains of Pa and pyocins S2 is shown to be able to eradicate Pa biofilms [39]. It is obvious that pyocins are potent antibiotics to be used on Pa biofilms.

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MATERIALS & METHODS

Cloning

In order to clone S1, S2, S3, and AP41 genes, plasmids from original sources (*E. Coli* cells) are extracted using QIAprep Spin Miniprep Kit (Table 3). Qiagen's original protocol is used to extract plasmids (Appendix A).

Strain	Species	Description
pYMSS11	*E. Coli* (XL-1 Blue)	Plasmid containing pyocin S1 gene in pUC119 (Ampicillin Resistant)
pYMPS1	*E. Coli* (XL-1 Blue)	Plasmid containing pyocin S2 gene in pUC119 (Ampicillin Resistant)
pYS3.3	*E. Coli* (XL-1 Blue)	Plasmid containing pyocin S3 gene in pUC8 (Ampicillin Resistant)
pYK211	*E. Coli* (XL-1 Blue)	Plasmid containing pyocin AP41 gene in pUC8 (Ampicillin Resistant)

Table 3: Source strains for pyocins and vectors provided.

Before extraction, *E. coli* cells possessing desired plasmids are cultured for 16 hours in 4ml of Luria Broth (LB) with 50ug/ml Ampicillin at a speed of 250rpm. After incubation, cells are centrifuged and pellet is lysed and lysate is loaded into the columns provided with the plasmid isolation kit. After plasmids are attached, column is washed with molecular biology grade water and desired plasmids are retained in 30ul water and stored at -20 Celsius degree freezer. (See Appendix A for full protocol)

Concentrations of plasmids are measured using Nanodrop Spectrophotometer at OD600. Nanodrop spectrophotometer is an advantageous device that can measure concentrations using very small amounts 1.5ug or less.

Extracted plasmids are confirmed by PCR reactions with Taq Polymerase. Primers are designed specifically for S1, S2, S3, and AP41 sequences in plasmids (Table 4). Primers are designed based on S-pyocin DNA sequences. The NCBI Genbank accession numbers of S-pyocin DNA sequences are D12707 for pyocin S1, D12708 for pyocin S2, X77996 for pyocin S3, D12705 for pyocin AP41. PCR reactions are run in 1% agarose gel and ethidium bromide labeled PCR fragments are monitored under UV light.

Genes	Primer Name	Primer Sequence
S1	S1 Forward	5' - ATGGCACGACCCATTGCT - 3'
	S1 Reverse	5' - TCACTT CCCTCCCTTGTG - 3'
S2	S2 Forward	5' - ATGGCTGTCAATGATTACGAA - 3'
	S2 Reverse	5' - TCACTTCCCTCCCTTGTG - 3'
S3	S3 Forward	5' - ATGGCTGATGCACCAC - 3
	S3 Reverse	5' - TCAGTACCACCCCTGTTC - 3'
AP41	AP41 Forward	5' - ATGAGCGACGTTTTTGAC - 3'
	AP41 Reverse	5' - TTATTTCTCCTTACGTTTAAGT - 3'

Table 4: Primers designed to verify and amplify pyocins S1, S2, S3, and AP41.

Another set of primers is designed for sequence needed to use for cloning using Novagen Xa/LIC Cloning Kit. LIC stands for "Ligase Independent Cloning" and as the name suggests using this kit, target gene can be inserted into the vector provided without restriction enzymes or ligation. New primers had the very same sequence as showed in Table 4 plus the sequence 5' - GGTATTGAGGGTCGC - 3' is added to 5' side of forward primer and sequence 5' – AGAGGAGAGTTAGAGCC- 3' is added to 5' side of a reverse primer to create overhangs that matches with Xa/LIC vector.

Plasmids acquired from *E. coli* are used as a template to generate desired overhangs using PCR. In this reaction Novagen KOD Hi-Fi DNA Polymerase is used instead of

Taq Polymerase because of its higher efficiency and lower mutation rate. Protocol provided by Novagen is used with temperatures provided by primer producing company (IDT). Primer annealing temperatures are later optimized for better results (Table 5).

Genes	TM	Denaturation	Extension
S1	57C / 5s	98C / 15s	72C / 40s
S2	57C / 5s	98C / 15s	72C / 40s
S3	57C / 5s	98C / 15s	72C / 40s
AP41	55C / 5s	98C / 15s	72C / 40s

Table 5: Melting, denaturation and extension temperatures and times for PCR of pyocins S1, S2, S3, and AP41

This method uses 3' to 5' endonuclease activity of T4 DNA Polymerase to create specific 12- or 15- base single stranded overhangs. Previously mentioned primers are designed to create overhangs that can match with overhangs of target vector. Vector and target DNA is complementary to each other and annealing is easily achieved (Figure 6).
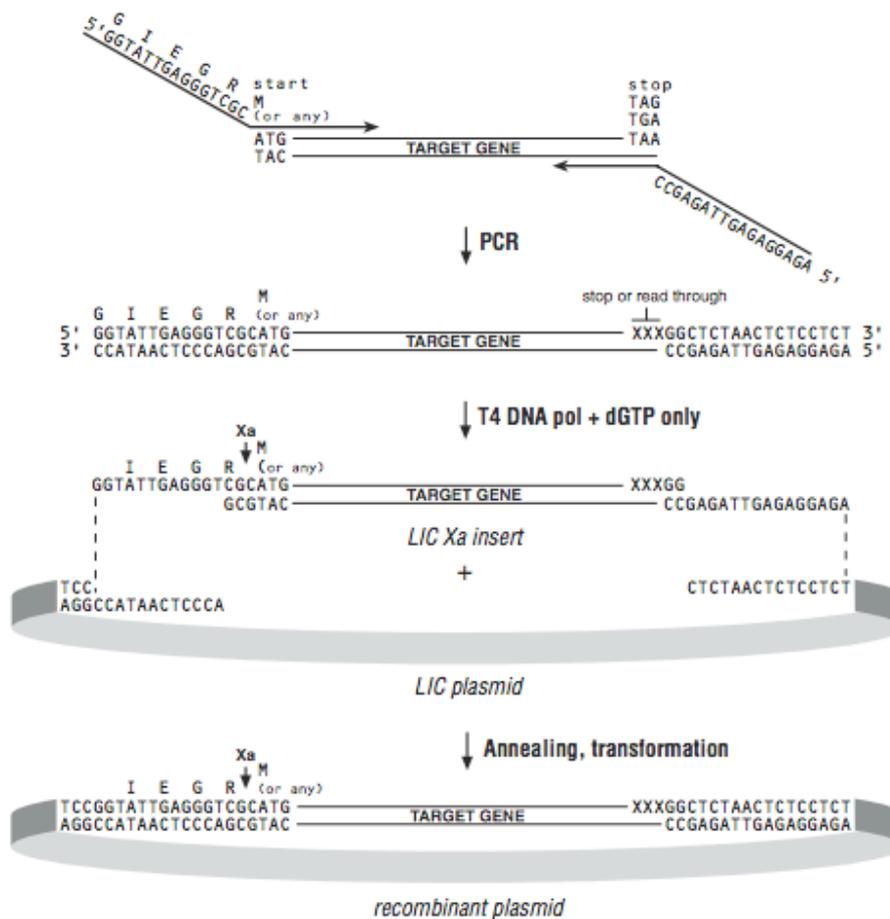
Figure 6: Xa/LIC strategy diagram. Target gene is treated with T4 DNA Polymerase to create overhangs that matches with vector overhangs. Anneal is achieved in transformant cell after transformation.

To prepare target DNA, double-stranded PCR product is treated with T4 DNA Polymerase and overhangs are created. Later enzyme is inactivated at 75 ^{0}C and later incubated with Xa/LIC vector for anneal. (See Appendix A for detailed protocol).

After anneal reaction is complete, transformation to NovaBlue GigaSingles Competent Cells is performed using the standard protocol of Xa/LIC Cloning Kit (See appendix). Transformation was unsuccessful despite several trials possibly because of handling during shipping. Therefore, using NovaBlue GigaSingles Competent Cells that do not retain competency, new competent cells are prepared using Calcium Chloride and stored in Calcium Chloride and Glycerol solution at -80^{0}C freezer.

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Newly prepared were used successfully afterwards and transformation is performed. Transformation results are controlled by culturing transformation reaction on LB agar plates with 30ug/ml Kanamycin in order to select successful transformants since Xa/LIC vector has Kanamycin resistance marker and therefore successful transformants possessed resistance against Kanamycin. NovaBlue cells are also checked on LB-agar plus Kanamycin plates as a negative control. Test plasmid provided by kit in order to test the efficiency of transformation efficiency is also used as a positive control.

Successful transformants are then grown overnight in 4ml LB broth with 30ug/ml Kanamycin and plasmid extraction is performed, again using QIAprep Spin Miniprep Kit. Using Nanodrop concentration is performed and plasmids are checked if desired gene transformed is present via PCR. Taq Polymerase kit and primers designed for control is used. PCR-positive plasmids are stored at -20^{0}C.

Plasmids are later transformed into *E. coli* BL21 DE3 expression cells. These cells are able to express T7 RNA Polymerase that can induce expression of desired gene on Xa/LIC vector that has T7 promoter upstream of target gene (Figure 7) . Plus, *E. coli* BL21(DE3) pLysS cells are also used for transformation. However, both expression cells were unable to be transformed like NovaBlue cells. Therefore, using these cells, new competent cells are prepared using previously mentioned competent cell protocol. Transformation reaction is tested on LB agar plates with 30ug/ml Kanamycin and successful transformants are grown overnight in LB broth with 30ug/ml Kanamycin and stored at -80^{0}C freezer in 15% glycerol solution for further use.
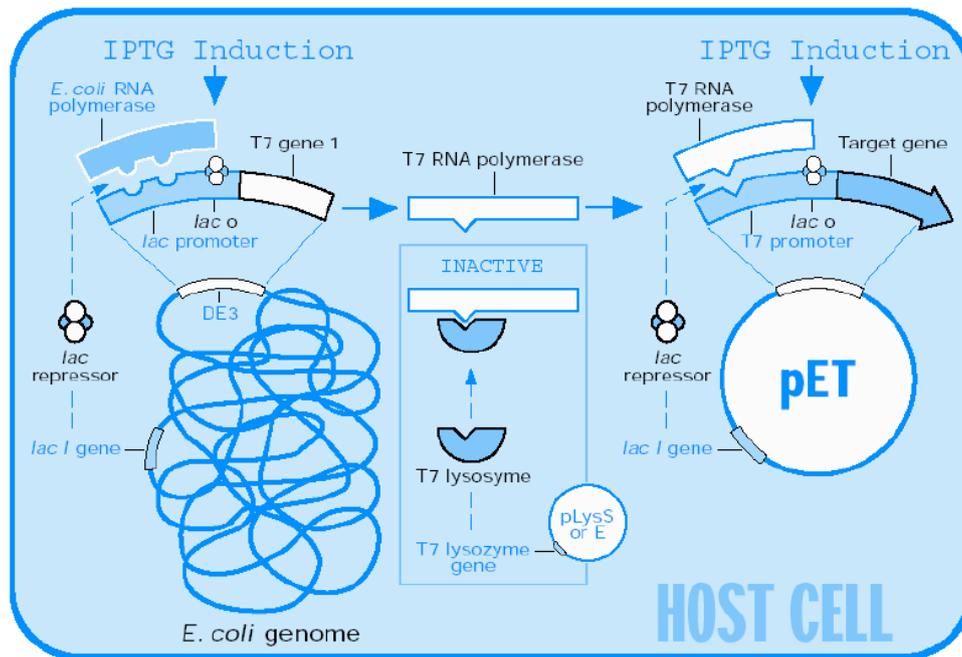
Figure 7: Control of expression of pET vector in BL21(DE3) cells.

Expression

Successful transformants selected are tested against sensitive strains Pa NIH3, Pa PML1516d, and Pa 7NSK2. Pa NIH3 and Pa PML1516d are known to be sensitive against pyocins S1, S2, and AP41 while Pa 7NSK2 is known to show sensitivity only to pyocin S3.

The test is called "Killing Assay". In Killing Assay, sensitive strains are equally distributed to a LB-agar plate including IPTG (1mM) and pyocin-producing strains are spotted on sensitive strain usually ranging from 2ul to 10ul. Spotted plates are left overnight in 37^0C incubator and killing zones, the area near the spotted producer bacteria where there is no bacterial growth, are observed. There are also negative controls (sensitive and non-producing strains) included.

