THERAPEUTIC POTENTIAL OF BIOENGINEERED

BACTERIOPHAGE FOR PSEUDOMONAS INFECTION CONTROL

by

Reuel Sandy

B.S. (Hunter College) 2010

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

January 2019

Accepted:

_____	_____
Ann Boyd, Ph.D. Committee Member	Ann Boyd, Ph.D. Director, Biomedical Science Program
_____	
Oney Smith, Ph.D. Committee Member	
_____	_____
Sathish Rajamani, Ph.D. Project Adviser	April Boulton, Ph.D. Dean of the Graduate School

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ACKNOWLEDGEMENTS

I would like thank Dr. Ann Boyd and Dr. Oney Smith for their valued insight and time taken reviewing the numerous iterations of this project. Dr. Sathish Rajamani, whose thoughtfulness and keen understanding of technical matters significantly guided the project.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

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Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
Therapeutic potential of bioengineered bacteriophage for Pseudomonas infection control

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle)	3b. DEGREE(S)	3h. eRA Commons User Name
Sandy, Reuel, Akil	B.S.	N/A

3c. POSITION TITLE
Graduate Student

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Department of Biology

3f. MAJOR SUBDIVISION
Biomedical Science Program

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E-MAIL ADDRESS:
reuels@gmail.com

4. HUMAN SUBJECTS RESEARCH	4a. Research Exempt	
☒ No ☐ Yes	N/A	
4b. Federal-Wide Assurance No.	4c. Clinical Trial	4d. NIH-defined Phase III Clinical Trial
N/A	☒ No ☐ Yes	☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes — 5a. Animal Welfare Assurance No. N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
10/01/2016	10/01/2019				

9. APPLICANT ORGANIZATION
Name
Address
Department of Biology
Hood College
401 Rosemont Ave
Frederick, MD 21701

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. N/A Cong. District N/A

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name Reuel Sandy
Title Graduate Student
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401 Rosemont Ave
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13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
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Title Graduate Student
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14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*
N/A

DATE
October 1, 2018

PROJECT SUMMARY (See instructions):

The bacterium *Pseudomonas aeruginosa* (*P. aeruginosa*) is a formidable pathogen that causes infections in animals and humans. In human hosts, infections can be widespread, chronic and difficult to treat. The prevalence of *P. aeruginosa* as a significant opportunistic pathogen is aided by its various virulence adaptations, including: production of biofilms, possession of efflux pumps and intrinsic resistance to several classes of antibiotics. Bacteriophages (phages), viruses that infect bacteria, can be used as effective agents to treat and kill resistant *P. aeruginosa*. In this study, the lysogenic Pf1-like *Pseudomonas* phage, Pf1B, will be used as a vector to deliver a histone-like nucleoid structuring (*hns*) gene to *P. aeruginosa*. Previous studies have shown that HNS overexpression in *Escherichia coli* is lethal. The *hns* gene will be placed downstream of the major coat protein (MCP) of Pf1B and the engineered phage will be tested against *P. aeruginosa*.

RELEVANCE (See instructions):

The increasing resistance of *P. aeruginosa* to traditional antibiotics calls for novel and effective counter measures; in the United States alone, *P. aeruginosa* causes 51,000 nosocomial infections each year. This bioengineered phage approach will offer new avenues of combining antibiotics with phage for complete elimination of *P. aeruginosa* and other drug resistant pathogens.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:		State:
Province:	Country:		Zip/Postal Code:
Project/Performance Site Congressional Districts:			

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Reuel Sandy	Graduate Student
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Hunter College, New York, NY	B.S.	2010	Laboratory Science
Hood College, Frederick, MD	M.S.	2019 (anticipated)	Biomedical Science

A. Positions and Honors

B. Selected peer-reviewed publications (in chronological order)

C. Research Support

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

N/A

Clinical:

N/A

Animal:

N/A

Computer:

N/A

Office:

N/A

Other:

N/A

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

- Centrifuges
- Thermal Cycler
- Incubators (37°C)
- Spectrophotometer-
- Refrigerator and freezer-
  media and reagents (2–8°C) (-20°C)
- Bio-Rad Electroporation System
- Nano Drop Spectrophotometer
- E-Gel Power Snap Electrophoresis Device

The following consumables will be purchased with grant funding:

- ATCC bacteria strains 15692,47085,25922
- DNA ladders / Primers/ Plasmids.
- 1% agarose ThermoFisher E-Gels
- ThermoFisher GN4F Sensititre plates
- Mueller Hinton Media
- Qiagen PCR/Plasmid purification kits
- NEB Quick Ligation Kit
- NEBNext Q5 PCR master Mix
- Polyethylene Glycol (PEG)
- Ampicillin/ Ciprofloxacin/ Tetracycline
- Restriction enzymes AflII,SbfI, AscI
- Isopropyl β-D-1-thiogalactopyranoside
- Molecular grade water

SPECIFIC AIMS

Spurio *et al.* (1992) showed the overexpression of HNS in *Escherichia coli* is lethal. The primary goal of this study is to reduce the viability of *P. aeruginosa* by using phage Pf1B as a vector for HNS overexpression. Phage Pf1B expressing HNS could be an effective therapeutic against drug resistant strains of *P. aeruginosa.*

The main objectives of this study are to:

1) Determine if overexpression of HNS protein reduces *P. aeruginosa* viability,
2) Isolate lysogenic phage Pf1B from *P. aeruginosa,* and
3) Examine the efficacy of Pf1B expressing HNS in *P. aeruginosa* and reduction of antibiotic minimum inhibitory concentration (MIC) in combination therapy.

BACKGROUND AND SIGNIFICANCE

Pseudomonas aeruginosa is an opportunistic pathogen with significant importance to human health. The organism causes both morbidity and mortality in humans. The bacterium is a hardy, fast growing, Gram negative rod with a sizable genome of 6.2 million base pairs (Fang *et al*. 2012). It is ubiquitous in the environment, inhabiting plants, animals, soil and water (Grosso-Becerra *et al*. 2014). In humans, *P. aeruginosa* inhabits mostly wet or moist regions of the body such as, the nose, armpits and mucosal membranes. It has a wide-ranging pathogenicity, causing infections of the skin, ears, lungs, urinary tract and bacteremia. The National Nosocomial Infections Surveillance System (NNIS) found *P. aeruginosa* to be the second most common cause of pneumonia (18.1%), the third most common cause of urinary tract infection (16.3%) and the eighth most frequently isolated pathogen from the bloodstream (3.4%) (Hirsch and Tam 2010). *Pseudomonas aeruginosa* is the major respiratory pathogen isolated from patients with cystic fibrosis; in these patients, it produces a chronic and intractable infection (Høiby *et al*. 2010). It can also be quite deadly in burn victims, where it is the most commonly isolated organism (Safaei *et al*. 2017). *Pseudomonas aeruginosa* is proficient as an opportunistic pathogen and has several adaptations for virulence; it forms biofilms, has efflux pumps and is intrinsically resistant to multiple classes of antibiotics.

