Using CRISPR-Cas9 to Modify *gyrA* to Remove Nalidixic Acid Resistance in Clinically Important Pathogens

Team SUPERBUG

Nicholas Breymaier, Joshua Kim, Neha Sripathi, Amber Rayford, Cristina Zhang

Mentored by Dr. Daniel Stein

Thesis submitted in partial fulfillment of the requirements of the Gemstone Honors Program, University of Maryland, 2025

Committee: Dr. Daniel Stein, Dr. Wade Winkler, Dr. Erin Tran, Dr. Stephen Mount, Dr. Kevin McIver

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. Here, we propose to use CRISPR-Cas9-based gene editing to restore antibiotic susceptibility in resistant *Escherichia coli*. Recent studies have used CRISPR-Cas9 gene editing to successfully target and modify resistance genes to increase antibiotic susceptibility. We chose this system to test proof-of-principle due to the implications towards treatment of nalidixic acid resistant *E. coli* urinary tract infections in women, which are a growing clinical problem. We bioinformatically designed a CRISPR-Cas9 construct that could revert *gyrA* mediated nalidixic acid resistance in *E. coli*, resulting in cells sensitive to antibiotics. Our goal is to develop a proof-of-concept antimicrobial strategy utilizing a CRISPR-Cas9 system delivered via bacteriophage M13 to edit a point mutation in the *gyrA* gene of nalidixic acid-resistant *E. coli*, thereby restoring antibiotic sensitivity and contributing to the broader effort to combat antimicrobial resistance. We isolated a series of mutants resistant to nalidixic acid, characterized them, and determined that our target was a single D87G point mutation in *gyrA*. The following thesis describes the progress made towards building, transforming, and testing this construct.

Acknowledgements

We would like to thank our faculty mentor, Dr. Daniel Stein; our team librarian, Ms. Nedelina Tchangalova; and the Gemstone Program staff for their guidance and support throughout our research.

Table of Contents

Abstract i

Acknowledgements ii

Table of Contents iii

List of Tables v

List of Figures vi

List of Abbreviations vii

Introduction 1

1. Social Overview *6*

2. Escherichia coli and Nalidixic Acid Resistance *8*

3. CRISPR Gene Editing *11*

a. Gene Editing as a Potential Treatment for Drug Resistance *11*

b. Overview of CRISPR Systems *14*

c. The Advantages of CRISPR-Cas9 *17*

d. Delivery Mechanisms for CRISPR-Cas9 *19*

e. The Advantages of a Bacteriophage Delivery System *22*

4. Bacteriophage Delivery System **24**

a. Bacteriophage Assembly *24*

b. Overview of Bacteriophage Delivery Systems *25*

Materials and Methods **28**

Results **37**

1. Selection of bacteriophage delivery system *37*

2. Overview of the CRISPR-Cas9 targeting mechanism and integration into chosen bacteriophage M13 *38*

3. Modification of the pCas plasmid to build the CRISPR system *40*

4. Using GFP as a screenable marker to measure Cas9 translation *41*

5. Designing a crRNA to target codon 83 *42*

6. Determining the minimum inhibitory concentration (MIC) of nalidixic acid for JM101 and JM109 *43*

7. Verifying Resistance Mutations *45*

8. Modifying the crRNA *49*

9. Designing the HDR Template *49*

10. Adding FseI restriction sites to GFP and pCas *50*

11. Modifying the pCas FseI primers due to poor PCR and ligation efficiency *51*

12. Transformation *52*

13. Control experiments to determine source of error *53*

Discussion **56**

References **63**

List of Tables

Table 1: Initial primers used for GFP insertion

Table 2: crRNA insertion primers

Table 3: *gryA* Sequencing Primers

Table 4: Modified crRNA insertion primers

Table 5: Modified pCas FseI primers

Table 6: Number of colonies in each transformation

Table 7: pUC-19 FseI primers

List of Figures

Figure 1. A graphical representation of the bioinformatic design of the final CRISPR-Cas9 construct used to edit gyrA.

Figure 2. MIC assay of JM101 and JM109 on nalidixic acid plates.

Figure 3. PCR of gyrA across JM101 and JM109 strains validating sequencing primers.

Figure 4. Validation of JM101 wildtype sequence and demonstrating spontaneous generation of JM101 mutations using sequencing chromatography.

Figure 5. Validation of JM109 resistance mutation through sequencing analysis.

Figure 6. Agarose gel to validate successful amplifications.

Figure 7. Agarose gel to validate successful amplifications after modification.

Figure 8. Agarose gel showing ligated and unligated samples after digestion with FseI of purified pUC-19 plasmid and GFPuv.

Figure 9. Agarose gel to validate successful digestion and ligation of the pFT180 plasmid.

Figure 10. A graphical representation of the project overview.

List of Abbreviations

AMR: Antimicrobial Resistance

FDA: U.S. Food and Drug Administration

GFP: Green Florescent Protein

HDR: Homology Directed Repair

KB: Kilobases

MDR: Multidrug Resistant

MIC: Minimum Inhibitory Concentration

NA: Nalidixic Acid

NCBI: National Center for Biotechnology Information

NEB: New England Biolabs

PAM: Protospacer Adjacent Motif

PCR: Polymerase Chain Reaction

UTI: Urinary Tract Infection

crRNA: CRISPR RNA

sgRNA: Single-guide RNA

trRNA: Trans-activating CRISPR RNA

Introduction

Since the introduction of the first antibiotics in the late 1930s, bacterial resistance has evolved in parallel with their development. Drug resistance to penicillin was identified in bacteria even before penicillin was formally approved as a therapeutic agent (Davies & Davies, 2010). The emergence of antimicrobial resistance (AMR) is not a new phenomenon; resistance genes predate the modern use of antibiotics. The ongoing presence of antibiotic resistance highlights an ongoing evolutionary arms race between bacteria and antibiotics. For instance, resistant bacteria over 2,000 years old have been isolated from glacial samples, and TEM-type β-lactamases (enzymes conferring resistance to β-lactam antibiotics) have been detected in metagenomic samples over 10,000 years old (Harbottle et al., 2006). More recently, AMR has emerged as a major global health crisis, complicating the treatment of infections and contributing to increased morbidity and mortality rates. In the United States, infections from resistant bacteria incur healthcare costs of up to $20 billion annually (Dadgostar, 2019). Globally, an estimated 5 million deaths were associated with bacterial AMR in 2019, including ~1.3 million deaths specifically attributed to resistant infections (Salam et al., 2023; Murray et al., 2022). Projections suggest that this number could surpass cancer-related deaths by 2050 (Kaur et al., 2021).

The evolutionary arms race between bacteria and antibiotics has been exacerbated by contemporary human antibiotic use and abuse, accelerating the spread of resistant strains. Key contributors to the AMR crisis include inappropriate antimicrobial use, limited access to effective treatments, and inadequate infection control measures (Murray et al., 2022). In 2017 the global market for antibiotics reached USD ~7.8 billion, and further increased by 5.6% in 2018, indicating ongoing demand for effective antibiotics. However, this demand is coupled with a predicted 200% surge in antibiotic consumption from 2015 to 2030, particularly in low- and

middle-income countries (Bell et al., 2014; Laxminarayan et al., 2020). Specific pathogens have been identified as significant players in the AMR landscape; for instance, the WHO European region recorded 541,000 deaths due to AMR in 2019, primarily linked to *Escherichia coli* and *Staphylococcus aureus*, followed by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Mestrovic et al., 2022). Alarmingly high resistance rates have been reported in China, with *K. pneumoniae* showing a 64.1% resistance rate and *Acinetobacter baumannii* at an 80% resistance rate against recommended antibiotics (Hu et al., 2019).

Limited treatment options for AMR infections also amplify the severity of AMR (Aminov, 2010). The term "superbugs" describes microbes that are highly resistant to the antibiotics intended to treat them (Davies & Davies, 2010). A notable example is methicillin-resistant *S.aureus* (MRSA), which initially referred to methicillin-resistant strains but has since become an umbrella term for multidrug-resistant *S. aureus*. MRSA is particularly significant due to its increased spread, demonstrating that superbugs are no longer restricted to healthcare settings (Davies & Davies, 2010). Bacteria resistant to first-line of-defense antibiotics were responsible for ~70% of the ~5 million deaths from AMR globally (Murray et al., 2022). Additionally, drug resistance is of even greater significance to those least equipped to face it with healthcare systems in areas with fewer resources to test for and treat these infections facing greater incidences of infection among their patients (Murray et al., 2022).

The accelerated rise of and impact of MDR and extensively drug-resistant (XDR) bacterial strains on global morbidity rates has necessitated the use of last-line defense antibiotics, such as colistin (Harbottle et al., 2006). Colistin is often used as a last resort due to its nephrotoxicity and neurotoxicity. The emergence of the mcr-1 gene, which confers colistin resistance, complicates the treatment landscape as colistin is a last-resort antibiotic used against

MDR bacteria (El-Sayed Ahmed et al., 2020). Despite the urgent need for new antibiotics, economic disincentives remain a significant barrier to pharmaceutical development. Large pharmaceutical companies are increasingly reluctant to invest in antibiotic research due to low profitability (Chokshi et al., 2019). Meanwhile, numerous strains of pathogenic bacteria are rapidly becoming resistant to currently available antibiotics (Aminov, 2010). The diversity of the global bacterial genome further complicates the situation as resistance to newly introduced antibiotics inevitably arises (Martinez, 2014).

Multiple mechanisms contribute to the acquisition of antibiotic-resistance genes in bacteria. These genes, known as antibiotic resistance genes, allow the bacteria to survive and proliferate despite antibiotic treatment (Alekshun & Levy, 2007). Bacteria can develop resistance through various mechanisms, and these resistance genes are often disseminated rapidly through mobile genetic elements such as plasmids, transposons, and integrons (Davies & Davies, 2010; Levy & Marshall, 2004). Plasmids, for example, can carry multiple resistance genes and facilitate horizontal gene transfer between bacterial species via conjugation (Harbottle et al., 2006). These plasmids, referred to as R plasmids or R factors, pose a significant threat due to their ability to transfer resistance across different bacterial genera. An illustrative case is the plasmid-mediated quinolone resistance (qnr) gene first identified in *K.pneumoniae*, which blocks the action of ciprofloxacin (Harbottle et al., 2006).

Transposons (DNA sequences that can jump between different genome locations) often carry antibiotic resistance genes. Integrons, another class of mobile DNA elements, can capture and express gene cassettes, conferring resistance to multiple antibiotics. Superintegrons, a specific form of integrons, can harbor hundreds of gene cassettes, enhancing bacterial adaptability and resistance (Harbottle et al., 2006). Bacteria can also acquire resistance through

intrinsic mechanisms, such as impermeable membranes, point mutations on specific genes, or acquired mechanisms via horizontal gene transfer (Harbottle et al., 2006). The latter includes transformation, transduction, and conjugation, with bacteriophages playing a crucial role in disseminating resistance genes. For instance, efflux pump systems, particularly in MDR *E. coli*, are major contributors to resistance. These pumps, such as those in the ATP-binding cassette (ABC) and resistance-nodulation-division (RND) families, effectively reduce drug efficacy (Harbottle et al., 2006). Understanding these mechanisms is vital for developing targeted strategies, such as CRISPR-Cas9 gene editing, which could disrupt efflux pump genes or other resistance pathways.

Another mechanism of resistance involves reduced permeability and active antibiotic efflux pumps. In *E. coli*, porins like OmpF and OmpC regulate the uptake of antibiotics. Mutations that affect these porins reduce antibiotic entry into the bacterial cell, contributing to resistance (Harbottle et al., 2006). This presents a significant challenge for traditional antibiotics, reinforcing the need for innovative solutions such as CRISPR-Cas9. Unlike antibiotics, CRISPR-Cas9 can bypass permeability barriers by delivering gene-editing tools directly to bacteria via bacteriophages, which do not rely on traditional uptake pathways. Efflux pumps, which actively expel antibiotics from bacterial cells, are another common resistance mechanism. These pumps, which can be encoded on chromosomal DNA or mobile genetic elements like plasmids, are often associated with multidrug resistance (Harbottle et al., 2006). Bacteriophage-mediated delivery of CRISPR-Cas9 offers a potential solution by targeting resistant bacteria without being affected by efflux pump mechanisms. The specificity of bacteriophages allows for the precise delivery of CRISPR components, bypassing traditional antibiotic resistance mechanisms and restoring antibiotic susceptibility.

Urinary tract infections (UTIs) caused by *E. coli* are an example of an urgent public health concern caused by MDR infections, accounting for ~150 million cases annually worldwide and 11 million in the United States (Griebling, 2005; Foxman, 2014). Typically, UTIs can be treated with common antibiotics, such as nalidixic acid. Yet, despite antibiotic treatment, 20–30% of women experience recurrent UTIs (Foxman, 2002). Quinolone resistance in nalidixic acid-resistant *E. coli* arises from mutations in the *gyrA* gene, which alters the binding efficiency of quinolone antibiotics, similar to the resistance observed in quinolone resistance-determining regions (QRDR) of the *gyrA*, *gyrB*, and *parC* genes (Harbottle et al., 2006). This is where the CRISPR-Cas9 system becomes particularly promising. Its precision allows for the targeted removal of specific mutations in resistance genes like *gyrA*, offering a novel therapeutic approach that leaves essential bacterial functions intact while rendering the bacteria susceptible to antibiotics once again. As such, this study aims to explore antimicrobial strategies through gene editing technology. We aim to develop a proof-of-concept for high-efficiency gene editing that can be applied in vivo. In doing so, we also consider the social and societal factors that contribute to the spread of resistance. Specifically, the research question guiding this study is: How can we effectively deliver a CRISPR-Cas9 gene editing system into a nalidixic acid-resistant bacterium to edit its resistance genes, making it susceptible to antibiotics? We hypothesize that introducing a homology-directed repair to edit codon 87 of the *gyrA* gene in nalidixic acid-resistant *E. coli*, delivered via bacteriophage M13, will significantly decrease the minimum inhibitory concentration of nalidixic acid required for bacterial cell death in strains that possess this mutation.

This approach, which focuses on selectively targeting and removing resistance genes, represents a significant step toward overcoming the AMR crisis by potentially restoring the

effectiveness of conventional antibiotics. Furthermore, understanding the interplay of genetic factors in AMR is essential for developing effective prevention strategies, including exploring alternatives to antibiotics such as vaccines and competitive exclusion products (Harbottle et al., 2006). By combining the precision of CRISPR-Cas9 with the specificity of bacteriophage delivery systems, we hope to contribute to novel therapeutic approaches to combating multidrug-resistant bacterial infections. Ultimately, this research underscores the potential of CRISPR-Cas9 as a transformative tool in the fight against antimicrobial resistance.

1. Social Overview

AMR poses a significant global threat, driven by the overuse and misuse of antibiotics in healthcare and agriculture. This issue is exacerbated by the inappropriate prescription of antibiotics, especially in critical care settings, and the use of antibiotics as growth promoters in livestock. These practices contribute to the emergence and spread of resistant bacterial strains, which can transfer from animals to humans. The implications of AMR are far-reaching, with millions of deaths attributed to resistant infections and a substantial economic impact forecasted for the future. Antibiotics are often prescribed for viral infections or other conditions where they are unnecessary, leading to the emergence of resistant bacterial strains (Tian et al., 2021). In many healthcare settings, identifying pathogens responsible for infections is poor, resulting in the broad and often incorrect use of antibiotics (Mohsin & Amin, 2023). In critical care, where infections are prevalent and timely treatment is essential, this practice is especially concerning, as the misuse of antibiotics accelerates resistance development. As such, this section will discuss the factors driving AMR, including antibiotic misuse in healthcare and agriculture, as well as the socioeconomic and global health consequences.

Compounding the issue, the availability of counterfeit or substandard antibiotics, particularly in low- and middle-income countries, further contributes to AMR. These low-quality medications may lack sufficient therapeutic effects, allowing bacteria to develop resistance at subtherapeutic levels (Zabala et al., 2022). The ease of obtaining antibiotics without a prescription, poverty, and limited access to health care foster antibiotic resistance in these regions (Poyongo & Sangeda, 2020). Healthcare providers must follow proper protocols and prescribe antibiotics judiciously to maximize clinical therapeutic effects while minimizing the risk of resistance development (English & Gaur, 2010).

