Bacteriophages against Colistin-resistant Klebsiella *pneumoniae*

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Klebsiella pneumoniae is one of the most common causes of hospital-acquired infections, and is becoming increasingly resistant to various agents including colistin, a last resort antibiotic. As there are limited treatment options for these types of infections, bacteriophages active against them are drawing attention as a potential treatment option. To identify such bacteriophages, environmental water samples were screened against five different colistin-resistant clinical strains. As a result, two individual bacteriophages, MC8 and MC9, with activity against a colistin-resistant *Klebsiella pneumoniae* strain were isolated. Both bacteriophages were exclusively active against *K. pneumoniae* Sequence Type (ST) 258 clade I strains, except for MC9, which was also active against an additional *K. pneumoniae* ST433 strain. Sequence analysis of the two bacteriophages showed identical sequences except for a 52 base pair nucleotide deletion in the tail spike gene in MC9. Generation of phage-resistant mutants to each bacteriophage was performed to identify their targets. Upon analysis of the phage-resistant mutant sequences, an insertion sequence was found in the *wcaJ* gene encoding undecaprenyl-phosphate glucose-1-phosphage transferase. This gene helps catalyze the synthesis of colanic acid, a polysaccharide in the extracellular membrane, and a known attachment point used for phage entry.

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1.0 Introduction

1.1 Antibiotic Resistance

The era of antibiotics started when Sir Alexander Fleming discovered penicillin in 1928. With the discovery of additional antibiotics through the 20th century, dubbed the "antibiotic era," scientists believed that infectious diseases could be eradicated (14). The number one cause of death in the United States in 1900, at 45.4%, was infectious disease. By 1997, only 4.5% of deaths were caused by infectious disease due to the availability of antibiotics, along with other factors such as improved hygiene and access to clean water (8).

However, increasing antibiotic resistance threatens this trend. Resistant strains against penicillin were discovered before the drug was used as a therapeutic. Once penicillin was used in clinics, resistance became widespread. By the mid-1950s, plasmids that contained genes conferring resistance to penicillin were discovered which led to a boom in resistant strains (14). These plasmids can horizontally transfer in bacteria and disseminate resistance genes (5).

Antibiotic resistance is an increasingly prevalent problem today as more and more bacteria are becoming multidrug-resistant. These types of bacteria are estimated to cause 2.8 million infections and 35 thousand deaths per year in the United States alone (5). Our laboratory is specifically interested in strains resistant to colistin.

Colistin is considered a last resort antibiotic. It is only used when there are no other treatment options left to treat infections due to its nephrotoxicity and neurotoxicity. However, resistance to colistin has also been increasing, leaving no additional treatment options for

multidrug-resistant organisms (12). This has led to the resurgence of interest in bacteriophage therapy against multidrug-resistant infections (11).

1.2 *Klebsiella pneumoniae*

K. pneumoniae is the most common nosocomial infection in the United States (12). It is a gram-negative rod bacterium that colonizes human gastrointestinal mucosal surfaces and can cause urinary tract infections, respiratory tract infections, meningitis, and sepsis. *K. pneumoniae* accounts for approximately 11.8% of all hospital-acquired pneumonia in the world (1).

Resistance of *K. pneumoniae* to extended-spectrum beta-lactams and carbapenems has increased in the past decade, leading to increased use of colistin as a last-resort treatment. From 2009 to 2019, colistin resistance in carbapenem-resistant *K. pneumoniae* has increased from less than 2% to 9% worldwide (12).

There are many sequence types (ST) within *K. pneumoniae* that divide the species into genetically related groups. Specifically, our laboratory focuses on ST258, an epidemic strain of *K. pneumoniae* that is the most prevalent ST in the United States. This ST is broken up into two clades, Clade 1 and Clade 2, that differ on their type of capsule polysaccharide that is encoded by the *cps* gene (18).

1.3 Bacteriophage Biology

Bacteriophages (phages) are viruses that infect bacteria by attaching to the bacterial cell surface by the tip of the bacteriophage tail (4). This term “bacteriophages” was coined back in 1917 by Frederick d’Hérelle while he was studying a dysentery breakout and noticed holes in the bacterial lawn. d’Hérelle hypothesized that these bacteriophages were viruses that could treat infections from bacteria, but this theory was met with a lot of controversy. In 1940 with the new discovery of the electron microscope, phages were able to be imaged and the existence of bacteriophages was widely accepted. But by this time, the use of antibiotics caused them to fall off the radar in the scientific community (4). Now that we are facing elevated levels of antibiotic resistance, research and development of bacteriophages as potential therapeutics are becoming of interest (16).

