# Spread Prevention and Eradication of Resistant Bacterial Growth (SUPERBUG)

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**Abstract**

Diseases caused by drug resistant bacteria are one of the leading causes of death in the United States, and they are becoming a pressing public health concern due to the lack of new antibiotics and the evolution of multidrug resistance. Drug resistance is an inequitable quandary, disproportionately affecting minorities and people of lower socio-economic status. Resistance is caused by genes on the chromosome or plasmids. CRISPR-Cas9 gene editing has been shown in recent studies to successfully edit resistance genes to increase susceptibility to antibiotics. The current study aims to use a bacteriophage delivery mechanism to insert a CRISPR-Cas9 system into nalidixic acid resistant *Escherichia coli* and sensitize it to nalidixic acid. We aim to improve upon the efficiency of previous studies.

*Keywords*: Bacteriophage, Gibson Assembly, CRISPR, Antibiotic Resistance, *Escherichia coli*, Nalidixic Acid

## Glossary

**ATTC** – The American Type Culture Collection

**Bacteriophage** – A virus which only infects bacteria.

**Capsid** – The head of a bacteriophage, wherein its genome is stored.

**Commensal Bacteria** – Bacteria residing within an organism which benefit rather than harm it.

**Conjugation** – A process by which a donor bacterium transfers a copy of some of its genetic material, usually in the form of a plasmid, to a recipient bacterium.

**Electroporation** – A lab technique that allows DNA to enter cells using electric shocks.

***Escherichia coli*** – a strain of bacteria from the family Enterobacteriaceae.

**Genome** – The aggregate genetic material contained within a population or an organism.

**Headful Packaging** – A mechanism by which, during bacteriophage construction from constituent proteins and nucleic acids, the capsid packages DNA until it is full.

***In vivo*** – Within a living organism.

**Microinjection** – Delivery of substances into a single cell via a very thin needle.

**Nephrotoxicity** – Toxicity to the kidneys.

**Nuclease** – An enzyme responsible for initiating and carrying out a cleavage bond between phosphodiester bonds in DNA replication of cells.

**Penicillin, Methicillin** – Names of two common antibiotics.

**Plasmid** – A piece of independently replicating circular DNA residing inside of a bacterium.

**Sticky Ends/Cohesive Ends** – Overhanging single-stranded DNA on one end of a chromosome that can bind to another complementary sticky end.

**Transduction** – The transfer of genetic material from one cell to another via a virus.

## Spread Prevention and Eradication of Resistant Bacterial Growth

Since the introduction of the first antibiotics in the late 1930s, bacterial resistance to those antibiotics has followed hand in hand. Drug resistance to penicillin was found in bacteria before penicillin was even formally approved as a therapeutic (Davies & Davies, 2010). The 'golden age' of antibiotic discovery is said to be 1950 to 1970, when all the classes of antibiotics that we know of today were discovered. After 1970, researchers have not discovered any new classes of antibiotics (Aminov, 2010). Coupled with this lack of new treatments is a rise in multidrug resistant bacteria (Nadimpalli et al., 2021).

In 2021, multidrug resistant infections were the third leading cause of death in the United States (Nadimpalli et al., 2021). Treatment choices are limited for multidrug resistant infections, often worsening the severity of these conditions (Aminov, 2010). 'Superbugs' are defined by J. Davies & D. Davies (2010) as microbes that are highly resistant to the antibiotics specifically designated for treating them. One significant superbug is methicillin-resistant *Staphylococcus aureus* or MRSA, which started as methicillin-resistant and has now become an umbrella term to describe multidrug-resistant *S. aureus*. This superbug is significant because of its increased community spread. It is an example of superbugs that are no longer restricted to the hospital setting (Davies & Davies, 2010).

A systematic review showed that in 2019 multidrug resistance was associated with 4.95 million deaths globally (Murray et al., 2022). Bacteria resistant to antibiotics traditionally considered as the first line of defense were responsible for 70% of those deaths. Healthcare systems and areas with fewer resources to test for and treat these infections saw greater incidence of infection in their patients (Murray et al., 2022). Thus, this already pressing problem of drug resistance is of even greater significance to those least equipped to face it.

Drug resistance is also inequitable in terms of who is at greatest risk of disease. According to Alividza and colleagues (2018), people of lower socio-economic status are at seven times greater risk of contracting *Streptococcus pneumoniae* and *Acinetobacter baumannii* infections compared to those in wealthy nations. Crowding, homelessness, and lack of education, all factors linked to lower socio-economic status, were also linked to resistant infections. Those of lower socio-economic status also tend to have worse patient outcomes, as they are less likely to have access to adequate healthcare (Alividza et al., 2018). There is also racial and ethnic inequality in the acquisition of drug resistant infections. Black and Hispanic people were also at greater risk for community acquired pathogens like MRSA (Nadimpalli et al., 2021). This may be due to the fact that minorities are more likely to live in crowded and multigenerational housing, which increases the risk of disease transmission in general. These minorities also tend to have more disease comorbidities that put them in hospital settings where they are more likely to acquire drug resistance (Nadimpalli et al., 2021).

