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Bacteriophages as Antimicrobial Agents and Synthetic Biology Scaffolds to Tackle Antimicrobial Resistance

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List of Abbreviations

≈	Approximately
Amp	Ampicillin
<i>ampR</i>	ampicillin resistance gene
AMR	Antimicrobial resistance
aTc	anhydrotetracycline
AUC	Area under the curve
<i>bla_{ndm-1}</i>	New Delhi metallo-β-lactamase-1 gene
<i>blaR</i>	β-lactam antibiotic resistance
bp	base pair
CC medium	Ceceal content media
CDS	Coding DNA sequence
CFU	colony forming unit
Cm	Cloramphenicol
<i>cmR</i>	chloramphenicol resistance gene
CRISPR	clustered regularly interspaced short palindromic repeats
CRISPRi	clustered regularly interspaced short palindromic repeats interference
crRNA	CRISPR RNA
Ct	cycle threshold
<i>dCas9</i>	dead Cas9
DSBs	double-stranded DNA breaks
e.g.	exempli gratia (for example)
EARS-Net	European Antimicrobial Resistance Surveillance Network
EPC	<i>European Pharmacopoeia Commission</i>
ESKAPE	<i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , <i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> , and <i>Enterobacter spp.</i>
<i>gfp</i>	green fluorescent protein gene
GFP	green fluorescent protein
GI tract	gastrointestinal tract
GTAs	phage-like gene transfer agents
HDR	homology-directed repair
HSL	N-3-oxohexanoyl-L-homoserine
i.e.	id est (in other words)
IPTG	isopropyl-β-D-1-thiogalactopyranoside
Kan	Kanamycin
LANL	Los Alamos National Laboratory
LB BS	Lysogenic Broth media supplemented with 0,4% (w/v) of bile salt
LB G	Lysogenic Broth media supplemented with 10 mM glucose

ln	natural logarithm
<i>mcr-1</i>	mobilized colistin resistance-1 gene
MDR	Multidrug resistant
MIC	Minimum Inhibitory Concentrations
MOI	Multiplicity of infection
NHEJ	non-homologous end joining
OD592	optical density at 592 nm
OD600	optical density at 600 nm
<i>ompA</i>	outer membrane protein A gene
ORI or ori	origin of replication
PAM	protospacer adjacent motif
PFU	plaque forming unit
pre-crRNA	pre-CRISPR RNA
qPCR	quantitative real-time PCR
RBP	receptor-binding proteins
RBS	ribosome binding site
RF	replicative form
RFP	red fluorescent protein
<i>rfp</i>	red fluorescent protein gene
RT-PCR	reverse transcription real-time PCR
sgRNA	single guide RNA
TAE	Tris-Acetate-EDTA
tracrRNA	trans-activating crRNA
UTIs	Urinary tract infections
WHO	World Health Organization
wt	wild-type
Δ AUC	Delta area under the curve
Δ Ct	delta cycle threshold
Φ	Phage

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Abstract

Antimicrobial resistance (AMR) is a global health emergency, with the World Health Organization (WHO) identifying the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*; sometimes extended to ESKAPEE to include *Escherichia coli*) as priority threats. The urgent need for new antimicrobial strategies has motivated the exploration of phages and synthetic biology as alternative tools to combat resistant bacteria. In this work, two complementary approaches were pursued. First, lytic phages were studied as direct antimicrobial agents against carbapenem-resistant *K. pneumoniae*. Their activity was evaluated in gut-like environments, where resistance genes are often exchanged, and in urine, due to the clinical relevance of urinary tract infections. Both *in vitro* and *in vivo* assays were performed, and phage cocktails were tested for efficacy. In parallel, the molecular determinants of host specificity, such as phage co-receptors, were investigated as potential targets for engineering. Second, a non-lytic phage-based delivery system was developed to introduce *clustered regularly interspaced short palindromic repeats interference* (CRISPRi) into pathogenic bacteria. To achieve this, genetic parts including constitutive and inducible promoters were tested in *A. baumannii*. The CRISPRi system was characterized by first repressing a red fluorescent protein (RFP), and later targeting an outer membrane porin implicated in virulence and biofilm formation. Preliminary delivery assays were also performed in *E. coli* using engineered M13 particles, first to transfer a reporter cassette and then to silence resistance genes to colistin and meropenem. Together, these results demonstrate the feasibility of combining phage biology and synthetic biology to design novel antimicrobial strategies, highlighting both the therapeutic potential and the technical challenges of such approaches.

Introduction

Antimicrobial resistance is a global threat. It occurs when microorganisms develop mechanisms that render them resistant to antimicrobial drugs. This phenomenon can lead to infections with high morbidity and mortality^{1,2}. Indeed, in 2019 WHO declared AMR as one of the top global health threats facing humanity. In addition, AMR severely impacts the economy, due to longer hospital stays and the need for more treatments³.

Currently, worldwide 700,000 deaths per year are linked to infections caused by multidrug resistance (MDR) pathogens². According to *The Review on Antimicrobial resistance* these microbes, called *superbugs*, are predicted to cause 10 million deaths by 2050⁴.

Data presented by the European Antimicrobial Resistance Surveillance Network (EARS-Net) highlights that each year more than 670,000 AMR infections occur in the European Union/European Economic Area. EARS-Net estimates that in the mentioned area 33,000 deaths are a direct consequence of AMR infections. Moreover, it reports that the costs for the health care system are around 1.1 billion euros⁵.

AMR has a crucial role in human health and its outbreak is linked to improper antibiotic usage and overuse in human clinical practice. On the other hand, the phenomenon is strictly related to other fields such as agriculture and the food industry. In fact, antibiotics have been extensively used in animal husbandry and agriculture for growth promotion, feed efficiency improvement, in addition to the classical application for treating infectious diseases^{6,7}. This wide usage in animal farming, industry and agriculture has direct effects on the development of AMR in bacteria, with newly AMR strains increasing their probability of entering the food chain and thus reaching humans^{2,7}.

AMR Pathogen Breakout

Antibiotic resistance is a natural phenomenon that occurs when microorganisms are exposed to antibiotics, and it may be an evolutionary advantage^{6,8}.

As illustrated by *Holmes et al.*⁹ the resistance mechanism developed against β -lactam antimicrobial drugs occurred naturally. It seems that saprophytic organisms can produce enzyme degrading β -lactam drugs as a competitive matter⁹.

Bacteria exhibit distinct levels of AMR: intrinsic resistance derives from inherent properties of the bacterium, such as the intrinsic Gram-negative glycopeptide resistance due to their outer membrane architecture. As shown in different studies *Acinetobacter baumannii* and *Pseudomonas aeruginosa* present a remarkable drug resistance due to the expression of pumps on the outer membrane extruding antimicrobial compound^{2,10,11}. Moreover, *P. aeruginosa* carries *ampC*, a chromosomal gene, which codify an inducible β -lactamase which hydrolyses a large quantity of β -lactam antibiotics¹¹. Acquired resistance develops in bacteria that were previously sensitive to a drug due to either a mutation or the acquisition of new genetic material through horizontal gene transfer. As illustrated by *Barbe et. al* in *Acinetobacter spp*, an organism that has evolved rapidly accumulating, in some cases, more

than 20 genomic islands encoding antibiotic resistance determinants and virulence functions^{2,6,10,11}.

AMR Mechanism: Mutations and Horizontal Gene Transfer

Antimicrobial resistance begins with mutations in bacterial DNA that occur randomly during DNA replication. Specifically, spontaneous mutations are found to occur at a common rate of 10^{-10} – 10^{-9} per nucleotide per generation for many bacteria and they can result in different scenarios such as: production of enzymes degrading the drug; an inhibitor that bind to the target becoming unreachable to the compound; an up-regulation efflux pumps that leads to the loss of the antibiotic in the bacteria cell; a pathway loss involved into the uptake of the antimicrobial.

However, mutations can represent a high fitness cost to the cell and result in the loss of genes involved in AMR. On the other hand, if bacteria are constantly exposed to the compound, they need the “gained function” to survive^{2,6,9,12}.

In addition to bacterial genome mutations, new genetic material can be taken up through *horizontal gene transfer* which can occur through three “canonical” mechanisms: conjugation, transformation, and transduction.

- The first mechanism requires a physical contact between two bacteria – a sex pilus – through which genetic material passes from the donor to the recipient bacteria.
- Alternatively, naked DNA can be released by cells in the environment and taken up by others through transformation; this mechanism has been exploited and optimized by scientists in recent years, becoming, a chemically or electrically induced process routinely adopted in molecular biology laboratories to clone genetic material into host cells.
- In addition, transduction takes place when a bacteriophage accidentally picks up bacteria host DNA within its genetic material. Consequently, when bacteriophage infects other cells, it can transfer foreign bacterial DNA from a donor cell to a receiver offspring.
- Lastly, to the classical mechanism, foreign DNA can be taken up via membrane vesicles, tiny pilus-like structures called nanotubes or phage-like gene transfer agents (GTAs)^{2,6,9,13}.

The foreign DNA with resistance genes can be carried on:

- plasmids – extra-chromosomal circular genetic elements;
- transposons – genetic elements that contain a gene or a set of genes flanked by two complementary inverted repeats associated with a transposase that enables the genes to be excised and relocate in DNA;
- integrons – mobile genetic elements, containing a recombination site, a 59-base element, and a set of gene expression cassettes, associated with a recombinase enzyme that operates the DNA translocation from the source to the target^{2,6}.

Horizontal gene transfer is favoured in dense communities such as the human gut where genetic exchanges occur between commensals and pathogens. Another crucial hotspot for genetic exchanges is biofilm, which is constituted by multicellular communities held together by a self-produced extracellular matrix. Normally the microbial aggregate adheres to each other or/and to the surface and it includes varied species ^{2,14,15}.

Microorganisms capable of forming biofilms exhibit a remarkable resistance to drugs due to several factors. First, the biofilm itself is a drug barrier which can dramatically reduce antimicrobial permeability. Specifically, it could happen that the antibiotic penetrates the biofilm, but it cannot reach the inner part of the aggregate because of differences in biofilm structure and composition. Furthermore, the biofilm architecture and organization determine a gradient of nutrient diffusion, oxygen absorption, and waste dispersion. As a result, each bacterium develops a different growth rate and metabolism. Non-growing and slowly growing bacteria are less susceptible to antibiotics due to the inactivity of antibiotic targets, a phenomenon known as “drug indifference.” This heterogeneity leads to distinct levels of antimicrobial resistance ^{12,15,16}.

Bacteria Involved in AMR: the ESKAPE

In 2017 the WHO put the ESKAPE pathogens in a priority status list. The acronym stands for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*, which are the most relevant bacteria involved in AMR¹. The acronym is sometimes extended to ESKAPEE to include *Escherichia coli*¹⁷. This research activity is focused on the last four families.

Acinetobacter baumannii is a pleomorphic coccobacillus, Gram-negative, strictly aerobic, and nonmotile. It is recognized as a nosocomial pathogen responsible for bacteremia, pneumonia, meningitis, and urinary tract infections in immunocompromised patients. *A. baumannii* represents a relevant challenge due to its potentiality resistance acquisition, intrinsic resistance, and capability to survive harsh conditions such as desiccation, UV, detergents, and disinfectants ^{18–20}.

Klebsiella pneumoniae is gaining attention due to the rise in case infection numbers and the increasing strains resistance. The microbe is an encapsulated Gram-negative bacterium. It causes serious infections including respiratory tract, urinary tract, and bloodstream infections. Usually, these infections occur in hospitalized or immunocompromised patients^{19–21}.

Although not formally recognized as part of the ESKAPE group of pathogens, AMR *Escherichia coli* is identified as responsible for more than 3 million deaths by 2050 ^{4,22}. The microbe is the most prevalent facultative anaerobic species in the gastrointestinal tract of humans and animals, and is usually a commensal but occasionally is responsible for intestinal and extra intestinal disorders such as diarrhoea, colitis, urinary tract infections, bacteremia, as well as sepsis ^{19,23}.

Need for Innovative Interventions: Bacteriophages

AMR is recognized as a global emergency, and in recent years, the WHO has called for prompt preventive and innovative therapeutic interventions to combat this growing phenomenon. At the beginning of the twentieth century, the introduction of antibiotics marked a revolutionary breakthrough. Phage therapy has been studied since 1919, with the Eliava Institute in Georgia playing a leading role²⁴.

However, the effectiveness of currently available antibiotic molecules has drastically decreased due to the emergence of AMR and MDR infections. To address this need, one solution is the use of bacteriophages in modern medical practice.

Bacteriophages are viruses that infect bacteria, obligate parasites that require the cellular machinery of the host bacteria to reproduce. Generally, infections begin with the specific binding of the viral co-receptor to the receptor on the bacterial cell surface. Subsequently, the bacteriophage releases its genome into the host cell. The virus replicates and expresses its genes using the cellular machinery. This is followed by the packaging of the genome (RNA or DNA) within a protein capsid and the release of the phage progeny extracellularly through various strategies^{25,26}.

Moreover, bacteriophages are species-specific, a characteristic that prevents phage interference with the human microbiota. However, this can also be a limitation, as a single phage preparation is not necessarily effective against bacteria of the same species. Additionally, bacteriophages are used in cocktails and in combination with antimicrobials to counteract host protection mechanisms and prevent the development of resistance²⁷.

A study published by *Cesta N et al.*²⁷ presents the case of an Italian patient with a *Pseudomonas aeruginosa* infection associated with a hip prosthesis. The authors demonstrate both *in vitro* and in a "real-life" case the efficacy of phage therapy as an adjuvant to antibiotic treatments to eradicate the AMR infection²⁸. Another case in the United States involved a 68-year-old patient with necrotizing pancreatitis complicated by multidrug-resistant *Acinetobacter baumannii* infection, which was resolved following the intravenous administration of a personalized phage cocktail in combination with antibiotic therapy and the patient recovered after a few months²⁹. Furthermore, the work produced by *Onsea J. et al.* lays the groundwork for the regulation, use, and control of phages in medical practice. In the study, a multidisciplinary group proposed an approach based on phage therapy to treat patients with severe infections and obtained hospital authorization to administer the personalized treatment³⁰.

These examples demonstrate the potential of phage therapy in conjunction with antibiotic administration to combat antibiotic-resistant infections.

Despite these successes, limitations still exist. In particular, phage therapy has shown promising results in non-randomized trials and in the aforementioned case studies; however, recent randomized controlled trials have had disappointing results, likely due to issues with regulatory, production, and logistical challenges²⁴. *Verbeken G.* and *Pirnay J* suggest the

development of phage-based medicinal products outside the traditional centralized marketing authorization pathway and propose an alternative *magistral preparation* pathway. In the context of phage therapy, a *magistral preparation* refers to a phage-based medicinal product prepared by a pharmacist using phage active pharmaceutical ingredients that follow their proposed *monograph*. This approach enables the production of personalized phage preparations, thereby avoiding the time and money wasted on pre-defined phage cocktails³¹. Regarding this, the *European Pharmacopoeia Commission* (EPC) adopted in March 2024 "Phage Therapy Medicinal Products (5.31)", which is a general chapter that provides a framework of requirements for the production and control of phage therapy products³².

However, clinical usage of phages is connected to several issues: the development of resistance mechanisms, such as CRISPR–Cas adaptive immune systems and undesired immune reaction¹¹. Phage usage leads to other limitations: difficulties penetrating barriers, e.g., biofilms, limited knowledge of phages except the ones infecting *Escherichia coli*. For this reason, broader research efforts across multiple scientific fields are needed to better understand phage biology and to develop effective solutions to these limitations.

Overall Aim

This thesis aims to explore the potential of bacteriophages as tools to combat bacterial infections caused by antimicrobial-resistant pathogens. The main goal is to exploit the natural ability of bacteriophages to selectively infect specific bacterial hosts and combine it with the advanced approaches offered by synthetic biology.

The work is divided into two main chapters. The first chapter summarizes research carried out during an international experience at the University of Chicago (Illinois, United States) in the laboratory of Professor Mark Mimee under the supervision of Dr. Ella Rotman. In this part, we investigated the ability of lytic bacteriophages to target and kill carbapenem-resistant *Klebsiella pneumoniae* strains in gut-like environments. Because the gastrointestinal tract is a critical niche where bacteria can exchange resistance genes, the use of bacteriophages represents a promising strategy to selectively eliminate resistant pathogens from the microbiome. To this end, both *in vitro* and *in vivo* experiments were performed to mimic the gut environment. Furthermore, given that *K. pneumoniae* is also capable of colonizing the urinary tract and causing AMR infections, phage cocktails were evaluated in urine media to assess their therapeutic potential. Finally, this section explores how bacteriophage host specificity is determined by receptor-binding proteins, and whether these determinants can be engineered or swapped to expand host range.

The second chapter focuses on a complementary synthetic biology-based strategy to tackle ESKAPE pathogens. Specifically, we developed a system that employs non-lytic bacteriophages as delivery platforms to introduce a CRISPR interference (CRISPRi) circuit into target bacteria. To establish this platform, a series of genetic components (*i.e.*, plasmids as well as constitutive and inducible promoters) was tested in *Acinetobacter baumannii*. CRISPRi functionality was then characterized in *A. baumannii* through two steps: first, by silencing a reporter gene (*rfp*), and second, by interfering with the expression of an outer membrane protein involved in virulence and biofilm formation. Finally, a preliminary assessment of the delivery system was carried out in *E. coli*. This included delivery of a reporter gene to validate phage-based transfer, followed by the delivery of CRISPRi constructs targeting genes responsible for resistance to colistin and meropenem.

Together, these two approaches combine classical bacteriophage biology with modern synthetic biology, aiming to develop novel therapeutic strategies that can restore antibiotic sensitivity and provide targeted weapons against multidrug-resistant pathogens.

1. Chapter: Bacteriophages for Direct Killing

Klebsiella pneumoniae is a Gram-negative bacterium and a member of the *Enterobacteriaceae* family. It is considered an opportunistic pathogen with a high degree of adaptability, shaped by its ability to acquire new genes, adapt to different hosts and environments, and evade or resist host immune defences and antimicrobial agents. This bacterium is a common cause of hospital-acquired infections, particularly affecting immunocompromised patients. It is also responsible for the illnesses mentioned in Bacteria Involved in AMR: the ESKAPE section'. Colonization of mucosal surfaces, such as the gastrointestinal (GI) tract, can occur following person-to-person transmission in hospital settings or through contact with contaminated surfaces and medical instruments. The transition from colonization to active infection by *K. pneumoniae* remains incompletely understood. Over the past few decades, *Klebsiella pneumoniae* has emerged as one of several bacterial species exhibiting a significant rise in antibiotic resistance. A variety of resistance mechanisms have been identified, with β -lactam resistance posing the greatest obstacle to effective treatment. This growing resistance is particularly concerning in hospital settings, where colonization with antibiotic-resistant *K. pneumoniae* has been linked to an increased risk of subsequent infections. Despite this correlation, the precise biological and clinical factors that drive the progression from colonization to active infection remain poorly understood. The gastrointestinal tract represents a key niche for a wide range of bacteria, serving as both a reservoir for antibiotic resistance genes and a site that facilitates their dissemination. In this context, colonization of the GI tract by *K. pneumoniae* may significantly contribute to the persistence and spread of resistance within healthcare environments^{33,34}.

Bacteriophage therapy represents an interesting approach not only for killing bacteria involved in antimicrobial-resistant infections but also for shaping the microbiota and eliminating bacteria considered harmful to human health. For instance, BiomX conducted a successful Phase 1 clinical trial using oral administration of the bacteriophage BX002, which targets *Klebsiella pneumoniae* associated with outbreaks of inflammatory bowel disease³⁵. Additionally, ELIGO Bioscience presented the development of the candidate EB003, a phage equipped with CRISPR-Cas9 designed to target and eliminate Shiga toxin-producing *E. coli* in the gut to reduce Shiga toxin levels and prevent haemolytic uremic syndrome³⁶.

Rotman *et al.* present a *Klebsiella* Phage Bank, a collection of bacteriophages isolated from raw sewage at the Stickney Water Reclamation Plant in Chicago. They investigated the efficacy of these phages in killing a panel of *Klebsiella pneumoniae* clinical and multi-drug-resistant isolates. Notably, among the multi-drug-resistant strains, *K. pneumoniae* JHCK1 and BAA2341 were the most susceptible to the phage collection. Furthermore, the authors tested a 5-phage cocktail composed of phylogenetically different phages predicted to depend on different host factors for infection. The authors demonstrated complete eradication of bacterial populations *in vitro* without the emergence of resistance. They then extended their study to a gut infection model, introducing *K. pneumoniae* into a defined murine gut community without causing a bloom of *Klebsiella* or significantly disturbing the microbial community structure. The phage cocktail successfully suppressed the *K. pneumoniae* burden in the mouse gut, although minor shifts in the overall microbiome

composition were observed. Importantly, the study highlights a discordance between *in vitro* characterization and *in vivo* outcomes. While phage cocktails can be optimized in controlled laboratory settings, their performance in the complex gut environment can vary substantially. For example, phages such as Φ ER15 and Φ ER46e demonstrated high virulence *in vitro* but were either undetectable or failed to be maintained at significant titres *in vivo*, suggesting that the gut environment is not permissive to their infectivity. In contrast, Φ ER39 maintained high titres in the gut and showed potential for co-evolution, indicating that phage efficacy and persistence can be shaped by host-associated conditions. These differences likely arise from factors such as the anaerobic environment, high bacterial density, and the competitive metabolic landscape of the gut, all of which differ dramatically from standard laboratory conditions. The study highlights the importance of understanding how phage-bacteria dynamics evolve in complex ecological contexts such as the gut microbiome³⁷.

Bacteriophages are known for their high specificity, meaning they recognize and bind to bacterial receptors with varying levels of selectivity. This specificity is advantageous because it allows phages to infect and kill only the target bacteria, without affecting non-target species such as beneficial members of the human microbiota. However, this high specificity can also be a limitation in clinical settings: when treating an infection caused by a particular bacterial strain, none of the phages in the available collection may be effective, as their host range is often defined at the species or even strain level. This necessitates the selection of different phages for personalized treatment. To overcome this limitation, wild-type phages can be genetically engineered to broaden their host range. For example, *Ando et al.*³⁸ engineered T7 phages by swapping in genes from a *Klebsiella* phage (K11), enabling the synthetic phage to infect *Klebsiella* instead of its original *E. coli* host. Conversely, *Klebsiella* phages were modified to infect *E. coli* by incorporating T7 tail components. These host-range transformations would not be possible with wild-type phages alone. Furthermore, *Yehl et al.*³⁹ developed a targeted engineering approach to expand and control the host range of bacteriophages. Instead of swapping out the entire tail fiber gene, they mutated specific, short regions of the phage tail fiber protein. This strategy generated a vast diversity of functional phages.

To carry out such precise genetic modifications, it is essential to know the exact gene sequences involved, particularly those encoding the tail fiber or other host-determining structures. These genes must be annotated to identify the specific regions responsible for host recognition. Without accurate annotation and sequence data, targeted mutations or gene swaps could disrupt essential phage functions or fail to produce the desired host-range changes.

The interaction between bacteriophages and bacteria begins when the phage receptor-binding proteins (RBPs), typically long tail fibers or shorter tailspikes, recognize and bind to specific receptors on the surface of the bacterial host cell. In some cases, RBPs also exhibit depolymerase activity, enabling them to degrade bacterial exopolysaccharides such as capsules and lipopolysaccharides, or components of the biofilm matrix. In the study by *Latka et al.*⁴⁰, the role of RBPs was investigated in detail, particularly focusing on the structure and genetic organization of *Ackermannviridae* phages. *Ackermannviridae* is a family of

bacteriophages characterized by distinctive structural features. While they share the long, contractile tails typical of classic *Myoviruses*, they differ at the end of the tail, which features "star-like" or "pronged" structures instead of the typical tail fibers. These specialized structures known as *tailspikes*, are essential for host adsorption and infection. Phages within the *Ackermannviridae* family present a conserved genome organization, particularly in the structural gene regions. However, they also encode multiple RBPs, many of which possess depolymerase activity. This multiplicity enables them to recognize and degrade various capsular polysaccharide types, thereby allowing for a broader host range and greater adaptability.

The genes responsible for encoding the tailspike proteins include genes 2, 3, 5, 7, and 8: protein 2 is a primary RBP, functioning as an anchor protein and plays a critical role in the initial attachment to the host; proteins 3, 5, 7, and 8 are additional tailspike proteins, each with a conserved N-terminal domain involved in phage attachment, a central depolymerase domain, a variable regions contributing to distinct host specificity. The presence or absence of certain RBPs varies among different phages within the group, allowing for modular assembly and functional diversity⁴⁰. This modularity is essential for targeting a wide array of bacterial hosts and is a key factor in the adaptability and therapeutic potential of these phages.

1.1 Aim

The primary aim of this section is to investigate the gut adaptability of bacteriophages that target *Klebsiella pneumoniae*. Specifically, we seek to understand how different phages behave when *in vitro* conditions (using LB medium) and the *in vivo* gut environment using monocolonized mice. Understanding these dynamics is essential to determine which phages are most effective and persistent in the GI tract, a critical step for developing effective phage-based therapies. Furthermore, since *Klebsiella pneumoniae* is a pathogen responsible for urinary infections specifically catheter-associated infections, the idea is to treat these resistant infections using phage therapy administered directly through the catheter. Therefore, we also aimed to assess the infectivity of the phages in urine.

To achieve this, we will employ a two experimental approach:

- Strategy 1: We will isolate and test individual phages representing different taxonomic or functional groups. These phages will be evaluated under a variety of *in vitro* conditions, including different media compositions and environmental parameters, to assess their growth dynamics. This will allow us to characterize how individual phages perform outside the host and provide a control for comparing the *in vivo* results.
- Strategy 2: In parallel, we will use the entire phage collection in both *in vivo* (mouse gut) and *in vitro* (LB media) enrichment settings. We will track the enrichment patterns of each phage population over time using high-throughput sequencing technologies. This strategy will allow us to compare the relative success of each phage in colonizing and persisting in the gut versus laboratory conditions, revealing selective pressures and host-environment interactions that may not be evident from individual phage testing.

Together, these strategies will provide insight into how *K. pneumoniae* phages interact in the gut environment and help identify phage candidates with optimal characteristics for therapeutic development.

Lastly, we will investigate the design and generation of chimeric bacteriophages to study the interactions between bacterial receptors and phage co-receptors, as well as explore potential approaches to broaden the host range. To achieve this, we will use homologous recombination to engineer chimeras of the Ackermann phage Φ ER12. Specifically, we will develop a three-plasmid system compatible with *K. pneumoniae* that carries homologous regions flanking the target genes involved in host receptor recognition and adsorption (genes 3, 5, and 7), which are predicted to encode tail fiber components. By selectively swapping these genes, we aim to identify which ones are necessary for host specificity and infection efficiency.

1.2 Material and Methods

In the following sections, the materials and methods employed in Chapter 1 are described in detail.

1.2.1 Bacterial Strains and Bacteriophage Collection

The experiments were conducted on clinical isolated *Klebsiella pneumoniae* JHCK1⁴¹, *K. pneumoniae* JHCK1 non-capsulated, the ATCC strain *K. pneumoniae* BAA2342⁴², *K. pneumoniae* MH258, *K. pneumoniae* UTI47, *K. pneumoniae* K6, *K. pneumoniae* iso-1 (KL106)³⁷. The strains were grown in Lysogeny Broth media (LB; BD Difco) or aerobically on plates (agar 15 g/L) (Fisher Scientific). According to specific experiment conditions, bacteria were grown in urine (Innovative Research) or caecal content obtained from gnotobiotic C57BL/6 mice prepared as follows: collected from mice and diluted with distilled water at a 1:5 weight-to-weight (w/w) ratio. Specifically, one part caecal content was mixed with five parts distilled water (e.g., 1 g caecal content + 5 g water), yielding a suspension containing 16.67% caecal material and 83.33% water. The mixture was thoroughly homogenized prior to use.

Low-salt plates contained: 10 g /L Bacto tryptone (BD), 5 g/L yeast extract (Gibco) and 5g/L NaCl (Acros Organics).

According to the experiment conditions, bacteria were grown in anaerobic conditions at 37°C, static or aerobic conditions at 37°C and 250 rpm shaking. Antibiotics and supplements were used as followed 100 mg/mL hygromycin (GoldBio); 10 mM glucose (Merck), 0.4 g/L bile salts (Millipore, Ox-bile 70168).

Lytic bacteriophages used in this work section originated from the Mimee Lab collection³⁷. They were isolated and amplified on LB 0.6% top agar plates poured over the LB agar plates. Serial dilutions were performed in 1X phosphate-buffered saline (PBS; Thermo Scientific).

1.2.2 Phage Isolation

New bacteriophages were isolated from filtered sewage obtained from the Stickney Water Reclamation Plant in Chicago, IL. A mixture of 300 µL of sewage and 300 µL of an overnight bacterial culture was added to 3 mL of LB top agar (0.6% w/v) and poured onto LB agar plates. The plates were incubated overnight at 37 °C. The following day, plaques resulting from phage infection were picked and subjected to three successive rounds of plaque purification on LB plates containing 0.6% (w/v) top agar to ensure clonal phage populations. The nucleic acids from the isolated bacteriophages were extracted and submitted for Illumina sequencing (Genewiz).

1.2.3 Phage Enrichment

Bacteriophages were titrated before use, and when the titre was less than 10⁹ PFU/mL, the phages were subjected to enrichment. Enrichment was performed using either a liquid or solid amplification method.

For liquid enrichment, the target overnight bacterial culture was diluted 1:100 and allowed to grow until it reached mid-log phase. Approximately 10–20 plaques were added to the culture, which was then incubated at 37 °C with shaking at 250 rpm for 3.5 hours. After incubation, the infected culture was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was filtered using a 0.20 µm filter. For solid enrichment, plaques were collected by scraping the top agar layer with an L-shaped spreader and transferring it to a 50 mL tube. To this, 4 mL of LB broth and 500 µL of chloroform were added. The mixture was vortexed for 10 seconds and incubated at room temperature for 10 minutes. The sample was then centrifuged at 10,000 rpm for 10 minutes, and the supernatant was filtered. The resulting enriched phage preparations were titrated by performing serial dilutions and spotting 2.5 µL of each dilution, in triplicate, onto LB agar plates overlaid with 0.6% top agar. The phage titre was determined by counting the resulting plaques and applying the appropriate calculation formula.

$$\text{CFU or PFU/mL} = \text{geometric mean} \times \frac{1,000}{(\text{spotted volume})} \times 10^{-(\text{dilution factor})}$$

1.2.4 Phenol Chloroform Extraction

Phage nucleic acids were extracted using a phenol:chloroform (PCI)-based method with enzymatic pre-treatment. 6.25 µL of 1M MgCl₂ (Merck) was added to a 1.5 mL microcentrifuge tube, followed by 500 µL of phage lysate. The mixture was gently mixed, and 0.5 µL of RNase A (100 mg/mL; Promega) and 0.5 µL of Turbo DNase I (Thermo Fisher) were added. After brief vortexing, the samples were incubated at 37 °C for 30 minutes. Next, the following reagents were added in the order listed: 20 µL of 0.5 M Ethylenediaminetetraacetic acid (EDTA; Gibco), 1.25 µL of Proteinase K (20 mg/mL; Promega), 25 µL of 10% Sodium dodecyl sulphate (SDS; Merck). The tubes were gently inverted and incubated at 55 °C for 60 minutes. An equal volume (~550 µL) of PCI (Merck) was added to each tube, and the samples were inverted several times to mix. Tubes were centrifuged at 13,000 rpm for 5 minutes at room temperature (RT). The lower organic phase and interphase were carefully discarded, 550 µL of chloroform (Merck) and centrifuged for 5 minutes at 13,000 rpm. DNA was precipitated by adding 1 mL of 95% ethanol (Merck) and 50 µL of 3 M sodium acetate (Merck), placed on ice for 5 minutes, and centrifuged at 13,000 rpm for 10 minutes at RT. Subsequently, the pellet was washed with 70% ethanol and centrifuged for 10 minutes at 13,000 rpm. The pellet was air-dried for 15 minutes. Finally, the DNA was resuspended in 50 µL of EB buffer (Qiagen). DNA was quantified with the Qubit Flex Fluorometer (Invitrogen) and the Quant-iT™ 1X dsDNA Assay Kits, high sensitivity (HS) and broad range assay (Invitrogen). The samples were stored at -20°C or submitted for Illumina sequencing (Genewiz).

1.2.5 Microplate Reader Assay

To characterize newly isolated bacteriophages and assess their infectivity under specific growth conditions, microplate reader assays were performed. The assays were conducted using the Sunrise™ microplate reader (Tecan) in kinetic mode. The kinetic cycle was set to 97 cycles, with a measurement interval of 10 minutes and a measurement time of 5 seconds per cycle. The total run time was 16 hours. Prior to each measurement, the plate was shaken for 5 seconds at high intensity, with no settling time. Between kinetic cycles, shaking was

performed for 540 seconds at normal intensity, also without settling time. A fresh bacterial culture, either *K. pneumoniae* JHCK1 or BAA2342, was inoculated in 3 mL of LB medium and incubated overnight at 37 °C, either under aerobic conditions with shaking at 220 rpm or under static conditions in an anaerobic chamber, depending on the experimental setup. Overnight bacterial cultures were diluted 1:100 and allowed to grow until they reached mid-log phase, corresponding to an approximate titre of $1-2 \times 10^8$ CFU/mL. The assays were performed in a clear, flat-bottom 96-well plate (Corning) by adding 180 µL of bacterial culture and 20 µL of phage suspension to achieve a multiplicity of infection (MOI) of 1.

The data obtained from the microplate reader assay were analysed using Microsoft Excel and plotted with GraphPad Prism. Heatmaps were generated by calculating the Area Under the Curve (AUC) in Prism, and the Delta Area Under the Curve (ΔAUC) was determined using the following formula:

$$\Delta AUC = AUC_{control\ strain} - AUC_{infected\ strain}$$

1.2.6 Sequence Analysis and Annotation

Bacteriophage genomes were assembled using the Velvet algorithm within Geneious Prime® 2024.0.7. Genome annotation was performed using Pharokka⁴³. Whole-genome alignments were performed using the Mauve algorithm, which is also implemented in Geneious Prime® 2024.0.7. Mapping to a reference genome or a reference genome from the phage collection was conducted using the Geneious Mapper tool within the same software platform.

1.2.7 In Vivo Experiments

The *in vivo* experiments were conducted on gnotobiotic C57BL/6 mice, which were housed in germ-free isolators and maintained on autoclaved food at the University of Chicago Gnotobiotic Research Animal Facility (GRAF). Faecal samples were collected prior to the start of the experiment to confirm the germ-free status of the animals. Mice were orally gavaged with 200 µL of *Klebsiella pneumoniae* strains JHCK1 or BAA2342 at a concentration of $1 - 4 \times 10^8$ CFU. Four days post-infection, faecal samples were collected to assess colonization by *K. pneumoniae*. On day 7 post-infection, additional faecal samples were collected, and mice were gavaged with 100 µL of sodium bicarbonate followed by 200 µL of the bacteriophage cocktail. One day after phage administration, mice were euthanized by CO₂ asphyxiation, followed by cervical dislocation as a secondary method to ensure death.

1.2.8 Mouse Faecal Collection

Faecal pellets were collected from individual mice, weighed, and recorded. For bacterial analysis, 1 mL of sterile PBS1X was added to each faecal sample, and the tubes were sealed and homogenized using a tissue homogenizer at 2,000 rpm for 2 minutes. Samples were then centrifuged at 300 g for 30 seconds.

For phage quantification, 0.6% LB top agar was melted in advance and maintained in a bead bath prior to faecal collection. Upon return, agar plates were overlaid with the appropriate bacterial host strain mixed with top agar (300 µL of the culture in 3 mL of top agar per plate). Following bacterial plating, the same faecal homogenates were centrifuged at 10,000 g for 10

minutes to separate bacteria. The supernatant for both phage and bacteria enumeration was serially diluted in PBS1X, and from each dilution in triplicate, 2.5 μ L was spotted onto agar plates. Plates were incubated at 37 °C overnight for CFU or PFU enumeration.

1.2.9 Cloning Procedure

Cloning was performed in 10-beta *E. coli* (New England Biolabs) using the Gibson Assembly method. The primers (Integrated DNA Technologies; listed *Table A-1*) used to amplify the desired DNA fragments were designed with two components: a 20–25 bp annealing region specific to the target sequence, and a 15 bp 5' overhang homologous to the ends of adjacent fragments. These overlapping regions enable assembly during the cloning process.

PCR amplification of the fragments for Gibson Assembly was performed using Q5® High-Fidelity DNA Polymerase (New England Biolabs) in a final reaction volume of 50 μ L, following the manufacturer's instructions. Thermal cycling conditions were as follows: an initial denaturation at 98 °C for 30 seconds, followed by 30 cycles of denaturation at 98 °C for 10 seconds, annealing at the recommended temperature (calculated using the New England Biolabs Tm calculator) for 20 seconds, and extension at 72 °C for 30 seconds per kilobase of target sequence. A final extension was performed at 72 °C for 2 minutes, followed by a hold at 10 °C. 5 μ L of each sample was run on a 1% agarose gel and visualized using a GelDoc imaging system (Bio-Rad). The remaining volume of the reaction was purified using the QIAquick PCR & Gel Cleanup Kit (Qiagen). The purified insert and vector fragments were mixed at an insert-to-vector molar ratio of either 5:1 or 10:1. A total of 10 μ L of Gibson enzyme mix containing T5 exonuclease, Phusion DNA polymerase, and Taq DNA ligase (all from New England Biolabs) was combined with 10 μ L of the DNA mixture. The final reaction was incubated at 50 °C for 1 hour and then transformed into 10-beta *E. coli* following the standard protocol mentioned *Klebsiella pneumoniae* Competent Cells and Electroporation Protocol section (Materials and Methods). The vector used for cloning was the *pZE4D-hyg-mC plasmid*. This plasmid carries a hygromycin resistance gene as a selection marker, functional in both *E. coli* and *Klebsiella pneumoniae*. It also includes the ColE1 origin of replication, which supports replication in both strains. Finally, the plasmid harbours the mCherry reporter gene. Plasmids purification was performed using the QIAprep Spin Miniprep Kit Plasmid Purification (Qiagen).

Briefly, three plasmid constructs (pZE4D-P3, pZE4D-P5, and pZE4D-P7) were generated to create chimeric phages using a two-step Gibson Assembly strategy as illustrate *Figure 1-1*. In the first step, the vector pZE4D-hyg-mC was amplified, excluding the mCherry cassette, while $\approx$ 800 bp regions flanking genes 3, 5, and 7 from ϕ ER12 were also amplified. For example, the pre-pZE4D-P3 construct consisted of pZE4D-hyg-mC lacking the mCherry cassette, with the cloning site replaced by the 800 bp upstream and downstream sequences of gene 3; analogous constructs were generated for genes 5 and 7. In the second step, the pre-pZE4D plasmids and the corresponding genes of interest were amplified: gene 3 from ϕ LilB, gene 5 from ϕ ER75, and gene 7 from ϕ LilB. These amplified genes were then cloned into the pre-pZE4D-P3, pre-pZE4D-P5, and pre-pZE4D-P7 backbones, respectively.

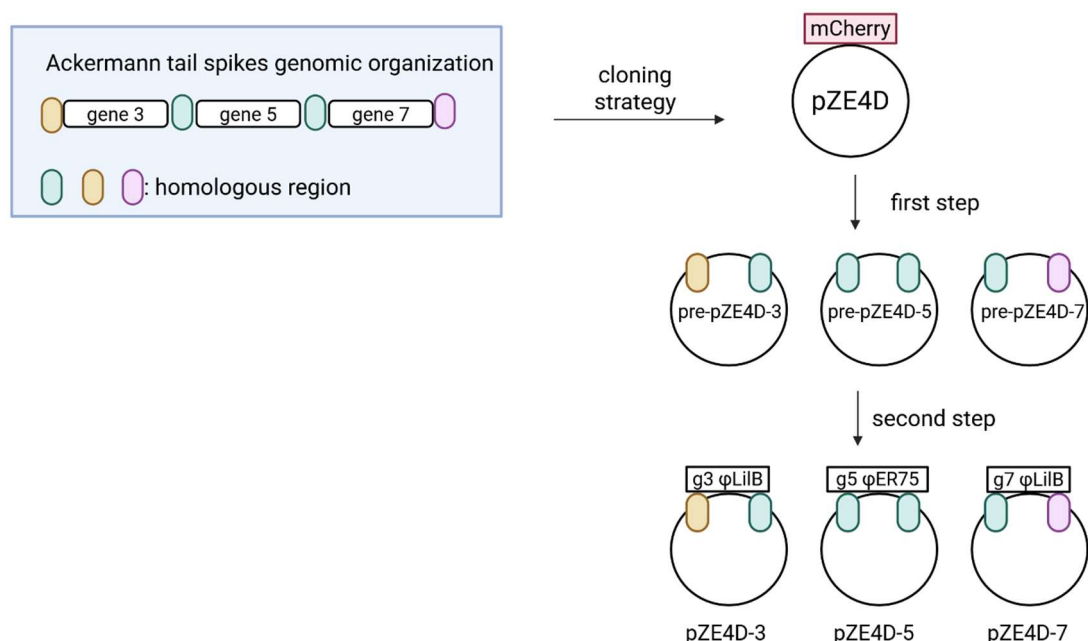


Figure 1-1: Cloning strategy generating pZE4D-3, pZE4D-5 and pZE4D-7.

