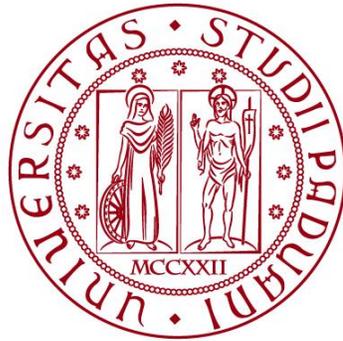


UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea magistrale in Molecular Biology



TESI DI LAUREA

Phage delivery of CRISPR interference system to aid AMR bacteria sensitization

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ANNO ACCADEMICO 2024/2025

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ABSTRACT

The spread of antimicrobial-resistant bacteria is a global issue that is taking a toll on the healthcare system and the world economy. The process of new antibiotic development is slow, costly, and unable to keep up with the emergence of new AMR strains. New strategies to combat this challenge need to be studied and developed to tackle it from different angles. By leveraging the synthetic biology toolset, the synthesis of new systems that can be employed to slow down the rise of this issue is possible. In this work, we studied, characterized, and exploited M13 bacteriophages as scaffolds to deliver a CRISPR interference system, which achieved partial re-sensitization of two laboratory strains resistant to Meropenem or Colistin. The AMR genes we targeted were *bla_{ndm1}* (Meropenem resistance), and *mcr1* (Colistin resistance). We also investigated two different approaches for evaluating the growth delay obtained from the circuit we employed. With this work, we shed light on the issue of AMR bacteria and investigate a new approach for their treatment, with the hope that it may one day be seen as a valuable alternative to complement traditional antibiotic treatment.

INTRODUCTION

THE ANTIMICROBIAL RESISTANCE ISSUE

The widespread use of antibiotics in the last century, particularly in fields such as medicine, agriculture, and intensive farming, has led to the emergence of antimicrobial-resistant bacteria (AMR). This issue has reached a state that can't be ignored anymore. The evolution of multidrug-resistant bacteria poses a significant threat to the control of infectious diseases globally, making it urgent to develop new therapeutic tools to combat this real threat.

“A post-antibiotic era in which minor injuries and common infections can kill because of the lack of drugs, or their ineffectiveness, is nowadays not an apocalyptic fantasy, but a real 21st-century threat”¹.

In 2019, the World Health Organization (WHO) identified AMR as "one of the top ten global threats to humanity"². Currently, there are 700,000 deaths associated with infections caused by antibiotic-resistant pathogens³. According to data collected in "The Review on Antimicrobial Resistance," antibiotic-resistant microorganisms, also known as superbugs, are projected to be responsible for over 10 million deaths by 2050.⁴

The WHO has compiled a list of six notable bacteria due to their high levels of antibiotic resistance and ability to evade conventional therapies. The ESKAPE acronym includes these six bacteria: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*⁵. In recent years, this group has been expanded due to the rise in the number of cases of antimicrobial-resistant *Escherichia coli*, resulting in the acronym being changed from ESKAPE to ESKAPEE.

Last-resort antibiotics comprise the last line of defense against bacteria that are resistant to the standard antibiotics. They should be employed only in cases of multidrug-resistant bacterial infections or in severe infections that require efficient and immediate treatment. Their use should be thoughtfully prescribed since they often cause side

effects, but more importantly, their careless use can and is leading to the rise of bacterial strains that are resistant. If last-resort antibiotics start to spread worldwide, we could reach a point where even our strongest defense is no longer enough.

The concept of One Health is relevant when discussing antimicrobial resistance, as it is an issue that affects more than just one system. The one health approach aims to tackle problems like the one we are discussing without focusing on just one topic, but with a broader spectrum of view, since it can affect not only human health but also fields like domestic and wild animal health, as well as the entire environment. With this approach, issues are addressed from more than one perspective with a holistic view, instead of compartmentalizing each issue to the niche it affects. ⁶

Meropenem and Colistin are two last-resort antibiotics that are at risk due to the emergence of resistance strains.

Meropenem is a member of the carbapenem group of β -lactams. It is a broad-spectrum antibiotic active against both Gram-positive and Gram-negative bacteria, used in cases of infection caused by multidrug-resistant strains that produce extended-spectrum β -lactamase (ESBL). Its mechanism of action is based on binding to Penicillin Binding Proteins (PBPs); this interaction inhibits the synthesis of the cell wall, specifically the cross-linking of peptidoglycan, leading to weakening of the cell wall and ultimately resulting in cell lysis and death. The resistance gene responsible for resistance to Meropenem is the New Delhi Metallo β -lactamase gene, denoted as *bla_{ndm1}*. The mechanism of action of this enzyme is based on two Zn^{2+} ions positioned in the active site, which can hydrolyze the β -lactam ring of β -lactam antibiotics, thereby conferring resistance to the bacteria. *Bla_{ndm1}* genes are mainly located on plasmids, which makes it possible for bacteria to easily spread the resistance gene through horizontal gene transfer, increasing the likelihood of the emergence of different multi-drug-resistant strains. ⁷

Another resistance gene that has recently started to spread is the one for Colistin, the mobilized Colistin resistance gene, *mcr-1*. Colistin is also known as Polymyxin E This antibiotic, mainly active against gram-negative bacteria, acts by attaching to the

bacterial cell wall, reducing its solubility and disrupting its integrity, ultimately leading to the formation of pores and cell death. The *mcr-1* gene confers resistance through the activity of an enzyme, phosphoethanolamine transferase, which modifies the target of Colistin, lipid A, thereby inhibiting the antibiotic's binding and ultimately reducing its activity. The *mcr-1* gene is also located on a plasmid, similar to *bla_{NDM1}*, and it is rapidly spreading worldwide, partially due to its distribution through animals. This phenomenon could be explained by the intensive use of Colistin that has been going on in the animal field.^{8,9}

SYNTHETIC BIOLOGY

Synthetic biology is an emerging discipline that combines aspects of different scientific fields, such as engineering and molecular biology, to create a new vision and tools for application in live cell systems. The goal is to borrow techniques from these different fields and apply them to biological systems to modify, module, and lastly exploit them to achieve a specific purpose. These biological systems can be employed to synthesize a particular product, sense a molecule or genetic element, or deliver a circuit to achieve a modification in another organism.

To reprogram biological systems, synthetic gene circuits are developed. These circuits are composed of modules, or parts, that are thoughtfully tuned and switched to achieve the desired goal. These parts are essentially DNA sequences with specific functions, such as promoters, ribosome binding sites (RBS), genes, terminators, activators, and repressors. All of these are used as building blocks for assembling the final circuit. Well-characterized libraries of all these parts exist and can be used to model the system starting from silico and then moving in vivo. The approach for developing a synthetic gene circuit is the DBTL cycle: design, build, test, and learn. This approach combines in-silico predictions and data analysis with experimental synthesis and testing to develop a system that is characterized and tuned to achieve the predisposed function.^{10,11}

In 2014, Bikard D. et al.¹² employed phage particles to deliver a circuit harboring a CRISPR-Cas9 system targeting the *aph-3* kanamycin resistance gene. This work, along with some other similar studies, stemmed the idea for our project.

CRISPR INTERFERENCE

In 2020, the Nobel Prize in chemistry was awarded to Emmanuelle Charpentier and Jennifer Doudna for their development of a method for genome editing based on CRISPR-Cas9 technology. The revolutionary aspect of this discovery is attributable to the capacity of performing specific, scarless gene modification using a system that is easily modifiable and modulable.

The CRISPR/Cas9 technology comprises the Cas9 nuclease, obtained from *Streptococcus pyogenes*, and the CRISPR array, which consists of short repeated sequences interspaced by spacers.

Cas9 is classified as a class 2 type II Cas nuclease. It functions as a single protein effector that recognizes only DNA and cuts both strands in specific positions, thanks to two nuclease domains: the HNH and the RuvC-like domain. The specificity of the system is determined by the spacers, which are viral genome sequences that are cut and integrated into CRISPR arrays following a primary infection. Subsequently, when the bacteria get re-infected by the same virus, it can use these elements to recognize and block the infection process as part of the bacterial immune system.

This system requires several maturation steps, including the transcription of the different elements and the activation of the immune response. First, the CRISPR arrays are transcribed into precursors-CRISPR RNAs (pre-crRNAs). Another non-coding RNA, named trans-activating crRNA (tracrRNA), is transcribed and is fundamental for the maturation process since it pairs with the repeats included in the pre-crRNAs. This pairing drives the host RNase III to recognize the hybrid and cleave it, forming singular complexes that are then further processed by host ribonucleases, which trim the 5' end, aiding in the maturation of the crRNA. This duplex crRNA:tracrRNA is identified by

the Cas9 nuclease that can bind to it and perform its nuclease activity. An important region inside the crRNA is the protospacer adjacent motif (PAM) that is placed downstream of the spacer and is fundamental to discriminate between self and non-self sequences. The PAM of the most commonly used *Streptococcus pyogenes* Cas9 is 5'-NGG-3' in which N stands for each of the four bases.^{13,14}

The duplex crRNA:tracrRNA has been synthetically combined into a singular sgRNA that retains the ability to bind to Cas9, avoiding the maturation step.

This discovery opened the door to the development of new technologies and tools, not only for genome editing but also for regulating gene transcription, as seen in CRISPR interference.

CRISPRi is a technique that leverages the specificity and tunability of the CRISPR/Cas9 technology, but rather than cleaving the DNA strand, it interferes with the transcription process. This interference activity is achieved through two point mutations, D10A and H840A, at the levels of the Ruv-C-like and HNH domains, which inhibit their nuclease activity. This modified Cas9 is referred to as dead-Cas9 (dCas9), and it retains its binding activity, allowing it to be employed to target specific regions of DNA and block RNA polymerase activity by physically blocking the elongation of the RNA transcript. According to the literature, targeting the gene promoter can increase repression by up to 300-fold; therefore, this strategy was chosen.¹⁵

PHAGES

Due to the continuous rise of new AMR strains, the need for novel tools to fight this emergency is becoming increasingly needed, and this should go hand in hand with a more stringent regulation on antibiotic use both in human health and in husbandry. One potential approach to overcome this issue is the incorporation of bacteriophages into contemporary medical treatments. Bacteriophages are viruses that specifically target bacteria; they are obligate parasites that rely on the host's bacterial cellular machinery for reproduction. Infections typically begin when the viral co-receptor binds to a

specific receptor on the bacterial surface. Following this, the bacteriophage injects its genetic material into the host cell. The virus then uses the host's cellular machinery to replicate and express its genes. This process is followed by the packaging of the viral genome (either RNA or DNA) inside a protein shell and the eventual release of new phages into the extracellular environment through various methods.^{16,17}

BACTERIOPHAGE M13

The phage used in this thesis is the M13 bacteriophage, which has an ssDNA genome and belongs to the family of filamentous phages. The M13 has a unique life cycle, as it is neither lysogenic nor lytic, since it does not integrate into the bacterial genome and does not lead to cell lysis. This is one of the reasons that make this bacteriophage a good candidate for use as a delivery tool.