Bacteriophages can enter the cell and either go through a lytic or lysogenic replication cycle. Lytic phages immediately start replicating by transcription and translation of their DNA followed by assembly of the phage and finally lysing the bacterial cell. On the other hand, lysogenic phages incorporate their DNA into the host genome and may or may not be induced to re-enter their lytic phage to reproduce. For this project, we were most interested in lytic phages, which can immediately reproduce and kill the bacterial host (3). This can eventually lead to clearance of bacterial infections in practice (8).

Since phages attach to a specific part of the bacterial cell wall, they are highly specific in the type of bacteria they infect (8). This is an advantage in using bacteriophages for treatment because the beneficial bacteria that live in the body are unaffected by treatment, unlike with

traditional antibiotics that have a broader host range (18). However, this specificity can also be a problem because the phage is very limited on the type of infections it can treat (11).

Another benefit of treatment with bacteriophages is that they can self-replicate in the body by infecting their target bacteria and proliferating. Therefore, fewer numbers of phages may be needed to have lasting treatment (11). Although bacteriophages are a promising treatment option, generation of phage-resistant bacteria is a problem. More research studying the generation of phage-resistant mutants is needed to understand how it can be mitigated (10).

2.0 Statement of Project and Specific Aims

The goal of this project is to isolate bacteriophages active against colistin-resistant pathogens. Colistin-resistant pathogens are left with limited treatment options. With the discovery of new lytic bacteriophages, a new treatment option can become available. First, characterization of the bacteriophages needs to be performed. Then, phage-resistant mutants are generated in order to identify the target of the bacteriophage and potentially resistance mechanisms that can happen in practice.

2.1 Aim 1

Aim 1 was to isolate and characterize bacteriophages as a potential therapeutic option against colistin-resistant gram-negative bacteria. Isolated active bacteriophages were screened to determine their host range. Finally, we analyzed the genomes of susceptible bacterial strains to predict which future strains the bacteriophage would be active against.

2.2 Aim 2

Aim 2 was to produce phage-resistant bacterial strains. Extracted DNA of phage-resistant mutants were sequenced and compared with the susceptible wild-type parent strain. Comparing the genomes of the phage-resistant mutants to the wild-type helps elucidate mutations in targets involved in the entry of the phage into the host bacteria.

3.0 Material and Methods

3.1 Screening of Environmental Water Samples for Active Phage

Environmental samples that were collected from water sources in the Pittsburgh area were initially centrifuged to collect debris in 50 mL aliquots. Then, the supernatant was collected and filtered through an Amicon® 100 kDa filter (MilliporeSigma, Burlington, MA) tube by centrifugation. Phage screening was performed using a soft agar overlay assay as described next. The bacterial strains were incubated overnight in Brain Heart Infusion (BHI) Broth. BHI bottom agar (1.5% agar + 1 mM $CaCl_2$ and 1 mM $MgCl_2$) was prepared in square bottom petri dishes. Bacterial overnight cultures (100 µL) and the filtered environmental water sample (100 µL) were mixed and incubated for 5 minutes at room temperature. After incubation, 5 mL of BHI top agarose (0.5% agarose + 1 mM $CaCl_2$ and 1 mM $MgCl_2$) that was cooled to 55°C was mixed with the water/bacterial mixture and immediately overlaid on the bottom agar plate. The plates were incubated overnight 35°C and the plates were inspected for zones of clearing that would indicate plaque formation.