In addition to healthcare, antibiotic misuse in agriculture accelerates the spread of resistance. In livestock production, antibiotics are frequently used as growth promoters and disease preventatives rather than solely for treatment (Dankar et al., 2022; Walia et al., 2023). This misuse in livestock and poultry creates a reservoir for resistant bacteria, which can be transmitted to humans through the consumption of contaminated meat (Levy & Marshall, 2004). Furthermore, the application of land manure containing antibiotic-resistant bacteria has contributed to the dissemination of resistance genes into the environment (Levy & Marshall, 2004). These findings highlight the transmission of antibiotic-resistant strains due to human activities and emphasize the impact of antibiotic resistance beyond healthcare settings.

The economic burden is equally alarming, with projections suggesting a 1% global GDP loss per year by 2050, disproportionately affecting developing nations (Hou et al., 2023). The COVID-19 pandemic has further complicated the issue, as the overuse of antibiotics during the pandemic has worsened the treatment of infections like tuberculosis, particularly in countries such as India and Russia (Dadgostar, 2019). Although antibiotic resistance develops naturally (Martinez, 2014), the misuse of antibiotics drastically accelerates this process. The discovery of

new antibiotics is consistently followed by the emergence of resistant strains (Davies & Davies, 2010). Nosocomial infections worldwide are predominantly caused by antibiotic-resistant bacteria from the ESKAPE-E group, which includes *E.faecium*, *S.aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *Enterobacter spp.*, and *E. coli* (Ayobami et al., 2022). These pathogens not only possess intrinsic resistance genes but also acquire multidrug resistance through selective pressure resulting from antibiotic overuse.

Antibiotic resistance, driven by misuse in healthcare and agriculture, presents a formidable challenge to global health. The spread of resistant bacteria through healthcare mismanagement, livestock production, and environmental contamination underscores the complexity of the problem. Furthermore, the socioeconomic burden of AMR, particularly in developing nations, compounds the urgency of addressing this issue. Targeted interventions, including stricter antibiotic stewardship, improved diagnostic practices, and policies regulating antibiotic use in agriculture, are essential to mitigating the spread of AMR and preserving the efficacy of existing antibiotics.

2. Escherichia coli and Nalidixic Acid Resistance

AMR is a growing global threat, exacerbated by the overuse of antibiotics in both healthcare and agriculture, particularly in treating infections caused by bacteria like *E. coli*, which increasingly exhibit resistance to multiple drugs. Gram-negative bacteria such as *E. coli*, which serve as reservoirs of resistance genes, are central to the discussion of AMR due to their prevalence in both clinical settings and livestock. This section outlines our rationale for selecting *E. coli* as the model organism for our research, explores the global rise of AMR in *E. coli*, and introduces innovative gene-editing technologies, such as CRISPR-Cas9, as a potential solution to combat drug-resistant bacterial strains.

We chose *E. coli* as the model organism for our research due to its biological characteristics, safety profile, and relevance to antimicrobial resistance. *E. coli* is a highly diverse bacterial species commonly found in the environment, food, and intestines of humans and animals (Blount, 2015). While some strains are pathogenic, most are harmless or commensal, classifying *E. coli* as a Biological Safety Level (BSL) 1 organism (Bayot & King, 2022). BSL 1 applies to low-risk microbes that pose minimal risk to lab personnel, allowing us to work with *E. coli* without excessive regulatory constraints.

E. coli has become an ideal model for proof-of-concept studies in antibiotic resistance research. The species is well-studied, with extensive background literature, including one of the earliest complete genome sequences (Blount, 2015). Its susceptibility to various antibiotics also provides flexibility in selecting antibiotic resistance genes to insert, target, and remove in experimental designs (Centers for Disease Control and Prevention & Brunette, 2019). Additionally, *E. coli's* ability to grow in both aerobic and anaerobic conditions, its rapid reproduction rate, and its optimal growth temperature of 37°C make it a practical choice for laboratory research (Blount, 2015).

AMR in *E. coli* has risen globally, particularly in food-producing animals. The extensive use of antimicrobials such as nalidixic acid and erythromycin in livestock has driven resistance in bacterial species like *E. coli*, *Salmonella sp.*, and *Campylobacter sp.* (Van Boeckel et al., 2017). *E. coli*, in particular, is a significant reservoir for resistance genes, making it a key indicator for monitoring AMR in agricultural systems (Poirel et al., 2018). For instance, high levels of resistance to erythromycin have been detected in poultry, pigs, and cattle due to its widespread use as a growth promoter (Byarugaba et al., 2011). In Africa, resistance to nalidixic

acid in sheep and poultry further highlights the selective pressure exerted by antimicrobial use in livestock production systems (Adesokan et al., 2015).

The transmission of resistant *E. coli* strains from animals to humans presents a significant risk to public health. Consumption of contaminated meat can lead to antibiotic-resistant infections in humans. As a result, efforts to reduce antimicrobial use in agriculture are essential in mitigating the spread of AMR. The One Health approach, which integrates human, animal, and environmental health strategies, is crucial for reducing the public health risks associated with AMR (Velazquez-Meza et al., 2022).

In clinical settings, drug-resistant Gram-negative bacteria, including *E. coli*, pose a major global threat. Resistance to fluoroquinolones, a critical class of antibiotics for treating *E. coli* infections, is rising due to mutations in the *gyrA* and *parC* genes and the activity of bacterial efflux pumps (Redgrave et al., 2014; Rodríguez-Martínez et al., 2016). Resistance to first-, second-, and third-generation cephalosporins, as well as carbapenems, has left colistin as one of the few remaining treatment options (Milano et al., 2022). However, the emergence of colistin resistance, driven by phospholipid modifications in bacterial outer membranes, presents a severe challenge to treating multidrug-resistant (MDR) infections (Le Guern et al., 2017).

Our research focuses on targeting nalidixic acid resistance in *E. coli*, as this resistance mechanism is well-defined and presents a singular genetic target suitable for CRISPR-Cas9 gene editing. Nalidixic acid resistance is commonly linked to a point mutation at codon 87 in the *gyrA* gene, which can be effectively targeted using homology-directed repair (Saenz, 2003). This research is particularly relevant to UTIs in females, where *E. coli* is the leading cause of infection. UTI cases resistant to nalidixic acid are increasingly common, with colistin often being the only alternative treatment (Lee et al., 2018). Colistin, however, is associated with severe side

effects, including nephrotoxicity and neurotoxicity, further emphasizing the need for novel treatments.

The rising prevalence of MDR *E. coli* strains underscores the need for innovative solutions. CRISPR-Cas9, a precise genome-editing tool, offers the potential to directly target resistance genes like *gyrA* in *E. coli* strains. By incorporating bacteriophage delivery systems, we aim to develop a targeted, precision-based alternative to traditional antibiotics. Bacteriophages, viruses that specifically infect bacteria, can be engineered to deliver the CRISPR-Cas9 system to resistant bacterial populations, selectively disrupting resistance mechanisms and restoring antibiotic susceptibility.

3. CRISPR Gene Editing

a. Gene Editing as a Potential Treatment for Drug Resistance

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems are integral components of prokaryotic immune defenses, first identified in *E. coli* in 1987 (Barrangou et al., 2007; Ishino et al., 1987). These systems defend bacteria against bacteriophages by recognizing and cleaving foreign DNA using sequences derived from previous viral infections. The CRISPR-Cas9 system, one of the most widely studied, consists of the Cas9 endonuclease, CRISPR-associated RNA (crRNA), and trans-activating crRNA (tracrRNA). These components form a single-guide RNA (sgRNA) complex that directs Cas9 to specific DNA sequences for degradation (Jinek et al., 2012). The advent of CRISPR-Cas technology marks a significant advancement in gene editing. Initially discovered as a bacterial immune defense mechanism, CRISPR-Cas has been repurposed for genome editing, including cases for targeting and excising drug-resistant genes from bacterial genomes. This capability holds promise for addressing the growing issue of MDR bacteria.

CRISPR-Cas9, the most well-known system, functions by utilizing a guide RNA (gRNA) to direct the Cas9 enzyme to a specific DNA sequence, where it induces a double-stranded break. The cell's natural repair mechanisms can then be leveraged to either disrupt or replace the targeted gene. Studies have successfully demonstrated the removal of resistance genes in bacteria such as *E. coli*. For example, Sun et al. (2017) showed that CRISPR-Cas9 could excise genes conferring resistance to last-resort antibiotics, making this system a promising tool in the fight against AMR. The discovery of CRISPR in *E. coli* in 1987 and its subsequent role in bacterial immunity paved the way for its modern applications in gene editing. By 2012, Doudna and Charpentier had simplified the system, demonstrating its potential for precise genome modification. Their work revolutionized genetic engineering, leading to the widespread use of CRISPR-Cas9 in various organisms, including humans (Jinek et al., 2012). The technology's precision in targeting specific DNA sequences allows for highly controlled genetic modifications, positioning CRISPR as a transformative tool in both research and therapeutic applications.

CRISPR systems provide a new approach to address AMR, particularly in multidrug-resistant bacteria. These systems have the potential to disrupt existing resistance mechanisms and reduce the need for traditional antibiotics. The development of MDR bacteria is often fueled by horizontal gene transfer and biofilm formation, making them difficult to treat with conventional antibiotics (Sun et al., 2019; Lewis, 2007). CRISPR-Cas systems can target specific genes responsible for resistance, providing a focused method to combat resistance.

One example is the CRISPR-CasIII-A system, which has demonstrated the ability to cleave active DNA and target RNA, disrupting bacterial defense mechanisms (Liu et al., 2018). This system, along with CRISPR-Cas9, represents a more precise and efficient alternative to

older gene-editing technologies such as transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs), which are limited by lower specificity and efficiency (Javaid & Choi, 2021; Kundar & Gokarn, 2022).

In the context of MDR bacterial strains, CRISPR-Cas technology offers an innovative approach. By targeting resistance genes, this technology can sensitize previously resistant bacteria to antibiotics. For instance, targeting *gyrA*, a gene that contributes to fluoroquinolone resistance, can restore antibiotic susceptibility in *E. coli* (Dong et al., 2019). Building on this concept, our project aims to resensitize nalidixic acid-resistant *E. coli* to nalidixic acid by specifically targeting the *gyrA* gene, with the goal of reestablishing the bacteria's vulnerability to this antibiotic. The ability to resensitize bacteria by disrupting genes like extended-spectrum beta-lactamase (ESBL)-producing strains has shown success in restoring the efficacy of antibiotics (Kim et al., 2016; Price et al., 2019). Moreover, CRISPR/Cas9 systems have proven effective in targeting plasmid-mediated AMR genes, such as mcr-1, which confers colistin resistance in *E. coli* (He et al., 2022). This highlights CRISPR's potential not only in targeting chromosomal resistance genes but also in eliminating mobile genetic elements that contribute to the rapid spread of AMR.

Another challenge is the potential for bacterial resistance to CRISPR-Cas-induced DNA breaks. Bacteria may evolve mechanisms, requiring multiple target sites to enhance bacterial killing efficiency and reduce the likelihood of resistance (Uribe et al., 2021). Additionally, CRISPR-Cas9 has faced issues with off-target effects, where Cas9 inadvertently cuts DNA sequences similar to the target site. These effects can be mitigated by optimizing gRNA design and using systems like λ-Red recombination to improve editing accuracy (Cho et al., 2014; Pyne et al., 2015).

The CRISPR-Cas9 system has demonstrated significant potential in treating bacterial infections by targeting and removing resistance genes. However, its application extends beyond bacterial AMR. CRISPR technology has been employed in various fields, including cancer treatment, genetic disorder correction, and viral infection control (Rautela et al., 2021). Compared to traditional antimicrobial approaches like small-molecule antibiotics and phage therapy, CRISPR offers more precise and effective solutions. Phage therapy, for instance, has had inconsistent success and faces challenges in characterization and regulatory approval (Bikard et al., 2014; Gholizadeh et al., 2020). In contrast, CRISPR-Cas9 provides a targeted approach that can enhance the effectiveness of existing treatments while directly addressing the genes responsible for resistance (Vaghari-Tabari et al., 2022).

b. Overview of CRISPR Systems

The classification of CRISPR systems includes three main types, distinguished by the specific Cas proteins involved, their structure, and the presence of specific signature genes.

Type I systems contain the *cas3* gene, which codes for a large protein with helicase activity. This helicase unwinds double-stranded DNA (dsDNA), facilitating gene modification (Makarova & Koonin, 2015).

Type II systems include the *cas9* gene, which encodes a multidomain protein (Cas9) responsible for both target recognition and DNA cleavage. Cas9 contains two nuclease domains essential for cutting the target DNA, ans is highly efficient for gene editing. The diversity within Type II systems is reflected in its three subgroups—II-A, II-B, and II-C—which differ based on additional genes and structural features (Makarova & Koonin, 2015).

Type III systems are characterized by the *cas10* gene, which codes for a multidomain protein. Type III systems exhibit greater diversity compared to Types I and II, allowing for more

flexibility and a broader range of gene modifications. This diversity makes Type III systems particularly useful for more complex genetic engineering applications (Makarova & Koonin, 2015).

Type II CRISPR systems, which utilize Cas9, are particularly notable for their applications in genome editing due to their simplicity and effectiveness (Makarova et al., 2011; Mohanraju et al., 2016). Within this context, the Class 2 subtype II systems have emerged as the most studied platforms for gene editing, particularly in efforts to eliminate AMR (Zhang et al., 2014).

The application of CRISPR-Cas technology in combating antimicrobial resistance has gained substantial attention due to its potential to precisely target and excise resistance genes. By designing a gRNA specific to resistance genes, CRISPR can efficiently disrupt the genetic mechanisms that confer resistance to antibiotics. One significant advantage of CRISPR gene editing systems is their adaptability, allowing for precision in editing bacterial genomes, which is critical in addressing the growing threat of multidrug-resistant bacteria (Wei et al., 2013; Xu & Li, 2020). However, a major challenge in utilizing CRISPR systems for AMR is the development of effective delivery methods. Delivery vehicles are crucial for guiding the CRISPR machinery to the target bacterial cells. Current delivery methods can be classified into two main categories: phage vectors and non-phage vectors.

Non-phage vectors include physical methods like electroporation, ultrasonic microbubbles, and laser microinjection, as well as chemical methods like lipofection and nanoparticles. While these methods offer some success in laboratory settings, they are limited in their application for in vivo studies due to low efficiency and delivery challenges (Martins & Ademola, 2023). Phage vectors offer greater efficiency in delivering CRISPR systems to

bacterial cells. However, some phage vectors pose potential risks of uncontrolled propagation and may cause additional mutations within the genome, limiting their use in clinical settings (Yang et al., 2022).

Although CRISPR systems are widely used in laboratories for gene modification, their clinical applications, particularly in AMR, remain limited. The technology is primarily used ex vivo, especially in stem cell research, due to concerns about safety and the efficiency of delivery systems (Martins & Ademola, 2023). To fully unlock the potential of CRISPR-Cas systems for AMR, further research is needed to develop safer and more effective delivery mechanisms. Despite these challenges, the accessibility, low cost, and high efficiency of CRISPR-Cas systems have made them a cornerstone of modern genetic engineering. Their ability to precisely modify pathogenic gene mutations provides hope for combating not only AMR but also a wide range of diseases. Studies have demonstrated the potential of CRISPR-Cas9 technology to enhance existing treatments by targeting specific genes involved in drug resistance, increasing the effectiveness of antimicrobial drugs (Vaghari-Tabari et al., 2022).

Before the advent of CRISPR, other methods, such as small-molecule antibiotics and phage therapy, were used to treat bacterial infections. However, these methods faced limitations in terms of effectiveness and consistency. Phage therapy, in particular, has shown variable success rates and remains poorly documented. The characterization of phages is often incomplete, further complicating their approval for use in humans (Gholizadeh et al., 2020).

c. The Advantages of CRISPR-Cas9

CRISPR-Cas9 has emerged as a revolutionary gene-editing technology, surpassing earlier tools such as ZFNs and TALENs. Its simplicity, versatility, and efficiency make it particularly effective for addressing antibiotic resistance, a global healthcare challenge. This section outlines

the advancements in CRISPR-Cas9, compares it to previous technologies, and highlights its clinical applications and successes (Ran et al., 2013).

The development of base-editing techniques represents a significant advancement in genome editing. These methods allow for precise DNA alterations without inducing double-stranded breaks, reducing the risk of unwanted mutations. Base editing relies on fusion proteins, such as dead Cas9 or nickase Cas9, coupled with nucleobase deaminases that act on single-stranded DNA. This technique enhances the precision of site-directed mutagenesis, improving the accuracy and efficiency of genome editing, particularly in the context of combating antibiotic resistance (Kantor et al., 2020; Rabaan et al., 2023).