Another pressing concern is the rise of resistance to last-line of defense antibiotics. Colistin is a drug that is often used only as a last resort due to side effects such as damage to kidney function and damage to the nervous system. With the rise of multidrug resistant infections, these last resort drugs are becoming necessary for use. However, the gene *mcr-1* was first described in 2015 and has been found to confer drug resistance to colistin (El-Sayed Ahmed et al., 2020). With the last-line of defense antibiotics now failing to adequately treat multidrug-resistant infections, new treatment options are desperately needed. This study focuses on improving an existing antimicrobial using gene editing technology, which could fill the treatment gap currently plaguing those infected with drug resistant bacteria. The research question this study is attempting to answer is: How can we effectively deliver a CRISPR-Cas9

gene editing system into a nalidixic-acid resistant bacterium in order to edit its resistance genes to make it susceptible to antibiotics? Our hypothesis is that introducing a homology directed repair edit to codon 83 of the *gyrA* gene in nalidixic-acid resistant *E. coli* through use of a CRISPR-Cas9 system delivered via bacteriophage M13 will significantly decrease the minimum inhibition concentration of nalidixic acid for bacterial cell death.

# Literature Review

## Antibiotic Resistance

### *1. Coping with Antibiotic Resistance*

If treatment of a bacterial infection by an antibiotic is rendered less effective by the expression of a gene harboured by the bacteria causing the infection, that strain of bacteria is said to be resistant to that antibiotic, and that gene is called an antibiotic resistance gene or a resistance gene (Alekshun & Levy, 2007). There are many mechanisms by which resistance genes may circumvent antibiotics, and thus many opportunities for bacteria to develop resistance to an antibiotic (Aminov, 2010). Resistance genes are disseminated most widely and rapidly when they are able to replicate and travel via mobile genetic elements such as plasmids (Davies & Davies, 2010), and there exist diverse means of this gene transfer (Levy & Marshall, 2004).

Today, there is a negative economic incentive for pharmaceutical companies to develop novel antibiotics (Chokshi et al., 2019), and numerous strains of pathogenic bacteria are rapidly becoming resistant to antibiotics currently in use (Aminov, 2010). Furthermore, the diversity of the planetary bacterial genome renders inevitable the proliferation of resistance to newly introduced antibiotics (Martinez, 2014). Therefore, recent research has focused heavily on novel means of combating bacteria which inherently avoid the development of resistance to them, such as phage therapy and gene editing technologies (Gholizadeh et al., 2020).

Our research focuses on the removal of resistance genes from bacteria via gene editing in order to allow such bacteria to be eliminated once more by antibiotics. We seek to develop a proof-of-concept demonstration of high efficiency gene editing which may

be applied *in vivo*. First, we considered the social and societal factors which contribute to the spread of resistance.

### *2. Social Overview*

Although antibiotic resistance develops naturally (Martinez, 2014), the misuse of antibiotics drastically accelerates this process, and every discovery of a new antibiotic leads to the appearance of antibiotic-resistant strains (Davies & Davies, 2010). Today, the strains of bacteria from which the majority of nosocomial infections worldwide originate are *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp., and Escherichia coli* (ESKAPE-E) (Ayobami et al., 2022). These bacteria not only contain intrinsic resistance genes, but also have the capability to acquire or develop multidrug resistance, especially as a result of selective pressure presented by the overuse of antibiotics. In hospitals, antibiotics are used not only to treat but also to prevent infection (Levy & Marshall, 2004). This overuse of antibiotics, coupled with the close proximity of patients and healthcare workers, contributes greatly to the spread of antibiotic-resistant infections (Chokshi et al., 2019). Furthermore, in countries where antibiotics are available over the counter, antibiotic misuse from self-medication is close to inevitable (Chokshi et al., 2019). Failure of patients to adhere to treatment regimens and instead relying on antibiotics to quickly resolve an illness should be addressed, as well as healthcare providers' judicious prescription of such antibiotics (English & Gaur, 2010). Healthcare providers must follow proper protocols and prescribe only in order to maximize clinical therapeutic effects while minimizing the risk of antibiotic resistance development.

Aside from the direct use of antibiotics through patient consumption, antibiotics incorporated in food-producing animals have been found to impact the development of antibiotic-resistant bacteria. In animals, antibiotics are often misused as preventatives against disease and as animal growth stimulants rather than treatment for disease (Chokshi et al., 2019). The complex relationship between humans and these animals has been found to facilitate the transmission of antibiotic-resistant strains into humans (Levy & Marshall, 2004). For example, the consumption of meat contaminated with antibiotic-resistant bacteria can lead to the acquisition of antibiotic-resistant infections in humans. It has also been established that the application of land manure has contributed to the spread of antibiotic resistance genes into the soil environment. (Levy & Marshall, 2004). This account emphasizes the swiftness of spreading antibiotic resistance accordant to human activity, and demonstrates how antibiotic resistance impacts society outside of healthcare.