Increasing Resistance to Antibiotics

Over the last decade, physicians have been challenged with the problem of bacteria developing increased antibiotic resistance (Figure 1). Recently, newly discovered resistant strains have emerged and have been given the name “superbugs” or more

correctly known as multidrug resistant organisms (MDRO). In many cases, physicians are left with no choice but to use last resort drugs that are harsh and prone to have undesirable side effects. Antibiotic resistance has emerged, in part, as a consequence of antibiotic overuse (Potron *et al*. 2015). In addition, when physicians treat one pathogen with an antibiotic, they inadvertently apply selective pressure to other organisms, resident in the same patient, which now develop a tolerance to that drug. MDRO have become a serious health problem with an adverse economic impact (Nolte 2014 Mar 31). Moreover, clinical isolates of *P.aeruginosa* have displayed resistance to several classes of antibiotics including: beta-lactams, aminoglycosides and fluoroquinolones (Hirsch and Tam 2010). A study by Morales *et al.* (2012) evaluated 402 confirmed cases of nosocomially acquired *P.aeruginosa* infections; their results showed that 62.9% of *P. aeruginosa* isolates were either resistant or multidrug resistant; the remaining 37.1% were non-resistant. They found resistant strains of *P. aeruginosa* cost 70% more to treat than non-resistant strains (Morales *et al*. 2012). A separate study by Sara Cosgrove illustrated that, globally, the cost to treat infections are much higher for resistant organisms than non-resistant ones (Cosgrove 2006).

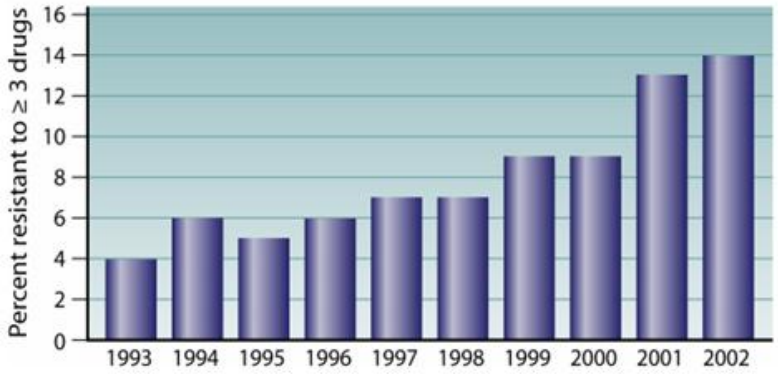

Figure 1. Increasing prevalence of multidrug resistance among *P.aeruginosa* from ICU patient in the United States. Data from 13,999 non-duplicated isolates collected from 1993 to 2002 (Reprinted from Lister *et al* 2009).

Efflux Pumps

Like many Gram-negative bacteria, *P. aeruginosa* has efflux pumps; these are primarily transmembrane protein systems that actively expel antimicrobial compounds that make their way into the bacterial cell (Figure 2). Efflux pumps are major contributors to *P. aeruginosa* being multidrug resistant. The largest and most common type of efflux pump is the Resistance Nodulation Division (RND) family (Lister *et al*. 2009). A single strain of *P. aeruginosa* may express multiple families of efflux pumps and some pumps have overlapping functions and can expel multiple classes of antibiotics. Recent studies have explored efflux pump inhibitors as a viable method of increasing *P. aeruginosa* sensitivity to previously resistant antibiotics (Rampioni *et al*. 2017). However, this approach has resulted in very limited success.

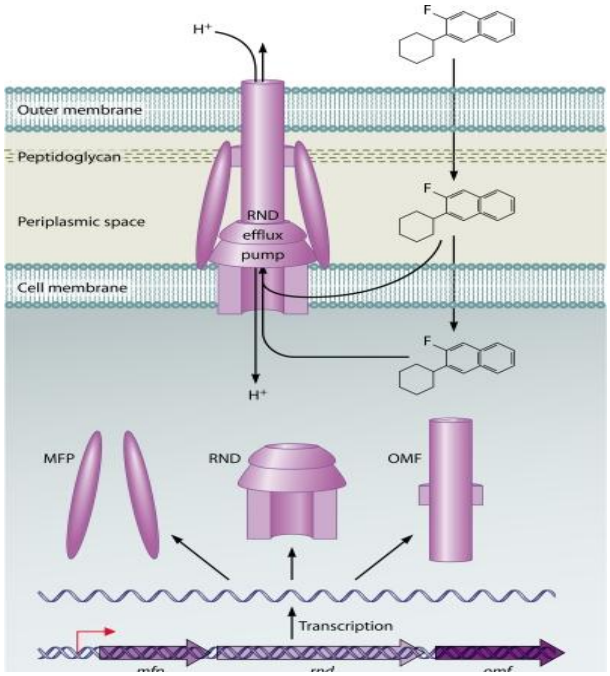

Figure 2. Structure and function of RND efflux pumps in *P. aeruginosa***.** This complex forms a channel spanning the entire membrane, allowing for the proton-driven transport of lipophilic and amphiphilic drugs from the cytoplasm of the cell across the cytoplasmic membrane **(**Reprinted from Lister *et al* 2009).

Formation of Biofilms.

The bacterial biofilm is a secreted protective matrix composed of polysaccharides, lipids proteins and extracellular DNA (Moradali *et al*. 2017). Biofilms form a biological shield and protects *P. aeruginosa* from unfavorable environmental conditions (Figure 3). This protective matrix renders the host's immune cells and other antimicrobial agents inaccessible to *P. aeruginosa.* The formation of biofilms in patients with cystic fibrosis is the major factor contributing to the chronic nature of this disease. *Pseudomonas*

aeruginosa biofilm can also adhere to fomites and other hospital equipment, thus complicating patient treatment and recovery (Høiby *et al*. 2010). Agents that alter the biofilm state and or agents that target bacteria embedded with the biofilm are limited and are highly useful.

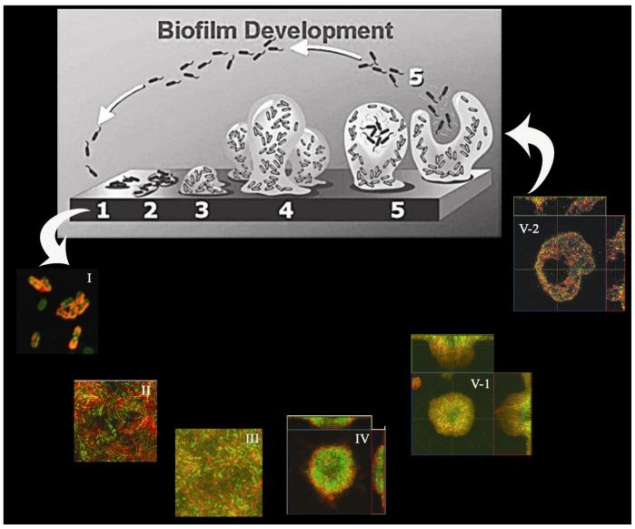

Figure 3. Biofilm development in *P. aeruginosa***.** Images showed how the matrix of polysaccharide **(**red fluorescence) enmeshes bacterial cells (green fluorescence) within bacterial communities during biofilm development (I: initial attachment; II: irreversible attachment; III: microcolony formation; IV: biofilm maturation; V: biofilm dispersion) (Reprinted from Wei *et al*. 2013).