CRISPR-Cas9 offers distinct advantages over its predecessors, TALEN and ZFN. Both TALEN and ZFN require custom-designed proteins to target specific DNA sequences, making these systems more expensive, time-consuming, and labor-intensive. In contrast, CRISPR-Cas9 utilizes gRNA, which is far simpler and more cost-effective to design. The ease of modifying gRNA enables CRISPR-Cas9 to target multiple genes simultaneously, offering flexibility that TALEN and ZFN cannot match. Moreover, CRISPR-Cas9's ability to address biofilm-based antibiotic resistance, a significant contributor to chronic infections, positions it as a superior tool for tackling antimicrobial resistance (Zuberi et al., 2022; Rabaan et al., 2023).

CRISPR-Cas9 can be employed in two distinct strategies to combat antibiotic resistance: pathogen-focused and gene-focused approaches. The pathogen-focused approach involves directly targeting and destroying harmful bacterial strains by cleaving essential chromosomal regions. This strategy reduces the overall bacterial load, addressing infections at their source. On the other hand, the gene-focused approach targets specific antibiotic-resistance genes, particularly those carried on plasmids, which can be horizontally transferred between bacterial

species. By disrupting these resistance genes, CRISPR-Cas9 sensitizes bacteria to antibiotics, potentially restoring the efficacy of conventional treatments (Shabbir et al., 2019; Allemailem, 2024).

Numerous studies have demonstrated the clinical efficacy of CRISPR-Cas9 in reducing antibiotic resistance. In mouse models, CRISPR interventions successfully decreased skin colonization by *S. aureus*, a common source of antibiotic-resistant infections (Rodrigues et al., 2019). Similarly, CRISPR-Cas9 was used to reduce erythromycin resistance in *E.faecalis*, another pathogen known for its resistance to antibiotics (Wang et al., 2019). Furthermore, CRISPR technology has been applied to multidrug-resistant *K. pneumoniae*, where it disrupted genes involved in resistance to colistin and tigecycline, two last-resort antibiotics for severe infections (Sun et al., 2019). These examples underscore CRISPR-Cas9's potential to mitigate the global threat posed by antimicrobial-resistant bacteria.

CRISPR-Cas9 represents a significant improvement over older gene-editing technologies. Unlike ZFNs and TALENs, which require the use of complex DNA-cutting enzymes, CRISPR-Cas9 requires only a simple RNA guide molecule to direct the system to its target. This reduces both the time and cost associated with gene editing, while increasing the system's efficiency and adaptability (Ran et al., 2013). Researchers can easily customize CRISPR-Cas9 to block transcription, create specific mutations, or fluorescently tag genetic sequences. These capabilities allow for a broader range of applications, from basic research to therapeutic interventions (Young et al., 2015).

Additionally, CRISPR-Cas9 is not only faster but also just as effective as traditional gene-editing techniques like embryonic stem cell microinjection. The system's ability to target multiple genes at once provides an unprecedented level of control in genetic modification. Its

wide adoption in clinical trials demonstrates its reliability and effectiveness in treating genetic disorders and potentially addressing antibiotic resistance (Young et al., 2015). Despite the promise of CRISPR-Cas technology, challenges remain, particularly in the efficient delivery of CRISPR systems to bacterial cells. Bacteriophage-based delivery has emerged as a viable solution. Bacteriophages, viruses that infect bacteria, can be engineered to carry CRISPR-Cas9 and deliver it directly to resistant bacteria, offering specificity and reducing off-target effects (Uribe et al., 2021).

d. Delivery Mechanisms for CRISPR-Cas9

For CRISPR-Cas9 to effectively target and remove antibiotic resistance genes from bacteria, it must first enter the bacterial cell to access its genetic material. Traditional delivery methods such as microinjection (Horii et al., 2015) and electroporation (Han et al., 2015) have been used in laboratory settings but are impractical for treating large-scale infections involving widespread bacterial populations. Therefore, in vivo delivery mechanisms such as probiotics and bacteriophages offer more practical solutions for the therapeutic application of CRISPR systems.

Probiotics, beneficial bacteria that modulate the human gut microbiome, are one approach to delivering CRISPR systems into the body. By engineering probiotic bacteria to carry plasmids encoding CRISPR systems that target antibiotic resistance genes, it is possible to introduce these CRISPR constructs into the gastrointestinal tract. Once inside the gut, these plasmids can be transferred between bacteria via bacterial conjugation. This allows the CRISPR system to spread throughout the bacterial population and eliminate resistance genes in bacteria that harbor them. Neil et al. (2021) demonstrated the feasibility of this approach in mice, showing that a CRISPR system delivered via a high-transfer-efficiency plasmid could effectively eliminate antibiotic-resistant bacteria from the gut microbiome. However, a significant drawback

is the risk associated with the uncontrolled spread of modified genetic material, making regulatory approval for therapeutic use difficult (National Institutes of Health, 2019). The safety and environmental impact of introducing genetically modified organisms must be carefully assessed before such a system can be widely implemented.

Bacteriophages (phages) are viruses that specifically infect bacteria, making them ideal candidates for delivering CRISPR-Cas9 into bacterial cells. Phages can be engineered to carry the CRISPR-Cas9 system and selectively infect target bacterial strains, allowing for precise genome editing. This specificity minimizes collateral damage to beneficial bacteria, addressing a major drawback of broad-spectrum antibiotics (Yosef et al., 2015). Phages also have the ability to disrupt biofilms, which are protective matrices formed by bacteria that bacteria form to resist antibiotics, by producing depolymerases that break down the biofilm structure (Lin et al., 2017). By breaking down biofilms and delivering CRISPR-Cas9 to specifically edit resistance genes in targeted bacterial strains, engineered phages offer a dual-action approach: weakening bacterial defenses while precisely restoring antibiotic susceptibility. This combination provides an alternative to conventional antibiotics, which often lack the specificity and biofilm-penetrating capability needed for the effective treatment of MDR infections.

The use of phage cocktails, which combine multiple phages targeting different bacterial strains, has shown promise in overcoming bacterial resistance and enhancing treatment outcomes. This approach has been particularly successful in regions like Eastern Europe, where phage therapy is actively practiced (Brown et al., 2017).

Phages replicate via two main cycles: the lytic and lysogenic cycles. In the lytic cycle, phages infect bacterial cells, hijack their machinery to replicate, and ultimately cause cell lysis, killing the bacteria. This is the cycle used in phage therapy, as it directly reduces bacterial

populations. The lysogenic cycle, on the other hand, involves phage DNA integrating into the bacterial genome, where it can remain dormant. While lysogenic phages can contribute to bacterial resistance, obligately lytic phages, which bypass the lysogenic phase, are preferred for therapeutic use (Sharma et al., 2022). Phage therapy, which uses lytic bacteriophages to target and destroy pathogenic bacteria, was first demonstrated in the early 20th century by d'Herelle, who successfully treated dysentery and bubonic plague using phages (Sharma et al., 2024). Today, phage therapy is still practiced in countries like Georgia and Poland, particularly for treating MDR infections.

One significant advantage of phage therapy lies in the use of phage-derived enzymes called endolysins. Endolysins break down the bacterial cell wall, causing osmotic lysis and cell death. They work in conjunction with holins, proteins that create pores in the bacterial membrane, allowing endolysins to access the cell wall. Because endolysins target specific bonds in the bacterial cell wall, they are highly effective against MDR pathogens and biofilms, without the risk of promoting resistance, as seen with traditional antibiotics (Sharma et al., 2022).

Despite its promise, phage therapy faces challenges, particularly the narrow host specificity of phages, which may limit their therapeutic range. Creating phage cocktails that target multiple bacterial strains is a potential solution, enhancing efficacy while preserving the beneficial microbiota (Sharma et al., 2024). Additionally, bacterial evolution poses a challenge as bacteria may develop resistance mechanisms against phages, requiring continuous adaptation of phage formulations. Future research should focus on genetically modified phages and phage-derived enzymes, like endolysins, which have shown promise in fighting antibiotic-resistant bacteria without fostering new resistance (Sharma et al., 2024).

Endolysins also offer an advantage by bypassing common bacterial defenses like efflux pumps and resistance mechanisms. This makes them a promising tool in the ongoing fight against antibiotic resistance. The development of endolysin-based drugs, such as SAL-1, which entered Phase I clinical trials in 2013, marks a significant step forward in exploiting phages and their derivatives as alternative antimicrobials (Sharma et al., 2022).

Both probiotics and bacteriophages offer viable delivery mechanisms for CRISPR-Cas9 systems aimed at eliminating antibiotic resistance genes in bacterial populations. While probiotics provide a natural method of gene transfer through bacterial conjugation, bacteriophages offer a highly specific and adaptable means of delivering CRISPR constructs directly into bacteria. Additionally, the use of phage-derived enzymes like endolysins enhances the effectiveness of phage therapy, particularly in combating MDR pathogens. As research continues to refine these delivery systems, they hold significant potential in addressing the global challenge of antibiotic resistance.

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.

e. The Advantages of a Bacteriophage Delivery System

Bacteriophage-based delivery systems have emerged as a promising method for delivering CRISPR-Cas components to bacterial targets, especially in combating AMR. Phages are highly specific, infecting only particular bacterial species, and can be engineered to carry CRISPR-Cas9. Once inside the target bacteria, these CRISPR constructs can disable resistance genes, making bacteria more susceptible to antibiotics. For instance, the M13 bacteriophage has been successfully used to package CRISPR constructs for AMR gene targeting in *E. coli* (Kiga et al., 2020). However, challenges such as mutations in phage receptors on bacterial surfaces can limit the efficacy of this delivery system. Ongoing research focuses on overcoming these challenges, with advances such as tail fiber protein modification improving phage adaptability (Wongpayak et al., 2021).

Bacteriophages offer several key advantages as delivery vehicles. Their species specificity ensures that they infect only the target bacterial species, preventing off-target effects (Kasman & Porter, 2022). Furthermore, phages naturally package their DNA into capsids, which can then inject it into the bacterial cells, allowing for replication and integration of foreign DNA through lytic or lysogenic cycles (Bikard et al., 2014). According to the International Committee on Taxonomy of Viruses, over 270 phage species have been classified that can infect *E. coli* (Olsen et al., 2020). The diversity of bacteriophages offers the flexibility to select different phages based on the size of foreign DNA that needs to be inserted. Phages also contain genetic markers that help identify infected cells, a useful feature for monitoring phage activity. Importantly, bacteriophages have been used in clinical trials for phage therapy, with the U.S. Food and Drug Administration (FDA) approving the first bacteriophage therapy in 2019 (Aswani & Shukla, 2021). This makes phages a viable option for in vivo applications, compared to alternatives like microinjection, which is impractical for large-scale use.

Despite these advantages, one of the major challenges in using bacteriophages as therapeutic agents is effective delivery to the site of infection. Phages may struggle to penetrate tissues, and without proper preparation, they may be degraded by enzymes or neutralized by the host immune system before reaching their target (Khambhati, 2022). To address this, researchers have developed encapsulation techniques aimed at protecting phages and improving their therapeutic efficacy. For instance, liposome encapsulation has been shown to shield phages from environmental stresses, such as acidic pH and enzymatic degradation. Encapsulated phages targeting *Salmonella* were able to survive simulated gastric fluid with a pH of 2.8, demonstrating improved stability compared to free phages (Khambhati, 2022). Other encapsulation strategies include hydrogels, such as alginate, which stabilize phages under acidic conditions and enable controlled release in applications like catheter infections (Khambhati, 2022). Additionally, fibers have been used to immobilize phages for wound dressings, providing an effective topical delivery method (Khambhati, 2022).

Bacteriophage delivery systems, particularly those employing CRISPR-Cas9, offer significant potential in addressing antibiotic-resistant bacterial infections. Phages provide a highly specific and adaptable means of targeting resistant bacteria, while advances in encapsulation and bioengineering are helping to overcome the challenges of phage instability and bacterial resistance.

4. Bacteriophage Delivery System

a. 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.

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 kilobases (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).

b. Overview of Bacteriophage Delivery Systems

Bacteriophages have long been a subject of study due to their ability to selectively infect bacterial hosts, making them ideal for gene and drug delivery. The bacteriophage λ, first studied in the 1950s, is one such virus of interest. It contains sticky ends at the termini of its linear chromosome, a feature significant in its assembly. During phage assembly, the precursor to the capsid is formed first, and a DNA translocase inserts the DNA. The λ phage has a double-stranded genome with a size of 48,502 base pairs (Casjens & Hendrix, 2015). To ensure capsid stability, it must contain 37 to 52 KB of DNA (Clark et al., 2019). Importantly, there is a non-essential section of the genome, approximately 15 KB in size, which can be removed to accommodate larger inserts (Clark et al., 2019).

Filamentous bacteriophages, such as M13, have shown particular promise in genetic engineering applications. Unlike other phages, M13 does not lyse its host, allowing for

non-lethal infections of Gram-negative bacteria like *E. coli* (Rami et al., 2017). Due to this feature, M13 is frequently employed in CRISPR-Cas9 delivery systems. M13 belongs to the Ff class of filamentous phages and is widely used in phage display technology—a technique pioneered by George P. Smith in 1985. Phage display allows for the presentation of peptides on the phage surface by fusing the desired peptide to gene III of the phage, making it a critical tool in the development of novel therapies and drug delivery systems because it enables the identification of peptides that can bind specifically to biological targets, facilitating the design of targeted therapeutics (Sioud, 2019; Ebrahimizadeh & Rajabibazl, 2014). The M13 bacteriophage has a single-stranded DNA genome consisting of only 6,400 base pairs (Lai et al., 2021). Despite its small size, M13 has been observed to accept inserts up to 42 KB, which is seven times the size of its genome. The replication process of M13 is distinctive as it does not inject its genome into the host cell like other phages. Instead, the p3 proteins on the bacteriophage bind to the F-pilus of male *E. coli*, and the pilus contracts, drawing M13 closer to the cell surface. This allows the phage DNA to penetrate and enter the host cell. The small genome size and non-lytic nature of M13 make it a viable candidate for gene delivery applications. However, M13 cannot infect female *E. coli* due to its reliance on the F-pilus (Smeal et al., 2017).

Several other phages were considered but ultimately eliminated from the study. The P2 bacteriophage, which has a genome size of 33,592 base pairs, consists of an icosahedral head and a tail filament with a nonspecific injection mechanism (Christie & Calendar, 2016). Despite its versatility in transducing multiple bacterial strains, P2 was dismissed due to its large genome size, which limits efficient and cost-effective DNA insertion.

Similarly, the T7 bacteriophage, which has a double-stranded DNA genome, was not suitable for this study due to its lytic life cycle. The T7 phage destroys the cells it infects, which

is incompatible with the goal of creating a sustainable delivery system for CRISPR-Cas9 (Cuervo et al., 2013).

In this research, we opted to use the M13 phage as a delivery vector for CRISPR-Cas9. Its small genome size and lysogenic nature make it cost-effective and efficient for gene insertion via restriction cloning. Although M13 cannot infect female *E. coli*, this limitation does not affect the current study's goal, which is to provide proof of concept that CRISPR-Cas9 can serve as an alternative antimicrobial strategy for sensitizing drug-resistant *E. coli* to antibiotics. Future research will address the challenge of M13's specificity for male *E. coli*.

Materials and Methods

1. Primer design

PCR Primers were designed to have 18–24 complementary bases, start and end with C/G pairs, have 40–60% G/C content, and have an annealing temperature of 50–60 ℃. We verified that the majority of these specifications were met and that the primers did not have significant potential to dimerize or form secondary structures using IDT DNA's OligoAnalyzer™ Tool. When using primers to add restriction sites to DNA, restriction sites were added to the 5' ends of the primers. Additional bases were then added to the 5' ends in order to improve restriction site cutting efficiency in the amplicon. Each set of primers was verified via PCR amplification and gel electrophoresis as described in Materials and Methods sections 4 and 5.

2. Sequencing and chromatography analysis

Two 20-base PCR primers were designed as described in Material and Methods Section 1 to amplify the first 571 bases of *gyrA*. PCR was performed on JM101 and JM109 chromosomes using these primers as described in Material and Methods Section 6. The resultant amplicons were sequenced by GENEWIZ (Research Triangle Park) with the same primers being used for PCR amplification.

Once the sequencing reads and chromatographs were received, we conducted chromatograph analysis to look for *gryA* point mutations in each sequence. We first aligned the forward and reverse reads using the European Molecular Biology Laboratory European Bioinformatics Institute EMBOSS Needle Pairwise Sequence Alignment online interface (Madeira et al., 2024) and trimmed non-complementary sequences at their 5' and 3' ends. We then located bases that were inconsistent between the forward and reverse reads, and analyzed

the chromatographs to determine which was correct by finding which chromatograph had the cleanest, clearest peak at each location.