## *Escherichia coli* and Nalidixic Acid Resistance

In order to carry out our research in combating antibiotic resistance, we first chose a model organism on which to perform our research, this being the bacterium *Escherichia coli*. *E. coli* is a large and diverse group of bacteria which are found in the environment, foods, and the intestines of people and animals (Blount, 2015). The primary reason why we chose to work with *E. coli* is that, while some strains of *E. coli* are harmful, most strains are harmless or commensal, making it a Biological Safety Level (BSL) 1 organism (Bayot & King, 2022). BSLs specify standards of protections for certain activities that take place in biological labs in order to protect laboratory personnel. Level 1 is the lowest biosafety level and applies to work with low-risk microbes that pose little to no threat of

infection (*Biosafety Levels*, 2015). This allows us to both procure and work with *E. coli* without having to go through cumbersome regulatory procedures or endanger ourselves. *E. coli* is, however, still a pertinent species, as antimicrobial resistance in *E. coli* has started to develop worldwide, which has caused treatment for infections by it to become more complicated (Rasheed et al., 2014). Harmless strains of *E. coli* can therefore be used in proof-of-concept experiments in order to find a method to combat antibiotic resistance in more harmful strains. *E. coli* is also a bacterium that is well researched, providing us with bountiful background literature on which to base our research (Blount, 2015). It was one of the earliest organisms to have its genome sequenced, allowing for thorough understanding of each gene and its function (Blount, 2015). *E. coli* is susceptible to many different antibiotic treatments, which allows us flexibility in choosing which antibiotic resistance gene to insert, target, and subsequently remove (Centers for Disease Control and Prevention & Brunette, 2019). *E. coli* also grows best at 37 degrees celsius, can grow with or without oxygen, and can reproduce at a fast rate (Blount, 2015), making it exceedingly flexible and easy to work with.

We chose to study nalidixic acid resistance in *E. coli* because it has a singular genetic target that would be ideal for CRISPR-Cas9 gene editing. The common mutation is a substitution at codon 83 in the *gyrase A* gene, which can be targeted through homology directed repair (Saenz, 2003). This also has direct clinical significance with regards to urinary tract infections (UTIs) in females, as UTIs caused by *E. coli* are the most common type of bacterial infection in females. Such infections are rapidly becoming nalidixic acid resistant, with colistin, a far harsher antibiotic with side effects

including nephrotoxicity and neurotoxicity, being the only current alternative treatment (Lee et al., 2018).

## CRISPR Gene Editing

### *1. Gene Editing as a Potential Treatment for Drug Resistance*

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems are a novel technology derived from the adaptive immune systems of bacteria and archaea. CRISPR has become widely used and researched due to its gene editing capabilities (Makarova & Koonin, 2015). A variety of studies have shown that it is possible to target a wide range of human genes and treat some human diseases with the CRISPR-Cas9 system. Prior to the introduction of CRISPR systems, treatments such as small molecule antibiotics or phage therapy were used as antimicrobial techniques (Gholizadeh et al., 2020). There have been many issues with these mechanisms: Phage therapy has had variable success rates and is still poorly documented (Gholizadeh et al., 2020). Moreover, phages used in phage therapy are often poorly characterized, making difficult their approval for use in humans (Bikard et al., 2014). CRISPR-Cas9 gene editing is one of the most effective genome manipulation techniques. Research has shown that CRISPR-Cas9 technology, in addition to having the potential to be used as a new therapeutic approach in the treatment of antibiotic resistant bacteria, can also be used to enhance the effectiveness of existing treatments (Vaghari-Tabari et al., 2022). The issue of drug resistance is one of the main obstacles in the treatment of multidrug resistant infections and disease. More studies have shown that the CRISPR-Cas9 technique can be used to target important genes involved in antibiotic resistance, thus increasing the effectiveness of antimicrobial drugs.

## 2. *Overview of CRISPR Systems*

CRISPR-Cas systems are relatively new genome editing technological systems (Wei et al., 2013) that are commonly used for the DNA modification of pathogenic gene mutations in laboratories around the world. These systems have become widely used due to their global accessibility, low cost, simple design, high efficiency, and good repeatability (Y. Xu & Li, 2020). Due to the large variety of CRISPR-Cas systems, they are organized into three different types based on the presence of signature Cas genes. Within each type, there are many subtypes that differ based on their signature genes and complementary enzymes, structural features, and additional factors (Makarova & Koonin, 2015).