Phages as Antimicrobials

Phages are viruses that infect bacteria. They were discovered in the early 20th century by William Twort and Felix d'Herelle and were used successfully to treat many bacterial infections (Pires *et al*. 2016). Bacteria have, over time, evolved numerous mechanisms of resistance towards antibiotics; a return to phage therapeutics could be a viable alternative. Like many viruses, phages are bacterial host specific and therefore only infect selective bacterial cells. The phage Pf1B is specific for the bacterium *P. aeruginosa*.

Pf1B Prophage of *P. aeruginosa*

Phages are the most abundant organism on the planet and are estimated to outnumber bacteria ten to one (Clokie *et al*. 2011). Many, if not all, bacteria are parasitized by or harbor phages in some form. A study by Knezevic *et al*. (2015) examined the genomes of 241 isolates of *Pseudomonas* and found that 60 % contained at least one Pf1-like element (Knezevic *et al*. 2015). Phages can be placed broadly into the categories of lytic and lysogenic. A phage that is only lytic, such as *E.coli* phage T4, replicates in its host and exits by cell lysis. In contrast, lysogenic phage Pf1B, exits *P.aeruginosa* without lysis or cell death. A BLAST query reveals *P. aeruginosa.* strain ATCC 15692, has two Pf1-like prophage regions. These Pf1-like genomic islands share between 92-98 % identity with *Pseudomonas* phage Pf1 and are located between nucleotides 4,720,898 and 5,248,482 within the *P. aeruginosa* genome (Figure 4).

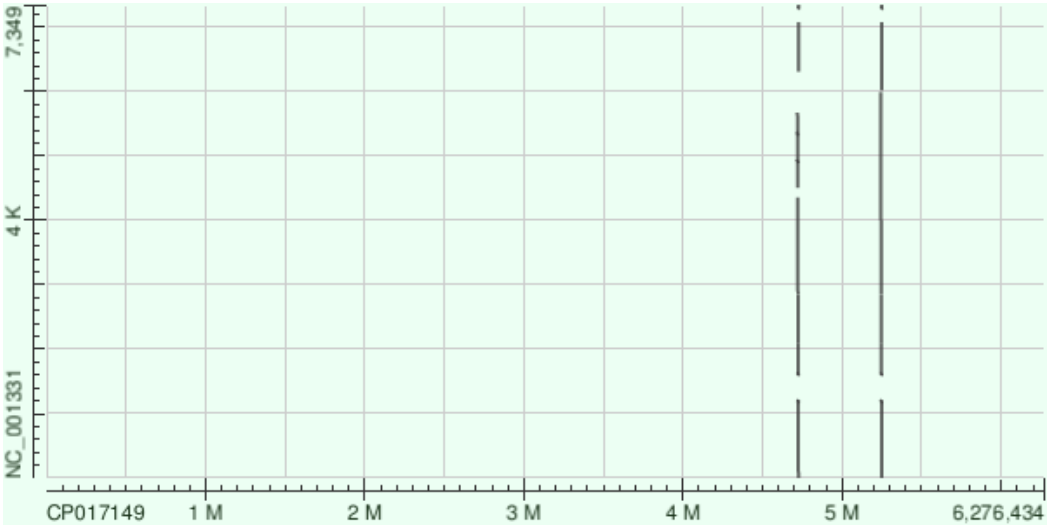

Figure 4. The Pf1-like prophage regions of *P.aeruginosa.* Two similar Pf1-like prophages present in the *P.aeruginosa* genome. Image created using NCBI nucleotide BLAST tool.

Filamentous *Inoviridae* Family

Pf1B is a member of the family *Inoviridae* (Ινα Greek; fiber). Virions in this family are filamentous and flexible. Notable members include *Pseudomonas* phages Pf1 and Pf3, *Escherichia coli* phage M13 and *Vibrio cholera* phage CTXφ (Fauquet *et al*. 2005). These phages are non-enveloped, rod shaped and contain circular, positive sense, single stranded DNA. Virions are approximately 7 nm in diameter and can be up to 2000 nm in length (Fauquet *et al*. 2005). Genome sizes range from 4.5-12.4 kbp and encode between 4 to 17 genes. All members of the family *Inoviridae* possess a similar morphology and replication cycle. (Fauquet *et al*. 2005) . Virion adsorption is facilitated by the interaction of attachment proteins gp3 and gp6 to bacterium pili (Figure 5). Once in the cytoplasm, the single stranded DNA is converted to a double stranded replicative form; new virion single stranded DNA is created by a rolling-circle replication. Progeny virions exit from various points of the bacterium cell membrane without causing cell lysis (Fauquet *et al*. 2005).

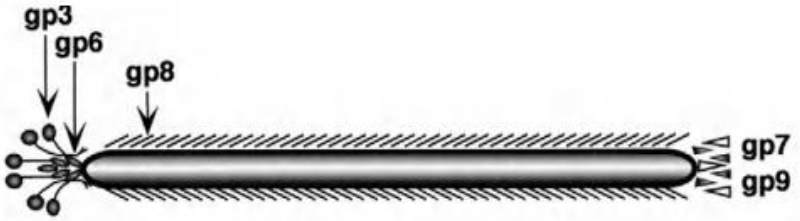

Figure 5. Virion representing all members of the *Inoviridae* family. Pili attachment proteins (gp3, gp6). Major coat protein (gp8). Minor coat proteins (gp7, gp9) **(**Reprinted from DNA Replication, 2nd edition. W.H. Freeman, New York 1992).

HNS Protein

Histone-like nucleoid structuring (HNS) protein belongs to a family of prokaryote DNA binding proteins. The primary function of HNS is gene regulation and genome structuring. High concentrations of HNS can be found in prokaryote nucleoid structures. HNS of *E .coli* is a small 15.5-kDa protein with orthologs present across several species of bacteria (Landick *et al*. 2015). Previous research has found that HNS plays a major role in bacterial DNA nucleation and gene suppression; it also shares similar DNA binding motifs with prokaryote RNA polymerase and affects RNA polymerase function by direct interaction, polymerase trapping and exclusion (Landick *et al*. 2015) (Figure 6).