1.2.10 Colony PCR

The obtained plasmids were screened by colony PCR using the following protocol: individual colonies were resuspended in 200 μ L of deionized water and incubated at 100 $^{\circ}$ C for 15 minutes to lyse the cells. The lysate was then centrifuged at 4,000 rpm for 10 minutes, and 2.5 μ L of the resulting supernatant was used as the DNA template for PCR. Reactions were carried out using GoTaq[®] DNA Polymerase (Promega) according to the manufacturer's instructions, in a final reaction volume of 10 μ L. Primers were bought from Integrated DNA Technologies and are listed in the *Table A-1*.

PCR amplification was performed under the following thermal cycling conditions: initial denaturation at 95 $^{\circ}$ C for 2 minutes (1 cycle), followed by 30 cycles of denaturation at 95 $^{\circ}$ C for 1 minute, annealing at the appropriate temperature (calculated using the Promega Tm calculator) for 1 minute, and extension at 72 $^{\circ}$ C for 1 minute per kilobase of target sequence. A final extension step was performed at 72 $^{\circ}$ C for 5 minutes (1 cycle), followed by an indefinite hold at 4 $^{\circ}$ C. The samples were run on a 1% agarose gel and visualized using a GelDoc imaging system (Bio-Rad). The positive plasmids were sent for whole plasmid sequencing to Plasmidsaurus Inc.

1.2.11 *Klebsiella pneumoniae* Competent Cells and Electroporation Protocol

A fresh *Klebsiella pneumoniae* colony was inoculated into 3 mL of LB medium and incubated overnight at 37 $^{\circ}$ C with shaking at 200 rpm. The following day, the overnight culture was diluted 1:100 into 10 mL of fresh LB medium and grown under the same conditions as described above. After 2 hours, the culture was placed on ice and centrifuged at 13,000 rpm

for 1.5 minutes. The resulting pellet was washed with 5 mL of cold sterile water and centrifuged again under the same conditions. The bacterial pellet was then resuspended in 500 μ L of cold 10% glycerol and centrifuged again. Almost all of the supernatant was discarded, leaving approximately 125 μ L to resuspend the final pellet. Aliquots of 50 μ L of competent cells were transferred into 1.5 mL tubes and used immediately or stored at -80°C .

The transformation of competent cells was carried out via electroporation. Approximately 100 ng (about 2.5 μ L) of the desired plasmid was added to the competent cells. The mixture was transferred into a pre-chilled 2 mm electroporation cuvette (Merck), gently flicked to avoid bubble formation, and electroporated using the Gene Pulser Xcell Total System (Bio-Rad) with the following parameters: 25 μ F, 200 Ω , 2500 V, and a 0.2 cm gap. Immediately after electroporation, 1 mL of LB medium was added to the cuvette, and the cells were incubated at 37°C with shaking at 250 rpm for 1 hour. Transformed cells were then plated on low-salt agar plates supplemented with hygromycin (100 μ g/mL; GoldBio) for selection and incubated at 37°C overnight. The transformed colonies were restreaked and used for the preparation of chimera phages.

1.2.12 Recombinant Phage Preparation

Recombinant phages were prepared using phage crosses based on homologous recombination^{44,45}. A fresh *Klebsiella pneumoniae* culture carrying the plasmid with the gene of interest (either tailer fiber *gene3*, *gene5*, or *gene7*) was inoculated in LB medium supplemented with hygromycin (100 μ g/mL; GoldBio) and incubated overnight at 37°C with shaking at 200 rpm. The overnight bacterial culture was then diluted 1:100 and allowed to grow for 2 hours and 30 minutes. Subsequently, 20 μ L of Φ ER12 phage was added to the culture, which was then incubated for 3 hours. After incubation, the infected bacterial culture was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was filtered. Bacterial lawns were prepared by mixing 3 mL of top agar with 300 μ L of the bacterial culture of interest and allowing the mixture to solidify for approximately 10 minutes. The phage preparation, containing both the wild-type and recombinant phages, was titrated by serial dilution in 1X PBS (Gibco) and spotted on bacterial lawns of either the wild-type target strain or the recombinant target strain. The plates were incubated overnight at 37°C . The following day, the appearance of clear plaques on the plate with the recombinant target strain indicated successful production of the recombinant phage.

Results

In the following sections, the results obtained in Chapter 1 are presented and discussed.

1.2.13 Isolation and Characterization of Three *K. pneumoniae* Bacteriophages

The bacteriophage collection was enriched by isolating and characterizing new bacteriophages from the *Klebsiella pneumoniae* host JHCK1. Bacteriophages isolated from raw sewage produced plaques with different morphologies, as shown in *Figure 1-2*. Three distinct plaques were selected and subjected to three rounds of restreaking to obtain purified, clonal phage populations.

The resulting bacteriophages were characterized by genome extraction and sent for sequencing. The sequencing data were assembled using Geneious Prime. To eliminate potential bacterial DNA contamination, the assembled reads were mapped to the JHCK1 genome. Taxonomic classification of the phages was performed using BLAST, and genome annotation was carried out with PharoKka⁴³.

The isolated bacteriophages SL1, SL2 and SL3 were identified as members of the *Drulisvirus* T7-like and *Slopekvirus* T4-like genera, respectively.

We evaluated the infectivity of these phages against the target strain using a dynamic growth assay in a plate reader (*Figure 1-3*). Since *K. pneumoniae* can express a polysaccharide capsule, we tested the phages on both the wild-type capsulated strain (JHCK1) and a non-capsulated mutant (JHCK1 NC).

As shown in *Figure 1-3* panel a, SL1 is unable to infect the non-capsulated JHCK1. In the capsulated JHCK1, the phage initially suppresses bacterial growth, but the population eventually recovers, possibly due to the emergence of phage-resistant variants; the SL2 (panel b) displays a similar trend, an initial period of stable growth followed by a drop in optical density at 600 nm (OD₆₀₀), then a slower recovery. This suggests a delayed phage effect and partial resistance. Finally, SL3 (panel c) shows an interesting behaviour, being able to infect both capsulated and non-capsulated strains, consistently reducing bacterial growth in both cases.

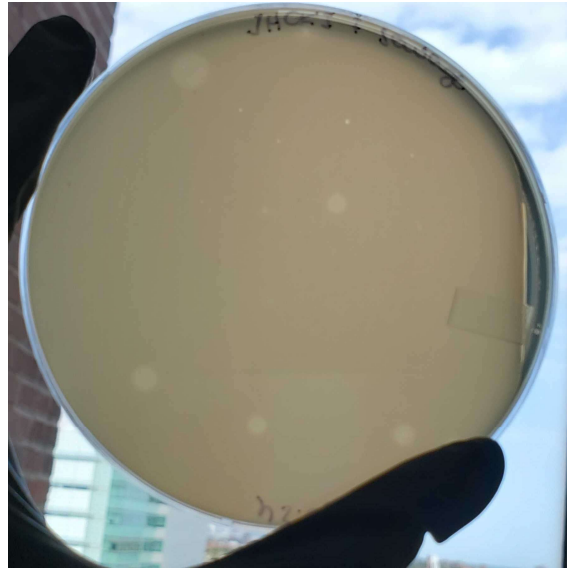


Figure 1-2: Plaques with different morphologies were produced by bacteriophages isolated from raw sewage on *Klebsiella pneumoniae* JHCK1.

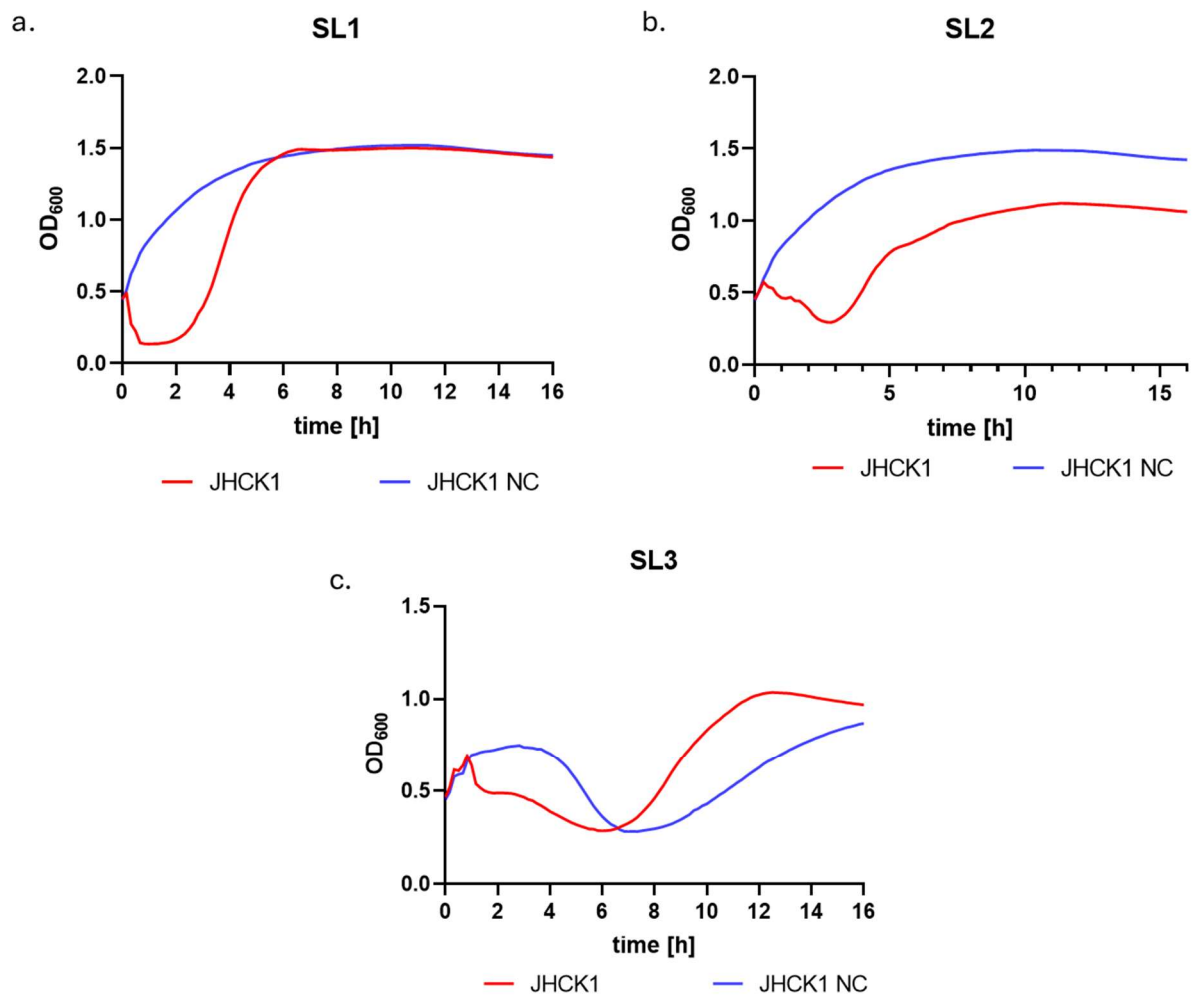


Figure 1-3: Infectivity of bacteriophages SL1 (a.), SL2 (b.), and SL3 (c.) against *Klebsiella pneumoniae*, evaluated using a microplate reader growth assay. Bacterial growth was monitored for the wild-type capsulated strain JHCK1 (blue curves) and the non-capsulated.

1.2.14 Phage Infectivity in Different Environmental Settings under Aerobic and Anaerobic Conditions against *K. pneumoniae*

These experiments focus on two specific *K. pneumoniae* strains of interest: JHCK1 and BAA2342, which are representative of clinically relevant lineages and, according to previous experiments, represent the two strains most susceptible to the bacteriophage collection. The tested phages include a diverse range of taxonomic groups, including T4-like (*Slopek*, *Jiaoda*, *Marfa*), T1-like (*Weber*), T5-like (*Demerec*), and others such as T7 (*Przondo*), as well as the Jumbo. The panel includes phages such as ER16e, ER15, MM3, MM1, ER43, Westerburg, Tostito, and others (Table 1-1), covering a broad phylogenetic spectrum and infectivity profiles across the two host strains.

Taxonomic Group	JHCK1	BAA2342
Slopek (T4)	ER16e, ER27, SL3	ER16e, ER27,SL3
Jiaoda (T4)	ER15, ER38	ER15, Er38
Marfa (T4)	MM3, MM14	MM3, MM14
Pseudo (T4)	-	-
Weber (T1)	MM23	-
Przondo (T7)	ER43	-
Demerec (T5)	ER46e, ER77	ER46e, ER77
Acker	Westerburg, Tostito	ER12, ER82
Purpura	MM1, MM19	MM1, ER39
Jumbo (Alcyon)	ER78	ER78
Drulis (T7)	SL1, SL2	SL1, SL2

Table 1-1: Tested bacteriophages including different taxonomic groups on *K. pneumoniae* JHCK1 and BAA2342.

Phages were tested in three different LB-based *in vitro* conditions designed to mimic various gut-relevant environments:

- LB plein (LB): baseline condition to assess standard phage activity.
- LB + 10 mM glucose(LB G): an media with a carbon source.
- LB + 0.4% (w/v) bile salts (LB BS): to simulate gut-like stress conditions, particularly those present in the small intestine.

Each condition was tested under both aerobic and anaerobic environments to better reflect the complex and oxygen-variable gut niche.

Phage infectivity was assessed by monitoring bacterial growth dynamics over time using OD₆₀₀ measurements. The effectiveness of each phage will be quantified by calculating the AUC of bacterial growth in the presence and absence of phages. A reduction in Δ AUC corresponds to poor infection and weak lytic activity, whereas larger Δ AUC values indicate high phage infectivity and strong lytic activity. This is because a reduction in the area under the curve reflects inhibited bacterial growth compared to physiological conditions, due to phage-induced lysis. Therefore, a high Δ AUC, calculated as the AUC of the control culture (bacteria without phage infection) minus the AUC of the phage-treated culture, represents a large difference between the two conditions, indicating effective lysis and strong bacteriophage killing activity.

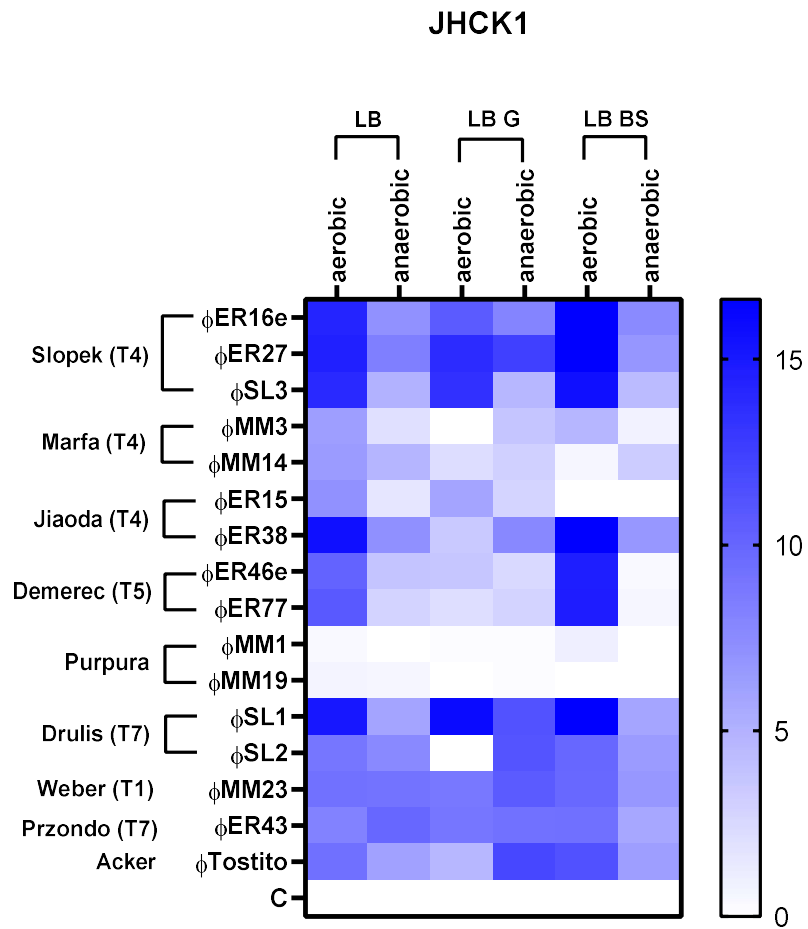


Figure 1-4: Heatmap of phage infectivity against *Klebsiella pneumoniae* JHCK1 across six growth conditions: LB, LB + glucose (LB G), and LB + bile salts (LB BS), each tested under aerobic and anaerobic conditions. Rows represent individual phages grouped by taxonomic classification, while columns represent the different culture conditions. Infectivity was quantified as bacterial growth reduction (ΔAUC), with darker blue indicating stronger lytic activity and white corresponding to no detectable infection.

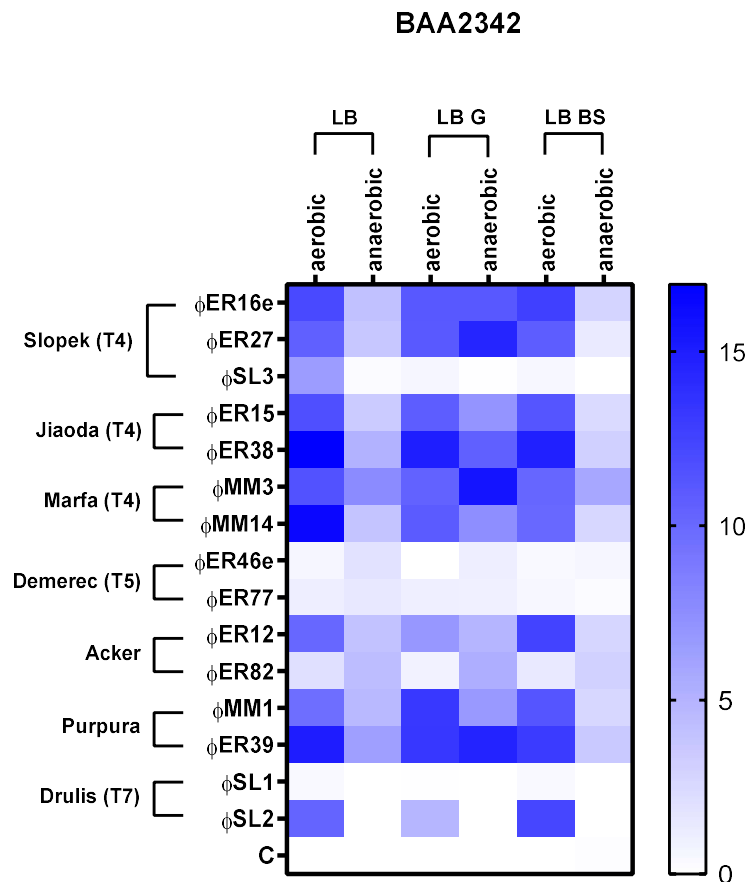


Figure 1-5: Heatmap of phage infectivity against *Klebsiella pneumoniae* BAA2342 across six growth conditions: LB, LB + glucose (LB G), and LB + bile salts (LB BS), each tested under aerobic and anaerobic conditions. Rows represent individual phages grouped by taxonomic classification, while columns represent the different culture conditions. Infectivity was quantified as bacterial growth reduction (ΔAUC), with darker blue indicating stronger lytic activity and white corresponding to no detectable infection.

The heatmaps (Figure 1-4; Figure 1-5) show phage activity quantified as bacterial growth reduction, with darker blue indicating stronger lytic effects. As the colour fades, infection becomes weaker, until white, which corresponds to no detectable infection.

Across both hosts, T4-like phages, specifically *Slopek* and *Jiaoda* (e.g., ΦER16, ΦER27, ΦER38), demonstrated broad and consistent lytic activity, particularly under aerobic conditions supplemented with bile salts, suggesting potential applicability in gut-relevant environments. Notably, ΦMM14 and ΦMM3 were consistently effective across all six tested conditions against strain BAA2342; by contrast, this effect was less pronounced in JHCK1. A general reduction in infectivity was observed under anaerobic conditions, indicating that oxygen availability may influence phage–host interactions or bacterial susceptibility.

ΦSL1, ΦSL2, and ΦSL3 exhibited more host-specific activity, being primarily effective against JHCK1 but largely inactive against BAA2342. This is consistent with their origin, as the SL phages were isolated using JHCK1 as the host. Their infectivity was also more sensitive to environmental conditions, with reduced activity in glucose-enriched and anaerobic media, possibly due to altered capsule expression or receptor accessibility.

Phages from the *Demerec* (T5-like) and *Ackermann* groups exhibited moderate to minimal lytic activity, indicating limited effectiveness under the tested conditions. Except for Φ Tostito (*Ackermannviridae*), which consistently showed high infectivity against the JHCK1 strain across all tested conditions. Interestingly, a few phages (e.g., Φ ER77, Φ ER12) displayed activity only under specific conditions, highlighting the influence of environmental context on phage performance. Overall, both target strains exhibited lower phage infectivity under anaerobic conditions, which is particularly relevant given that the gastrointestinal tract is predominantly anaerobic. The reduction in lytic activity suggests that phage performance may be compromised in gut-like environments, underlining the importance of screening phages under physiologically relevant conditions.

Even so, *Weber* (T1-like) and *Prozondo* (T7-like) phages displayed moderate but consistent infectivity against JHCK1 across all six tested conditions, suggesting they may be interesting candidates for *in vivo* evaluation. Conversely, in strain BAA2342, none of the tested phages exhibited a relevant infection profile, underscoring the need to isolate phages that specifically target this taxonomic group. Furthermore, phages from the *Purpura* group exhibited high infectivity against BAA2342 under aerobic conditions; however, their activity dropped markedly in anaerobic conditions, which warrants further investigation. Unfortunately, no *Purpura* phages have been isolated for the JHCK1 strain yet.

In both strains, the Jumbo Φ ER78 was unable to infect. However, it may represent an interesting candidate for genetic engineering, as Jumbo phages typically have genomes larger than 200 kb, compared to the ~40–60 kb genome size of most tailed bacteriophages. The large DNA content of Φ ER78 likely includes intergenic regions without coding sequences that could be exploited for the introduction of new genes.

1.2.15 *In Vivo* Bacteriophage Enrichment

In addition to individual phage testing, we assessed the behaviour of the entire phage collection, referred to as Garbage Soda, under both *in vivo* and *in vitro* conditions. While single-phage assays provide infectivity profiles, testing the full community allowed us to avoid missing phages that may only be active under specific conditions or in the presence of other phages.

Garbage Soda is a sequenced mixture of 106 phages belonging to different taxonomic groups of Φ *K. pneumoniae* and isolated from various *Klebsiella pneumoniae* strains, as reported by Rotman et al³⁷. The mixture was used here as a defined input population. For the *in vivo* experiment, the phage mixture was administered to monocolonized mice, which carried only a single *K. pneumoniae* strain. Caecal contents were collected post-treatment, and phage DNA was extracted to profile the output population shaped by the phage-bacteria interaction in this controlled environment.

In parallel, *in vitro* incubations were performed by culturing the same phage mix overnight (O/N, 37°C) with *K. pneumoniae* in either LB or caecal content (CC) medium. After incubation, phage DNA was extracted from the supernatant to determine the *in vitro* output.

By comparing input and output populations across both systems, this strategy enabled us to explore how individual phages persist or are selectively amplified depending on the environmental context and host interaction.

For the *in vivo* experiment, four mice were colonized with *K. pneumoniae* JHCK1 and treated with the Garbage Soda phage cocktail, while another four were colonized with *K. pneumoniae* BAA2342 and treated similarly. An additional three mice served as a control group and received only the Garbage Soda without prior bacterial colonization. To verify the gnotobiotic status of the mice and confirm successful colonization, faecal pellets were collected and bacterial loads were quantified using colony-forming units per gram (CFU/g) of faeces. Faecal samples were collected the day after *K. pneumoniae* colonization to assess bacterial colonization, and again the day after phage treatment to evaluate the cocktail's ability to reduce bacterial burden.

Sample name	Treatment	Day 7 CFU/g	Day 8 CFU/g
281	<i>K.pneumoniae</i> JHCK1+ Garbage Soda	1.08×10^{10}	1.61×10^7
282	<i>K.pneumoniae</i> JHCK1+ Garbage Soda	7.24×10^9	2.25×10^5
283	<i>K.pneumoniae</i> JHCK1+ Garbage Soda	1.86×10^{10}	1.07×10^7
284	<i>K.pneumoniae</i> JHCK1+ Garbage Soda	6.23×10^9	1.46×10^8
3060	<i>K.pneumoniae</i> BAA2342+ Garbage Soda	1.13×10^{10}	6.09×10^7
3061	<i>K.pneumoniae</i> BAA2342+ Garbage Soda	1.79×10^{10}	2.08×10^8
3062	<i>K.pneumoniae</i> BAA2342+ Garbage Soda	9.08×10^9	5.51×10^7
3063	<i>K.pneumoniae</i> BAA2342+ Garbage Soda	1.13×10^{10}	1.24×10^8
348	Garbage Soda	0	0
349	Garbage Soda	0	0
350	Garbage Soda	0	0

Table 1-2: Bacterial loads in mice colonized with *K. pneumoniae* JHCK1 or BAA2342 before (Day 7) and after (Day 8) treatment with the phage cocktail Garbage Soda. Phage treatment reduced bacterial counts in all colonized animals, while phage-only controls showed no detectable CFU.

As a preliminary step before analysing phage dynamics within the Garbage Soda mixture, we confirmed successful colonization of mice with *K. pneumoniae* strains JHCK1 and BAA2342, and evaluated whether phage treatment led to measurable reductions in bacterial burden. Mice were colonized for 7 days before receiving the phage cocktail, and faecal samples were

collected on Day 7 (pre-phage) and Day 8 (24 hours post-phage treatment) for CFU quantification.

All colonized animals exhibited high bacterial loads on day 7, ranging from approximately 6×10^9 to 1.9×10^{10} CFU/g, confirming stable colonization. By day 8, bacterial loads had decreased across all animals, indicating that the phage cocktail was active *in vivo*. Although complete clearance was not observed, as expected for this time point, the data confirmed that the phages reached the colonization site and were able to infect and kill the bacteria cells (

Table 1-2).

Importantly, this step was designed not to test therapeutic efficacy, but to verify that phage treatment was functional in each strain background. These data support the validity of the model and justify subsequent analysis of phage community composition in the output (caecal) samples. Control mice that received only the phage cocktail (no bacterial colonization) remained culture-negative, confirming the gnotobiotic status of the experimental system.

The mice were sacrificed one day after phage treatment, and caecal contents were collected and processed to purify the phage population. Phage suspensions were then plated on *K. pneumoniae* to assess their presence. Since the entire phage cocktail was administered as a mixture, the resulting data did not represent quantitative titres for individual phages but rather served as a qualitative readout confirming that viable phages were present and active in the gut environment. Based on these results, we proceeded with phage DNA extraction from the caecal content of treated mice. The sequencing data were then assembled using Geneious Prime for downstream analysis.

To assess changes in phage population structure following *in vivo* exposure, we compared the composition of the input phage community (Garbage Soda) with that of the phage DNA recovered from the caecal content of monocolonized mice (output). Sequencing reads were assembled and taxonomically classified, and the relative abundance of each phage genus was expressed as a percentage of normalized reads.

In mice colonized with *K. pneumoniae* JHCK1, the phage community composition recovered 24 hours after *Garbage Soda* gavage showed modest but clear shifts compared to the input cocktail (Figure 1-6). Most taxonomic groups present in the input remained detectable, indicating that the mixture was stable *in vivo*. However, specific groups, including *Tunavirinae* T1-like, *Slopekvirus* T4-like, *Drulisvirus* T7-like, and *Webervirus* T1-like, were enriched in the output, suggesting selective amplification of these phages in the gut. This pattern may reflect differences in host range, replication efficiency, or competition among phages, and highlights the ability of certain taxonomic groups to expand *in vivo*. Such selective enrichment is relevant for therapeutic application, as it demonstrates that some members of the cocktail can effectively replicate within the host environment, potentially driving bacterial suppression over time.

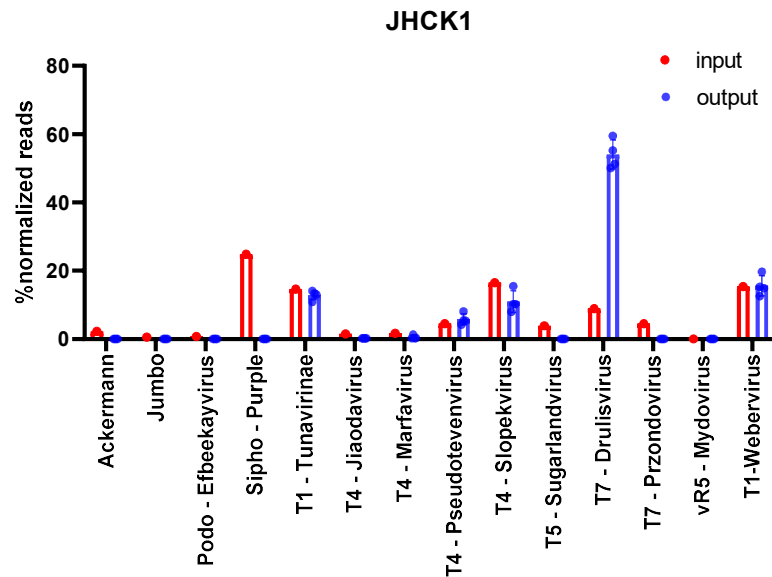


Figure 1-6: Phage community composition before (input, red) and after 24 h (output, blue) of Garbage Soda administration in mice colonized with *K. pneumoniae* JHCK1.

In contrast, in mice colonized with *K. pneumoniae* BAA2342, the phage profile revealed more pronounced changes (Figure 1-7). While most lineages present in the input were still detectable after 24 h, several groups, including *Slopevirus* T4-like, *Purpura*, *Pseudotevenvirus* T4-like, and *Tunavirinae* T1-like, were enriched in the output. These shifts indicate more effective replication or retention of specific phage groups in this host background, underscoring host-dependent differences in phage dynamics *in vivo*.

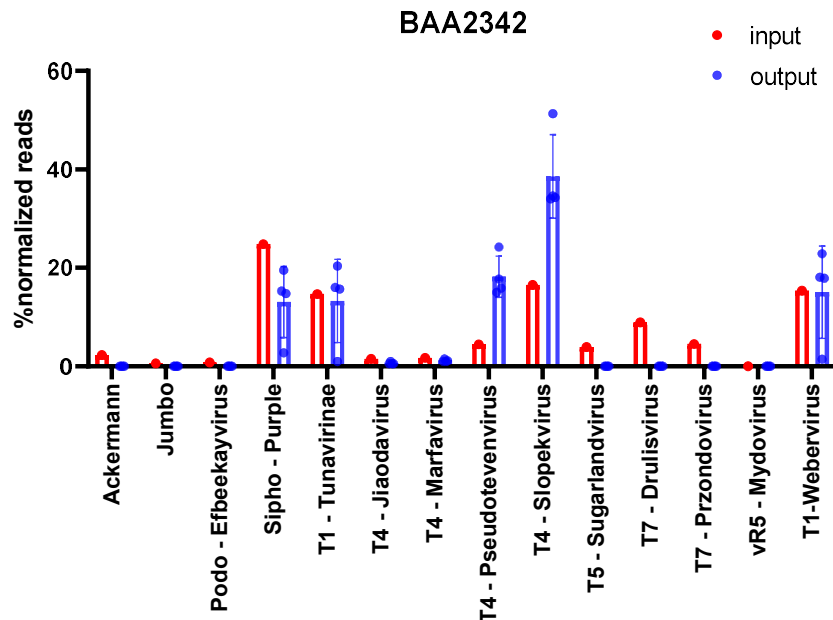


Figure 1-7: Phage community composition before (input, red) and after 24 h (output, blue) of Garbage Soda administration in mice colonized with *K. pneumoniae* BAA2342.

1.2.15.1 *In Vivo* versus *In Vitro* Bacteriophage Enrichment

To evaluate phage population dynamics under controlled laboratory conditions mimicking the gut environment, we compared the genera phage composition across three conditions: *in vitro* in LB, *in vitro* in caecal content medium, and *in vivo* for mice monocolonized with *K. pneumoniae* strains JHCK1 and BAA2342. Normalized sequencing reads were mapped to known phage taxa to quantify changes in composition.

In the JHCK1 group, both *in vivo* and *in vitro* conditions resulted in the enrichment of specific phage genera, particularly *Drulisvirus* (T7-like), *Webervirus* (T1-like), and *Sugarlandvirus* (T5-like, except *in vivo*), indicating that these phages efficiently replicated in this host background. In addition, *Slopekvirus* (T4-like) and *Tunavirinae* (T1-like) were moderately enriched *in vivo* but not *in vitro*, either in CC medium or LB medium (Figure 1-8).

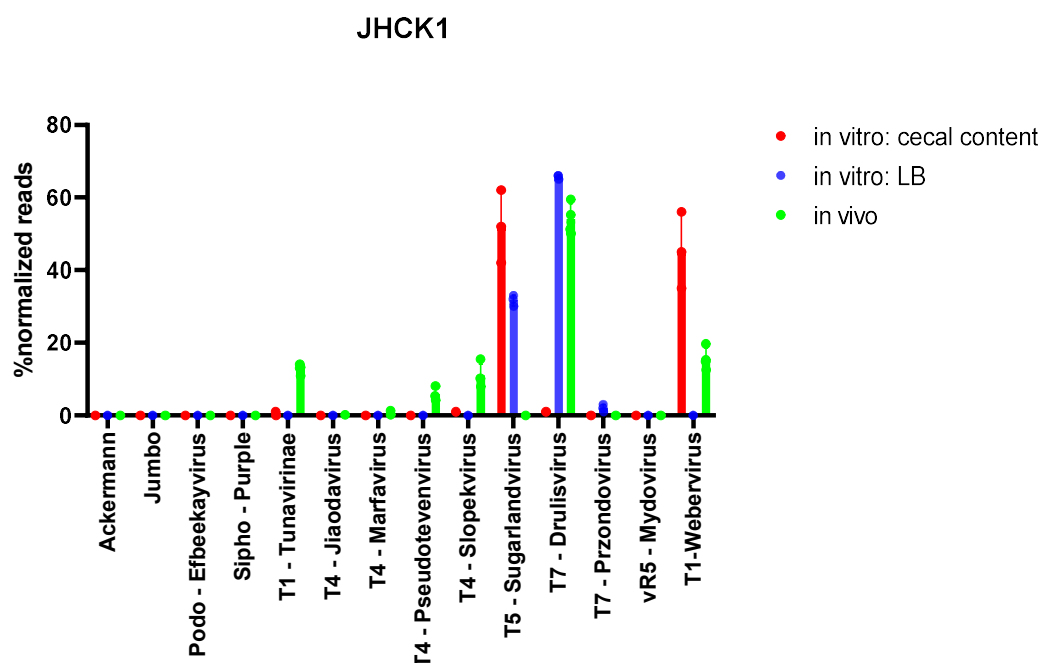


Figure 1-8: Phage composition in *K. pneumoniae* JHCK1 under *in vivo* (red), *in vitro* LB (blue), and *in vitro* CC medium (green) conditions after Garbage Soda exposure.

In contrast, the BAA2342 group showed clear differences between *in vitro* and *in vivo* conditions. While *Purpura* (Sipho-Purple) phages dominated in both *in vitro* media, they were less abundant in the *in vivo* output. Instead, the *in vivo* samples were enriched in *Slopekvirus* T4-like and *Webervirus* T1-like, indicating that these phages were better adapted to persist or replicate in the gut environment (Figure 1-9).

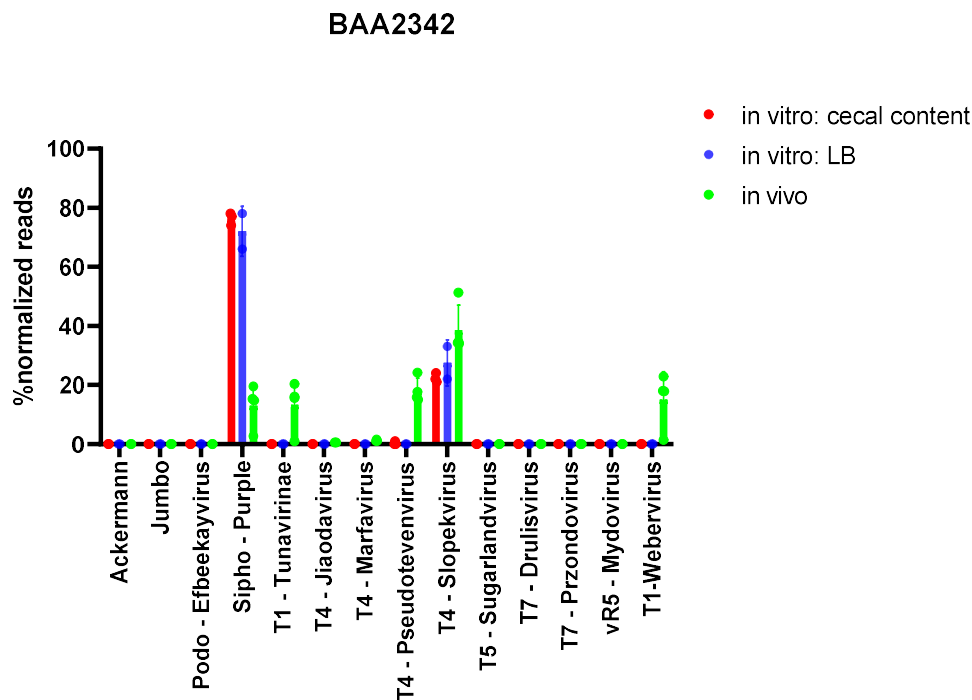


Figure 1-9: Phage community composition in *K. pneumoniae* BAA2342 under *in vivo* (red), *in vitro* LB (blue), and *in vitro* CC medium (green) conditions following exposure to the Garbage Soda cocktail.

These findings highlight the influence of both the bacterial host and environmental conditions on phage fitness, demonstrating that *in vitro* systems, although useful, may not always accurately predict phage behaviour *in vivo*, especially in more complex or physiologically relevant contexts.

1.2.16 Consideration on Bacteriophage-Bacteria Interaction in Urine under Aerobic and Anaerobic Conditions against *K. pneumoniae*

We performed experiments under both aerobic and anaerobic conditions to simulate the different environments that phages may encounter *in vivo*, particularly within the urinary tract. This approach was motivated by the potential therapeutic use of phages to treat urinary tract infections (UTIs), where oxygen availability can vary. Assessing phage infectivity in both conditions allowed us to evaluate their stability and effectiveness in a setting that more closely mimics clinical applications.

In these assays, the control bacteria (non-infected cultures) exhibited relatively slow growth in urine, most likely due to the nutrient limitations of this medium (Figure 1-10). Although urine was not supplemented in the present study, bacterial growth could potentially be enhanced in future experiments by adding small amounts of nutrient-rich medium (e.g., LB), carbon sources such as glucose, or amino acid or yeast extract supplements. Before investigating bacteriophage–bacteria interactions in this medium, it would therefore be advisable to optimize bacterial growth conditions.

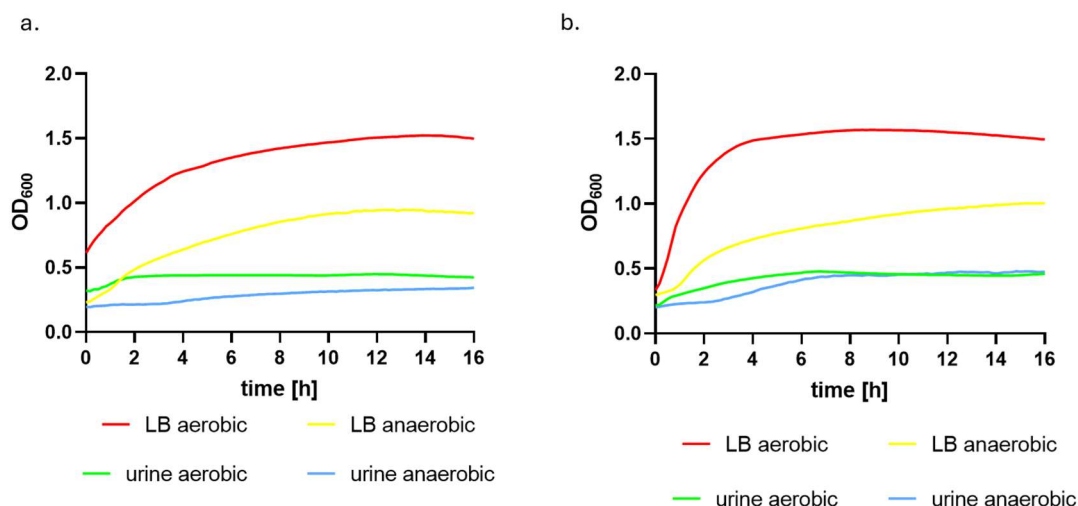


Figure 1-10: Growth inhibition of *Klebsiella pneumoniae* strains JHCK1 (a) and BAA2342 (b) in urine under aerobic and anaerobic conditions. Bacterial growth was monitored by OD₆₀₀.

1.2.17 Generating Ackermann Phage Chimeras

To construct phage variants with modified host specificity, we engineered Ackermann phage chimeras by targeting key structural genes involved in host recognition specifically gene 3, gene 5, and gene 7. These genes were identified within the phage genome as potential determinants of tail fiber tips which play a central role in defining host range⁴⁰.

Each gene of interest (GOI) was cloned into a plasmid and introduced into *K. pneumoniae* MH255 host cells. Each GOI was flanked by sequences homologous to the corresponding region in the phage genome, enabling homologous recombination upon phage infection (Figure 1-11; panel a.). Upon infection with wild-type Ackermann phage, homologous recombination between the plasmid-encoded gene and the native phage genome allowed for the generation of chimeric phages (Figure 1-11; panel b.). This directed swapping of specific tail fiber genes resulted in the generation of chimeric phages with modified host tropism, while preserving the rest of the phage genome (Figure 1-11; panel c). This strategy enabled the modular assembly of a library of phage chimeras, which could then be screened for altered infectivity profiles across different *Klebsiella* strains.