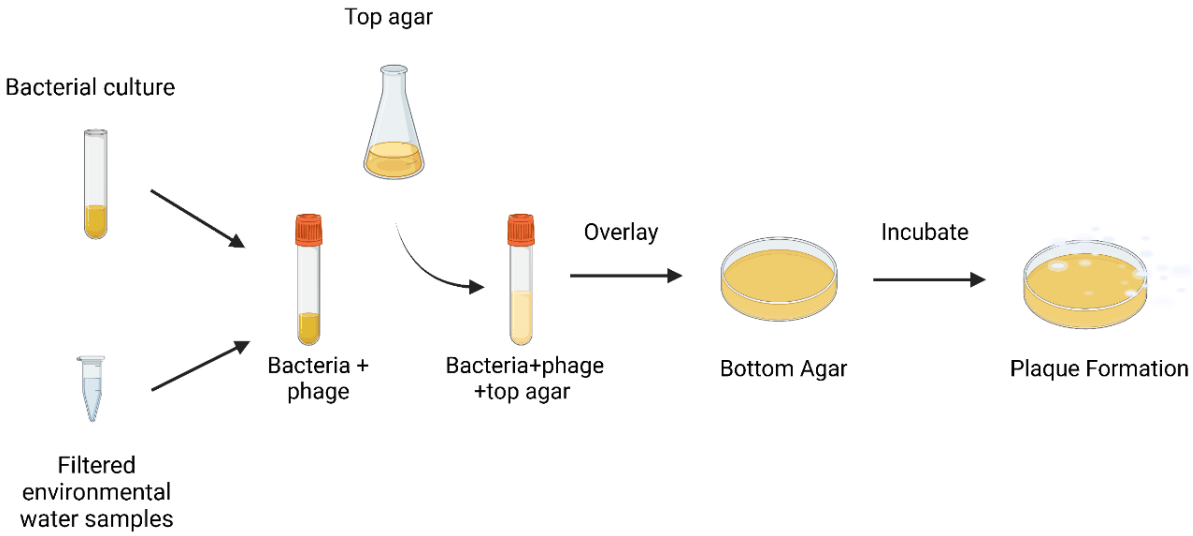

Figure 1: Protocol for Screening of Filtered Environmental Water Sample with Bacterial Culture of Interest

3.2 Passaging Plaques

The plaques were picked using a 200 µL pipet tip. The pipet tip was dropped into a 15 mL conical tube containing 100 µL of SM buffer (50 mM TrisCl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$) and incubated overnight shaking at 35°C. Also, the host bacterial strain was inoculated in BHI broth and grown overnight shaking at 35°C. The next day, plates of bacterial lawns were made by mixing 100 µL of the overnight bacterial culture with 5mL of molten BHI top agarose cooled to 55°C. The top agarose was overlayed on BHI bottom agar plates. The picked plaques grown overnight were diluted into a 10-fold serial dilution series using SM buffer. Five microliters of each dilution were plated onto the bacterial lawn and left to dry. The plates were then incubated overnight at 35°C and observed the next day for zones of clearing that would indicate plaque

formation. A second round of plaque passaging was performed by picking a single plaque and growing in SM buffer using the same method. This procedure was performed four times to generate pure phage stock. Then, titering and phage stock generation was performed.

3.3 Preparation of High-Titer Stocks of Phage

Bacterial cultures were made of the host bacterial strain by inoculating tryptic soy broth (TSB) and incubating overnight shaking at 35°C. A single plaque picked from the fourth round of passaging was incubated in 100 µL of SM buffer overnight. The overnight bacterial culture (1 mL) was pelleted by centrifugation at 8,000 rpm for 10 minutes and resuspended in 1 mL of SM buffer. The bacterial sample was diluted 1:10 fold in SM buffer and 100 µL of the 1:10 diluted bacteria was mixed with 100 µL of the single phage plaque. This mixture was incubated for 5-10 minutes at room temperature. Bottom agar plates were prepared with TSB and 1.5% agar in large 150 mm petri dishes. Molten top agarose with TSB (10 ml) and 0.5% agarose was prepared and mixed with the bacterial and phage sample. This was overlaid on top of the bottom agar and left to incubate overnight at 35°C. The next day, the plate was observed for a clearance of a lawn of bacteria. SM buffer (10 ml) was applied to the top of the plate and incubated at 35°C for 1 hour with occasional swirling. After incubation, the top supernatant was collected with a serological pipette and centrifuged at 8,000 rpm for 10 minutes to pellet the bacterial cells from the solution. The supernatant containing the phage was filtered through a 0.22-µm filter to completely remove any remaining bacteria.