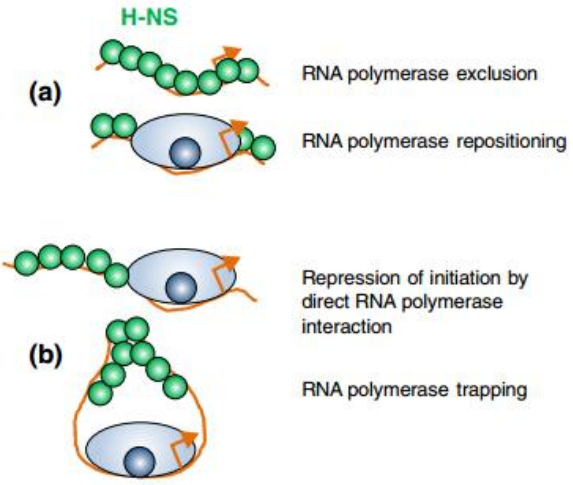

Figure 6. HNS interaction with RNA polymerase**.** (a) HNS can exclude RNA polymerase by occluding promoter DNA elements or bind DNA in conjunction with RNA polymerase to influence its position on the DNA. (b) Transcription initiation may be blocked by HNS mediated stalling of RNA polymerase (Reprinted from Landick *et al*. 2015).

PRELIMINARY REPORT / PROGRESS REPORT

In a study by Spurio *et al.*(1992) the overproduction of HNS protein in *Escherichia coli* was proven to be lethal. Following the overexpression of HNS, a strong and nearly immediate inhibition of RNA and protein synthesis was observed (Spurio *et al*. 1992). Briefly, the native *hns* gene was placed under the control of an inducible lamda promoter in plasmid pPLc2833. As a control, a mutant of HNS was created by restriction digest and blunt-end ligation. The incorporation of radioactive thymidine, uridine, histidine and acetlyglucosamine was used to access the effects of overexpressing both the wild type and mutant HNS. Their results showed that only the overexpression of wild type HNS affected RNA and protein synthesis (Spurio *et al*. 1992).

Several genetic modifications have been made to bacteriophages; some examples include, adaptations for antibody display as well as delivery of antimicrobial peptides (Pires *et al*. 2016). Lu and Collins showed that filamentous phage M13 can be genetically modified to overexpress the gene *lexA3,* which suppresses bacterial DNA damage repair systems. The overexpression of *lexA3* rendered *Escherichia coli* more susceptible to the quinolone antibiotic ofloxacin (Lu and Collins 2009).

There has been recent success in the use of phages to treat multidrug resistant organisms. Shooley *et al.* (2017) describes the successful treatment of a 68 year-old patient who contracted a strain of multidrug resistant *Acinetobacter baumannii*. After several weeks of failed antibiotic therapy, permission was obtained to use a lytic phage cocktail as an emergency investigational new drug. The phage cocktail was administered percutaneously, at the primary site of infection, as well as intravenously. After only a few weeks, the patient made a full recovery.

RESEACH DESIGN / METHODS

AIM 1: Determine if overexpression of HNS protein reduces *P. aeruginosa* viability.

Histone-like nucleoid structuring protein is small protein with only 137aa; it has been extensively studied and the complete sequence is available (Landick *et al*. 2015). The *hns* gene sequence from *Escherichia coli* (Figure 7) will be cloned into the broad host range vector pBBR1MCS-4 (Figure 8). The vector pBBR1MCS-4 will be supplied to GenScript for custom cloning of the *hns* sequence. To drive expression of *hns*, the inducible promoter *tac* (Ptac) will be included upstream of the *hns* gene (Soldati *et al*. 1987). A second vector, similar in design but with a mutation (12 bp deletion of nucleotides 337- 348 with a loss of amino acids Gly_{112}-Arg-Thr-Pro_{115}) in the *hns* gene will also be obtained for use as a control (Spurio *et al*. 1992).

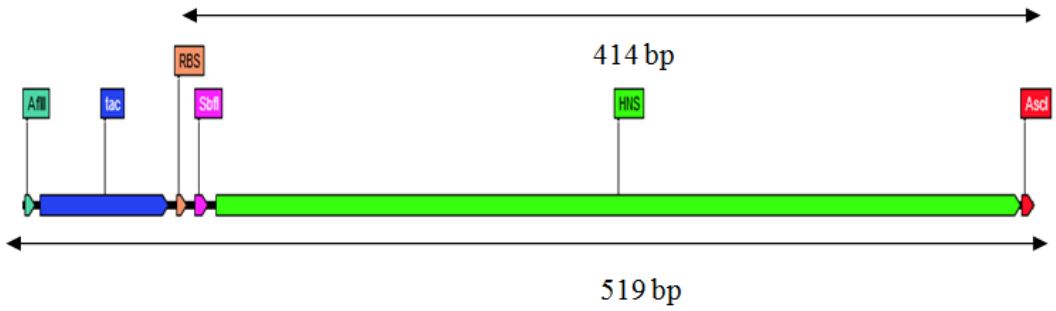

Figure 7. GenScript synthesized *hns* insert. The *Escherichia coli hns* gene is flanked by restriction sites SbfI and AscI. The complete insert is flanked by restriction sites AflII and AscI and includes a ribosome-binding site (RBS) and the *tac* promoter sequence. Image created using molbiotools.com/WebDSV

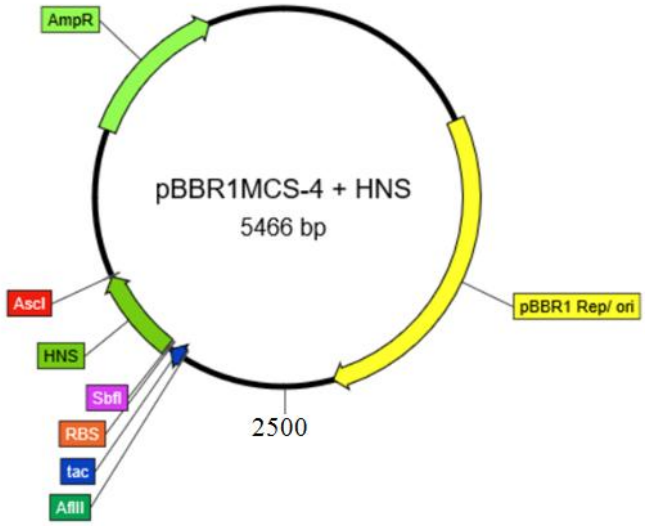

Figure 8. Cloning vector pBBR1MCS-4 with hns insert. The *hns* insert is flanked by restriction sites AflII and AscI. The ribosome-binding site (RBS) is upstream of the *hns* gene. Image created using molbiotools.com/WebDS.