M13 can infect specifically male *E. coli* that express the F-episome through the interaction of one of its capsid proteins located at the base of the phage, the p3. After recognition of the receptor, the ssDNA genome of the phage is injected into the bacterial cell, where it is converted into circular dsDNA by the host's replication machinery. This circular dsDNA form is called the replicative form (RF), and it is both transcribed into mRNA to synthesize the phage proteins and replicated through rolling circle replication to form copies of ssDNA that will be packaged into the progeny. The M13 genome encodes 11 proteins, 3 of which are implicated in the replication process (pII, pV, pX), 5 compose the capsid (pIII, pVI, pVII, pVIII, pIX) and the last 3 are involved in the capsid assembly process. Still, they are not incorporated in it (pI, pIV, pXI). Once enough proteins have been synthesized, specifically pV, replication starts to decrease due to the action of pV, which sequesters ssDNA by cooperatively binding to the newly synthesized (+) strand. The pV-ssDNA complex is capable of recognizing a specific assembly site in the bacterial membrane, where the capsid-forming proteins are located. The pV is subsequently detached from the ssDNA while the coating proteins are assembled around it, encapsidating it, while extruding from the bacterial membrane. Thanks to this particular way of encapsidation, which does not depend on a preformed capsid, the length of the ssDNA that can be incorporated is not restricted, as long as there are sufficient coating proteins to complete the process. One specific

element, known as the packaging signal, located in a -508 bp intergenic region, is fundamental for this process. This packaging signal is responsible for interacting with the proteins involved in capsid assembly. ^{18,19}

PHAGEMIDS

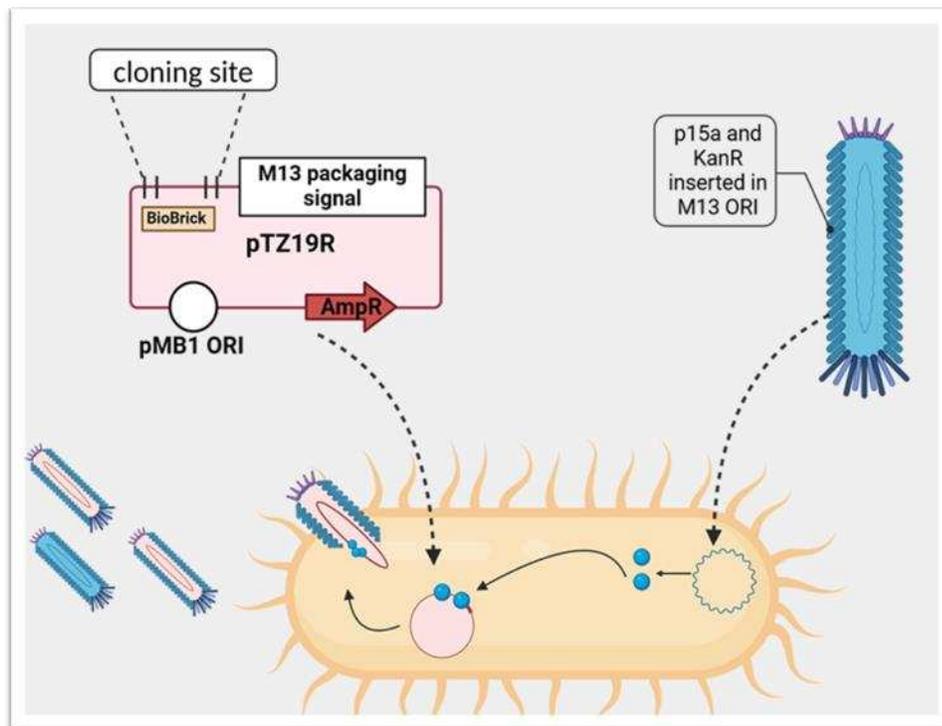


Figure 1 phage production: illustration representing the schematic process of phage particle production through the aid of a phage helper (on the right) and a phagemid (on the left). The phagemid pTZ19R circuit, used as backbone in this work, is represented with its different specifics.

The -508 bp intergenic region has been cloned into plasmids to develop the so-called phagemids. Phagemids can be inserted by transformation into F' *E. coli* that can subsequently be infected by the so-called helper phage (figure 1), that are responsible for the transcription of the phage proteins needed for the progeny production. Once the proteins for the capsid and the assembly are synthesized, the phagemid intergenic region will be recognized, and it will be encapsidated, yielding phage particles that harbor the phagemid rather than the helper phage genome. The phagemid will be predominantly incorporated due to an ad hoc mutation at the packaging signal level of

the helper phage, which lowers its affinity for the encapsidation machinery. This system can be leveraged to yield phage production in for the insertion of a desired construct. Due to the unique packaging mechanism of M13 phages, phagemids of various sizes and contents can be incorporated into the phage particle, thereby making this system an ideal platform for delivering synthetic circuits.

AIMS

My thesis project aims to understand better, characterize, and improve the use of phage particles as a platform for delivering the CRISPRi system to re-sensitize antibiotic-resistant *E. coli*.

By leveraging the methods and technologies of synthetic biology, we aim to create innovative solutions that can reduce the spread of antibiotic-resistant bacteria and improve the effectiveness of current antibiotics, since the development process required for new antibiotics takes years, costs millions, and can't keep up with the AMR emergency.

Our focus is to employ the recombinant phage M13 as a delivery platform to transfer the CRISPRi circuit (dCas9 protein and gRNA) targeting AMR genes in *E. coli*. To do this, first, we will evaluate and characterize the delivery ability of the phage system. To achieve this, we will deliver a reporter gene, a red fluorescence protein cassette (*rfp*). Next, we will test the system by delivering a more complex circuit containing the CRISPRi system against the RFP. Lastly, we will employ this toolkit to target two AMR genes, the *ndm-1* and the *mcr-1*, which confer resistance to Meropenem and Colistin, respectively, in two *E. coli* laboratory strains.

MATERIALS AND METHODS

STRAINS

Escherichia coli DH10-beta (New England Biolabs) for cloning, *Escherichia coli* TOP10 F'(Invitrogen) as phage target. All the strains were grown in Lysogeny broth (10g/L Sodium chloride, 10g/L Bacto-Tryptone, 5g/L Yeast extract) at 37°C with shaking at 200rpm. Antibiotics were added to the growth medium at the following final concentrations: 12.5 µg/mL chloramphenicol (Cm), 25 µg/mL kanamycin (Kan), 100 µg/mL ampicillin (Amp), and 100 µg/mL carbenicillin (Carb). Bacteria were grown on LB agar (1.5% w/v; Thermo Fisher Scientific) supplemented with the appropriate antibiotic when needed.

The engineered phage particles generated with the phagemid pTZ19R (ThermoFisher Scientific) and the M13KO7 Helper Phage (Invitrogen) were produced in grown in Lysogeny broth (10g/L Sodium chloride, 10g/L Bacto-Tryptone, 5g/L Yeast extract) at 37°C with shaking at 250 rpm and with the required antibiotics to the following final concentrations: 75µg/mL kanamycin and 100µg/mL ampicillin.

PLASMIDS

All the plasmids and phagemids used in the study were obtained by either classic digestion and ligation cloning or Gibson Assembly cloning. After cloning, the plasmids were verified via restriction enzyme gel screening and then whole plasmid sequencing.

All the phagemids we used in this work are based on the pTZ19R (Thermo Fisher Scientific) phagemid backbone. This backbone contains the pMB1 origin of replication, the *blaR* gene that confers resistance to β-lactam antibiotics, and the phage f1 intergenic region, which is necessary for replication and packaging into phage particles. This phagemid also contained a cloning site, where we specifically inserted the cassettes required for this work.

The first phagemid we used was the pTZ-I13521. In the cloning site, the I13521 (BBa_I13521) cassette, composed of the pTet promoter, *mrfp* gene, and a terminator,

was inserted by digesting the pTZ backbone and pSB1A2_I13521 with EcoRI (New England Biolabs) and PstI (New England Biolabs). The two digested parts were then ligated with T4 Ligase (Thermo Fisher Scientific) overnight at 16°C.

In the pTZ19 backbone, the dCas9 cassette was then inserted, allowing us to create a plasmid for CRISPRi. pTZ19 and pSB3K3 carrying *dCas9* under the J23109 promoter were digested with EcoRI (New England Biolabs) and PstI (New England Biolabs), ligated, transformed, and verified.

pTZ19_109dCas9 was then used to obtain two of the phagemids used for the CRISPRi treatment, the pTZ-ndm1-mcr1 and the pTZ-ndm1. Both these plasmids were obtained via Gibson assembly. The cassettes containing the ndm1:mcr1 and ndm1 CRISPR arrays were obtained from two plasmids described in Chiacchiera et al. 2025²⁰. Three amplicons were obtained using the primers listed in *Table 2*: the pTZ backbone from pTZ19_109dCas9, the *dcas9* under the J23109 promoter from pTZ19_109dCas9, and lastly the ndm1:mcr1 and ndm1 CRISPR arrays, respectively, from OriT_gNDM1-gMCR1_{array}+dCas9 and OriT_gNDM1_{array}+dCas9. The three amplicons were then assembled through Gibson assembly, transformed, and then verified.

The last phagemid used for the CRISPRi targeting the *mcr1* gene was obtained differently. The CRISPR array targeting *mcr1* was synthesized via the Genscript services with an EcoRI restriction site at the 5' end and an XbaI restriction site at the 3' end. The synthesized mcr1 CRISPR array and pTZ19_109dCas9 were digested with EcoRI (New England Biolabs) and XbaI (New England Biolabs) and then ligated with T4 Ligase (Thermo Fisher Scientific) overnight at 16°C, transformed, and verified.

To generate pTZ19 harbouring CRISPRi targeting *rfp*, pTZ19 and pSB3K3 carrying *dCas9* under the J23109 promoter were digested with EcoRI (New England Biolabs) and PstI (New England Biolabs), ligated, transformed, and verified. Subsequently, pTZ19_109dCas9 was digested with EcoRI (New England Biolabs) and XbaI (New England Biolabs), and pSB4C5 harbouring the HSL-inducible sgpTet was digested with EcoRI (New England Biolabs) and SpeI (New England Biolabs). The two

digestion products were then ligated with T4 Ligase (Thermo Fisher Scientific) overnight at 16°C, transformed, and verified.

The two target plasmids for the CRISPRi system, pSB3K3-ndm1 and pSB3K3-mcr1 were obtained via Gibson assembly. The two resistance genes were obtained from two plasmids, pGDP1-ndm1 and pGDP2-mcr1, taken from Chiacchiera et al. 2025²⁰. The amplicons were obtained by using the primers listed on *table 2*. Amplicons were then assembled through Gibson assembly, transformed and then verified.