Bacteria that are able to kill sensitive bacteria by creating a killing zone are grown overnight in 10 ml LB with an antibiotic to preserve plasmid until it reaches to OD600 ~3.00. Afterwards, they are transferred to a 1 L LB with the same amount of antibiotic and let grow in 37^0C shaker until it reaches OD600 ~0.5-0.6. When desired

OD600 is reached, expression of S-pyocins is induced with IPTG (1mM) After induction, bacteria are grown until it reaches OD600 1.2.

After reaching desired OD, bacteria are transferred to 0.5L Sorvall centrifuge bottles on the ice. Using Sorvall centrifuge and SLA-3000 rotor, bacteria are centrifuged for 30 minutes at 7,000rpm. Pellet, including bacteria and pyocins, is transferred to plastic beakers and weighed. After centrifugation pellets are always worked on ice. Pellets are stored in -80^{0}C until the next step.

The next step after first centrifugation is lysis where pellet is lysed and all bacterial proteins are spread in solution. Lysis is done using both chemicals and physical disruption. First, pellet is resuspended in 20ml Buffer A, which is used in purification step. Buffer A is composed of 50mM Tris, 200mM NaCl, and pH of Buffer A is set to 8.00. After resuspension, 1 Complete Tablet without EDTA (protease inhibitor), 0.5mM PMSF (protease inhibitor), and 1mg/ml Lysozyme (Fluka Chicken Egg White) is added to solution and in cold room, where temperature is 4C, solution is stirred with a magnetic stirrer for 1 hour.

Solution is sonicated with a pulse 6 seconds on, 9 seconds of with amplitude of 36%. Sonication was performed for 30 minutes and held in cold room (+4^{0}C degree).

As an alternative way for lysis, BugBuster Protein Extraction Reagent of Novagen is used. The difference between two methods were, in BugBuster method, pellet is resuspended in BugBuster solution at room temperature and Benzoase nuclease and r-Lysozyme is added and solution is stirred at room temperature. No physical disruption is required. However, this method is abandoned because of low activity of lysate extracted using this method compared to previously explained method.

Solution is then transferred to 50ml pre-cold centrifuge bottles and centrifuged at 15,000rpm for 1 hour. Since S-type pyocins are known to be soluble, supernatant is fully saved and immediately taken to purification step. Pellet is resuspended in sterile water and 1ml pellet is saved for Killing Assay, and SDS-PAGE analyses.

Purification

Purification is performed using AKTA FPLC (Fast Protein Liquid Chromatography) machine. AKTA FPLC machine is used for liquid chromatography to separate desired proteins from a pool of proteins using a proper column (Figure 8). Since S-pyocins were tagged with 6xHistidine-tag, 1ml and 5ml nickel-affinity columns from GE Healthcare were used. Since nickel has an affinity for 6xHistidine-tag, it is expected that proteins possessing desired tag would bind to column while others are passing through.
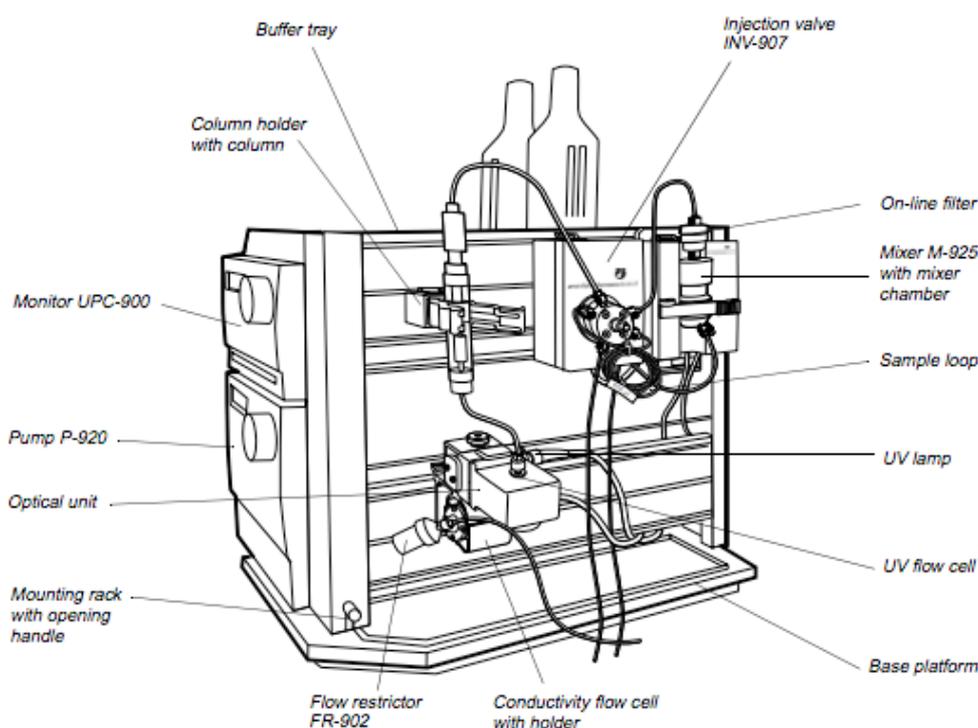
Figure 8: Diagram of AKTA-FPLC Machine retrieved from manual

There are two buffers used for purification; Buffer A and Buffer B. Buffer A is very same buffer used for resuspension of bacterial pellet. Buffer B is Buffer A and imidazole (500mM) added. Buffer A is used to pass all protein solution through column to make desired protein to bind to column and Buffer B is later used to elute desired protein from affinity column.

First, supernatant from lysis reaction is loaded into 50ml loop. FPLC Machine (the path of solution including column) is washed with sterile water, and buffer A. After washing was complete, solution in loop is injected into the column. During passage from column, UV and conductivity is monitored and recorded. Flow-through is collected for further testing to be sure if desired protein is successfully bound to the column. After all solution is passed through the column and UV and conductivity values were stabilized, Buffer B (including imidazole) is added with an increasing concentration starting from 0-100% until UV values gave a peak. The peak usually means that there is another set of proteins passing the UV reader, therefore one of the peaks given theoretically include the desired protein. Fractions corresponding to peaks and other control points were collected and stored at $+4^0$C refrigerator for SDS-PAGE analysis.

Fractions collected are run in SDS-PAGE gel along with protein markers and pre-purification samples (pellet after lysis, and flow-through). SDS-PAGE gel consisted of two parts: 12% Separating Gel, and 5% Stacking Gel (See Appendix for Ingredients). Before loading into SDS-PAGE gel, samples are boiled at 95C for 10 minutes with a loading dye (beta-mercaptoethanol) in 1:1 concentration.

SDS-PAGE gel is run for 45 minutes at 26mA and gel is later boiled twice and stained with Coomassie Brilliant Blue. Staining is performed 5 minutes and later gel is boiled with water in microwave several times before letting gel overnight for destaining. After overnight destaining, samples are compared with protein marker and results are photographed. Fractions with desired molecular weight are stored at $+4^0$C refrigerator.

Biofilm Formation

For biofilm formation, Calgary Biofilm Device (CBD) is used. CBD is a special assay for determining antibiotics susceptibilities of biofilms. Biofilms are formed on 96-well plate lid containing 96 pegs after exposure to planktonic cell culture for a certain period of time.
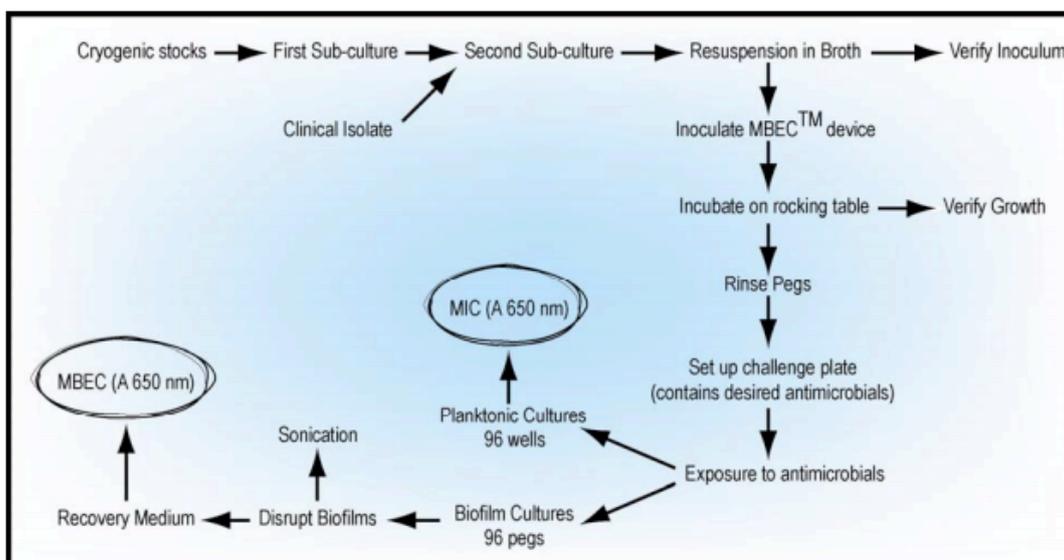
Figure 9:Diagram of Calgary Biofilm Device framework.

In order to start a new experiment, a LB-agar plate was streaked with biofilm-producing bacteria and plate was incubated overnight at 37^0C incubator. A single colony is spread to a new LB-agar plate and incubation is again done in same conditions overnight. In the third day, bacteria is scraped with sterile toothpick and resuspended in LB. Resuspension is diluted to 1 MacFarland standard as stated in protocol provided by CBD kit (See Appendix for protocol). 1 MacFarland standard is OD600 0.257 in 600nm wavelength. 1ml of MacFarland standard of resuspension is diluted to 1:30 and 150ul is added to each well of CBD and lid having 96 pegs is closed. CBD is then incubated in a VWR Signature Incubating Rocking Platform Shaker at a 7-degree tilt angle, 10rpm speed, and 37^0C degree for 6 hours. After biofilms were formed, new 96-well plate with a desired antibiotic gradient is prepared and biofilms produced is exposed to antimicrobial agents in, what is called, challenge plate after pegs are washed with sterile physiological saline solution (0.9% NaCl, autoclaved) (Figure 9).

Interaction Testing

MBEC values of biofilms are determined using CBD. Biofilms on the pegs of 96-well plate are exposed to challenge plate with a gradient of antibiotics and later remaining biofilms are recovered on a fresh 96-well plate with LB without antibiotics.

Planktonic cell MIC values are also determined by creating a gradient of antibiotics. Later, gradient showing MIC values are duplicated and other antimicrobial agent (pyocins, or antibiotics) are added. Then two results are compared to see if there is a shift in MIC values. According to the result, it can be concluded that interaction is synergistic, antagonistic, or additive.

RESULTS

Cloning

Vectors isolated from pyocin sources are run on 1% agarose gel and extraction is confirmed (Figure 10)
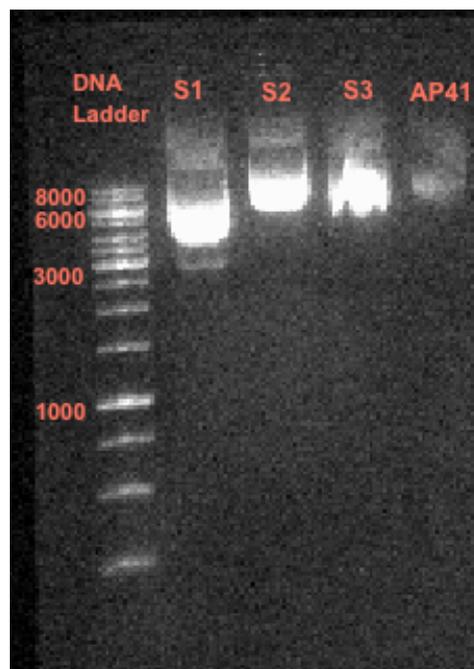

Figure 10: Template vector confirmations from source *E. Coli* possessing S1, S2, S3, and AP41 genes in pUC plasmids. Vector DNA's are extracted with Miniprep Kit of QIAGEN.