Electroporation Transformation of *P.aeruginosa*

Pseudomonas aeruginosa is not a naturally competent cell, however, genetic material such as expression vector pBBR1MCS-4 or other plasmid DNA can be introduced into *P.aeruginosa by* electroporation; electroporation utilizes a high-intensity electric charge to permeabilize cell membranes (Filloux and Ramos 2014). *Pseudomonas aeruginosa* ATCC 47085 ($lacI^q$) will be inoculated in 30 mL of Mueller Hinton Broth (MHB) and incubated at 37°C with shaking at 200 rpm for 24 h. This bacterium produces the repressor protein of the *lac* operon which binds to the operator and suppresses gene expression until induced by the addition of IPTG. After 24 h, this culture will be centrifuged for 30 min at 3,000 x g and 25°C. The supernatant will be discarded and the pellet retrained. Fifteen mL of 300 mM sucrose solution will be used to resuspend the pellet before centrifuging at 3,000 x g for 30 min, the supernatant will be discarded and the pellet retained. The pellet will then be resuspended for a second time in 7.5 mL of 300 mM sucrose solution and centrifuged at 3,000 x g for 30 min. The supernatant will be discarded and the pellet retained. (Filloux and Ramos 2014). One hundred µL of 300 mM sucrose solution will be added to the bacterial pellet. Fifty µL of the cell pellet will be added to 0.2-mm electroporation cuvettes. One µL of vector pBBR1MCS-4 containing the *hns* mutant will be added to cuvette A. Similarly, vector pBBR1MCS-4 containing the *hns* wild-type (wt) will be added to cuvette B. Electroporation will be conducted using the Bio-Rad® Gene Pulser Electroporation System. Immediately after electroporation, 50 µL of cells, from cuvettes A and B will be placed in 1 mL of super optimal broth and transferred to news tubes. The cells will be recovered in an incubator at 37°C with shaking at 200 rpm for 1 h. After 1 h incubation,

100 µL of cells from A and B will be plated onto individual Mueller Hinton Agar (MHA) plates containing 500 µg/mL ampicillin for selection of transformants. Plates A and B will be inverted and incubated at 37°C for 24 h. After 24 h of incubation, 3 colonies from plate A and B will be selected and inoculated in individual 5 mL of MHB with 500 µg/mL ampicillin. A third culture labeled "C" of *P.aeruginosa* ATCC 47085 (untransformed) will be inoculated in 5 mL of MHB. Cultures A (#1 to #3), B (#1 to #3) and C will be incubated at 37°C with shaking at 200 rpm for 24 h.

HNS Gene Expression and *P.aeruginosa* Viability

After 24 h, a representative culture of A, B and wild type untransformed C will be added to individual 95 mL of MHB for a final volume of 100 mL and incubated at 37°C with shaking at 200 rpm for 30 min. After 30 min of incubation, 1 mL will be removed from each culture and the optical density OD_{600nm} will be measured using a Bio-Rad® spectrophotometer. Twenty µL of culture A, B, and C will be plated onto MHA plates to determine viability and colony forming units (CFUs). At this point 50 µL of 1 M stock IPTG will be added to cultures A, B and C (0.5 mM final ITPG concentration) (Derouazi *et al*. 2008). Incubation will then resume at 37°C with shaking at 200 rpm. At 30 min intervals, the OD_{600nm} will be measured as well as 20 µL of each culture plated onto MHA plates. The experiment will be terminated at the end of 240 min and the growth curve data assessed. All plates will be inverted and incubate at 37°C for 16 h. After 16 h, the CFUs corresponding to each time point will be recorded.

Expected Results

The optical density, observed at 600 nm, is a measure of turbidity and an indication of bacterial cell growth. The OD_{600nm} value of culture A and C is expected to

increase over the 240 min period. The OD_{600nm} values for culture B (HNS/wt) should decrease (Figure 9). A correlation is expected between the OD_{600nm} and CFUs present on the MHA plates; for culture B, HNS wild type, there should be fewer colonies present as the time point progresses (Figure 10).

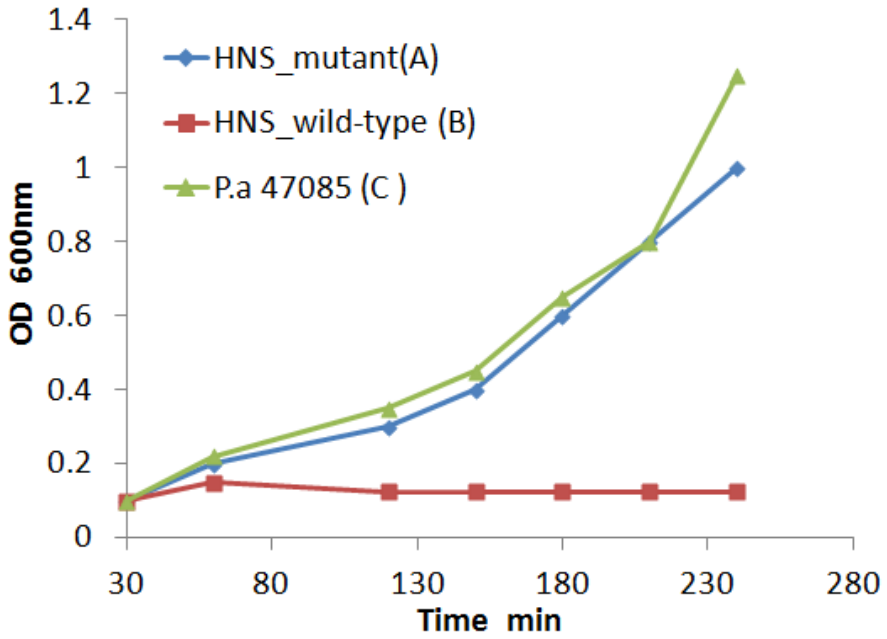

Figure 9. The expected effects of HNS and HNS-mutant overexpression in *P.aeruginosa.* The optical density measured at 600 nm was recorded every 30 min over a period of 240 min.

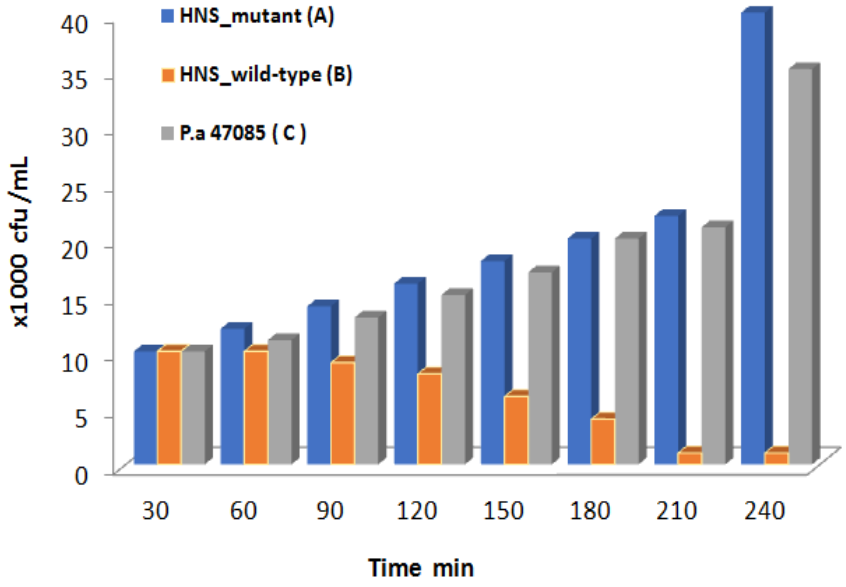

Figure 10. The expected effects of HNS and HNS-mutant overexpression on *P.aeruginosa* cell viability. Cell viability quantified as the number of colony forming units (CFUs) present on MHA plates.