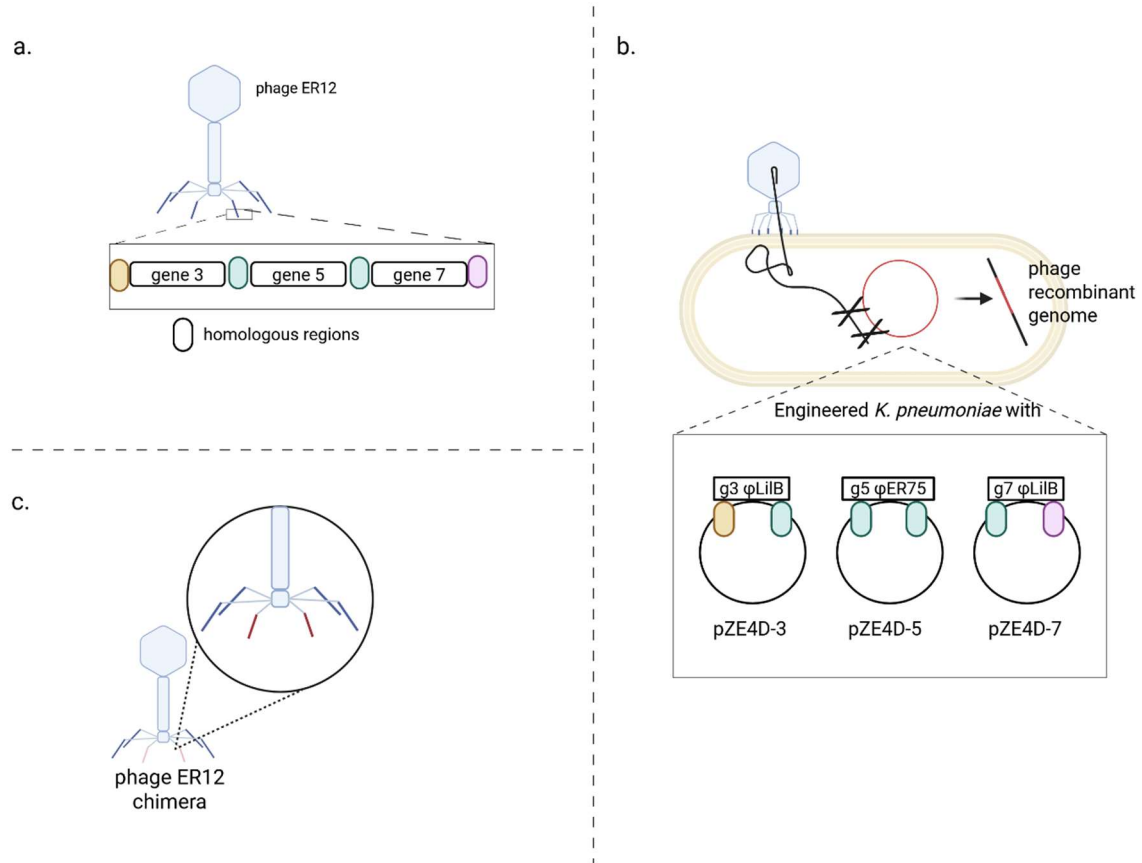


Figure 1-11: Strategy for generating Ackermann phage ER12 chimeras with modified host specificity. (a) Structural genes involved in host recognition (genes 3, 5, and 7) were identified in the phage genome. (b) Each gene of interest (gene 3 from Φ LilB, gene 5 from Φ ER75 and gene 7 from Φ LilB) was cloned into plasmids with homologous flanking regions. The three plasmids (pZE4D-3, pZE4D-5, and pZE4D-7) were individually transformed into *Klebsiella* cells, generating three engineered bacterial strains, each carrying a different plasmid. Upon infection with wild-type ER12, homologous recombination occurred between the plasmid-encoded gene and the phage genome. (c) This process yielded chimeric phages carrying swapped tail fiber genes, creating variants with altered host tropism.

The Ackermann phage Φ ER12, from the Mimee Lab collection, along with its host *K. pneumoniae* MH528, was used as the chassis for generating chimeric phages. We aimed to determine which host-recognition gene from the wild-type Φ ER75 was responsible for its ability to infect the *K. pneumoniae* strain UTI47, a strain not susceptible to Φ ER12. While Φ ER75 is known to infect UTI47, it was unclear whether this tropism was mediated by gene 3, 5, or 7.

To investigate this, we constructed three Φ ER12-derived chimeric phages by replacing gene 3, gene 5, or gene 7 with the corresponding sequences from Φ ER75. Each gene was introduced via plasmids containing homologous flanking regions to facilitate recombination during phage replication in MH528.

The resulting chimeric phages were tested on UTI47 bacterial lawns. Only the chimera carrying Φ ER75's gene 5 produced clear lysis plaques, indicating that gene 5 is necessary for Φ ER75's infectivity toward UTI47. Neither gene 3 nor gene 7 from Φ ER75 conferred infectivity when swapped into the Φ ER12.



Figure 1-12: Φ ER12::p5_ER75 identification of the host recognition determinant in ER75 via chimeric phage construction. On the left, the chimeric Φ ER12::p5 is able to infect the host *K. pneumoniae* UTI-47; On the right, the wild type phage Φ ER12 is unable to recognize and infect *K. pneumoniae* UTI-47.

As shown in the Figure 1-12 Φ ER12 chimeric phages carrying individual genes (gene 3, 5, or 7) from Φ ER75 were tested for their ability to infect *K. pneumoniae* strain UTI47. Lysis plaques were observed only for the Φ ER12 chimera containing gene 5 from Φ ER75, indicating that gene 5 is responsible for enabling infection of UTI47. On the right, the wild-type Φ ER12 phage was used as a negative control and, as expected, did not produce plaques on UTI47, confirming that Φ ER12 lacks the necessary host-recognition determinant without the gene 5 swap. Although plaque formation was observed for the Φ ER12::p5_ER75, the plaque density was relatively low, which is consistent with expectations for recombination-based chimera production. Since the wild-type phage remains the dominant genotype after infection, the recombinant chimeras represent only a minority population within the lysate.

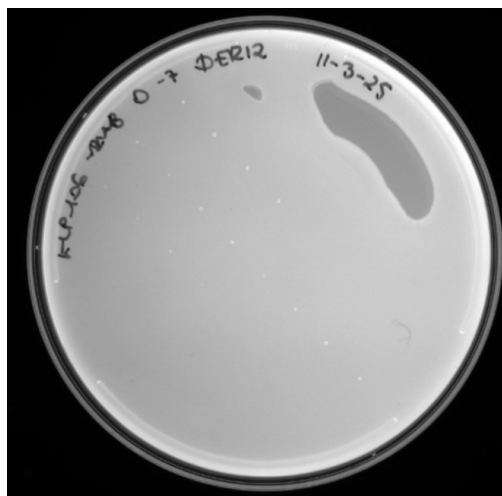


Figure 1-13: Negative control for host-range specificity on *K. pneumoniae* KLP106.

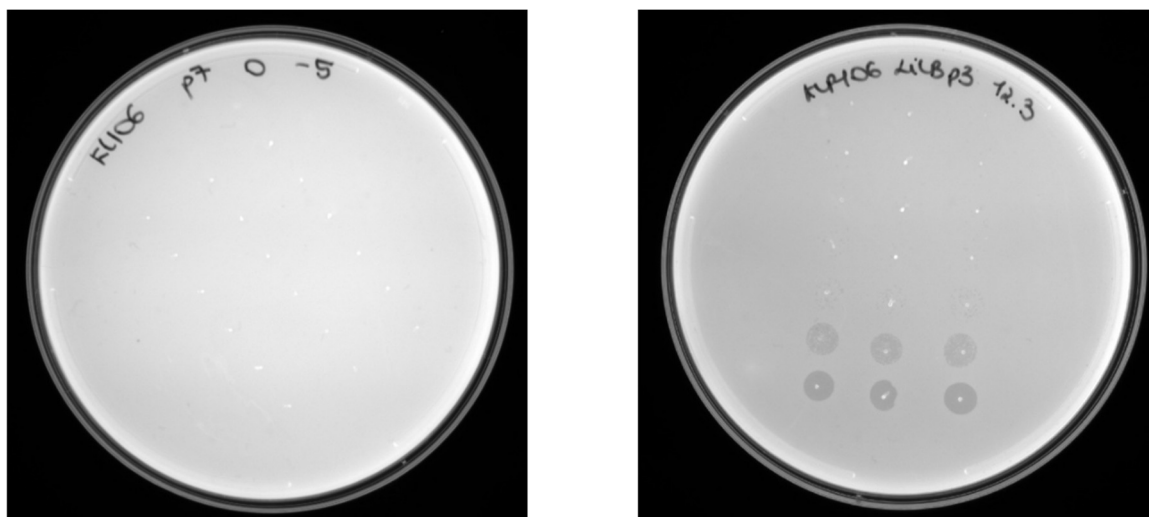


Figure 1-14: Identification of the host-recognition gene from Φ LilB phage enabling infection of *K. pneumoniae* KLP106; on the left plaque assays comparing Φ ER12 chimeras carrying gene 3 or gene 7, on the right, from the Φ LilB phage on *K. pneumoniae* KLP106.

A similar approach was used to generate an additional chimeric phage, Φ ER12::p3_LilB, in order to investigate the host recognition determinants of the Φ LilB, which naturally infects *K. pneumoniae* KLP106, a strain that is not susceptible to wild-type Φ ER12. In this case, we hypothesized that either gene 3 or gene 7 from Φ LilB could be responsible for host specificity. To test this, we constructed Φ ER12 chimeras by individually replacing gene 3 or gene 7 of Φ ER12 with the corresponding gene from Φ LilB, using plasmids containing the Φ LilB gene flanked by Φ ER12-homologous regions to enable recombination during phage replication.

When tested on *K. pneumoniae* KLP106, only the chimera carrying gene 3 from Φ LilB (Φ ER12::p3_LilB) was able to form lysis plaques, while the gene 7 chimera and the wild-type Φ ER12 control failed to infect (Figure 1-13 and Figure 1-14). This result clearly indicates that gene 3 from Φ LilB is sufficient to confer infectivity toward KLP106, and functions as the key host-recognition determinant in this strain background. As with previous recombination-based chimeras, plaque density was relatively low, indicating that the engineered variant represents a minor subset of the overall phage population.

1.3 Discussion

Bacteriophages represent an interesting weapon against antimicrobial-resistant infections. Since their discovery in the early 20th century by Twort and d'Hérelle, they have been studied and applied as antibacterial agents. With the advent of antibiotics, phage therapy was largely abandoned, except in the Soviet Union, particularly in Armenia, where George Eliava founded the Eliava Institute, which became a pioneering centre for phage research worldwide.

This first chapter of this thesis focuses on studying the *Klebsiella pneumoniae* phage collection isolated and preserved over the years by the Mimee Lab³⁷. In particular, we aimed to clarify how bacteriophages can be selected for formulation of a phage cocktail, and whether *in vitro* screening of their infectivity provides a reliable approximation of their behaviour *in vivo* within the gastrointestinal tract. To address this, we developed a combined approach to test phages under both conditions, mimicking the gut environment as closely as possible.

The first step consisted of selecting one or two bacteriophages from each taxonomic group, based on previous experiments³⁷, that could successfully infect the chosen bacterial strains. These phages were then tested under different media conditions supplemented with glucose, bile salts, and in both anaerobic and aerobic environments to better replicate the gut environment. The addition of a carbon source such as glucose provides an accessible energy supply, which can alter bacterial metabolic activity and potentially influence bacteria–phage interactions.

The second strategy was developed because *in vitro* screening alone did not reliably identify the most effective bacteriophages. To overcome this limitation, the entire phage collection, *Garbage Soda*, was tested against two strains of *K. pneumoniae* under both *in vivo* and *in vitro* conditions. Following bacteriophage infection in both settings, the resulting viral populations were recovered, extracted, and sequenced. Instead of aligning the recovered genomes to individual phages, the analysis was performed at the taxonomic group level. From these experiments, two *K. pneumoniae* strains, BAA2342 and JHCK1, were identified as the most sensitive to the phage collection.

In vitro, several taxonomic groups from the collection emerged as promising candidates, including *Webevirus* (T1-like), *Slopekvirus* (T4-like), *Purpurea*, and *Ackermannviridae*. These phages demonstrated strong stability in single-bacteriophage assays. Although their infectivity was only moderate, this trait remained consistent under both aerobic and anaerobic conditions and across different environments, including glucose, caecal content, and LB medium. This stability represents their major strength, making them particularly valuable despite their moderate infection efficiency. In particular, the *Purpurea* group had previously been identified as effective in earlier experiments³⁷, especially for their ability to replicate within the gastrointestinal tract. However, while they performed well under aerobic conditions in LB supplemented with glucose and bile salts, we observed a reduction in phage infectivity under anaerobic conditions. Additionally, the current phage bank does not contain *Purpurea* phages with strong activity against JHCK1, indicating that new strategies will be needed to isolate *Purpurea* phages targeting this strain, which appears to be an interesting candidate; however, only limited information is currently available in the literature,

highlighting the need for further studies to better characterize its biology and potential applications³⁷. Furthermore, the addition of glucose to the medium did not show any relevant differences, except for strain BAA2342, where the T4-like bacteriophages did not exhibit the drop in infectivity under anaerobic conditions that was observed for the other bacteriophages. This suggests that a connection between glucose supplementation and the maintenance of T4-like phage infectivity under anaerobic conditions may exist, but it requires further investigation. The hypothesis is supported by findings that T4 phages in *E. coli* can enter a reversible dormant state in metabolically inactive host cells. In that study, the lytic cycle was reactivated by the addition of glucose, which supplied the energy required for phage replication and host cell lysis. The added carbon source may act as an essential co-factor for lytic infection efficacy in environments where host metabolism is suppressed⁴⁶.

The differences observed between anaerobic and aerobic conditions raise important questions, particularly for those bacteriophages that performed well under aerobic conditions in media mimicking the gut. This leads to the question of whether *K. pneumoniae* behaves differently in the gut compared to aerobic *in vitro* conditions. For instance, reduced infection could be related to altered bacterial surface factors (capsule, lipopolysaccharides, outer membrane proteins) or metabolic regulators that affect susceptibility.

Interestingly, neither the *in vivo* nor the *in vitro* Garbage Soda experiments recovered a single taxonomic group, as might be expected from the principle of competitive exclusion, but rather multiple groups. The experiments consistently highlighted the same major taxonomic groups: *Webervirus* (T1-like), *Slopekvirus* (T4-like), and *Purpurea*, although some differences were noted between conditions. The outcomes of these experiments are similar to those of *Pyenson et al.*⁴⁷, although the settings differ. They examined combinations of 10–12 phages and recovered individual phages over 12 days of passage. In contrast, we tested over 100 phages simultaneously against *K. pneumoniae* strains JHCK1 and BAA2342, even though these are not their natural hosts, and our analysis was conducted at the taxonomic group level rather than the detection of individual phages. Nevertheless, both approaches revealed a similar pattern: multiple phages or phage groups were maintained, with at least two or three persisting instead of a single type outcompeting all others.

Notably, differences between *in vitro* and *in vivo* conditions were also observed. While we expected differences between LB-based *in vitro* results and *in vivo* outcomes, we expected the *in vitro* assays using caecal content to be more similar to the *in vivo* environment, since the gut conditions were mimicked as closely as possible. However, this was not the case. Even so, the bacteriophage taxonomic group that was recovered closely resembled the most successful phages identified in the array experiment. This underlines how host and environmental factors influence phage fitness and highlights that *in vitro* systems, while useful, may not always predict phage behaviour *in vivo*, particularly in complex or physiologically relevant contexts.

In the Phage Collection³⁷, while *Slopekvirus* and *Purpurea* bacteriophages display a broad host range, those belonging to the *Ackermannviridae* family exhibit a much narrower host range. One promising way to overcome this limitation in developing broad-host-range phages is through genetic engineering and synthetic biology, as reported by *Ando et al.*³⁸. To pursue this approach, it is necessary to precisely identify the genetic determinants involved in

bacterial receptor recognition. *Latka et al.*⁴⁰ proposed a structural model for phages in the *Menlow* group of the *Ackermannviridae* family. With this information, it becomes possible to design cocktails that combine wild-type bacteriophages with engineered chimeras to expand host coverage. Creating chimeras with different genes codifying for receptor-binding proteins, for instance, may allow a formulation to effectively target multiple *K. pneumoniae* strains.

To begin addressing this, we focused on Ackermann phages from the Mimee Phage Bank collection³⁷, including Φ ER12, Φ LilB, and Φ ER75, whose genomes were isolated, sequenced, and annotated, with particular attention to their tail fiber spikes that determine host recognition. We specifically aimed to determine whether genes 3, 5, or 7 are involved in receptor recognition of different bacterial strains. Using a three-plasmid system based on phage crosses, we generated chimeras of Φ ER12 by swapping gene 3, 5, or 7 with the one from other Ackermann phages in the collection through homologous recombination. This approach enabled us to identify the structural gene involved in the recognition of a specific *K. pneumoniae* strain. Following this strategy, we found that gene 5 of Φ ER75 is necessary for the infection of *K. pneumoniae* UTI47, while gene 7 enables the infection of strain KLP106 by Φ LilB. These results pave the way for the creation of more complex chimeras, such as Φ ER12 carrying multiple variants of gene 3 or combinations of different tail fiber genes, to broaden the host range.

1.3.1 Future Perspectives

In vivo experiments would be particularly informative if, from the Garbage soda cocktail, the most successful phages were removed to evaluate how the remaining phages perform once the dominant competitors are absent. Furthermore, the experiments conducted here represent only a snapshot at a single time point (24 hours post-infection). Repeating these experiments at earlier and later time points would provide a more dynamic picture of phage–bacteria interactions. Finally, regarding the *in vitro* assays with individual phages, more effort is required to understand the lifestyle of *K. pneumoniae* under anaerobic conditions, potentially through RNA-seq approaches, to uncover differential expression of bacterial determinants involved in bacteriophage infection. To gain a broader understanding of the potential of our phage collection for phage therapy, the natural or human host environment must be further explored. We began collecting data on phage–bacteria interactions in urine. First, we attempted to grow the two bacterial strains in urine, but this was not successful (i.e., the bacteria grew overnight, but after subculture, they failed to reach the exponential phase within a typical subculture period). This process likely requires optimization, for example, by adding supplements such as carbon sources or amino acids. Furthermore, it would be valuable to investigate phage–bacteria interactions in the bloodstream; to this end, infections could be assessed in whole blood.

Regarding the investigation of host range, although the described framework is promising, phage crosses have limitations. Screening recombinant phages is straightforward when focusing on a single gene, but it becomes significantly more complex when multiple variables are introduced. For example, designing a chimera that expresses two versions of gene 3 from different phages would make interpretation and screening challenging using this method. Fortunately, recent advances in phage engineering, such as BRED (Bacteriophage Recombineering of Electroporated DNA)^{48,49} and yeast-based vector systems³⁸, are becoming increasingly available, offering more precise tools for building and studying synthetic phages.

2. Chapter: Bacteriophages for Target Delivery

Lytic bacteriophages are an interesting alternative to antibiotics because they can specifically target and kill pathogenic bacteria, including strains that are resistant to conventional drugs. In phage therapy, lytic phages are generally preferred over temperate phages, because temperate phages can integrate into the bacterial genome and transfer additional genes to their host. This horizontal gene transfer can provide the bacterium with new components in its genetic repertoire, sometimes including virulence factors or antibiotic resistance, leading to a gain of function that makes the infection more severe⁵⁰.

An example is *Corynebacterium diphtheriae*: the bacterium itself does not naturally produce the diphtheria toxin. The toxin gene is carried by a temperate bacteriophage, β -phage. When this phage infects *C. diphtheriae* and integrates into its genome, the bacterium acquires the toxin gene and becomes capable of causing diphtheria disease⁵¹.

Similarly, certain pathogenic *Escherichia coli* strains, such as enterohemorrhagic *E. coli* O157:H7, carry temperate phages that encode the *Shiga toxin*. The acquisition of these phages transforms otherwise non-toxigenic *E. coli* into virulent pathogens capable of causing hemorrhagic colitis and haemolytic uremic syndrome⁵².

Therefore, using temperate phages in therapy requires comprehensive genomic analysis to confirm the absence of any genes that could enhance bacterial pathogenicity or resistance, thereby avoiding the risks associated with lysogenic conversion.

Synthetic biology, a field that combines principles of biology and engineering to design and construct new biological systems or reprogram existing ones, can help overcome issues related to the lysogenic lifestyle. By applying genome-editing tools such as CRISPR circuitry (Cas proteins and gRNA) or recombineering, temperate phage genomes can be precisely modified to remove genes responsible for integration, lysogeny, or the carriage of harmful cargo, including toxins. These modifications can convert temperate phages into safe, obligately lytic variants or into neutral delivery platforms for beneficial genetic payloads.

2.1 Challenges in Phage Genome Engineering

While studying and testing wild-type bacteriophages *in vitro* and *in vivo* has become a well-established and rapidly expanding field, the engineering of these viruses for therapeutic use through synthetic biology is still in its early stages and continues to evolve. Although recent advances have demonstrated the feasibility of modifying phage genomes to enhance lytic efficiency, broaden host range, or remove undesirable genes, the practical implementation of these approaches remains challenging.

One major unmet need is the development of reliable and standardized methods for phage genome engineering. Techniques such as CRISPR–Cas systems, recombineering, and cell-free assembly have enabled targeted genetic modifications, but many phages remain difficult to engineer because, despite powerful sequencing technologies, accurate annotation of their genetic elements remains a major bottleneck, even with advanced bioinformatic tools. Developing a general pipeline or protocol for engineering phages in a standardized manner would help address this challenge. Such a framework would include systematic identification

of virulence factors to prevent the introduction of novel harmful traits into wild-type bacteria, as well as the easy recognition of essential phage genetic elements, such as packaging signals and receptor determinants, which are critical for maintaining phage viability and infectivity. Expanding our understanding of phage genomes and functional elements through standardized approaches is therefore essential to fully harness their therapeutic potential.

Finally, regulatory and biosafety frameworks for synthetic or genetically modified phages are still underdeveloped. Establishing standardized evaluation criteria, particularly regarding horizontal gene transfer, ecological safety, and long-term genetic stability, is crucial for ensuring their safe and effective use in therapeutic applications.

2.1.1 M13 phage biology and its usage as a vector

One group of phages that are having a major impact on synthetic biology are the *filamentous phages*. These phages, such as M13, have small circular single-stranded DNA genomes (<10 kb) and are secreted from the bacterium without lysis^{53,54}.

M13 is a member of a family of filamentous bacteriophages (Ff) *Inoviridae* with a 6,400-bases genome which has been completely sequenced. M13 phages are viruses characterized by their long-rod shape and linear nanostructure. Their genome is organized in nine open reading frames encoding eleven proteins belonging to three classes of proteins responsible for the viral replication, morphogenetic and structural functions. The genes are transcribed in the same direction and are organized into two operons^{55,56}.

Among these eleven proteins, five are coat proteins located on the capsid. The major coat protein, pVIII, appears in approximately 2,700 copies along the phage's side wall, while the minor coat proteins—pIII, pVI, pVII, and pIX—are present in five copies each, located at the phage's ends (pIII and pVI at one end, pVII and pIX at the other). Due to their wide range of compatibility, pVIII (g8p) and pIII (g3p) are frequently modified for peptide display applications^{57,58}.

The coat proteins have several vital roles: minor coat proteins are involved in biorecognition, whereas pVIII proteins contribute to the structural integrity of the phage. Filamentous phages, including M13, predominantly infect Gram-negative bacteria. M13 phages specifically target bacteria that express the F-pilus (sex pilus), such as *Escherichia coli*, from which they can produce up to 10^{12} phage particles per millilitre overnight. Unlike lytic phages, M13 phages do not lyse the host cells but rather produce progeny virions while maintaining the viability of the infected bacteria^{56,59}.

A small noncoding region is positioned between genes VIII and III, while a larger noncoding region of 508 base pairs is located between genes II and IV. This intergenic region presents all the essential cis-acting elements of the bacteriophage genome. It includes sequences that regulate the packaging and orientation of DNA within bacteriophage particles, as well as sites for the initiation and termination of both (+) strand and (-) strand DNA synthesis, a signal for transcription termination^{56,59,60}.

The absorption of M13 phages starts when the minor coat protein pIII attaches to the F-pilus of a bacterial cell. This interaction triggers pilus retraction, which enables the pIII to interact with the secondary receptor TolA, a periplasmic protein. However, while the F pilus is not absolutely required for infection, the secondary receptor TolA is essential for infection^{55,61}. Subsequently, the phage is transported to the surface of the host cell, where it undergoes disassembly and the phage genome is ejected into the cytoplasm. The (+) circular single-stranded DNA of the phage enters the bacterial cytoplasm, where it is first used as a template for the synthesis of the (-) strand, resulting in a double-stranded replicative form (RF). The replication process begins with *E. coli* DNA polymerase when the pII protein generates a nick in a specific site of the (+) strand, and it proceeds until the entire (+) strand is synthesized, and the 5' and 3' ends are joined by pII. The RF generates linear single-stranded DNA through the movement of the replication machinery around the (-) strand template, a process known as *rolling circle replication*. The ssDNA is then coated by the phage-encoded ssDNA-binding protein pV, forming the packaging substrate. During assembly, pV is progressively released from the ssDNA, and the major coat protein pVIII associates with the DNA at the inner membrane. The assembly is completed by the addition of the minor coat proteins pIII and pVI, which release the phage from the host cell without causing lysis or its death^{55,59,61}.

Since the viral genome is not inserted into a preformed structure, there is no fixed limit to the size of the single-stranded DNA that can be packaged. Instead, the length of the filamentous particle adjusts according to the amount of DNA it contains. Foreign DNA inserts up to seven times the length of the wild-type viral genome has been successfully cloned and propagated in M13⁵⁹.

Although lysogenic phages can occasionally switch to the lytic cycle under certain environmental conditions, M13 phages are generally harmless, inducing only mild immune responses as they do not disrupt the cell wall or membrane. Their precise replication and targeted infection suggest that M13 phages can be safely used in human applications, offering significant potential in therapeutic and biotechnological fields^{56,59}.

The larger intergenic region known as the f1 origin contains sequences that regulate the packaging and orientation of the DNA. Due to its small size, it is possible to clone this part of the phage genome into a plasmid vector known as a phagemid. When cells transformed with these phagemids containing an f1 origin are infected by a helper vector or mutant helper bacteriophage M13, the single-stranded phagemid DNA is preferentially packaged and secreted into the culture medium. It happens because the gene II carried by the helper is mutated. Consequently, pII interacts less efficiently with the bacteriophage origin of replication carried on its own genome (due to the insertion of *lacZ* sequences) than with the origin cloned into the phagemid vector. This preference results in the production of more (+) strands from the phagemid than from the helper virus, ensuring that the virus particles produced by the cell contain a majority of single-stranded DNA derived from the phagemid^{55,59}.

The helper can be either a helper virus generated by mutation of the M13 wild type or a helper vector harbouring M13 genome.

The helper virus and specifically M13KO7 is a derivative of bacteriophage M13 that carries a plasmid origin of replication (derived from p15A), the kanamycin-resistance gene from the transposon Tn903, and a mutated version of gene II⁵⁹.

The vector plasmids created by *Chasteen et al.* are based on M13mp19, with the phage packaging signal origin replaced by p15A, an additional chloramphenicol resistance gene and the nine M13 genes⁶². The absence of packaging signals in these helper plasmids prevents their DNA from being packaged, providing significant advantages over the use of standard and modified helper phages, such as the contamination of the recombinant phage preparation with the standard helper virus.

2.1.2 An Innovative Approach: Usage of Engineered Phage Particles as Delivery Platforms

Recent advances in phage engineering and CRISPR-Cas technology have opened new avenues for developing highly specific antimicrobials that target and disable antibiotic resistance or virulence genes directly within bacterial populations.

Citorik J. R. et al. demonstrated the use of type II CRISPR-Cas system of *Streptococcus pyogenes* technology to create antimicrobials that can selectively target and kill bacteria with specific DNA sequences, such as antibiotic resistance and virulence genes. They engineered RNA-guided nucleases that can be delivered efficiently to microbial populations using bacteriophages or conjugative plasmids. Specifically, they developed phagemid vectors by combining RNA-guided nucleases, which target antibiotic resistance genes such as *bla* NDM-1 or *bla* SHV-18, with an f1 origin to enable packaging into M13 phage particles. These engineered phage particles were able to effectively transduce an entire population of *E. coli* cells⁶³.

Moreover, *Bikard et al.* developed a sequence-specific antimicrobial platform based on phage engineering and CRISPR-Cas9. They produced a phagemid system to deliver the Cas9 nuclease and its RNA guide targeting AMR chromosomal and plasmidic genes to *Staphylococcus aureus*. To develop the phagemid system suitable for *S. aureus*, the authors built a phagemid carrying genes and a packaging site from the Φ NM1 phage. Overall, the phagemid delivery system provides an appropriate approach for delivering the CRISPR-Cas9 antimicrobial, although difficulties associated with purity, large-scale production and narrow host range could limit its extensive use⁶⁴.

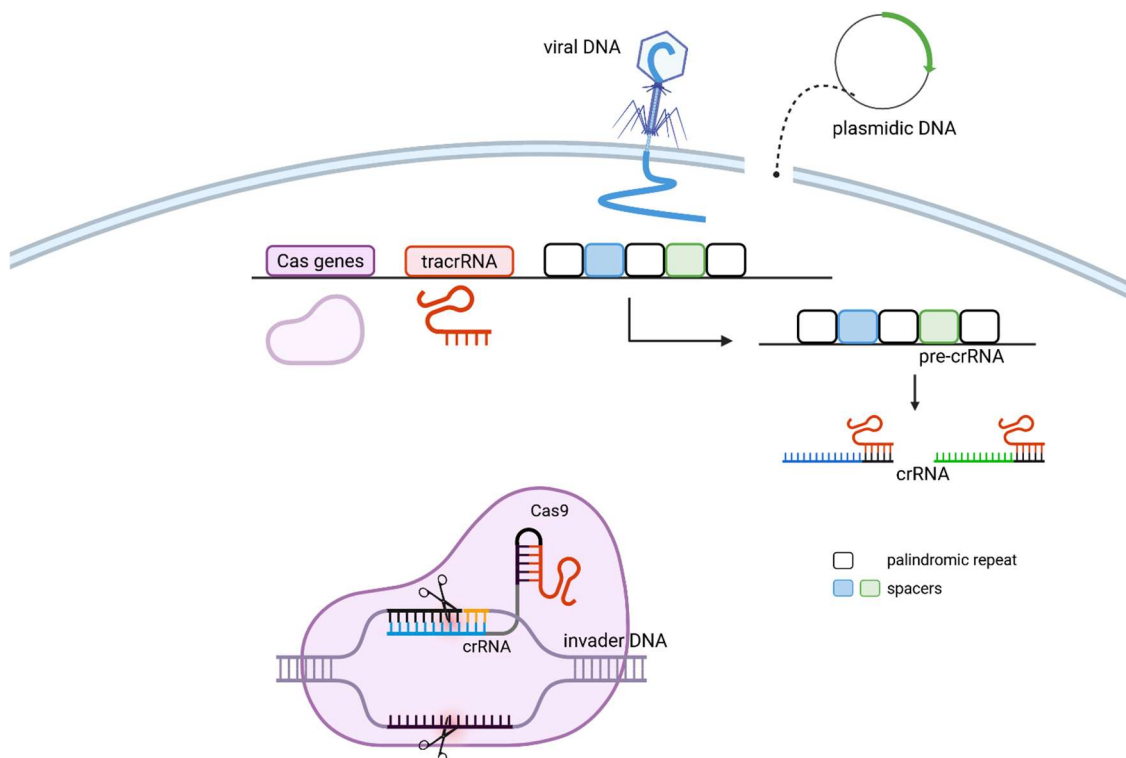
These innovative strategies highlight the potential of phage-based delivery systems in overcoming antibiotic resistance, paving the way for more effective and targeted antimicrobial therapies.

2.1.3 How to Act: CRISPR Interference

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas is an adaptive immunity against viruses and other genetic elements. About 40% of bacterial and 70% of archaeal genomes contain CRISPR sequences, which act as an immune memory by storing fragments of invading viral and plasmid DNA in spacer sequences within the CRISPR array.

The CRISPR locus includes *cas* genes, which work with the spacer-repeat array to target and neutralize foreign DNA^{65,66}.

CRISPR-Cas systems were discovered in the 1980s in *E. coli*, the function of CRISPR wasn't understood until 2007, when it was demonstrated that *Streptococcus thermophilus* could incorporate phage DNA sequences into its CRISPR array, granting immunity against subsequent infections. When a new infection occurs, the CRISPR array is transcribed into pre-crRNA, which is processed into crRNAs. These crRNAs guide Cas nucleases, like Cas9, cleave foreign DNA complementary to the spacer sequence, preventing infection⁶⁷. Briefly as displayed in *Figure 2-1*, when exogenous DNA from sources such as plasmids or bacteriophages enters the cell, Cas nucleases process it into short fragments called protospacers. These are integrated into the CRISPR array in the bacterial genome, where they act as a genetic memory of past infections. The array is made up of the invader sequences called spacers, separated by short, conserved repeats, and is accompanied by CRISPR-associated (Cas) genes organized in operons. Upon reinfection, the spacer-repeat array is transcribed into a long pre-CRISPR RNA (pre-crRNA), which is then processed into individual CRISPR RNAs (crRNAs). These guide RNAs bind to Cas nucleases and direct them to DNA sequences matching the stored spacers. The Cas nuclease then cleaves the matching viral or plasmid DNA, effectively stopping the infection⁶⁷.



*Figure 2-1: Schematic representation of the native CRISPR-Cas9 immune defence mechanism. When exogenous DNA from plasmids or bacteriophages enters the bacterial cell, short fragments (protospacers) are integrated into the CRISPR array as spacers, separated by conserved palindromic repeats. The array is transcribed into a precursor CRISPR RNA (pre-crRNA), which is processed into mature crRNAs. Together with *tracrRNA* and *Cas* nucleases, these crRNAs guide the *Cas9* protein to complementary invader DNA sequences, leading to targeted cleavage and neutralization of the infection.*

The CRISPR-Cas system comprises three main types (I, II, and III) that employ distinct molecular mechanisms to achieve nucleic acid recognition and cleavage. The Type I and Type III systems utilize a large, complex array of Cas proteins for crRNA-guided targeting, whereas the Type II system requires only a single protein, Cas9, for RNA-guided DNA recognition and cleavage. The Type II CRISPR-Cas9 system from *Streptococcus pyogenes* utilizes a guide RNA made up of crRNA and a trans-activating crRNA (tracrRNA) to direct the Cas9 endonuclease to cleave target DNA sequences. This two-component system was further engineered into a single guide RNA (sgRNA) that retains the key features for Cas9 binding and target DNA recognition, making it a practical and programmable tool for genome editing and gene expression in a wide variety of organisms such as bacteria, yeast, fish, mice and human cells^{63,68,69}.

When crRNA and tracrRNA or the sgRNA are loaded into the wild-type Cas9, it can specifically introduce double-stranded DNA breaks (DSBs). These DSBs can result in nonspecific mutations that inactivate a gene via the error-prone non-homologous end-joining (NHEJ) pathway. Alternatively, if a donor template with homology to the target locus is provided, the DSB can be repaired through the homology-directed repair (HDR) pathway, allowing for precise replacement mutations. By directing cells toward one of these repair mechanisms, CRISPR can generate gene knockouts or knock-ins in desired cells. The targeting specificity is determined by the base pairing between the sgRNA and the target DNA, and as well as the binding of the Cas9 protein to a short DNA motif commonly found at the 3' end of the target DNA, called the protospacer adjacent motif (PAM). This PAM sequence, which consists of a 2- to 5-bp recognition site varying with the CRISPR system and the host organism, for example, the *Streptococcus pyogenes* Cas9 recognizes a 5'-NGG-3' PAM. The binding of the Cas9-sgRNA complex to the target DNA induces double-strand breaks within the target region due to the endonuclease activity of Cas9^{67,69,70}.

The CRISPR-Cas system can be repurposed to tune gene expression by regulating their transcription. This system is called CRISPRi and it exploits a RNA-guided DNA-binding system to interfere with the transcription of a target gene. To achieve this, a modified version of Cas9 is used, which is called dead Cas9 or dCas9. RuvC-like and HNH nuclease domains are two endonuclease domains of native Cas9, which respectively cleave the target DNA strand non-complementary with the sgRNA and the DNA strand complementary with the sgRNA. The dCas9 derived from *S. pyogenes* includes two modifications in its RuvC-like and HNH nuclease domains, which, rather than cleaving the targeted sequence, can modulate transcript levels^{69,71}.

In addition to dCas9, RNase III and trans-acting RNA are two components required for the maturation of crRNA, guiding dCas9 to the desired target. To further simplify this technology, a modification can be made by using synthetic sgRNA instead of trans-acting RNA and crRNA, thereby bypassing the maturation step. The sgRNA is a 102- nucleotide-long RNA and it is composed of:

- the spacer, a 20 nucleotide-long variable site which specifically binds to the target,
- the scaffold, a 42 nucleotide-long constant sequence which binds to dCas9,
- the terminator, a 40-nucleotide region which signals the end of the transcription.

sgRNAs mimics the crRNA-tracrRNA complex, enabling them to form a complex with dCas9 and altering site-specifically the gene expression⁷¹.

The CRISPRi technology can target the promoter region or the coding sequence of the candidate gene. The efficiency of CRISPRi-mediated repression is generally higher when targeting the promoter compared to the coding sequence. Specifically, targeting the promoter can more effectively prevent the binding of transcription factors and RNA polymerase, thereby inhibiting transcription initiation. In contrast, targeting the coding region depends on RNA polymerase encountering the DNA-bound dCas9/sgRNA complex, which blocks transcription elongation but may be less efficient⁷¹.

2.1.4 CRISPRi in Non-Conventional Model Bacteria: pSGAb

The CRISPRi circuitry needs to be delivered into the target bacterial cell, a process that can be achieved through phage delivery. Phage particles can be engineered, following methods established by *Bikard et al.* and *Citorik et al.*, to transfer the CRISPRi associated DNA to specific pathogens^{63,64}. However, prior to phage engineering, the CRISPRi platform must undergo testing in the target pathogen. To achieve this, *in vitro* assays should assess the functionality by cloning the CRISPRi components into a broad-host-range expression plasmid. Specifically, pWH1266 is an *Escherichia coli*-*Acinetobacter species* shuttle vector is a cloning vector and an expression plasmid suitable for gene expression in *A. baumannii*. The pWH1266 was generated by combining the pWH1277 natural cryptic plasmid of *A. calcoaceticus* BD413 with the *E. coli* pBR322 vector. Subsequently, pSGAb was obtained by replacing the selection marker *bla*, conferring resistance to β -lactams, with a kanamycin resistance cassette. The pSGab-km plasmid has the following features:

- two origins of replication, one from the *E. coli ColE1* and one from the *A. calcoaceticus* BD413 plasmid pWH1277, allowing them to replicate in both *E. coli* and *Acinetobacter spp*;
- kanamycin as antibiotic resistance marker;
- an estimated copy number of 56-70 copies per chromosome in *A. baumannii* and *E. coli*, respectively^{72,73}.

The pWH1266 shuttle vector has been employed in various studies to manipulate *Acinetobacter baumannii*. For example, *Wang et al.* utilized the pSGAb plasmid for genome editing in *Acinetobacter baumannii*. The system employs a dual-plasmid setup consisting of pSGAb and pCasAb. The pCasAb-pSGAb system integrates a CRISPR-Cas9 genome cleavage mechanism with the RecAb recombination system native to *A. baumannii*. They demonstrated the platform's versatility in achieving efficient gene deletion, insertion, and point mutation in *A. baumannii* strains⁷³.

Brychcy et al. developed a CRISPRi system in *A. baumannii* targeting a reporter gene providing a powerful tool to study gene regulation and essential genes in this pathogen. Specifically, they built two plasmids derived from the pWH1266 plasmid of *A. calcoaceticus*:

- pMB04, contains an androtetracycline (ATc)-inducible *dCas9* gene, fluorescence reporter genes (*eGFP* and *mCherry*), and constitutively expressed sgRNAs targeting the fluorescence genes.
- pMB05, contains the same fluorescence genes but no-targeting nonsense sgRNAs.

They observed that in ATc-treated cells with pMB04, the fluorescence signal was significantly repressed, but not in cells carrying the pMB05 with the nonsense sgRNA. Increasing the ATc concentration intensified this effect, demonstrating the effective use of the CRISPRi platform in *A. baumannii*⁷⁴.

2.2 Aim

The primary aim of this chapter is to contribute to the fight against antibiotic resistance through an innovative synthetic biology-based approach, addressing current unmet needs in targeted bacterial therapies.

Specifically, this chapter focuses on the design and evaluation of a standardized system for generating recombinant bacteriophages in *Escherichia coli*, capable of delivering CRISPR interference (CRISPRi) tools to silence antibiotic resistance genes. This strategy targets two clinically relevant and highly resistant members of the ESKAPEE group: *Acinetobacter baumannii* and *Escherichia coli*. Their rapid dissemination and ability to acquire new resistance determinants highlight the urgent need for precise, effective, and targeted antimicrobial interventions.