The target plasmid for the CRISPRi system against pTet was pSGAb-I13521. It was obtained by digesting the pSGAb medium copy and the pS1A2 carrying the RFP cassette Bba I13521 with EcoRI (Thermo Scientific) and PstI (Thermo Scientific). The

Plasmid	Source	Usage
pSGAb-km	Addgene #121999	shuttle vector for <i>A. baumannii</i> and <i>E.coli</i>
pSGAb	Gibson Assembly	pSGAb-km with the <i>ColE1 E.coli</i> origin of replication replaced with the p15a origin of replication
pSB3K3	iGem Registry	origin of replication
pSGAb-I13521	this work	expression of RFP under pTet promoter in the shuttle vector
pSB3K3_dCas9	iGem Registry	to mutagenize the EcoRI site in the dCas9 sequence
pSB3K3_dCas9mut	this work	to express a compatible BioBrick standard dCas9
<i>pSB1A2_I13521</i>	iGem Registry	expression of Red Fluorescent Protein under the pTet promoter
pTZ19R	Thermo Fisher Scientific	phagemid backbone
pTZ-I13521	this work	phagemid expressing the Red Fluorescent Protein under the pTet promoter
pTZ19R_109dCas9	this work	phagemid expressing <i>dCas9</i> under the J23109 promoter
pTZ-ndm1	this work	phagemid to target <i>ndm1</i> through CRISPRi
pTZ-ndm1-mcr1	this work	phagemid to target <i>ndm1</i> and <i>mcr1</i> through CRISPRi
pTZ-mcr1	this work	phagemid to target <i>mcr1</i> through CRISPRi
OriT_gNDM1-gMCR1 _{array} +dCas9	Chiacchiera et al. 2025 ²⁰	to amplify the <i>ndm1-mcr1</i> CRISPR array
OriT_gNDM1 _{array} +dCas9	Chiacchiera et al. 2025 ²⁰	to amplify the <i>ndm1</i> CRISPR array
pSB4C5	Thermo Fisher Scientific	to digest the HSL-inducible <i>sgpTet</i>
pSB3K3-ndm1	this work	plasmid expressing the <i>bla_{ndm1}</i> resistance gene
pSB3K3-mcr1	this work	plasmid expressing the <i>mcr1</i> resistance gene
pGDP1-ndm1	Chiacchiera et al. 2025 ²⁰	to amplify the <i>bla_{ndm1}</i> gene
pGDP2-mcr1	Chiacchiera et al. 2025 ²⁰	to amplify the <i>mcr1</i> gene

Table 1 plasmids: list of plasmids employed in this work

two digestion products were then ligated with T4 Ligase (Thermo Fisher Scientific) overnight at 16°C, transformed, and verified.

PRIMERS

Primers 5'-3' sequences and synthetic strand	Features/Name	Usage
taacgcttacaattccattc	FW_RFP_phage	To assess the delivery of <i>rfp</i> in the transduced colonies
agctctaatacagctcactatagg	RV_RFP_phage	
tattacgccagctggcga	FW_seq_pTZ19_NEB	to sequence the cloning site of pTZ19
acaggttcccactgga	RV_seq_pTZ19_NEB	
cactaagggttagttagttttacagctagctcagctcctagga	FW_DFdCas264	to amplify the <i>dcas9</i> from the backbone pTZ19_J109dCas9
ttaccgaagctctaatacagctcactataggaaagcttgc	RV_DFdCas264	
gtcagcaggtgtaaacgatatactagagttgacggctagctc	FW_DFnewarray	to amplify the double array (<i>ndm1-mcr1</i>) from <i>oriT_gNDM1-gMCR1_{array}+dCas9</i> and the single (<i>ndm1</i>) from <i>oriT_gNDM1_{array}+dCas9</i>
ggactgagctagctgtaaaaactaactaaccttagtactcct	RV_DFnewarray	
gtattagagctggcgtaatcatgg	FW_DFnewback264	to amplify the pTZ19 backbone from pTZ19_J109dCas9
cgttttacaacgctgactg	RV_DFback264	
gctcactcaaaaggcgtaat	FW_backbone3K3	to amplify the backbone pSB3K3
cagaaatcatccttagcgaag	RV_backbone3K3	
Taaggatgattctgtatcggtgattcattctgctaac	FW_MCR-1	to amplify the <i>mcr-1</i> from pGDP2-mcr-1
cgccttgagtgaccatgagcggatacatatttgatg	RV_MCR-1	
taaggatgattctgattaacgcttacaatttaggtggc	FW_NDM-1	to amplify the <i>bla_{ndm1}</i> from pGDP1-ndm-1
cgccttgagtgagcgtcgtatgagtttttaagaattaat	RV_NDM-1_new1_P39	
atccagctattccatggtgc	FW_seq_3K3back	to sequence the cloning site of the pSB3K3 backbone
gcagtttcatttgatgctcg	RV_seq_3K3back	
gaattctactagagttgacggctagctcagctcaggtacag tgctagcggaaaccattcaaacagcatagcaagttaaaa taaggctagtcggtatcaactgaaaaagtggcaccgagt cggctcttttttcgcaaaaaaccccgttcggcggggttttc gcactagtaattgtgagcgataacaattgacattgtgagcg gataacaagatactgagcacagtttttagagctatgctgtttg aatgtcccaaacgccacaagaacaacggactgacc gagcgtgttttagagctatgctgtttgaatgtcccaaac gatctaaaaaaaaaccccggccctgacagggcggggttt tttttctaga	synthesized <i>mcr-1</i> array	to clone cutting EcoRI-XbaI the single crRNA <i>mcr-1</i> in the pTZ19_J109dCas9

Table 2 **primers**: list of primers employed in this work

CLONING

Competent cells used for cloning were *Escherichia coli* TOP10 F' (Invitrogen) and *Escherichia coli* DH10b (New England Biolabs).

In this project, two different cloning strategies were employed: classic digestion-ligation and the Gibson Assembly.

Regarding the classic digestion-ligation approach, this was performed according to the BioBrick RFC [10] standard. This assembly standard defines the structure of the parts inserted in the construct, but does not define any functional characteristics of the parts. Each part is flanked on both sides by two sequences: at the 5' end a prefix (sequence that comprises the EcoRI and XbaI restriction sites), at the 3' end a suffix (sequence that comprises the SpeI and PstI restriction sites). During classic digestion-ligation cloning, the prefix is cut with XbaI and the suffix with SpeI. These restriction enzymes generate compatible sticky ends that can be ligated together, resulting in a scar that cannot be recognized by either restriction enzyme, allowing for stable assembly. After digestion, a gel extraction was performed utilizing Wizard®SV Gel and PCR Clean-Up System (Promega). Ligation was achieved using T4 DNA ligase (Thermo Fisher Scientific). A 30µL reaction was prepared by adding 1µL of T4 DNA ligase, 3µL of T4 DNA ligase buffer, and a volume of insert and vector to achieve a ratio that was respectively of either 3:1 or 5:1. The reaction was incubated overnight at 16°C. After ligase inactivation at 65°C for 10 minutes, the product was transformed into *E. coli* DH10-beta.²¹

For the Gibson Assembly strategy of cloning, we employed the NEBuilder® HiFi DNA Assembly Master Mix kit (New England Biolabs). This method requires a step of PCR amplification followed by the Gibson Assembly reaction, which allows for the seamless assembly of multiple DNA fragments. The PCR step was performed using the Q5® High-Fidelity 2X Master Mix (New England Biolabs) kit. A 50µL reaction was prepared by adding 25µL of master mix, 2.5µL of each primer (forward and reverse), 1-10 ng of DNA template, and nuclease-free water to the final volume. Thermal cycling conditions were set by following the kit protocol, and the specific

melting temperature for each primer couple was calculated using the NEB Tm calculator. After PCR, the product was purified utilizing the Wizard®SV Gel and PCR Clean-Up System (Promega). The Gibson assembly reaction was performed following the NEBuilder® HiFi DNA Assembly Master Mix kit (New England Biolabs) protocol with a vector-to-insert molar ratio of either 1:5 or 1:10. The reaction was finally used to perform a transformation into *E. coli* DH10-beta.

COMPETENT CELLS PREPARATION

In a 250mL flask 50mL of LB and 750µL of MgCl₂ to a final concentration of 15mM were added. 500µL of bacteria from a pre-inoculum grown overnight were diluted 1:100 into the flask. The preparation was grown over a day at 37°C with shaking at 200rpm until an OD of 0.4-0.6 (max 0.65) was reached. The flask was transferred to ice, and the bacteria were poured into 50mL conical tubes. The bacteria were centrifuged at 3500g, 4°C for 15 minutes. The supernatant was discarded and the pellet resuspended gently with 12.5mL of solution A (50mM of CaCl₂ * 2H₂O, 10mM MnCl₂ * 4H₂O, 10mM MES pH 6.3). The tubes were kept on ice for 15 minutes to incubate. The bacteria were centrifuged again at 4000g, 4°C, for 15 minutes, and then the supernatant was discarded. The pellet was gently resuspended in 1.25mL of cold solution B (solution A + 15% glycerol). 25 tubes were placed in dry ice, and then 50µL of competent cells were aliquoted into each one of them. The Eppendorf with the competent cells was stored at -80°C.

DNA ISOLATION

A miniprep is a laboratory technique used to extract plasmid DNA from a bacterial culture. For this protocol, the QIAprep Spin Miniprep Kit from Qiagen was used.

The bacterial strain from which the plasmid DNA was to be extracted was first streaked on a selective LB plate and grown overnight. The following day, a single colony was picked from the plate, and an inoculum was prepared with the selective media and grown overnight. The inoculum was centrifuged at 5000rpm for 15 minutes at room temperature. The pellet was resuspended in 250 µL of buffer P1 (containing RNase A)

and then transferred to a microcentrifuge tube. After the addition of 250 μ L of buffer P2, the microcentrifuge tube was inverted 5 to 6 times. After no more than 5 minutes, 350 μ L of buffer N3 was added, and the solution was mixed immediately by inversion. The solution was then centrifuged at 13,000 rpm, for 10 minutes at room temperature. 800 μ L of the supernatant was then transferred to a spin column provided with the kit. The spin column was then centrifuged at 13,000 rpm for 1 minute at room temperature. The flow-through was discarded, and the column was washed with 500 μ L of buffer PB. The spin column was then centrifuged again at 13,000 rpm for 1 minute at room temperature, and the flow-through was discarded. The column was then washed again with 750 μ L buffer PE (containing EtOH). The spin column was then centrifuged again at 13,000 rpm for 1 minute at room temperature, and the flow-through was discarded. The column was then spun again to remove any residue. The column was placed in a 1.5mL Eppendorf and then 30 μ L of buffer EB were added to extract the plasmid DNA from the filter. The Eppendorf tube with the column was centrifuged at 13000rpm for 1 minute at room temperature (RT) one last time to elute the plasmid DNA. Plasmid DNA concentration was measured using the NanoDrop™ One/OneC (Thermo Fisher Scientific).

TRANSFORMATION

The tubes containing the competent cells needed for transformation were thawed on ice. 1 μ L of miniprep was added to the competent cells and then left in ice to incubate for 30 minutes. In the meantime, a selective LB agar plate and tube with sterile LB medium were pre-warmed at room temperature. The thermoblock was prewarmed and set to a temperature of 42°C. Once the 30 minutes of incubation had passed, the competent cells with the miniprep were subjected to a heat shock in the heat block. The tubes were placed in the heat block at 42°C for 45 seconds and then transferred to ice for 1 minute. 200 μ L of sterile, warm LB was added to the cells, and then the Eppendorf tube was placed in a hot bath at 37°C for 1 hour. After the incubation time had passed, 200 μ L of the culture was plated on a selective agar plate and then grown overnight (O/N) at 37°C.

COLONY PCR

A 1.5µL microcentrifuge tube for each colony to be tested was picked and 200µL of filtered autoclaved MilliQ water was added. A single colony was picked from a plate and carefully resuspended in the tube. A heat block was turned on and the temperature set to 100 °C. When the desired temperature was reached, the tube containing the colony was placed in the heat block for 15 minutes to break the cell wall and allow the genetic material to resuspend. The tube was then centrifuged at 4,000 rpm for 10 minutes at room temperature. 5µL of the supernatant was added to the PCR mix to perform the PCR.

The DNA polymerases used for the colony PCR were the Phusion™ High-Fidelity DNA Polymerase (New England Biolabs).

PHAGE PRODUCTION

A streak of the *E. coli* F' bearing the phagemid with the circuit of interest was prepared and grown O/N.