After determining the true amplified sequences, we aligned them with the wildtype K-12 substrain MG1655 *gyrA* sequence in the National Center for Biotechnology Information (NCBI) Nucleotide Database to determine the location of codons 83 and 87. We then noted mutations between the wildtype sequence and our amplicons.

The first attempt at sequencing JM109 *gyrA* using these primers resulted in a noisy chromatographs, Hence, two more 20-base PCR primers were designed as described in Material and Methods Section 1 to amplify bases 104–411 *gyrA*. PCR and sequencing were performed as with JM101, and the received chromatographs were normal amplitude and clean. Analysis was done as with JM101.

3. Plasmid purification using Monarch plasmid miniprep kit

Plasmids were purified according to New England Biolabs (NEB) protocol #T1010 using the Monarch Plasmid Miniprep Kit (New England Biolabs, Cat. No. #T1010). Bacterial plate cultures were swabbed for growth and transferred into 200 µL of resuspension buffer in microcentrifuge tubes. 200 µL of lysis buffer was then added, and the microcentrifuge tubes were inverted 5–6 times and then incubated at room temperature for 1 minute. 400 µL of neutralization buffer was added and the tubes were inverted until neutralized. The tubes were then incubated at room temperature for 2 minutes. The lysate was centrifuged at 160,000 rotations per minute (RPM) for 5 minutes. The supernatant was transferred to a spin column and centrifuged for one minute. Once the flow through was discarded, 200 µL of wash buffer 1 was added, and the tubes were centrifuged for 1 minute. 400 µL of wash buffer 2 was added and the tubes were centrifuged for 1 minute. The columns were transferred to clean microcentrifuge

tubes and 30 µL of elution buffer was added. The tubes were centrifuged for 1 minute after a 1 minute incubation period to elute DNA.

4. PCR amplification of GFP

The GFP PCR was carried out using Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Cat. No. M0494S). Four PCR reactions were prepared, each using varying concentrations of template DNA. Serial dilutions of the GFP plasmid were performed to create these template concentrations.

a. Serial Dilution of GFP Plasmid:
    i. An initial 1:1000 dilution was prepared by adding 1 µL of GFP plasmid to 999 µL of nuclease-free water. Next, a 1:10 dilution series was performed by transferring 1 µL of the 1:1000 dilution into 9 µL of nuclease-free water, creating the first 1:10 dilution. This process was repeated three additional times to generate four successive 1:10 dilutions, which were used as the experimental samples.
b. Preparation of Primers:
    i. 10 µM forward and reverse primers were prepared by diluting the GFP FseI primers at 1:10 by adding 1 µL of the forward primer to 9 µL of water in a microcentrifuge tube. Similarly, 1 µL of the reverse primer was added to 9 µL of water in another labeled microcentrifuge tube.
c. GFP PCR Reaction Setup:
    i. Each 50 µL GFP PCR reaction contained: 10 µL of 5X Q5 reaction buffer, 2.5 µL each of forward and reverse primers (10 µM), 1 µL of template DNA (one of the serial dilutions), 1 µL of dNTPs, 0.5 µL of Q5 High-Fidelity DNA Polymerase, and Nuclease-free water to a final volume of 50 µL.

d. Thermocycling Conditions for GFP PCR

i.

5. PCR amplification of pCas plasmid

The pCas PCR was performed using the LongAmp PCR Kit from New England Biolabs (New England Biolabs, Cat. No. E5200S). Serial dilutions of the pCas plasmid were prepared using the same method as for GFP as described in Materials and Methods Section 4, generating four pCas dilutions. Similarly, 10 µM forward and reverse primers were prepared using the same 1:10 dilution method as for GFP.

a. pCas PCR Reaction Setup:

ii. Each 50 µL pCas PCR reaction contained 25 µL of LongAmp Taq 2X Master Mix, 2 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 1 µL of template DNA, and nuclease-free water to a final volume of 50 µL

e. Thermocycling Conditions for pCas PCR:

i. Initial Denaturation: 94°C for 30 seconds

ii. 30 Cycles of: Denaturation: 94°C for 30 seconds, annealing: 55°C for 30 seconds, and extension: 65°C for 11 minutes

iii. Final Extension: 65°C for 10 minutes

iv. Hold: 4°C indefinitely

***6. PCR amplification of* gyrA**

The *gyrA* PCR was performed using Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Cat. No. M0494S). Serial dilutions of the JM101 and of JM109 were prepared using the same method as for GFP as described in Materials and Methods Section 4, generating

four dilutions of each. Similarly, 10 µM forward and reverse primers were prepared using the same 1:10 dilution method as for GFP.

b. *gyrA* PCR Reaction Setup:

    v. Each 50 µL *gyrA* PCR reaction contained: 10 µL of 5X Q5 reaction buffer, 2.5 µL each of forward and reverse primers (10 µM), 1 µL of template DNA (one of the serial dilutions), 1 µL of dNTPs, 0.5 µL of Q5 High-Fidelity DNA Polymerase, and Nuclease-free water to a final volume of 50 µL.

f. Thermocycling Conditions for *gyrA* PCR:

    i.

7. Gel electrophoresis

Following amplification, PCR products were analyzed using agarose gel electrophoresis to confirm successful amplification. A 1% agarose gel was prepared by dissolving 1 g of agarose in 100 mL of 1X TBE buffer in an Erlenmeyer flask. A 1x TBE buffer was made by adding 100 mL of 10x TBE buffer stock and 900 mL of deionized water. The solution was heated in a microwave until the agarose was fully dissolved. After heating, the flask was allowed to cool until it was slightly warm. Once cooled, 10 µL of ethidium bromide (EtBr) (10 mg/ml stock) was added. The solution was poured into a gel casting apparatus, and the gel was left to solidify at room temperature.

Once the gel had solidified, it was placed in an electrophoresis chamber filled with 1X TBE buffer. Next, 10 µL of DNA ladder was loaded into the first lane as a size reference. PCR samples were prepared for loading by mixing 2 µL of PCR product, 5 µL of 5X orange loading buffer, and 13 µL of nuclease-free water

Each sample was carefully pipetted into separate wells of the gel. Electrophoresis was performed at 100 mV constant voltage for 30 minutes.

8. Purification of PCR amplified DNA using Agilent StrataPrep PCR purification kit

After PCR amplification, the samples were analyzed by gel electrophoresis as described in Materials and Methods Section 5. Amplicons were purified using the Agilent StrataPrep PCR purification kit. A volume of DNA binding solution was added to each tube in an equal volume to the sample, and this mixture was transferred to a spin column. The column was centrifuged at 160,000 RPM speed for 30 seconds. The flow through was discarded and 750 µL of wash buffer was added to the column. The tube was centrifuged for 30 seconds. The spin column was transferred to a new tube, and 50 µL of elution buffer was added. The tube was incubated at room temperature for 5 minutes and then centrifuged for 30 seconds to elute DNA.

9. Preparing growth media

To determine the Minimum Inhibitory Concentration (MIC) of nalidixic acid-resistant *E. coli*, LB agar media was prepared and supplemented with nalidixic acid (NA), kanamycin, or ampicillin at varying concentrations. A total of 37 g of LB agar powder was measured using an analytical balance and transferred to a sterile 250 mL glass flask. Distilled water (1 L) was added to the flask, which was then covered with aluminum foil. The flask was autoclaved at 220°C for 1 hour at 15 psi in liquid mode, ensuring it was placed in a bin containing 1 inch of water to minimize evaporation. Following autoclaving, the flask was transferred to a water bath and allowed to cool to approximately 50°C to prevent premature solidification of the agar and degradation of the antibiotics.

To prepare NA-supplemented plates, a stock solution was prepared by dissolving 20 mg of NA powder in 1 L of ethanol, yielding a 20 µg/mL stock solution. Once the LB agar had

cooled, the required volumes of NA stock solution were added to achieve final concentrations of 2.5, 5, 10, and 20 μg/mL. Specifically, 1.875 mL, 3.75 mL, 7.5 mL, and 15 mL of stock solution were added per 50 mL of agar, respectively. The medium was mixed thoroughly before being poured into sterile petri dishes and allowed to solidify overnight at room temperature. Control plates without NA were also prepared.

To prepare kanamycin-supplemented plates, a stock solution was prepared by dissolving 0.5 g of kanamycin in 10 mL of sterile water. A volume of 1 mL of this stock solution was added to the LB agar to achieve a final concentration of 50 μg/mL. The medium was thoroughly mixed before being poured into sterile petri dishes and allowed to solidify overnight at room temperature.

To prepare ampicillin-supplemented plates, a stock solution was prepared by dissolving 0.6 g of ampicillin in 20 mL of sterile water. A volume of 1 mL of this stock solution was added to the LB agar to achieve a final concentration of 30 mg/mL. The medium was mixed thoroughly before being poured into sterile petri dishes and allowed to solidify overnight at room temperature. All plates were labeled with the name, date, and antibiotic concentration and stored at 4°C until use.

10. Conducting minimum inhibitory concentration (MIC) assays

To determine the MIC of NA-resistant *E. coli*, JM101 and JM109 strains were resuspended and cultured on antibiotic-supplemented plates. Lyophilized cultures were rehydrated by removing the vial cap and rubber stopper, then adding 1 mL of recovery broth using a micropipette. The rubber stopper and cap were replaced, and the contents were mixed by gently pipetting up and down to ensure homogeneity.

For MIC testing, JM101 and JM109 strains were streaked onto agar plates supplemented with varying concentrations of NA. Each plate was divided into two sections, with JM101 streaked on one half and JM109 on the other. Control plates without antibiotics were also streaked. All plates were labeled with the strain, date, and antibiotic concentration before being incubated at 37°C for 24 hours. Following incubation, plates were photographed to document bacterial growth at each antibiotic concentration.

11. Generation of spontaneous mutants

To identify spontaneous mutants with increased resistance to NA, colonies that exhibited growth above the MIC were selected. These colonies were presumptively identified as mutants based on their ability to survive at higher antibiotic concentrations. Selected colonies were subcultured onto fresh NA-containing plates to confirm their resistance phenotype. DNA was extracted from confirmed resistant colonies using the NEB Monarch purification kit according to the manufacturer's protocol. The presence of mutations conferring NA resistance was validated through polymerase chain reaction (PCR) amplification followed by DNA sequencing. Sequence data were analyzed to identify mutations associated with NA resistance.

12. Digestion and ligation with restriction enzymes

After adding the Fse1 restriction sites to the ends of the GFP fragment and pCas plasmid as described in Material and Methods Section 4, a digestion was performed. 20 µL of the pCas plasmid and 20 µL of the GFP fragment were combined in a 2 mL tube along with 5ul of rCutSmart digestion buffer and 1 µL of FseI enzyme (New England Biolabs, Cat. No. R0588S). This digestion mixture incubated overnight at 37°C. 2ul of the samples were then run on an agarose gel to confirm digestion. The remaining sample was heat inactivated at 80°C for 20 minutes. 2ul of 10x ligase buffer was then added and the sample was split into 2 aliquots. 1 µL of

T4 ligase was then added to one of the aliquots, and no ligase was added to the other as a negative control. The samples were then incubated at 37°C overnight. 4 µL of each sample was run on an agarose gel to confirm ligation.

13. Transformation

Following ligation of the GFP fragment and pCas Plasmid, a transformation was conducted. NEB 5-alpha competent *E. coli* was thawed on ice and separated into 3 tubes each containing 100 µL. While on ice, 5 µL of ligation mix, 5ul of the ligation mix containing no ligase(negative control) and 1 µL of purified pCas was then each added to 1 of the tubes respectively, and left on ice for 30 minutes. The 3 samples were then heat shocked at 42°C for 2 minutes. 900 µL of S.O.C medium was then added to each tube and incubated for 1 hour on a rotator drum. 200 µL of the ligated sample mix was then transferred to 5 LB plates with kanamycin(section 7) and distributed using the spread plate method. This was repeated to make 1 plate for the ligated sample containing no ligase, and 3 plates with the pCas9 using 100, 10, and 1 µL of sample. The plates were then left to incubate at 30°C.

Results

1. Selection of bacteriophage delivery system

Several bacteriophages were considered as our delivery vehicle. Many factors such as the size of the genome and the nature of the phage infection were taken into consideration. The P2 bacteriophage, which has a genome size of 33,592 base pairs, consists of an icosahedral head and a tail filament with a nonspecific injection mechanism (Christie & Calendar, 2016). Despite its versatility in transducing multiple bacterial strains, P2 was dismissed due to its large genome size, which limits efficient DNA insertion via restriction cloning.

Similarly, the T7 bacteriophage, which has a double-stranded DNA genome, was not suitable for this study due to its lytic life cycle. The T7 phage destroys the cells it infects, which is incompatible with the goal of creating a sustainable delivery system for CRISPR-Cas9 (Cuervo et al., 2013).

Each of these phages were eliminated from the study due to their larger genome sizes, which would limit the efficiency of the packaging process, or their lytic life cycles, which would not allow for the sustained expression of CRISPR components.

For the delivery of the CRISPR system, we chose bacteriophage M13, a non-lytic filamentous phage. The primary reasons for selecting M13 were its small genome size of 6,400 base pairs and its capacity to carry large DNA inserts, such as the CRISPR construct (Lai et al., 2021). The small genome size of M13 is advantageous since it allows for the efficient packaging of the phage genome along with the inserted CRISPR system and pCas plasmid backbone without spatial constraints. The non-lytic nature of M13 ensures that the host cell is not immediately destroyed, allowing for prolonged expression of the CRISPR system, which is necessary for sustained gene editing activity.

Despite the limitation that M13 can only infect male *E. coli* strains, this constraint was not significant for this proof-of-concept experiment, as we used a strain of *E. coli* specifically designed to support M13 infection (Smeal et al., 2017). Future research will address the challenge of M13's specificity for male *E. coli*.

2. Overview of the CRISPR-Cas9 targeting mechanism and integration into chosen bacteriophage M13

Our experiment uses CRISPR-Cas9 along with homology directed repair (HDR) to edit the resistance mutation because CRISPR-Cas9 presents advantages over older systems in its efficiency and adaptability (Ran et al., 2013). Using Cas9 to introduce double stranded breaks near the resistance mutation allows the cell's endogenous HDR mechanisms to repair the damaged region using our HDR template, which contains the nalidixic acid sensitive sequence of the *gyrA* gene.

Once the CRISPR-Cas9 system and associated HDR template has been introduced into the resistant bacteria, our experiment will confirm that the resistance mutation has been edited using PCR and DNA sequencing to amplify and sequence the target region. Our experiment then uses nalidixic acid MIC assays to confirm that the edited bacteria have been resensitized to nalidixic acid.

The CRISPR-Cas9 system was designed to target a specific antibiotic resistance gene in *E. coli*, disrupt its function, and restore the bacterium's susceptibility to antibiotics. First, a guide gRNA sequence was designed to specifically target the resistance gene of interest. This sequence was chosen based on the analysis of conserved regions in the gene, ensuring that it would not cross-react with other genes in the genome. The gRNA was designed to target a gene region essential for its function, minimizing the potential for off-target effects. To facilitate the delivery

of the gRNA and Cas9 endonuclease to the target bacteria, the CRISPR system was designed to be cloned into a plasmid backbone designed for packaging into a bacteriophage genome.

The plasmid backbone includes a sequence encoding the Cas9 endonuclease and a sequence encoding the gRNA scaffold (Jiang et al., 2015). Once the plasmid containing the CRISPR components was designed, with additional elements designed to be added through restriction cloning, it would be prepared for incorporation into the bacteriophage genome.

We planned to measure the success of the CRISPR-Cas9 delivery system using a combination of PCR-based assays and antibiotic susceptibility testing. PCR would be used to verify the presence of the CRISPR-induced mutations in the target gene, confirming that the gene editing process was successful. Additionally, antibiotic susceptibility testing would be conducted before and after CRISPR treatment to assess whether the disruption of the resistance gene restored susceptibility to the targeted antibiotic.

The experimental design involved several key steps to successfully demonstrate the CRISPR-Cas9 delivery system using bacteriophages. First, the M13 bacteriophage would be prepared by packaging the CRISPR-Cas9 plasmid into the phage genome. This would be achieved through transduction, using a helper phage to facilitate the incorporation of the CRISPR construct into the M13 genome. Following this, the *E. coli* JM101 strain would be infected with the modified M13 bacteriophage. The infection will allow for the delivery of the CRISPR system into the bacterial cells, where the Cas9 protein and gRNA are expressed. This expression enables the targeted disruption of the antibiotic resistance gene in the bacterial genome.