The novelty of this work lies in integrating synthetic biology tools to develop engineered phages that function both as delivery vehicles and programmable actuators. The methodology addresses two main aspects: (I) the design and optimization of the delivery system, i.e., engineered phage particles capable of efficiently infecting target bacteria, and (II) the development of a genetic actuator library, including the identification and characterization of functional parts active in the targeted strains, alongside the transformation protocols required to implement these systems.

By providing a standardized and modular framework, this chapter aims to establish a scalable platform for the rational design of therapeutic phages, enhancing the precision and efficacy of antimicrobial strategies while offering innovative solutions to combat antibiotic resistance.

2.2.1 The Actuator Machinery

The actuator machinery is the technology we will use to counteract AMR in bacteria, namely CRISPRi. We propose to utilize CRISPR interference instead of traditional CRISPR for several reasons. First, conventional CRISPR systems that induce DNA double-strand breaks exert strong selective pressure, often leading to the evolution of resistance against the CRISPR components themselves. Second, CRISPR-escape mutants, which arise through mutations in the target gene, may still encode functional resistance proteins, thereby giving rise to new resistance variants. By employing CRISPRi, which silences gene expression without altering the DNA sequence permanently, we can effectively target antimicrobial resistance genes while minimizing these risks.

To establish this technology in *Acinetobacter baumannii*, we will first identify an expression shuttle vector functional in both *A. baumannii* and *E. coli*, allowing constructs to be cloned in *E. coli* and subsequently tested in *A. baumannii*. Next, we will evaluate and implement a reliable transformation protocol to express a reporter gene in *A. baumannii* using the shuttle vector. In addition, we will include studying and screening genetic parts such as expression vectors, antibiotic selection markers, and both constitutive and inducible promoters

functional in *A. baumannii*. If necessary, we will construct a library of these elements to systematically evaluate them in *A. baumannii*.

Subsequently, we will assess the potential of CRISPRi in *A. baumannii*. The CRISPRi system will first be cloned to target a reporter gene (*rfp*) in the shuttle vector, and its activity will be characterized in *E. coli* and then in *A. baumannii* using microplate reader assays measuring red and green fluorescence. In parallel, a *gfp* reporter under a constitutive promoter (J23100) will be incorporated into the circuit to serve as an indicator of metabolic burden.

Finally, we will target and silence a gene involved in *A. baumannii* virulence and AMR. This target gene will be non-essential but associated with multiple mechanisms, including epithelial cell attachment, antibiotic transport, and biofilm formation. A functional evaluation will be conducted using a biofilm assay based on crystal violet quantification and real-time PCR analysis.

2.2.2 The Design and Optimization of the Delivery System

The primary objective of this study is to evaluate a standardized system for generating recombinant phages in *Escherichia coli*. Specifically, we aim to optimize the delivery platform based on engineered M13 phages. This involves evaluating and determining the most effective method for generating phage particles. Specifically, we would like to compare the efficacy and suitability of two approaches:

- a helper plasmid and phagemid, this method involves the co-transformation of a helper plasmid and a phagemid;
- a helper phage and phagemid, which relies on the infection of a helper phage in a *E. coli* F' harbouring a phagemid to generate phage particles.

Our goal is to assess which method best fits our application needs by examining its efficiency and ease of manipulation. Given our ultimate aim of altering the tropism of the engineered M13 phages, we hypothesize that utilizing a helper plasmid may offer significant advantages. The plasmid-based system provides greater flexibility and simplicity in DNA manipulation, which is crucial for the genetic modifications required to alter phage tropism.

Additionally, we aim to develop a reliable transduction assay to verify the efficiency of phage delivery of the reporter gene to its natural host. This will involve creating a standardized protocol to ensure consistent and accurate results. In parallel, we aim to develop a robust quantification method for engineered phage preparations. To achieve this, we intend to optimize a quantitative real time PCR protocol coupled with the treatment of the phage preparation with DNase prior to DNA extraction to accurately quantify the copy number of amplified phage DNA. This method will provide precise and reproducible measurements.

Moreover, we are interested in developing a phagemid that carries CRISPRi technology to target genes involved in AMR outbreaks, thereby preventing the use of last-resort antibiotics such as colistin and meropenem. Our goal is to demonstrate a proof of concept by exploiting phage-based delivery of CRISPRi to silence *bla_{NMD-1}* and *mcr-1* in laboratory strains carrying these genes on plasmids. We aim to evaluate silencing efficiency through growth inhibition

assays and quantify the growth delay in silenced strains. Furthermore, we plan to develop an inducible CRISPRi system, specifically a homoserine lactone (HSL)-inducible sgRNA. To achieve this, we will design and construct a phagemid carrying a significant genetic cargo. To measure the associated metabolic burden, a green fluorescent protein (GFP) reporter will be introduced into the system. The new circuit will be validated using a microplate reader assay to measure both red and green fluorescence.

This will enable us to measure the effectiveness of CRISPRi-mediated gene silencing in a quantitative and high-throughput manner, while also studying and evaluating strategies to modulate the tropism, making the platform usable in *A. baumannii*.

2.3 Materials and Methods

In the following sections, the materials and methods applied in Chapter 2 are described in detail.

2.3.1 Strains and Culture Conditions

Escherichia coli 10-beta (New England Biolabs) for cloning, *Escherichia coli* TOP10 F'(Invitrogen) as phage target, *Escherichia coli* DH5 α as initial carrier of the *m13cp* helper plasmid⁶², *Acinetobacter baumannii* ATCC 19606. All the strains were grown in Lysogeny broth (10 g/L Sodium chloride, 10 g/L Bacto-Tryptone, 5 g/L Yeast extract) at 37°C with shaking 200 rpm. Antibiotics were added to the growth medium to the following final concentrations: 12,5 μ g/mL chloramphenicol (Cm), 25 μ g/mL kanamycin (Kan), 100 μ g/mL ampicillin (Amp), 100 μ g/mL carbenicillin (Carb). Bacteria were grown on LB agar (1.5% w/v; Thermo Fisher Scientific) supplemented with the appropriate antibiotic when needed.

The Kirby-Bauer test was assessed on *A. baumannii* ATCC 19606 using Müller-Hinton agar (300 g/L beef infusion; 17.5 g/L casamino acids; 1.5 g/L starch; 17 g/L agar).

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

The engineered phage particles generated with the phagemid pTZ19R (Thermo Scientific) and the *m13cp* helper plasmid⁶² were produced in grown in Lysogeny broth (10 g/L Sodium chloride, 10 g/L Bacto-Tryptone, 5 g/L Yeast extract) at 30°C with shaking 250 rpm and with the required antibiotics to the following final concentrations: 12.5 μ g/mL chloramphenicol and 100 μ g/mL ampicillin.

2.3.2 Plasmids

To express genes in *Acinetobacter baumannii* ATCC 19606 the shuttle vector pSGAb-km (Addgene #121999) and pME6032, kindly provided by the laboratory of Prof. Rampioni (University of Rome), were employed. pSGAb-km contains a resistance marker, kanamycin; two different replication regions: *ColE1* for replication in *E. coli* and *WH1266* for replication in *A. baumannii* and *K. pneumoniae*. pME6032 harbours the tetracycline resistance gene, p15a origin of replication for *E.coli* and pVS1 for *A. baumannii*.

The *pSB1A2* backbone is a high-copy-number plasmid that carries ampicillin resistance. It features a replication origin derived from the pUC19-derived pMB1 (with a copy number of 100-300 per cell) and was obtained from the iGEM Registry. This plasmid backbone was employed in our project as carrier for the Red Fluorescent Protein (*RFP*) expression cassette BBa_I13521, with the insert consisting in the mRFP1 gene under the control of the pTet promoter BBa_R0040, the RBS BBa_B0034, and the double transcriptional terminator

BBa_B0015; all these parts and codes can be found in the iGEM Registry of Standard Biological Parts (https://parts.igem.org/Main_Page).

The *pSB3K3* backbone is a medium-copy plasmid with kanamycin resistance. It has a p15A pMR101-derived replication origin, with a copy number of 20-30, and was obtained from the iGEM Registry. The vector is used in our study as in [<https://doi.org/10.3389/fbioe.2021.743950>] to express the *dCas9* under the control of the BBa_J23116 promoter from the Anderson promoter collection. The same backbone was also utilized to harbor the sgRNA targeting the pTet promoter, which controls *rfp* expression.

The *pSB4C5* is a vector with a low-copy replication origin, with an expected copy number of ~5 per cell, and a chloramphenicol antibiotic resistance marker, obtained from the iGEM Registry. The backbone was employed with the sgRNA targeting the pTet controlling the *RFP* expression (*pSB4C5*-sgpTetRFP).

The helper plasmid *m13cp* was gifted by *Andrew M. Bradbury*. It carries M13 phage genes, chloramphenicol resistance, and it lacks M13 origin of replication, which is replaced by p15a origin, which provides ~15 copies per cell⁴⁵.

The M13KO7 Helper Phage (Invitrogen) is a derivative of bacteriophage M13 that carries a plasmid origin of replication (derived from p15A), the kanamycin-resistance gene from the transposon Tn903, and a mutated version of gene II.

The phagemid pTZ19R (Thermo Scientific) harbours the F' M13 phage intergenic region (IG), which contains the packaging signal. It includes the gene encoding for ampicillin resistance and the origin of replication pMB1.

2.3.3 Chemically Competent Cells

Chemically competent *E. coli DH5alpha* or *E.coli NEB 10-beta* cells were prepared using a modified calcium-manganese method. An overnight culture was started by inoculating 5 mL of LB with a single fresh colony and incubated at 37°C with 220 rpm shaking. The following day, 2,5 mL of the overnight culture was used to inoculate 100 mL of fresh LB medium in a 500 mL flask. Cultures were grown at 37°C with 220 rpm shaking until they reached an OD₆₀₀ of 0.4–0.6. Cells were harvested by centrifugation at 3,500 rpm for 15 minutes at 4°C and resuspended in 60 mL of ice-cold Solution A (10 mM MnCl₂·4H₂O, 50 mM CaCl₂, 10 mM MES pH 6.3; Merck). After incubation on ice for 20 minutes, cells were pelleted again under the same conditions and resuspended in 6 mL of Solution B (Solution A supplemented with 15% glycerol; Merck). Both solutions were filter-sterilized and stored at –20°C before use.

The cell suspension was aliquoted into sterile 1.5 mL microcentrifuge tubes, snap-frozen in dry ice, and stored at –80°C until use.

2.3.4 Transformation

Plasmid DNA was introduced into chemically competent *E. coli* either *coli DH5alpha* or *E.coli NEB 10-beta* cells using a standard heat-shock transformation protocol. Briefly, 50 µL of competent cells were thawed on ice and gently mixed with plasmid DNA (if the DNA was a

plasmid purified via miniprep, 1–5 ng was used; if it was a ligation product, 20 µL was added; for Gibson Assembly products, $\frac{1}{20}$ of the competent cell volume was used). The mixture was incubated on ice for 30 minutes, followed by a 45-second heat shock at 42°C, and then placed on ice for 2 minutes. Immediately after the heat shock, 200 µL of LB medium was added to the cells, and the mixture was incubated at 37 °C for 1 hour in a water bath to allow for the expression of antibiotic resistance.

After recovery, the entire volume of the transformation mixture was plated onto LB agar plates containing the corresponding antibiotic for selection. Plates were incubated overnight at 37 °C, and the resulting colonies were screened the following day.

2.3.5 Plasmids Construction and General Cloning Procedures

The desired plasmids purified with the QIAprep Spin Miniprep Kit (Qiagen) were digested with Restriction Enzymes (New England Biolabs).

The cloning procedures, based on traditional digestion and ligation, were performed according to the BioBrick RFC[10] standard. In this system, each genetic part is flanked by a prefix (containing the restriction sites EcoRI and XbaI) at the 5' end, and a suffix (containing SpeI and PstI) at the 3' end. These standardized overhangs enable modular and seamless assembly of parts. The XbaI and SpeI sites generate compatible sticky ends that can ligate together, but the resulting mixed site is no longer recognized by either enzyme, preventing re-cleavage and allowing stable assembly of multiple parts in a defined order⁷⁵. The obtained digested bands were gel extracted with the Wizard®SV Gel and PCR Clean-Up System (Promega). Ligation was performed in a 20 µL reaction mixture using T4 DNA Ligase (Thermo Scientific) and incubated overnight at 16 °C, with a vector-to-insert molar ratio of either 1:3 or 1:5. The ligation products were chemically transformed into *E.coli* 10-beta.

Mutations in plasmids were introduced using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs), following the manufacturer's instructions. Briefly, plasmid DNA was purified and used as a template in a PCR reaction with Q5 polymerase and specific primers. After amplification, 5 µL of the PCR product was run on a 1% agarose gel to verify amplification, and 1 µL was treated with KLD buffer (Kinase, Ligase, DpnI mix) for 5 minutes at room temperature to phosphorylate, ligate, and degrade the template DNA. The resulting mixture was directly transformed into competent *E. coli* cells, which were then plated on selective LB-agar and incubated overnight at 37 °C.

Plasmid constructs were also assembled using Gibson Assembly⁷⁶, carried out with the NEBuilder® HiFi DNA Assembly kit (New England Biolabs), a PCR-based, one-step isothermal DNA assembly method. PCR amplification of the DNA fragments required for assembly was performed using Q5® High-Fidelity DNA Polymerase (New England Biolabs) in a 50 µL reaction volume, following the manufacturer's instructions. Thermal cycling conditions included an initial denaturation at 98 °C for 30 seconds, followed by 30 cycles of denaturation at 98 °C for 10 seconds, annealing at the recommended temperature (calculated using the NEB Tm calculator) for 20 seconds, and extension at 72 °C for 30 seconds per kilobase of target sequence. A final extension was carried out at 72 °C for 7 minutes, followed by a hold

at 4°C. A 5 µL aliquot of each PCR product was run on Tris-Acetate-EDTA (TAE) 1% agarose gel and visualized using a GelDoc imaging system (Bio-Rad). The remaining PCR product was purified using the Wizard®SV Gel and PCR Clean-Up System (Promega) before proceeding to assembly. In the assembly reaction, the purified overlapping fragments were combined with the NEBuilder HiFi Master Mix in a single isothermal reaction. A 5' exonuclease chews back DNA ends, generating single-stranded overhangs that allow complementary regions to anneal. DNA polymerase then fills in gaps, and DNA ligase seals the nicks, producing a seamless, circular plasmid ready for transformation

The colonies carrying the newly constructed plasmids were checked using Sanger sequencing by Eurofins Genomics and/or the Whole Plasmid Sequencing service offered by Eurofins Genomics, and then stored in 20% glycerol (Merck) at -80°C.

Primers used for Gibson Assembly, mutagenesis PCR, colony PCR screening, and Sanger sequencing were synthesized by Merck and are listed in *Table A-2* and *Table A-3* in the *Appendix*. The plasmids used in this work are listed in *Table A-4* and *Table A-5*.

2.3.5.1 Shuttle Vector *E. coli*-*A. baumannii* Expressing *rfp*

To generate the *pSGAb-km_rfp* we created an intermediate vector by cutting a 118 base pair synthetic double stranded DNA, bearing a Bio-Brick standard cloning site, (*Table A-2*) and *pSGAb-km* with HindIII (Thermo Scientific) and SacI (Thermo Scientific). Next the construct was digested with SpeI (New England Biolabs) and PstI (New England Biolabs), and the insert I13521⁷⁷, *rfp*, with XbaI (New England Biolabs) and PstI (New England Biolabs) to obtain *pSGAb_pTet*.

The high-copy pMB1/pUC19 origin of replication of *pSGAb* was replaced with the medium-copy p15a origin. This change was made by Gibson assembly. The amplicons to be assembled were obtained with the primers reported in *Table A-2, Appendix*.

The expression vector with the *rfp* under the control of the inducible promoter pTet (*pSGAb_pTet*) was obtained by digesting the *pSGAb* medium copy and the pS1A2 carrying the RFP cassette Bba I13521 with EcoRI (New England Biolabs) and PstI (New England Biolabs).

To construct the *pME6032_blaR* plasmid, the *pME6032* backbone was amplified by PCR, excluding the *tetA/tetR* genes, while the *blaR* gene was amplified from the pTZ19R plasmid (Addgene plasmid #4468). Both PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and assembled using the NEBuilder® HiFi DNA Assembly kit. Subsequently, *pME6032_blaR* was further modified to insert the *rfp* gene driven by pTet promoter flanked by prefix and suffix sequences to ensure compatibility with the BioBrick RFC[10] standard. To facilitate this, a PCR was performed to remove a small segment of the plasmid backbone containing an unwanted *EcoRI* restriction site, which could interfere with future cloning steps. The RFP cassette, under the control of the pTet promoter, was amplified from a pS1A2-based plasmid that included the BioBrick flanking regions. Both PCR fragments were purified using the Wizard® SV Gel and PCR Clean-Up System and assembled using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs), resulting in the final construct *pME6032_pTet*.

Primers used for Gibson Assembly, mutagenesis PCR, colony PCR screening, and Sanger sequencing are listed in *Table A-2, Appendix*. The mentioned plasmids are available in *Table A-4, Appendix*.

2.3.5.2 Constitutive and Inducible Promoters in the Shuttle Vector *E. coli-A. baumannii* Targeting *rfp*

A series of constitutive *rfp* expression plasmids (pSGAb_J23XX_RFP see *Table A-4*) was constructed by digesting a set of promoter-RFP expression cassettes from the Anderson Library⁷⁸ and the pSGAb_pTet backbone with EcoRI (New England Biolabs) and PstI (New England Biolabs).

To generate the pSGAb_pLuxR plasmid, the RFP cassette controlled by an HSL-inducible system was excised from a donor plasmid (pE47, kindly provided by the BMS Laboratory, University of Pavia) using EcoRI (New England Biolabs) and PstI (New England Biolabs), and ligated into the pSGAb_pTet backbone digested with EcoRI (New England Biolabs) and PstI (New England Biolabs). Similarly, the aTc-inducible system was assembled by digesting both the donor plasmid (pSB4C5_aTc, kindly provided by the BMS Laboratory, University of Pavia) and pSGAb_pTet with the same restriction enzymes.

The IPTG-inducible construct was created in a two-step process. First, an RFP expression cassette (lacking a promoter⁷⁹) was excised from an existing plasmid using SpeI (New England Biolabs) and PstI (New England Biolabs) and ligated into a plasmid carrying the IPTG-inducible promoter (Y32_pLac, kindly provided by the BMS Laboratory, University of Pavia) digested with the same enzymes. The resulting IPTG-inducible RFP construct and the vector pSGAb_pTet were digested using EcoRI (New England Biolabs) and PstI (New England Biolabs), following the same strategy used for the other inducible systems.

Primers used for colony PCR screening, and Sanger sequencing are listed in *Table A-2, Appendix*. The mentioned plasmids are available in *Table A-4, Appendix*.

2.3.5.3 CRISPRi in The Shuttle Vector *E.coli-A. baumannii* Targeting *rfp*

The pSGAb_dCas9 plasmid was built in a two-step cloning procedure. Firstly, the *dCas9* gene from the pSB3K3_dCas9 plasmid was modified by site-directed mutagenesis to remove the EcoRI restriction site, which is incompatible with the RFC[10] standard. Mutagenesis was performed using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs), resulting in the pSB3K3_dCas9mut construct. Finally, the pSGAb_dCas9 plasmid was generated by digesting the pSGAb medium copy and the pSB3K3 carrying the *dCas9_mut* (pSB3K3_dCas9mut) under the control of the J23116 promoter with EcoRI (New England Biolabs) and PstI (New England Biolabs), see *Table A-4*.

The pSGAb-CRISPRi plasmid was obtained by digesting dCas9-pTet-RFP-pSGAb with EcoRI (New England Biolabs) and XbaI (New England Biolabs), and the pSB4C5-sgpTetRFP with EcoRI (New England Biolabs) and SpeI (New England Biolabs), see *Table A-4*.

The pSGAb_sgplac plasmid was obtained by replacing the sgRNA spacer targeting the pTet promoter with one targeting the pLac promoter using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs), following the manufacturer's instructions.

Primers used for mutagenesis PCR, colony PCR screening, and Sanger sequencing are listed in *Table A-2, Appendix*. The mentioned plasmids are available in *Table A-4, Appendix*.

2.3.5.4 CRISPRi in The Shuttle Vector *E. coli*- *A. baumannii* Targeting *ompA*

The sgRNAs (sg157 and sg211) targeting the *ompA* gene were amplified using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher) with the following PCR cycles: 98°C for 30 seconds; (98°C for 7 seconds, 63°C for 20 seconds, 72°C for 20 seconds) × 40 cycles; 72°C for 7 minutes; and a final hold at 4°C. The 300 bp amplicons were resolved on a 2% agarose gel, gel-extracted using the Wizard® SV Gel and PCR Clean-Up System (Promega), digested with EcoRI (New England Biolabs) and SpeI (New England Biolabs), and ligated with pSGAb_dCas9, which had been previously digested with EcoRI (New England Biolabs) and XbaI (New England Biolabs), resulting respectively in the pSGAb_CRISPRi157 and pSGAb_CRISPRi211

The plasmid carrying both sgRNAs (sg157 and sg211) was created by digesting the vector pSGAb_CRISPRi211 with EcoRI (New England Biolabs) and XbaI (New England Biolabs) and the insert with EcoRI (New England Biolabs) and SpeI (New England Biolabs).

Primers used for mutagenesis PCR, colony PCR screening, and Sanger sequencing are listed in *Table A-2, Appendix*. The mentioned plasmids are available in *Table A-4, Appendix*.

2.3.5.5 pTZ19 Cloning

The commercial phagemid pTZ19R was modified through PCR mutagenesis using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) to remove the existing multiple cloning site and replace it with the prefix and suffix of the Standard Assembly (i.e., restriction sites for EcoRI, XbaI, SpeI, and PstI, see <https://parts.igem.org/Help:Prefix-Suffix>). The resulting plasmid (pTZ19) and pS1A2 carrying pTetRFP were digested with EcoRI (Thermo Scientific) and PstI (Thermo Scientific), ligated, transformed, and verified by Sanger.

To mitigate the toxicity of dCas9 in *E. coli* when using higher copy plasmids than the pSB3K3 the J23116 promoter upstream of the *dCas9* gene was replaced with the J23109 promoter using PCR mutagenesis with the Q5® Site-Directed Mutagenesis Kit (New England Biolabs).

Phagemids carrying CRISPRi systems targeting the *bla_{ndm-1}* and *mcr-1* genes were constructed using Gibson Assembly. Briefly, the assembly strategy involved three fragments: the backbone (pTZ19), the *dCas9* gene under the control of the J23109 promoter, and the tracrRNA-crRNA cassette. The *bla_{ndm-1}* array was amplified from plasmid T10-ORIT-AedCas9-ARRAYndm1, and the dual-target *ndm-1/mcr-1* array was amplified from plasmid T10-ORIT-AedCas9-ARRAYndm1mcr1, both kindly provided by Prof. Pasotti⁸⁰. PCR amplification of the fragments and their assembly were performed as described in the corresponding section of Materials and Methods, Plasmids Construction and General Cloning Procedures.

The phagemid carrying the single-spacer *mcr-1* cassette was obtained using classical digestion–ligation. The *mcr-1* targeting tracrRNA–crRNA sequence was newly synthesized by GenScript with 5' EcoRI and 3' XbaI sites, and cloned into a pUC57 high-copy backbone. This plasmid and the phagemid containing *dCas9* under the J23109 promoter were digested with EcoRI and XbaI and then ligated. All three newly constructed plasmids were verified by whole-plasmid sequencing.

Target plasmids (*mcr-1*_3K3 and *ndm-1*_3K3) carrying *mcr-1* or *bla_{ndm-1}* were also generated using Gibson Assembly. Each assembly consisted of two fragments: the pSB3K3 backbone and the gene of interest, amplified respectively from plasmids pGDP2-*mcr-1* and pGDP1-*ndm-1*, both kindly provided by Prof. Pasotti from the BMS laboratory, University of Pavia, Italy⁸⁰. PCR amplification and assembly were performed following the procedures described in the *Materials and Methods, Plasmids Construction and General Cloning Procedures*. Both plasmids were confirmed by whole-plasmid sequencing.

To generate pTZ19 harbouring CRISPRi targeting *rfp* (pTZ19_CRISPRi_HSL), 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, transformed, and verified using Sanger sequencing.

Primers used for Gibson Assembly PCR, colony PCR screening, and Sanger sequencing are listed in *Table A-3, Appendix*. Furthermore, all the modified phagemids are listed in *Table A-5*.

sgRNA Design

To design a reliable sgRNA, various design tools were employed, including Benchling, CHOPCHOP, Cas-Designer, and CRISPOR. To obtain efficient and robust target-specific specific the following parameters were set up:

- GC content has to be between min: 30% and max: 70%;
- within the first 20% of the TSS of the gene
- lowest Self-complementarity
- off-targets with 0, 1, 2, or 3 mismatches.

The sgRNAs (see *Table A-2*) targeting *OmpA* in *Acinetobacter baumannii* ATCC 19606, both sg157 and sg211, were designed using the same components: the restriction enzyme recognition sites for EcoRI and XbaI, the J23119 promoter, ribosome binding site (RBS), spacer, terminator, and scaffold, the restriction enzyme recognition sites for SpeI and PstI.

The sgRNAs targeting the promoter pTet and pLac were the same as those designed in *Bellato et al.*⁸¹

2.3.6 Kirby-Bauer Disk Diffusion Susceptibility Test

To evaluate the sensitivity or resistance of the ATCC bacterial strain, Kirby-Bauer disk diffusion susceptibility tests were conducted. Bacteria reaching a 0,5 McFarland standard (between 1×10^8 and 2×10^8 CFU/ml) were inoculated on Müller-Hinton agar. The surface of the agar plates was allowed to dry, and diffusion disks impregnated with chloramphenicol, ampicillin, and kanamycin were placed on it. After an overnight incubation at 37°C, the inhibition zones were checked.

2.3.7 Physical Transformation of *A. baumannii*

Bacterial transformation via electroporation is a method to introduce plasmid or linear DNA into a recipient bacterium by applying an electric field to the cells, which changes their permeability and allows DNA to enter. Before performing electroporation cells need to be washed three times with a non-ionic solution such as glycerol 10% to remove all traces of ions present in the growth media.

We followed *Jacobs et al.*⁸² protocol to prepare and electroporate the *A. baumannii* cells. Briefly, overnight *A. baumannii* 19606 cultures were diluted 1 : 100 into fresh LB and grown at 37 °C, 220 rpm until OD₆₀₀ around 5. Cells were harvested (10,000 g, 10 min, room temperature), washed twice with 10 % (v/v) sterile glycerol kept at ambient temperature (no ice), and resuspended to 50 µL per transformation. For each electroporation, 50 µL of competent cells was mixed with 1 µL of plasmid DNA (~25 ng) and transferred to a room-temperature 1 mm gap cuvette. Cells were pulsed at 1,800 V, 100 Ω, 25 µF (Gene Pulser Xcell™; time constant ≈ 5). Immediately, 950 µL of pre-warmed (37 °C) SOC was added, and the suspension was incubated for 1 h at 37 °C and 220 rpm. Finally, 100 µL were plated on LB agar containing the appropriate antibiotic and incubated overnight at 37 °C. The transformed colonies were picked and inoculated in the appropriate growth medium. Plasmids were purified from the *A. baumannii* host using the *Qlprep Spin Miniprep Kit* (Qiagen), quantified through the *Nanodrop Spectrophotometer* (Thermo Scientific), and sequenced by *Eurofins Genomics* (see Table A-2, Appendix).

2.3.8 Microplate Reader Assay

Bacterial growth (optical density - OD, absorption at 600 nm), RFP fluorescence (excitation 535 nm and emission 610 nm), GFP fluorescence (excitation 485 nm and emission 520 nm) were determined through *Varioskan™ LUX multimode microplate reader* (Thermo Scientific). The overnight culture was diluted 1:100 in LB media containing the appropriate antibiotics in a 96-well lidded plate. The plates were maintained at 37 °C with shaking at 300rpm every 10 minutes, immediately after the sampling step. The OD₆₀₀ and fluorescence of each well were acquired for either 14 hours or 16 hours. Growth rate was computed through linear fitting of the log(OD) over the exponential growth phase OD₆₀₀ between 0.05 and 0.18 for *E.coli* and between 0.02 and 0.2 for *A. baumannii*, upon subtraction of the background absorption of the media provided by cells filled with only media without cells, while RFP and GFP production were determined as average RFP/OD₆₀₀ and GFP/OD₆₀₀ signal along the same phase.

2.3.8.1 Crystal Violet Quantification

Air-dried, crystal-violet-stained biofilms were resuspended in 30% acetic acid to release the dye. The absorbance of the resulting solution was recorded as optical density at 592 nm (OD₅₉₂) using a Varioskan™ LUX multimode microplate reader (Thermo Scientific). Values were normalized to the OD₆₀₀ of the corresponding planktonic cultures.

2.3.8.2 Inducible Promoters Assays

To evaluate the functionality of inducible promoters, reporter constructs were tested in *Varioskan™ LUX multimode microplate reader* (Thermo Scientific) under increasing concentrations of their respective inducers. Serial dilutions of selective media were prepared with *N*-3-oxohexanoyl-*L*-homoserine, lactone (HSL; Sigma Aldrich), isopropyl-β-*D*-1-thiogalactopyranoside (IPTG; Sigma), or anhydrotetracycline (aTc; Thermo Fisher). Final concentrations in the assay ranged from 0.1 to 500 nM for HSL; 0.05 to 2 mM for IPTG, and 0 to 500 ng/mL for aTc. Bacterial cultures were harvested and prepared as described in *Microplate Reader Assay* section and inoculated into the assay plates at a 1:100 dilution. Fluorescence output was measured over time and normalized to OD₆₀₀, allowing the determination of promoter activity and induction dynamics across the tested concentration ranges.

2.3.8.3 Growth Inhibition Assays

To evaluate the growth inhibition caused by infection with engineered phages carrying either a dual-spacer CRISPRi cassette targeting both *bla_{ndm-1}* and *mcr-1* (ΦM13::CRISPRi_ndm-1_mcr-1), or a single-spacer cassette targeting *bla_{ndm-1}* (ΦM13::CRISPRi_ndm-1), or a single-spacer cassette targeting *mcr-1* (ΦM13::CRISPRi_mcr-1), kinetic growth inhibition assays were performed by measuring OD₆₀₀ over 16 hours, following the cycle described in the *Microplate Reader Assay* section. Three colonies from each condition, (I) transduced with the phage, (II) negative control *E. coli* F', and (III) the target strain *E. coli* F' carrying either *mcr-1_3K3* or *ndm-1_3K3*, were inoculated overnight and diluted 1:100 in LB medium supplemented with ampicillin and kanamycin. For the growth inhibition assay, the *mcr-1* strain was tested in the presence of 8, 4, 2, 1, 10⁻¹, and 0 µg/mL colistin (Merck; dissolved in sterile water). The growth inhibition assay for the *ndm-1* strain was performed using 500, 200, 100, 10, 1, and 0 µg/mL meropenem (Merck; dissolved in DMSO). Growth delay (Δt) was calculated by setting an OD₆₀₀ threshold of 0.1 and computing the difference between the time required to reach this threshold (*t*_{0,1}) for CRISPRi-transduced samples and the corresponding resistant control strain at the same antibiotic concentration:

$$\Delta t = t_{0,1;CRISPRi} - t_{0,1;resistant\ control}$$

The data obtained with the *Varioskan™ LUX multimode microplate reader* (Thermo Scientific) were analysed in Microsoft Excel and plotted using GraphPad Prism.

2.3.9 Confocal Microscopy

Square coverslips placed in a 6-well plate were coated with poly(D-lysine; Thermo Scientific). Specifically, 80 µL of poly(D-lysine) was added to each coverslip and incubated at room

temperature for 30 minutes. After incubation, the excess was removed and the coated coverslips were left to dry. Then, 200 μ L of an overnight bacterial culture was added to the coated coverslips. The plates were incubated at 37°C for 45 minutes to allow the adhesion of *A. baumannii* cells to the coverslips. Subsequently, the plates were washed with 1XPBS (Dulbecco) buffer and fixed for 10 minutes at room temperature using 4% paraformaldehyde solution. Finally, the coverslips were mounted onto slides by adding 70% glycerol.

These slides were first examined using the Leica DM IL LED Microscope to verify the presence of bacteria, and were subsequently visualized and imaged using the Nikon TE200-C1 (x60) for GFP (using a 488 nm laser) and RFP (using a 594 nm laser). The obtained pictures were analysed with ImageJ.

2.3.10 Biofilm Assay

Wild-type and engineered strains were cultured overnight in their respective media. The next morning, the OD₆₀₀ was recorded, and each culture was diluted to an OD₆₀₀ of 0.5 in 12 $\times$ 75 mm polystyrene tubes (Falcon). After incubation, the planktonic fraction was removed and its OD₆₀₀ measured. Biofilms that had developed at the air–liquid interface and adhered to the tube walls were rinsed once with 1XPBS (Dulbecco), then stained for 10 min with 0.1 % crystal violet in 20 % ethanol (Carlo Erba). Excess stain was removed by two additional 1XPBS washes. Biofilm biomass was subsequently quantified with a microplate reader, as detailed *Microplate Reader Assay* section.

2.3.11 RNA Extraction and Gene-Expression Analysis

To verify CRISPRi-mediated repression of *ompA*, total RNA was isolated from overnight cultures using the GRS Total RNA Kit – Bacteria (Grisp Research Solution). RNA concentration was determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific). Transcript levels were analysed via one-step reverse transcription real-time PCR employing the iTaq Universal SYBR Green One-Step Kit (Bio-Rad). For each sample, a 10 μ L reaction mix was prepared consisting of 5 μ L of iTaq™ Universal SYBR® Green Reaction Mix 2X, forward and reverse primers (*Table A-2*) diluted 1:100 from the original 100 μ M stock, 0.125 μ L of iScript™ Reverse Transcriptase, 50 ng of RNA, and nuclease-free water to the final volume. Thermal cycling was performed on a QuantStudio™ 3 System (Thermo Fisher Scientific) using the following conditions: reverse transcription at 50 °C for 2 minutes, reverse transcriptase inactivation and initial denaturation at 95 °C for 10 minutes, followed by 40 amplification cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. Melt curve analysis was then performed at 95 °C for 15 seconds, 60 °C for 1 minute, and at 95 °C for 1 second. Relative expression was calculated from Δ Ct values, normalized to a housekeeping gene, and expressed using the $\Delta\Delta$ Ct method, with the *A. baumannii* wild-type strain serving as the reference.

The data were analysed in Microsoft Excel and plotted using GraphPad Prism.

2.3.12 Phage Production

The production of phage particles varies depending on whether a helper plasmid and phagemid or a helper phage and phagemid are used. Consequently, the protocols for phage production differ, though the purification process remains distinct.

2.3.12.1 Phage Production Based on Helper Plasmid and Phagemid Interaction

The phagemid pTZ19R carrying the circuits of interest was transformed chemically in *E. coli* DH5 α harbouring the helper plasmid m13cp and plated on Amp⁺-Cm⁺-2XYT agar and maintained at 30°C for 48 hours. The obtained colonies were inoculated in 3 mL Amp⁺-Cm⁺-2XYT, incubated at 30°C and 250 rpm overnight. 0.5 ml of the overnight culture was inoculated into 50 ml Amp⁺-Cm⁺-2XYT in a 250 ml flask and incubated for an additional 24 hours at 250 rpm shaking overnight.

2.3.12.2 Phage Production Based on Helper Phage and Phagemid Interaction

A colony of *E. coli* TOP10 F' harbouring the phagemid pTZ19R, carrying the circuits of interest, was inoculated into 50 ml of LB + Amp⁺ medium and incubated at 37°C with shaking at 250 rpm until the OD₆₀₀ was above 0.05. When the culture appeared slightly turbid, 50 μ l of the helper phage M13KO7 (Invitrogen) was added and incubated for 60 to 90 minutes at 37°C with shaking at 250 rpm. After the incubation, 75 μ l of Kan was added to the flask, which was then incubated for an additional 14-18 hours at 37°C with shaking at 250 rpm.

2.3.13 Phage Purification

The overnight inoculum was pelleted and the supernatant fluid containing the phagemid particles was passed through a 0.2 μ m filter. For the purification, the engineered particles were precipitated by adding 10% polyethylene glycol (PEG-8,000, Thermo Scientific) and 1M NaCl (Carlo Erba), incubated overnight at 4°C, and centrifuged at 12,000 g for 1 hour. The pellet was resuspended with $\frac{1}{100}$ volume SM buffer (50 mM Tris-HCl [pH 7.5]), Merck; 100 mM NaCl, Carlo Erba; 10 mM MgSO₄, Merck) and stocked at -80°C.

2.3.14 Transduction assay

To evaluate the efficiency of the transduction-based delivery method, a transduction assay was performed by infecting *E. coli* TOP10 F'. The bacterial culture was grown to an OD₆₀₀ of 0.6 (avoiding growth to saturation, as this increases the risk of losing the pili encoded by the F' plasmid). At this point, 100 μ L of the phage resuspension was mixed with 100 μ L of the bacterial culture. A mock infection was also performed to assess bacterial viability by mixing 100 μ L of the bacterial suspension with 100 μ L of SM buffer. After a static incubation at room temperature for 30 minutes, the transduced cells were serially diluted (10^{-3} , 10^{-4}) in PBS 1X (Dulbecco) or LB medium, and both undiluted and diluted samples were plated on selective LB agar. Plates were incubated statically at 37 °C overnight.

To assess the repression efficiency of CRISPRi constructs targeting *mcr-1* and *bla_{ndm-1}*, *E. coli* F' strains harbouring either *mcr-1*_3K3 or *ndm-1*_3K3 plasmids were infected with engineered phage suspensions carrying either a dual-spacer CRISPRi cassette targeting both *bla_{ndm-1}* and *mcr-1* (ΦM13::CRISPRi_nda-1_mcr-1), a single-spacer cassette targeting *nda-1* (ΦM13::CRISPRi_nda-1), or a single-spacer cassette targeting *mcr-1* (ΦM13::CRISPRi_mcr-1). The resulting colonies were analysed using a kinetic microplate reader to determine the growth inhibition by measuring OD₆₀₀, as described in *Microplate Reader Assay* section.

To evaluate the delivery and silencing ability of CRISPRi targeting RFP, the same transduction assay was performed by infecting *E. coli* TOP10 F' carrying pSGAb_pTet, a plasmid containing the target gene for silencing. Colonies growing on selective plates were analysed using a kinetic microplate reader to quantify emitted red and green fluorescence.

Transduction efficiency for the *rfp* delivery was calculated with the following formula:

$$Efficiency (\%) = \frac{CFU/mL (red colonies transduced on LB)}{CFU/mL (red + white colonies transduced on LB)} \times 100$$

Purity of the phage preparation was calculated as:

$$Purity (\%) = \frac{CFU/mL (colonies transduced on LB Amp^+)}{CFU/mL (colonies transduced on LB Kan^+)} \times 100$$

2.3.15 Colony PCR

To verify the expression of the *rfp* and possible contamination of the transduced cells, we performed colony PCR after the transduction assay.

The transduced cells were inoculated in 200 µL of deionized water and incubated at 100°C for 15 minutes in a water bath. The lysate cells were centrifuged at 3.000 rpm for 10 minutes. 5 µL were added to the PCR mix Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific), and the amplified sequences were run in a 1% agarose gel (see *Table A-3*).

2.3.16 Phage Genome Extraction and Colony PCR

To evaluate potential contamination during phage production, the engineered phage genome was extracted using the Maxwell® RSC 48 (Promega) extractor and the Maxwell® RSC Viral Total Nucleic Acid (Promega) kit. 5 µL of the extracted DNA were added to the PCR mix Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific). The amplified sequences were run on a 1% agarose gel.

Additionally, to test for possible contamination from residual phagemid or bacterial DNA, a treatment with DNase I (Roche) was performed. The phage suspension was treated with 2U of DNase I, incubated at room temperature for 1 hour, and 1 µL of EDTA 5M, pH 8, was added. The mixture was inactivated at 75°C for 10 minutes, and 2 µL was loaded into the PCR mix.

2.3.17 Quantification of Phage Titre via qPCR

Firstly, a standard curve with pTZ19 carrying the *rfp* gene was performed. The template samples for the calibration curve were serially diluted in PCR-grade water with 1% Salmon Sperm DNA to stabilize the plasmid. Each 25 μ L reaction mix consisted of 12.5 μ L of TaqMan Universal PCR master mix (Applied Biosystems), 10 μ L of DNA (sample or quantification control), and forward and reverse primers and probe (*Table A-3*) at a concentration of 600 nM. The PCR parameters were 50°C for 2 minutes, 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 62°C for 1 minute. All assays were performed on a QuantStudio 7 Pro Real-Time PCR System thermal cycler.

2.4 Results

The results are presented in two sections: (SI) the actuator machinery and (SII) the delivery platform.

2.4.1 Section I: The Actuator Machinery

In this section, all results concerning the actuator machinery are reported, particularly the characterization of genetic parts, such as expression plasmids in *A. baumannii*, and the CRISPRi system in a shuttle vector, first tested in *E. coli* and subsequently in *A. baumannii*. Finally, the CRISPRi system was applied to target a virulence factor in *A. baumannii*.

2.4.1.1 Development of a Toolkit for *A. baumannii*: Characterization of Genetic Parts

Successful implementation of CRISPRi in *Acinetobacter baumannii* required the prior development of a well-characterized genetic toolkit. Considering the limited availability of standardized parts and systems in *A. baumannii* compared to model organisms such as *E. coli*, we undertook the task of assembling and validating a modular library of genetic elements that would support robust and tuneable CRISPRi functionality.