AIM 2: Isolation of lysogenic phage Pf1B from *P. aeruginosa*

Phages that are lysogenic, such as Pf1B, can undergo spontaneous induction into their lytic phase, however, the lytic phase can also be induced by environmental changes such as, exposure to UV light or chemicals which promote DNA damage (Clokie 2009). The induction of phage Pf1B will be achieved using a minimum inhibitory concentration (MIC) of ciprofloxacin (Brazas and Hancock 2005). *Pseudomona aeruginosa* strain ATCC 15692 will be grown in 50 mL of MHB overnight at 37°C with shaking at 200 rpm. At 24 h, 5 mL of the overnight *P.aeruginosa* culture will be added to a new flask containing 95 mL of MHB with ciprofloxacin at a final concentration of 0.25 µg/mL. This 100-mL of *P.aeruginosa* plus ciprofloxacin culture will be incubated at 37°C with shaking at 200 rpm for 6 h. As a control, an identical culture will be prepared minus the addition of ciprofloxacin. After 6 h, both cultures will be centrifuged for 30 min at 3,000 x g and 25°C to collect the cell free media and the bacterial pellet. The bacterial pellet will be discarded, while the cell-free supernatant will be filtered through a 0.22-µm filter and the filtrate stored at 4°C.

Phage Pf1B Precipitation from Supernatant Filtrate

Filamentous phages can be separated from supernatant filtrate by precipitation using a solution of polyethylene glycol (PEG) and sodium chloride (NaCl) (Bonilla *et al*. 2016). One hundred mL stock solution of PEG 8000 with 2.5M NaCl will be prepared and stored at 4°C. In an ice bucket, 25 mL of the PEG8000/NaCl solution will be added to the 100 mL of phage supernatant filtrate in a 250-mL centrifuge bottle. The bottle will be inverted a few times to thoroughly mix the filtrate with the PEG8000/NaCl. The centrifuge bottle will be incubated on ice for 1 h. After 1 h on ice, the bottle will be

centrifuged in a precooled centrifuge at 10,000 x g for 20 min. After centrifugation, the supernatant will be discarded and the phage pellet collected at the bottom of the centrifuge bottle will be resuspended in 5 mL of phosphate buffered saline (PBS) and stored at 4°C.

Purification of Phage Pf1B by Plaque Assay

Bacteriophages can be isolated and purified by doing a plaque assay; the plaque assay also permits quantification of phage Pf1B and verifies phage infectivity (Clokie 2009). Five mL of MHB will be inoculated with *P.aeruginosa* strain ATCC 15692 and incubated at 37°C with shaking at 200 rpm for 24 h. At 24 h, 100 µL of this overnight culture will be added to a 50 mL conical tube. Five µL of phage Pf1B (from above phage prep) will be added to the 100 µL of *P.aeruginosa*. Eight mL of 1.5% Mueller-Hinton Agar (MHA) at 50°C will be added to the *P.aeruginosa*/Pf1B mixture. The entire content will be mixed a few times by gently swirling the tube. This mixture will then be poured onto a fresh MHA plate at room temperature. An identical control plaque assay plate will be prepared minus the addition of 5 µL of phage Pf1B. Once the top agar has solidified, both plates will be inverted and incubated at 37°C for 24 h.

Isolating Pf1B Phage from a Single Plaque

Phages in the family *Inoviridae* do not lyse their host and therefore form turbid plaques due to retarded bacterial cell growth (Clokie 2009). After 24 h of incubation at 37°C, a sterile pipette tip will be used to pick cells from the center of a single phage plaque. These infected cells will be added to 100 mL of MHB and incubated for 24 h at 37°C with shaking at 200 rpm. As a control, a sterile pipette tip will be used to pick cells from an uninfected region of the plate. These cells will be treated similarly to the cells

taken from the center of the plaque. After 24 h, both cultures will be centrifuged at 3,000 x g for 30 min and the supernatant will be filtered through a 0.2-µm filter. The supernatant filtrate containing phages will be stored at 4°C. This filtrate should contain a high concentration of phage and can be retained as a pure phage stock; the remaining pellet will be stored at -20°C and will be used to isolate circular double stranded Pf1B DNA.

Isolation of double stranded Pf1B DNA

Pseudomonas aeruginosa cells infected with phage Pf1B contain a circular double stranded form of DNA, similar to a plasmid, termed the replicative form (Sambrook and Russell 2001). The QIAprep Spin Miniprep Kit by Qiagen will be used to isolate molecular grade phage DNA from the *P.aeruginosa* pellet. The DNA isolation will follow the manufacturer's specific protocol; briefly, the bacterial pellet will be lysed using a lysis buffer. The lysate is then centrifuged and the phage DNA, retained in the supernatant, is added to a QIAprep Spin column which binds the phage DNA. The column is washed to remove impurities and the bound DNA is then eluted from the column using a low salt concentration buffer. The Pf1B DNA will be quantified using the ThermoFisher NanoDrop spectrophotometer. The purified Pf1B DNA will be store at -20°C until needed.

Expected Results

Prophage Pf1B should be induced by the addition of 0.25 µg/mL of ciprofloxacin and a phage pellet should be present in the centrifuge bottle. The control culture that received no ciprofloxacin is expected to have no induction and therefore no phage pellet.

Phage Pf1B is expected to be infectious and visible plaques should be present in the plaque assay, in contrast, the control plaque assay should be void of plaques (Figure 11).

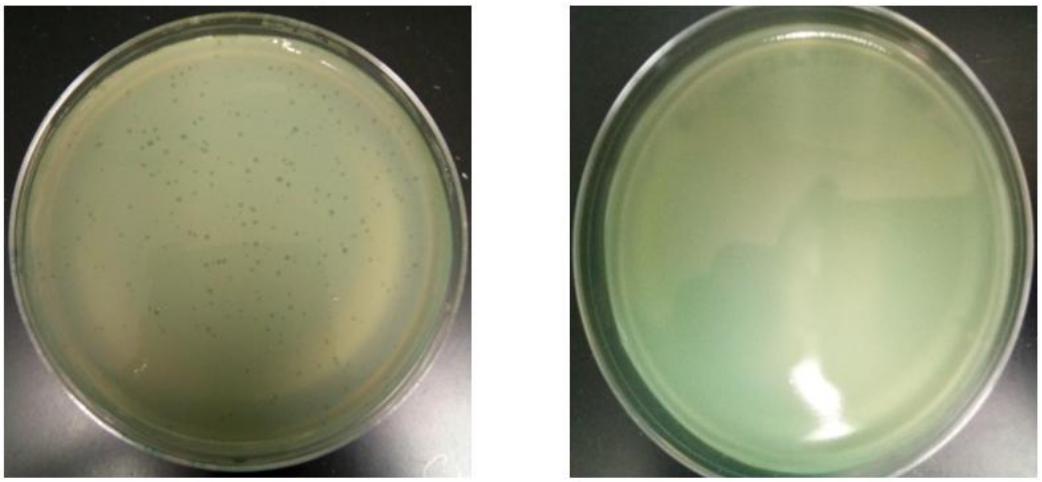

Figure 11. *Pseudomonas aeruginosa* plaque assay. a) Positive assay, several Pf1B plaques present. b) Control plate without addition of phage, no plaques present.