Later, template DNA is prepared using designed primers for creating overhangs.
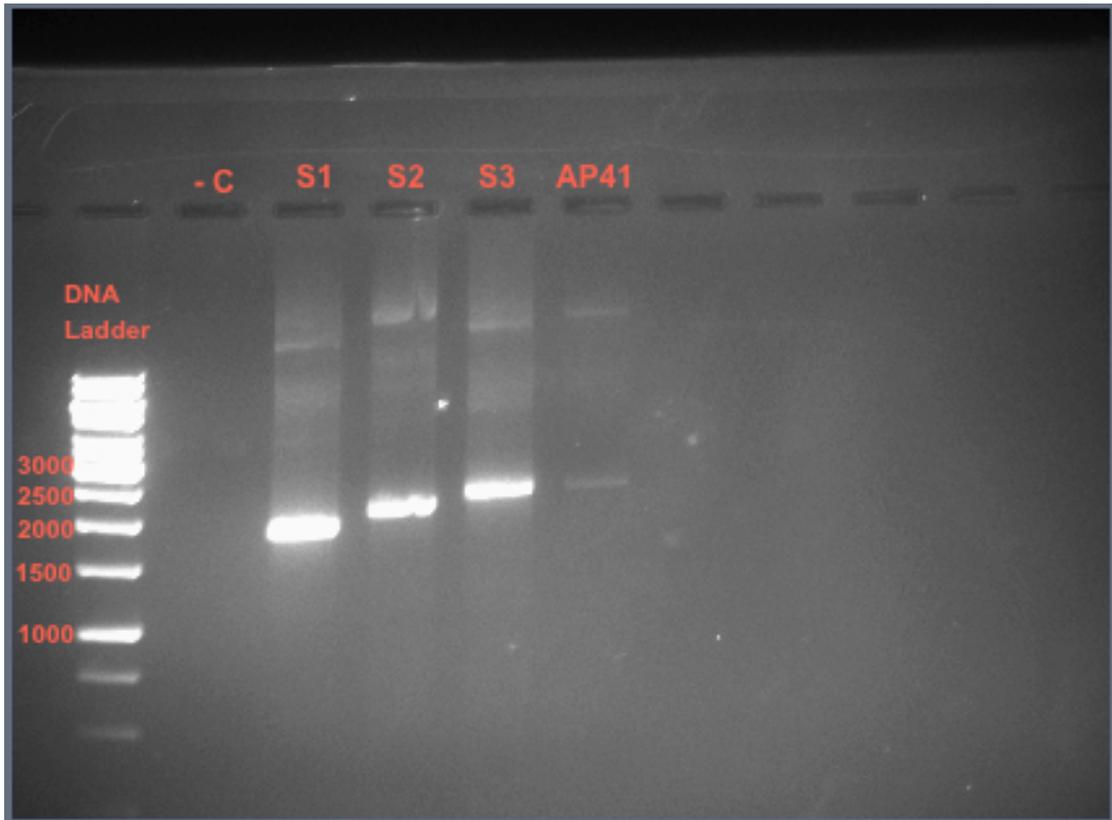
Figure 11: Template DNA amplification from vector DNA's isolated from source. Expected lengths for PCR products were 1856bp, 2056bp, 2300bp, and 2333bp for S1, S2, S3, and AP41, respectively. Negative control didn't include template DNA. C: Negative Control.

PCR reaction for pyocin AP41 is repeated with a melting temperature of 57^0C since acquired DNA after first PCR was low for pyocin AP41 compared to others. (For S1, S2, and S3 suggested melting temperature was 55^0C). Results are shown in Figure 12.
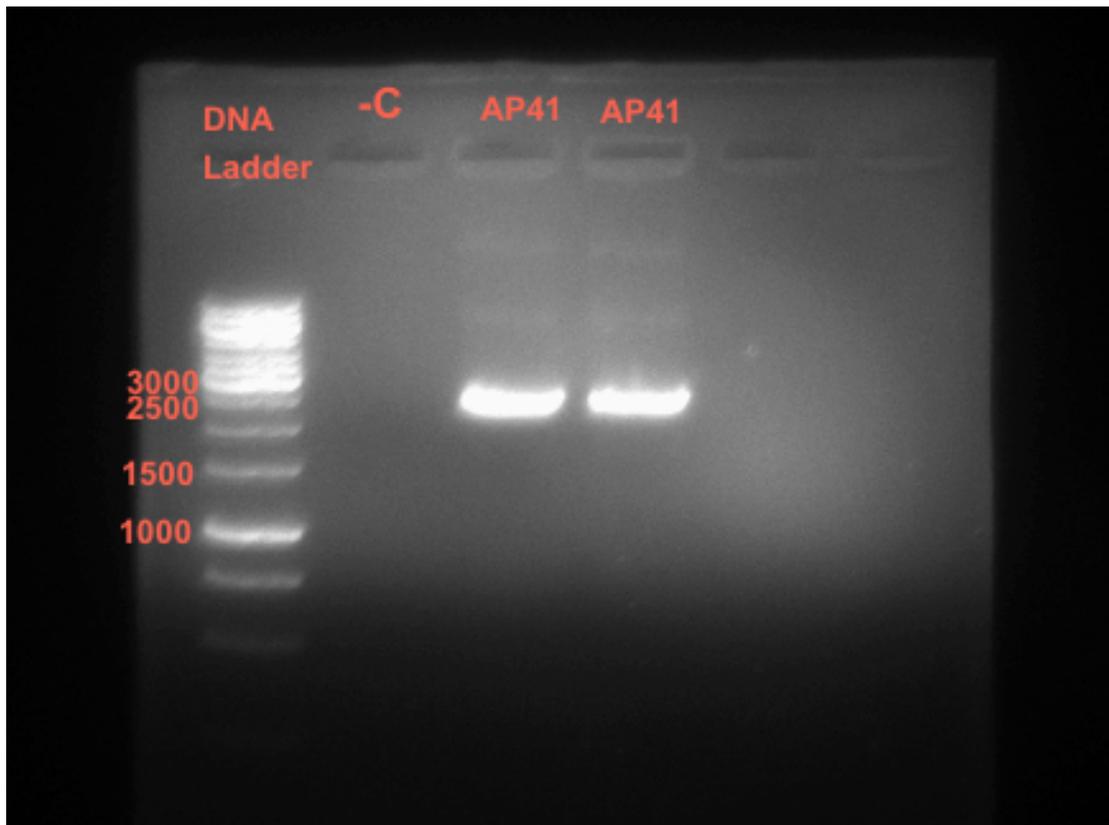
Figure 12: Template DNA amplification for AP41 for melting temperature of 57C. Expected size is 2333bp for AP41. -C: Negative Control.

Later, confirmed target DNA is annealed with Xa/LIC vector and transformed into Nova GigaSingles cells after creating overhangs with T4 DNA Polymerase enzyme. Transformed colonies are picked and grown overnight to extract their plasmid and verify successful anneal.
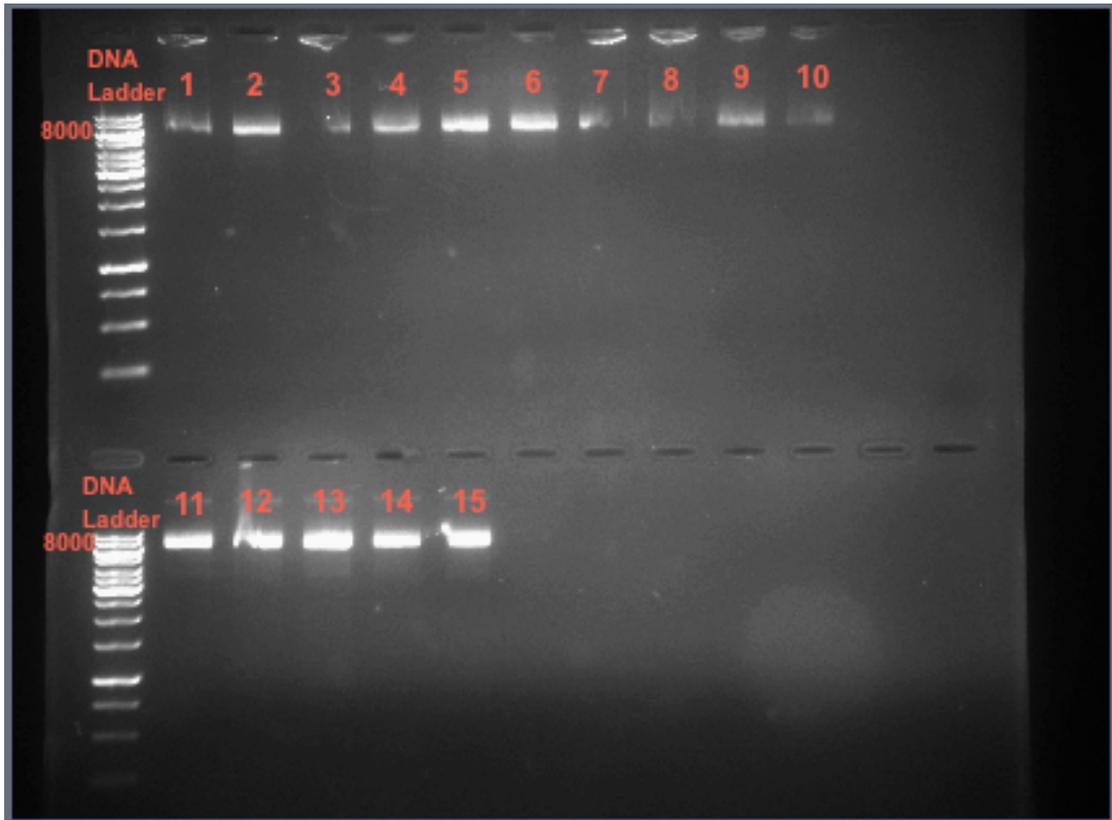
Figure 13: AP41 + Xa/LIC plasmid confirmation for 15 samples. Xa/LIC vector is ~5.6kb and expected size for final product is 8100bp.

Later, vectors isolated are verified with PCR using primers specific to related pyocin gene.
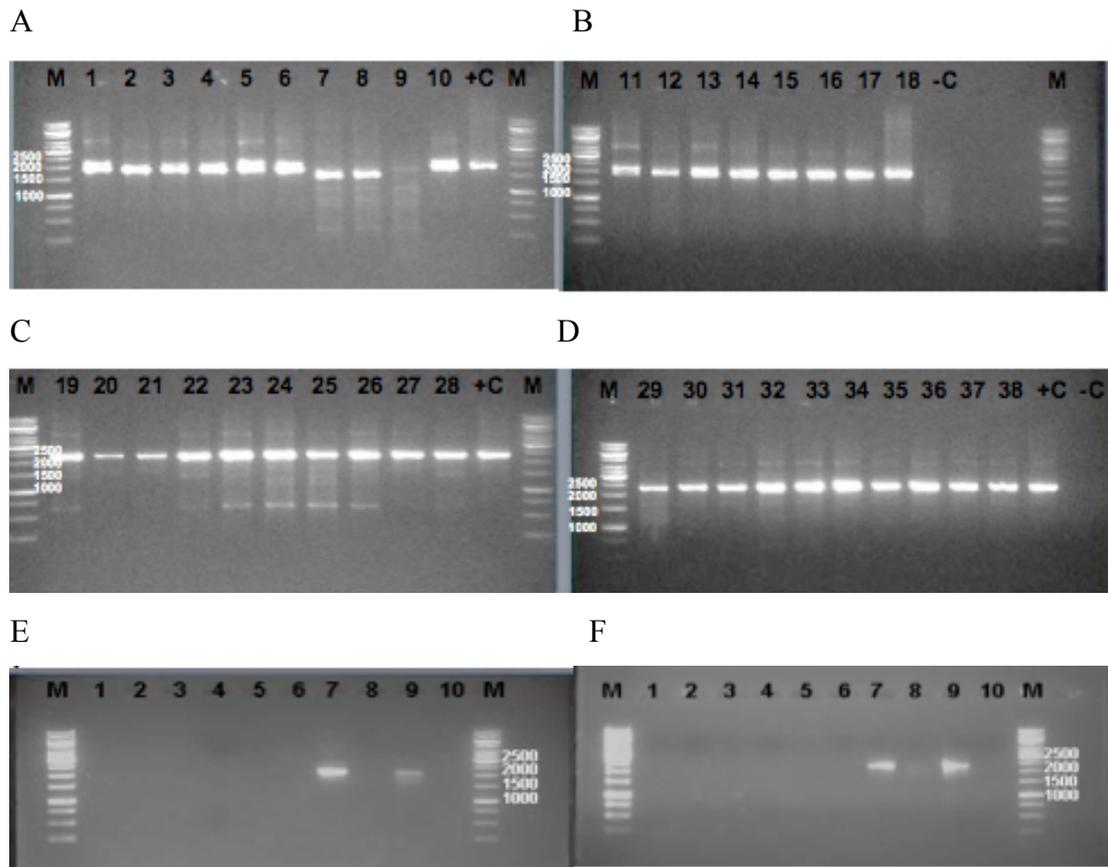
Figure 14: Vector confirmation with PCR for S1, S2, S3, and AP41. A. Lanes 1-10 PCR product of Xa/LIC+S1. B. Lanes 11-18 PCR product of Xa/LIC+S1 C. Lanes 19-28 PCR product of Xa/LIC+AP41 D. Lanes 29-38 PCR product of Xa/LIC+AP41 E. Lanes 1-10 PCR product of Xa/LIC+S2 F. Lanes 1-10 PCR product of Xa/LIC+S3. +C: Positive Control, -C: Negative Control.