A single colony was picked from the plate and inoculated in 50mL of LB medium with ampicillin in a 250mL flask and grown at 37°C, 220 rpm until it reached OD₆₀₀ equal to 0.05. When the set OD₆₀₀ was reached, 50 µL of helper phage M13KO7 (Invitrogen) was added, and the culture was allowed to grow at 37°C and 220 rpm for an additional 90 minutes. After the incubation time passed, 75 µL of kanamycin was added to the culture, and it was allowed to grow at 37°C and 220 rpm overnight.

The following day, the culture was transferred into a 50mL conical tube and centrifuged at 5,000 rpm for 15 minutes at room temperature. The supernatant was then filtered through a 0.2 µm filter to remove the bacterial pellet into a new sterile 50mL conical tube. 10% polyethylene glycol (PEG-8000; Merck) and 1 M NaCl (Carlo Erba) were added and mixed thoroughly until the solution appeared clear. It was then left to incubate overnight at 4°C.

The day after preparation, the sample was centrifuged at 12,000g for 1 hour at 4°C. The supernatant was discarded, being careful not to touch the pellet area in order to

maintain as many phage particles as possible. The pellet was subsequently resuspended with 500 μ L of SM buffer (50mM Tris-HCl, pH 7.5, 100mM NaCl, 10mM MgSO₄). The phage production was then transferred to a 2mL screw cap tube and stored at -80°C.

TRANSDUCTION ASSAY

This assay was performed to deliver the desired construct into the bacterial strain to be tested via the phage particle previously produced.

A pre-inoculum of the target bacterial strain was prepared and grown overnight at 37°C with shaking at 200rpm. The next day, a dilution of 1:100 was prepared from the pre-inoculum and allowed to grow until it reached the mid-exponential phase (approximately an OD₆₀₀ of 0.4-0.5 for 2-3 hours) at 37°C and 200rpm. It is essential to ensure that the OD₆₀₀ does not exceed 0.6, as F' *E. coli* tends not to express the pilus at levels above this state.

100 μ L of the grown culture was transferred into a 2mL screw cap tube, and 100 μ L of the phage production to be tested was added. The mixed cultures were allowed to grow statically at room temperature for 30 minutes.

After 30 minutes, dilutions of the transduction assay, from 0 to 10⁻⁸, were spotted on both LB and LB + Amp agar plates.

COLISTIN GROWTH INHIBITION ASSAY

To test the efficiency of the CRISPRi system in decreasing the resistance of AMR bacteria, we used a growth inhibition assay. For the Colistin resistance, six concentrations of Colistin were chosen: 8 μ g/mL, 4 μ g/mL, 2 μ g/mL, 1 μ g/mL, 0.1 μ g/mL, 0 μ g/mL.

Colistin sulphate (Merck) was dissolved in Milli-Q water and then filtered using a 0.2 μ m filter. Different preparations of LB with added Colistin were prepared to reach the desired concentrations. An inoculum for each strain to be tested was prepared the previous day. In a 96-well plate, 198 μ L of LB with added Colistin, 2 μ L of bacteria

were added in order to have a 1:100 dilution. The OD₆₀₀ was measured using a Varioskan™ LUX (ThermoFisher Scientific). The growth was measured every 5 minutes, with shaking at 300rpm in between for 15 seconds on and 4 minutes 45 seconds off, for a total of 16 hours.

Data analysis was performed first on Excel to blank the results, removing the background noise, and then on Prism to create the graph displayed in this work.

MEROPENEM GROWTH INHIBITION ASSAY

The same assay was also performed to test the CRISPRi system's efficiency to repress the Meropenem resistance. The six concentrations of Meropenem that were chosen were: 500µg/mL, 200µg/mL, 100µg/mL, 10µg/mL, 1µg/mL, and 0µg/mL.

The Meropenem (Merck) powder was dissolved in autoclaved DMSO.

RESULTS

PHAGEMID DEVELOPMENT

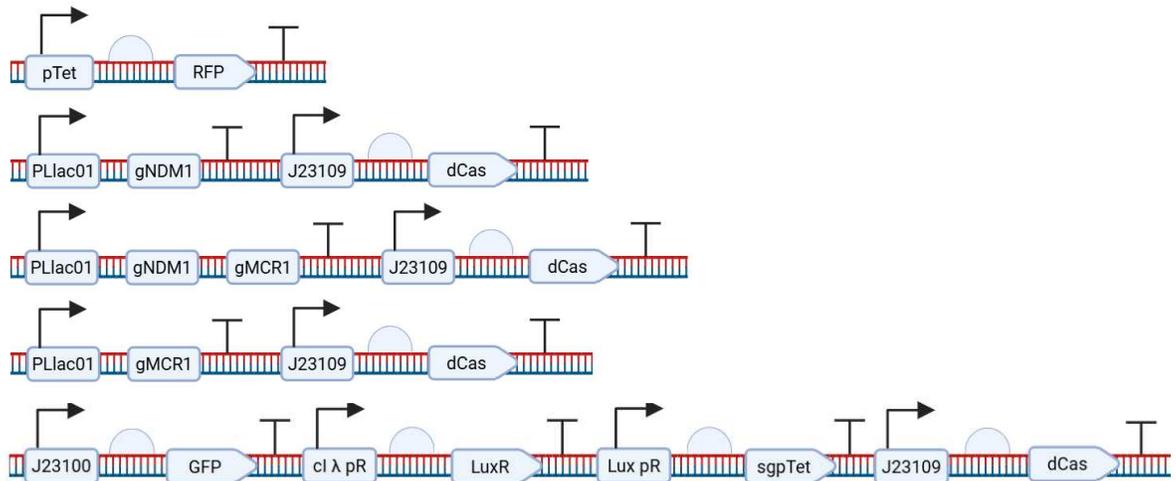


Figure 2 **phagemids**: graphic illustration representing the phagemids employed in this work

The backbone for the synthesis of the phagemids comes from the commercially available pTZ19R (Thermo Fisher Scientific). This phagemid contains the pMB1 origin of replication and the *bla* gene, which is responsible for producing β -lactamase and confers resistance to ampicillin.

Starting from this vector backbone, we constructed five different phagemids (*figure 2*).

The first one is the pTZ-I13521, which was used as a means of characterizing the process. This phagemid carries the *rfp* gene into F' *E. Coli* as a reporter, thanks to the color of the protein, to recognize the infected bacteria. pTZ-I13521 harbors the *mRFP* gene under the control of the pTet promoter.

Then we moved onto a more complex circuit that was developed to test the possibility of sensitizing AMR bacteria through the delivery of a CRISPRi system. Three phagemids were employed in this process: one harboring a spacer to target the *bla_{ndm1}* gene (pTZ-ndm1), one harboring two spacers targeting both the *bla_{ndm1}* gene and the *mcr1* gene (pTZ-ndm1-mcr1), and lastly one harboring a spacer to target exclusively

mcr1 (pTZ-mcr1). For this CRISPRi system, we placed the *dCas9* gene under the constitutive promoter J23109 from the Anderson library. For the guide RNAs (gRNAs), we chose to use the system via CRISPR arrays, constituted of spacers, flanked by non-coding repeats, under the Isopropyl β -d-1-thiogalactopyranoside (IPTG) inducible promoter PLlacO1, which is constitutively active without the need for induction. The other part of the CRISPR array consists of the tracrRNA, which is under its own promoter and terminator.

The last phagemid we used, pTZ-AegpTet, was designed to test the construct's capability to bear a heavier metabolic burden and also to investigate how changes in the promotion of the different modules would affect repression. In this last phagemid, we also changed the approach for the CRISPRi system, relying on an sgRNA with a spacer and tracrRNA in a unique structure. In this construct, we added the *gfp* gene under the control of the constitutive promoter J23100 from the Anderson library as a metabolic burden indicator. The targeting was achieved by using the sgpTet, which targets the pTet promoter. For the promotion of this sgRNA, we decided to use the (N-3-oxohexanoyl-homoserine lactone) HSL-inducible LuxI promoter. Lastly, we have the *dCas9* gene under the control of the J23109 promoter.

CHARACTERIZATION OF PHAGE DELIVERY SYSTEM

We decided to approach the phage production by employing a method that involves the use of a phagemid and a phage helper. In our project, we used the M13KO7 helper phage (Invitrogen), which carries a Kanamycin resistance gene and the p15A origin of replication. F' *E. coli* culture harboring the desired phagemid was infected by the M13KO7 in order to develop a progeny of phage particles carrying the desired construct.

To characterize the efficiency of the phage delivery system, we began by delivering a phagemid that harbored an *rfp* cassette under the control of the pTet promoter. The *rfp* was chosen as a reporter gene to recognize and distinguish the infected and non-

infected colonies easily. The phagemid pTZ-I13521 holds *blaR*, a β -lactam antibiotic resistance gene, involved in Ampicillin resistance, and the pMB1 origin of replication.

We performed a transduction assay to test the ability of the phages particles to infect the F' *E. coli*. We also tested the phage production on the *E. coli* TOP10 strain as a negative control (*Figure 3b*) because of its lack of the F' episome. The transduction was spotted in Amp plates at different dilutions (from 0 to 10^{-6}) to display the specificity of the system (*figure 3a*). We also spotted and plated the transduced F' *E. coli* on an LB plate (*figure 3c, 3e*) to compute the efficiency of transduction by comparing the red colonies on the LB Amp plate with the total population grown on plain LB plate (red and white colonies). After counting the red and white colonies from the LB plates in which the transduced F' *E. coli* grew, we estimated that the efficiency of the system was around 92%.

In *figure 3b* we can notice that the transduced TOP10, lacking the F' episome, spotted with no dilution (10^0), was able to grow in LB ampicillin plate. Although the colonies were able to grow on ampicillin, they did not display any red shade, which would be an indication of the pTZ-I13521 transduction. To understand this issue, we investigated whether the ability of *E. coli* TOP10 to grow on ampicillin was due to the acquisition of *blaR* from the phagemid or if the high cell density allowed the bacteria to grow. Indeed, ampicillin is connected to the satellite colonies phenomenon. During ampicillin selection, β -lactamase secreted by resistant colonies can diffuse into the medium and locally inactivate the antibiotic, but this shouldn't be the case since the *E. coli* Top10 wt shouldn't have any resistance gene. We decided to spot the non-transduced *E. coli* TOP10 strain in an LB Ampicillin plate, and we noticed that when not diluted, the strain was able to grow even though it didn't possess any Ampicillin resistance gene. We hypothesize that the huge bacterial load could be the explanation for this phenomenon.

Besides this phenomenon, we can see how the phage particles infect only the F' *E. coli*, demonstrating the specificity of the system.

Another parameter of the protocol we wanted to test was the purity of the phage production, to understand if, in the process, some helper phage would still be produced and in what quantity. To obtain this information, we compared the colonies that grew on LB plates with added ampicillin with those that grew on LB plates with added kanamycin (*Figure 3d*), which is indicative of the resistance carried in the helper phage genome. In the Kanamycin plate, only two colonies were visible in the non-diluted spot, which, compared to the Ampicillin plate, demonstrated a purity of over 99.9%.