AIM 3: Examine the efficacy of Pf1B expressing HNS in *P.aeruginosa* and reduction in antibiotic minimum inhibitory concentration (MIC) in combination therapy.

Isolation of the *hns* Gene Sequence from Vector pBBR1MCS-4

The *hns* gene, including the *tac* promoter region, will be excised from vector pBBR1MCS-4 by restriction digest. The region of interest, which includes the 414 bp *hns* gene is 519 bp and flanked by restriction sites AflII and AscI. The standard New England Biolabs (NEB) restriction digest protocol will be used along with NEB restriction enzymes AflII and AscI. The restriction digest will be a 50-µL reaction containing 5 µL of 10X NEB CutSmart buffer, 1 µg of vector pBBR1MCS-4/*hns* wild type, molecular grade water and 1 µL each of restriction enzymes AflII and AscI. After incubation for 1 h at 37°C, the fragment of interest (519 bp) will be isolated from the remaining vector using the ThermoFisher E-Gel electrophoresis system (G8100) with 1% agarose E-Gel. The 519-bp fragment will be purified using the Qiagen QIAquick PCR purification kit (28104) and stored at -20°C.

Adding restriction Sites AflII and AscI to Phage Pf1B

Restriction enzyme sites AflII and AscI will be added to phage Pf1B by polymerase chain reaction (PCR) using primers: AflII_P1 (forward) and AscI_P2 (reverse). Primers will be obtained from ThermoFisher and PCR will be performed using NEBNext Ultra II Q5 PCR master mix (M0544S) in a 50-µL reaction containing 25 µL of 2X Q5 master mix , 0.5 µM each of primers P1 and P2, 0.5 µg of Pf1B dsDNA and molecular grade water. The PCR reaction will follow NEB standard PCR protocol (M0544) and use the ThermoFisher SimpliAmp thermal cycler (A24811). The PCR

product, obtained as linearized Pf1B, will be digested with enzymes AflII and AscI using the standard NEB restriction digest protocol in a 50-µL reaction containing 5.0 µL of 10X NEB CutSmart buffer, 1 µg of PCR product, molecular grade water and 1 µL each of restriction enzymes AflII and AscI. After 1 h incubation at 37°C, the digested product will be subjected to electrophoresis. The expected 7.3 kbp Pf1B fragment will be collected and purified using the Qiagen QIAquick PCR purification kit (28104) and stored at -20°C until needed for subsequent ligation reaction.

Ligation of Pf1B with *hns*

Ligation of Pf1B with *hns* will be achieved using NEB Quick ligation kit (M2200S) and NEB Quick ligase protocol (M2200) in a 20 µL reaction containing 10 µL of 2X Quick ligase buffer, 2 µg of Pf1B (vector) and 424 ng of P_{tac}-*hns* (insert), molecular grade water and 1 µL of T4 DNA Ligase. The reaction will be set up on ice and then incubated for 5 min at room temperature. The ligation product will be stored at 4°C until needed. Successful ligation will be confirmed by two independent restriction digest reactions. In reaction I, the ligation product, novel construct Pf1B/*hns*, will be digested with AscI only. In reaction II, Pf1B/*hns* will be digested with enzymes AflII and AscI. The digest products of reaction I and II will be subjected to electrophoresis to compare and verify the length of fragments produced by each reaction.

Expected Results

The restriction digest of cloning vector pBBR1MCS-4/*hns* wild type with enzymes AflII and AscI should produce two fragments during electrophoresis, one fragment should be 4.9 kbp and the other 0.5 kbp. PCR using primers P1 and P2 should produce a linearized Pf1B of 7.3 kbp. Ligation of Pf1B with P_{tac}-*hns* should produce the novel construct Pf1B/*hns* that is 7.8 kbp (Figure 12). When Pf1B/*hns* is digested with AflII, a single fragment of 7.8 kbp should be produced; however, when digested with both AflII and AscI, one fragment will be 7.3 kbp and the other 0.5 kbp (Figure 13).

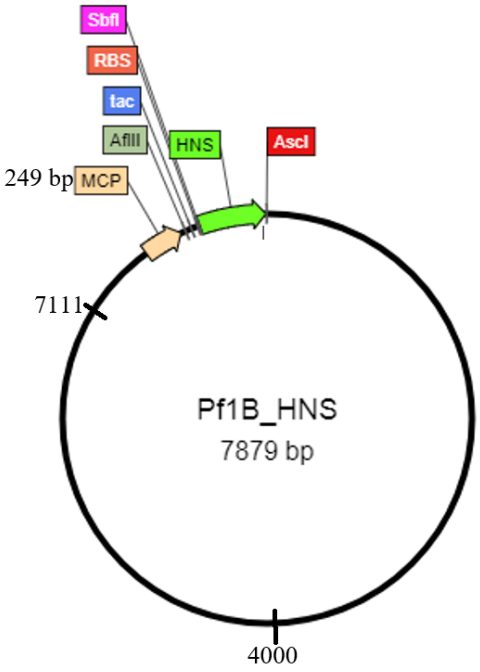

Figure 12. Expected Pf1B/*hns* construct. The *hns* gene is flanked by restriction sites SbfI and AscI. The complete insert is downstream of the Major Coat Protein (MCP). Image created using molbiotools.com/WebDSV.