Expression

Using 1mM IPTG, cells containing recombinant plasmids are induced and tested against sensitive cells via Killing Assay. Later, successful killers (cells producing effective killing proteins) are collected and stored -80^{0}C. However, since killing was weak and thus it was not possible to take photographs.

In the next steps, samples are induced in 1L cultures with an IPTG concentration of 1mM. Later cells are centrifuged and lysed with BugBuster reagent. Lysates (Cell Free Extracts) are tested against sensitive bacteria via Killing Assay. Cell Free Extracts (CFE) of competent cells are also spotted on sensitive lawn as a negative control.
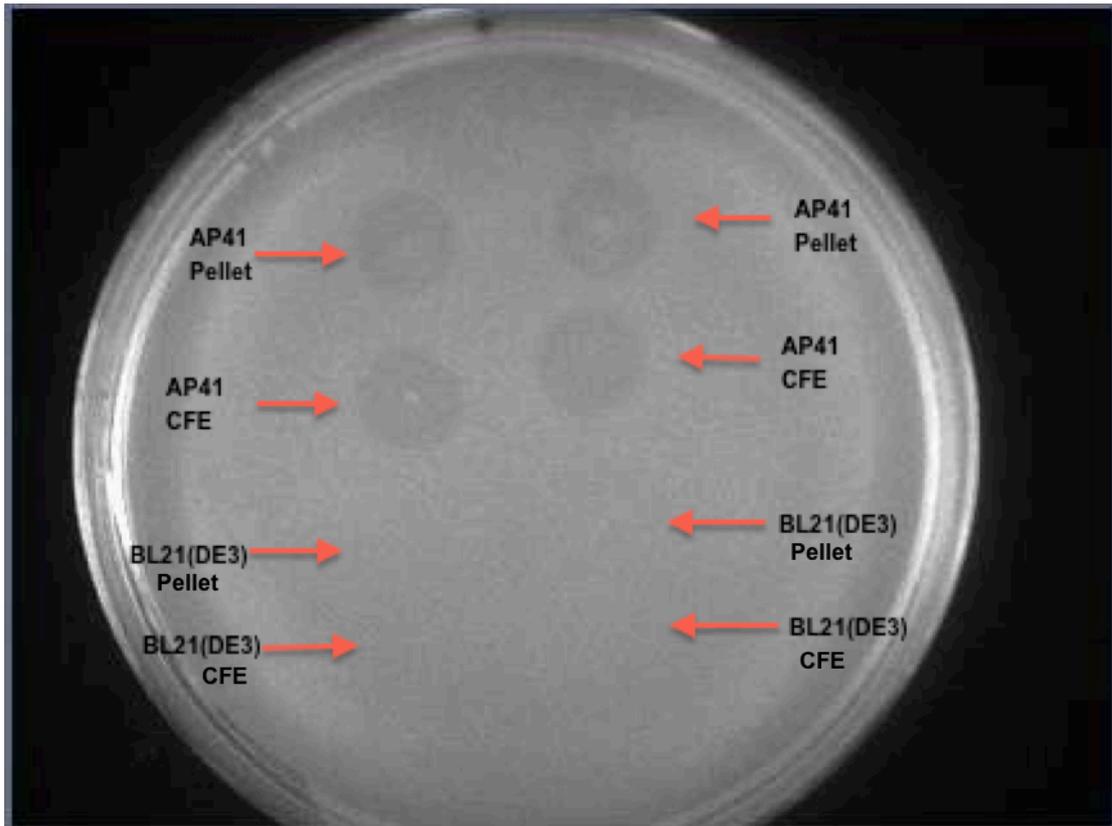
Figure 15: Cell Free Extract activity control. AP41 pellet and CFE are tested on the lawn of NIH3 sensitive cells. BL21(DE3) cells are tested as a negative control.

Pyocins S1, S2, S3 samples did not show a strong killing activity as seen in pyocin AP41. Therefore, cloning steps are repeated and optimized further. However, despite having positive results for vectors possessing the desired gene, expression products were able to show a little or not activity. For pyocins S1 and S3 there were a little activity compared to pyocins AP41 and for pyocin S2 killing activity were not visible on sensitive lawn.

In order to solve the problem, expression steps are optimized further. Three different temperatures (26^0C, 30^0C, 37^0C), and IPTG concentrations (0.4mM, 0.8mM, 1mM) are tested for pyocins S1, S3, and AP41 and results are scored. Novagen BugBuster solution is used for lysis in this experiment.

Temperature	26C			30C			37C		
IPTG (mM)	0.4	0.8	1	0.4	0.8	1	0.4	0.8	1
185-S1	++	++	++	++	++	++	++	++	++
195-S3	+	+++	+++	++	++	++	+	+	+
205-AP41	+	+	+	+++	+++	+++	+	+	+

Table 6: Temperatures, and IPTG concentrations for S1, S3, and AP41 samples. + is the smallest killing zone, +++ is the best killing zone observed.

Later, expressions carried out with the information acquired from Table 6 however, activity of expression products were very low. However, samples showing the best activity compared to the others tested were stored and used in purification steps.

Purification

Purification result for S1:
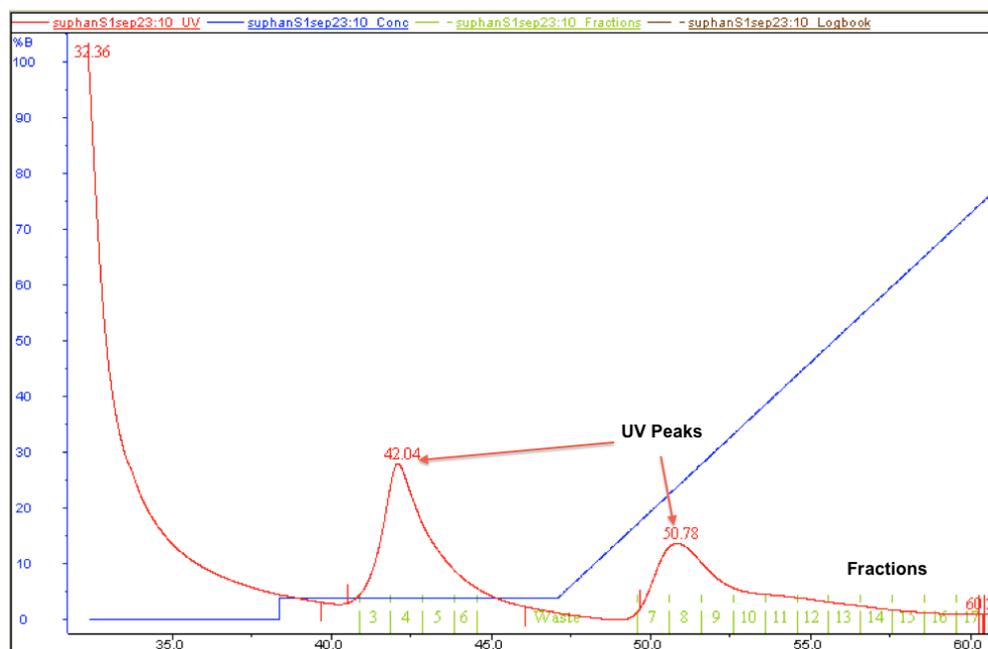
Figure 16: UV peaks of purification using AKTA-FPLC for S1 There are two peaks at ~10% buffer B concentration and ~20% buffer B concentration. Fractions underneath are collected and loaded into SDS gel to find out sizes.
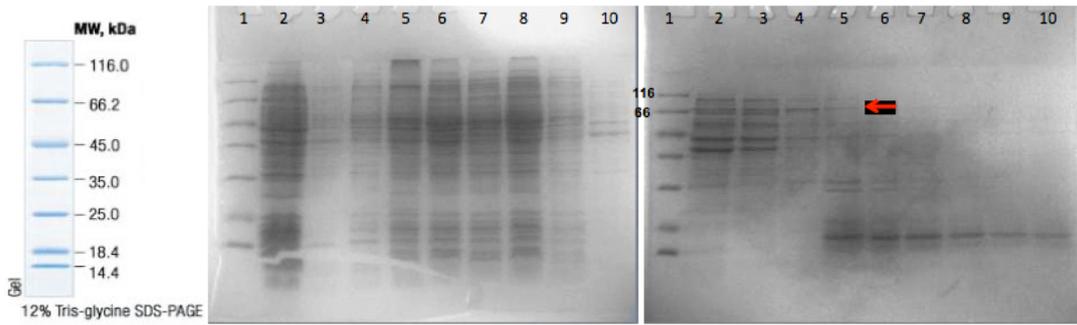
Figure 17: SDS gel result for S1. Length shown with red arrow is expected size of S1 protein ~66kDa.

Purification result for S3:
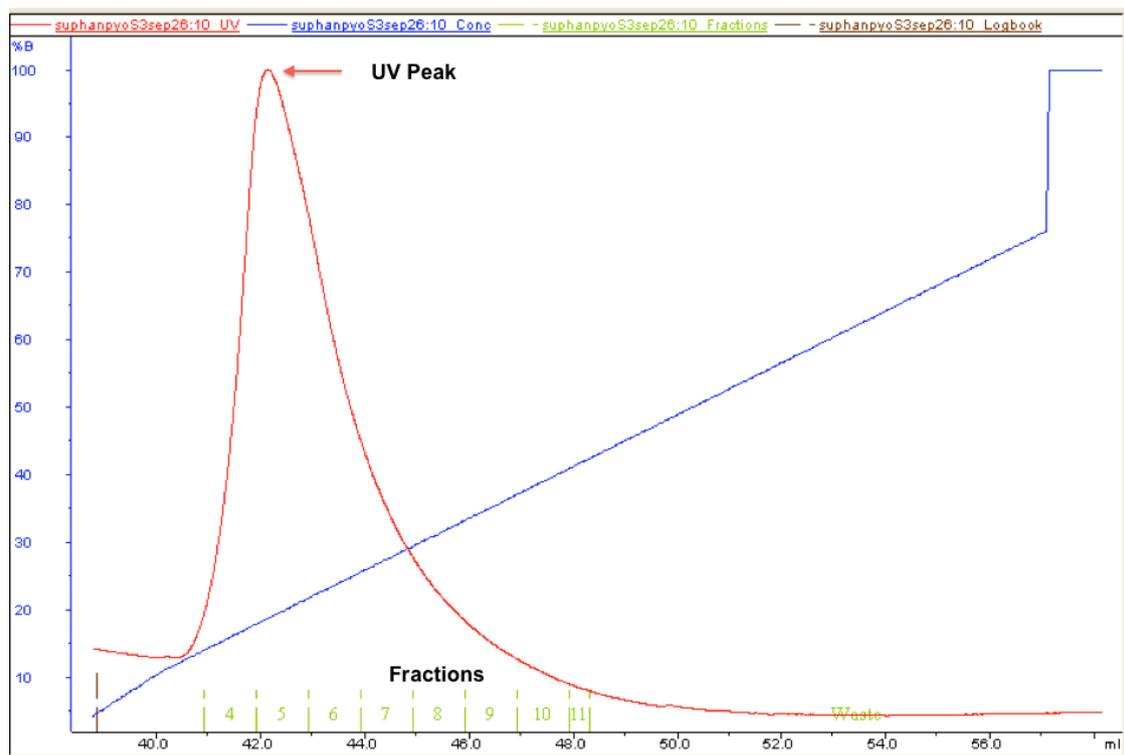
Figure 18: UV peak of purification using AKTA-FPLC for S3 There is one peak at ~20% buffer B concentration. Fractions underneath are collected and loaded into SDS gel to find out sizes.
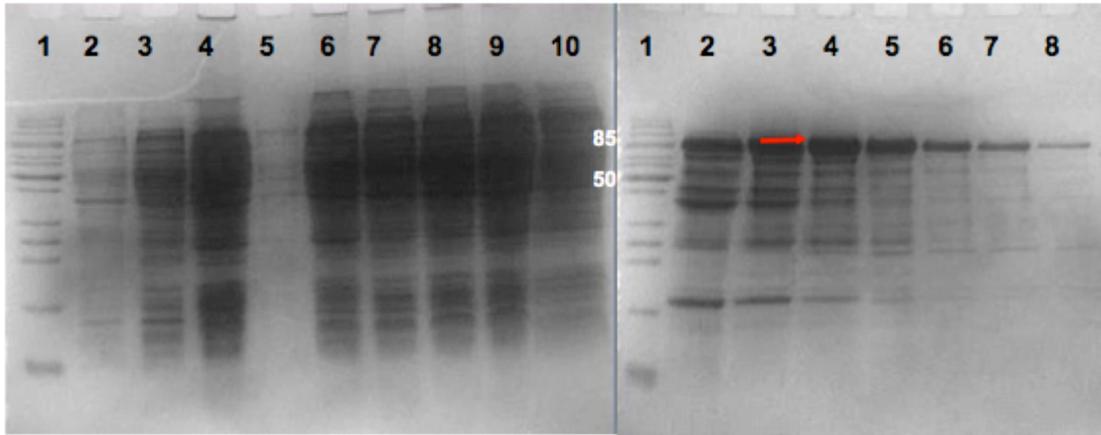
Figure 19: SDS gel result for S3. Length shown with red arrow is expected size of S1 protein ~85kDa.