2.4.1.1.1 Electroporated *A. baumannii* Expressed a Red Fluorescent Protein

To express genes and study genetic parts in *A. baumannii*, a suitable plasmid for expression in the pathogen was identified. Given that *A. baumannii* presents a complex antimicrobial resistance profile, we performed a Kirby-Bauer test to determine its susceptibility to ampicillin, chloramphenicol, and kanamycin. As shown by the inhibition zones in the Figure 2-2, the strain is sensitive only to kanamycin, which we selected as the marker for the expression plasmid.

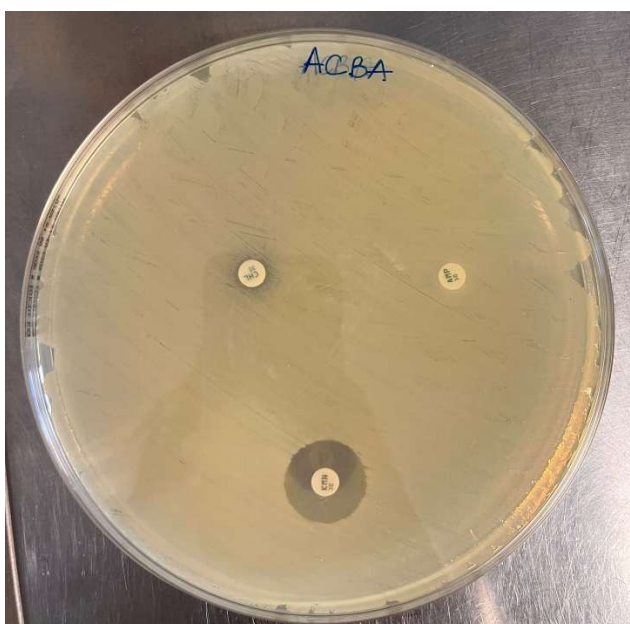


Figure 2-2: Kirby-Bauer test performed on *A. baumannii* ATCC 19606.

We then electroporated *A. baumannii* following the protocol proposed by Jacobs *et al.*⁸². Firstly, we electroporated *A. baumannii* with the expression plasmid pSGAb, which carries a red fluorescent protein under two different promoters: pTet, a strong inducible promoter in *E. coli*, and J23119, a weaker constitutive promoter in *E. coli*. Successful electroporation of *A. baumannii* was confirmed by the appearance of red colonies on the selective plates (Figure 2-3).

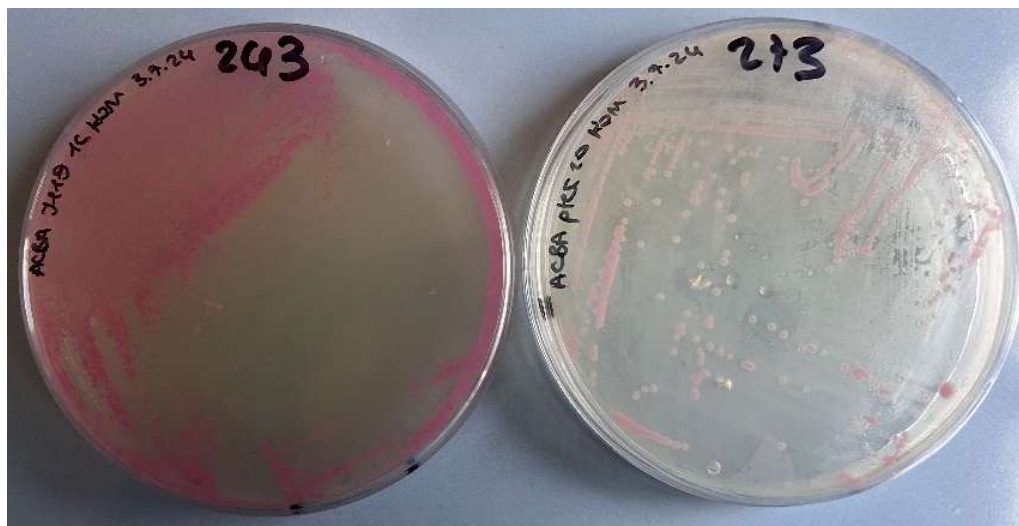


Figure 2-3: Electroporated *Acinetobacter baumannii* with the *E. coli*-*A. baumannii* shuttle vector. On the right: *A. baumannii* expressing *rfp* under the control of J23119 (pSGAb_J23199) on the left: *A. baumannii* expressing *rfp* under the control of pTet (pSGAb_pTet).

To confirm the results, we picked three colonies from each plate and tested their fluorescence using a kinetic plate reader assay. RFP fluorescence was normalized to OD₆₀₀ values measured during the exponential growth phase (OD₆₀₀ between 0.02 and 0.2) to ensure accurate per-cell expression analysis and to avoid artifacts associated with low signal or nonlinear OD readings at higher cell densities. Interestingly, the expression of *rfp* under the constitutive promoter (pSGAb_J23119 see the Appendix A4) was six-fold higher than that of *rfp* expressed under the control of the inducible promoter (pSGAb_pTet see the Appendix A4). The pSGAb_pTet strain exhibits moderate fluorescence, demonstrating that the inducible promoter is functional but less effective than the constitutive promoter under the conditions tested. Furthermore, the *A. baumannii* wt control group (in green in Figure 2-4) shows no fluorescence signal, confirming the specificity of *rfp* expression in the experimental groups. In addition, a high variability in the expression of the *rfp* is observed, but it still needs to be clarified.

The red fluorescence signal was also confirmed through visualization at 60x confocal microscopy of *A. baumannii* bearing expression plasmids pSGAb_pTet (see the Appendix A4).

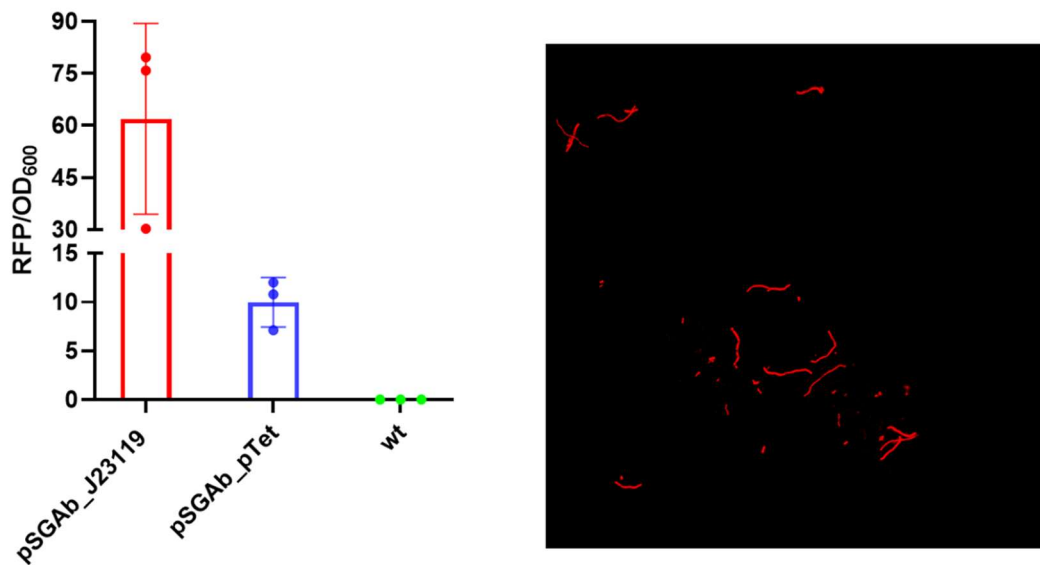


Figure 2-4: Expression of the red fluorescent protein in *A. baumannii*. On the right the pTet *rfp* strain exhibits moderate fluorescence than the constitutive promoter under the conditions tested. On the left, a visualization at 60x confocal microscopy of *A. baumannii* expressing the *rfp* gene under the control of pTet.

In addition to pSGAb-km⁷³, pME6032⁸³ is one of the two plasmids with origins of replication reported in the literature to function reliably for replication and gene expression in *A. baumannii*. pME6032 is an *E. coli*–*Acinetobacter* shuttle vector commonly used for gene expression in both hosts. pME6032 is a *E. coli*–*Acinetobacter* shuttle vector used for gene expression in both organisms. It was modified to create pME6032_pTet, which includes the *blaR* gene for resistance to ampicillin and carbenicillin. This plasmid has the p15A origin of replication for *E. coli* and pVS1 for *A. baumannii*⁸³.

Both plasmids were modified in order to express the same reporter gene, specifically, pSGAb_pTet and pME6032_pTet (see the *Appendix A4*) were engineered to express the same red fluorescent protein cassette under the control of the pTet promoter. Since they both harbor the same constitutive expression cassette, they can be directly compared. As shown in *Figure 2-5*, in both strains, pSGAb_pTet (blue bars, see the *Appendix A4*) produced similar RFP/OD₆₀₀ values. However, when *rfp* was carried on the pME6032 backbone (red bars, see the *Appendix A4*), expression of the red fluorescent protein was markedly lower, specifically about 6-fold lower in *A. baumannii* compared with pSGAb_pTet (see the *Appendix A4*), likely due to the reduced copy number of pME6032 in this species.

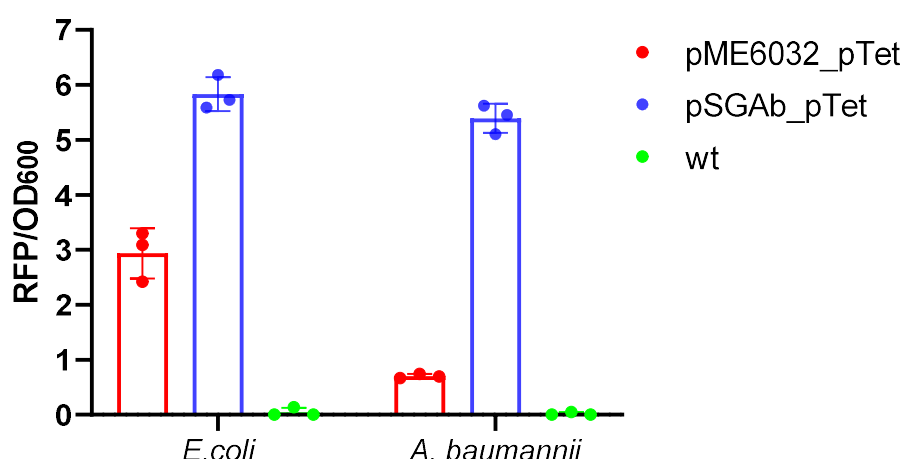


Figure 2-5: *rfp* expression from different plasmid backbones in *E. coli* and *A. baumannii*. pSGAb_pTet produced similar RFP/OD₆₀₀ values in both strains, whereas pME6032 showed markedly reduced expression (~6-fold lower in *A. baumannii*).

2.4.1.1.2 Constitutive Promoters

A selection of the promoters from the Anderson library in the iGEM Registry⁷⁸ were tested in *Acinetobacter baumannii* to characterize their strength and assess their potential for future synthetic biology applications. The Anderson library, developed by Chris Anderson, consists of 20 promoters that have been previously characterized in *E. coli*. To evaluate their relative strength in *A. baumannii*, fluorescence output was measured for each promoter. In this work, 13 different promoters driving expression of the *rfp* gene were tested. While both the iGEM Registry and the Anderson promoter library are based on the BioBrick standard⁸⁴, a difference lies in the placement of the *SpeI* restriction site, which results in different cloning architectures. In standard iGEM Registry plasmids, the *SpeI* site is located downstream of the gene terminator, following the conventional BioBrick format. In contrast, the Anderson library places the *SpeI* site between the promoter and the ribosome binding site. This design allows for a different cloning setup, enabling the easy removal of the RBS, gene, and terminator as a single fragment, effectively isolating the promoter along with the plasmid backbone. This makes the Anderson library particularly useful when swapping or characterizing promoters independently of downstream elements, as illustrated in Figure 2-6.

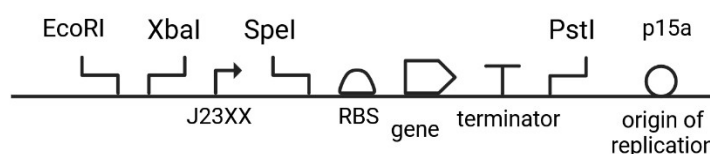


Figure 2-6: Anderson library construct showing the *SpeI* site placed between the promoter (J23XX) and the RBS, allowing easy removal of the RBS, gene, and terminator while retaining the promoter and backbone.

The pSGAb-set of plasmids carrying the selected Anderson promoter upstream the *rfp* gene (see the *Appendix A4*) were tested to evaluate promoter activity, using RFP/OD₆₀₀ fluorescence per cell as a measure of relative promoter strength. Fluorescence values for each promoter were normalized to the pSGAb plasmid carrying the promoter J23100. The results, shown in *Figure 2-7*, indicate that all tested promoters except J23112, J23113, and J23117 are functional in *A. baumannii*. Among them, J23100, J23101, and J23118 produced the highest fluorescence signals.

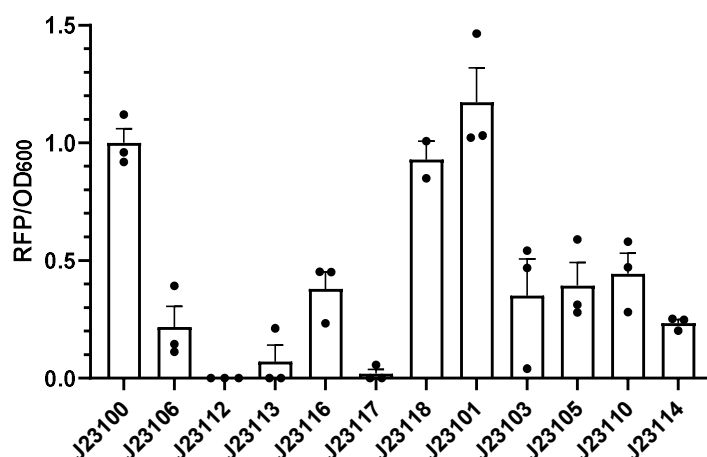


Figure 2-7: Activity of Anderson promoters in A. baumannii. RFP/OD₆₀₀ values were normalized to promoter J23100. All promoters except J23112, J23113, and J23117 were active, with J23100, J23101, and J23118 showing the strongest expression.

When compared to promoter strength data reported in the iGEM Registry of Standard Biological Parts (*Table 2-1*), J23100, J23101, and J23118 consistently exhibit high expression in both *A. baumannii* and *E. coli*. Notably, J23103, which is weak in *E. coli*, shows moderate strength in *A. baumannii*, suggesting species-specific differences in promoter performance.

Promoter	<i>A. baumannii</i> promoter strength	<i>E.coli</i> promoter strength
J23100	1	1
J23106	0.22	0.47
J23112	0	0
J23113	0	0.01
J23116	0.38	0.16
J23117	0	0.06
J23118	0.93	0.56
J23101	1.17	0.7
J23103	0.35	0.01
J23105	0.39	0.24
J23110	0.44	0.33
J23114	0.23	0.1

Table 2-1: Comparison of relative promoter strengths between *A. baumannii* and *E. coli*. Promoter activity was measured using normalized RFP/OD600 values, with J23100 set as the reference in both organisms. While some promoters (e.g., J23100, J23101, J23118) show strong expression in both species, others (e.g., J23103, J23116) display different activity profiles, highlighting species-specific variations in promoter performance.

2.4.1.1.3 Inducible Gene Expression Systems

To enable tuneable gene expression in *Acinetobacter baumannii*, we developed and validated three inducible systems based on commonly used bacterial regulators: aTc, IPTG, and HSL. Each system was designed to control the expression of a *rfp* reporter and serves as a template for future applications, such as controlled expression of *dCas9* or guide RNAs.

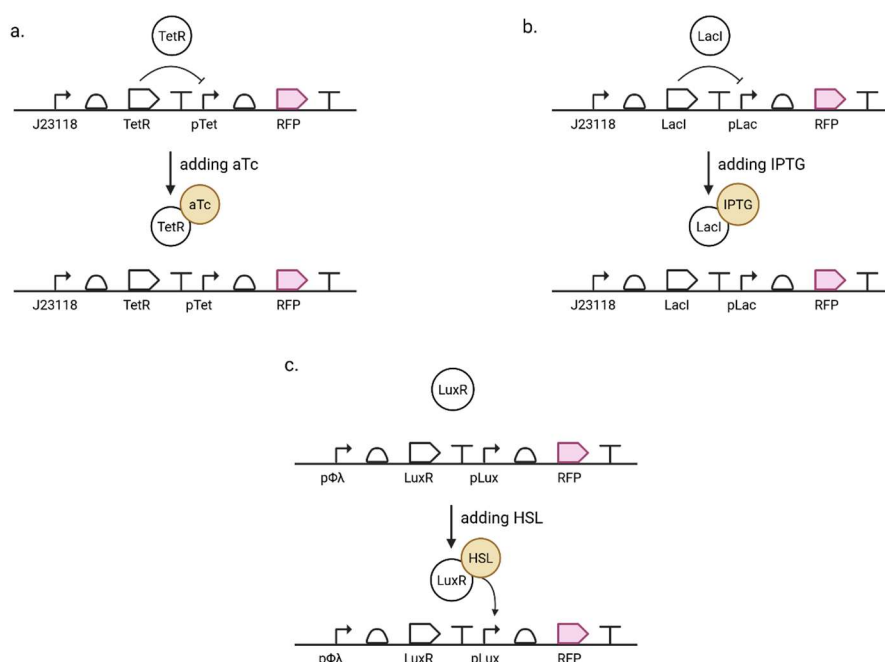


Figure 2-8: Inducible systems controlling *rfp* expression. (a) aTc induces *rfp* by inhibiting TetR repression on *pTet*. (b) IPTG induces *rfp* by inhibiting LacI repression on the *pLac* promoter. (c) HSL activates LuxR, which induces *pLux*, leading to *rfp* repression via *pLux*.

aTc-Inducible System

This system uses the tetracycline repressor (*tetR*), which is constitutively expressed and keeps the downstream pTet promoter inactive under normal conditions. *rfp* is placed under the control of the pTet promoter. In the absence of aTc, TetR binds to pTet and prevents transcription. When aTc is added, it binds to TetR and prevents it from repressing the promoter, allowing *rfp* expression to be turned on (*Figure 2-8*, panel a).

To characterize the functionality of the aTc-inducible cassette in *A. baumannii*, we tested a range of aTc concentrations from 0 to 500 ng/mL. Since aTc is an analogue of tetracycline and can cause toxic, antibiotic-like effects at high concentrations, we evaluated the growth curve over 16 hours using a kinetic plate reader assay. OD₆₀₀ measurements showed that the growth of engineered *A. baumannii* strains remained largely unaffected up to 100 ng/mL but decreased notably at 500 ng/mL (*Figure 2-8*, panel a). Based on these results, we measured the fluorescence emitted by RFP to investigate the performance of the aTc-inducible system in *A. baumannii*. RFP fluorescence was normalized to OD₆₀₀ values within the exponential growth phase (OD₆₀₀ = 0.2–0.2). Despite the reduction in growth rate at higher concentrations, we observed a clear increase in RFP/OD₆₀₀ values with rising aTc levels, indicating effective induction of *rfp* expression (*Figure 2-8*, panel b). When we compared the RFP/OD₆₀₀ of bacteria carrying the inducible cassette to that of the strain carrying pSGAb_pTet (*rfp* under the constitutive promoter), we found that the inducible system did not reach the same expression levels, even at the highest aTc concentration (see *Figure 2-9*, panel b and *Figure 2-4*). This suggests functional differences between the two expression systems.

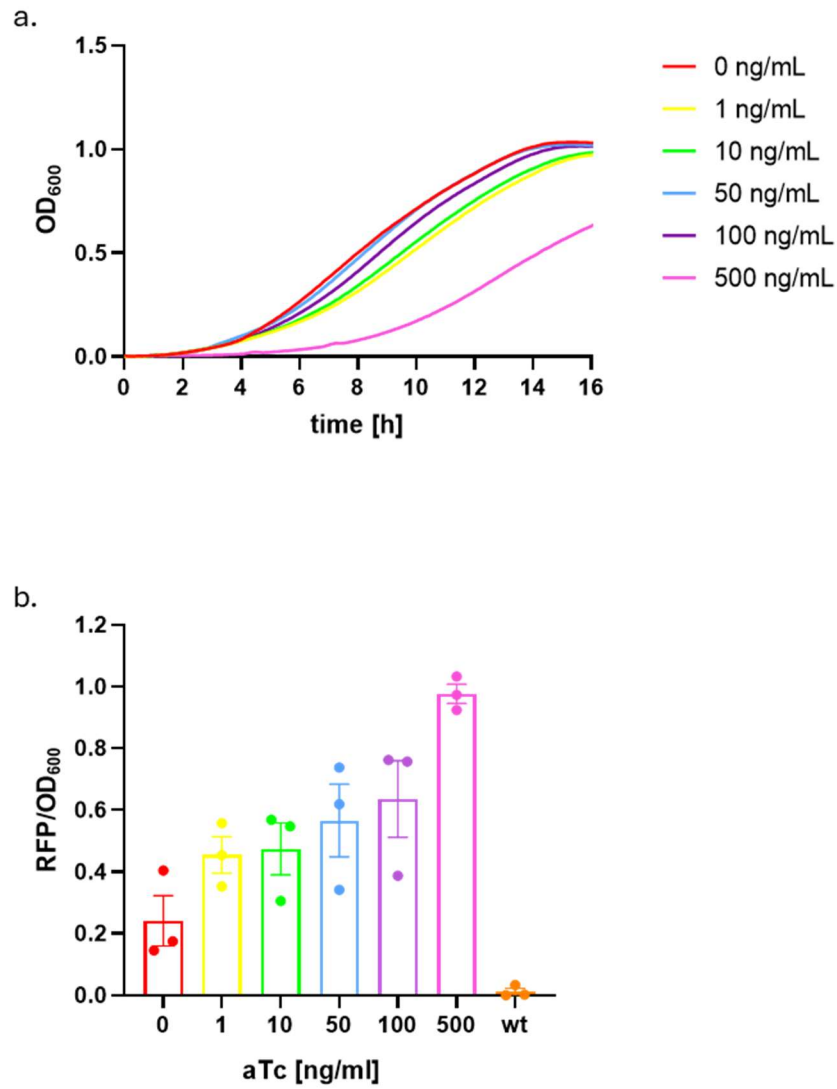


Figure 2-9: Effect of aTc induction on growth and *rfp* expression in engineered *A. baumannii*. (a) Growth curves monitored over 16 h at OD₆₀₀ in the presence of aTc concentrations ranging from 0 to 500 ng/mL. (b) *rfp* expression normalized to OD₆₀₀ during the exponential phase (OD₆₀₀ 0.02–0.2).

Finally, we examined the dynamics of RFP production and degradation over time, rather than considering only the exponential phase. By analysing RFP/OD₆₀₀ values across the growth curve (Figure 2-10), we identified the phases during which each system reached steady-state expression. As expected, the constitutive system (pSGAb_pTet) showed stable expression mainly during exponential growth, followed by a plateau in the stationary phase. In contrast, the aTc-inducible system maintained consistent RFP/OD₆₀₀ values throughout both exponential and stationary phases, suggesting more sustained activity across the entire growth curve.

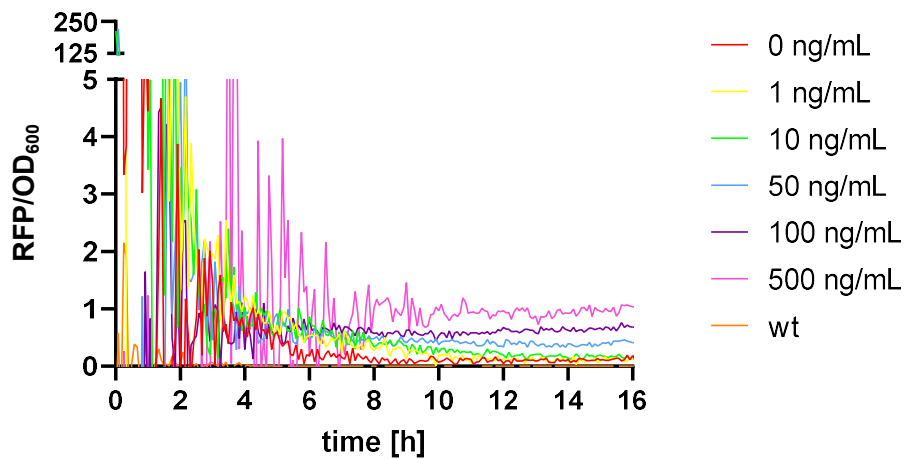


Figure 2-10: Normalized RFP/OD₆₀₀ values measured across the entire growth curve of engineered strains exposed to different aTc concentrations (0–500 ng/mL) compared to wild type.

IPTG-Inducible System

The LacI repressor is constitutively produced and inhibits the pLac promoter upstream of the *rfp* gene. In the absence of IPTG, LacI binds to pLac and blocks transcription. Upon IPTG addition, LacI is inactivated, releasing the promoter and enabling *rfp* expression (Figure 2-8, panel b). *A. baumannii* carrying the IPTG-inducible system was tested in the presence of IPTG to evaluate changes in RFP fluorescence compared to the wild-type *A. baumannii* strain. The experiment was performed across a range of IPTG concentrations (0–2 mM), and RFP fluorescence was normalized to OD₆₀₀ values within the exponential phase. As shown in Figure 2-11 panel a, increasing IPTG concentrations did not produce a consistent trend in RFP/OD₆₀₀. Moreover, even without IPTG (no induction), the engineered strain exhibited significantly higher RFP/OD₆₀₀ values than the wild type. These findings suggest that the *lacI* gene, expressed under the chosen ribosome binding site, is only weakly active in *A. baumannii*, leading to insufficient repression and consequently elevated RFP/OD₆₀₀ levels. Time-course analysis of RFP/OD₆₀₀ further showed that the system remains active throughout exponential growth, maintaining high expression across all tested IPTG concentrations (Figure 2-11, panel b).

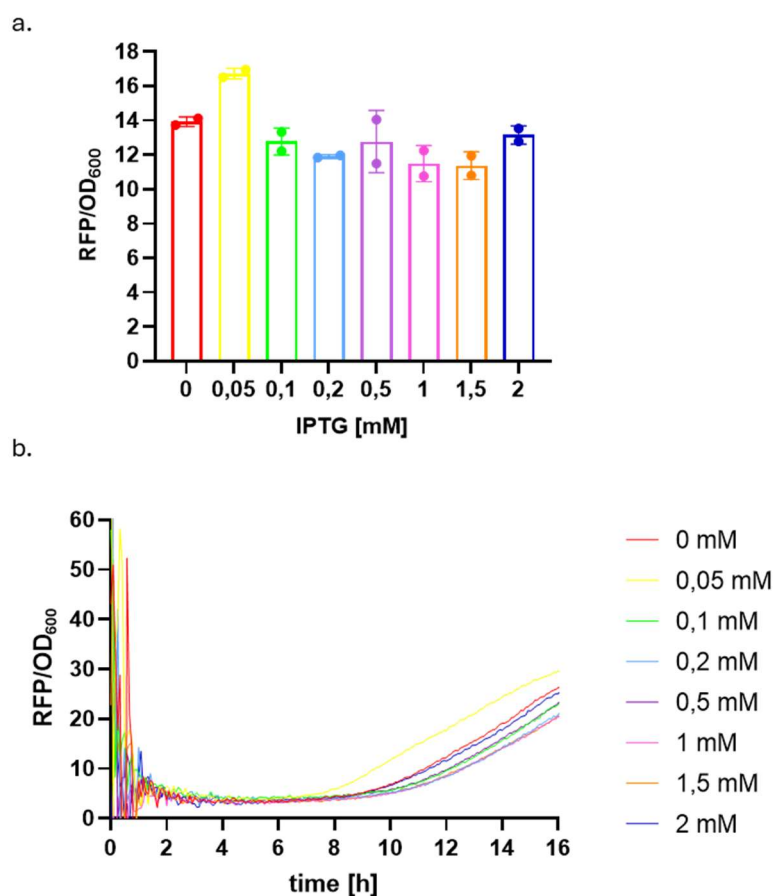


Figure 2-11: Characterization of the IPTG inducible system in *A. baumannii*. (a) Normalized RFP/OD₆₀₀ values measured in the exponential phase of engineered and wild-type strains over 16 h in the presence of increasing IPTG concentrations (0–2 mM). (b) Normalized RFP/OD₆₀₀ values measured across the entire growth curve.

HSL-Inducible System

This system is based on quorum sensing. The LuxR transcriptional regulator is constitutively expressed and, upon binding to its signal molecule HSL (homoserine lactone), forms an active complex that can bind to and activate the pLuxR promoter (Figure 2-8, panel c). *rfp* is expressed from this promoter only in the presence of both LuxR and HSL, enabling precise control based on cell–cell signalling inputs. A range of HSL concentrations, from 0 to 10⁴ nM, was tested. The experiment shown in the Figure 2-12 demonstrates that the HSL-inducible promoter effectively induces *rfp* expression in *A. baumannii*. Both very low (1 nM and 10 nM) and very high (10⁴ nM) concentrations of HSL result in high *rfp* expression, indicating strong promoter activation. Specifically, the cells harbouring the HSL-circuit induced with 10³ and 10⁴ nM HSL exhibited up to a 50-fold increase in RFP fluorescence compared to the uninduced control, which showed minimal *rfp* expression, confirming the specificity and inducibility of the HSL-responsive system.

This data supports the conclusion that the HSL-inducible system is functional in *A. baumannii* and capable of robustly driving gene expression in response to the signalling

molecule. As shown, the promoter exhibits strong responsiveness across a broad range of HSL concentrations, with the highest expression levels observed at both low (1 nM, 10 nM) and high (10^4 nM) inducer concentrations.

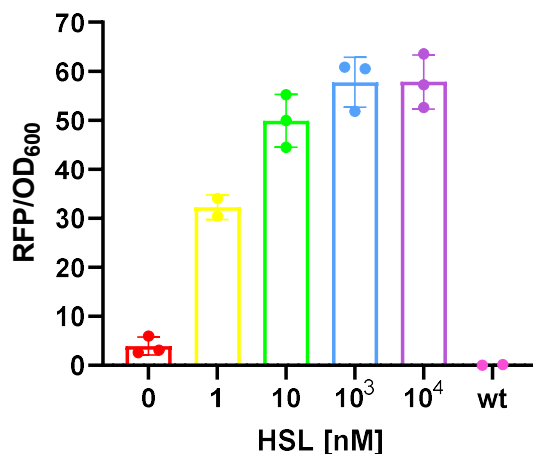


Figure 2-12: Normalized RFP/OD₆₀₀ values of engineered *A. baumannii* strains exposed to increasing concentrations of HSL (0–500 nM). Fluorescence was measured during the exponential growth phase and represents the induction of *rfp* expression in response to HSL.

2.4.1.2 The CRISPRi in *A. baumannii*

The ongoing advancements in CRISPR interference technology have shown promising potential in the study and manipulation of bacterial genomes, including that of the pathogen *Acinetobacter baumannii*. This bacterium is known for its multi-drug resistance and its ability to cause severe infections, making it a critical target for novel therapeutic approaches. In the following section, we examine the significant advancements in utilizing CRISPRi technology with *A. baumannii*.

2.4.1.2.1 Characterization of CRISPR Interference

Once the optimization protocol was established and the plasmid's functionality in *A. baumannii* was confirmed, we proceeded to clone all the necessary cassettes to study and investigate CRISPR interference. Initially, we tested the constructs in *E. coli*, where all the cloning procedures were assessed. Subsequently, we moved on to *A. baumannii* for further evaluation. To characterize the system, we decided to silence the *rfp*. To achieve this:

- a two-plasmid system can be designed and built in which the CRISPRi can be cloned in a backbone and the target in another one;
- a single plasmid system bearing the *dCas9* cassette, the sgRNA(s), and the *rfp* cassette.

We opted to develop the single plasmid system due to the limited understanding of the functional components and backbone structure in *A. baumannii*.

The circuits were built in the *E.coli-A. baumannii* shuttle vector (pSGAb) are represented in the *Figure 2-13* and are the following:

- pSGAb_dCas9: the *dCas9* cassette under the constitutive promoter J23116, and it was constructed to assess its toxicity, as it is known that the *dCas9* alone, without the sgRNA, can be toxic to the bacterial cells (see the *Appendix A4*).
- pSGAb_pTet: *rfp* gene under the inducible promoter pTet, the target (see the *Appendix A4*);
- pSGAb_CRISPRi the CRISPRi made of a *gfp* cassette as a metabolic burden indicator, an sgRNA targeting the *rfp* (specifically the pTet promoter responsible for the expression of the reporter gene) under the LuxI promoter inducible by HSL, and a *dCas9* under the control of the constitutive promoter J23116 and the *rfp* under the pTet promoter (see the *Appendix A4*);
- pSGAb_sgpLac harbours a *gfp* as a metabolic burden indicator, a sgRNA targeting the pLac promoter under the LuxI promoter inducible by HSL, and a *dCas9* under the control of the constitutive promoter J23116 and the *rfp* under the pTet promoter (see the *Appendix A4*).

This latter plasmid is designed as a control to investigate whether the reduction in gene expression (such as lower RFP signal) observed in the CRISPRi system is due to the actual gene repression or simply a consequence of metabolic burden caused by the expression of CRISPRi components. Specifically, metabolic burden refers to the stress placed on the cell when it has to produce large amounts of non-essential proteins, such as *dCas9*, sgRNAs, or reporter genes like *rfp*. This extra demand on cellular resources such as energy, ribosomes, and amino acids can slow down growth and reduce the expression of other genes, not because of targeted repression, but because the cell is simply overwhelmed. To distinguish this effect, the sgRNA in this plasmid targets an unrelated promoter (pLac) that does not control any gene in the system. This means the CRISPRi machinery is active (*dCas9* binds sgRNA and searches for a target), but no actual gene repression occurs. If a decrease in GFP (the burden indicator) is observed, it suggests that the CRISPRi system itself imposes a metabolic burden. Conversely, if only the targeted CRISPRi system (e.g., sgRNA targeting *rfp*) causes a drastic reduction in RFP but not in GFP, then the effect is likely due to true gene repression.

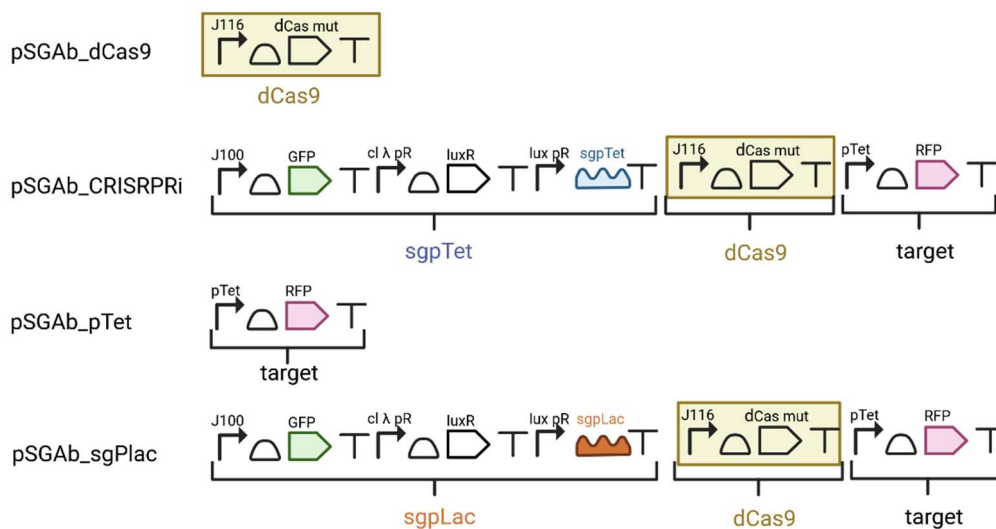


Figure 2-13: The circuits built in the *E.coli*-*A. baumannii* shuttle vector (pSGAb) to perform the silencing of the *rfp* through CRISPRi.

2.4.1.2.2 CRISPRi Characterization in *E. coli*

The Figure 2-14 - panel a presents the results of a microplate reader assay measuring the expression levels of red fluorescent protein normalized to the optical density at 600 nm in three different *E. coli* samples: the engineered with the pSGAb_CRISPRi (see the Appendix A4), with the pSGAb_pTet plasmid (positive control, see the Appendix A4), and the wild-type (negative control, see the Appendix A4). The RFP/OD₆₀₀ ratio of the pSGAb_CRISPRi is close to zero, indicating that the CRISPRi system effectively repressed the *rfp* expression in *E. coli*. This demonstrates that the CRISPRi system is functioning correctly and is able to repress the target gene expression. The RFP/OD₆₀₀ ratio of the strains carrying the pSGAb_pTet is higher compared to the other two samples. This serves as a positive control, confirming that the *E. coli* is capable of producing RFP when the pSGAb_pTet plasmid is present. In addition, the RFP/OD₆₀₀ ratio of the negative control is close to zero, indicating the absence of *rfp* expression. This validates that in the absence of the pSGAb_pTet plasmid, *E. coli* does not produce RFP.

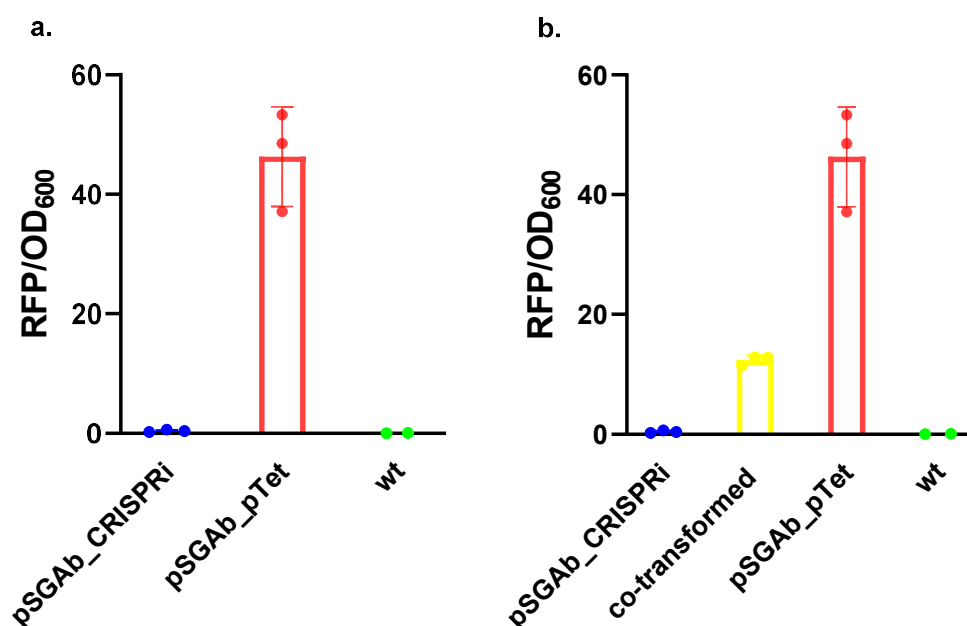


Figure 2-14: CRISPRi-mediated silencing of *rfp* expression in *E. coli*. (a.) Characterization of the CRISPRi targeting *rfp* in *E. coli*; (b.) Co-transformed *E. coli* with the CRISPRi system and a high-copy plasmid carrying an additional target.

To demonstrate that CRISPRi effectively silences the target gene and that the absence of the signal is not only due to the plasmid burden, we co-transformed *E. coli* with the CRISPRi plasmid (which carries a medium copy origin of replication, see the *Appendix A4*) and pSB1A2_I13521, a plasmid harbouring the *rfp* driven by pTet (with a high copy origin of replication, a pUC19-derived pMB1, see the *Appendix A4*). In this setup, we expected to detect lower repression due to the target gene being carried on a high-copy plasmid while the dCas9-sgRNA complex is on a medium-copy plasmid. In Figure 2-14 - *panel b*, the RFP/OD₆₀₀ ratio of the co-transformed strain is ≈ 15 , indicating a moderate level of *rfp* expression. This suggests that CRISPRi is still effective in reducing *rfp* expression in the co-transformed cells but not as completely as in the pSGAb-CRISPRi cells, likely due to the high copy number of the pSB1A2_I13521.

2.4.1.2.3 CRISPRi Characterization in *A. baumannii*

Based on the results obtained in *E. coli*, we proceeded to test the CRISPRi system in *Acinetobacter baumannii*. Firstly, we evaluated the effects of the constructs, particularly the one involving dCas9, on the growth of *A. baumannii*. As reported for *E. coli*, the expression of dCas9 alone in *A. baumannii* (red curve in Figure 2-15, see the Appendix A4) negatively impacts bacterial growth. However, the combined expression of dCas9 and the transcription of the sgRNA has a milder effect on growth. This observation suggests that while dCas9 expression alone can be harmful, the presence of sgRNA may mitigate some of these adverse effects.

To evaluate red fluorescence expression, we typically normalize the RFP signal to the corresponding OD₆₀₀, focusing on measurements taken during the exponential growth phase. This is crucial because cells in this phase are actively dividing and exhibit consistent physiological and metabolic states, providing more reliable and comparable fluorescence data.

For *E. coli*, the exponential phase is generally considered to fall within the OD₆₀₀ range of approximately 0.01 to 0.15. However, since wild-type *Acinetobacter baumannii* tends to grow more rapidly, we aimed to verify whether the exponential phase occurs over a different range.