Type I CRISPR-Cas systems contain the *cas3* gene, coding for a large protein and helicase along with single stranded DNA. The presence of the helicase leads to the unwinding of the double helix shaped DNA, and gene modifications follow this action (Makarova & Koonin, 2015).

Type II CRISPR-Cas systems contain the *cas9* gene, coding for a multidomain protein which merges jobs of effector complexes and target DNA cleavage action. It is also important to note that the *cas9* protein is particularly large and contains two nucleases which are essential for the target DNA cleavage. Type II systems range so drastically that there are three subgroups, II-A, II-B, and II-C that all contain additional genes within this system type (Makarova & Koonin, 2015).

Type III CRISPR-Cas systems contain the *cas10* gene which codes for a multidomain protein. Along with the encoding of the Cas10 protein, each type III locus also encodes for other small subunits and receptor-activity modifying proteins. It has also

been found that in comparison to type I and II systems, the type III systems are more diverse due to multiple gene modifications, allowing for more flexibility and variety between variants (Makarova & Koonin, 2015).

### *3. The Advantages of CRISPR-Cas9*

CRISPR-Cas9 is the newest form of gene editing technology and it is far simpler than most others. Compared to previous gene editing systems such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) where DNA-cutting enzymes were needed, CRISPR-Cas 9 is faster in comparison (Ran et al., 2013). For CRISPR-Cas 9 systems, researchers only have to create their own RNA guide molecules, which makes it easier to design, more specialized, efficient, and adaptable than previous technologies. Additionally, due to the popularity of this new system, there are enough clinical trials to corroborate with and demonstrate the system's reliability.

CRISPR-Cas9 is faster and just as efficient as the microinjection of embryonic stem cells, another form of gene disrupting technology. CRISPR-Cas9 is the most efficient form of gene editing. The chosen system can target multiple genes with a variety of different methods such as blocking transcription, creating mutations at certain points and fluorescent tagging (Young et al., 2015). Overall, CRISPR-Cas9 systems are faster, easier to use, just as efficient as previous technology, capable of both adaptability and specialization and have been used in enough clinical trials to be a reliable form of gene editing technology.

### *4. Delivery Mechanisms for CRISPR-Cas9*

For CRISPR-Cas9 to target and eliminate antibiotic resistance genes within a bacterium, it must first be present inside of the bacterium in order to access its genetic

material. CRISPR systems may be introduced into a cell via physical mechanisms such as microinjection (Horii et al., 2015) and electroporation (Han et al., 2015), but such methods operate only on minute populations of bacteria. Our research seeks to alleviate *in vivo* antibiotic resistant infections, which involve large populations of bacteria which are often spread throughout a region of the body, rendering such methods obsolete. The two delivery mechanisms for administration of modified CRISPR-Cas9 into infected patients which we considered were probiotics and bacteriophages.

A probiotic is a bacterium which is beneficial to humans by modifying the gastrointestinal microbiota, which is composed of the microorganisms residing in the digestive tract. If such a bacterium was to contain a plasmid harboring DNA encoding a CRISPR system which targets and eliminates an antibiotic resistance gene, this bacterium may be ingested by a human host, and via bacterial conjugation the plasmid of interest may be transferred unto other bacteria within the gut. Each of the bacteria to whom the plasmid is transferred may then transfer it to other bacteria, and once the DNA harbored within the plasmid is expressed, the CRISPR system will remove the antibiotic resistance gene of interest should it be present within the expressing bacterium. Neil et al. (2021) demonstrated that if such a CRISPR system is added to a plasmid with very high transfer efficiency, the above process may be used with good efficacy to eliminate one or more types of antibiotic resistant bacteria within the gut microbiome of mice. A significant drawback of this delivery mechanism is its reliance on the cloning of the DNA sequence encoding the CRISPR system: the uncontrolled reproduction and spread of modified DNA is highly regulated (National Institutes of Health, 2019), making approval for this delivery mechanism for therapeutic use very difficult.

Bacteriophages (phages) are viruses which infect bacteria. There are many species and variants of bacteriophages, with each having a specific host range as determined by the presence or absence of sites on the surface of a bacterium onto which they may bind (Lin et al., 2017). This makes phages an appealing candidate for treating infections by known strains of bacteria, as a phage may be chosen which is only capable of infecting the strain of interest (Yosef et al., 2015). This specificity minimizes side effects *in vivo* such as commensal bacteria death, one of the main drawbacks of traditional antibiotic treatments. The use of unmodified phages to kill bacteria via the lytic cycle is known as phage therapy. An overview of such is given in *Figure 1*. One of the major drawbacks of this treatment is that it produces, as a byproduct of the elimination of bacteria, a large quantity of bacteriophages, which is undesirable *in vivo* due to possible side effects or spread if the phage used has undesirable properties. Tridgett and colleagues (2021) demonstrated that the mass production of "phage-like particles," which are essentially phages with their reproductive DNA removed, is possible, and that such phage-like particles may be packaged with other genetic material of choice. Modified bacteriophages thus make an ideal candidate for transduction of non-replicative CRISPR systems to be expressed in a recipient population of pathogenic bacteria.