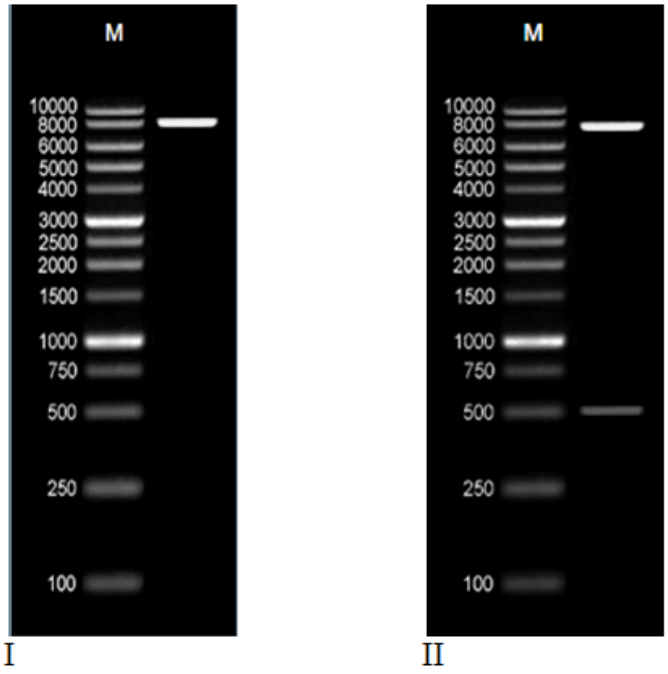

Figure 13. *In silico* electrophoresis of Pf1B/*hns* DNA. I) Pf1B/*hns* cut with AflII only. II) Pf1B/*hns* cut with both AflII and AscI. Gel images created with gene sequences using molbiotools.com/WebDSV.

Propagation of Phage Pf1B/*hns*

To ensure the novel phage Pf1B/*hns* is infectious and replicative, Pf1B/*hns* phage will be generated by transfecting Pf1B/*hns* DNA into *P. aeruginosa* strain ATCC 47085 by electroporation, as previously described. *Pseudomonas aeruginosa* ATCC 47085 is a modified *P. aeruginosa* strain with genotype $LacI^q$; thus, it will permit replication of phage Pf1B/*hns* while suppressing *hns* gene expression. This bacterium produces the repressor protein of the *lac* operon, which binds to the operator and suppresses gene expression until induced by the addition of IPTG. One µL of purified Pf1B/*hns* dsDNA will be added to cuvette I. DNA is not added to cuvette II. Electroporation will be done using the Bio-Rad® Gene Pulser Electroporation System. Immediately after electroporation, the 50 µL content of each cuvette will be added to 1 mL of super optimal broth and incubated at 37°C with shaking at 200 rpm for 1 h. After 1 h, the entire content of each tube will be added to 100 mL of MHB and incubated for 24 h at 37°C with shaking at 200 rpm. After 24 h, both cultures I and II will be centrifuged at 3,000 x g for 30 min. The bacterial pellet will be discarded, while the cell-free supernatant will be filtered through a 0.22-µm filter and the filtrate stored at 4°C.

Verifying Pf1B/*hns* Infectivity

The presence and infectivity of phage Pf1B/*hns* will be confirmed by a simple phage spot test (Clokie 2009). One hundred µL of a 24-h culture of *P. aeruginosa* ATCC 15692 will be confluently spread across the surface of two MHA plates labeled I and II, corresponding to supernatant filtrates I and II, previously isolated; both plates will be air dried for 30 min. Five µL of each supernatant filtrate will be spotted onto its respective plate in triplicate. As a control, 5 µL of MHB will be spotted onto each plate in

duplicated. Both plates will again be air dried for 30 min, then inverted and incubated at 37°C for 24 h. After 24 h, the plates will be observed for the presence or absence of plaques.

Pf1B/*hns* MIC assay

The concentration of phage particles, plaque forming units per mL (pfu/mL), in the filtrates of Pf1B and Pf1B/*hns* will be determined by plaque assay, as previously described. *P. aeruginosa* antibiotic MIC in the presence of phages Pf1B and Pf1B/*hns* will be evaluated using ThermoFisher Sensititre GN4F antimicrobial sensitivity plates. Sensititre GN4F plates contain lyophilized antibiotics at concentrations suitable for clinical sensitivity testing. Sensititre GN4F plates require 50 µL of the subject organism, at an inoculum concentration (IC) of 1.0 x 10^5 cfu/mL, to be added to the appropriate wells (Gould and Meer 2011). The plates are incubated at 37°C and read after 18 to 24 h. A 24-h culture of *P.aeruginosa* ATCC 15692 will be used to start thee 10 mL inoculum cultures, labeled A, B and C in MHB. Culture A will contain *P. aeruginosa only* at the required IC. Culture B will contain *P. aeruginosa* at IC along with phage Pf1B in a 1:1 ratio. (5 mL of *P. aeruginosa* at 2X IC + 5 mL of Pf1B at 2X IC). Culture C will contain *P. aeruginosa* at IC along with Pf1B/*hns* in a 1:1 ratio. To verify the integrity of the Sensititre GN4F plates, a control organism, such a *Escherichia coli* ATCC 25922, with known MIC values, will be setup in an identical manner to culture A. Sensititre GN4F plates with each inoculum culture will be setup in duplicate. The plates will be incubated for 24 h at 37°C. The MIC values will be read after 24 h.

Expected Results

Pseudomonas aeruginosa strain ATCC 47085 should permit the propagation of phage Pf1B/*hns* without any loss of cell viability. The novel phage Pf1B/*hns* is expected to be infectious and viable, the plaque assay should produce visible plaques. *P. aeruginosa* cells which received no DNA, cuvette II, should produce no phage plaques; the negative controls of the phage spot test, 5 µL of MHB, should also produce no plaques. Unmodified Pf1B should stymie the growth of *P. aeruginosa* in culture B and this is expected to produce a downward shift in the MIC when compared to an absence of phage, as in culture A. A more pronounced decline in the MIC is expected in the presence of phage Pf1B/*hns* (Table 1)

Table 1. Expected minimum inhibitory concentration (MIC) in µg/mL of antibiotics in the presence of Pf1B and Pf1B expressing *hns*

Antibiotic	**(A)*P. aeruginosa***	**(B)Pf1B**	**(C)Pf1B/*hns***
Ampicillin	16	8	**0.5**
Aztreonam	8	2	**1**
Cefazolin	32	16	**0.5**
Ceftriaxone	64	32	**1**
Minocycline	8	4	**1**
Nitrofurantoin	64	16	**0.25**
Tetracycline	16	8	**1**
Tigecycline	16	8	**0.1**

Future Directions: Determine the full effective range of novel phage Pf1B/*hns*.

To evaluate the effective range of novel phage Pf1B/*hns*, it should be tested against numerous clinical isolates of multidrug resistant *P. aeruginosa*. The full range of clinically relevant antibiotics should be tested as well, since Pf1B/*hns* might now render *P. aeruginosa* sensitive to previously unusable antibiotics.

SUMMARY

New therapeutics are needed to combat the increasing resistance of bacterial pathogens to traditional antimicrobials; using bacteriophages will be an effective countermeasure. Shooley *et al.* (2017) demonstrated that phage therapy can be effective in a human patient when traditional antibiotics have failed. This project will utilize a genetically modified *Pseudomonas* phage , Pf1B, to eliminate strains of *Pseudomonas* that are untreatable by traditional antibiotics. To reduce the chances of *Pseudomonas* developing resistance ,this approach uses native bacterial component; a phage induced from *Pseudomonas* itself and an innate HNS bacterial protein. This project proposes a valuable tool for the complete eradication of resistant *Pseudomonas aeruginosa*.

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