To determine this, we analysed the growth curve of *A. baumannii* and plotted the natural logarithm (ln) of OD₆₀₀ to identify the exponential phase, which appears as a straight line in this representation. Based on this analysis, we decided to use a slightly broader OD₆₀₀ range from 0.02 to 0.2 that includes the linear region (Figure 2-15, panel b) as well as points just before and after it. This allows us to account for slight variations and ensures we capture the general dynamics of exponential growth without being restrictive.

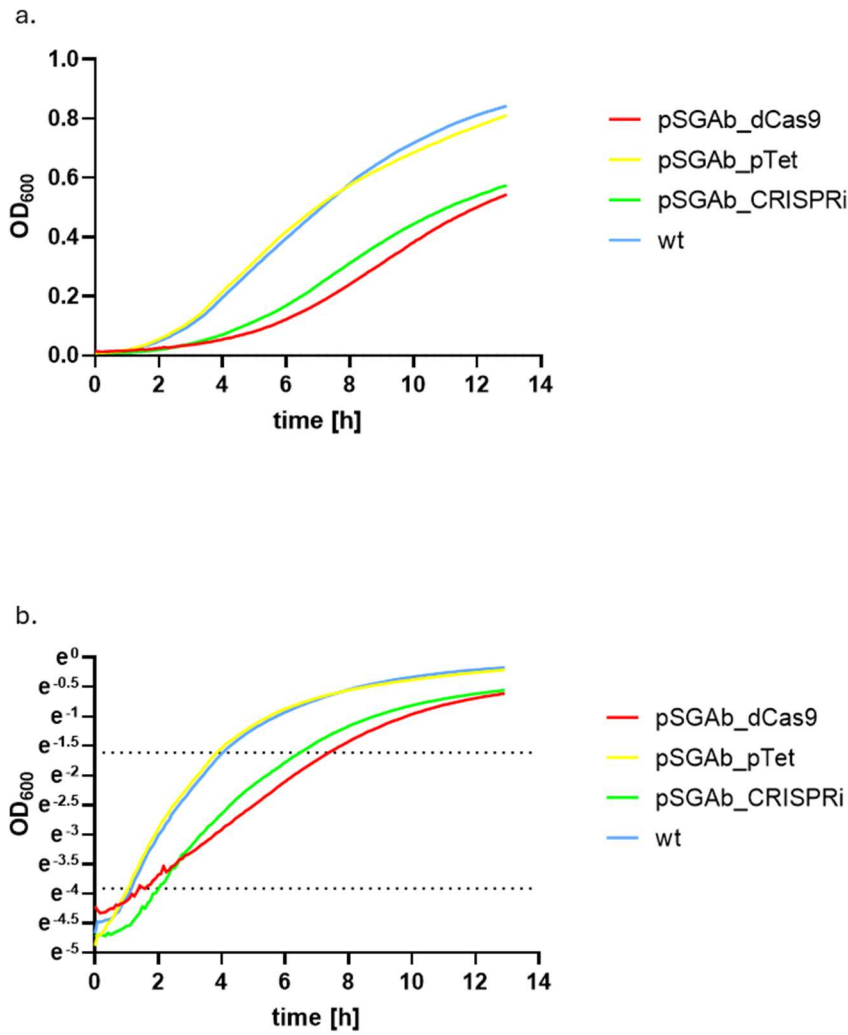


Figure 2-15: *A. baumannii* curve affected by the expression of the dCas9. a) Growth curves with natural (linear) scale on the y-axis (OD_{600}). b) Growth curves with logarithmic ($\ln$) scale on the y-axis (OD_{600}).

To evaluate the repression effect of the CRISPRi machinery, we analysed four different *A. baumannii* strains:

- pSGAb_CRISPRi, carrying the CRISPRi system, specifically a sgRNA targeting the promoter upstream of the *rfp* gene (pTet) and the target (*rfp*), used as the repressor strain (see the Appendix A4);
- pSGAb_pTet, used as the positive control for *rfp* expression without repression (see the Appendix A4);
- pSGAb_sgpLac, used as a negative control for repression, carrying a sgRNA targeting the unrelated pLac promoter (and not the pTet promoter upstream of *rfp*, see the Appendix A4);
- and the wild-type (wt) strain, which lacks any plasmid (see the Appendix A4).

To quantify gene expression, RFP fluorescence was normalized to optical density (RFP/OD_{600}) to account for differences in cell density. As shown in Figure 2-16, the pSGAb_CRISPRi strain

exhibited a two-fold decrease in *rfp* expression compared to the positive control. Furthermore, the positive control and the negative control (pSGAb_sgPlac) showed similar levels of *rfp* expression, indicating that the observed repression is not due to potential metabolic burden caused by the presence of the CRISPRi system components.

Metabolic burden was monitored via *gfp* expression (Figure 2-16, panel a; Figure 2-17), which serves as a burden reporter. The increased *gfp* expression in the pSGAb_sgPlac strain confirms that the CRISPRi system introduces a metabolic load, even when no gene is being targeted. However, *rfp* expression remains high in this condition, suggesting that metabolic burden alone does not affect *rfp* expression. In contrast, the reduced RFP fluorescence the pSGAb_CRISPRi strain is primarily the result of specific CRISPRi-mediated repression, rather than non-specific effects due to cellular stress or resource competition.

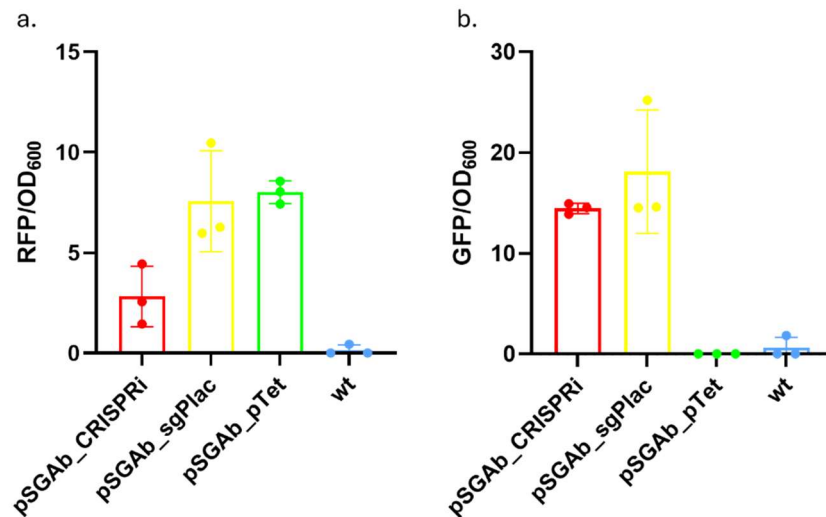


Figure 2-16: *rfp* and *gfp* expression in *A. baumannii* strains with CRISPRi constructs. RFP/OD₆₀₀ (left) shows that pSGAb_CRISPRi significantly reduces *rfp* expression compared to controls, indicating effective repression. GFP/OD₆₀₀ (right) reveals increased metabolic burden in both pSGAb_CRISPRi and pSGAb_sgPlac, confirming that repression is not due to burden alone.

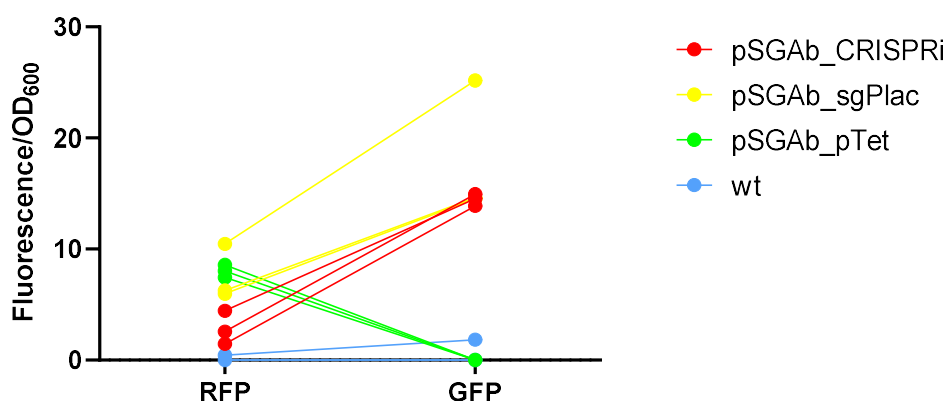


Figure 2-17: Paired analysis of *rfp* and *gfp* expression to assess CRISPRi repression and metabolic burden. Each line connects RFP/OD₆₀₀ and GFP/OD₆₀₀ values for individual replicates across *A. baumannii* strains. The CRISPRi strain (red) shows reduced RFP and moderate GFP levels, indicating targeted repression with a manageable burden. The sgPlac negative control (yellow) shows high GFP but no RFP repression, confirming the burden is not responsible for *rfp* silencing.

To further validate the fluorescence measurements obtained with the plate reader assays, samples were also mounted on glass slides and analysed via confocal microscopy, providing visual confirmation of fluorescence expression at the single-cell level. Specifically, the engineered CRISPRi *A. baumannii* strains were first examined using a fluorescence microscope to verify the presence of bacteria and the expression of red fluorescent protein on the slides (for more detail see A.7 Supplementary Material in Appendix Section), and then images were acquired using a confocal microscope at 60X magnification, capturing both green and red fluorescence. The results revealed that bacteria expressing red fluorescent protein were fewer in number than those expressing green fluorescent protein. Additionally, some bacteria exhibited both green and red fluorescence, indicating overlap between GFP and RFP signals. These observations suggest that while *rfp* repression is occurring, it is not uniformly effective across all cells (Figure 2-18). Although we acknowledge that confocal microscopy provides qualitative data, the observed fluorescence patterns support the quantitative results obtained from the microplate reader. Together, both approaches consistently show reduced *rfp* expression in the CRISPRi condition, while *gfp* expression patterns reflect the metabolic burden associated with the system. These combined findings reinforce the effectiveness of CRISPRi in repressing target gene expression in *A. baumannii*.

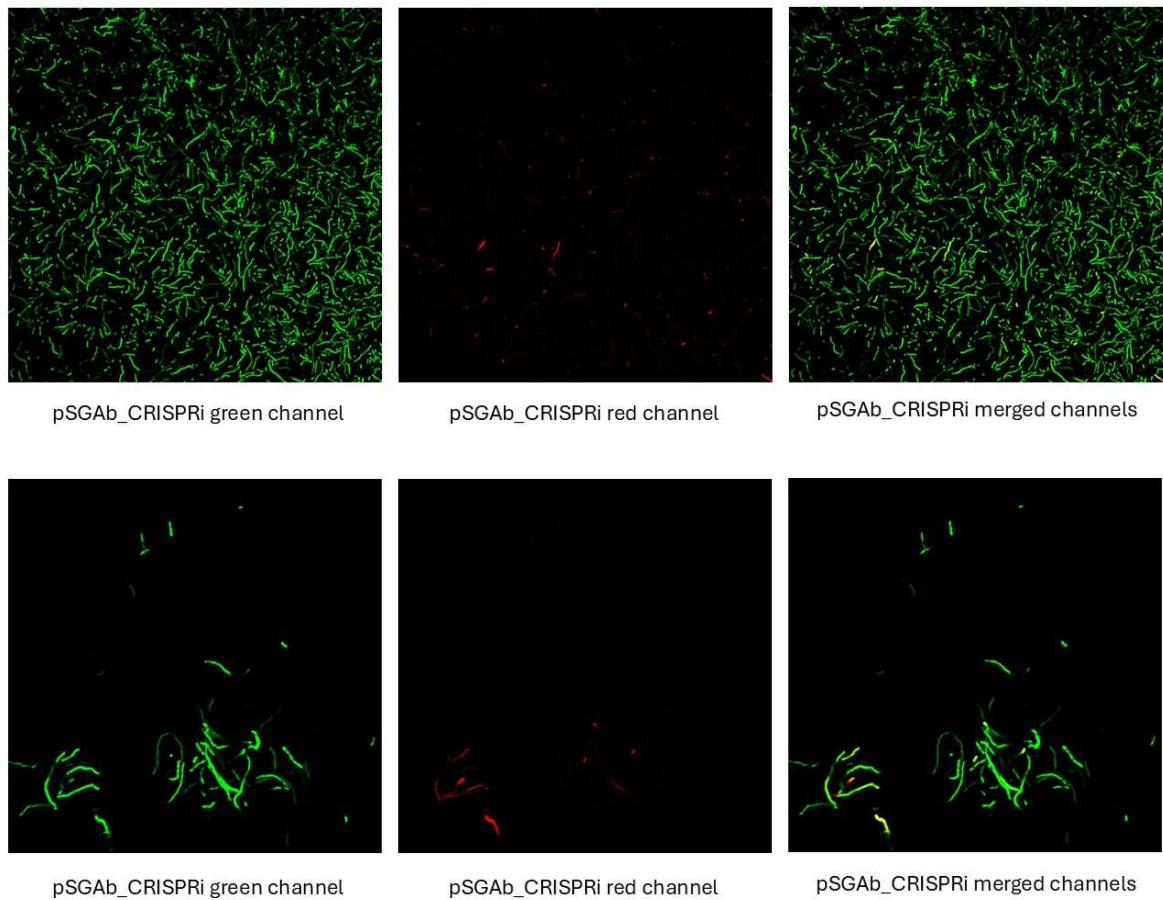


Figure 2-18: The engineered CRISPRi *A. baumannii* strains were visualized using confocal microscopy at 60X magnification. The bottom panel is a zoomed-in views. Left: *A. baumannii* carrying the CRISPRi system, as indicated by *gfp* expression (green channel). Center: *rfp* expression (red channel) showing that repression is functional but not complete, as some bacteria still express *rfp*. Right: A merged image of the two channels, highlighting some bacteria that co-express both *gfp* and *rfp*, suggesting partial overlap and incomplete repression of *rfp*. The three panels below show the same regions at the original magnification, providing an overall view of the bacterial population and fluorescence distribution.

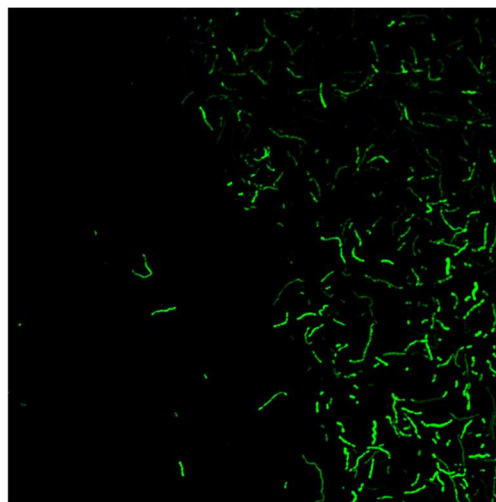


Figure 2-19: Zoom-in view of the coating edge observed on coverslips treated with D-lysine.

Regarding the confocal microscopy experiments, the Figure 2-19 highlights the necessity of the coating step for fixing the bacteria to the coverslips. Specifically, the image is divided by

the non-uniform coating: the left side shows fewer bacteria, whereas the right side is densely populated with bacteria.

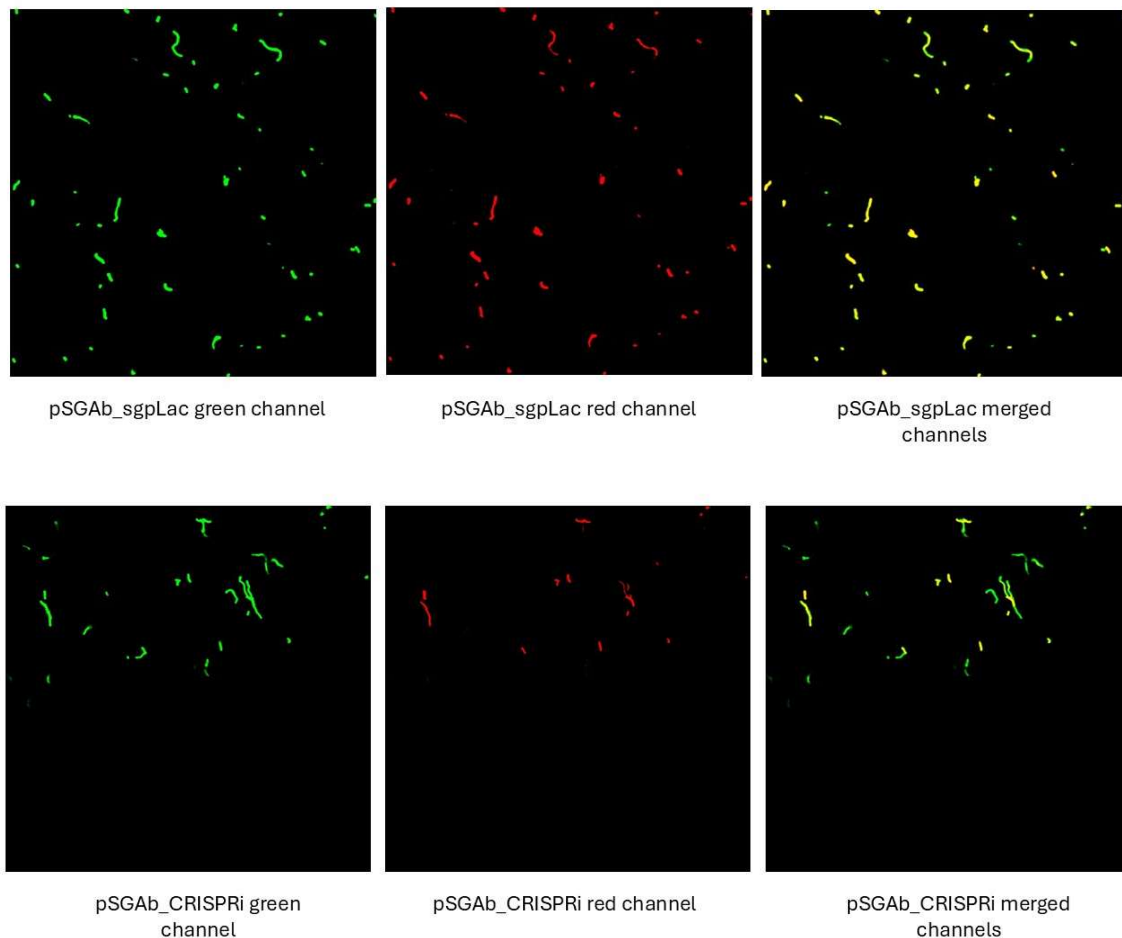


Figure 2-20: Confocal microscopy images of *A. baumannii*: upper panel, positive control strain carrying pSGAb_sgplac (non-targeting sgRNA to pLac); lower panel, CRISPRi strain carrying pSGAb_CRISPRi targeting the pTet promoter upstream of *rfp*.

In addition to the previous data, a positive control was tested at the confocal microscopy, specifically a non-sense sgRNA (pSGAb_sgplac, see the *Appendix A4*), which targets the pLac promoter unrelated to the *rfp* gene; strong red fluorescence is observed across nearly all cells. The merged confocal image reveals numerous yellow cells, indicating robust co-expression of *gfp* and *rfp*, consistent with the absence of repression. In contrast, the pSGAb_CRISPRi strain, which expresses a sgRNA targeting the pTet promoter upstream of the *rfp* gene, displays a weaker *rfp* signal. Fewer cells exhibit red fluorescence, and many appear green-only or weakly yellow in the merged image (Figure 2-20). This pattern suggests effective, though incomplete, repression of *rfp* expression in a substantial portion of the population.

These confocal microscopy images provide qualitative evidence supporting the repression of *rfp* in the pSGAb_CRISPRi strain compared to the control pSGAb_sgplac. To complement this visual analysis, we attempted to obtain quantitative data using Fiji/ImageJ by splitting the merged images into separate green and red channels (in grayscale) and analysing pixel intensity to estimate the proportion of fluorescence. However, due to the incomplete

repression of *rfp*, the red fluorescence remained strong across many cells, limiting the accuracy of pixel-based quantification and making it challenging to extract reliable numerical data from the images alone. Nonetheless, these qualitative confocal microscopy data align with the trend observed in the plate reader assay measuring fluorescence (Figure 2-16), further supporting the effectiveness of the CRISPRi-mediated repression in *A. baumannii*.

Based on the repression findings, we aimed to enhance the system by inducing the HSL promoter upstream of the sgRNA, with the expectation of achieving complete repression (Figure 2-21). We evaluated five different induction conditions to assess the potential full efficacy of the CRISPRi system in *Acinetobacter baumannii*. The *rfp* expression across these five conditions was similar to that observed in the non-induced strain from the Figure 2-16 (panel a), this suggests that varying HSL induction levels did not affect the engineered *A. baumannii* strain. This observation together with the data presented in the Figure 2-7 and

Table 2-1 leads us to hypothesize that the J23116 promoter upstream of the *dCas9* controlled the expression of the aforementioned gene but it is insufficiently strong to achieve complete silencing. Consequently, the dCas9-sgRNA complex may be saturated.

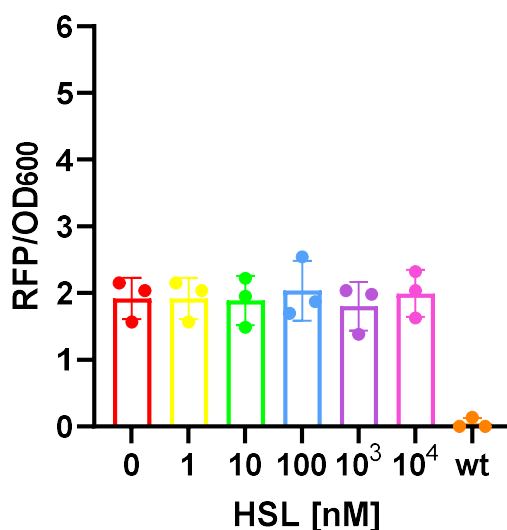


Figure 2-21: sgRNA induced with a range of HSL induction did not affect the repression of the *rfp* in *A. baumannii*.

To enhance CRISPRi efficiency, we aimed to replace the J23116 promoter upstream of *dCas9* in the pSGAb_CRISPRi plasmid with a stronger promoter J23101 based on the data presented in the constitutive promoter section (Table 2-1, in bold). Site-directed mutagenesis, performed via PCR, was employed for this modification. However, the resulting plasmids displayed unexpected mutations or partial loss of the CRISPRi construct. This outcome may be attributed to limitations of the mutagenesis protocol, such as suboptimal primer design, or to the high strength of the J23101 promoter, which may lead to excessive *dCas9* expression and impose a metabolic burden on the host cells.

2.4.1.2.4 CRISPRi Targeting *ompA*

Based on the CRISPRi characterization results, we focused on targeting a specific bacterial gene. Although the current system does not achieve full repression and the promoter upstream of the *dCas9* is weak, these are not necessarily limitations when considering the application of the project, which is to restore antimicrobial sensitivity. In therapeutic contexts, complete silencing of genes involved in antimicrobial resistance or virulence is often unnecessary; a partial reduction in gene expression may be sufficient to weaken the bacteria and make them more susceptible to existing antibiotics.

For this purpose, we selected *ompA* as a genomic target. OmpA is a porin, an outer membrane protein, implicated in multiple functions related to environmental interaction and bacterial adaptation. In addition to its role in nutrient transport, OmpA also contributes to antibiotic resistance by participating in the extrusion of antibiotics from the periplasmic space in conjunction with inner membrane efflux systems. Moreover, OmpA is involved in epithelial cell adhesion and biofilm formation, further underlining its relevance as a therapeutic target.

To investigate CRISPRi-mediated repression of a genomic target, we designed two sgRNAs, sg157 and sg211, both targeting the coding DNA sequence (CDS) of the *ompA* gene in *A. baumannii* (Figure 2-22). While we are aware that targeting the CDS is generally less efficient than targeting promoter regions^{85–87}, this strategy was chosen because the *ompA* promoter is not annotated. Suitable PAM sites were difficult to identify within the putative promoter region. Rather than selecting an uncertain upstream region, we targeted well-defined CDS sites to ensure reliable sgRNA binding. The expression of both sgRNAs was driven by the J23119 promoter, which, based on our *rfp* expression analysis (Figure 2-4), is a strong promoter in *A. baumannii*. The guides were cloned into the pSGAb_dCas9 plasmid, which also includes an *rfp* cassette, allowing red fluorescence to serve as a convenient visual marker for plasmid presence (see the Appendix A4).

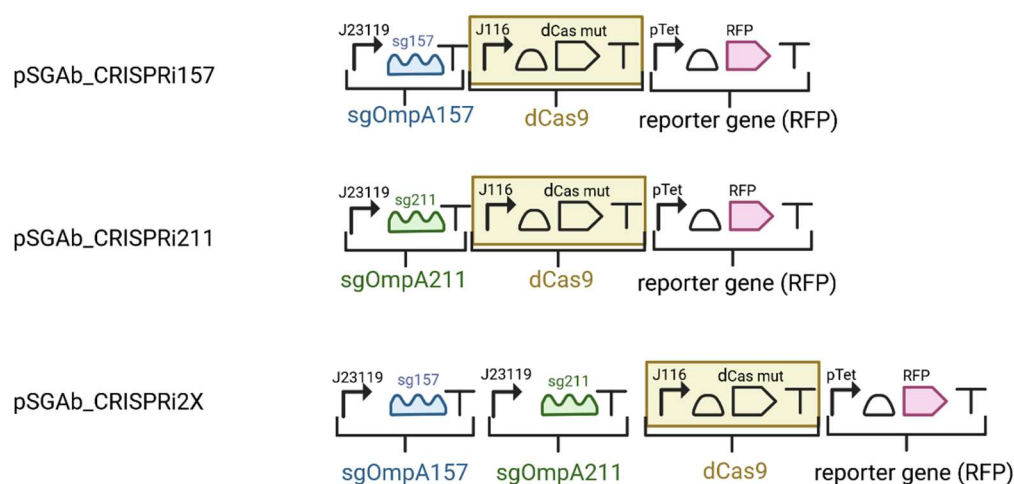


Figure 2-22: Schematic representation of CRISPRi plasmid constructs used to target the *ompA* gene in *A. baumannii*.

The resulting two CRISPRi strains were tested in a biofilm formation assay to evaluate whether silencing *ompA* would impair biofilm development. Unfortunately, the results showed no difference between the wild-type strain and the strain carrying the CRISPRi targeting *ompA*. As a next step, we constructed a multiplex CRISPRi system (Figure 2-22), combining both sgRNAs as independent operons on the same plasmid to enhance silencing efficiency. Control strains included wild-type *A. baumannii* and *A. baumannii* carrying the pSGAb_pTet plasmid, to assess whether plasmid carriage and antibiotic selection (used for plasmid maintenance) had any influence on biofilm formation.

Biofilm formation was first assessed using a crystal violet staining assay (Figure 2-23, panel a). To account for possible differences in bacterial growth, OD₅₉₂ values from the crystal violet assay were normalized to OD₆₀₀ measurements, ensuring that biofilm quantification reflects true biofilm production rather than variations in cell density. The pSGAb_CRISPRi2X strain (see the Appendix A4) showed a reduction in OD₅₉₂, indicating reduced biofilm biomass. The strain expressing only sg157 exhibited a mild reduction, while sg211 alone did not alter biofilm formation compared to the control strains. It is worth noting that, although the pSGAb_CRISPRi2X strain showed the most pronounced reduction in biofilm formation, some variability was observed across replicates, suggesting that the efficiency of multiplex CRISPRi repression may fluctuate under different conditions. Normalizing biofilm levels to cell density (OD₅₉₂/OD₆₀₀) yielded similar trends (Figure 2-23), confirming that the observed differences were not due to growth issues.

To ensure the observed effects were due to CRISPRi-mediated repression and not a plasmid-related artifact, we included pSGAb_pTet as a control. This strain, which carries the plasmid harbouring the *rfp* cassette under antibiotic selection, showed a slight increase in biofilm formation compared to the wild-type. This suggests that plasmid carriage and antibiotic selection may influence biofilm formation but does not account for the reduction observed in the CRISPRi strains.

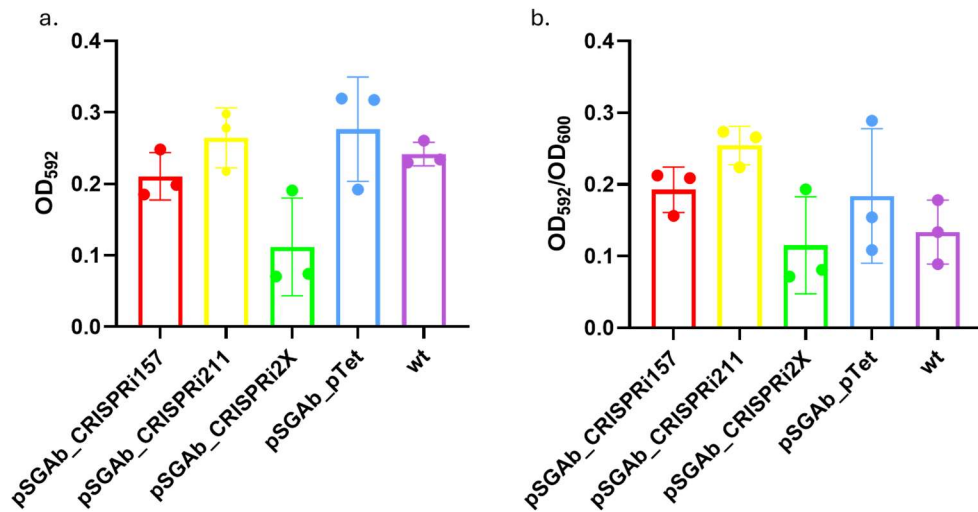


Figure 2-23: Biofilm formation (OD_{592} , left) and normalized biofilm (OD_{592}/OD_{600} , right) of *A. baumannii* strains after 24h. Strains carrying CRISPRi_2X and CRISPRi_157 show reduced biofilm levels, supporting *ompA* silencing effects.

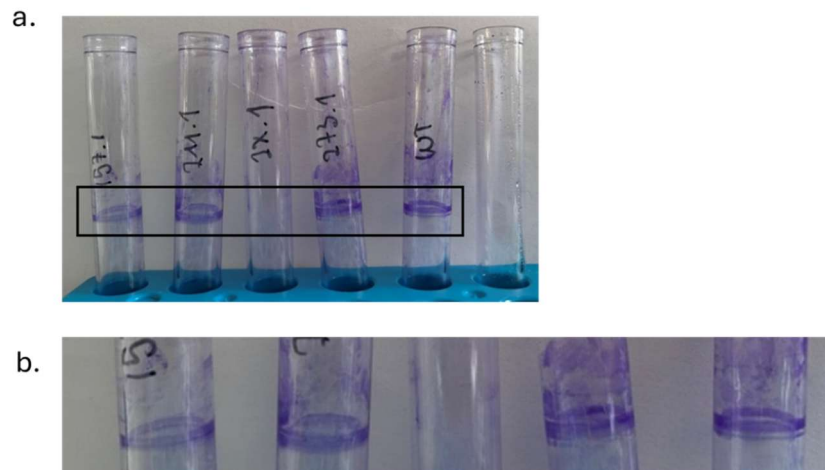


Figure 2-24: Crystal violet-stained tubes showing biofilm formation from right to left: (a.) pSGAb_CRISPRi157, pSGAb_CRISPRi211, pSGAb_CRISPRi2X, pSGAb_pTet, wild-type (wt), and LB control. (b.) Zoomed region of the same tubes highlighting biofilm formation, from right to left: pSGAb_CRISPRi157, pSGAb_CRISPRi211, pSGAb_CRISPRi2X, pSGAb_pTet, and wild-type (wt)

The engineered strains were subjected to total RNA extraction, and the expression levels of *ompA* mRNA were assessed by reverse transcription real-time PCR (RT-PCR). The data were analysed using the ΔC_t (delta cycle threshold) method, which enables the relative quantification of gene expression. In this approach, the C_t value of the target gene (*ompA*) is normalized to the C_t value of a housekeeping gene (*rpoB*)⁸⁸ within the same sample to account for variability in RNA quantity and quality. The resulting ΔC_t values were then compared across samples, using the wild-type strain as the reference condition. A lower ΔC_t value indicates higher expression, while a higher ΔC_t suggests reduced expression of the target gene. The relative expression levels were then expressed as fold changes, providing a straightforward comparison of gene silencing efficiency. This analysis confirmed that *ompA*

expression was reduced in strains expressing sg157 and the multiplex (2X) CRISPRi construct, but not in the sg211 strains (Figure 2-24 and Figure 2-25).

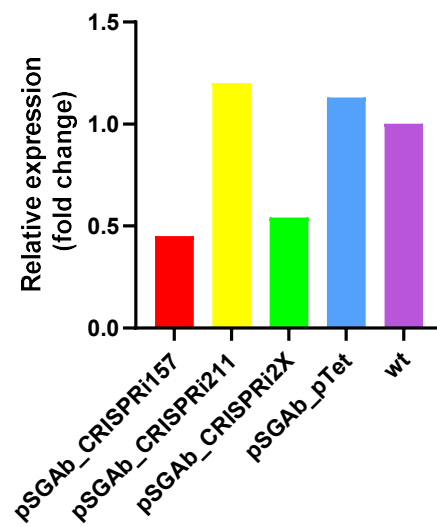


Figure 2-25: Relative expression of *ompA* mRNA in engineered *A. baumannii* strains, analysed by RT-PCR. Data are shown as fold change normalized to the wild-type strain and housekeeping gene *rpoB*.

2.4.2 Section II: The Delivery Platform

In the following section, we present data regarding the advances made in the development of the delivery platform in *E. coli*. First, we characterized the system by transferring a red fluorescent protein, reporting on transduction efficiency, system specificity, and the purity of phage preparations. Secondly, we delivered the actuator technology, i.e., CRISPRi, targeting genes involved in colistin resistance (*mcr-1*) and meropenem resistance (*bla_{ndm-1}*). Moreover we explored the tunability of the silencing platform by evaluating an HSL-inducible sgRNA for transfer. Finally, considerations regarding titration assays are discussed.

2.4.2.1 Engineered Phage Particles as Delivery Platform

Filamentous phages such as M13 are commonly used as transducing vectors to deliver DNA payloads to bacteria. In this perspective, we aimed to explore this delivery platform to transfer CRISPRi machinery and tackle AMR.

We implemented a strategy for producing and characterizing phage particles using a phagemid carrying the *rfp* cassette in combination with an M13 helper plasmid (see *Appendix A5*). This approach was selected for its versatility, which relates to our goal of expanding phage tropism.

By using a plasmid-based system, we aimed to achieve greater flexibility in manipulating the phage particles compared to using a mutated helper phage.

The helper plasmid was specifically designed and constructed to overcome the limitations associated with helper phage contamination. One of the key features of the helper plasmid is its inability to package itself. This design ensures that only the phagemid particles are produced, resulting in pure preparations, avoiding any contaminating helper phage. Moreover, the helper plasmid system proved to be highly efficient as demonstrated by Chasteen *L. et al*⁶².

The Engineered Phage Particles Delivered a Reporter Gene

We produced phage particles based on the interaction between a phagemid carrying the *rfp* and a helper plasmid, detailed in the Phage Production section (Materials and Methods). To demonstrate the production and the ability of the engineered phages to transfer DNA to the host, we conducted a transduction assay.

We infected the natural host, *E. coli* harbouring the F' episome, and used *E. coli* TOP10 as a negative control. As shown in *Figure 2-26*, the engineered phage successfully infected the natural host, transferring the *rfp*, but did not infect *E. coli* TOP10.

Furthermore, to confirm the delivery of the reporter gene, we performed colony PCR. Six colonies were picked from the transduction plate, and *E. coli* F' that was not transduced was used as a negative control. The transduced samples exhibited the expected bands $\approx 1,000$ bp, whereas the negative control did not display any bands. Additionally, as a positive control,

we performed colony PCR on *E. coli* carrying the phagemid, which also showed the expected bands.

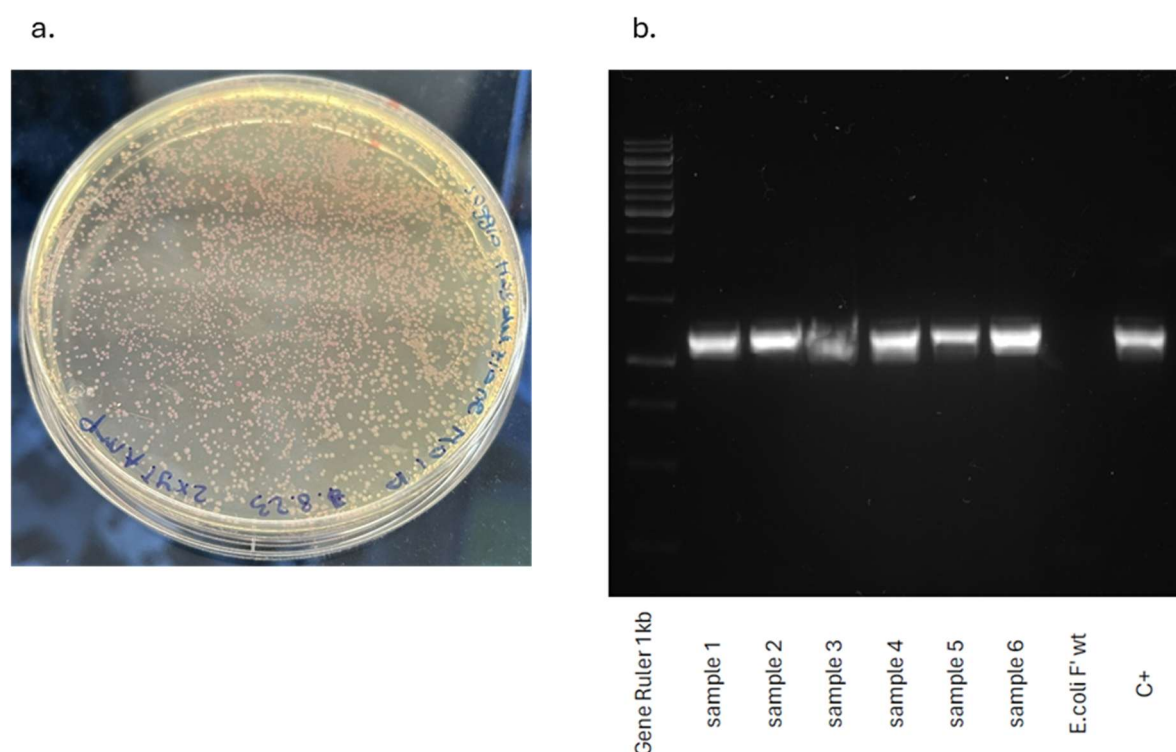


Figure 2-26: Engineered phage *rfp* delivery. a) The engineered phage successfully infected the natural host, transferring the *rfp* gene, and b) colony PCR confirmed the presence of *rfp* in the recipient cells.

The helper plasmid mutated easily

To produce robust and reliable data, we initiated a new phage production followed by a transduction assay. Despite numerous attempts and extensive protocol optimization, we were unable to produce viable phage particles using this method. The transduction assays performed (see Figure 2-27) resulted in a lawn of white colonies. This inability to consistently produce phage particles led us to hypothesize that there may be an issue with the assembly of the phage particles. Given the results from the initial transduction assay, where no reporter gene expression was visually detected, we conducted a PCR check to better understand the underlying issue. Single colonies obtained from the transduction assay were picked and processed for colony PCR. Several gene targets were amplified, representative of all the genetic constructs involved, to check the presence of both constructs:

- *ampR*, *RFP* cassette, F' region, and the ORI for the phagemid,
- as well as the *cmR* for the helper plasmid.

The PCR conducted on the picked colonies from the transduction plates, confirmed the presence of all the genetic constructs, suggesting that the issue was related to the inability of the phage assembly process.



Figure 2-27: The transduction assays performed resulted in a lawn of white colonies.

To further investigate the issue, we performed whole plasmid sequencing on our two plasmids: the phagemid carrying the reporter gene and the helper plasmid. Interestingly, the whole phagemid sequence perfectly aligned with the given phagemid map. However, in the helper plasmid sequence, several mutations and deletions were detected:

- Deletion in the selection gene coding for chloramphenicol resistance (*cmR* gene);
- Multiple mutations in the backbone (possible mutations in the endogenous promoters);
- 15-base deletion in gene III, which codes for the minor coat protein pIII required for adsorption and phage DNA penetration;
- A mutation in gene IV, resulting in an aminoacidic substitution R87.

Moreover, gene IX presented a mutation producing a stop codon, causing the production of a truncated protein. pIX is a minor coat protein of M13 that interacts with the packaging signal located in the intergenic region of M13 DNA. These findings demonstrate the inability of the M13 genes to be expressed and to interact with the packaging signal, thus preventing the assembly process.

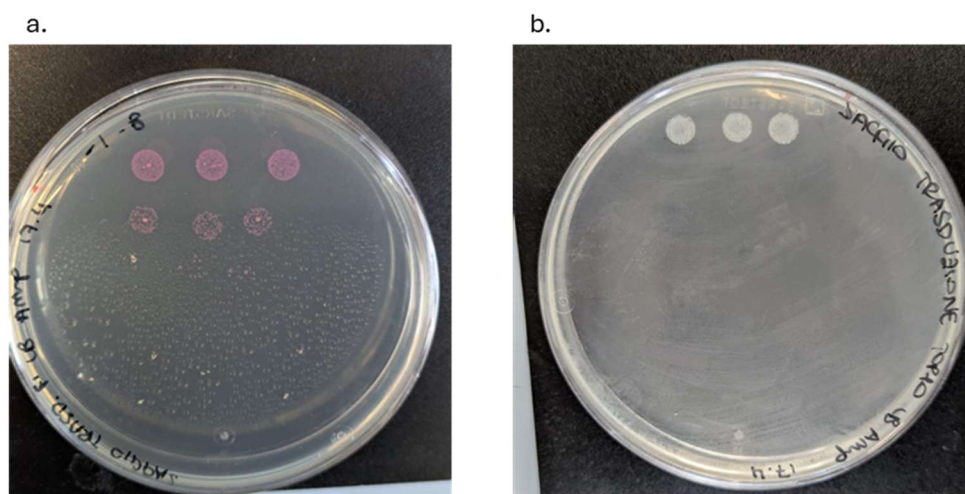
In conclusion, the helper plasmid employed for the phage production was unstable, and the repeated steps of streaking and inoculating the construct led to the emergence of mutations.