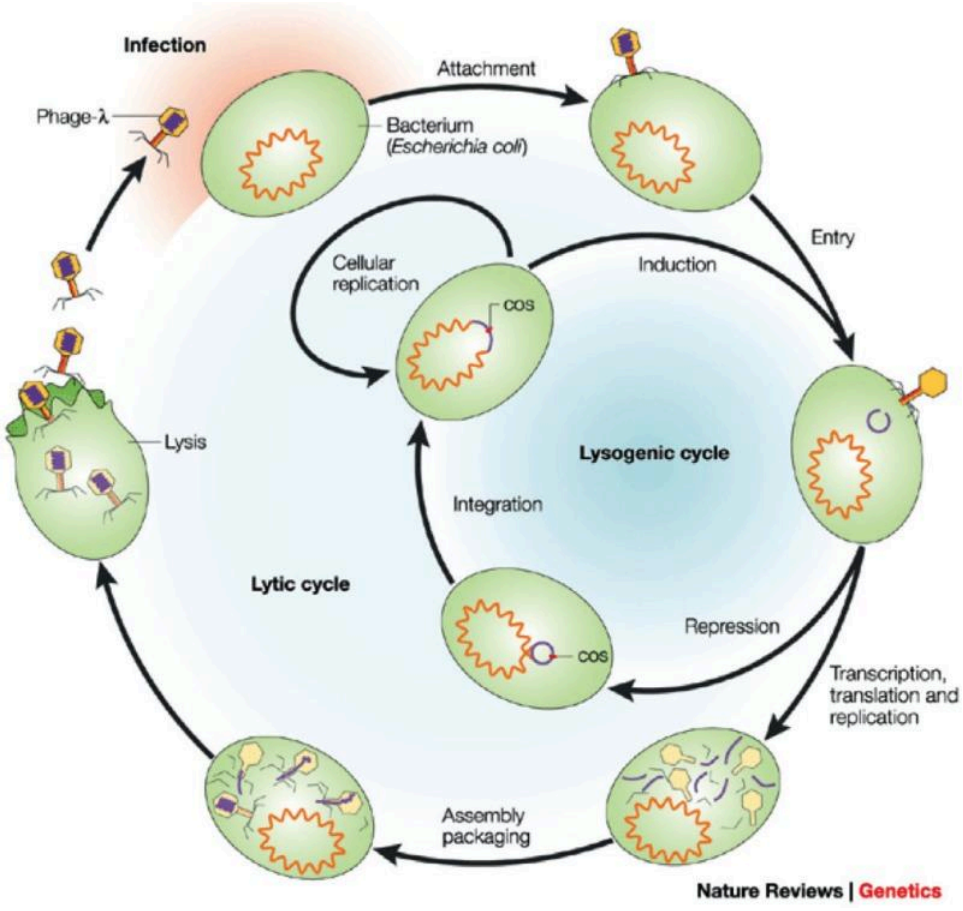


*Figure 1.* Steps in the combined lytic and lysogenic life cycles of a bacteriophage with images and labels for major events.

Image retrieved from Campbell A. 2013. The future of bacteriophage biology. Nat Rev Gen 4:471–7.

### *5. The Advantages of a Bacteriophage Delivery System*

There are many advantages to using bacteriophage as a delivery system. Bacteriophages are species specific and only infect a single bacterial species. This allows for specific targeting of the bacteria cells (Kasman & Porter, 2022). The phage also naturally packages their DNA into capsids in order to inject it into the target bacteria (Bikard et al., 2014). This causes the phage to undergo either the lytic or lysogenic

replication system, which replicates itself and integrates its DNA into the bacterial cell chromosomes (Kasman & Porter, 2022). According to the International Committee on Taxonomy of Viruses, there are currently over 270 classified phage species able to infect *E. coli* (Olsen et al., 2020). The abundance of various phages allows for the use of different phages to be used depending on the need. Each bacteriophage also has a varying insert size allowing different options of phages to choose from depending on the amount of foreign DNA needed to be inserted. Phages also contain genetic markers which can be used to identify infected cells. The use of bacteriophage in clinical trials such as in bacteriophage therapy have also been approved by the FDA. with the first FDA approval being announced in 2019, and is a viable option in vivo compared to alternative methods such as microinjection, which is impractical (Aswani & Shukla, 2021). We plan to use bacteriophage transduction in order to deliver the DNA encoding our CRISPR-Cas9 system into the bacteria.