Purification result for AP41:
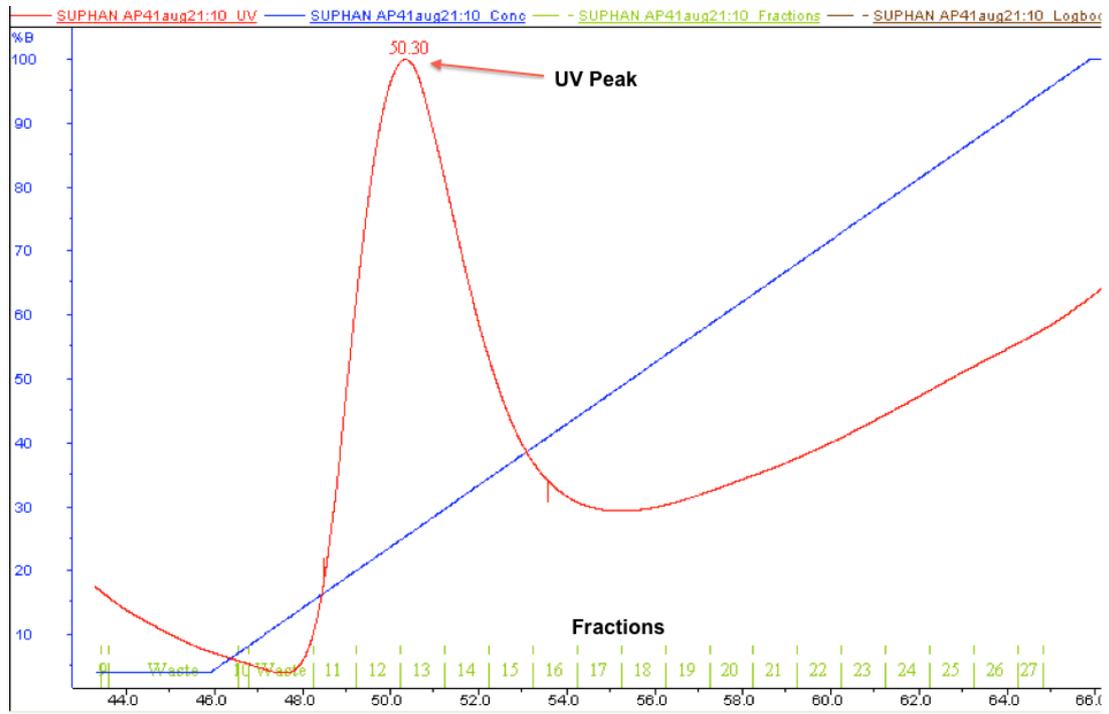
Figure 20: UV peak of purification using AKTA-FPLC for AP41 There is one peak at ~20% buffer B concentration. Fractions underneath are collected and loaded into SDS gel to find out sizes.
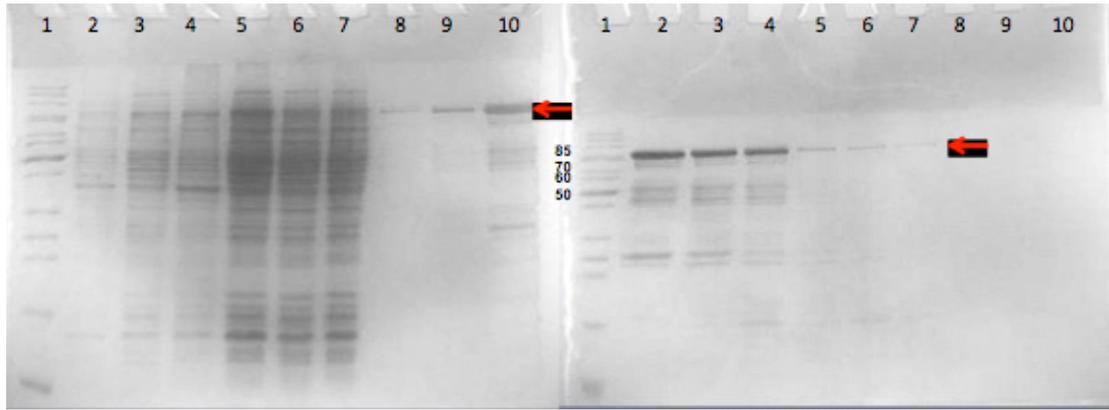
Figure 21: SDS gel result for AP41. Length shown with red arrow is expected size of AP41 protein ~85kDa.

Contrary to expectations that low activity of proteins can be caused by low concentrations of protein and purification may lead to a better killing activity on sensitive lawn, purified proteins didn't show strong killing activity on sensitive Pa lawns.

The reason is proposed to be the method of lysis and during lysis; proteins may be degraded or damaged. Therefore, lysis is repeated with lysozyme, PMSF, and complete tablets.
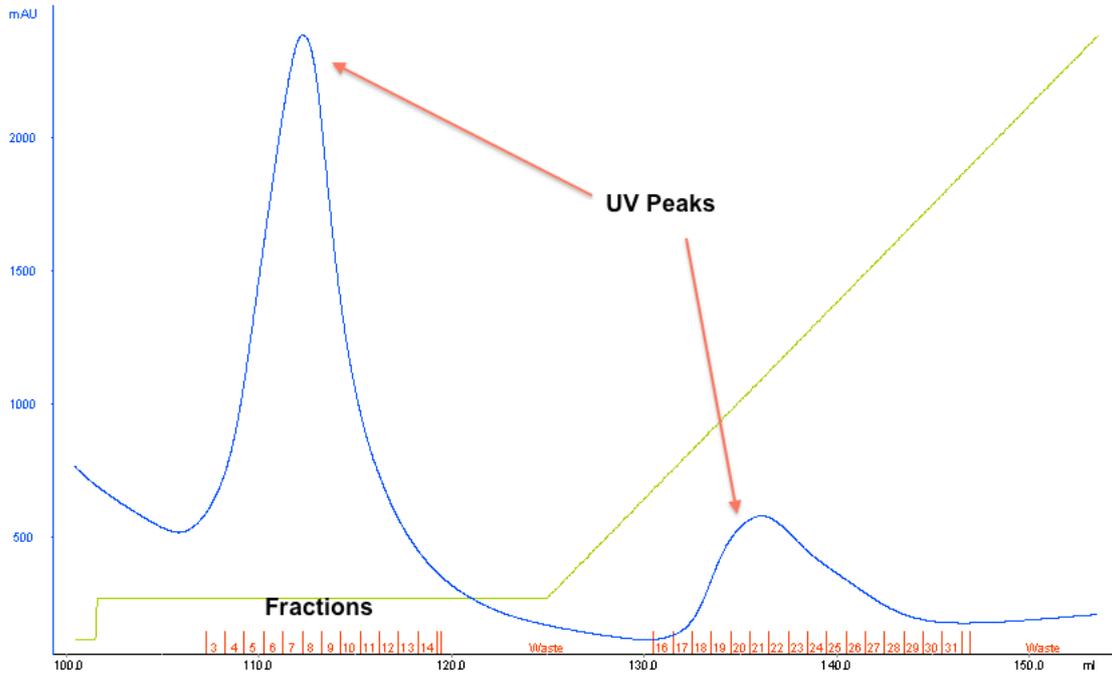
Figure 22: Repeated AP41 purification with a lysis method using lysozyme
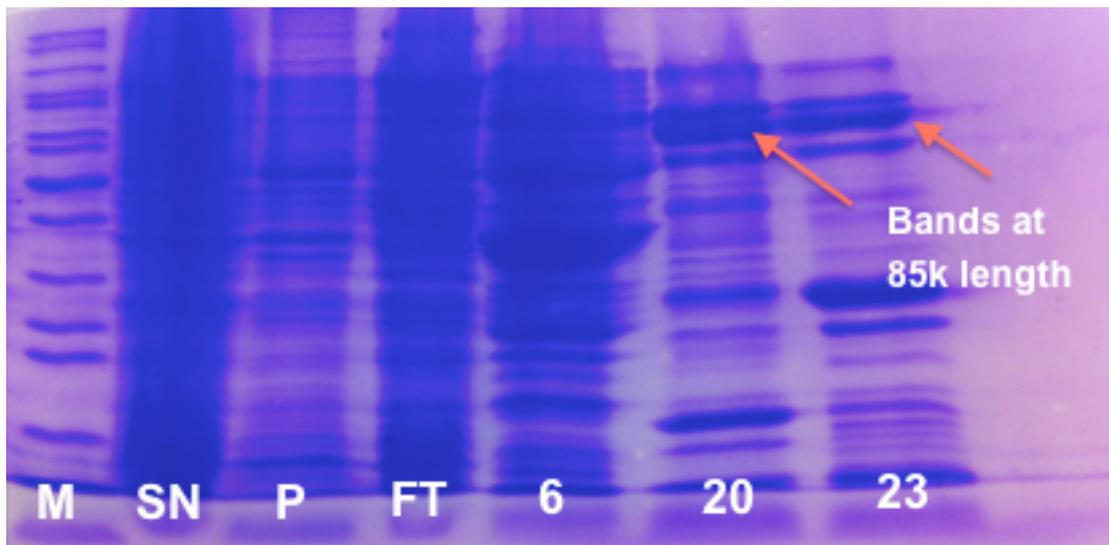
Figure 23: SDS gel results for AP41 purified with a lysis method using lysozyme. M: Marker, SN: Supernatant, P: Pellet, FT: Flowthrough, 6, 20, and 23: Fractions.

Expected size bands and low killing activity showed that there is a functioning protein even though it is not enough in concentration; therefore Anion Exchange Chromatography is performed on fractions number 17 to 30 in order to have more purified protein.
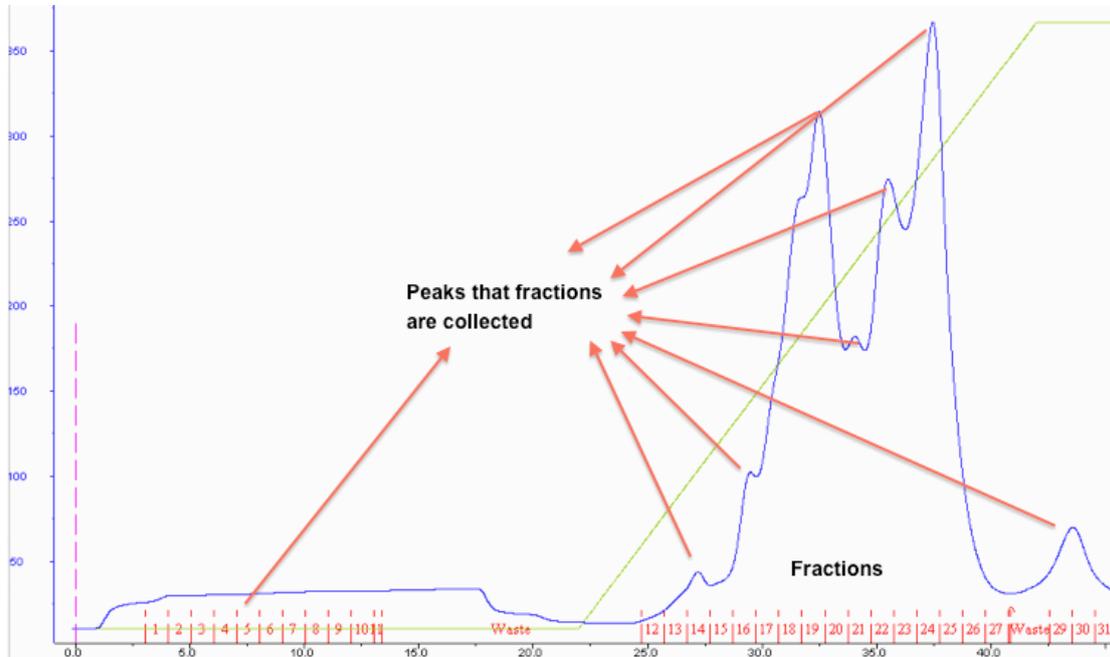
Figure 24: Anion exchange for AP41 for fractions collected from affinity chromatography. Several peaks are observed for theoretically different charged proteins.