Changing Approach in Phage Production and Characterization

Consequently, we opted for a different approach: producing phage particles by combining a phagemid and helper phage. This strategy, based on M13KO7, required particular attention due to the ability of the mutated phage to replicate independently. M13KO7 can replicate in the absence of phagemid DNA. However, in the presence of a phagemid bearing a wild-type M13 or f1 origin, single-stranded phagemid DNA is preferentially packaged and secreted into the culture medium.

Successful Delivery via Φ M13::RFP

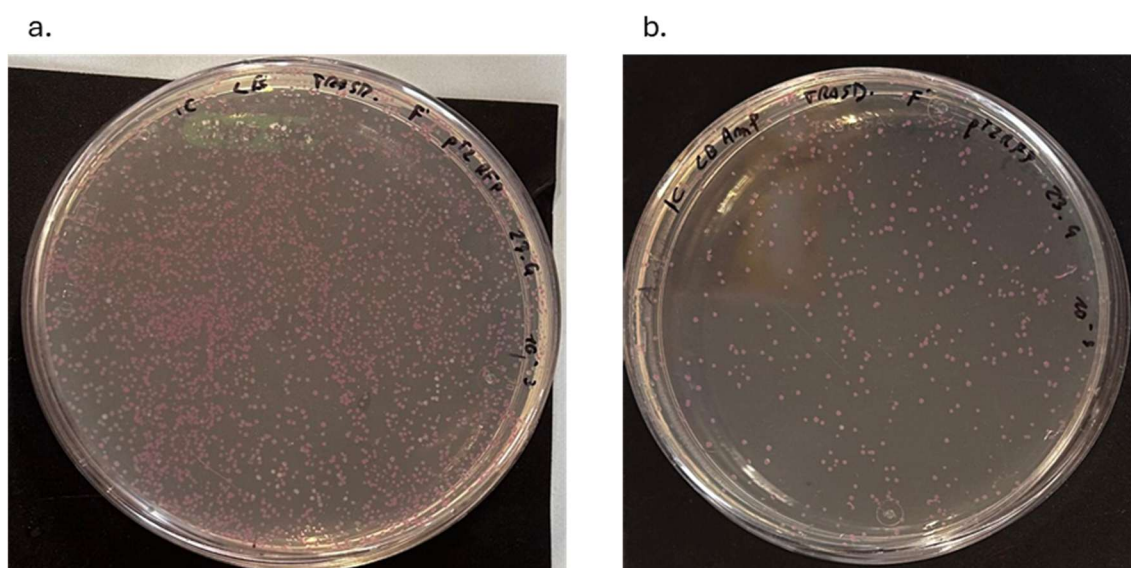
We implemented an established protocol for producing phage particles using a helper phage and a phagemid containing the reporter gene *rfp*. This approach allowed the generation of engineered phage particles that, instead of carrying the M13 genome, packaged the phagemid harbouring *rfp* (Φ M13::RFP see *the Appendix A6*). To evaluate phage production and *rfp* transfer, we performed two different transduction assays. The first assay was designed to assess the ability of the engineered phages to infect the target strain *E. coli* TOP10 F'. The second assay aimed to evaluate the specificity of infection by attempting to transduce *E. coli* TOP10 cells that do not express the receptor recognized by M13, the F'-pilus named the sex pilus or conjunctive pilus. The transduced bacterial cultures were serially diluted (10^{-1} to 10^{-8}) and spotted on ampicillin-containing agar plates (*Figure 2-28*). Growth and pink coloration on the selective medium indicated successful infection of the natural host strain and transfer of the *rfp* marker to recipient cells. The growth observed at the 10^{-1} dilution is likely due to the high density of bacteria in the spotted area, which may have blocked the activity of the antibiotic. In fact, at higher dilutions, no colony growth was observed. This interpretation is supported by *Figure 2-28*, where the transduced cells in the left panel are pink, while the white spots in the right panel are not transduced bacteria but bacteria that grew on the selective media due to the high density in the spotted area.



*Figure 2-28: Spot assay of phage-mediated transduction on selective media. (a.) E. coli TOP10 F' showed growth and pink coloration at lower dilutions, indicating successful *rfp* transfer. (b.) E. coli TOP10 lacking the M13 receptor showed limited growth only at the lowest dilution, consistent with reduced infection efficiency and confirming receptor-dependent specificity.*

Based on the previous results, we aimed to obtain more reliable data on transduction efficiency. To this end, we performed two additional transduction assays in which the engineered Φ M13::RFP (see *Appendix A6*) infected *E. coli* TOP10 F'. In one assay, the cultures were plated on plain LB agar to allow growth of both wild-type and transduced cells; in the other, they were plated on ampicillin-containing agar to select only transduced cells. Following the approach used in the previous experiment, where colonies were countable on full plates at the 10^{-3} dilution, both assays in this study were plated as full plates at a 10^{-3}

dilution to improve count accuracy. Colonies were counted, and transduction efficiency was calculated by dividing the red colonies expressed as CFU/mL obtained on LB plates by the white colonies CFU/mL obtained on LB plates and expressing the result as a percentage. Based on the colony counts, approximately 92% of the total bacterial population consisted of transduced cells (*Figure 2-29*). In other words, out of all colonies growing on non-selective LB plates (representing the entire population so transduced and non-transduced cells), 92% of the counted colonies were red, indicating that they had successfully received and expressed the *rfp*-carrying phagemid delivered by Φ M13::RFP. This proportion represents the calculated transduction efficiency.



*Figure 2-29: Transduction of Φ M13::RFP into *E. coli* TOP10 F' at a 10^{-3} dilution. (a.) colonies grown on non-selective LB agar (total bacterial population= transduced + non transduced colonies). (b.) colonies grown on LB-Amp⁺ agar.*

The Φ M13::RFP Production is Highly Specific for the Natural Host

Since this phage production method is known to yield a mixed population of recombinant and wild-type M13 particles, we tested whether wild-type phages were present in the Φ M13::RFP preparation (see *Appendix A6*). *E. coli* TOP10 F' cells were infected with the phage preparation and plated, via spot assay at serial dilutions from 10^0 to 10^{-4} , on agar containing either ampicillin or kanamycin. The different antibiotic selections allowed distinction between recombinant and wild-type infections, as the wild-type M13 genome carries a kanamycin resistance cassette. Growth of colonies on kanamycin plates indicated the presence of wild-type phages within the preparation.

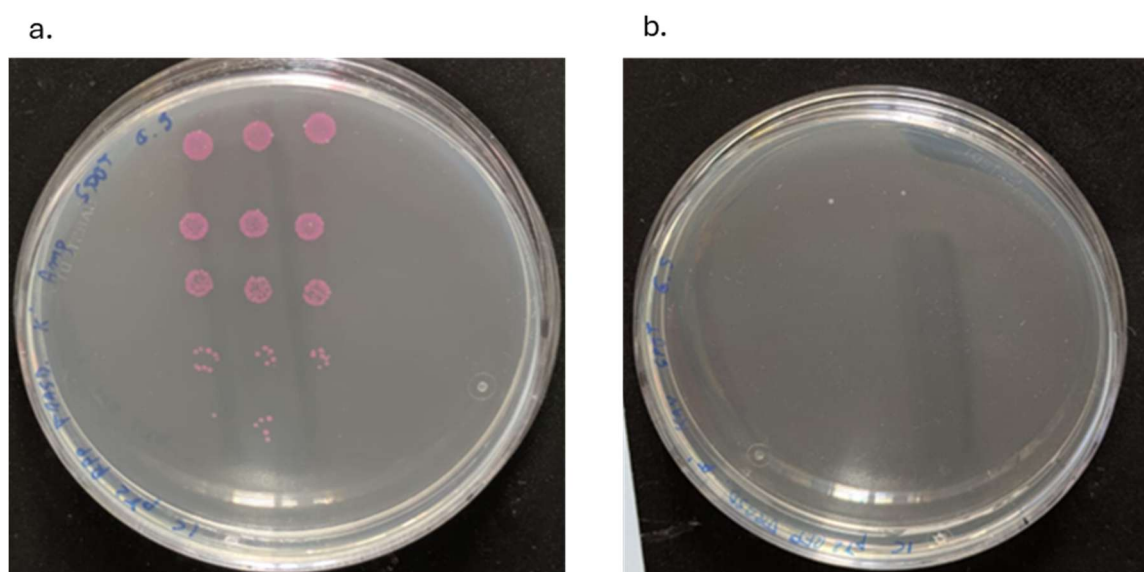


Figure 2-30: Spot assay to assess phage preparation purity. *E. coli* TOP10 F' infected with Φ M13::rfp was plated on ampicillin-containing agar (left) and kanamycin-containing agar (right) at serial dilutions (10^0 – 10^{-4}).

The purity of the Φ M13::RFP preparation was calculated by comparing the CFU/mL obtained on ampicillin plates (representing recombinant phages) with the CFU/mL obtained on kanamycin plates (representing wild-type phages). As expected, ampicillin plates showed numerous colonies across dilutions, whereas only a few colonies were observed on kanamycin plates, indicating minimal presence of wild-type M13 carrying the kanamycin resistance cassette. This corresponds to a calculated phage preparation purity of 99.9% (Figure 2-30).

2.4.2.2 Restore Antimicrobial Resistance in Laboratory Strains with Phage Delivery

The main goal of the project is to transfer CRISPRi into antibiotic-resistant bacteria by exploiting the ability of phages to infect them, to resensitize those cells, and restore the effectiveness of commercially available antibiotics. Meropenem and colistin are, respectively, a carbapenem antibiotic and a polymyxin antibiotic, both considered last-resort treatments for severe infections. However, resistance to carbapenems significantly limits the available options to combat antimicrobial-resistant bacteria, and the emergence of isolates carrying the *mcr-1* gene, which confers resistance to colistin, is alarming. To address this issue, we investigated the potential of delivering, via phage particles, a CRISPRi system targeting the *mcr-1* (mobilized colistin resistance-1 gene) and *bla_{ndm-1}* (New Delhi Metallo- β -lactamase-1 gene) genes, which are respectively responsible for colistin and meropenem resistance.

To do so, we employed the CRISPRi array described in *Chiacchera et al.*⁸⁰ to silence these genes in two laboratory strains carrying *mcr-1* and *bla_{ndm-1}*. To assess the restorative effect, we performed a kinetic growth inhibition assay on the transduced colonies by measuring OD₆₀₀. The growth delay was determined as the difference in growth curves between the transduced bacteria and the resistant strain. We set 0.1 as the OD₆₀₀ threshold because, under our growth conditions, this value corresponds to the early exponential phase, when

bacterial cells are actively dividing and growth differences due to genetic or environmental factors are most pronounced. Measuring the delay in reaching this phase provided a sensitive indicator of growth delay caused by CRISPRi-mediated silencing of the resistance genes.

The resistant strains were constructed as described in *pTZ19 Cloning* section (Materials and Methods). We were unable to use the backbones described by Chiacchiera *et al.*⁸⁰ (*PGDP2 mcr-1* and *PGDP1 ndm-1*) because they harbour the *ColE1* replicon, which is incompatible with the *pMB1* replicon carried by the phagemid. During the transduction assay, the coexistence of the *ColE1*-based resistance plasmid and the *pMB1*-based phagemid delivered by the recombinant phage would result in incompatibility issues, potentially compromising plasmid stability and experimental outcomes. Therefore, the resistance genes were cloned into the medium-copy backbone pSB3K3, which harbours the *p15A* replicon generating pSB3K3_ndm1 and pSB3K3_mcr1. The three plasmids carrying the CRISPRi circuits (*Figure 2-31*) share the same overall structure: a tracrRNA for gRNA maturation, either a single or double spacer, and a final cassette containing *dCas9* driven by the J23109 promoter. The original cassette used the J23116 promoter, but given that the backbone, pTZ19, has a high-copy-number replicon, we replaced it to reduce *dCas9* expression, which at high levels can be toxic.

Briefly, the target strain pSB3K3_ndm-1 was infected with either the phage-based CRISPRi single-spacer *ndm-1* tool (Φ M13::CRISPRi_ndm-1, see *Appendix A6*) or the double-spacer tool (Φ M13::CRISPRi_ndm-1_mcr-1, see *Appendix A6*) in two separate infections. Similarly, the *mcr-1*-carrying strain was infected with either the CRISPRi single-spacer *mcr-1* tool (Φ M13::CRISPRi_mcr-1, see *Appendix A6*) or the same double-spacer tool (Φ M13::CRISPRi_ndm-1_mcr-1, see *Appendix A6*), following the procedures described in the *Transduction assay* section. The transduced cells were then plated on double-antibiotic selective media to maintain both the transduced construct and the target plasmid. At least three colonies from each experiment were picked and tested for kinetic growth inhibition determination.

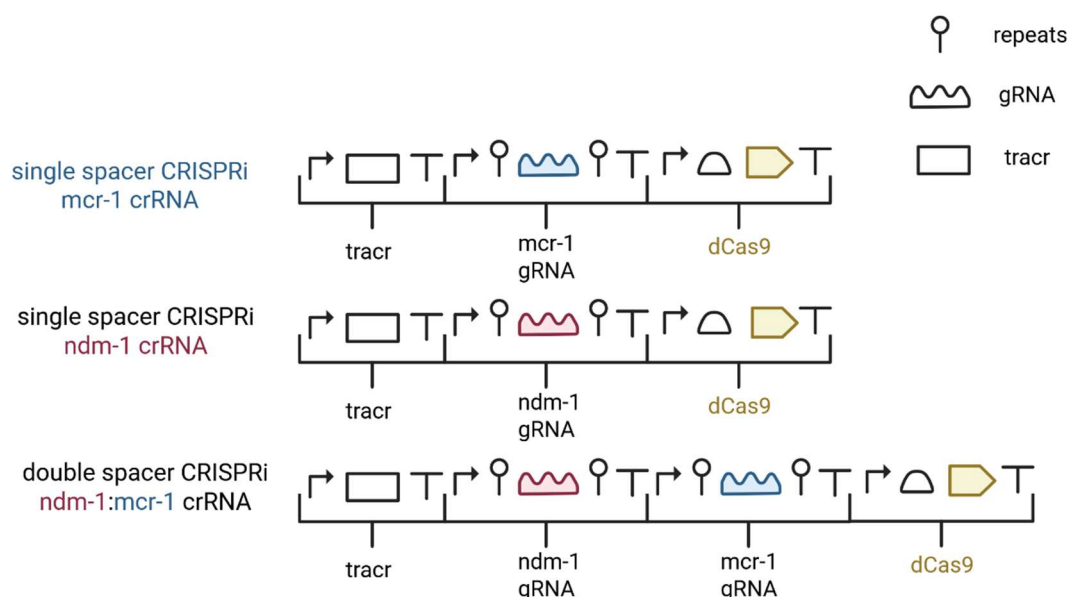


Figure 2-31: CRISPRi constructs with single or double spacers targeting *mcr-1* and *bla_{ndm-1}*. Schematic representation of CRISPRi arrays containing single spacers targeting either *mcr-1* (blue) or *ndm-1* (red), and a double spacer targeting both *bla_{ndm-1}* and *mcr-1*. The tracrRNA, gRNA spacers, and repeats are indicated, along with the dCas9 module.

Single and Double Spacer against Meropenem Resistance (*bla_{ndm-1}*)

The natural host strain of Φ M13 (Figure 2-31, panel a) was tested under a range of meropenem concentrations, and only at 0 μ g/mL did it displays a typical growth curve. Conversely, the resistant laboratory strain, *E. coli* F' harbouring the *ndm-1* gene (plasmid pBS3K3_ndm-1; Figure 2-31 panel b), which confers resistance to meropenem, was able to grow at all concentrations tested except the highest (500 μ g/mL). A growth delay was also observed at 200 μ g/mL and 100 μ g/mL.

The engineered strain carrying pBS3K3_ndm-1 (see Appendix A5) was transduced with either a phage harbouring the single spacer (Φ M13::CRISPRi_ndm-1; Figure 2-32 panel c) or the double spacer (Φ M13::CRISPRi_ndm-1_mcr-1; Figure 2-32 panel d). The single spacer construct yielded notable results, as bacteria were unable to grow at concentrations of 100 and 200 μ g/mL. In contrast, growth was observed at the lower concentrations of 10 and 1 μ g/mL. This approach led to an almost complete re-sensitisation of meropenem susceptibility in the tested cells. The double spacer system was less effective in restoring susceptibility. In particular, bacteria failed to grow at 200 μ g/mL but were able to grow at 100 μ g/mL, although with a noticeable delay. This could be explained by the fact that in the double spacer system, the dCas9 protein must be divided between two different gRNAs, potentially reducing silencing efficiency for *bla_{ndm-1}*, whereas in the single spacer system, dCas9 binds exclusively to the *ndm-1* guide, maximizing gene silencing potential.

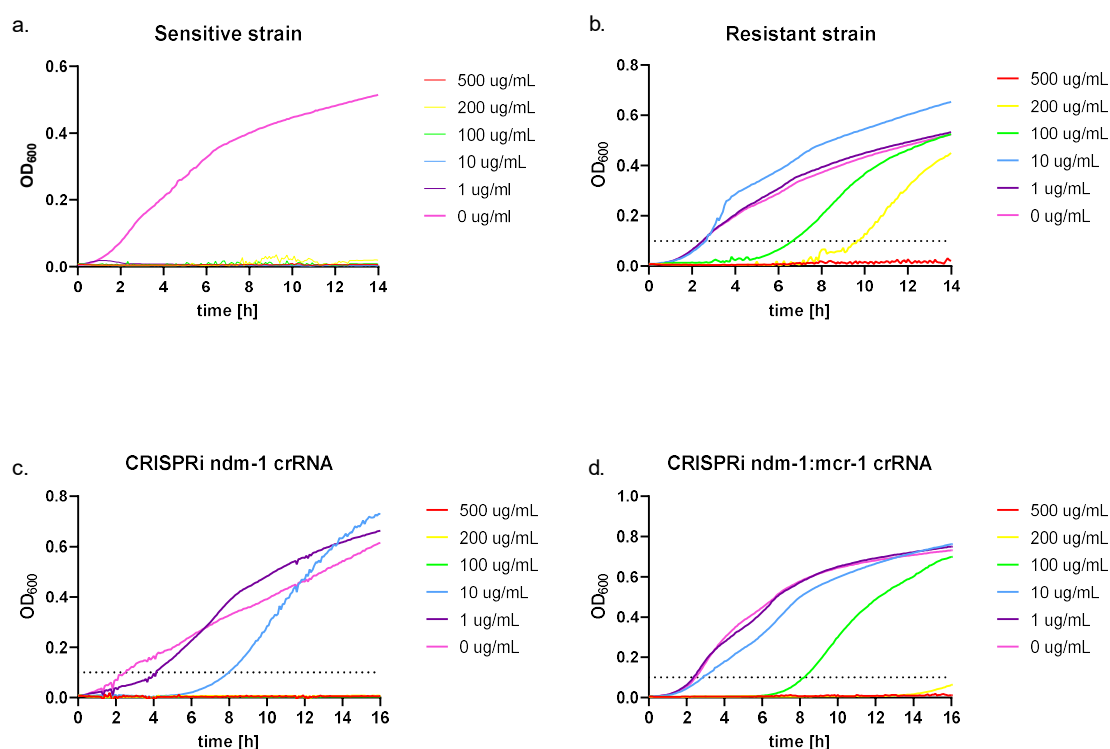


Figure 2-32: Growth curves of *E. coli* strains under different meropenem concentrations (0- 500 µg/mL). (a) The sensitive strain *E. coli* F', the natural Φ M13 host strain grows only at 0 µg/mL. (b) The resistant strain (*E. coli* F' pBS3K3_nda-1) grows at all concentrations except 500 µg/mL, with delays at 100–200 µg/mL. (c) Transduction with Φ M13::CRISPRi_nda-1 restores susceptibility, with no growth at 100–200 µg/mL. (d) Φ M13::CRISPRi_nda-1_mcr-1 reduces growth at 200 µg/mL but allows delayed growth at 100 µg/mL.

Growth Delay Caused by Φ M13::CRISPRi_nda-1

Given the clear silencing effect observed in the growth curves, particularly for the single spacer construct, we next quantified the actual growth delay caused by the phage-based single spacer CRISPRi system. We selected an OD₆₀₀ value of 0.1 as the threshold, as this corresponds to the early exponential growth phase and minimizes potential measurement artifacts. We compute the Δt as the time required for the transduced cells to reach this OD. This value was determined for each meropenem concentration and compared with the time required for the resistant strain to reach the same OD at the corresponding meropenem concentration. As expected, no growth was detected at 500 µg/mL meropenem, as even the resistant strain was unable to grow under these conditions. Similarly, no growth was observed at 200 µg/mL or 100 µg/mL. At 10 µg/mL, however, cells exhibited a growth delay of approximately 5 hours *Figure 2-33*.

The *Figure 2-33* shows the time (in hours) required for the resistant strain and the strain transduced with the phage-based single-spacer CRISPRi system to reach an OD₆₀₀ of 0.1 at each meropenem concentration tested. At 10 µg/mL, the resistant strain reached this threshold in about 3 hours, whereas the transduced strain required nearly 8 hours. At 1 µg/mL, a moderate difference in growth time between the two strains was observed. These results demonstrate that the phage-based CRISPRi system targeting *nda-1* can affect bacterial growth in the presence of meropenem, suggesting a potential strategy for restoring susceptibility in antimicrobial-resistant strains.

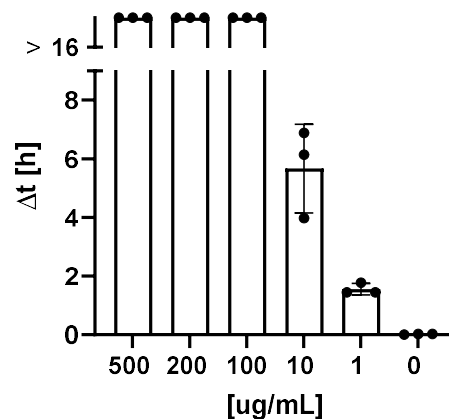


Figure 2-33: Growth delay (Δt) of resistant and CRISPRi-transduced *E. coli* at different meropenem concentrations, expressed as the time to reach $OD_{600} = 0.1$.

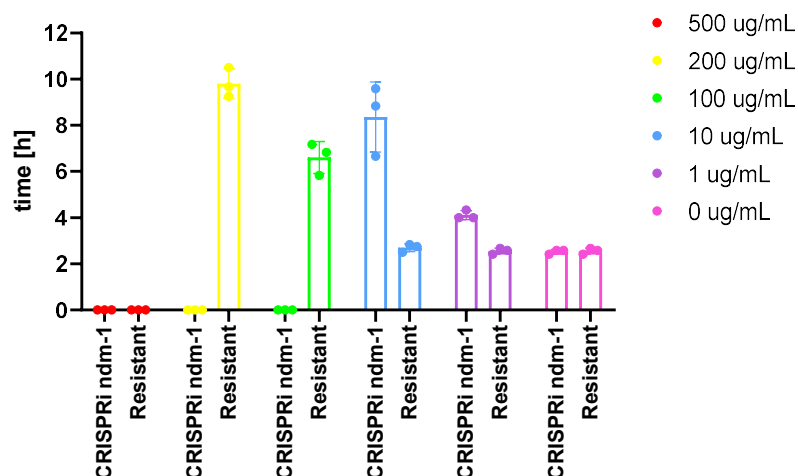


Figure 2-34: Time required for resistant and single-spacer CRISPRi-transduced *E. coli* to reach $OD_{600} = 0.1$ at different meropenem concentrations.

Single and Double Spacer against Colistin (*mcr-1*)

The sensitive strain (Figure 2-35 panel a) was tested under different concentrations of colistin. As expected, the sensitive strain was able to grow only at very low concentrations (0 $\mu\text{g/mL}$ and 0.1 $\mu\text{g/mL}$). Conversely, the resistant strain *E. coli* F' (pSB3K3_*mcr-1*; Figure 2-35, panel b) was able to grow at all concentrations tested except the highest (8 $\mu\text{g/mL}$). At 4 $\mu\text{g/mL}$, a slight reduction in growth compared with lower concentrations was observed, indicating a delay in growth under this condition.

Panel c in Figure 2-35 shows the bacterial growth profiles at different colistin concentrations for the resistant strain transduced with $\Phi\text{M13}::\text{CRISPRi_mcr-1_crRNA}$ (single spacer targeting *mcr-1*). At the two lowest concentrations (0.1 and 0 $\mu\text{g/mL}$), growth was faster than at higher concentrations, with similar behaviour observed between the two. Notably, the

transduced strain at 0 $\mu\text{g/mL}$ colistin was expected to reach an OD_{600} equal to 0.1 at the same time as the resistant strain; however, it reached this threshold later. This delay may be partly explained by the fact that, under infection conditions (*Figure 2-35*, panel c and d), the bacteria required two antibiotics to maintain both the phagemid and the resistance plasmid, whereas the resistant control required only one. At intermediate concentrations (2 and 1 $\mu\text{g/mL}$), growth was slower than at the lowest concentrations, while at 4 $\mu\text{g/mL}$ growth began only after 8 hours. No growth was detected at 8 $\mu\text{g/mL}$ in either the resistant or sensitive strains. *Figure 2-35-panel d* shows the growth profiles of cells transduced with $\Phi\text{M13}::\text{CRISPRi_ndm-1:mcr-1_crRNA}$ (double spacers). Although the three-group trend was still visible, it was less pronounced than in *Figure 2-35-panel c*, consistent with results from the meropenem experiments, suggesting that the double-spacer system is less efficient than the single-spacer system, likely because dCas9 must interact with two guide RNAs instead of one. Interestingly, in the presence of 2 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ colistin, both transduced strains displayed a more pronounced slowdown in growth compared to the two lowest. The double spacer strain *Figure 2-35-panel d* exposed to the range of colistin concentration requires more time to reach an OD_{600} equal to 0.1.

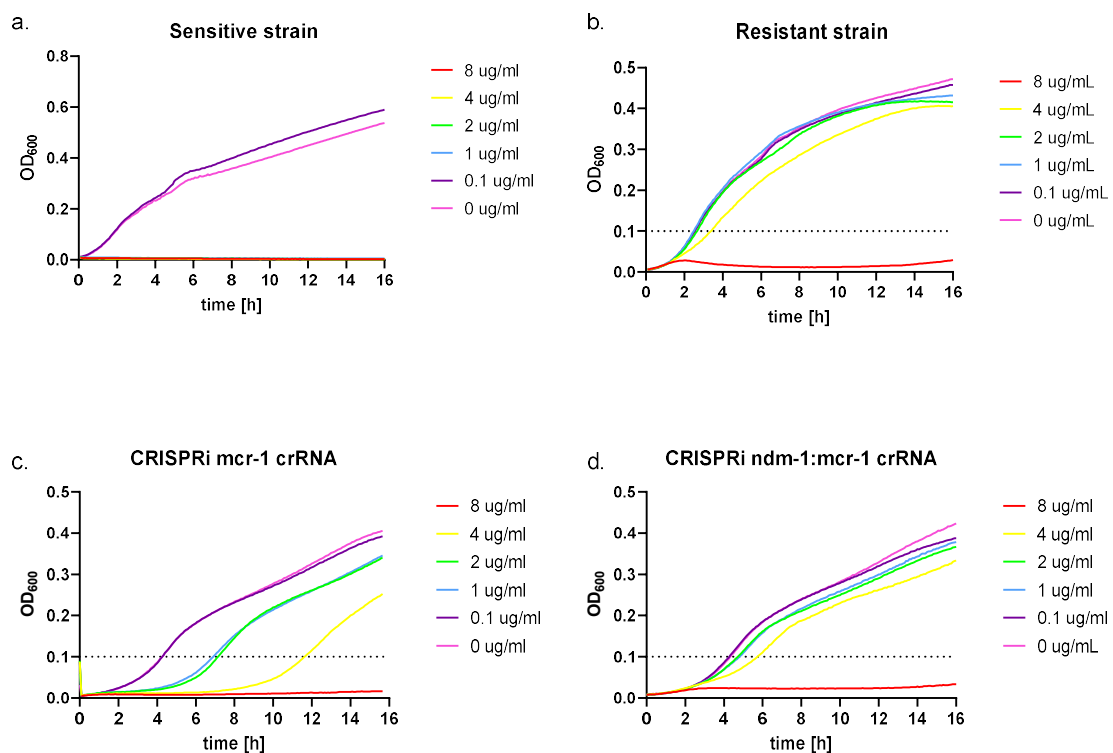


Figure 2-35: Growth curves of E. coli F' strains at different colistin concentrations (0–8 $\mu\text{g/mL}$). a) Sensitive strain; (b) resistant strain (pSB3K3_mcr-1); (c) resistant strain transduced with $\Phi\text{M13}::\text{CRISPRi_mcr-1_crRNA}$ (single spacer); (d) resistant strain transduced with $\Phi\text{M13}::\text{CRISPRi_ndm-1:mcr-1_crRNA}$ (double spacers).

Growth Delay Caused by $\Phi\text{M13}::\text{CRISPRi_mcr-1}$

As with the meropenem experiments, we calculated Δt for colistin, even though a difference in the time to reach OD_{600} equal to 0.1 was already present between the resistant strain and the transduced single-spacer strain at 0 $\mu\text{g/mL}$ colistin (*Figure 2-37*). At 4 $\mu\text{g/mL}$, we observed an approximate 8 hour growth delay, while at 1 and 2 $\mu\text{g/mL}$ the delay was about 4

hours. Comparing the time to reach the threshold at 0 $\mu\text{g/mL}$ colistin, we noted a baseline difference of approximately 2 hours between the two strains (Figure 2-37). We hypothesize that this delay is not only due to CRISPRi-mediated silencing itself, but may instead result from the additional burden of maintaining both the phagemid and the resistance plasmid in the presence of two antibiotics. To account for this potential bias, we subtracted the baseline shift from the values at 0, 10^{-1} , 1, 2, and 4 $\mu\text{g/mL}$ (Figure 2-38). Although this correction reduced the magnitude of the observed differences, a substantial growth delay remained between the CRISPRi-*mcr-1* strain and the resistant control, the cause of which remains unclear.

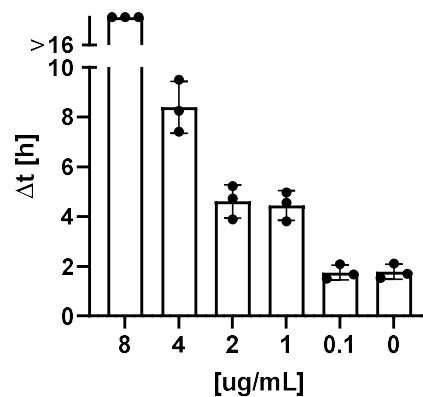


Figure 2-36: Growth delay of *E. coli* F' (pSB3K3_ *mcr-1*) transduced with the phage-based CRISPRi system targeting *mcr-1*. Bars indicate the time required to reach an OD_{600} equal to 0.1 at different colistin concentration.

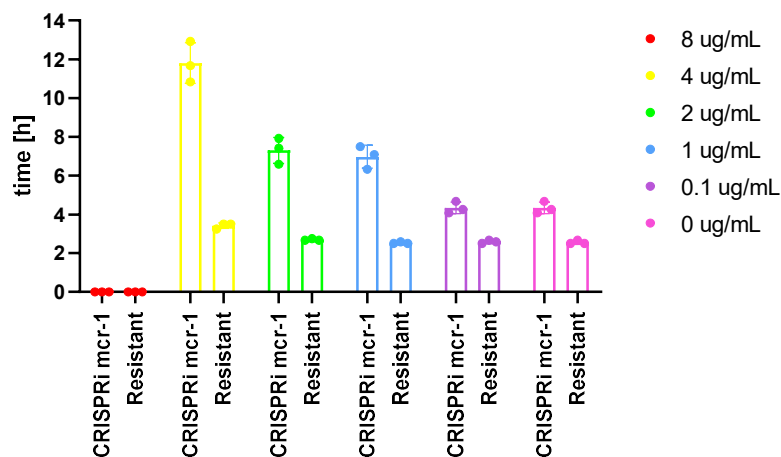


Figure 2-37: Time required for resistant and CRISPRi-*mcr-1* strains to reach $OD_{600} = 0.1$ at different colistin concentrations. Bars represent mean values, with error bars indicating standard deviation.

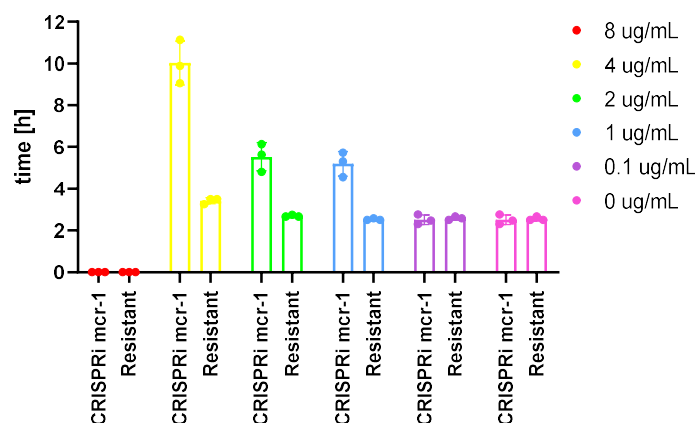


Figure 2-38: Time required for resistant and CRISPRi-mcr-1 strains to reach $OD_{600} = 0.1$ at different colistin concentrations after subtracting the baseline difference observed at 0 $\mu\text{g/mL}$. Bars represent mean values, with error bars indicating standard deviation.

2.4.2.3 Development of an Inducible Phagemid

As the system described in the previous section produced successful results, further optimization is needed to achieve a higher level of repression, particularly for the colistin-targeting silencing system. In general, more effort should be directed toward tuning the expression of the components involved in CRISPRi. In particular, we are interested in evaluating the effect of introducing an inducible single-guide RNA (sgRNA) into the system. Furthermore, adding a reporter gene to the phage-based CRISPRi tool would provide an immediate visual indication that the process is likely working, allowing us to more easily identify promising colonies. To achieve this, a CRISPRi circuit targeting a reporter gene (*rfp* under the control of the inducible promoter pTet) was designed and cloned into the phagemid cloning site generating pTZ19_CRISPRi_HSL (see Appendix A5). The circuit comprises a *gfp* as a metabolic burden indicator, an sgRNA targeting the *rfp* (specifically the pTet promoter responsible for the expression of the reporter gene) under the LuxI promoter inducible by HSL, and a *dCas9* under the control of the constitutive promoter J23109 (Figure 2-39). The metabolic burden indicator is required as we are adding the inducible system which add more potential burden to the high-copy phagemid. In contrast to the restored antimicrobial resistance section, we opted for a single sgRNA instead of a CRISPRi array (tracrRNA and pre-crRNA) in order to reduce the metabolic burden, thereby avoiding the gRNA maturation steps.

In addition, an *E. coli* F' strain carrying the CRISPRi target was generated. For this purpose, a plasmid compatible with the phagemid was utilized, specifically pSGAb carrying the *rfp* under the control of the pTet promoter (Figure 2-39). This plasmid contains a *p15a* origin of replication, which is compatible with the *pMB1* origin of the phagemid. Moreover, the two plasmids have different selection markers: ampicillin resistance for the phagemid and kanamycin resistance for pSGAb, respectively.

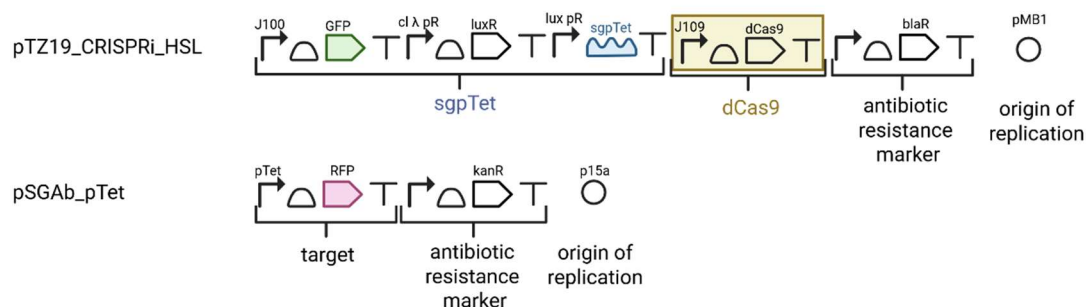


Figure 2-39: Schematic representation of the genetic circuits involved in the inducible CRISPRi tool based on phage delivery. The upper diagram shows the phagemid (pTZ19_CRISPRi_HSL) carrying the inducible CRISPRi system. The lower diagram shows the target construct harbouring the RFP reporter under the control of the pTet promoter, which is targeted by the CRISPRi system (pSGAb_pTet).

Recombinant bacteriophages containing the pTZ19_CRISPRi_HSL engineered phagemid were produced using the helper phage M13KO7 and purified according to the optimized protocol (see Phage Production, Materials and Methods) obtaining the Φ M13::CRISPRi_HSL (see Appendix A6). Two transduction assays were performed with an *E. coli* F' strain harbouring the pSGAb_pTet target plasmid (see Appendix A4). In the first assay, the target strain was infected with Φ M13::CRISPRi_HSL and plated on LB agar Amp⁺Kan⁺ (Figure 2-40 panel a). In the second assay, SM buffer was added as a mock infection, and cells were plated at a 10^{-4} dilution on LB agar Amp⁺ (Figure 2-40 panel b). This setup was designed to compare *rfp* expression in the target strain with and without phage-mediated transduction. In the transduction plate (Figure 2-40 panel a), two types of colonies were observed. The majority appeared transduced and silenced, showing no red fluorescence but a greenish shade. However, a few red colonies were present on the plate and required further investigation. Three red colonies from Figure 2-40 (panel a) were picked and grown in liquid LB Amp⁺Kan⁺, but they failed to grow successfully. Since these colonies were clustered in a specific area of the plate, we hypothesize that they represent satellite colonies. The surrounding green transduced colonies carry both *kanR* and *blaR* genes as selection markers. During ampicillin selection, β -lactamase secreted by resistant colonies can diffuse into the medium and locally inactivate the antibiotic. Under conditions of high plating density or prolonged incubation, this allows ampicillin-sensitive

satellite colonies to appear near resistant ones. We therefore hypothesize that the red colonies are target cells harbouring the plasmid pSGAb_pTet.

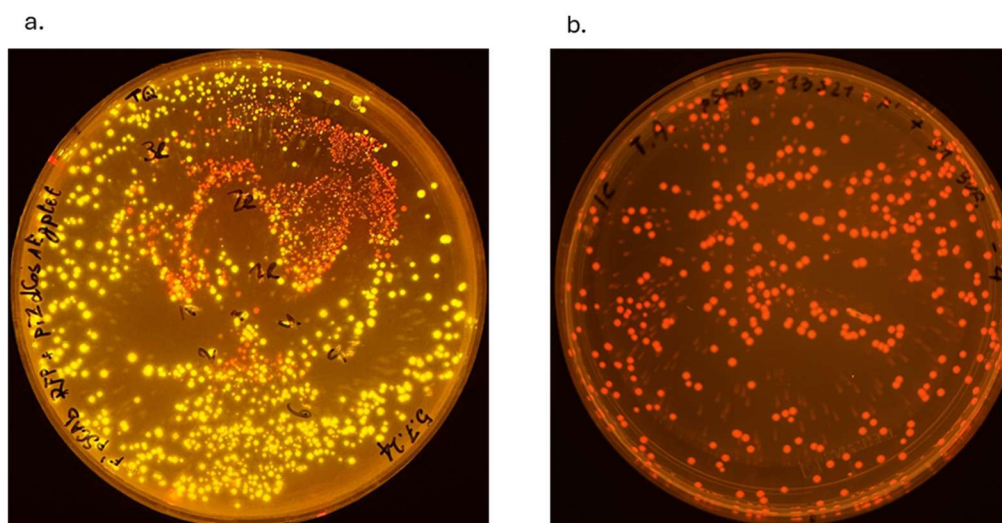


Figure 2-40: Transduction assay with a phage delivery based system carrying a HSL-inducible CRISPRi. (a.) *E. coli* F' harbouring *rfp* successfully transduced with the CRISPRi system. (b.) *E. coli* F' harbouring *rfp* (negative control), plated first at a 1:100 dilution and last at a 10^{-4} dilution.

Besides the qualitative data, we also aimed to obtain a quantitative measurement of red and green fluorescence. A plate reader kinetic assay was performed on three colonies of:

- *E. coli* F' harbouring the target and transduced with Φ M13::CRISPRi_HSL (see Appendix A6),
- *E. coli* F' pSGAb_pTet, the positive control harbouring the target plasmid (see Appendix A4),
- *E. coli* F' as a negative control (wt).

In Figure 2-41 panel a, the cells transduced with Φ M13::CRISPRi_HSL (red bar) exhibited a phenotype reduction in *rfp* expression compared to the positive control (blue bar). This suggests that the engineered phage particles can successfully deliver CRISPRi machinery to silence *rfp*. However, it is important to note that the repression is not completely effective. Two transduced colonies showed a 6-fold reduction in *rfp* repression, whereas the third colony was characterized by a weaker silencing effect, approximately 3-fold less than the other two. Moreover, where the repression of *rfp* is not completely effective, the expression of *gfp* is relatively low compared to the colonies where repression is more effective, resulting in higher *gfp* expression (Figure 2-41 panel b). This observation suggests that the circuit is not fully expressed under conditions of incomplete *rfp* repression. The reason for this difference remains unclear.