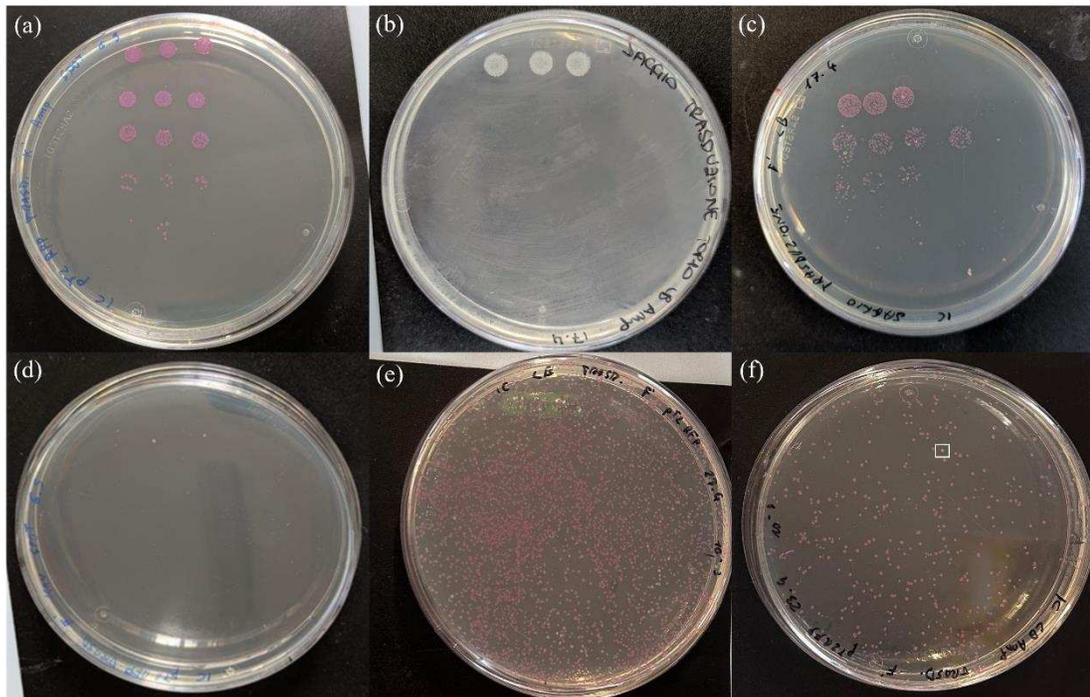


Figure 3 Phage production characterization: transduction assay performed with pTZ-I13521 phage production in different conditions. (a) transduction assay on *E. coli* F' spotted on LB amp (dilution 10^0 - 10^{-6}), (b) transduction assay on *E. coli* TOP10 spotted on LB amp (dilution 10^0 - 10^{-6}), (c) transduction assay on *E. coli* F' spotted on LB ((dilution 10^0 - 10^{-6}), (d) transduction assay on *E. coli* F' spotted on kanamycin (dilution 10^0 - 10^{-6}), (e) transduction assay on *E. coli* F' plated on LB (dilution 10^{-3}), (f) transduction assay on *E. coli* F' plated on LB amp, white square represent a single white colony (dilution 10^{-4}).

When we plated the transduced F' *E. coli* on a full plate of LB ampicillin (*figure 3f*) we noticed the unusual presence of a single white colony (white square). Different explanations could be attributed to this event, so we decided to investigate them.

Our first hypothesis was that the white colony could be the result of an F' *E. coli* infected by the M13KO7 helper phage, since we observed that, even if minimal, some helper phage particles were present during phage production. The growth of ampicillin

can be explained by the satellite effect or by a double infection event from two different phages, resulting in the coexistence of both pTZ-I13521 and M13KO7. To test the presence of the helper phage in the colony, we decided to test the growth of the colony in three different LB conditions: with ampicillin, with kanamycin, and with both together.

The isolated white colony was able to grow only in the absence of kanamycin, leading us to conclude that the helper phage genome was not present in the bacteria. To further confirm this result, we decided to perform a colony PCR, testing the isolated white colony with either primers specifically for the phagemid pTZ-I13521 or the primers for the M13KO7 helper phage. After the colony PCR, we ran a gel electrophoresis and noticed that only the pTZ-I13521 primers were able to amplify some genomic material. The length of the amplicon was slightly smaller compared to the amplicon from the phagemid miniprep.

This result confirmed that the M13KO7 genome was not present in the isolated white colony. To finally understand the reason for the lack of red shade in the colony, we decided to send the isolated plasmid DNA extracted from the white colony for sequencing using the Sanger method. From the sequencing, we were able to identify a deletion at the level of the pTet promoter for the *mRFP* gene, which explains the absence of red fluorescence. This riddle made us come to another conclusion: the fact that we could estimate an incidence of mutants lower than 0.01% during the transduction assay protocol.

DELIVERY OF CRISPRi SYSTEM TARGETING ANTIMICROBIAL RESISTANCE (AMR) GENES *bla* (*ndm-1*) AND *mcr-1*

After we completed the stage of characterization of the system we decided to move forward with the main aim of the project, the delivery of a CRISPRi system with the objective of decreasing AMR bacteria resistance to antibiotic treatment.

To test the system, we had to first create two constructs that would represent the targets, meaning two plasmids holding the resistance genes *bla_{ndm1}* and *mcr-1*. For the targets we had to choose a plasmid that possessed an origin of replication compatible with the pMB1 ori of the pTZ phagemids backbone. For this purpose, we decided the pSB3K3 as the backbone of the target plasmids. This plasmid contains a p15A origin of replication that is compatible with the pMB1 origin.

The two targets were obtained by starting from two plasmids kindly provided to us by Prof. Pasotti from the BMS laboratory at the University of Pavia, Italy. These plasmids were constructed on the pGDP backbone, which was not compatible with our system.

The two genes, *bla_{ndm1}* and *mcr-1*, responsible for the resistance in the target strains, under the control of two different promoters, pBla and pLacI, respectively, were inserted into the pSB3K3 backbone, yielding the pSB3K3-ndm1 and the pSB3K3-mcr1 target plasmids. These two plasmids were transformed into F' *E. coli* cells, allowing them to be employed for phage delivery testing, thanks to the presence of the F pilus.

REPRESSION OF bla_{ndm1} RESISTANCE GENE

Two phagemids targeting the *bla_{ndm1}* coding DNA sequence (CDS) region were assembled, pTZ-ndm1 and pTZ-ndm1-mcr1. The first was constructed with a single spacer to target the CDS of the *bla_{ndm1}* gene through the CRISPRi system. The second circuit contained two different spacers, one for the *bla_{ndm1}* and one for the *mcr-1* CDS, to test whether targeting two different genes in a single system would decrease the efficiency of repression.

Two phage productions, one for each phagemid, were achieved, and a transduction assay was performed on F' *E. coli* harboring the pSB3K3-ndm1 target plasmid. Colonies resulting from each transduction assay were then tested with a growth inhibition assay to extrapolate the MIC value and growth delay. *E. coli* TOP10 F' wild type (wt; sensitive strain) and F' *E. coli* harboring the pSB3K3-ndm1 target plasmid (resistant strain) were also tested as positive and negative controls for the antimicrobial activity of Meropenem.

Six concentrations of Meropenem were tested for each strain: 500 μ g/mL, 200 μ g/mL, 100 μ g/mL, 10 μ g/mL, 1 μ g/mL, 0 μ g/mL.

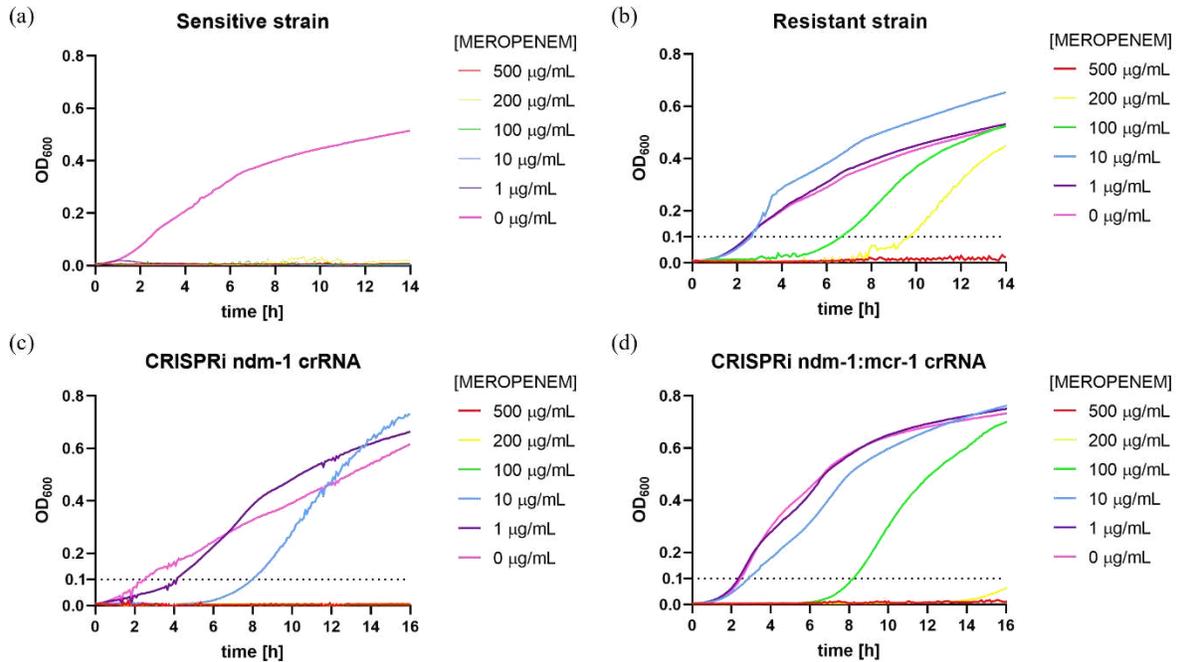


Figure 4 growth profiles for meropenem treatment: growth profiles of four different strains treated with meropenem at 6 different concentrations, dotted line indicates the threshold for the evaluation of the growth delay (a) *E. coli* TOP10 F' wild type (wt; sensitive strain), (b) F' *E. coli* harboring the pSB3K3-ndm1 target plasmid (resistant strain), (c) F' *E. coli* harboring the pSB3K3-ndm1 transduced with pTZ-ndm1 to target resistance gene via CRISPRi, (d) F' *E. coli* harboring the pSB3K3-ndm1 transduced with pTZ-ndm1-mcr1 to target resistance gene via CRISPRi.

In figure 4 we can see the growth of each strain represented in relation to the Meropenem concentration of the treatment. OD₆₀₀ of 0.1 was chosen as the threshold to evaluate the growth delay of each strain tested.

We can see that the Meropenem efficacy is confirmed, as the sensitive strain was unable to grow at any of the concentrations tested.

We observed that the resistant strain exhibited a MIC of >200 μ g/mL, whereas the sensitive strain had a MIC of <1 μ g/mL, representing a 200-fold increase. For the two CRISPRi-treated strains, the *ndm1* array and the *ndm1-mcr1* array, we obtained MICs of >10 μ g/mL and >100 μ g/mL, respectively. These values can already partially show

the effectiveness of the treatment in interfering with the resistance to Meropenem activity.

The effect can be demonstrated and validated even more comprehensively by analyzing the growth delay observed after CRISPRi treatment, as we will discuss later.

Another important point to notice is the difference between the system carrying two crRNAs (*ndm1:mcr1*) and the system with a single crRNA (*ndm1*). The single one shows a substantial increase in interference activity, allowing Meropenem to completely block growth even at a concentration of 100µg/mL, while the double guide was able to achieve strong sensitization to Meropenem only at 200µg/mL. This suggests that a significant portion of the dCas9 in the case of the double guide was unutilized due to binding to the *mcr1*-crRNA, resulting in a decrease in the overall activity of the CRISPR interference system.

In the future, thanks to the synthetic biology approach, a construct with a stronger promoter for dCas9 could be developed, allowing for a system that simultaneously targets different resistance mechanisms in the treatment of MDR bacteria.