## Bacteriophage Delivery System

### *1. Bacteriophage Assembly*

The process to create the bacteriophage delivery particles used to deliver the CRISPR-Cas9 system into the cell is known as transduction (Tridgett et al., 2021). In the transduction process, helper phage is used to package the desired DNA into the phage capsids of interest inside a research strain of bacteria, and the bacteriophage life cycle causes the release of this non-replicative phage packaged with the DNA of interest along with helper phage particles. Tridgett and colleagues (2020) describe a system where the DNA of interest is inserted into the phage genome using Gibson assembly (*Figure 2*).

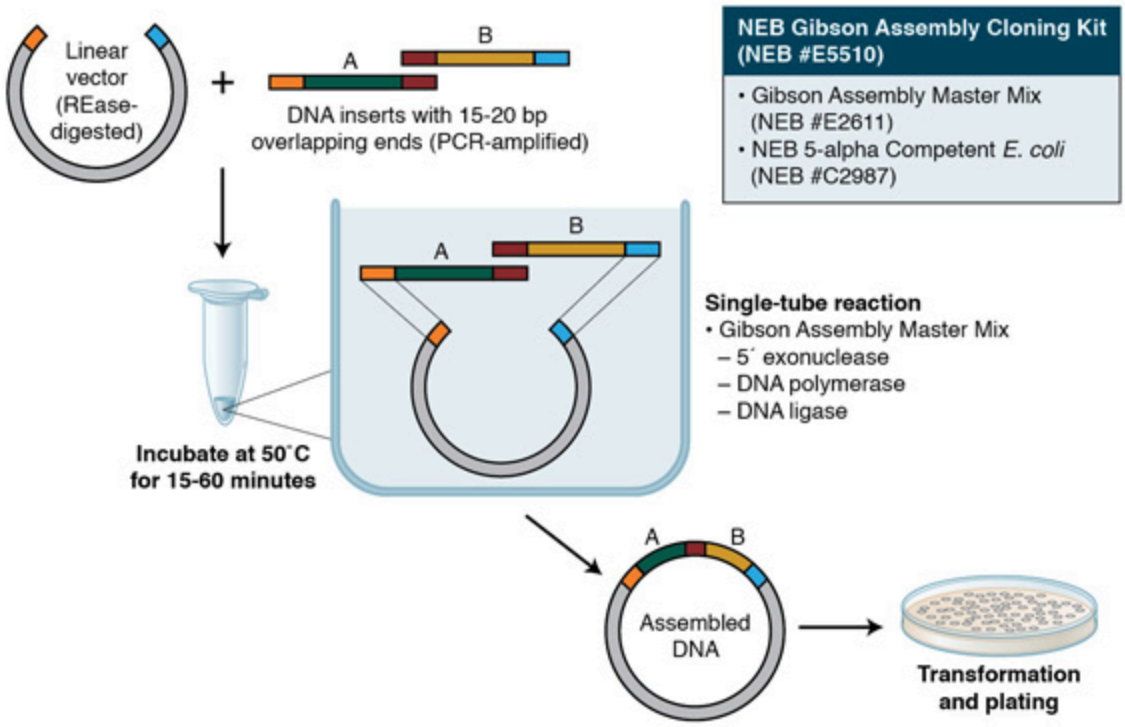


*Figure 2*. Overview of Gibson assembly by New England Biolabs (2022). The linear vector and desired insert DNA both have complementary ends to each other, and they are incubated in a single tube PCR reaction with the Gibson assembly specific reagent mix, resulting in a recombined DNA sequence.

There are many considerations necessary when determining the components of a transduction system. Helper phage is replicative and any contamination can therefore hinder the use of the modified phages in a clinical setting, but this is less of a consideration for a proof-of-concept experiment (Tridgett et al., 2021). Another component of interest is the insert size of the bacteriophage delivery system. Xu and colleagues (2019) describe the most commonly used spCas9 system as having a size of approximately 4.2 kb. Because bacteriophages are assembled using headful packaging, meaning that they can only assemble properly when filled with a certain amount of DNA,

the insert size that the phage can accommodate must be taken into consideration (Coren et al., 1995).

### 2. *Overview of Bacteriophage Delivery Systems*

Bacteriophage λ has a long history in research and has been studied since the 1950's. One feature of note is the sticky ends present at the ends of the linear chromosome. When the λ phage is being assembled, the precursor to the capsid is assembled first and a DNA translocase is used to insert the DNA afterwards. Lambda also has a double stranded genome size of 48,502 base pairs (Casjens & Hendrix, 2015). The capsid head for bacteriophage λ must contain 37 to 52 kb of DNA in order to be stable (Clark et al., 2019). There is a section of the genome that is not considered essential and has a size of approximately 15 kb. This can be removed in order to accommodate larger inserts (Clark et al., 2019).