Fractions collected didn't show any killing activity. In order to find out the reason there is no activity, Western Blot is applied for fractions under the peaks.

Figure 25: Western blot results for AP41 lysates

Results showed that 6xHis tag is available in lysates suggesting proteins somehow lost the activity during protein purification.

Since it was not possible to purify His-tagged protein for further studies, Cell Free Extract (CFE) is used. Before using CFE, producer bacteria are spot on sensitive lawn and grown overnight.
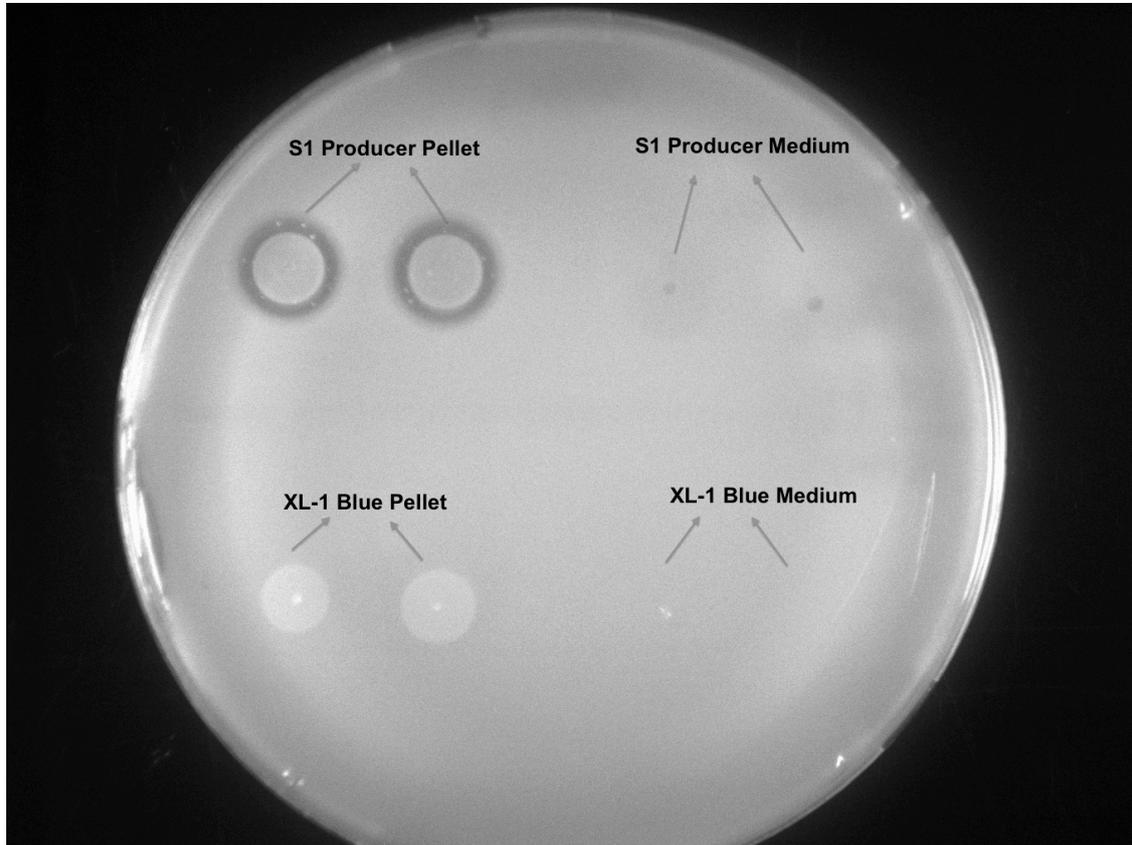
Figure 26: Killing assay for S1 producer. 3ml overnight cultures are centrifuged and resuspended in 100ul LB and spotted on sensitive lawn (NIH3). Medium of overnight cultures are spotted as well. XL-1 Blue pellet and medium are spotted as negative control.
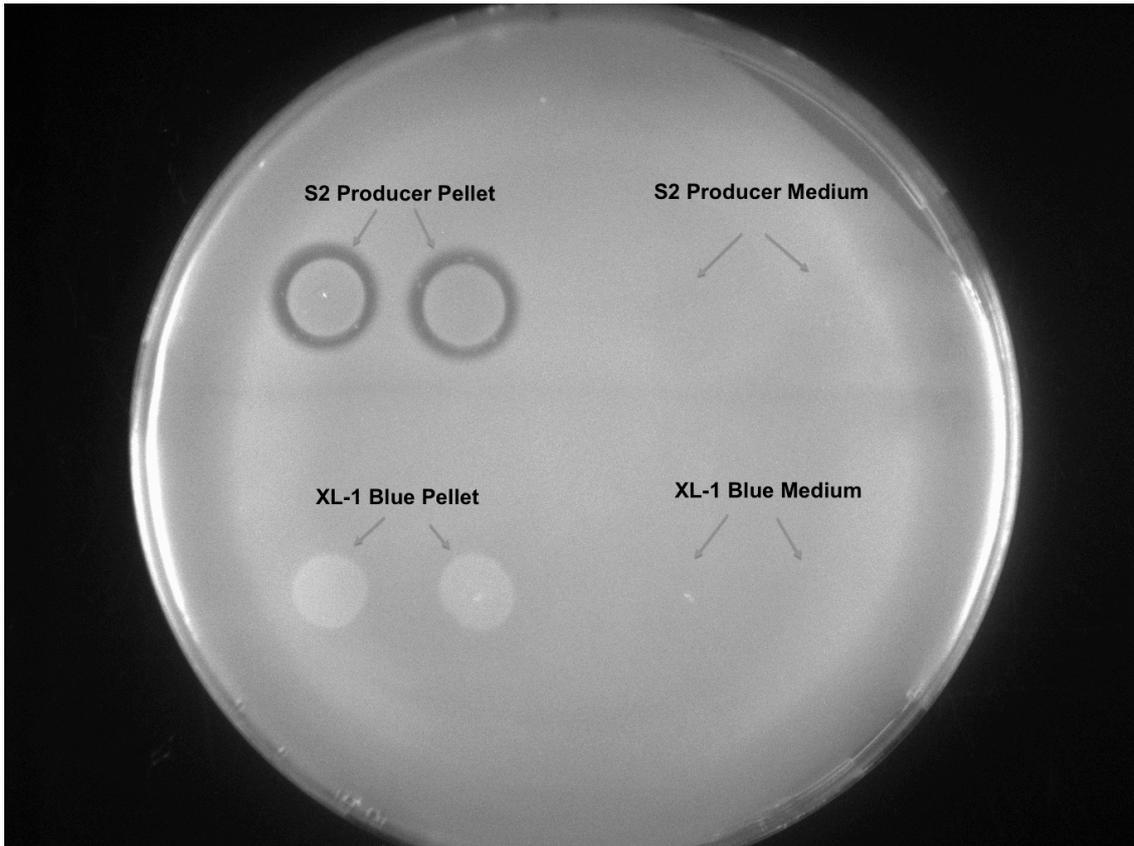
Figure 27: Killing assay for S2 producer. 3ml overnight cultures are centrifuged and resuspended in 100ul LB and spotted on sensitive lawn (NIH3). Medium of overnight cultures are spotted as well. XL-1 Blue pellet and medium are spotted as negative control.
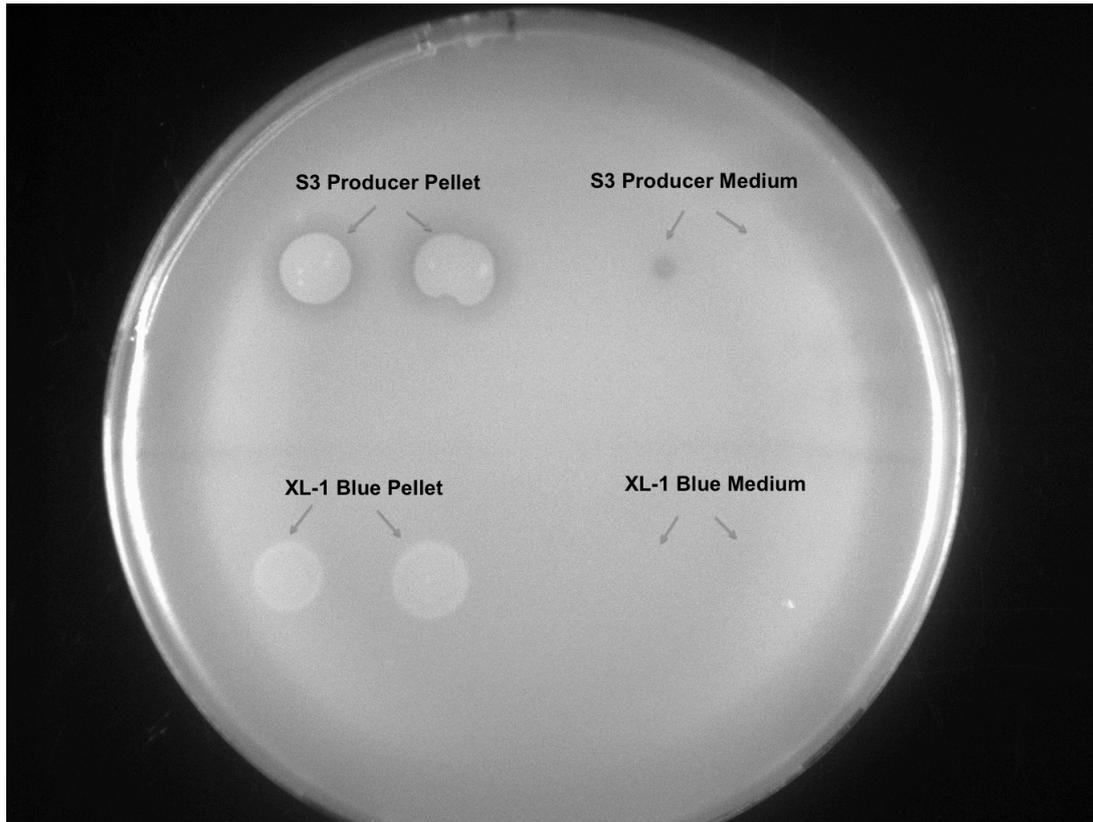
Figure 28: Killing assay for S1 producer. 3ml overnight cultures are centrifuged and resuspended in 100ul LB and spotted on sensitive lawn (7NSK2). Medium of overnight cultures are spotted as well. XL-1 Blue pellet and medium are spotted as negative control.