3.4 Spot Dilution of Plaques

Bacterial cultures of strains of interest were made by inoculating BHI broth and incubating overnight shaking at 35°C. The next day, plates of bacterial lawns were made by mixing 100 µL of the overnight bacterial culture with 5 mL of molten BHI top agarose cooled to 55°C. The top agarose/bacterial mixture was overlaid onto BHI bottom agar plates. High-titer stocks of phages were diluted 10-fold to a 1 x 10^{-15} dilution in SM buffer. Each dilution (5 µL) was plated onto the plates containing the bacterial lawn and left to dry. The plates were incubated overnight at 35°C and observed for plaque formation. Plaques were counted for each dilution and used to determine the plaque forming units of each phage for each strain tested. Plaque forming units were determined by dividing the number of plaques by the dilution factor.

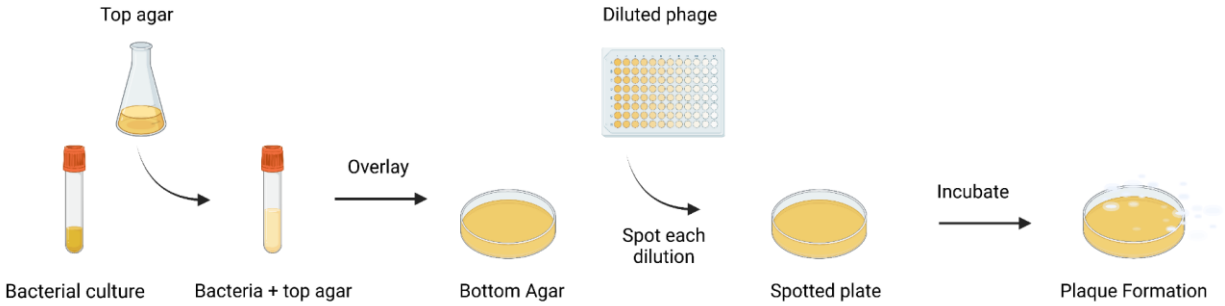

Figure 2: Protocol for Spot Dilution of Phages Against Bacterial Strains

3.5 Phage DNA Extraction and Sequencing

The phage lysate (250 µL) was incubated with 0.25 µL of DNase (1000 U/mL) (Promega, Madison, WI) and 0.25 µL of RNase (10 mg/mL) (ThermoFisher Scientific, Waltham, MA) for 10 minutes at room temperature. After this incubation, DNA was extracted using the Qiagen DNeasy Blood and Tissue kit. The extracted phage DNA samples were sent to SeqCenter (Pittsburgh, PA) for Illumina sequencing. The samples were indexed using IDT 10-bp UDI indices and sequenced to a depth of 200 Mbp (Illumina, San Diego, CA). Further analysis comparing the genomes to each other was performed using CLC Genomics Workbench (Qiagen, Hilden, Germnay) and Mauve (DNASTAR, Madison, WI).

3.6 Generation of Phage Resistant Bacterial Mutants

Phage-resistant bacterial mutants were generated using the colistin-resistant *K. pneumoniae* clinical strain I2. I2 was incubated overnight shaking at 35 µL in BHI broth. The next day, 100 µL of the overnight culture was added to 5 mL of molten BHI top agarose cooled to 55°C and plated onto a square petri dish containing a bottom layer of BHI bottom agar. Undiluted phage lysate (10 µL) was spotted on top of the top agarose. The plates were incubated at 35°C for at least 24 hours. Bacterial colonies that grew in the kill zone of the phage were restruck onto Luria-Bertani (LB) agar. An overnight culture of the restruck bacterial cultures were grown in BHI broth. Bacterial lawns of both the wildtype bacterial strain and the candidate mutant strains were made using BHI top agarose on top of BHI bottom agar square plates. Ten microliters of the phage-

resistant bacterial mutant candidate strains were spotted onto a lawn of the wildtype bacteria strain to check for competitive action at the border. Competitive action between the cultures would cause any prophages to be released. True phage-resistant bacterial mutants should have no zone of inhibition from prophages. Undiluted phage (10 µL) was spotted onto a lawn of the candidate phage-resistant bacterial mutants to check for a zone of inhibition. The plates were incubated overnight at 35°C and observed the next day. After confirming the phage-resistant mutant bacterial strains had no zone of inhibition from the phage, they were frozen at -80°C in a nutrient broth and glycerol stock and retested at a later date to ensure that the mutant did not revert back to susceptibility to the phage. DNA was extracted from the phage-resistant mutant bacterial strains using the Blood and Tissue kit (Qiagen) and sent for Illumina sequencing at SeqCenter (Pittsburgh, PA).