To verify the success of the gene editing process, PCR assays would be conducted to confirm the presence of mutations at the target gene, and antibiotic susceptibility testing would be performed before and after CRISPR treatment to assess whether the disruption of the

resistance gene restored the bacteria's sensitivity to the targeted antibiotic. These tests serve as the primary means of validating the effectiveness of the CRISPR-Cas9 system in disrupting the antibiotic resistance gene and restoring antibiotic susceptibility in the *E. coli* strain.

The overall design of the project involved the construction of a CRISPR-Cas9 system that could be delivered via bacteriophage to disrupt antibiotic resistance genes in *E. coli* (*Fig. 1*). The selection of the M13 phage as the delivery vector for gene insertion via restriction cloning and *E. coli* JM101 as the host strain was based on their compatibility with the CRISPR-Cas9 system and the requirements for efficient gene editing. This project design aims to provide proof of concept for using bacteriophage-based delivery systems to combat antibiotic resistance.

3. Modification of the pCas plasmid to build the CRISPR system

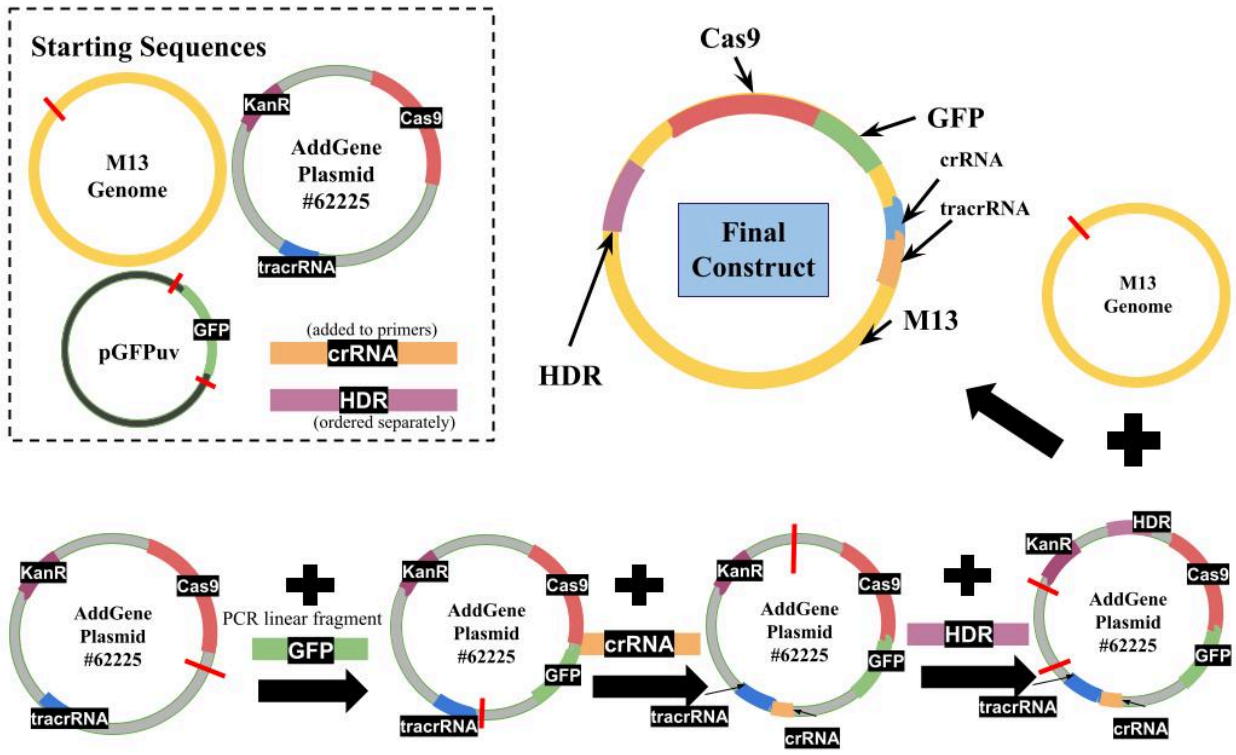

Figure 1. A graphical representation of the bioinformatic design of the final CRISPR-Cas9 construct used to edit *gyrA*.

We designed a recombinant DNA construct that can use CRISPR-Cas9 and homology directed repair to edit *gyrA* codon 83 and resensitize *E. coli* to nalidixic acid. We chose plasmid #62225 (pCas) from AddGene (Jiang et al., 2015) as the backbone since it includes the *cas9* gene, a tracrRNA (trRNA) sequence, and a kanamycin resistance gene *KanR* transcribed constitutively. The trRNA sequence can be used to guide Cas9, while *KanR* can be used as a selectable marker.

Our modifications of the pCas plasmid were planned in four steps. Our first step was to add a screenable marker that would show us that Cas9 is being appropriately expressed. Our second step was to add a crRNA that targets *gyrA* codon 83 to the existing trRNA on the plasmid. Our third step was to introduce a Homology Directed Repair (HDR) template into our plasmid to induce the intended edit to codon 83. Our fourth step was to introduce the modified pCas plasmid into the polycloning site into the bacteriophage M13 genome. *Figure 1* outlines this process.

4. Using GFP as a screenable marker to measure Cas9 translation

We used the *pGFPuv* plasmid vector to obtain the *gfp* gene sequence (Crameri et al., 1996). We chose to insert the *gfp* into the pCas plasmid following the *cas9* sequence so they would be co-transcribed and co-translated. Detection of fluorescence from GFP would therefore imply *cas9* translation. To insert *gfp,* our design uses restriction cloning. We chose the restriction enzyme Fse1 since it uses an 8 base pair cut site that did not already exist in the *pCas* plasmid.

Our design uses PCR cloning to add the restriction sites to the *pCas* plasmid where we had chosen to insert *gfp* and to the amplified *gfp* fragment. As a result, restriction sites were added flanking the linearized *gfp* fragment as well as directly after the *cas9* gene. *Table 1* contains the initial primers used.

Table 1

Initial primers used for GFP insertion

Primer Function	**Forward Sequence**	**Reverse Sequence**
Adding pCas plasmid FseI restriction site	5'-attGGCCGGCCttttagatgaagattatttct-3'	5'-actGGCCGGCCtatacttcagtcacctccta-3'
Adding FseI restriction sites to GFP sequence	5'-cagtcaGGCCGGCCaacaattcacacaggaaacagctatga-3'	5'-ctgacaGGCCGGCCggaattcattatttgtagag-3'

5. Designing a crRNA to target codon 83

Our design uses an sgRNA for efficiency and simplicity given that other options would require multiple DNA molecules (Jinek et al., 2012). The pCas plasmid contains a gRNA scaffold sequence designed such that attaching a 20 nucleotide crRNA sequence at its 5' end forms a sgRNA that recruits the Cas9 protein and guides it to the target sequence (Anders et al., 2014).

A 5'-NGG-3' protospacer adjacent motif (PAM) sequence directly downstream of the sgRNA is necessary for Cas9 activity (Jinek et al., 2012). To design our crRNA, we chose an antisense PAM sequence upstream of the target site in codon 83, as this placed the target site within the Cas9 endonuclease activity window of 3–8 base pairs from the PAM sequence (Jinek et al., 2012). The resulting crRNA sequence was **5'-CCGCCGAGTCACCATGGGGA-3'**, and the primers designed to insert it adjacent to the tracrRNA in the pCas plasmid are shown in Table 2 below. The highlighted nucleotide is the location of the codon 83 we targeted.

Table 2

crRNA insertion primers

Primer Function	Forward Sequence	Reverse Sequence
Adding codon 83 crRNA upstream of gRNA scaffold	5'-CCGCCGAGTCACCATG GGGAggaaccattcaaaacagc-3' (42bp)	5'-aacaagattattttataact-3' (20bp)

6. Determining the minimum inhibitory concentration (MIC) of nalidixic acid for JM101 and JM109

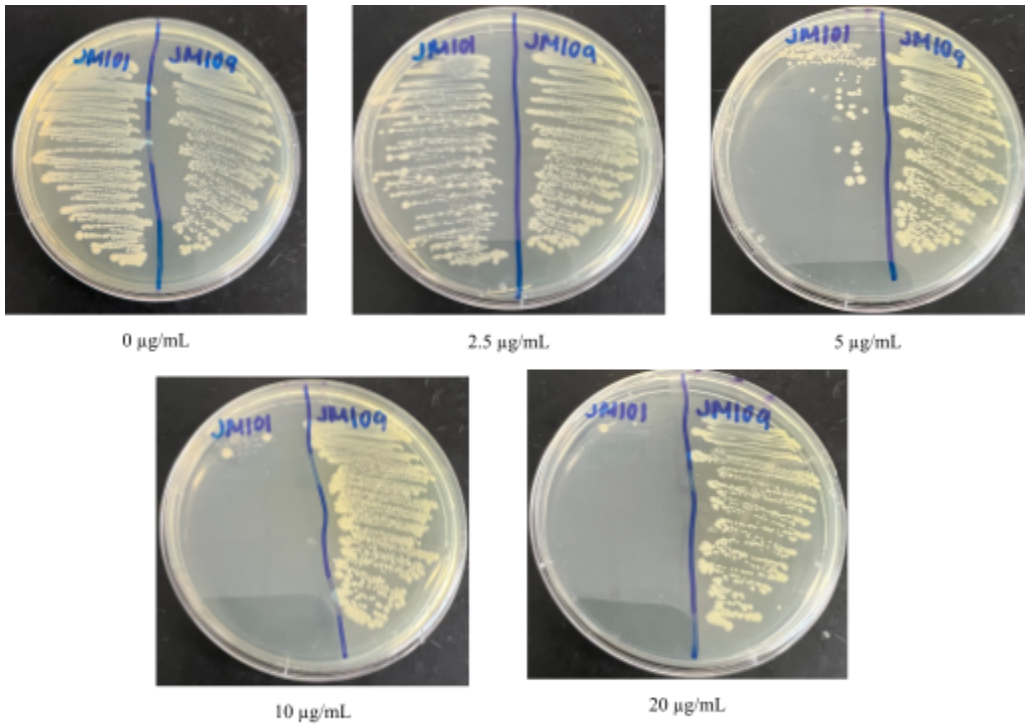

Figure 2. MIC assay of JM101 and JM109 on nalidixic acid plates.

For the bacteriophage to successfully infect and deliver the CRISPR-Cas9 system, it was crucial to select an appropriate host strain of *E. coli*. The host strain chosen for this study was *E. coli* JM101, which is a commonly used laboratory strain known to be compatible with phage M13 (Blount, 2015). *E. coli* JM101 is a male strain that expresses the F-pilus, the receptor

required for M13 infection, making it an ideal candidate for the M13-based CRISPR delivery system. This strain also harbors mutations that are relevant to the experimental design, such as antibiotic resistance mutation that allows for the testing of CRISPR-mediated gene disruption and restoration of antibiotic susceptibility.

The host strain JM101 was also engineered to carry the resistance gene of interest *gyrA*, which was selected as a target for CRISPR-mediated disruption. This gene was present in the *E. coli* genome as part of a resistance cassette, allowing for the measurement of CRISPR-Cas9 effectiveness by assessing changes in antibiotic susceptibility following treatment.

The MIC assay was utilized to determine the susceptibility of the *E. coli* Strains JM101 and JM109 to nalidixic acid. We plated the bacteria on various concentrations and determined the MIC, and in doing so, we identified a small number of spontaneous mutants. The results from the MIC assay provide clear evidence of NA resistance in *E. coli* strains JM101 and JM109 (*Fig. 2*). As shown in the images, bacterial growth patterns varied based on antibiotic concentration, with JM109 exhibiting a consistently high level of resistance, while JM101 initially showed susceptibility but later developed resistant colonies.

At 0 μg/mL NA, which was the control plate, both JM101 and JM109 demonstrated robust bacterial growth across the plate, as expected in the absence of selective pressure. The colonies were dense, indicating normal bacterial proliferation. At 2.5 μg/mL NA, there was no significant inhibition observed for either strain. JM101 and JM109 grew comparably, suggesting that this concentration was insufficient to suppress bacterial growth. At 5 μg/mL NA, JM101 displayed partial inhibition, with fewer and smaller colonies compared to JM109. However, some JM101 colonies still persisted, suggesting that a subset of the population may have begun developing resistance. JM109 remained unaffected, further supporting its pre-existing resistance

to NA. At 10 µg/mL NA, JM101 showed significant inhibition, with only a few isolated colonies present, which were noticeably smaller and more dispersed. In contrast, JM109 continued to grow without apparent inhibition. The presence of JM101 colonies at this concentration strongly indicates the emergence of spontaneous resistant mutants. At 20 µg/mL NA, JM101 was nearly completely inhibited, with very few or no visible colonies, whereas JM109 remained unaffected, demonstrating its high-level resistance to NA.

7. Verifying Resistance Mutations

After designing the theoretical construct that we planned to use to deliver the gene editing system, we needed to verify that the JM101 and JM109 strain that we selected for this experiment contained an appropriate resistance mutation. We derived 4 independent JM101 nalidixic acid mutants at 5 µg/mL, JM109, and JM101 colonies from the MIC assay described above. The initial goal was to amplify the region around codon 83 for sequencing, as this was the most common codon known to carry the mutation that causes nalidixic acid resistance in a study of 80 resistant *E. coli* strains from a variety of samples (Saenz, 2003). We designed the primers seen in Table 3 according to the known sequence of *gyrA* (NC_000913.3):

Table 3

gryA Sequencing Primers

Forward Sequence	Reverse Sequence
5’-CAGATGTCCGAGATGGCCTG-3’	5’-CGGCCATCAGTTCATGGGCA-3’

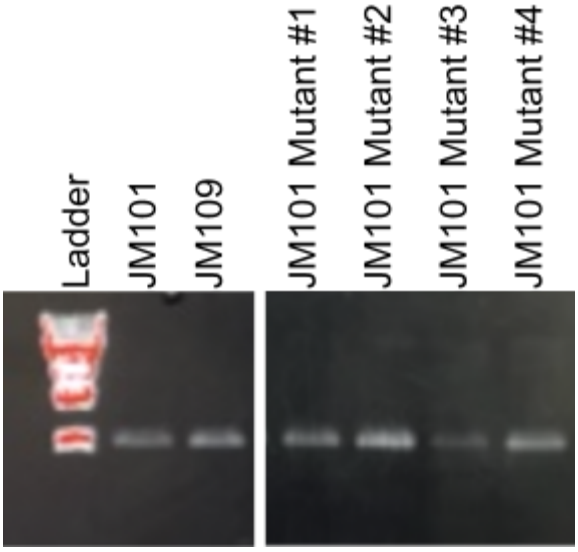

Figure 3. PCR of *gyrA* across JM101 and JM109 strains validating sequencing primers. Extraneous lanes were cropped out.

Once the primers were verified and amplification had been conducted, we sent the samples for JM109, JM101, and the JM101 mutants 1–4 for sequencing. PCR amplification of gyrA also validated the primers (*Fig. 3*).