REPRESSION OF mcr1 RESISTANCE GENE

The same approach was carried on to test the CRISPRi system on the *mcr1* resistance gene. Two phage productions were developed starting from the phagemids pTZ-*mcr1* and pTZ-*ndm1-mcr1*. To test the CRISPRi system, a transduction assay was performed on F' *E. coli* holding the target plasmid pSB3K3-*mcr1* for Colistin resistance.

A growth inhibition assay was performed on colonies resulting from both transduction assays. We also previously tested *E. coli* TOP10 F' wild type (sensitive strain) and F' *E. coli* holding pSB3K3-*mcr1* (resistance strain) as positive and negative controls.

Six concentrations of Colistin were tested for each strain: 8 $\mu\text{g/mL}$, 4 $\mu\text{g/mL}$, 2 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$, 0.1 $\mu\text{g/mL}$, 0 $\mu\text{g/mL}$.

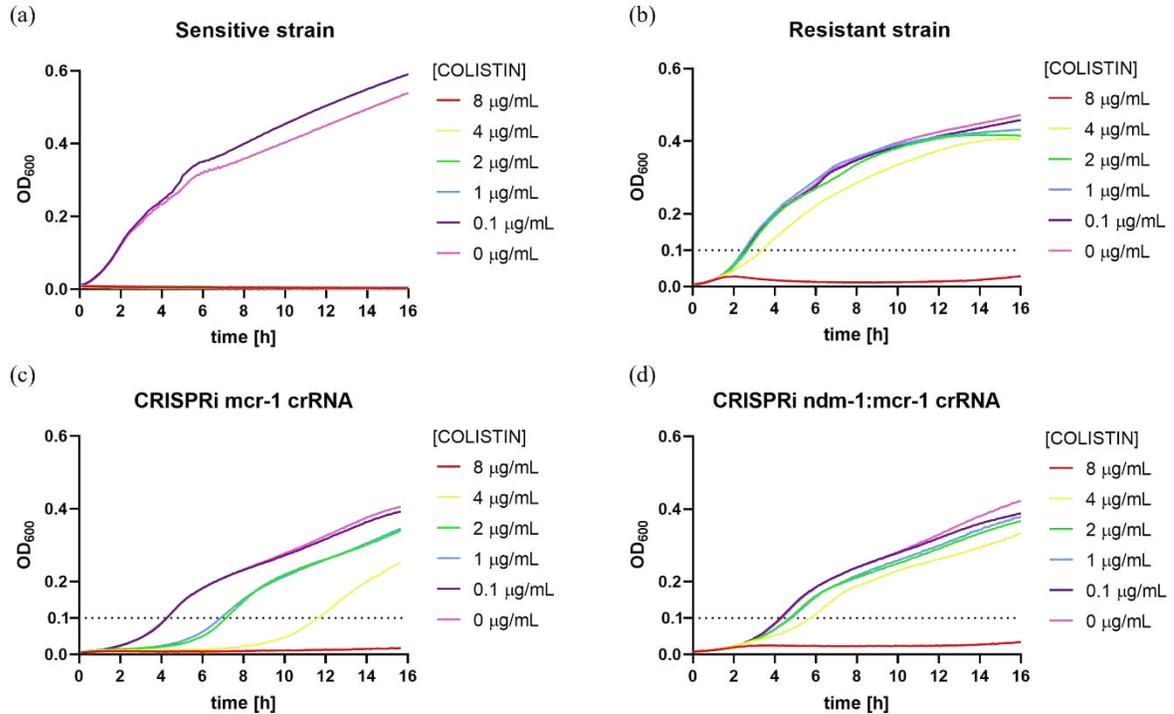


Figure 5 growth profiles for colistin treatment: growth profiles of four different strains treated with colistin at 6 different concentrations, dotted line indicates the threshold for the evaluation of the growth delay (a) *E. coli* TOP10 *F'* wild type (*wt*; sensitive strain), (b) *F'* *E. coli* harboring the *pSB3K3-mcr1* target plasmid (resistant strain), (c) *F'* *E. coli* harboring the *pSB3K3-mcr1* transduced with *pTZ-mcr1* to target resistance gene via CRISPRi, (d) *F'* *E. coli* harboring the *pSB3K3-mcr1* transduced with *pTZ-ndm1-mcr1* to target resistance gene via CRISPRi.

In this case, the first concentration of Colistin tested, 0.1 $\mu\text{g/mL}$, was not effective even on the sensitive strain, so this value is not significantly informative for the purpose of the thesis.

In *figure 5* the growth at the different Colistin concentrations tested is displayed alongside the threshold of OD_{600} 0.1, chosen for the growth delay evaluation.

In this case, we can assess a $\text{MIC} > 4 \mu\text{g/mL}$ for both the CRISPRi-treated strains and the resistant strain. Although the MIC value is the same for all three tested strains, a significant difference in the growth profile is clearly evident, confirming the activity of the CRISPRi system. As previously noted, we can observe a difference in the

repression of the resistance mechanism between the system with a single crRNA (*mcr1*) and the one with a double crRNA (*mcr1:ndm1*), where the single crRNA shows a more pronounced sensitization of the bacteria to Colistin activity.

GROWTH DELAY EVALUATION

When it came to evaluating the growth delay after treatment with the CRISPRi system, we encountered a crossroads, as no gold standard for this type of data analysis exists. This pushed us to evaluate the data obtained with two different approaches.

In the first method, we evaluated the growth delay (Δt CRISPRi - resistant) by subtracting the time point at which the CRISPRi-treated strain reached the threshold from the time point at which the resistant strain reached the threshold, comparing the same concentrations of Antibiotic.

In the second method, instead (Δt CRISPRi +Ab -Ab), we decided to do the difference from the threshold timepoint of the CRISPRi-treated strain at a specific Antibiotic concentration and the CRISPRi-treated strain with no added Antibiotic (0 μ g/mL).

These two methods give us similar but slightly different results. This does not mean that one is better or worse than the other, but they can be used to enlighten different aspects of the whole experiment.

With the first method, we can make a clearer comparison between the two arrays tested for CRISPR interference: the one with a single crRNA and the one with a double crRNA. This Δt calculating approach already takes into account the difference in growth delay achieved against the resistant strain, so it is more suitable to compare the efficiency of the two constructs directly.

The second approach, however, is more suitable for understanding the efficiency of the CRISPRi system in repressing the resistance gene, as we can directly compare the growth delay of the CRISPRi strain with that of the resistant strain. In this case, the Δt takes into account that the CRISPRi system in itself could imply changes to the growth

profile even when no antibiotic is added, so by taking out of the equation this variable, we can have a more specific measurement of the effectiveness of the system.

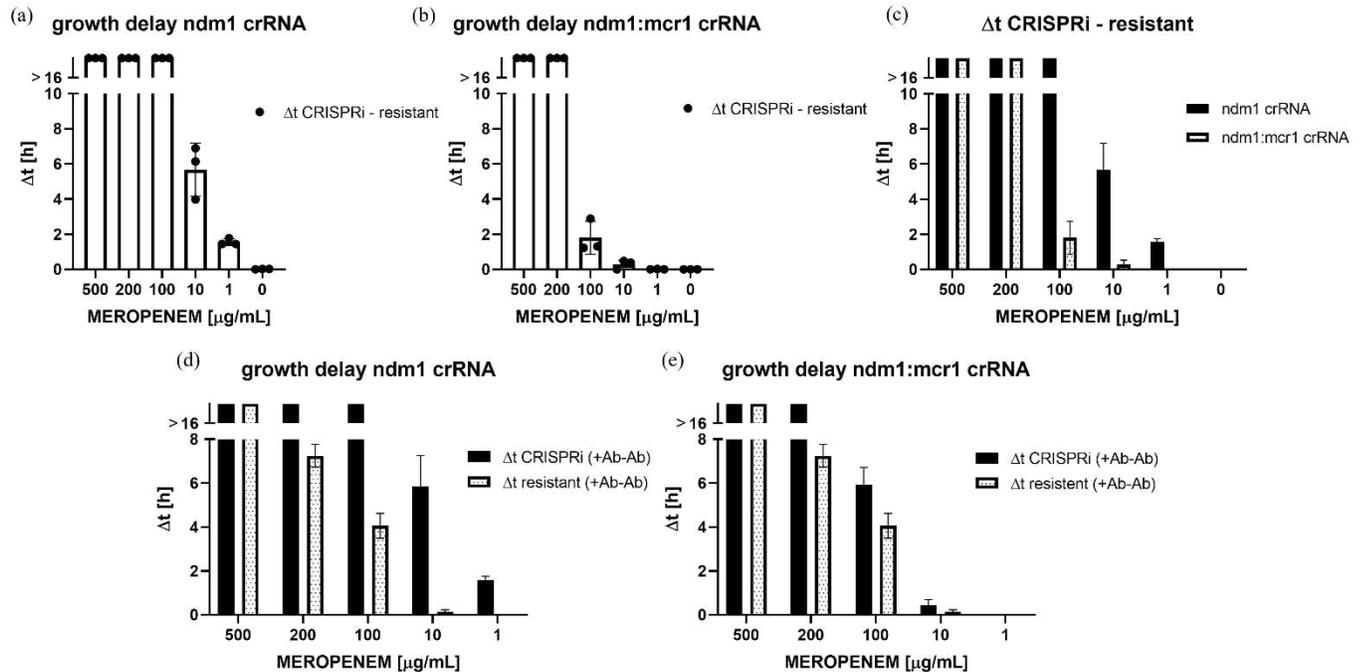


Figure 6 meropenem induced growth delay: graphical representation of growth delay obtained after meropenem treatment calculated via two different methods: Δt CRISPRi – resistant (a,b,c), Δt CRISPRi +Ab -Ab (d,e). (a) growth delay for the transduction assay with pTZ-ndm1, (b) growth delay for the transduction assay with pTZ-ndm1-mcr1, (c) comparison between growth delay of achieved with pTZ-ndm1 and pTZ-ndm1-mcr1. (d) comparison between resistant strain transduced with pTZ-ndm1 and non-transduced, (e) comparison between resistant strain transduced with pTZ-ndm1-mcr1 and non-transduced.

In figure 6 we can analyze the results for the targeting of bla_{ndm1} . For the ndm1 crRNA system we can see already at the 10 $\mu\text{g/mL}$ concentration the increase in the repression activity (figure 6a) and the difference with the ndm1:mcr1 crRNA system (figure 6b). This difference is demonstrated even more clearly at the 100 $\mu\text{g/mL}$, where the single crRNA is capable of completely suppressing the growth of the bacterial strain, while the double crRNA only reaches a growth delay of almost 2 hours. As we can see from the histogram the double crRNA becomes a reliable system only for high concentrations of Meropenem (> 200 $\mu\text{g/mL}$), this can let us conclude that having a system built like ours with two spacers targeting different resistant gene seems to be not the best choice since when compared with the single crRNA system we can clearly see a strong decrease in the activity.

When we look at the second approach for the Δt measurement (figure 6d, 6e), we can have a clearer comparison of the efficiency of the system regarding the change of the growth delay in comparison to the resistant strain. We can see how the ndm1 crRNA system is already efficient in sensitizing the strain to Meropenem action at 10 μ g/mL, delaying the time to reach the threshold OD₆₀₀ by 6 hours. In contrast, in the resistant strain, Meropenem has almost no repressing activity. As for the ndm1-mcr1 crRNA, we can clearly see that even at a 100 μ g/mL Meropenem concentration, the antibiotic's capability in blocking growth is not significantly different.