3.7 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed using Sensititre Gram Negative AST Plates (GNX3F) and Sensitire Gram Negative RUO Susceptibility Testing Plates (MDRGNX2F) (ThermoFisher Scientific, Waltham, MA). Phage-resistant bacterial mutants and the parental bacterial strain were grown overnight on Mueller-Hinton agar at 35°C. A 0.5 MacFarland solution of each strain was made in 0.85% sodium chloride using a colorimeter. A 1:100 dilution was made of each 0.5 MacFarland solution in cation-adjusted Mueller Hinton broth. Each well of the Sensititre microdilution plate was inoculated with 50 µL of the bacterial dilution. The plates were incubated at 35°C for 20-24 hours and observed for growth.

4.0 Results

4.1 Screening of Environmental Water Samples for the Presence of Active Phage Against Gram-Negative Bacteria

Five previously identified colistin-resistant gram-negative clinical strains that included *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, were screened against 12 water samples collected from around the Pittsburgh area to isolate potential active phages. Two phages isolated from water sample MC8 and one phage isolated from water sample MC9 were active against the colistin-resistant *Klebsiella pneumoniae* strain I2. High titer stocks were produced of these three phages for further testing and sequencing.

Table 1: Environmental Water Sample Screening Against Colistin-Resistant Gram-Negative Bacteria

	Environmental Water Sample											
Bacterial Strain	#1	#2	#3	#4	#5	#6	#7	E12	E25	MC6	MC8	MC9
E. cloacae DVT 899	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa DVT 402	-	-	-	-	-	-	-	-	-	-	-	-
A. baumannii DVT 1458	-	-	-	-	-	-	-	-	-	-	-	-
A. baumannii DVT 1459	-	-	-	-	-	-	-	-	-	-	-	-
K. pneumoniae I2	-	-	-	-	-	-	-	-	-	-	+	+

4.2 Genome Analysis of Phages

The three isolated phages were sequenced by Illumina sequencing and the reads were assembled using CLC Workbench (Qiagen, Hilden, Germany). The phage genomes were compared to sequences in the GenBank database through Basic Local Alignment Search Tool (BLAST), and were shown to be closely related to other Myoviridae phages. The two phages isolated from water sample MC8 were determined to be the same phage due to identical phage sequences using Mauve alignment through the MegAlign Pro software (DNASTAR, Madison, WI). MC9 was closely related to the phages from MC8, except for a 52 nucleotide base pair deletion in the phage tailspike protein.

4.3 Host Range

After high titer stocks were obtained, the phages were tested against additional *K. pneumoniae* strains to determine their host range. A variety of sequence types (STs) were selected and tested. The phages were mostly active against ST258, the most common ST type of multidrug-resistant *K. pneumoniae* in the United States. In particular, the phages were active only against strains in Clade 1 of ST258. The phage isolated from MC9 was also active against an ST433 strain, where the phage isolated from MC8 was not. The additional STs tested also included a hypervirulent *K. pneumoniae* strain (ST86) and both phages were not active against this strain. The activity of the phages was also calculated by determining the phage forming units (pfu) of each phage against the various *K. pneumoniae* strains.