M13 is a filamentous bacteriophage with single-stranded DNA that has been extensively researched and used for studies related to gene editing and cloning in bacteria, specifically *E. coli*. M13 is one of the smallest phages of the ones considered and has a genome that is only 6400 base pairs long (Lai et al., 2021). Yet, despite the small size of M13, the bacteriophage has been seen to accept inserts up to 42kB, or seven times the size of its own genome. The process of replication in M13 is different from the other phage options as it does not inject its genome into the host. Instead, the p3 proteins on the end of the bacteriophage bind to the F pilus of male *E. coli*. The pilus then contracts, drawing M13 closer to the cell surface and allowing it to penetrate and inject phage DNA into the host cell. Because of its small size, ease of use, and lysogenic nature, M13 is a viable candidate for our research study. One caveat is that M13 is incapable of

infecting female *E. coli* due to its method of infection relying on its binding onto the bacterial F-pilus (Smeal et al., 2017).

The bacteriophage P2 virion consists of an icosahedral head and a tail filament with a nonspecific injection mechanism, making it a promising candidate for uses in which multiple bacterial strains must be transduced upon (Christie & Calendar, 2016). It is a temperate phage with a non-inducible prophage, making it ideal as a transduction agent since it has no chance of lysing its target bacteria. Its genome, which has been fully mapped, consists of 33,592 base pairs of deoxyribonucleic acid (Christie & Calendar, 2016). It has been thoroughly researched for decades, and it is still frequently used in bacteriophage application research (Tridgett et al., 2021).

Bacteriophage T7 is made-up of double-stranded DNA, with a short non-contractile tail that assembles sequentially on the viral head after DNA packaging. The T7/T7 capsids act like a sphere of uniform density with an outer radius of 301 ± 2Å. The tail is short, positively charged, and is a complex of ~2.7 MDa. The capsid envelopes are icosahedral and negatively charged. Capsid I contains a smaller radius than capsid II, indicating an increase in the internal volume during DNA packaging. The internal volume occupied by the anhydrous protein in the envelope of capsid II is approximately $2.2 \pm (0.2)10^{-17}$ mL (Cuervo et al., 2013). Thus, the protein in the T7 envelope needs to be very tightly packed together. The T7 bacteriophage also follows a lytic life cycle, so it destroys the cell it infects which is incompatible with our goal.

We also chose to eliminate T-type phages because they are lytic in nature and thus do not serve our purposes as a delivery vector. We also chose to eliminate P2 and λ, both of which are too large in genome size for us to cost-effectively insert our desired DNA via

Gibson assembly. We chose to work with M13 phage as a delivery vector, as it has a small genome size, is lysogenic, and can take in a large piece of insert DNA. The small genome size makes it cost-effective for Gibson assembly, and though it does not infect female *E. coli*, the goal of this experiment is to provide a proof-of-concept showing that CRISPR-Cas9 can be used as an alternative antimicrobial to sensitize drug-resistant *E. coli* to antibiotics, so this is a hurdle that can be tackled in later iterations of research.

## Methodology

We propose a proof-of-concept lab research experiment in which we resensitize nalidixic acid resistant *E. coli* to nalidixic acid by reverting the antibiotic resistant *gyr*A gene from which this resistance originates to its original sequence. This shall be accomplished using homology directed repair facilitated by a repair template and CRISPR-Cas9 delivered via a bacteriophage. We seek to do so in order to contribute towards the development of a novel clinical treatment to be used to eliminate antibiotic resistance from bacterial infections in humans and improve health outcomes in those with such infections.

Prior to conducting our research, all the necessary materials and technology needed to perform our experiments will be acquired. We will acquire our nalidixic-acid-resistant *E. coli* and phages online and they will be delivered to our laboratory directly. The nalidixic-acid-resistant *E. coli* will be acquired from the ATCC seed stocks and cultured in our laboratory. It is also necessary to design a specific CRISPR-Cas9 guide RNA for our targeted gene: we will order the DNA encoding this system from the plasmid repositories at Addgene and deliver it to our laboratory. The transcribed CRISPR-Cas9 DNA shall be inserted into the bacteriophages via Gibson assembly. We will then culture our modified bacteriophage population to an appropriate volume for administration unto our *E. coli*.

Following these preparations, we will administer varying concentrations of our recombinant phages to the nalidixic acid-resistant *E. coli*. We will then sequence the *gyrA* gene in the affected *E. coli* to ensure the reversion of codon 83 to its non-resistant state, thus verifying the successful delivery of the DNA encoding our CRISPR-Cas9 system. Nalidixic acid will then be administered to our *E. coli* plates. We will observe its efficacy through either tracking cell deaths via plaque assays or an inhibitory concentration assay, which will inform us whether we

have successfully re-sensitized our *E. coli* to nalidixic acid. The experimental observations and data will be recorded and analyzed to determine experimental success.