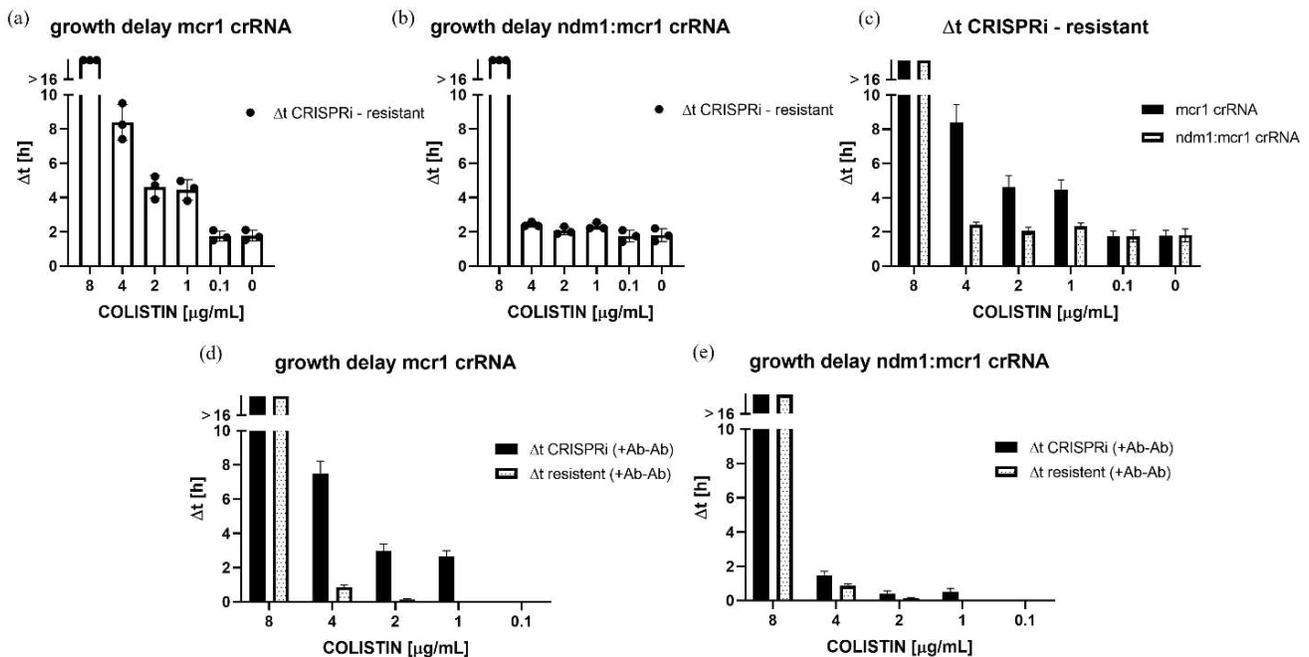


Figure 7 **colistin induced growth delay**: graphical representation of growth delay obtained after colistin treatment calculated via two different methods: Δt CRISPRi – resistant (a,b,c), Δt CRISPRi +Ab -Ab (d,e). (a) growth delay for the transduction assay with pTZ-mcr1, (b) growth delay for the transduction assay with pTZ-ndm1-mcr1, (c) comparison between growth delay of achieved with pTZ-mcr1 and pTZ-ndm1-mcr1. (d) comparison between resistant strain transduced with pTZ-mcr1 and non-transduced, (e) comparison between resistant strain transduced with pTZ-ndm1-mcr1 and non-transduced.

When we move on to the Colistin growth delay (figure 7) we can clearly see a difference in the first approach for growth delay since even at the Colistin concentration of 0 μ g/mL we achieved a growth delay of almost two hours. This enlightens the fact that in this case, the construct we used for the CRISPRi affects the growth of the

bacterial strain even without taking into account the presence of the antibiotic. Several reasons could explain this growth delay; the metabolic burden of the construct may be too heavy on the cell, resulting in an increased time to reach exponential growth. On the other hand, the maintenance of the phagemid inside the cell during the growth inhibition assay requires the presence of Ampicillin, which resistance is conferred by the construct, and this could also be a reason for the growth delay observed at 0 $\mu\text{g}/\text{mL}$. Ampicillin needs to be present because it ensures the maintenance of the phagemid, which could otherwise be lost since it is not vital for the survival of the bacteria. What is more important to note is that if we had decided to follow up with just the Δt CRISPRi +Ab -Ab approach for the analysis, we would not have been able to gain this information by examining the histograms exclusively. This highlights the importance of testing both approaches to gain a more comprehensive overall view.

Now that we have explained the importance of the two approaches, we can analyze the data. Let's take a look at Δt CRISPRi - resistant graphs (*figure 7a, 7b, 7c*). We can observe that the *ndm1:mcr1* crRNA system only resulted in a two-hour growth delay for all Colistin concentrations tested, except for 8 $\mu\text{g}/\text{mL}$, which was also effective against the resistant strain, indicating that the effect is not achieved through the CRISPRi system. As we mentioned earlier, the 2-hour growth delay we observe cannot be attributed to interference with the resistance gene but is more likely due to the metabolic burden of the construct itself. In conclusion, we can affirm that in this case, the system containing the two crRNAs was ineffective in sensitizing the bacteria to the antibiotic's effect.

To further confirm the results obtained from the *ndm1:mcr1* crRNA system (*figure 7e*) we tested them by running a two-tailed, two-sample unequal variance t-test. This test yielded p-values < 0.05 for concentrations ranging from 1 to 4 $\mu\text{g}/\text{mL}$, indicating that there was no statistically significant difference in growth delay between the CRISPRi-treated strain and the resistant strain.

Regarding the single guide *mcr1* crRNA system (*figure 7d*) instead, we can see that a growth delay of almost two hours was achieved already at 1 $\mu\text{g}/\text{mL}$, with an increase to

7 hours when scaling up to 4 μ g/mL, resulting in a 5-fold increase in growth delay when compared to the resistant strain.

TESTING THE CAPABILITY AND LIMITS OF THE SYSTEM

To conclude this study and try to understand the limits of the system we employed, we decided to build and deliver a more complex circuit in which we implemented a reporter gene (*gfp*), under the J23100 promoter, as a metabolic burden indicator. Not only that, but we also decided to switch from the CRISPR array system to a sgRNA system, which would require one fewer maturation step. The sgRNA was placed under the HSL-inducible LuxI promoter. This promoter has a constitutive level of induction but it can also be induced via the Homoserine lactones (HSL) that binds to the LuxR, when the complex is formed, this can lead to a stronger induction of the LuxI promoter. The whole circuit was placed into the pTZ phagemid backbone, yielding the pTZ-AegpTet phagemid. This is a more complex construct, designed to target the promoter (pTet) of the *mRFP* gene, which was chosen as a target due to its simplicity in recognizing efficacy, as it would be based on the change in the colonies' shade, evaluated by measuring the absorbance. The plasmid used as a target was pSGAb-II13521, a low-copy plasmid that carries the Kanamycin resistance gene and expresses *mRFP* under the pTet promoter.

Phage production was performed starting from F' *E. coli* carrying the pTZ-AegpTet plasmid.

It is essential to note that when we streaked the strain containing the pTZ-AegpTet to select the colony for phage production, two distinct types of colonies emerged. Some colonies expressed the GFP protein, which we used for production, while others appeared to be red. We couldn't explain the presence of red colonies since no *rfp* genes were present in the construct. Although we noticed this problem, we decided to continue by picking exclusively the green colony, due to the lack of time that would be

required to start from the beginning with the cloning of the whole phagemid from scratch.

The phage particles were used to carry on a transduction assay to infect F' *E. coli* carrying the target plasmid pSGAb-I13521. We plated the transduction assay on LB plate with added Ampicillin and Kanamycin (figure 8a). When visualized at the transilluminator, we observed different types of colonies: some were completely red, some were completely green, and finally, we noticed some colonies in which the edges were red and the center of the colony was green.

Other than the different shades of color, it is important to mention that compared to the previous transduction assays we did, in this case, the growth of the culture was significantly slower and the number of colonies much lower. This phenomenon could be attributed to the fact that, in this case, we have a strong metabolic burden in the construct that could interfere with and slow down the growth of the colonies.

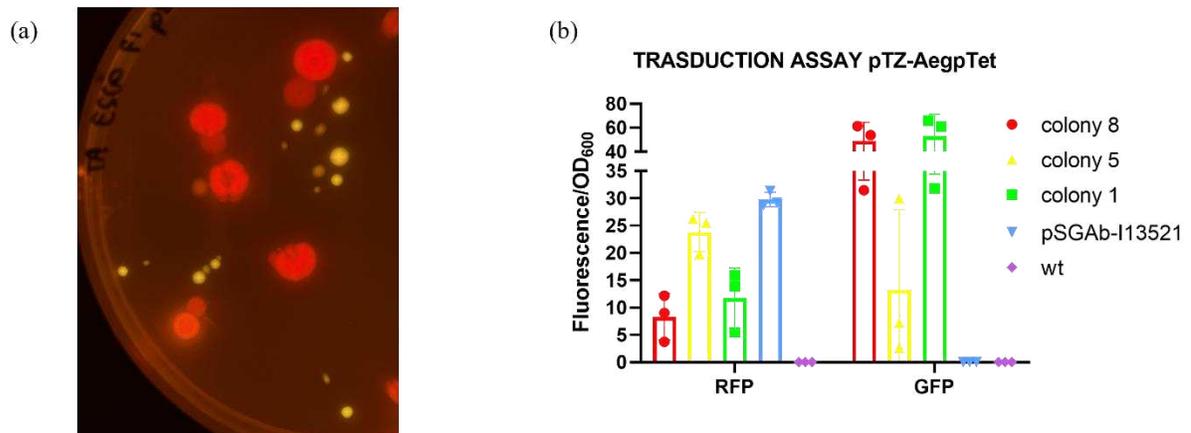


Figure 8 **pTZ-AegpTet** evaluation: (a) transduction assay performed with pTZ-AegpTet on F' *E. coli* carrying the p-SGAb-I13521, plated on LB ampicillin, (b) graph representing the fluorescence/OD₆₀₀ of three colonies isolated from the transduction assay (colony 8, 5 and 1), a positive control (F' *E. coli* carrying pSGAb-I13521), and a negative control (F' *E. coli* wt).

From the plate in figure 8a we decided to pick colonies, of each of the three typologies, and isolate them in LB plates with added ampicillin and kanamycin. Interestingly, the colonies that grew the best on double antibiotic plates were those that appeared

exclusively red, while green and mixed ones either formed just a few colonies or didn't grow at all.

Then, from the colonies we isolated, we performed inocula in three conditions (ampicillin alone, ampicillin + kanamycin, and kanamycin alone) to gain a clearer understanding of the content of the colonies and to use it for a microplate reader assay. After transitioning to liquid media, we noticed that only the red colonies (colonies 1, 5, and 8) were able to grow on double antibiotic.

We decided to evaluate the fluorescence/OD₆₀₀ of these colonies compared to the target pSGAb-I13521 (positive control) and the *E. coli* TOP10 F' wild type (negative control) (figure 8b). From the results, we can notice that two of the isolated colonies tested (colony 1 and 8) achieved a repression of the *rfp* expression that was similar (2-fold decrease in *rfp* fluorescence), while the third one (colony 5) did not achieve an analogous repression. What is noteworthy is the relation with the *gfp* expression; the first two colonies achieved a good expression of *gfp* while the third one (colony 5) did not show as much *gfp* expression. For colony 5, the decrease level of *gfp* fluorescence, in conjunction with the decrease repression of the *rfp*, can lead to the assumption that the metabolic burden resulted in a decreased expression of the whole construct.