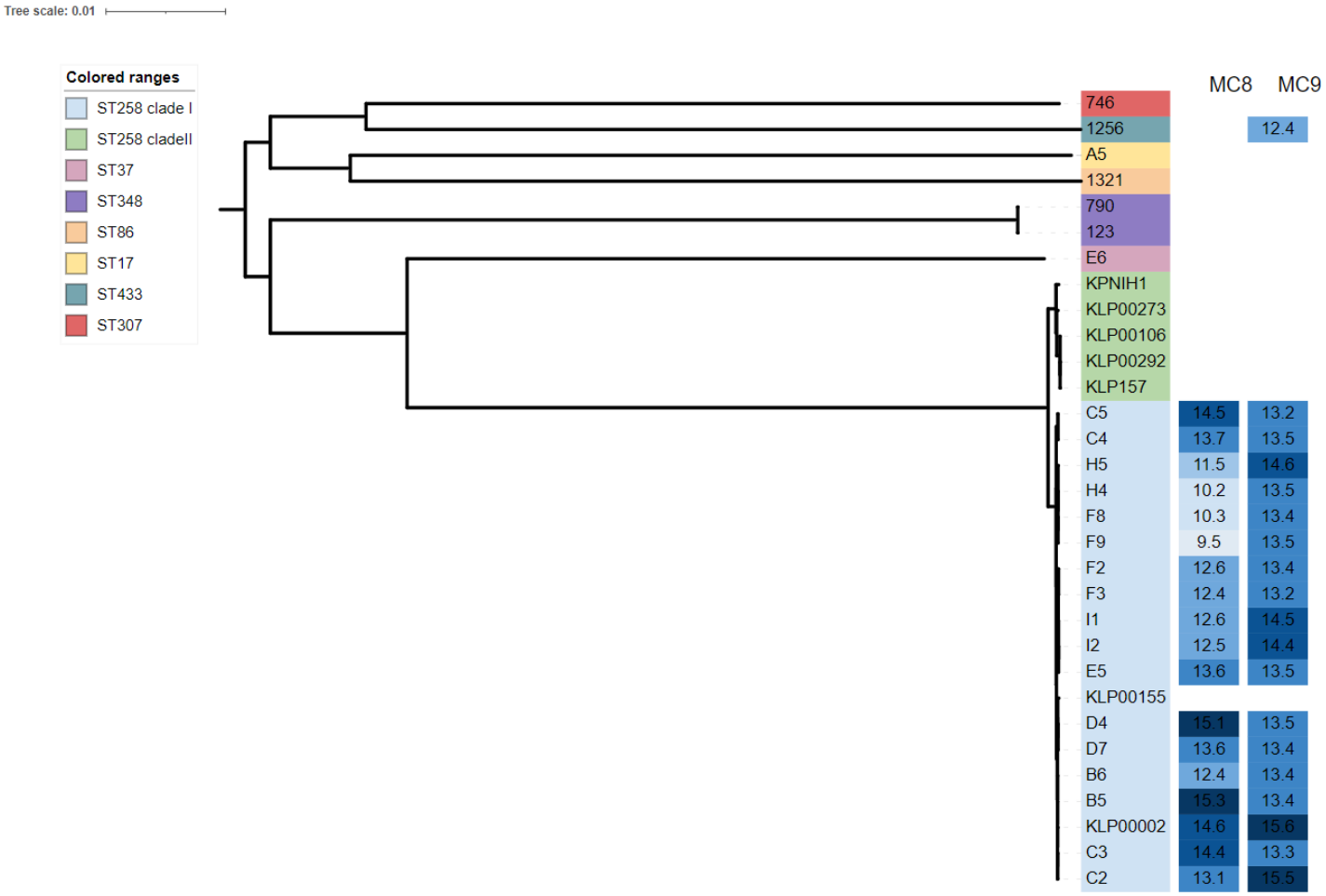

Figure 3: Infectivity of Isolated Phages Against K. *pneumoniae* Strains

K. pneumoniae strains are organized by phylogeny based on their genomes and labelled by color according to their responding ST. Infectivity is shown as the $\log_{10}$ titer (PFU/mL) of each phage against active strains. The blue shading correlated with phage activity, with the darker shading being more active and the lighter shading being less active.

4.4 Generation of Phage-Resistant Mutants

Phage-resistant mutants of *K. pneumoniae* I2 were generated using spontaneous mutagenesis of the phages from the MC8 and MC9 water samples. A mutant generated from each phage was sent for Illumina sequencing. Breseq analysis was performed on the sequencing data comparing the mutant sequences against the parent strain I2 (7). Analysis showed that there was an insertion sequence within *wcaJ*, encoding an enzyme that helps catalyze the production of colonic acid in the membrane. Colanic acid is polysaccharide that is found on the outside of the cell membrane (15).

Our lab had previously made a knockout of *wcaJ* in strain F8 (Figure 2). The knockout strain had a loss of function of the gene *wcaJ* in strain F8. Both phages were active against F8, but not active against the knockout. This leads us to believe that synthesis of colanic acid of *K. pneumoniae* is important for entry into the cell, and disruption of this can lead to phage resistance.

It has been noted in the literature that mutations in antibiotic-resistant bacteria that lead to phage resistance can cause conversion back to susceptibility to various conventional antibiotics (2, 13). Using commercially available plates, the antibiotic resistance profile of the phage-resistant mutants was compared to the parent strain (Sensititre, Thermo Fisher, Waltham, MA). There was no significant difference observed with any of the tested antibiotics between strains.