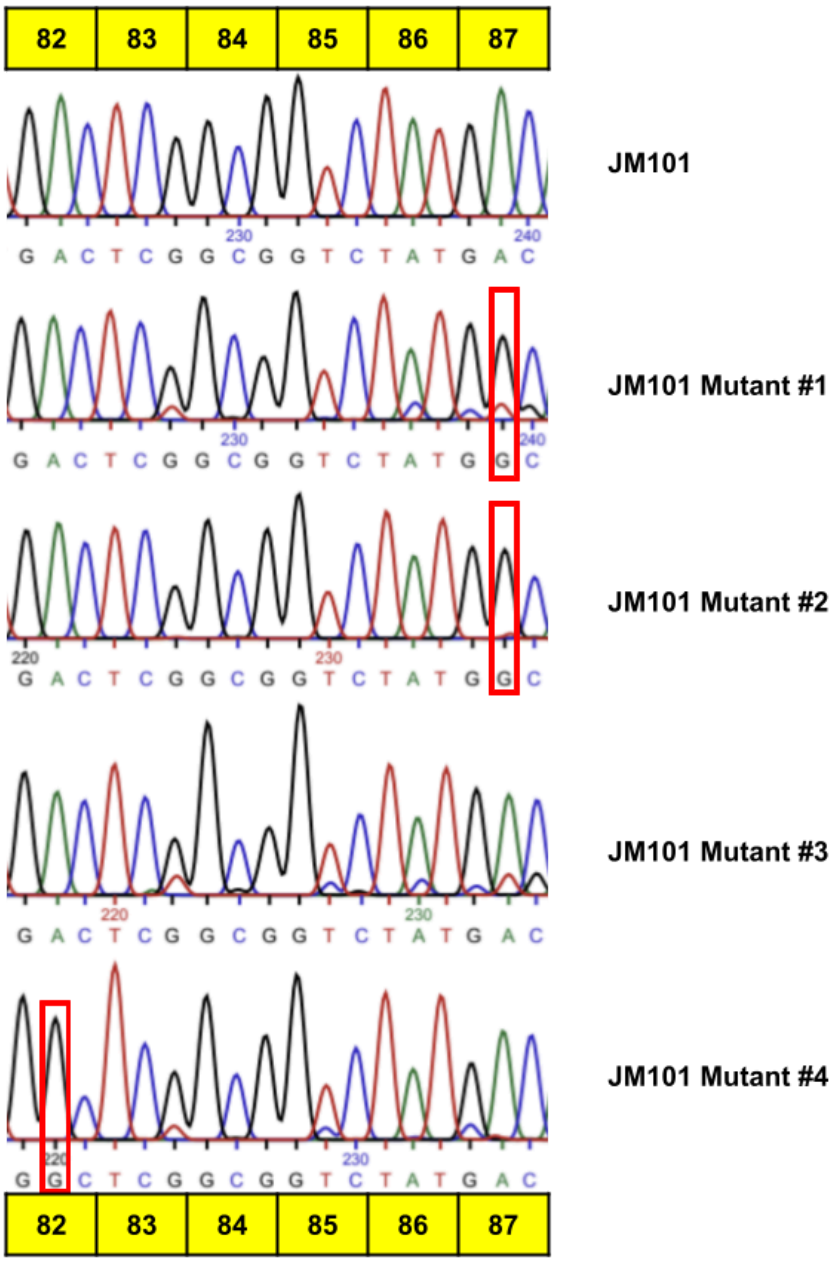

Figure 4. Validation of JM101 wildtype sequence and demonstrating spontaneous generation of JM101 mutations using sequencing chromatography.

Once the sequences were received, we conducted sequencing analysis to determine where the *gryA* point mutation occurred in each sequence. We noted that mutants 1 and 2 of JM101 naturally derived nalidixic acid resistance at codon 87, as opposed to codon 83, when compared

to the wildtype (*Fig. 4*). This appeared to be a D87G point mutation in both mutant strains. The JM101 Nal3 mutation was not noted in the *gyrA* sequence, so we posited that it was most likely in another gene. The Nal4 mutant showed the wildtype sequence at both codons 83 and 87, and showed a D82G mutation, which has been detected previously, but was noted to be a rare mutation (Johnning et al., 2015).

The JM109 sequencing shows a mutation in codon 87 (*Fig. 5*). Interestingly, this was a D87N mutation. These results were unexpected given our previous understanding that the majority of nalidixic acid resistance mutations occurred at codon 83, but a return to the literature showed that in *gyrA* D87 mutations were also reported, and that both D87G and D87N mutations had been previously seen (Saenz, 2003).

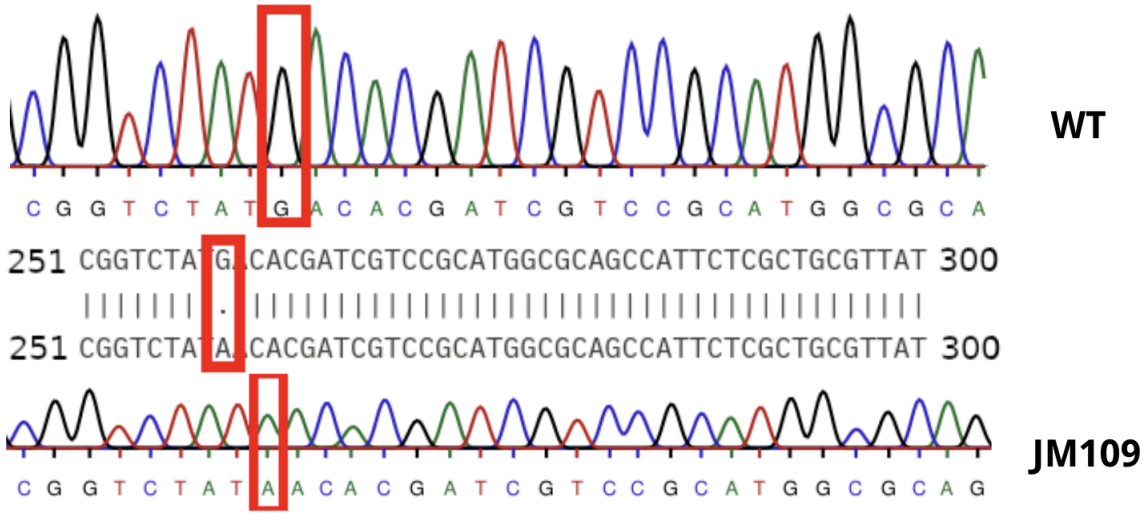

Figure 5. Validation of JM109 resistance mutation through sequencing analysis. Chromatographs from the wildtype and JM109 sequences show the presence of a point mutation at codon 87.

Given that our JM109 strain and JM101 naturally occurring mutants largely showed mutations at codon 87 following sequencing analysis, we chose to modify our existing construct

design in order to edit the mutation present at codon 87 and revert it to the original aspartate. The D82G mutation was noted to be rare, so we chose not to focus on it for this study.

8. Modifying the crRNA

We designed new primers to amplify the pCas plasmid and insert our modified crRNA adjacent to the tracrRNA sequence. Similar to the original crRNA, this sequence was designed to be complementary to the target sequence around codon 87 and to flank a 5'-NGG-3' PAM sequence on the opposite strand that is downstream of the target (Anders et al., 2014). The crRNA includes the D87N mutation, and is represented by:

5'-CTATAACACGATCGTCCGCA-3'. The primers for insertion of this sequence are seen in Table 4.

Table 4

Modified crRNA insertion primers

Forward Sequence	Reverse Sequence
5'-**CTATAACACGATCGTCCGCA**ggaaccattcaaaacagc-3'	5'-aacaagattattttataacttt-3'

9. Designing the HDR template

Once the crRNA was designed with the correct target sequence, we were able to determine the sequence of the HDR template to be used for targeted editing. We determined that we were unable to use the crRNA as the HDR template, as homology arms were required (Qadros et al., 2017). We instead chose to insert the HDR template into the pCas plasmid through restriction digest with NotI, as a NotI restriction site was already present on the pCas plasmid.

The HDR template was designed to have 30-50 bases on each homology arm surrounding the target site which contained the corrected D87 sequence. NotI restriction sites flank each homology arm, resulting in the following HDR template sequence:

5’ATTGCGGCCGCTTGGTGACGTAATCGGTAAATACCATCCCCATGGTGACTCGGCGGT CTATGACACGATCGTCCGCATGGCGCAGCCATTCTCGCTGCGTTATGCGGCCGCTTA-3’

10. Adding FseI restriction sites to GFP and pCas

In order to construct our plasmid insert, restriction cloning was used to insert a GFP sequence into the Cas9 Plasmid. Primers were first designed to add restriction sites to the ends of the linearized GFP fragment as well as the Cas9 Plasmid. Then, initial PCRs were run to amplify the GFP fragment and Cas9 Plasmid individually. Agarose gel electrophoresis was used to verify that the PCR reaction was successful. The PCR products were purified and visualized again using agarose gel electrophoresis. Initial results of the PCR showed successful amplification of the GFP fragment and unsuccessful amplification of the Cas9 Plasmid (*Fig. 6*).

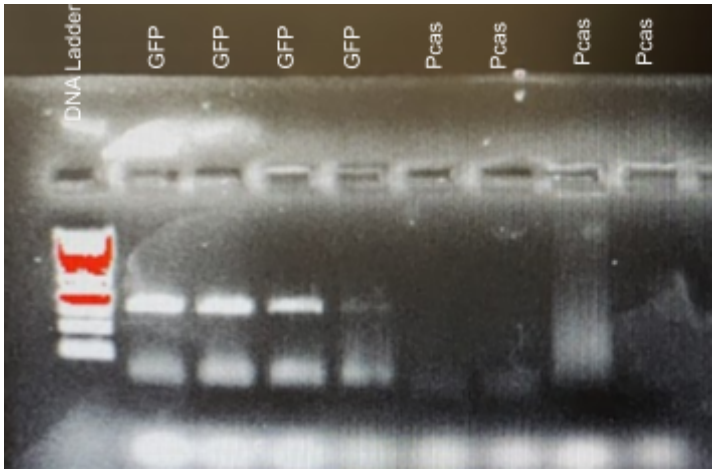

Figure 6. Agarose gel to validate successful amplifications. Lanes 2-5 contain successfully amplified GFP products, and Lanes 6-9 contain Unsuccessful pCas products.

11. Modifying the pCas FseI primers due to poor PCR and ligation efficiency

Several attempts at PCR amplification of the pCas plasmid using the primers designed to introduce FseI cut sites resulted in limited to no clear amplification of our target DNA. We considered the possibility of low amplification efficiency due to insufficient primer binding to the large plasmid, and due to the non-complementary sequence we were attempting to add. We thus redesigned the primers to add six additional complementary bases for stronger adhesion (*Table 5*). This was successful, as seen in *Figure 7* below.

We then conducted digestion and ligation of the purified GFP and pCas fragments as described in materials and methods Section 10. We noted a lack of efficiency when attempting to ligate GFP and pCas following FseI digestion.

We considered that this was possibly due to insufficient cutting by FseI of the inserted restriction sites. We, therefore, chose to add more non-complementary nucleotides to the 5' end of the pCas FseI primers to ensure cutting, with the number of 5' nucleotides after the FseI restriction site increasing from 3 to 7 (*Table 5*).

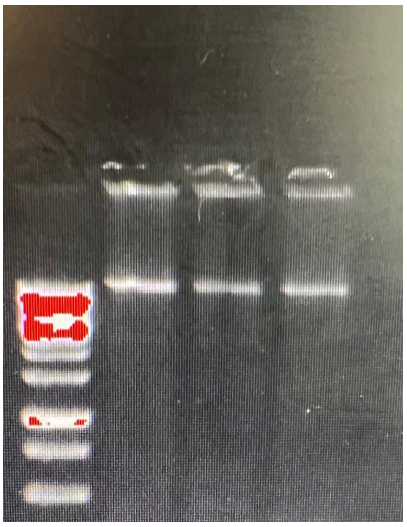

Figure 7. Agarose gel to validate successful amplifications following modification of primers.

Table 5

Modified pCas FseI primers

Primer Set	Forward Sequence	Reverse Sequence
Initial Modification	5’-attGGCCGGCCttttagatgaagatta tttcttaatctagacatgag-3’	5’-actGGCCGGCCtatacttcagt cacctcctagctgactcaaat-3’
Secondary Modification	5’-agatattGGCCGGCCttttagatgaa gattatttcttaatctagacatgag-3’	5’-ttcaactGGCCGGCCtatactt cagtcacctcctagctgactcaaat-3’

12. Transformation

Initial transformation of the ligated product was conducted resulting in growth of a few colonies. These colonies were then screened using fluorescent microscopy which depicted no colonies expressing GFP. A second transformation of the ligated products and pCas was performed which resulted in unsuccessful transformations with transformants only appearing in samples transformed with pCas and pUC-19 (positive controls) and no colonies growing in transformations done with the ligated plasmid (*Table 6*).

Table 6

Number of colonies in each transformation

Sample used in transformation	Number of Colonies
pCas 100 µL	74
pUC19 Control 100 µL	79
Ligated mix	0
Ligation mix no ligase	0

13. Control experiments to determine source of error

Because the positive controls showed growth, we determined that there was likely an error in the digestion or ligation. We chose to design a control experiment using the pUC-19 plasmid in order to test the digestion and ligation protocols. We obtained a new batch of FseI enzyme and designed primers that would add the FseI cut site to the polycloning site of the pUC-19 plasmid, according to the primer design specifications outlined in Materials and Methods section 1 (Table 7). We digested the plasmid with FseI and ligated according to the protocol specified in Materials and Methods section 11. When we ran the samples on an agarose gel, we noted that there was no change between the ligated and unligated samples, confirming that there was an error in either the digestion or ligation (*Fig. 8*).

Table 7

pUC-19 FseI primers

Forward Sequence	Reverse Sequence
5’-ttataCCGGCCGGACTGGCCGTCGTTTTACAAC-3’	5’-tttaaCCGGCCGGGGCGTAATCATGGTCATAGC-3’

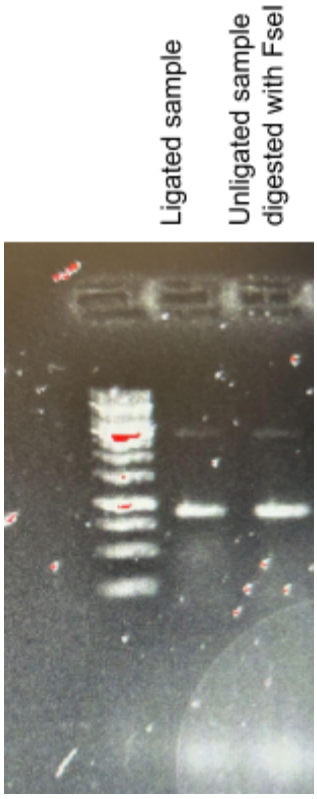

Figure 8. Agarose gel showing ligated and unligated samples after digestion with FseI of purified pUC-19 plasmid and GFPuv. Extraneous lanes were removed.

In order to determine if the digestion specifically was the point of failure that resulted in the unsuccessful transformation, we ran another control experiment using the pFT180 plasmid and HindIII restriction enzyme, a control enzyme that had a previously existing restriction site in pFT180. Gel electrophoresis of the ligation revealed a successful digestion and ligation of the pFT180 plasmid (*Fig. 9*), as the mobility of the band changed in the ligated sample.

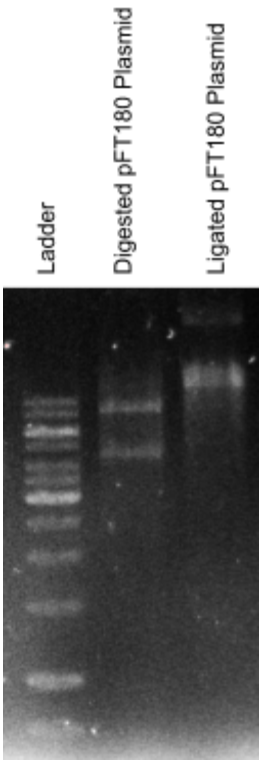

Figure 9. Agarose gel to validate successful digestion and ligation of the pFT180 plasmid. Lane 2 contains HindIII digest of pFT180, and lane 3 contains ligated pPT180.

Discussion

Antibiotic resistance is a rising threat, causing millions of deaths globally each year. *E. coli* is one of the leading pathogens associated with the AMR burden, and fluoroquinolone and beta-lactam antibiotics, largely the first line of defense, account for over 70% of AMR-related deaths (Murray et al., 2022). The emergence of muti-drug resistance and resistance to colistin, one of the last line of defense treatments, has resulted in a need for new treatment options for AMR (El-Sayed Ahmed et al., 2020).