## Timeline

For our timeline we plan to have finished our thesis proposal and literature review by the end of this Fall of 2022. During the spring of 2023 we will finalize our methodology and budget, complete lab training and begin to collect data in the lab. We will also begin to collect funding by participating in Launch UMD. In the fall of 2023, we intend to continue our work in the lab to collect and analyze data and present our initial findings at the do-good showcase. By the end of Spring of 2024, we expect to have finished our lab work and began writing our thesis. We will also present a poster at the undergraduate research day. During the fall of 2024 our team will continue writing and receive expert feedback on our thesis. In the Spring of 2025, we will complete and defend our thesis.

## Budget

| Expense & Justification | Estimated Cost | Funding Source |
|---|---|---|
| **Bacterial Growth Media:** contains nutrients, energy sources, minerals, etc. to support growth and differentiation of bacteria | $0.00 | Stein Lab |
| **Biosafety Equipment/PPE:** personal protective equipment when operating in lab to prevent | $0.00 | Stein Lab |

| | | |
|---|---|---|
| exposure to hazardous materials and harmful microorganisms (primarily bacteria) | | |
| **Laboratory Incubators:** provide a temperature-controlled environment to support growth of microbiological cultures (bacteria) | $0.00 | Stein Lab |
| **Flasks, Pipettes/Tips, Shakers, Other Miscellaneous Equipment** | $0.00 | Stein Lab |
| **Assorted Recombination Enzymes** | $0.00 | Stein Lab |
| **CRISPR DNA** | $0.00 | Donation |
| **Agar Plates (~1,000, $2/Plate):** a thin layer of growth medium solidified with agar nutrient gel in a petri dish, to culture bacteria | $2,000.00 | Stein Lab (Mentor's Budget) |
| **Bacteriophage (λ, P2) Stock** | $238.00 | TBD |

| | | |
|---|---|---|
| **DNA Sequencing:** to check CRISPR accuracy | $100.00/Sample, ~4-5 Trials | TBD |
| **PCR Materials ($10/Set, 20 Sets Of Primers & Amplification Kits)** | $200.00 | TBD |
| **E-Coli - Nalidixic Acid** | TBD | TBD |

The entirety of the budget will be allocated towards laboratory supplies. Basic materials will be funded via the Stein Lab. Specific systems such as CRISPR DNA and PCR will be donated, or fundraised through sources like Launch UMD, institutional discounts, or other resources. Additional expenses are still to be determined and researched for a definitive price.

## Equity Impact Statement

Antibiotic resistant infections may impact anyone of any population, but existing disparities between minority and majority populations with regards to economic and healthcare-related metrics put minority populations in the United States at a significantly greater risk of acquiring antibiotic resistant infections. Conditions such as crowded housing, expense barriers to diagnostic treatment, and working in industries wherein there exists heightened risk of the transmission of diseases all contribute to this risk. Once antibiotic resistant infections are acquired, they are significantly more costly to treat compared to ones which may be treated with common antibiotics, disproportionately burdening groups with lower socioeconomic status (Nadimpalli et al., 2021). However, through our method of removing antibiotic resistance genes, susceptibility to antibiotics is once again induced in multi-drug resistant bacteria, allowing for the treatment of such infections to be successful with currently available, low-cost antibiotics. Thus, our mission to improve upon methods of antibiotic-resistant infection treatment, which would in turn imply lessened rates of the transmission of such infections, directly seeks to reduce the disparate burdens associated with such infections.

One of the primary reasons for higher morbidity and mortality rates of antibiotic resistant infections amongst racial and ethnic minorities in the United States is the higher poverty rates experienced by such communities. Such poverty discourages frequent visitation to physicians, in part for fear of being prescribed expensive treatments out of the realm of affordability of the patient. As previously mentioned, antibiotic resistant infections are especially expensive to treat in current clinical settings: this directly presents a barrier to treatment for groups of lower income. Our research seeks to contribute towards the development, refinement, and proliferation of novel methods of treating antibiotic resistant infections, which, according to economic theory,

should lower the cost of treatment of such infections. This will, hopefully, alleviate some of the especial difficulty afforded to affected minorities.

Furthermore, UTIs caused by *E coli* are the most common types of infections in persons of female sex, and the proportion of these which are antibiotic resistant is increasing. For decades, females have been critically underrepresented in medicine and were excluded from clinical trials from fear that their hormones would interfere with the "proper" study of the effects of drugs (Merkatz, 1998). Many pharmaceuticals have therefore been marketed with little knowledge as to their specific effects on females. Our research seeks to contribute to the development of novel treatment for antibiotic resistance with special focus on *E coli*, which will hopefully lead to it being used in some way to combat UTIs in females. The mechanism by which our treatment is delivered is via bacteriophage, which should mean that it does not affect the cells of the person it is administered to at all. Our treatment should therefore be agnostic with regards to sex, preventing further disparities between males and females in medicine.

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