Table 2: Breseq Output of I2-MC8-R and Wildtype I2

Predicted mutations						
evidence	seq id	position	mutation	annotation	gene	description
RA	1	1,091,073	C→A	L233M (CTG→ATG)	*ycaD_1* →	putative MFS-type transporter YcaD
RA	1	3,529,349	G→A	intergenic (-47/-145)	*fadB* ← / → *pepQ*	Fatty acid oxidation complex subunit alpha/Xaa-Pro dipeptidase

Unassigned new junction evidence											
		seq id	position	reads (cov)	reads (cov)	score	skew	freq	annotation	gene	product
*	?	1	1549482 =	0 (0.000)	139 (0.810)	49/260	0.6	65.40%	coding (1142/1152 nt)	*wcaJ*	UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase
–	?	3	= 19622	147 (0.850)					intergenic (+15/-147)	*ACFLOFFG_05319/merC*	IS1 family transposase ISKpn14/Mercuric transport protein MerC
*	?	1	= 1549489	15 (0.090)	141 (0.820)	43/262	1	60.50%	coding (1135/1152 nt)	*wcaJ*	UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase
–	?	3	18855 =	169 (0.970)					coding (690/711 nt)	*ACFLOFFG_05317*	hypothetical protein
*	?	5	38226 =	86 (0.390)	108 (0.540)	41/236	1.4	51.30%	coding (867/2418 nt)	*ACFLOFFG_05521*	hypothetical protein
–	?	5	41751 =	128 (0.650)					intergenic (-74/-50)	*ACFLOFFG_05523/hin_2*	hypothetical protein/DNA-invertase hin
*	?	5	= 38238	86 (0.390)	120 (0.600)	39/236	1.6	53.90%	coding (879/2418 nt)	*ACFLOFFG_05521*	hypothetical protein
–	?	5	= 41737	128 (0.650)					intergenic (-60/-64)	*ACFLOFFG_05523/hin_2*	hypothetical protein/DNA-invertase hin

Table 3: Breseq Output of I2-MC9-R and Wildtype

Predicted mutations						
evidence	seq id	position	mutation	annotation	gene	description
RA	1	3,529,349	G→A	intergenic (-47/-145)	*fadB* ← / → *pepQ*	Fatty acid oxidation complex subunit alpha/Xaa-Pro dipeptidase
MC JC	3	47,032	Δ2,376 bp		*[ACFLOFFG_05343]–[ACFLOFFG_05345]*	*[ACFLOFFG_05343]*, *proQ_2*, *[ACFLOFFG_05345]*

Unassigned new junction evidence											
		seq id	position	reads (cov)	reads (cov)	score	skew	freq	annotation	gene	product
*	?	1	1549482 =	5 (0.020)	218 (0.980)	65/256	0.3	64.60%	coding (1142/1152 nt)	*wcaJ*	UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase
–	?	3	= 19622	234 (0.960)					intergenic (+15/-147)	*ACFLOFFG_05319/merC*	IS1 family transposase ISKpn14/Mercuric transport protein MerC
*	?	1	= 1549489	16 (0.080)	181 (0.810)	54/258	0.9	56.50%	coding (1135/1152 nt)	*wcaJ*	UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase
–	?	3	18855 =	263 (1.070)					coding (690/711 nt)	*ACFLOFFG_05317*	hypothetical protein

Table 4: Minimum Inhibitory Concentrations (µg/ µL) of Antibiotics of Phage-resistant and Wild-type Strains