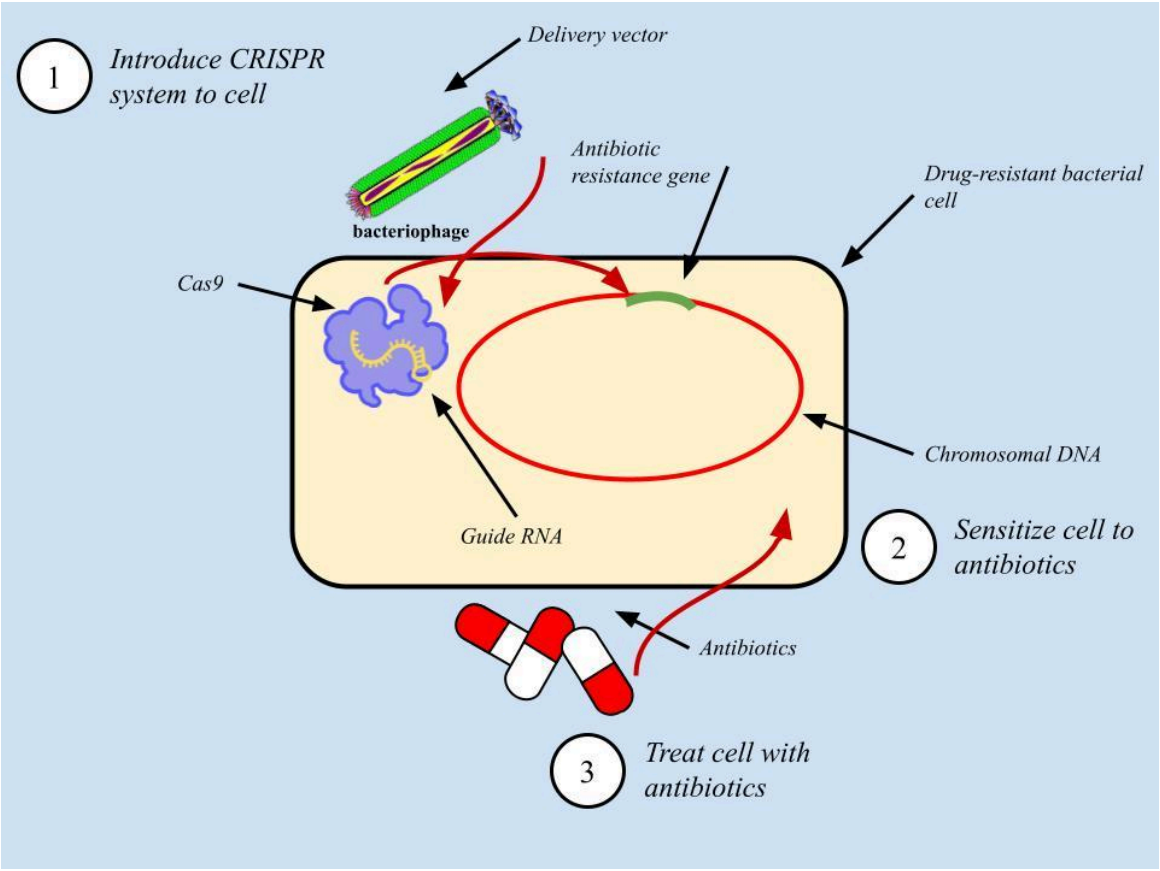

Figure 10. A graphical representation of the project overview.

The overall goal of the current study was to use bacteriophage M13 to deliver a CRISPR-Cas9 system that could successfully edit a single point mutation in the *gyrA* gene in

order to revert nalidixic acid resistant *E. coli* to cells sensitive to antibiotics (*Fig. 10*). The cells could then be treated with nalidixic acid and killed. This was a proof of principle experiment with the goal of adding to the growing body of literature demonstrating the potential for CRISPR-Cas9 editing in reverting antibiotic resistance genes. We chose to study *E. coli* due to optimal growth conditions for laboratory research, its classification as a BSL-1 organism, and the fact that it has a well-studied and fully sequenced genome (Blount, 2015, Bayot & King, 2022). Additionally, *E. coli* antibiotic resistance is a rising threat in agricultural and clinical realms (Van Boeckel et al., 2017, Velazquez-Meza et al., 2022). We chose to study nalidixic acid resistance in *E. coli* due to two factors. First, nalidixic acid is commonly linked to single point mutations in the *gyrA* gene, and this is an ideal target for a proof of principle gene editing study (Saenz, 2003). In addition, nalidixic acid resistant *E. coli* infections have significant clinical relevance in the context of rising rates of drug resistant UTIs in women, lending direct clinical applications to this study (Lee et al., 2018). Finally, we chose to use bacteriophage M13 as a delivery mechanism for this system due to the ability to produce non-lethal infections and small genome size (Rami et al., 2017).

We formed our initial construct design based on the literature, which indicated that an S83L mutation in *gyrA* was most prevalent, making this a strong point mutation target for our CRISPR-Cas9 system. In a study comparing the *gyrA* sequences of 80 nalidixic acid resistant *E. coli* strains from a variety of sources including humans, 54 showed S83L mutations, two showed other S83 mutations, and five showed some form of D87 mutation. In 19 strains with a higher MIC, 18 had both S83 and D87 mutations present, and 1 had a single S83 mutation (Saenz et al., 2003). In another study of environmental samples from wastewater treatment plants in India and Sweden, Johnning et al. (2015) found S83L mutations at a frequency of 86% in *gyrA*. D87N

mutations were present at a frequency of 32%, and D82G mutations were also noted though they were much rarer at a frequency of <1%. Additionally, the authors noted that the D87N mutations largely occurred with the S83L mutations.

When we conducted MIC assays of JM101 and JM109, we generated spontaneous mutants of JM101 which were resistant to nalidixic acid at concentrations of >5 μg/mL (*Fig. 3*). We characterized these mutants through DNA sequence analysis in order to validate the S83L sequence that our system would be targeting. We picked and characterized four mutants. Surprisingly, sequence analysis revealed that none of these four mutations showed a S83L mutation (*Fig. 5*). Mutants 1 and 2 revealed a D87G mutation. This was a low frequency mutant in Saenz et al. (2003)'s study, and was also noted at a high MIC of 24 μg/mL in an *E. coli* UTI clinical isolate by Komp Lindgren et al. (2003). D87 mutations in general are present throughout the literature previously discussed. Mutant 3 did not exhibit a mutation in the *gyrA* sequence, so we posited that it was likely in another gene. Komp Lindgren et al. (2003), Saenz et al. (2003), and Johnning et al. (2015) all noted *parC* mutations associated with fluoroquinolone resistance, a gene we did not sequence as it was outside the scope of our study. Saenz et al. (2003) and Johnning et al. (2015) did note that *parC* mutations were less prevalent than *gyrA* mutations. Perhaps most interestingly, mutant 4 revealed a D82G *gyrA* mutation. This was noted by Johnning et al. (2015) to be a rare mutation at a frequency of <1%. Upon further review of the literature, this D82G mutation was noted in 3 out of 6 quinolone resistant *E. coli* isolates that were also cross-resistant to ampicillin in a study by Kohanski et al. (2010). Perhaps this particular mutation becomes more prevalent in isolates exhibiting greater resistance or multi-drug resistance, though this requires further study.

Overall, we were surprised to see that our mutants, despite being a small sample, were found in rare or less common sites, and that the most common S83L mutation was not seen at all. It is perhaps worth noting that our MIC assays generated spontaneous mutants at a higher concentration (5 μg/mL) than the mutants studied by Saenz et al. (2003), which were generated at 2–4 μg/mL, and that the D87G mutation noted by Komp Lindgren et al. (2003) was at a very high concentration of 24 μg/mL. A direction for future research could be to explore the correlation between fluoroquinolone concentration and the emergence of rare resistance mutations. Because the majority of our spontaneous mutants contained a D87 mutation and our nalidixic acid resistant JM109 strain showed a well-studied D87N mutation after sequencing, we chose to move forward with codon 87 as our targeting sequence. In addition, D87G mutations were seen in *E. coli* UTI clinical isolates, which is the frame and potential application for this study. However, this small pool of mutants showing a variety of rare mutations or mutations not present in *gyrA* perhaps implies that a multi-target model might be more effective at resensitizing *gyrA* to nalidixic acid than a system targeting a single point mutation, and this is a recommended future direction of study.

As we began to construct our CRISPR-Cas9 system, our first step was to introduce FseI restriction sites to the pCas plasmid and *gfp* sequence via site-directed mutagenesis in order to ultimately translationally fuse a GFP as a fluorescent reporter to the *cas9* gene. This would allow us to confirm that the Cas9 protein was being expressed after transforming competent cells with our plasmid. While the *gfp* amplification was successful, we noted poor PCR efficiency when amplifying pCas with primers designed to introduce FseI restriction sites (*Fig. 7*). FseI requires an 8 base pair cut site, so this meant we were using primers with a significant amount of non-complementary DNA. We initially modified the primers to add additional complementary

nucleotides at the 3' end for better adhesion, as our original primers had around 20 homologous 3' nucleotides which was slightly under the optimal amount (Costa et al., 1996). This resulted in successful amplification.

Following successful pCas and *gfp* amplification with primers designed to insert the FseI restriction site, digestion and ligation of the fragments was unsuccessful. We hypothesized that this could be due to the restriction site sequence being too close to the end of the linear fragments, as it has been noted that restriction endonucleases have decreased efficiency at the ends of linear molecules (Carson et al., 2019). Our original primers were designed with 3 non-complementary 5' nucleotides, but we extended this to a total of 7 non-complementary 5' nucleotides. Our digestion and ligation continued to be unsuccessful, as noted through transformation where colonies grew on control plates but did not grow on experimental plates (*Table 6*).

We chose to run control experiments in order to identify the basis of the failed ligation. We first ran a digestion and ligation experiment using the FseI enzyme with the pUC19 control plasmid that was amplified to introduce FseI into the polylinker region of pUC19. We did not observe any ligation as we did not observe any change in mobility of the bands between ligated and unligated samples (*Fig. 9*). To determine if the DNA ligase was functional. We ran a digestion and ligation control experiment with the pFT180 plasmid, using different restriction enzymes. pFT180 has two HinDIII restriction sites (Stein et al., 1983). Digesting it with a HinDIII gave the two expected bands (*Fig. 9*). By ligating this digested DNA, we would then be able to determine if the ligation protocol was functioning. We were able to successfully digest with HindIII and then ligate with the same ligase and buffer used in our experimental protocol as

described in Materials and Methods section 10 (*Fig. 9*). This indicated that the ligation protocol was successful. From this, we concluded that the digestion with FseI was the point of error.

There are a few potential reasons for this, one being impaired FseI enzyme functionality. FseI is known to be impaired by high concentration salts which could have resulted from PCR cleanup, as purification with spin columns could potentially result in increased salts, though this is unlikely (NEB "Troubleshooting Guide for Cloning", 2025; NEB “FseI”, 2025). In addition, a longer incubation time may be required to ensure successful digestion (NEB “Troubleshooting Guide for Cloning”, 2025). Contamination of the enzyme is unlikely but possible, and all of these potential sources of error require further investigation. As we were investigating the source of error in our digestion and ligation experiments, we noted that it was difficult to assess the enzyme functionality of FseI directly, as it requires a unique 8 bp restriction site that is not readily found in control plasmids such as pFT180. As a result, analyzing FseI activity would require additional site directed mutagenesis, which adds additional potential points for error and makes directly assessing the enzyme activity more difficult. Because adding *gfp* to the pCas plasmid through restriction cloning requires a unique enzyme with no other cut site in the plasmid, perhaps future experiments should evaluate other methods to screen for Cas9 expression that allow for more direct analysis.

In conclusion, this thesis describes progress toward designing and constructing a CRISPR-Cas9 system that will edit a single point mutation in the *gyrA* gene in nalidixic acid resistant *E. coli* in order to resensitize the cell to antibiotics. We generated spontaneous mutants exhibiting nalidixic acid resistance when conducting MIC assays, and characterized them using sequencing analysis. Interestingly, none of the four mutants showed the most common S83L mutation described in the literature. Instead we noted a codon 82 mutation, known to be very

rare, two D87G mutations, and a mutation not present in *gyrA*, indicating that further research may be required in order to better characterize resistance producing *gyrA* mutations due to such a wide variety of changes in a small sample size. As a result, we designed a crRNA targeting codon 87, as well as a series of primers designed to translationally fuse GFP as a screenable marker to the Cas9 protein through restriction cloning. This was unsuccessful, and we were able to isolate digestion with FseI as the point of error in the experiment. We are currently working to identify the cause of this, but note that future study could potentially focus on alternative methods to restriction cloning that allow researchers to more directly assess success. Finally, this study describes a theoretical construct design that introduces a crRNA to an existing tracrRNA scaffold using site-directed mutagenesis, and a homology-directed repair template designed to revert a cell to antibiotic sensitivity.

In this thesis, we described successful characterization of rare spontaneous mutants of JM101 with nalidixic acid resistance. We also described the design of a CRISPR-Cas9 construct containing a specific crRNA and HDR template designed to edit a D87 mutation to revert a nalidixic acid resistant cell to sensitivity. We outlined successful PCR amplification and site-directed mutagenesis of the pCas plasmid and *gfp*. We demonstrated through control experiments that failure of transformation following digestion and ligation of these fragments was due to a failure in digestion with the FseI enzyme. Our next steps to continue this work include identifying the reason behind failure of the FseI enzyme, modifying the experimental design accordingly, and moving forward with restriction cloning of the crRNA and HDR into the pCas plasmid.

References

Alekshun, M. N., & Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell*, *128*(6), 1037–1050. https://doi.org/10.1016/j.cell.2007.03.004

Aminov, R. I. (2010). A brief history of the antibiotic era: Lessons learned and challenges for the future. *Frontiers in Microbiology*, *1*. https://doi.org/10.3389/fmicb.2010.00134

Anders, C., Niewoehner, O., Duerst, A., & Jinek, M. (2014). Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature, 513*(7519), 569–573. https://doi.org/10.1038/nature13579

Aswani, V. H., & Shukla, S. K. (2021). An early history of phage therapy in the United States: Is it time to reconsider? *Clinical Medicine & Research*, *19*(2), 82–89. https://doi.org/10.3121/cmr.2021.1605

Ayobami, O., Brinkwirth, S., Eckmanns, T., & Markwart, R. (2022). Antibiotic resistance in hospital-acquired ESKAPE-E infections in low- and lower-middle-income countries: A systematic review and meta-analysis. *Emerging Microbes & Infections*, *11*(1), 443–451. https://doi.org/10.1080/22221751.2022.2030196

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A., & Horvath, P. (2007). CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science*, *315*(5819), 1709–1712. https://doi.org/10.1126/science.1138140

Bayot, M. L., & King, K. C. (2022). Biohazard Levels. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK535351/

Bell BG, Schellevis F, Stobberingh E, Goossens H, Pringle M. A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. BMC Infect Dis. 2014 Jan 9;14:13. doi: 10.1186/1471-2334-14-13. PMID: 24405683; PMCID:

PMC3897982.

Bikard, D., Euler, C. W., Jiang, W., Nussenzweig, P. M., Goldberg, G. W., Duportet, X., Fischetti, V. A., & Marraffini, L. A. (2014). Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nature Biotechnology*, *32*(11), 1146–1150. https://doi.org/10.1038/nbt.3043

Blount, Z. D. (2015). The natural history of model organisms: The unexhausted potential of E. coli. *ELife*, *4*, e05826. https://doi.org/10.7554/eLife.05826

Brown, R., Lengeling, A., & Wang, B. (2017). Phage engineering: How advances in molecular biology and synthetic biology are being utilized to enhance the therapeutic potential of bacteriophages. *Quantitative Biology*, *5*(1), 42–54. https://doi.org/10.1007/s40484-017-0094-5.

Carson, S., Miller, H. B., Witherow, D. S., & Srougi, M. C. (2019). Completion of Vector Preparation and Polymerase Chain Reaction Amplification of egfp. In *Molecular Biology Techniques* (pp. 23–30). Elsevier. https://doi.org/10.1016/B978-0-12-815774-9.00003-4

Casjens, S. R., & Hendrix, R. W. (2015). Bacteriophage lambda: Early pioneer and still relevant. *Virology*, *479–480*, 310–330. https://doi.org/10.1016/j.virol.2015.02.010.

Centers for Disease Control and Prevention, & Brunette, G. W. (Eds.). (2019). *CDC yellow book 2020: Health information for international travel*. Oxford University Press.