pYK211, producer of AP41, didn't show a killing activity on sensitive strain, therefore it is cloned in BL21(DE3) cells from original plasmid from pYK211. New clones showed killing activity.
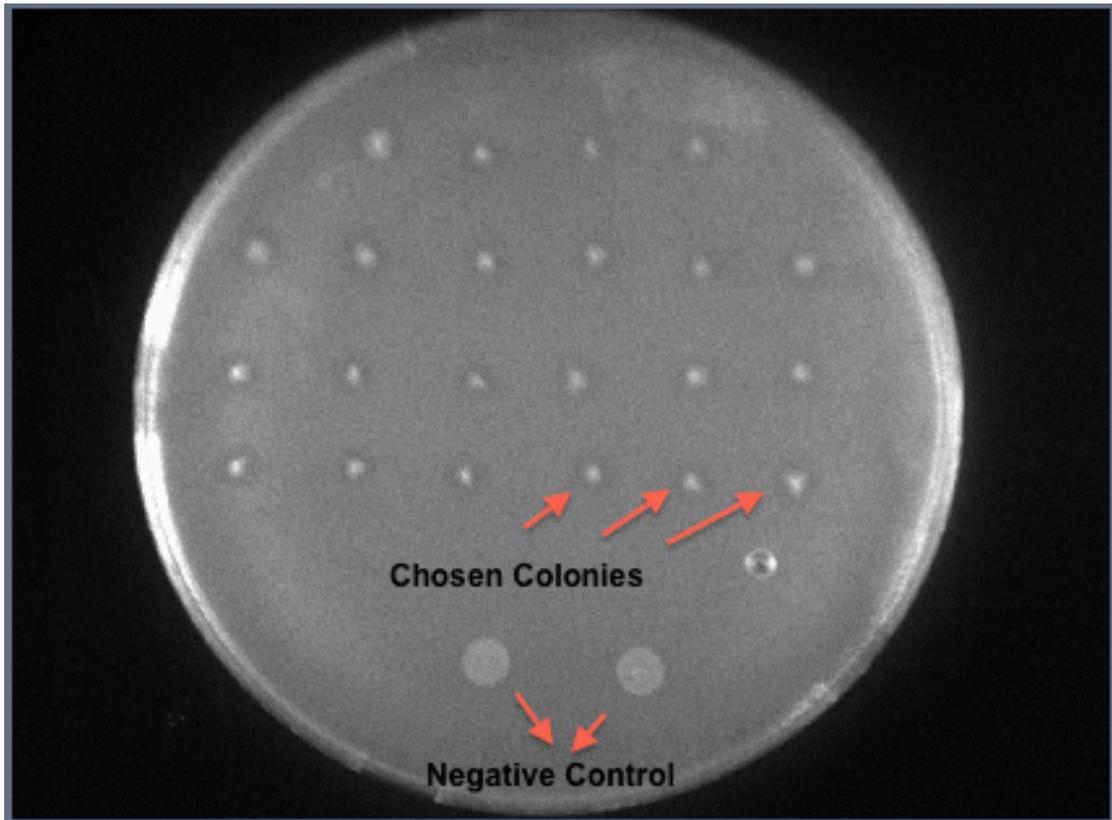
Figure 29: Transformants killing assay verification for AP41. Colonies are collected from transformation plate with sterile toothpicks and spotted on sensitive lawn of Pa NIH3. Best killers are cultured overnight and stored for later usage. NIH3 pellet is spotted as a negative control.

After confirmation, all producers are grown in 1L LB culture and pellet is lysed with lysozyme and centrifuged. Supernatant after lysis, Cell Free Extract, is used for further experiments.

A   B
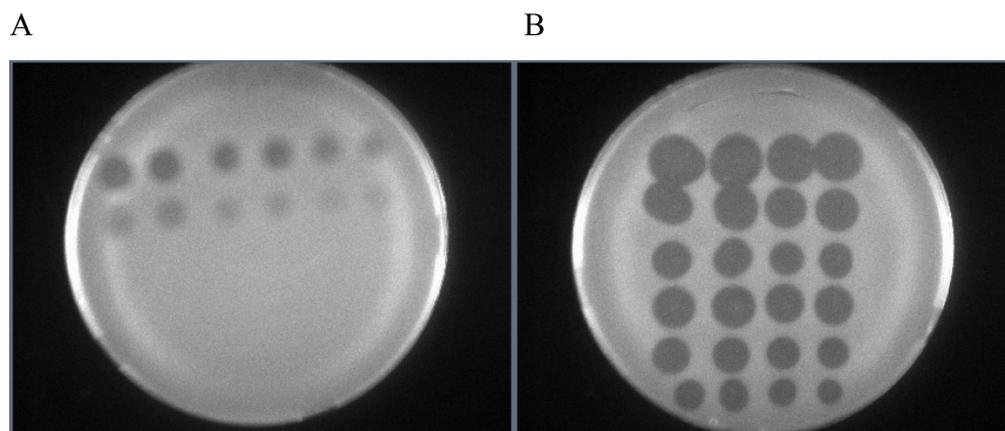
Figure 30: A. AP41 CFE spotted on Pa NIH3 lawn. B. S1 CFE spotted on NIH3 lawn.
Both plates are spotted with decreasing concentrations of CFE.
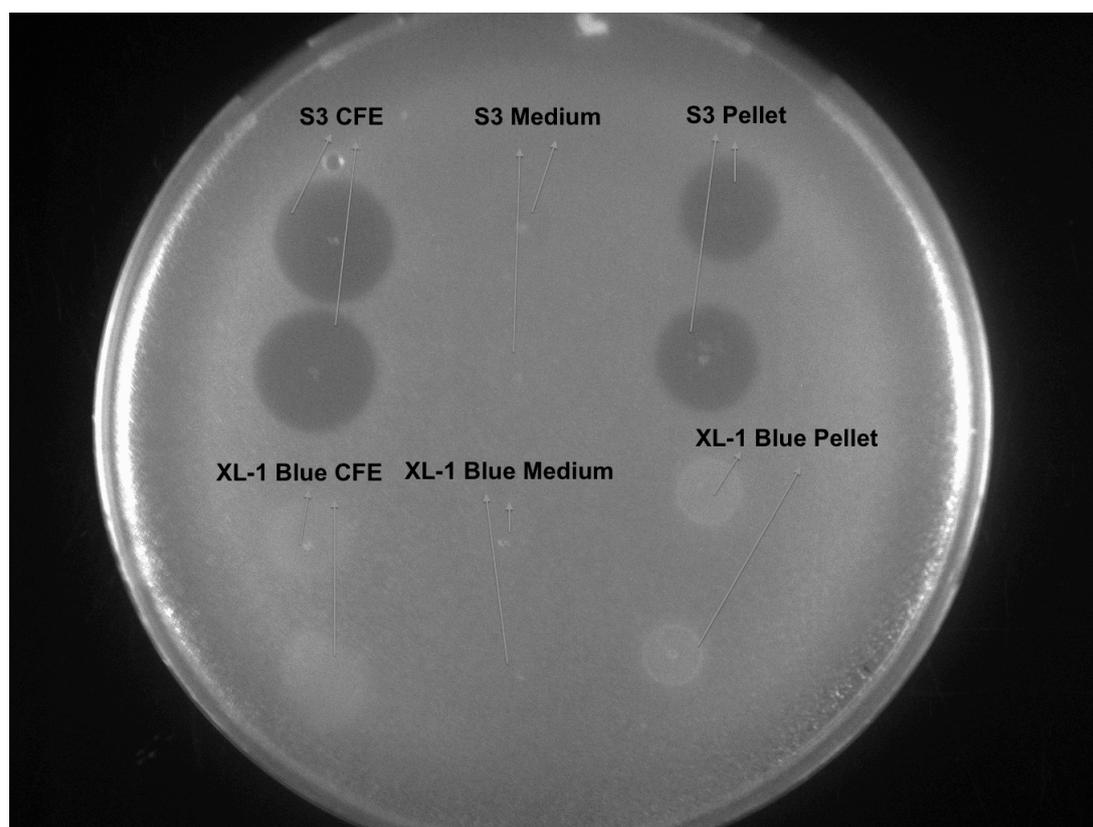
Figure 31: S3 CFE, medium, and pellet are tested on sensitive lawn of 7NSK2. XL-1
Blue cells are used as a negative control.

Interactions

Before testing CFE on biofilms it is aimed to determine Minimum Inhibitory Concentration (MIC) for planktonic cells and Minimum Biofilm Eradication Concentration (MBEC). Tobramycin, Gentamicin, Colistin, Piperacillin, Ceftazidime, and Ciprofloxacin are tested on biofilm cells as a gradient of concentrations. At the same time for further optimization of biofilm formation time, biofilms at different hours are tested.
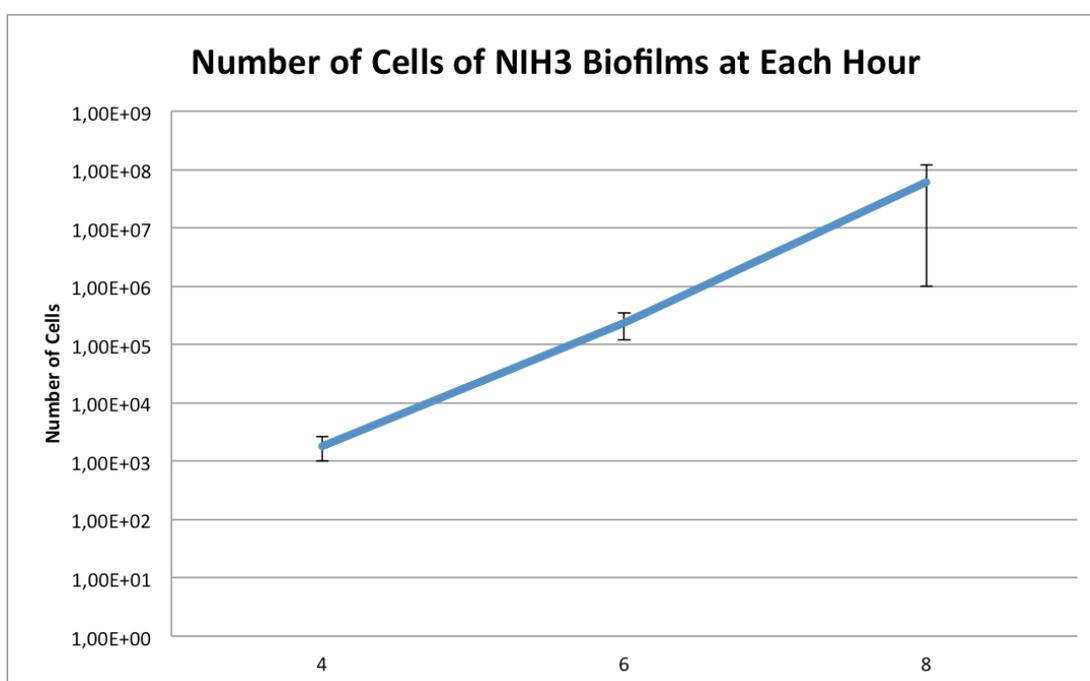
Figure 32: Number of cells in biofilms of NIH3 at 2, 4, and 6 hours respectively
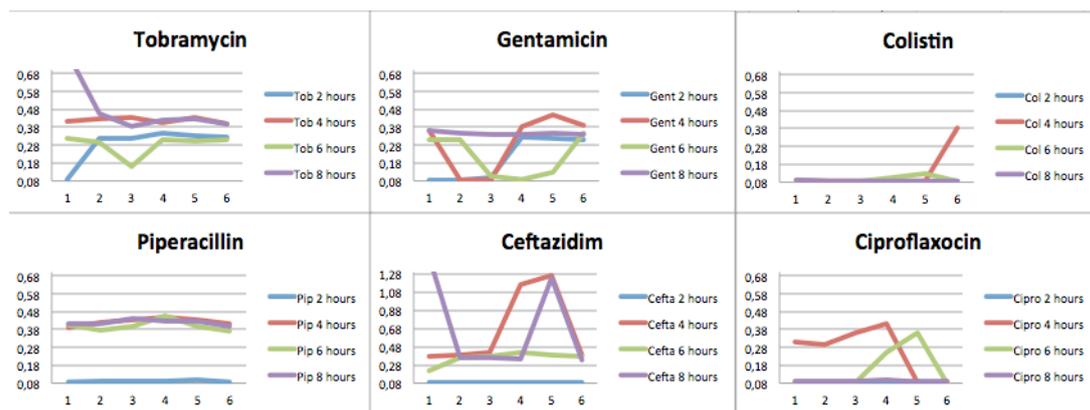
Figure 33: Antibiotic response of NIH3 biofilms at different hours. Biofilms grown 2, 4, 6, and 8 hours are exposed to decreasing gradient of antibiotics. Growths of each concentration are shown in Y-label as OD 595 measurements. Antibiotic concentrations are diluted by 50% in each dilution. Starting antibiotic concentrations were 10mg/ml, 50mg/ml, 50mg/ml, 50mg/ml, 50mg/ml, and 6mg/ml for Tobramycin, Gentamicin, Colistin, Piperacillin, Ceftazidime, and Ciprofloxacin, respectively.