Since we had some doubts about the starting culture used for pTZ-AegpTet phage production due to the presence of a mixed culture, we decided to investigate it thoroughly. We began by attempting to isolate single green colonies from the initial plate. Although we picked single green colonies for isolation, we still obtained a mixed culture of green and reddish colonies. This led us to assume that during the cloning process of the pTZ-AegpTet, an error occurred, and we possibly obtained two different phagemids: one carrying the correct construct with the *sgpTet* guide for the CRISPRi, and one that could hold a phagemid expressing the *mrfp* gene. To evaluate this hypothesis, we attempted to isolate DNA so that it could be sequenced using both the Sanger method and Whole Plasmid Sequencing (WPS). The results from both sequencing methods displayed only the correct pTZ-AegpTet and did not show the presence of any other phagemids. These results were unexpected since we still couldn't

explain the presence of the red colonies. The most probable hypothesis was still that the stock prepared after the cloning was contaminated but the presence of the second phagemid was too low to be visualized even through WPS. Unfortunately, this issue brought us to a halt in testing the system's limits due to the ambiguity of the system we were using. This also meant that we couldn't test the inducibility of the system by adding the HSL, as we couldn't determine if the obtained results were authentic or misleading due to the inaccuracy in phagemid development.

Nevertheless, the issue we encountered sparked a question in our mind; the possibility that a co-infection of a single F' *E. coli* could happen in the case of a mixed phage production. While examining the literature in search of information about bacteriophage co-infection, only a few works were found, and none specifically addressed bacteriophage M13. The fortuitous event of obtaining a phagemid pTZ-GFP, expressing solely the *gfp* gene under the J23109 promoter, lacking both the *dCas9* cassette and the *sgpTet* cassette, while we were trying to clone the pTZ-AegpTet, pushed us to try to investigate the co-infection theory since we also had another phagemid that could be easily visualized thanks to the color of the colonies, the pTZ-I13521.

To evaluate this possibility, we performed phage production using the helper phage method, starting with F' *E. coli* carrying the pTZ-GFP, to yield phage particles containing the desired phagemid. We set up a transduction assay by modifying the protocol slightly. Since we had two phage production that needed to be employed, we decided to add them in a 1:1 ratio, so 50 μ L of phage particles containing pTZ-I13521 were added to 50 μ L of phage particles containing pTZ-GFP and 100 μ L of F' *E. coli*.

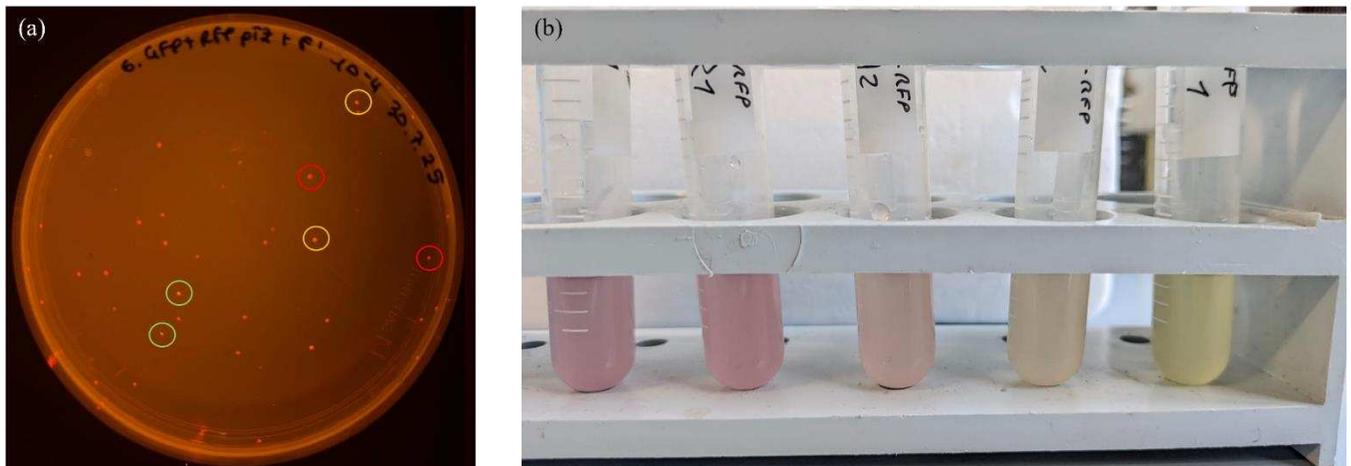


Figure 9 **transduction assay with two phage productions:** pTZ-I13521 and pTZ-GFP were used to perform two different phage production and then mixed in a single transduction assay on *E. coli*, plated on LB amp (a), red circles represent colonies that at the transilluminator appeared red, green circles appeared green, and orange circles were shades of colors in between the two. (b) liquid cultures of the isolated colonies from the transduction assay plate representing the variety of shades.

When plated on LB with added ampicillin (figure 9a), we obtained a mixed culture in which colonies with different shades could be visualized; these encompass both green and red colonies, but we could also spot colonies that were of shades in between the two. This phenomenon, together with the display of different shades we could see in liquid media as well (figure 9b), could be an indication of possible co-infection or the variability of the expression of each phagemid.

The last experiment we performed should not be taken as final or complete results, as many variables could interfere with the analysis. To be evaluated, some changes should be made to the circuits employed. First, both phagemids carry the same *blaR* resistance gene for ampicillin. To achieve a more accurate differentiation and confirm the presence of both phagemids, a different resistance gene should be introduced in one or the other. Additionally, both phagemids contain the pMB1 origin of replication, so even if they were present simultaneously, it is unclear whether they could both be expressed simultaneously or if one would prevail over the other. The origin of replication of either one should be swapped for a compatible one.

In conclusion, this last step of the work should be viewed exclusively as a starting point for a future in-depth analysis on the matter.

DISCUSSION

The issue of antimicrobial resistance, as we elucidated, is becoming a more tangible threat each year. With this work, we aim to address the emerging problem and explore new potential strategies to mitigate the possibility of a world where antibiotics are no longer a valuable resource. New antibiotic production is a process that takes years, is costly, and more importantly, it can't keep up with the emergence of new antimicrobial resistant strains. The use of phage particles as a delivery shuttle for a CRISPRi system that resensitizes antibiotic-resistant bacteria to already established antibiotics would help mitigate the issue and provide more time for new antibiotic research.

In this work, we effectively achieved and partially characterized the delivery of a phagemid circuit into *E. coli* carrying the F' episome by testing the efficiency and the specificity of the phage targeting. To achieve a comprehensive understanding of the system, it would also be beneficial to develop a protocol for effectively measuring and standardizing the quantity of phage particles in a phage production. In the case of lytic bacteriophages, this is achieved through the Plaque Assay, in which diluted aliquots of bacteriophages are mixed with host bacterial cells in soft agar, which is then distributed on an agar plate. A lawn of bacteria will grow, and plaques (zones of cleared bacterial growth) will form in it. By counting the number of plaques and relating it to the respective dilution, the number of plaques forming units per milliliter can be computed.²² In the case of the Bacteriophage M13, since it does not lead to cell lysis, this assay cannot be performed. The first set of experiments we conducted involved delivering a circuit carrying a reporter gene. Although this approach could be used to assess the efficiency of phage particle production, it remains an indirect measurement, as we are exclusively counting the phages that have effectively infected a host bacterial cell. A way to perform a direct measurement of the viable phage particles produced could be by treating the phage production with a DNase, to exclude the possibility of free DNA presence, and then extract the phage genome to perform a real time PCR to establish the quantity of genome present, that related to the data obtained by the transduction assay could give a fairly precise measurement.

After establishing the protocol for the phage production and testing it through the characterization, we moved on to the delivery of a CRISPRi system to target two AMR genes, *bla_{ndm1}* and *mcr-1*, in an attempt to re-sensitize resistant strains to the specific antibiotics, respectively, Meropenem and Colistin. We chose to exploit a CRISPR array system, composed of a tracrRNA and a crRNA, that, after transcription, requires a maturation step before linking with dCas9 to achieve transcription interference of the target gene. Three arrays were constructed, one targeting exclusively the *mcr1* CDS, one targeting the *bla_{ndm1}* CDS, and one that contained both crRNAs to have a single construct that could be used in the treatment of both resistance genes. In this work, we showed that the system consisting of the CRISPR array targeting both resistance genes was significantly less effective than the one with the single crRNA. This could be explained by the fact that the transduced dCas9 protein was insufficient to form a crRNA:dCas9 complex in sufficient quantities to effectively repress target gene transcription. A way to overcome this issue could be by redesigning the construct with a more potent promoter for the *dCas9* gene. This should be tested to determine if higher dCas9 expression could lead to cell toxicity due to the potential presence of free dCas9 protein. Another strategy to work around the decreased efficiency of the system with both crRNAs could be the use of two different inducible promoters for each crRNA, yielding a circuit that is modulable depending on the specifics of the treatment needed. Regarding the circuits containing the two single crRNAs, we achieved an efficient repression of the target AMR gene, with a more substantial effect in regard to the *bla_{ndm1}* gene compared to the *mcr1*. By employing the system targeting the Meropenem resistance gene, we were able to achieve a growth delay > 16 h for concentrations of Meropenem > 100µg/mL, while at the 10µg/mL concentration, a 6-fold increase in growth delay was obtained when compared to the resistant strain. In the treatment for colistin resistance, a 7-fold increase in growth delay was achieved at a 4µg/mL Colistin concentration, and a 3-fold increase was observed at 2µg/mL.

An interesting perspective for the future of this line of research would be to move on to clinical isolates and test the efficiency of the system to understand the possibility of adding Bacteriophages to our arsenal used to fight antimicrobial resistance.

Overall, the system was effective in repressing the transcription of the two AMR genes tested and in re-sensitizing the resistant bacteria to the antibiotic effect, but only at higher concentrations. The system can still be refined to improve its performance and increase sensitivity to antibiotic treatment, even at lower concentrations. A way to achieve this would be to adjust the various components, such as promoters, ribosomal binding sites, and the origin of replication, so that the circuit can be finely tuned to accomplish our goals. Thanks to the synthetic biology approach, this can be fulfilled through the DBTL cycle.

APPENDIX

Before starting to test the CRISPRi system, a concern arose in our minds. Since Meropenem is part of the β -lactams, we thought that the *blaR* gene present in the phagemid we use, which is necessary for plasmid retention, could potentially interfere with the test and yield artificial results that do not accurately represent the test's reality. To verify the likelihood of this hypothesis, we decided to do a growth inhibition assay comparing the growth of: F' *E. coli* carrying pTZ-I13521, F' *E. coli* carrying pSB3K3-ndm1, and TOP10 F' *E. coli* wild type.

For each condition we tested six concentrations of Meropenem: 500 μ g/mL, 200 μ g/mL, 100 μ g/mL, 10 μ g/mL, 1 μ g/mL, 0 μ g/mL.

For the sensitive strain, we assessed a MIC > 1 μ g/mL; meanwhile, for the pTZ-I13521, the MIC was > 10 μ g/mL. This result could be related to the presence of the *blaR* gene in the phagemid we used. This result did not convince us to change our resistance to the phagemid, as we had already constructed it, and the process would require a considerable amount of time. Therefore, we decided to proceed with the experiment, keeping this result in mind when analyzing the data from the CRISPRi system.

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