Cho, S. W., Kim, S., Kim, Y., Kweon, J., Kim, H. S., Bae, S., & Kim, J.-S. (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Research*, *24*(1), 132–141. https://doi.org/10.1101/gr.162339.113

Chokshi, A., Sifri, Z., Cennimo, D., & Horng, H. (2019). Global contributors to antibiotic resistance. *Journal of Global Infectious Diseases*, *11*(1), 36.

https://doi.org/10.4103/jgid.jgid_110_18

Christie, G. E., & Calendar, R. (2016). Bacteriophage P2. *Bacteriophage*, *6*(1), e1145782. https://doi.org/10.1080/21597081.2016.1145782

Clark, D. P., Pazdernik, N. J., & McGehee, M. R. (2019). Cloning genes for synthetic biology. In *Molecular Biology* (pp. 199–239). Elsevier. https://doi.org/10.1016/B978-0-12-813288-3.00007-0

Coren, J. S., Pierce, J. C., & Sternberg, N. (1995). Headful packaging revisited: The packaging of more than one DNA molecule into a bacteriophage P1 head. *Journal of Molecular Biology*, *249*(1), 176–184. https://doi.org/10.1006/jmbi.1995.0287

Costa, G. L., Bauer, J. C., McGowan, B., Angert, M., & Weiner, M. P. (1996). Site-Directed Mutagenesis Using a Rapid PCR-Based Method. In M. K. Trower, *In Vitro Mutagenesis Protocols* (Vol. 57, pp. 239–248). Humana Press. https://doi.org/10.1385/0-89603-332-5:239

Crameri, A., Whitehorn, E. A., Tate, E., & Stemmer, W. P. C. (1996). Improved Green Fluorescent Protein by Molecular Evolution Using DNA Shuffling. *Nature Biotechnology*, *14*(3), 315–319. https://doi.org/10.1038/nbt0396-315

Cuervo, A., Pulido-Cid, M., Chagoyen, M., Arranz, R., González-García, V. A., Garcia-Doval, C., Castón, J. R., Valpuesta, J. M., van Raaij, M. J., Martín-Benito, J., & Carrascosa, J. L. (2013). Structural characterization of the bacteriophage T7 tail machinery. *Journal of Biological Chemistry*, *288*(36), 26290–26299. https://doi.org/10.1074/jbc.M113.491209

Dadgostar, P. (2019). Antimicrobial Resistance: Implications and Costs. *Infection and Drug Resistance*, *Volume 12*, 3903–3910. https://doi.org/10.2147/IDR.S234610

Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiology and*

Molecular Biology Reviews, *74*(3), 417–433. https://doi.org/10.1128/MMBR.00016-10

El-Sayed Ahmed, M. A. E.-G., Zhong, L.-L., Shen, C., Yang, Y., Doi, Y., & Tian, G.-B. (2020). Colistin and its role in the era of antibiotic resistance: An extended review (2000–2019). *Emerging Microbes & Infections*, *9*(1), 868–885. https://doi.org/10.1080/22221751.2020.1754133

English, B. K., & Gaur, A. H. (2010). The use and abuse of antibiotics and the development of antibiotic resistance. In A. Finn, N. Curtis, & A. J. Pollard (Eds.), *Hot Topics in Infection and Immunity in Children VI* (Vol. 659, pp. 73–82). Springer New York. https://doi.org/10.1007/978-1-4419-0981-7_6

Foxman, B. (2002). Epidemiology of urinary tract infections: Incidence, morbidity, and economic costs. *The American Journal of Medicine*, *113*(1), 5–13. https://doi.org/10.1016/S0002-9343(02)01054-9

Foxman, B. (2014). Urinary Tract Infection Syndromes. *Infectious Disease Clinics of North America*, *28*(1), 1–13. https://doi.org/10.1016/j.idc.2013.09.003

Gholizadeh, P., Köse, Ş., Dao, S., Ganbarov, K., Tanomand, A., Dal, T., Aghazadeh, M., Ghotaslou, R., Ahangarzadeh Rezaee, M., Yousefi, B., & Samadi Kafil, H. (2020). How CRISPR-Cas system could be used to combat antimicrobial resistance. *Infection and Drug Resistance*, *Volume 13*, 1111–1121. https://doi.org/10.2147/IDR.S247271

Griebling, T. L. (2005). Urologic diseases in America project: Trends in resource use for urinary tract infections in women. *The Journal of Urology*, *173*(4), 1281–1287. https://doi.org/10.1097/01.ju.0000155596.98780.82

Han, X., Liu, Z., Jo, M. chan, Zhang, K., Li, Y., Zeng, Z., Li, N., Zu, Y., & Qin, L. (2015). CRISPR-Cas9 delivery to hard-to-transfect cells via membrane deformation. *Science*

Advances, *1*(7), e1500454. https://doi.org/10.1126/sciadv.1500454

Harbottle, H., Thakur, S., Zhao, S., & White, D. G. (2006). Genetics of Antimicrobial Resistance. *Animal Biotechnology*, *17*(2), 111–124. https://doi.org/10.1080/10495390600957092

Horii, T., Arai, Y., Yamazaki, M., Morita, S., Kimura, M., Itoh, M., Abe, Y., & Hatada, I. (2015). Validation of microinjection methods for generating knockout mice by CRISPR/Cas-mediated genome engineering. *Scientific Reports*, *4*(1), 4513. https://doi.

Hu, F., Guo, Y., Yang, Y., Zheng, Y., Wu, S., Jiang, X., Zhu, D., Wang, F., & China Antimicrobial Surveillance Network (CHINET) Study Group. (2019). Resistance reported from China antimicrobial surveillance network (CHINET) in 2018. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology*, *38*(12), 2275–2281. https://doi.org/10.1007/s10096-019-03673-1

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., & Nakata, A. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *Journal of Bacteriology*, *169*(12), 5429–5433. https://doi.org/10.1128/jb.169.12.5429-5433.1987

Jiang, Y., Chen, B., Duan, C., Sun, B., Yang, J., & Yang, S. (2015). Multigene Editing in the Escherichia coli Genome via the CRISPR-Cas9 System. *Applied and Environmental Microbiology*, *81*(7), 2506–2514. https://doi.org/10.1128/AEM.04023-14

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science*, *337*(6096), 816–821. https://doi.org/10.1126/science.1225829

Johnning, A., Kristiansson, E., Fick, J., Weijdegård, B., & Larsson, D. G. (2015). Resistance Mutations in gyrA and parC are Common in Escherichia Communities of both Fluoroquinolone-Polluted and Uncontaminated Aquatic Environments. *Frontiers in microbiology*, 6, 1355. https://doi.org/10.3389/fmicb.2015.01355

Kasman, L. M., & Porter, L. D. (2022). Bacteriophages. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK493185/

Kaur, J., Dhama, A. S., Buttolia, H., Kaur, J., Walia, K., Ohri, V., Kumar, V., Lynn, A. M., Srivastava, A., & Singh, H. (2021). ICMR's Antimicrobial Resistance Surveillance system (i-AMRSS): A promising tool for global antimicrobial resistance surveillance. *JAC-Antimicrobial Resistance*, *3*(1), dlab023. https://doi.org/10.1093/jacamr/dlab023

Khambhati, K., Bhattacharjee, G., Gohil, N., Dhanoa, G. K., Sagona, A. P., Mani, I., Bui, N. L., Chu, D.-T., Karapurkar, J. K., Jang, S. H., Chung, H. Y., Maurya, R., Alzahrani, K. J., Ramakrishna, S., & Singh, V. (2023). Phage engineering and phage-assisted CRISPR-Cas delivery to combat multidrug-resistant pathogens. *Bioengineering & Translational Medicine*, *8*(2), e10381. https://doi.org/10.1002/btm2.10381

Komp Lindgren, P., Karlsson, Å., Hughes, D. (2003). Mutation Rate and Evolution of Fluoroquinolone Resistance in Escherichia coli Isolates from Patients with Urinary Tract Infections. *Antimicrob Agents Chemother*, 47. https://doi.org/10.1128/aac.47.10.3222-3232.2003

Kohanski, M., DePristo, M., Collins, J. (2010). Sublethal Antibiotic Treatment Leads to Multidrug Resistance via Radical-Induced Mutagenesis. *Molecular Cell, 37*(3), 311-320. https://doi.org/10.1016/j.molcel.2010.01.003

Lai, J. Y., Inoue, N., Oo, C. W., Kawasaki, H., & Lim, T. S. (2021). One-step synthesis of M13

phage-based nanoparticles and their fluorescence properties. *RSC Advances*, *11*(3), 1367–1375. https://doi.org/10.1039/D0RA02835E

Laxminarayan, R., Van Boeckel, T., Frost, I., Kariuki, S., Khan, E. A., Limmathurotsakul, D., Larsson, D. G. J., Levy-Hara, G., Mendelson, M., Outterson, K., Peacock, S. J., & Zhu, Y.-G. (2020). The Lancet Infectious Diseases Commission on antimicrobial resistance: 6 years later. *The Lancet Infectious Diseases*, *20*(4), e51–e60. https://doi.org/10.1016/S1473-3099(20)30003-7

Lee, D. S., Lee, S.-J., & Choe, H.-S. (2018). Community-Acquired urinary tract infection by Escherichia coli in the era of antibiotic resistance. *BioMed Research International*, *2018*, 7656752. https://doi.org/10.1155/2018/7656752

Levy, S. B., & Marshall, B. (2004). Antibacterial resistance worldwide: Causes, challenges and responses. *Nature Medicine*, *10*(S12), S122–S129. https://doi.org/10.1038/nm1145

Lin, D. M., Koskella, B., & Lin, H. C. (2017). Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World Journal of Gastrointestinal Pharmacology and Therapeutics*, *8*(3), 162–173. https://doi.org/10.4292/wjgpt.v8.i3.162

Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J. J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F. J. M., Wolf, Y. I., Yakunin, A. F., van der Oost, J., & Koonin, E. V. (2011). Evolution and classification of the CRISPR-Cas systems. *Nature Reviews. Microbiology*, *9*(6), 467–477. https://doi.org/10.1038/nrmicro2577

Makarova, K. S., & Koonin, E. V. (2015). Annotation and classification of CRISPR-Cas systems. In M. Lundgren, E. Charpentier, & P. C. Fineran (Eds.), *CRISPR* (Vol. 1311, pp. 47–75). Springer New York. https://doi.org/10.1007/978-1-4939-2687-9_4

Martinez, J. L. (2014). General principles of antibiotic resistance in bacteria. *Drug Discovery*

Today: Technologies, *11*, 33–39. https://doi.org/10.1016/j.ddtec.2014.02.001

Mestrovic, T., Robles Aguilar, G., Swetschinski, L. R., Ikuta, K. S., Gray, A. P., Davis Weaver, N., Han, C., Wool, E. E., Gershberg Hayoon, A., Hay, S. I., Dolecek, C., Sartorius, B., Murray, C. J. L., Addo, I. Y., Ahinkorah, B. O., Ahmed, A., Aldeyab, M. A., Allel, K., Ancuceanu, R., … Naghavi, M. (2022). The burden of bacterial antimicrobial resistance in the WHO European region in 2019: A cross-country systematic analysis. *The Lancet Public Health*, *7*(11), e897–e913. https://doi.org/10.1016/S2468-2667(22)00225-0

Mohanraju, P., Makarova, K. S., Zetsche, B., Zhang, F., Koonin, E. V., & van der Oost, J. (2016). Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems. *Science (New York, N.Y.)*, *353*(6299), aad5147. https://doi.org/10.1126/science.aad5147.

Murray, C. J., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S. C., Browne, A. J., Chipeta, M. G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B. H., Kumaran, E. A. P., McManigal, B., … Naghavi, M. (2022). Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. *The Lancet*, *399*(10325), 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0

National Institutes of Health. (2019). *NIH guidelines for research involving recombinant or synthetic nucleic acid molecules*. 149.

Neil, K., Allard, N., Roy, P., Grenier, F., Menendez, A., Burrus, V., & Rodrigue, S. (2021). High-efficiency delivery of CRISPR-Cas9 by engineered probiotics enables precise microbiome editing. *Molecular Systems Biology*, *17*(10), e10335. https://doi.org/10.15252/msb.202110335

New England Biolabs. (2025). *FseI*. New England Biolabs.

https://www.neb.com/en-us/products/r0588-fsei

New England Biolabs. (2025). *LongAmp*® Taq *PCR Kit* . New England Biolabs. https://www.neb.com/en-us/products/e5200-longamp-taq-pcr-kit

New England Biolabs. (2025). *Monarch® Plasmid Miniprep Kit* . New England Biolabs. https://www.neb.com/en-us/products/t1010-monarch-plasmid-miniprep-kit

New England Biolabs. (2025). *Q5® Hot Start High-Fidelity 2X Master Mix.* New England Biolabs. https://www.neb.com/en-us/products/m0494-q5-hot-start-high-fidelity-2x-master-mix

New England Biolabs. (2025). *Troubleshooting Guide for Cloning*. New England Biolabs. https://www.neb.com/en-us/tools-and-resources/troubleshooting-guides/troubleshooting-guide-for-cloning

Olsen, N. S., Forero-Junco, L., Kot, W., & Hansen, L. H. (2020). Exploring the remarkable diversity of culturable Escherichia coli phages in the Danish wastewater environment. *Viruses*, *12*(9), 986. https://doi.org/10.3390/v12090986

Pyne, M. E., Moo-Young, M., Chung, D. A., & Chou, C. P. (2015). Coupling the CRISPR/Cas9 System with Lambda Red Recombineering Enables Simplified Chromosomal Gene Replacement in Escherichia coli. *Applied and Environmental Microbiology*, *81*(15), 5103–5114. https://doi.org/10.1128/AEM.01248-15

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*, *8*(11), 2281–2308. https://doi.org/10.1038/nprot.2013.143

Rautela, I., Uniyal, P., Thapliyal, P., Chauhan, N., Bhushan Sinha, V., & Dev Sharma, M. (2021). An extensive review to facilitate understanding of CRISPR technology as a gene editing

possibility for enhanced therapeutic applications. *Gene*, *785*, 145615. https://doi.org/10.1016/j.gene.2021.145615

Saenz, Y. (2003). Mutations in gyrA and parC genes in nalidixic acid-resistant Escherichia coli strains from food products, humans and animals. *Journal of Antimicrobial Chemotherapy*, *51*(4), 1001–1005. https://doi.org/10.1093/jac/dkg168

Salam, Md. A., Al-Amin, Md. Y., Pawar, J. S., Akhter, N., & Lucy, I. B. (2023). Conventional methods and future trends in antimicrobial susceptibility testing. *Saudi Journal of Biological Sciences*, *30*(3), 103582. https://doi.org/10.1016/j.sjbs.2023.103582

Smeal, S. W., Schmitt, M. A., Pereira, R. R., Prasad, A., & Fisk, J. D. (2017). Simulation of the M13 life cycle I: Assembly of a genetically-structured deterministic chemical kinetic simulation. *Virology*, *500*, 259–274. https://doi.org/10.1016/j.virol.2016.08.017

Tridgett, M., Ababi, M., Osgerby, A., Ramirez Garcia, R., & Jaramillo, A. (2021). Engineering bacteria to produce pure phage-like particles for gene delivery. *ACS Synthetic Biology*, *10*(1), 107–114. https://doi.org/10.1021/acssynbio.0c00467

Vaghari-Tabari, M., Hassanpour, P., Sadeghsoltani, F., Malakoti, F., Alemi, F., Qujeq, D., Asemi, Z., & Yousefi, B. (2022). CRISPR/Cas9 gene editing: A new approach for overcoming drug resistance in cancer. *Cellular & Molecular Biology Letters*, *27*(1), 49. https://doi.org/10.1186/s11658-022-00348-2

Wei, C., Liu, J., Yu, Z., Zhang, B., Gao, G., & Jiao, R. (2013). TALEN or Cas9—Rapid, efficient and specific choices for genome modifications. *Journal of Genetics and Genomics = Yi Chuan Xue Bao*, *40*(6), 281–289. https://doi.org/10.1016/j.jgg.2013.03.013

Xu, C. L., Ruan, M. Z. C., Mahajan, V. B., & Tsang, S. H. (2019). Viral delivery systems for CRISPR. *Viruses*, *11*(1), 28. https://doi.org/10.3390/v11010028

Xu, Y., & Li, Z. (2020). CRISPR-Cas systems: Overview, innovations and applications in human disease research and gene therapy. *Computational and Structural Biotechnology Journal*, *18*, 2401–2415. https://doi.org/10.1016/j.csbj.2020.08.031

Yang, W., Yan, J., Zhuang, P., Ding, T., Chen, Y., Zhang, Y., Zhang, H., & Cui, W. (2022). Progress of delivery methods for CRISPR-Cas9. *Expert Opinion on Drug Delivery*, *19*(8), 913–926. https://doi.org/10.1080/17425247.2022.2100342

Yosef, I., Manor, M., Kiro, R., & Qimron, U. (2015). Temperate and lytic bacteriophages programmed to sensitize and kill antibiotic-resistant bacteria. *Proceedings of the National Academy of Sciences*, *112*(23), 7267–7272. https://doi.org/10.1073/pnas.1500107112

Young, S., Aitken, R., & Ikawa, M. (2015). Advantages of using the CRISPR/Cas9 system of genome editing to investigate male reproductive mechanisms using mouse models. *Asian Journal of Andrology*, *17*(4), 623–627. https://doi.org/10.4103/1008-682X.153851

Zhang, Q., Doak, T. G., & Ye, Y. (2014). Expanding the catalog of cas genes with metagenomes. *Nucleic Acids Research*, *42*(4), 2448–2459. https://doi.org/10.1093/nar/gkt1262