Colistin and Ciprofloxacin were chosen since these drugs in a soluble range achieved eradication of biofilms. Pa NIH3 biofilms are tested with Colistin and Ciprofloxacin in 4, 6, and 8 hours.
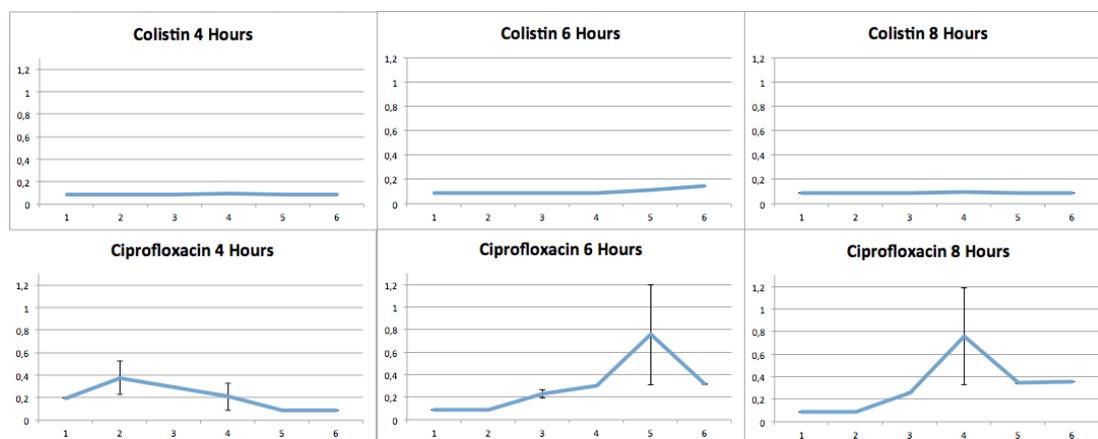
Figure 34: Growths of NIH3 biofilms in decreasing concentration gradient of Colistin and Ciprofloxacin. Concentrations are highest in X-axis value (1) and decreasing by 10% until concentration 6. Y-axis represents growth measured by OD 595 of cell culture. Starting concentration for Colistin at 80ug/ml and for Ciprofloxacin 20ug/ml. OD<0.1 is considered as no growth.

After killing ranges are detected, drugs are tested on Pa NIH3 planktonic cells with pyocin AP41.
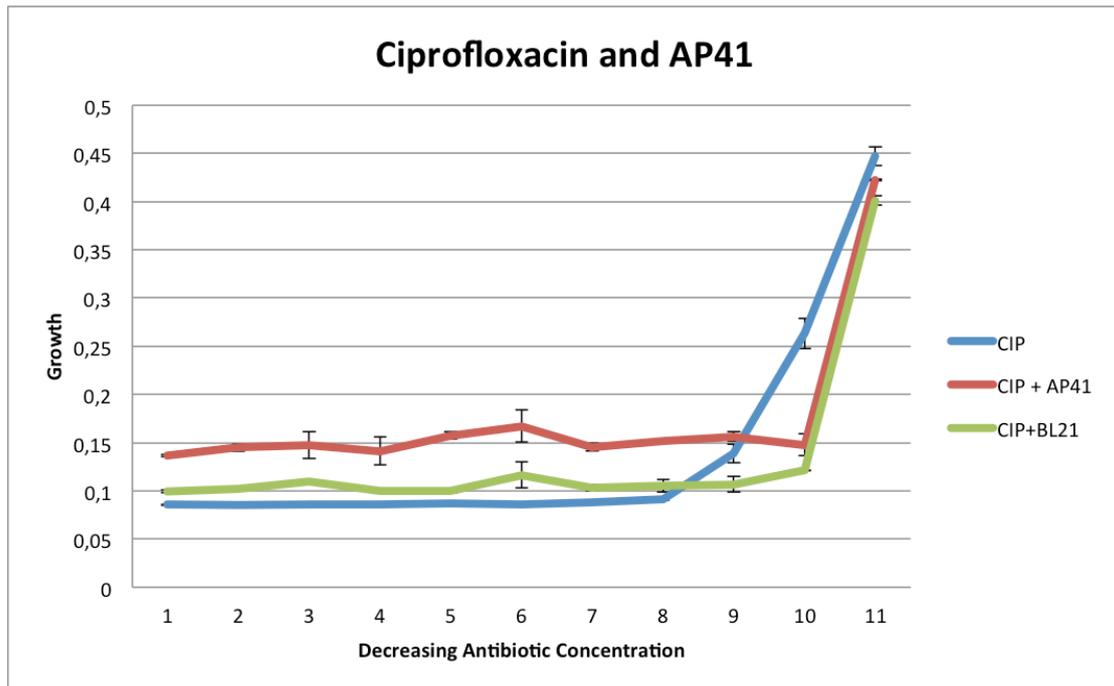
Figure 35: Shift Assay for Ciprofloxacin and AP41. Ciprofloxacin concentration is decreased by 10% as a gradient starting from X-axis label (1) until (10). X-axis label (11) is used as a growth control without an antibiotic. For CIP+AP41, 10ul CFE of AP41 is added to each well of Ciprofloxacin gradient. CIP+BL21 is negative control where 10ul CFE extract of BL21(DE3) cells are added to each dilution of antibiotic gradient of Ciprofloxacin. Ciprofloxacin concentration is started from 0.8ug/ml
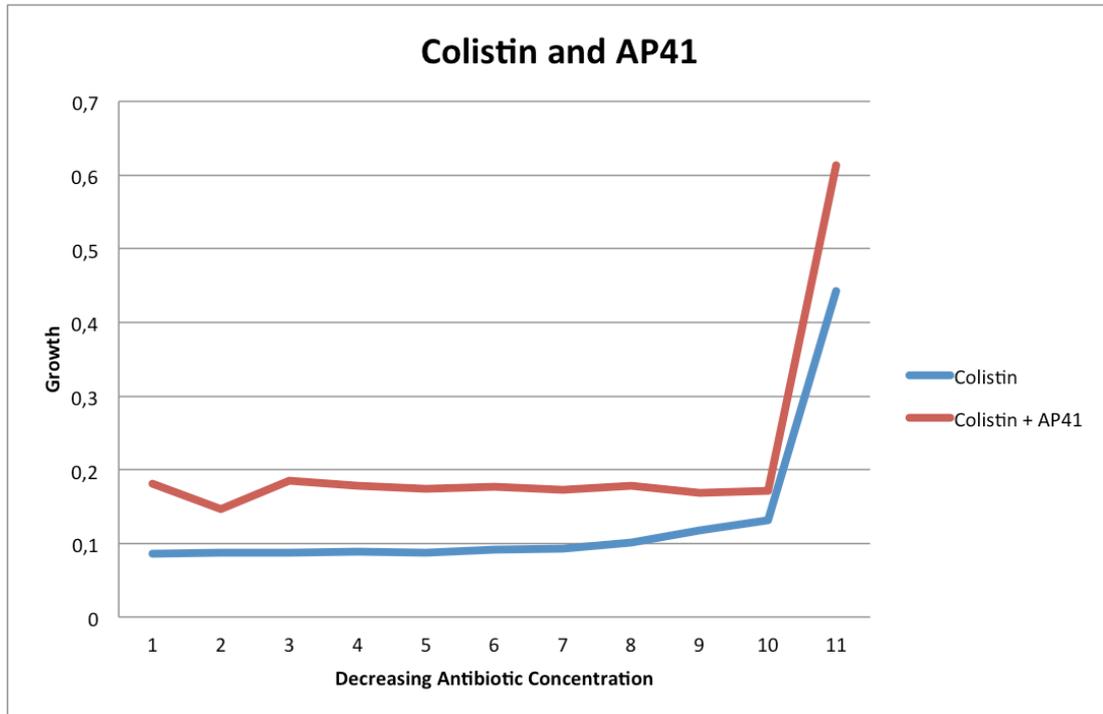
Figure 36: Shift Assay for Colistin and AP41. Colistin concentration is decreased by 10% as a gradient starting from X-axis label (1) until (10). X-axis label (11) is used as a growth control without an antibiotic. For Colistin+AP41, 10ul CFE of AP41 is added to each well of Colistin gradient.

DISCUSSION

Anti-pseudomonal drugs Tobramycin, Gentamicin, Colistin, Piperacillin, Ceftazidime, and Ciprofloxacin have been used for the treatment of Pa infections for a long time. However, increasing antibiotic resistance observed in Pa strains causes a significant need for investigating novel therapies such as drug-drug interactions, and other antimicrobial elements such as bacteriocins.

Since we know that pyocins can be used for eradication Pa biofilms [39] we hypothesized pyocins and synergistic pyocin-drug interactions could be used for inhibition of free-floating Pa cells, and also eradication of Pa biofilms. Therefore, in this study, we tested narrow-spectrum killer proteins, S-pyocins, against antibiotics Ciprofloxacin and Colistin.

In order to have pure pyocins, S-pyocin genes are cloned into Novagen pET-30 Xa/LIC System and expression is induced by adding 1mM IPTG concentration. After extraction of the cells, amount of protein for performing killing assays or MIC/MBEC

tests was not enough, or activity of extracted proteins were too low to see a visible killing. Therefore, several optimization steps are performed. IPTG concentrations at 0.4mM, 0.8mM, and 1mM were tried however there was no change in protein activity observed. Three temperatures (26^0C, 30^0C, and 37^0C) are tested along with IPTG concentrations for pyocins S1, S3, and AP41. However there is no significant change in killing activity observed. Possible optimization could be cloning with a system other than pET-30 Xa/LIC considering the reason of inactivity could've been cloning system, buffer and pH arrangements since the reason of inactivity could've been unfolding or degradation of pyocin in the buffers where extracts are stored. Using CFEs from *E. coli* cells which possess S-pyocin genes (Table 3) is more feasible for this study instead of further optimization of cloning and expression in pET30 Xa/LIC system.

We tested killing activity of CFEs obtained from *E. coli* cells on S-pyocin sensitive Pa cells to eliminate the potential interaction between proteins of *E. coli* and S-pyocin sensitive Pa cells. Results of these tests showed that proteins of *E. coli* cells do not kill S-pyocin sensitive Pa NIH3 cells.

Quantification of S-pyocin amount in CFE is one of the challenges. It is not possible to determine the S-pyocin amount as µg in the CFE obtained from *E. coli* cells with plasmids possessing S-pyocin genes. For this reason instead of testing different S-pyocin concentration gradients with drug gradients, constant amount of CFE is used against antibiotic gradient. The aim was to make MIC value of antibiotic shift to both sides that would lead observing a synergy or antagonism (Shift Assay). If MIC value for bacteria is higher when S-pyocin and antibiotic were added together compared to only antibiotic condition, the interaction is considered as antagonistic since addition of the second molecule (in this case S- pyocin or CFE containing S-pyocin) prevent killing activity of antibiotic. If MIC is shifted the other way, i.e. it is decreased; there is a possible synergy between two molecules.

Results of this study suggested a potential antagonistic interaction between pyocin AP41-Ciprofloxacin and pyocin AP41-Colistin drug combinations (Figures 35 and 36).

We also tested antibiotics and S-pyocins on Pa NIH3 biofilms. However, only Ciprofloxacin, Colistin, and Gentamicin were able to show killing activity. S-pyocins were also not effective against Pa NIH3 biofilms formed. Nevertheless, S-pyocins may be more effective on biofilms if they are more pure and concentrated.

FURTHER STUDIES

For the next period, all possible drug-pyocin pairs will be tested using shift assay and possible synergies and antagonisms will be monitored. Same assay will also be applied to biofilms.

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APPENDIX

Protocol: Plasmid DNA Purification using the QIAprep Spin Miniprep Kit and a Microcentrifuge

This protocol is designed for purification of up to 20 μg of high-copy plasmid DNA from 1−5 ml overnight cultures of *E. coli* in LB medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on page 34.

Please read "Important Notes" on pages 12–18 before starting. Note: All protocol steps should be carried out at room temperature (15–25°C).

Procedure

1.   Resuspend pelleted bacterial cells in 250 μl Buffer P1 and transfer to a micro- centrifuge tube.   Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet. If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2.   Add 250 μl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.   Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addi- tion of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3.   Add 350 μl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.   To avoid localized precipitation,

mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless sus- pension indicates that the SDS has been effectively precipitated.

4.   Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.

5.   Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.

6.   Centrifuge for 30–60 s. Discard the flow-through.

7.   Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.   This step is necessary to remove trace nuclease activity when using *endA+* strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5®α do not require this additional wash step.

8.   Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.

9.   Discard the flow-through, and centrifuge at full speed for an additional 1 min to remove residual wash buffer.   Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10.   Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 μl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

Protocol: Annealing Reaction for Xa/LIC Cloning Kits

1. For each insert, assemble the following components in a sterile 1.5-ml micro centrifuge tube

- 1 µl Xa/LIC Vector
- 2 µl T4 DNA Polymerase treated Xa/LIC insert (0.02 pmol)

Incubate at 22°C for 5 min, then add:

- 1 µl 25 mM EDTA
- 4 µl Total volume

2. Mix by stirring with the pipet tip and incubate at 22°C for 5 min.

Notes:

a) Greater volumes of the treated insert may be used; however, the concentration of vector in the reaction will decrease and subsequently fewer nanograms will be plated if a constant amount of annealing reaction is used for transformation.

b) Annealing is complete within 5 min of incubation; reactions can be incubated up to 1 h with equivalent results.