	Strain		
Antibiotics	MC8-1-R-I2	MC9-R-I2	I2 Parent
Amikacin	>32	>32	>32
Doxycyline	4	4	4
Gentamicin	2	2	2
Minocycline	8	4	8
Tobramycin	>8	>8	>8
Ciprofloxacin	>2	>2	>2
Trimethoprim/ Sulfamethoxazole	>4/76	>4/76	>4/76
Levofloxacin	>8	>8	>8
Aztreonam	>16	>16	>16
Cefepime	>16	>16	>16
Meropenem	>8	>8	>8
Colisitin	>4	>4	>4
Polymixin B	>4	>4	>4
Ceftazidime	>32	>32	>32
Cefotaxime	>32	>32	>32
Ampicillin/ Sulbactam 2:1 ratio	>64/32	>64/32	>64/32
Doripenem	>4	>4	>4
Piperacillin/ Tazobactam	>64/4	>64/4	>64/4
Ticarcillin/ Clavulanic acid	>128/2	>128/2	>128/2
Ceftazidime/ Avibactam	1/4	1/4	1/4
Ceftolozane/ tazobactam	>8/4	>8/4	>8/4
Delafloxacin	>1	>1	>1
Ervacycline	0.5	0.5	1
Cefiderocol	16	16	16
Imipenem	>16	>16	>16

Imipenem/ Relebactam	0.25/4	0.25/4	0.25/4
Fosfomycin+glucose-6-phosphate	128	128	>128
Meropenem/ Vaborbactam	0.5/8	1/8	0.5/8
Omadacycline	4	4	8
Plazomicin	1	1	1

5.0 Discussion

Colistin-resistant *K. pneumoniae* is a prevalent problem in our healthcare system that is only increasing. Bacteriophages show that they are a promising treatment option for those infections with no other option. In this study, two different phages, MC8 and MC9, were isolated from environmental water samples that were mainly active against *K. pneumoniae* ST258 clade I strains, an epidemic multidrug-resistant strain in United States.

The only difference in host range between the two phages was that MC9 was also active against an ST433 strain. This could be due to a small deletion in the tailspike protein of MC9. The tailspike protein in bacteriophages is important for the attachment of the bacteriophage to the bacterial cell, so an altered protein could cause this difference. It has been noted in the literature that the tailspike protein is vital to help determine the host range (8, 17).

We identified a mutation in the *wcaJ* gene in our generated *K. pneumoniae* phage-resistant mutant strains. This is a gene in the *cps* locus in the membrane that encodes a glycosyltransferase. This functions as an enzyme that initiates the biosynthesis of colanic acid. It has been reported that several phages use colanic acid as a receptor for entry into the cell. Since *wcaJ* functions to catalyze colanic acid, we believe that the entry point for the bacteriophage is in the membrane of the bacteria (15). This also explains the limited host range to only specifically *K. pneumonia*e ST258 clade I since the *cps* locus varies by ST. Previously in a study by Tan *et al*., they demonstrated that a mutation in *wcaJ* can lead to phage resistance in *K. pneumoniae* (15).

The absence of *wcaJ* reportedly also causes an increase in polymyxin resistance (15). It is well documented that phage-resistant mutants can revert to susceptible or have a different antibiotic resistance profile. Since the absence of *wcaJ* seems to increase resistance, this mutation is not likely to revert the bacteria to susceptible which was found through our antibiotic susceptibility testing.

Some limitations include not having additional *K. pneumoniae* ST433 strains to test against bacteriophage MC9 to help confirm the host range. In the genomic characterization, there were some portions of the bacteriophage sequences that were not well resolved. Since these experiments were all done *in vitro,* further testing *in vivo* testing should be performed in order to determine clinical therapy.

6.0 Conclusions

Two novel bacteriophages were isolated from environmental water samples. The bacteriophages were identical except for a 52 nucleotide base pair deletion in the tail spike protein. These bacteriophages were selectively active against *K. pneumoniae* ST258 Clade I. One bacteriophage, MC9, showed activity against *K. pneumoniae* ST433. Generation of phage-resistant mutants showed an insertion sequence in *wcaJ,* an enzyme that catalyzes production of colanic acid in the polysaccharide of the extracellular membrane. This suggests that the entry point of the bacteriophages is colanic acid, an entry point also confirmed by a study by Tan *et al*. (15).

7.0 Public Health Significance

Antibiotic-resistant bacteria poses a huge challenge in the future of public health. These types of infections will only increase and become more drug resistant. As multi-drug resistant strains are becoming more common, treatments options remain limited (5). Specifically, *K. pneumoniae* is one of the most common healthcare acquired infections. This type of bacteria alone burdens public health and is increasing in resistance to carbapenems and colistin (12). Bacteriophages are a promising treatment option for multi-drug infections. They can help cure infections which have no available susceptible antibiotics left as treatment options. The discovery of novel lytic bacteriophages can help ease the burden of antibiotic-resistant infections on public health (16).

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