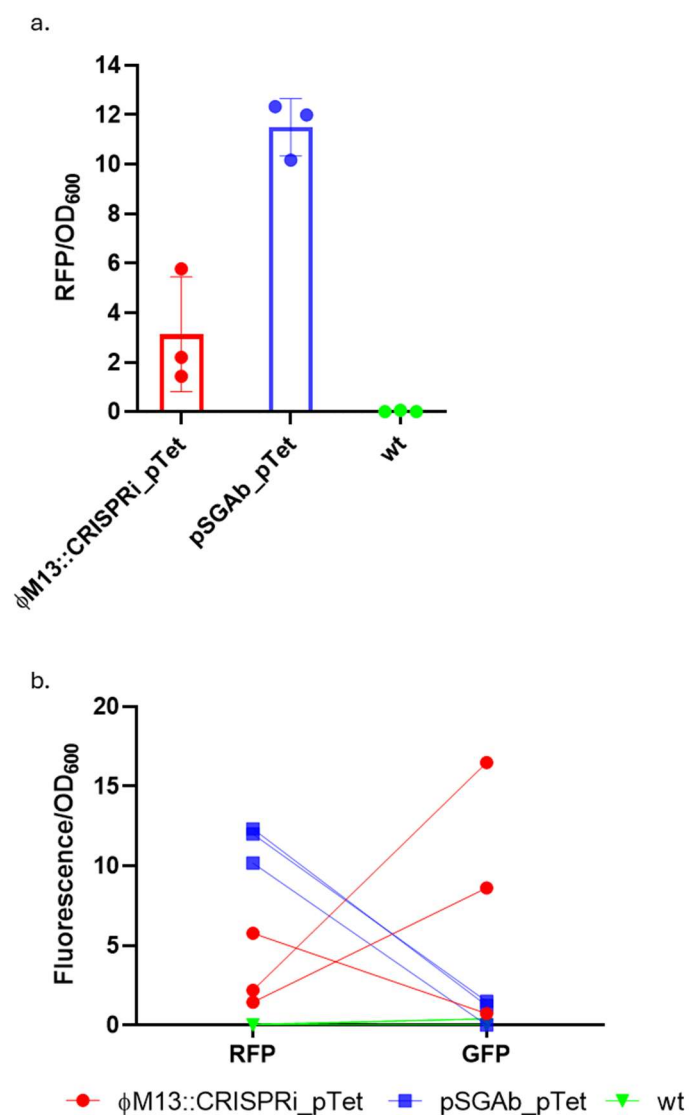


Figure 2-41: Quantification of red and green fluorescence. (a.) Bar graph with error bar showing RFP data of the transduced cells (red bar), the strain carrying pSGAb_pTet (positive control; in blue), the wild-type strain (wt; in green); (b.) Paired comparison presenting RFP and GFP data of the transduced cells (red bar), the strain carrying pSGAb_pTet (positive control; in blue), the wild-type strain (wt; in green).

Based on these considerations, further experiments are required to evaluate the reproducibility of the observations and to investigate the potential of an inducible CRISPRi phage-based system. We performed two different transduction assays: the first to evaluate the ability of ϕ M13::CRISPRi_HSL to transfer the payload, and the second to assess the repression effect of the CRISPRi delivered by the phage. For the first assay, infected bacteria were plated at a 10^0 dilution on ampicillin plates, the selection antibiotic required to maintain the phagemid. For the second assay, bacteria were

plated on LB agar Amp⁺Kan⁺ to maintain both the phagemid and the target plasmid carried by the *E. coli* F' strain.

As shown in *Figure 2-42* (panel a), the phage successfully infected bacteria, producing predominantly green colonies corresponding to bacteria carrying the phagemid with the CRISPRi circuit. A few bright red colonies were also observed, which could represent target cells harbouring the reporter plasmid and expressing *rfp*, as seen in the transduction assay performed previously. The prolonged incubation may have further facilitated β -lactamase diffusion and local antibiotic inactivation, allowing ampicillin-sensitive colonies to grow without effective selection, thereby permitting the target strain carrying the plasmid to proliferate. Additionally, some non-fluorescent whitish colonies were present, likely arising from extended incubation. Prolonged incubation at 37 °C was necessary because, after the first overnight, colonies appeared very small. This observation suggests that the construct transferred via transduction imposes a metabolic burden, slowing cell growth.

Figure 2-42 (panel b) shows the repression assay, whitish colonies were not observed, supporting the hypothesis described in the previous paragraph; in this case, the plates contained double-antibiotic selection, making it unlikely for non-resistant colonies to grow. In this assay, colonies were grown under double-antibiotic selection, and the number of green colonies was fewer than that of red ones. In addition, compared to the previous experiment, a lower overall density of bacterial colonies was detected. Closer inspection revealed that many red colonies displayed a whitish centre surrounded by a red periphery, a phenotype associated with partial repression of *rfp* (*Figure 2-43*). Seven colonies from this assay were picked and re-streaked on double-antibiotic agar; five of these grew, producing both red and green colonies, indicating incomplete repression, as observed in the previous experiment (*Figure 2-43*). Three of these colonies were further analysed using a plate reader assay to quantify fluorescence from the silenced target (*rfp*) and from *gfp*, used as a metabolic burden indicator.

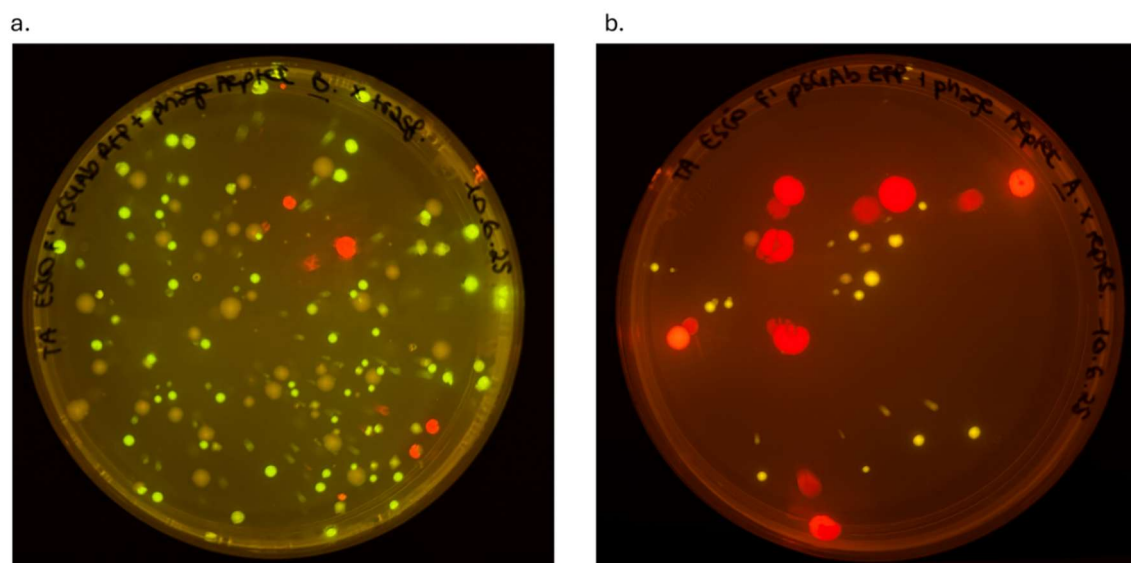


Figure 2-42: Transduction assays infecting *E. coli* F' carrying pSGAb_pTet with Φ M13::CRISPRi_HSL; (a.) Transduction assay plated on LB Amp⁺ to verify the delivery efficiency; (b.) Repression assay under double-antibiotic selection, with fewer green colonies and many red colonies showing a whitish center, indicating partial *rfp* repression.

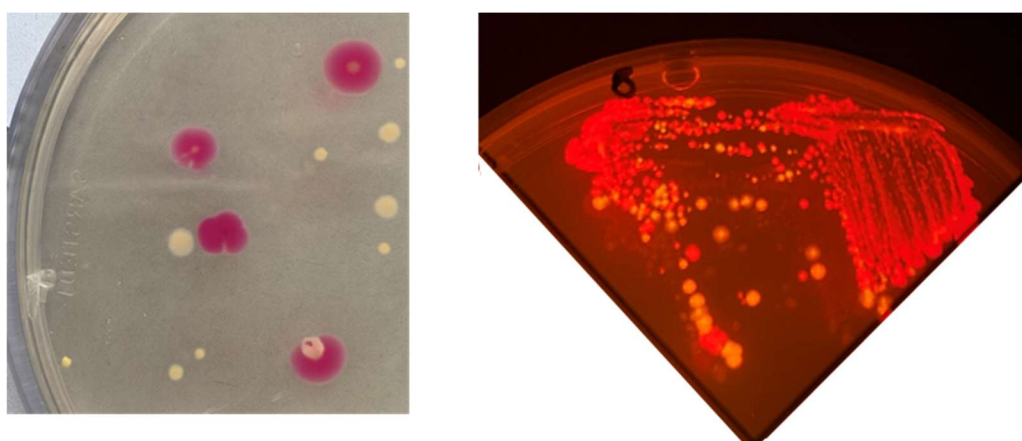


Figure 2-43: Detailed view of the transduction repression assay. On the right, a zoomed view highlights a colony with a white centre and red periphery. On the left, a zoomed detail of colony 8, restreaked from the repression transduction assay, shows an incomplete *rfp* repression phenotype.

As shown in Figure 2-44 (panel a) the growth curves indicate that all three transduced colonies (colony 1, colony 5 and 8) exhibited slower growth than the controls, with colony 1 showing the longest lag phase a delay in growth. However, the analysis of the two fluorescence signals was performed within the OD₆₀₀ range of 0.05–0.18, as described in the Microplate Reader Assay section (Materials and Methods and indicated in Figure 2-44, panel a, dashed lines). This approach allows *rfp* expression to be compared within the same growth phase, ensuring that observed differences in fluorescence reflect variations in reporter expression rather than differences in growth stage. In this context, the fluorescence quantification in Figure 2-44 (panel b) shows that colonies 1 and 8 displayed an

approximately twofold reduction compared to the pSGAb_pTet positive control. In contrast, colony 5 exhibited a reduction, but not as marked as the other two. For *gfp* expression, as expected, the strain harbouring the pSGAb_pTet plasmid did not emit green fluorescence. Colonies 1 and 8 expressed high levels of *gfp*, whereas colony 5, except one replicate, expressed approximately tenfold less *gfp*. The relative fluorescence comparison in *Figure 2-44* (panel c) highlights the same pattern observed in the previous experiment: when *rfp* is repressed, *gfp* expression is high, while when *rfp* levels are not effectively repressed, *gfp* expression is reduced.

We hypothesized that this phenotype could be related to mutations in the plasmid that prevent *rfp* repression, for example, mutations in the CRISPRi circuit. To investigate this, we performed whole-plasmid sequencing of the three strains; however, no point mutations nor massive mutations such as deletions were detected. We therefore hypothesize that the expression cassettes carried on the high-copy phagemid backbone may be subject to bacterial regulation due to the metabolic burden imposed by high copy number. This remains unclear, and further investigation is needed, for instance, by changing the origin of replication to a medium-copy version and performing redesign and tuning of the genetic parts before proceeding to develop an inducible CRISPRi tool based on phage delivery.

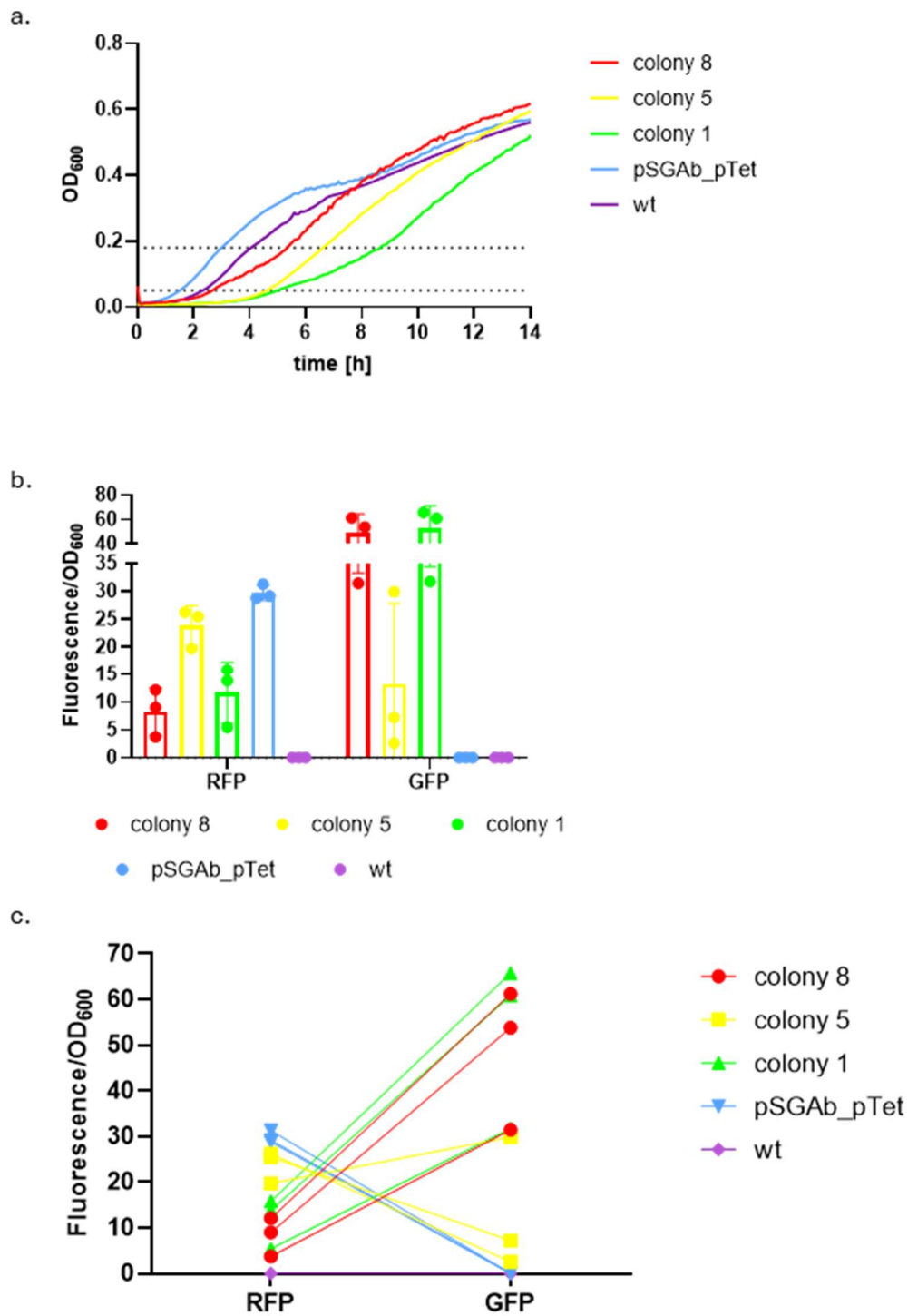


Figure 2-44: Repression transduction assay infecting *E. coli* F' carrying pSGAb_pTet with Φ M13::CRISPRi_HSL. Colonies 1, 5, and 8 are transduced; pSGAb_pTet indicates the *E. coli* F' strain carrying only the target plasmid (pSGAb_pTet), and wt is the unmodified *E. coli* F' strain. (a) Growth curves showing a delay in growth for all three transduced colonies. (b) Expression levels of *rfp* and *gfp* measured in the OD₆₀₀ range 0,05–0,18. (c) Paired comparison of *rfp* and *gfp* fluorescence for each colony, highlighting the inverse relationship between *rfp* repression and *gfp* expression.

2.4.2.4 Observations on Engineered Phage Particle Titration

Comparing results from different phage production batches, such as those described in the previous results section (*Figure 2-42* and *Figure 2-44*), can be difficult when the exact titre of each preparation is unknown. For this reason, we developed a quantification method based on quantitative real-time PCR (qPCR). A major issue with using qPCR to quantify viruses is that it does not measure viable virions, but rather the total amount of viral DNA, including DNA released from particles during infection, which can lead to an overestimation of production. In our case, recombinant phage preparations are unable to generate viable particles, as they lack the complete M13 genome and instead package a phagemid that does not contain the genes required for producing infectious phage. To avoid overestimation caused by free phagemid DNA, we treated the samples with DNase I before phage DNA extraction, thereby eliminating all traces of external DNA. The treated samples were then processed for DNA extraction and quantified by qPCR.

The standard curve was established using the phagemid pTZ19, which carries the target of the real-time PCR system: *rfp* under the control of the pTet promoter. Serial dilutions of the phagemid preparation were performed and amplified according to the protocol outlined in the Quantification of Phage Titre via qPCR section (Materials and Methods). The calibration curve points were averaged to produce a mean calibration curve. A sample of the phage extract was loaded into the real-time PCR system, and once the resulting Ct value was obtained, this value was plotted on the calibration curve (see *Figure 2-45*). The concentration of the target DNA was determined to be 32 ng/μL. This data is preliminary and lacks meaningful interpretation without a comparison to the number of transduced colonies and to other phage preparations.

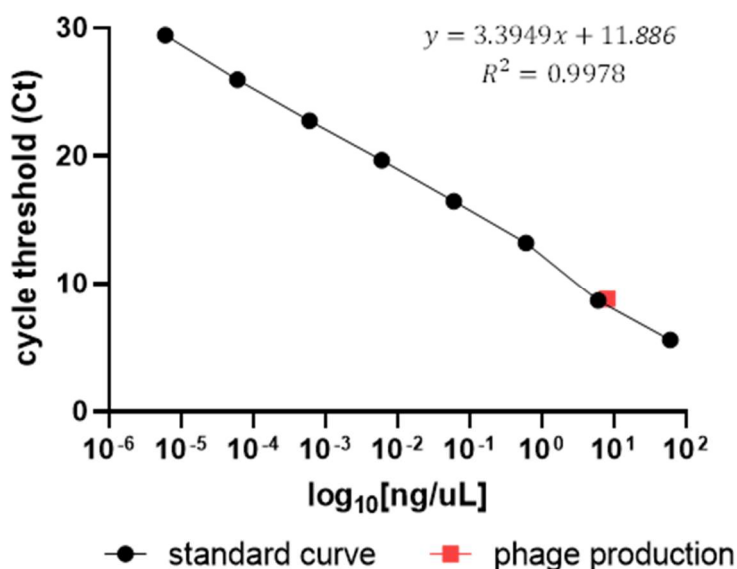


Figure 2-45: Standard curve and preliminary phage particles quantification.

2.5 Discussion

Bacteriophages are an interesting tool that can be used not only to shape the microbiota but also to fight infections caused by antimicrobial-resistant bacteria. Furthermore, they can be exploited for additional purposes. For instance, bacteriophage lysins can be used as antimicrobial molecules to kill bacteria⁵⁴. In addition, bacteriophages can serve as scaffolds to deliver payloads to their hosts. Specifically, essential genetic elements can be cloned into a plasmid known as a phagemid, enabling the generation of recombinant bacteriophages. When a wild-type phage recognizes the packaging signal carried by the phagemid, a new recombinant bacteriophage is produced^{63,64}.

Bikard et al. developed a phagemid based on the *Staphylococcus aureus* ΦNM1 phage, equipped with a CRISPR system targeting *S. aureus* strains carrying plasmid-borne resistance genes⁶⁴. Similarly, *Citorik et al.* designed a delivery system based on the filamentous phage M13 to transfer a CRISPR construct and control carbapenem resistance in *Escherichia coli*⁶³.

Based on these findings, we develop a phage-based platform for the delivery of a CRISPR interference system capable of silencing virulence factors and genes associated with multidrug resistance. We chose CRISPRi instead of nuclease-based CRISPR approaches because DNA cleavage by Cas9 can unintentionally promote the emergence of resistant mutants. When Cas9 introduces a double-strand break in a non-essential gene, the bacterial DNA repair machinery, often through error-prone mechanisms, can restore the break while introducing mutations. These mutations may generate new variants with altered or enhanced resistance traits. In contrast, CRISPRi does not cleave DNA but blocks transcription, therefore repressing gene expression without creating mutations. For this reason, we designed our phage delivery system around CRISPR interference rather than DNA cleavage. Moreover, by avoiding direct bacterial killing and employing a non-lytic phage platform, we reduce the likelihood of selecting for phage-resistant mutants. In other words, since we are not eradicating bacteria but instead attenuating their pathogenicity, we minimize the selective pressure that typically drives bacteria to evolve resistance against lytic phages. Final eradication can then be achieved by administering conventional antibiotics, to which the bacteria have been resensitized. The target bacteria that we are focused on are the ESKAPEE and specifically *A. baumannii*.

To achieve this goal, we first needed to identify a plasmid capable of expressing *dCas9* and transcribing sgRNA in *A. baumannii*. The plasmid pSGAb-km, used by *Wang et al.*, was originally developed as part of a genome-editing platform in *A. baumannii*. This system involved an exogenous recombination system from *A. baumannii*, a *Cas9* from *S. pyogenes*, and an sgRNA module. Each plasmid was designed to replicate in both *E. coli* and *A. baumannii*⁷³. Furthermore, *Bai et al.* used the same a high-copy-number plasmid derived from pWH1266 to develop a CRISPRi system in *A. baumannii*, where *dCas9* was integrated into the bacterial genome⁸⁹.

As a first step, we performed an antibiogram for ampicillin, kanamycin, and chloramphenicol to determine the resistance profile of our strain. Since it was sensitive to kanamycin, we selected that antibiotic marker. We also cloned a red fluorescent protein into the pSGAb-km plasmid and followed the electroporation protocol for *A. baumannii*.

We tested two promoters: the constitutive J23119 and the inducible pTet, though the latter was evaluated without induction. In *E. coli*, J23119 is considered a medium-strength constitutive promoter, while pTet is known as a strong inducible promoter. Interestingly, in *A. baumannii*, we observed the opposite behaviour: J23119 drove stronger expression than pTet. Based on this observation, we decided to further characterize the Anderson promoter library⁷⁸ in *A. baumannii* to determine relative promoter strengths. While some promoters (e.g., J23100, J23101, J23118) exhibited strong expression in both *E. coli* and *A. baumannii*, others (e.g., J23103, J23116) showed different activity profiles, highlighting species-specific variations in promoter performance.

In addition, we evaluated three inducible systems in *A. baumannii*: the anhydrotetracycline system, the homoserine lactone system, and the isopropyl β -D-1-thiogalactopyranoside system. For the aTc system, we observed an increase in *rfp* expression with increasing inducer concentration. However, even at the highest tested concentration, fluorescence levels did not reach those of the non-induced control, for reasons that remain unclear. The HSL-inducible *luxR* transcription factor driving the pLux promoter was shown to be functional in *A. baumannii*. By contrast, the IPTG system requires further optimization: the *lacI* gene, expressed under the selected ribosome-binding site, was only weakly active in *A. baumannii*, leading to insufficient repression and consequently elevated basal *rfp* expression.

Considering these findings, we proceeded to clone *dCas9* into the *A. baumannii* plasmid. We selected the J23116 promoter to drive expression of the inactive nuclease because it exhibits weak activity, and high expression levels of *dCas9* are known to be toxic in *E. coli*. Additionally, we inserted an HSL-inducible sgRNA cassette upstream of *dCas9*, targeting *rfp*. This design aimed to initiate a characterization step of CRISPRi in *A. baumannii* to evaluate the potential silencing effect. To achieve this characterization, we first cloned the target gene (*rfp*) into a second plasmid with a replication origin compatible with the pWH1266-based plasmid. However, the resulting strain carrying plasmid pME3062 with *rfp* exhibited only a low level of fluorescence, insufficient to reliably assess gene silencing. Therefore, we decided to clone the target directly into the same plasmid as the CRISPRi system. This resulted in the construction of a relatively large plasmid of approximately 14 kilobases. In addition to these genetic parts, the plasmid carried a *gfp* cassette upstream of the CRISPRi components, serving as a metabolic burden indicator. Our concern was that introducing such a large construct into the strain could impose a significant genetic burden, and that any observed *rfp* repression might result from this metabolic stress rather than a true CRISPRi effect. We demonstrated that the designed CRISPRi plasmid was able to repress *rfp* expression, although not completely, when compared to the strain carrying a non-sense sgRNA. Furthermore, the expression of the metabolic burden indicator in the strain harboring the CRISPRi system targeting *rfp* was similar to that in cells carrying the non-sense sgRNA, suggesting that the repression was target-specific rather than due to a metabolic burden.

To attempt complete repression, we induced sgRNA expression with HSL. However, none of the tested induction levels resulted in full repression of the target. This led us to hypothesize that *dCas9* expression levels were too low to achieve complete repression. In other words, while the ribonucleoprotein complex was formed, insufficient dCas9 protein was available to generate enough complexes for full repression.

Despite this limitation, we decided to move forward and target a genomic gene in *A. baumannii*. We selected the *ompA* gene for silencing. Previous knockdown studies had demonstrated its non-essential role for bacterial survival, making it a suitable candidate⁹⁰. *ompA* encodes an outer membrane porin protein in *A. baumannii* that plays important roles in virulence, including epithelial cell adhesion, biofilm formation, and antibiotic resistance. Specifically, *ompA* knockdown significantly decreases the minimal inhibitory concentrations of several antibiotics (chloramphenicol, aztreonam, and nalidixic acid), suggesting that OmpA participates in antibiotic extrusion from the periplasmic space through the outer membrane, in coordination with inner membrane efflux systems^{91,92}. To target *ompA*, three plasmids were designed and constructed. The first two carried a single-spacer sgRNA targeting the *ompA* coding sequence at two different positions, while the third combined both guides in a multiplex spacer arrangement. To evaluate the silencing effect of the system, we performed a biofilm assay to determine whether *ompA* repression could impact biofilm formation. The strains carrying the single-spacer sgRNAs were still able to form biofilm, although differences were observed between the guides. Specifically, sgRNA₁₅₇ resulted in less biofilm compared to sgRNA₂₁₁. Interestingly, the multiplex guide had a much stronger impact on biofilm formation, with no visible biofilm detected in the tubes or only a weak, non-adherent matrix. These results were further confirmed by crystal violet quantification.

Additionally, reverse transcription real-time PCR analysis was performed to quantify *ompA* expression levels. The RT-qPCR data aligned with the phenotypic results: strains carrying the single-spacer sgRNAs showed partial downregulation of *ompA*, whereas the multiplex sgRNA construct led to a marked reduction in *ompA* transcript abundance, consistent with the observed loss of biofilm formation.

In parallel, we investigated a phage-delivered platform to transfer CRISPRi into target bacteria. The literature describes M13 phage as a versatile platform capable of encapsidating nearly 42 kilobases of DNA, with the possibility of packaging foreign DNA using a phagemid that carries the payload, along with a helper M13 plasmid that provides the phage genes^{59,63}. ΦM13 naturally infects *E. coli*. However, by using a helper plasmid, it is possible to delete part or all of gene 3, which encodes the receptor-binding protein, and replace it with a homolog from an *Acinetobacter baumannii* filamentous phage, as demonstrated by Yehl *et al.*³⁹

To evaluate this platform, we first performed a transduction assay to test whether the recombinant phage could deliver a reporter gene. A successful transfer would result in the appearance of red colonies on the plate, since the phage is non-lytic and we used an *rfp* marker to allow for a rapid and simple readout. Initially, the experiment appeared highly successful, with nearly all transduced colonies exhibiting red fluorescence. However, when we repeated the assay multiple times, the efficiency of transduction declined, and the results became inconsistent. We hypothesized that, for some reason, the phage particles were not being correctly assembled or produced. To investigate this, we checked whether both the

phagemid and the helper plasmid were still present in the transduced cells using PCR amplification of their respective antibiotic resistance markers. PCR confirmed their presence, so we proceeded with whole plasmid sequencing. Sequencing revealed that while the phagemid remained intact and matched the expected reference map, the helper plasmid had accumulated multiple mutations in structural Φ M13 genes. These mutations impaired the assembly of functional phage particles, indicating that the helper-phage-based system was genetically unstable.

As a result, we switched to producing particles using helper phages rather than helper plasmids. This system relies on a mutated version of the wild-type Φ M13. The main limitation of this approach is that, although the majority of the produced phages are recombinant, a small fraction of wild-type phages is also generated.

To assess the purity of the preparation and quantify contamination by wild-type helper phages, we performed a transduction assay using a recombinant phage carrying *rfp* and plated the infected cells on LB plates supplemented with ampicillin and on LB plates supplemented with kanamycin. On ampicillin plates, we expected to see only red colonies infected with the recombinant phage, whereas colonies appearing on kanamycin plates would indicate infection by wild-type phages. We determined purity by calculating the ratio of colonies growing on ampicillin over the total colonies observed on both ampicillin and kanamycin plates. This experiment revealed a purity of 99%, meaning that approximately 1% of the phage population consisted of wild-type phages.

Furthermore, we investigated the specificity and efficiency of the transduction process. We demonstrated that the system is specific for strains carrying the F' pilus, which is the receptor required for infection. The efficiency of transduction was about 92%, indicating that most cells received and expressed *rfp*.

Encouraged by these results, we proceeded to transfer a more complex system: a CRISPRi construct targeting two antibiotic resistance genes, *bla_{ndm-1}* and *mcr-1*, which confer resistance to the last-resort antibiotics meropenem and colistin, respectively. The goal was to restore sensitivity to these antibiotics in laboratory strains harbouring *bla_{ndm-1}* or *mcr-1*. To achieve this, we used three different phagemid constructs carrying CRISPRi arrays: one with a single spacer targeting *mcr-1*, one with a single spacer targeting *bla_{ndm-1}*, and one multiplex array targeting both genes simultaneously. No additional characterization of the CRISPRi arrays was required, as they were directly adapted from a previously published study⁸⁰.

In laboratory strains carrying *bla_{ndm-1}*, we observed a reduction in the MIC: resistant strains normally grew at 100–200 μ g/mL meropenem, while transduced strains showed impaired growth even at 10 μ g/mL, with a growth delay of $\approx$ 5 hours compared to resistant controls. Interestingly, when strains were transduced with the multiplex construct, the resensitisation effect was less pronounced: the strains were able to grow at 100 μ g/mL, although with a $\approx$ 2-hour growth delay. This reduced effect may be explained by competition for dCas9 between the two guide RNAs in the multiplex array. Such limitations should be carefully considered when designing CRISPRi systems against MDR bacteria carrying multiple resistance genes.

Regarding colistin resistance, CRISPRi targeting *mcr-1* did not reduce the MIC, as transduced strains still grew at the same concentrations as resistant controls. However, we observed a substantial growth delay: ≈ 4 hours at 1–2 $\mu\text{g/mL}$ colistin and ≈ 8 hours at 4 $\mu\text{g/mL}$.

In summary, these results highlight both the potential and the challenges of using phage-delivered CRISPRi systems for resensitizing antibiotic resistant bacteria.

Finally, we investigated the possibility of using a phage-based delivery platform to transfer an inducible CRISPRi system. In particular, we designed an HSL-inducible sgRNA, and upstream of it we introduced a metabolic burden indicator to monitor the stress imposed by the new phagemid. To simplify the readout, we engineered an *E. coli* F' strain carrying a plasmid with an *rfp* cassette and targeted the *rfp* promoter to repress fluorescence expression. The experiment was repeated twice. However, in addition to the silenced cells, some cells continued to express *rfp*. Interestingly, the non-silenced cells not only displayed high levels of red fluorescence but also showed reduced expression of *gfp*. Despite sequencing the entire plasmid in the non-silenced cells, no mutations were detected in the phagemid. This led us to hypothesize that expression cassettes carried on a high-copy phagemid may be subject to bacterial regulation due to the metabolic burden imposed by elevated plasmid copy numbers. This limitation requires further investigation. Possible next steps include switching to a medium-copy origin of replication and redesigning and fine-tuning the genetic parts before moving forward with the development of an optimized inducible CRISPRi tool delivered by phages.

2.5.1 Future Perspectives

Overall, the promising results obtained with the actuator machinery highlight the importance of this platform, which can be used not only to silence virulence or resistance genes, but also as a tool to study the role of specific genes in pathogens by interfering with their expression. The possibility of fine-tuning the CRISPRi system represents an important step forward, as it would allow investigation of different expression levels in the pathogen.

An important next step would be to replace the current promoter of *dCas9* in the HSL-inducible system with a medium-to-weak promoter in *A. baumannii*, to establish an inducible platform and test it against the genomic target *ompA*. Although the phage-based delivery system was not specifically designed for *A. baumannii*, we obtained encouraging results in *E. coli*, which is also recognized as an ESKAPEE pathogen. Extending this work to *E. coli* clinical isolates would be particularly interesting. The first step would be a screening assay to determine whether clinical isolates can be infected by the recombinant phage. For this purpose, an *rfp* reporter could be delivered to provide a rapid readout, as infected strains would display red fluorescence. Positive isolates could then be tested further by delivering CRISPRi constructs targeting their resistance genes, according to the resistance profiles of each strain. To assess whether this platform is feasible for clinical application, *in vivo* testing will be required. For example, evaluating the CRISPRi phage-based platform in an UTI (urinary tract) mouse model would provide valuable insight into its therapeutic potential.

Finally, the delivery platform must be adapted specifically to *A. baumannii*. One possible strategy involves exploiting synthetic biology to modify the ΦM13 helper phage. In particular,

replacing gene 3 (responsible for host recognition) with the corresponding gene from a newly characterized filamentous *A. baumannii* phage⁹³, followed by rebooting of the recombinant phage genome in *A. baumannii*, could enable infection of this clinically relevant pathogen. To achieve this, the helper phage genome could be engineered in a yeast vector and subsequently rebooted, generating a modified helper phage specifically targeting *A. baumannii*. In parallel, to produce recombinant phage particles capable of infecting *A. baumannii*, a compatible phagemid must be designed and built. This can be achieved by cloning the Φ M13 508 bp intergenic region, which contains the packaging signal, into the pSGAb backbone, while introducing the desired CRISPRi circuit into the cloning site. This strategy would represent a crucial step toward the development of a versatile and effective phage-based delivery platform capable of addressing the urgent need for alternative therapies against multidrug-resistant *A. baumannii*.

Conclusion

The work presented in this thesis explores complementary strategies for harnessing bacteriophages in the fight against antimicrobial-resistant pathogens. In Chapter 1, naturally occurring bacteriophages were investigated for their ability to infect *Klebsiella pneumoniae* under both *in vitro* and *in vivo* conditions, while Chapter 2 focused on engineering bacteriophages as delivery platforms for CRISPR interference systems to repress bacterial virulence and resistance determinants. Together, these two approaches illustrate both the potential and the challenges of deploying bacteriophages as versatile tools to combat multidrug-resistant bacteria.

The first strategy relied on the selection and characterization of naturally isolated bacteriophages, evaluating how environmental and physiological factors influence their infectivity. Testing under conditions that mimic the gut environment revealed that *in vitro* assays often fail to accurately represent the actual infectivity of the tested phages. Specifically, bacteriophage performance appeared to vary markedly between aerobic and anaerobic conditions. However, phage groups such as *Webervirus*, *Slopekvirus*, and *Purpurea* demonstrated remarkable stability across different environments, indicating that robust and persistent phages can be identified through careful screening. These findings highlight that successful phage cocktail design must account for both phage–host interactions and the ecological context in which infection occurs.

The second approach explored the feasibility of repurposing phages as scaffolds for delivering CRISPRi systems to repress genes involved in multidrug resistance. The construction of a CRISPR interference system in *Escherichia coli* carrying the *bla_{ndm-1}* or *mcr-1* gene, and its subsequent transfer using a filamentous phage-based platform, represents a significant step toward programmable modulation of bacterial gene expression. Unlike nuclease-based CRISPR systems, CRISPRi offers a reversible and non-lethal means of silencing target genes, thereby reducing selective pressure for resistance. Furthermore, the successful silencing of biofilm determinants, *ompA*, in *Acinetobacter baumannii*, as well as the development of phage-delivered CRISPRi systems targeting resistance genes in *E. coli*, confirmed the potential of this approach to combat antimicrobial resistance.

When considered together, the two strategies highlight the complementary nature of natural and synthetic phage-based approaches. Naturally occurring phages, selected for their environmental stability and infectivity, act as potent agents capable of eradicating multidrug-resistant pathogens from the host. However, concerns remain regarding their fate once introduced into the circulatory system, as they may be recognized and cleared by the host immune response. Further investigation into phage–host immune interactions is therefore needed to determine whether the *K. pneumoniae* bacteriophages tested in this work would remain effective in complex environments such as human blood. Moreover, an interesting direction for future research would be to test the isolated bacteriophages in urine, given the potential application of the cocktail for treating urinary tract infections.

Another major challenge for the therapeutic application of lytic phages is the potential emergence of bacterial resistance to the phages themselves, an issue similar to antibiotic resistance. This limitation can be mitigated through the formulation of phage cocktails; yet,

as demonstrated in this work, designing effective mixtures is far from trivial. Numerous factors must be considered, including the physiological environment of the host and the resident microbiota, both of which can influence therapeutic outcomes.

While many questions remain regarding the therapeutic use of lytic bacteriophages, the field is rapidly evolving. In parallel, the rise of disciplines such as synthetic biology is driving innovation in bacteriophage research. Recombinant and engineered phages offer precision and adaptability, enabling the reprogramming of bacterial functions beyond simple lysis. The approach developed in this thesis demonstrates the potential of such engineered systems as versatile tools to combat antimicrobial resistance. By reprogramming wild-type phages as delivery scaffolds rather than lytic agents, bacteria are not subjected to strong selective pressure to develop resistance, since the goal is to restore antibiotic sensitivity rather than to kill the cells directly.

Further progress will depend on expanding our understanding of bacteriophage genomics and improving predictive models to support rational phage design. Comprehensive annotation of phage genomes will be essential for creating recombinant phages with predictable and controllable properties. This will, in turn, require iterative refinement of experimental models, standardization of phage characterization methods, and the integration of computational tools for predictive design.

Overall, the combination of natural and synthetic phage-based strategies may enable the development of advanced phage therapies that combine environmentally robust phage backbones with programmable genetic payloads, allowing precise modulation of bacterial resistance and virulence.

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A. Appendix

A1. Primers Used in Chapter 1: Bacteriophages for Direct Killing

Primers sequences (5'-3')	Name	Usage
ctcagaggtgaagacgaaaggg	sID1 p3vector_fwd	to amplify the vector pZE4D-hyg-mC
cctaggcggtcggctgcg	sID2 p3vector_rev	
gccgcagccgaacgcctaggGGATTATACCAGCCGCCC	sID3 prot2-p3_fwd	to amplify the 800 homologous base pair from ϕ ER12 gene 2
taagcttgatccccgggAAGGCGTTACCAATATGGGG	sID4 prot2-p3_rev	
ttccccgggaTCCAAGCTTATGAATCCAC	sID5 prot5-p3_fwd	to amplify the 800 homologous base pair from ϕ ER12 gene 5
cctttcgtttcacctcgagATCTTGAGCGTGGTAAAAC	sID6 prot5-p3_rev	
gccgcagccgaacgcctaggCTGATAACCGGCTGTTGAG	sID7 prot3-p5_fwd	to amplify the 800 homologous base pair from ϕ ER12 gene 5
TCCCCGGGGATGAATCTC	sID8 prot3-p5_rev	
aggagattcatccccggggaTCCAAGCTTATGAAAACGCA GTTTAAC	sID9 prot7-p5_fwd	to amplify the 800 homologous base pair from ϕ ER12 gene 5
cctttcgtttcacctcgagAATGCCACGGCCATCCGA	sID10 prot7-p5_rev	
gccgcagccgaacgcctaggTATACAACGGCTGCGAATA ATGCCTATATCCAGC	sID11 port5-p7_fwd	to amplify the 800 homologous base pair from ϕ ER12 gene 5
taagcttgaTCCCCGGGGGGTGGTTCC	sID12 port5-p7_rev	
cccccggggaTCCAAGCTTATGCAACAATTG	sID13 prot8-p7_fwd	to amplify the 800 homologous base pair from ϕ ER12 gene 8
cctttcgtttcacctcgagCCGTCTTTGATCTTTGCC	sID14 prot8-p7_rev	
TCCAAGCTTATGAATCCACAATTA	sID46-FW_backbone-pZE4Dp3	to amplify backbone p3
AAGGCGTTACCAATATGGGGTA	sID47-RV_backbone-pZE4D-p3	
TATTGGTAACGCCCTTATGGCCAGAAACATAGAAGAAATC	sID48-FW_ER20_Gib	to amplify ϕ ER20 gene 3
GATTCATAAGCTTGGATTATTCCTCCAAATATAACGCCAC	sID49-RV_ER20_Gib	
GATTCATAAGCTTGGATTATGCCCTTAATCTTGCGTTAAT	sID50-RV_LiBp3_Gib	to amplify ϕ LiB gene 3
ATATTGGTAACGCCCTTATGGGGTATTTCAAATGACCAGA	sID51-FW_LiBp3_Gib	
AAGCTTATGAAAACGCAGTTTAAC	sID52-FW_backbone-pZE4Dp5	to amplify backbone p5
GATGAATCTCCTGTTGTTTTCTG	sID53-RV_backbone-pZE4Dp5	
CAACAGGAGATTATCATGAATAAGATGTTTCAGTCAGCCTG	sID54-FW_prot5-ER75_Gib	to amplify ϕ ER75 gene 5
TGCGTTTTTCATAAGCTTTCAATTCACCTTTGTATCTACTGGA	sID55-RV_prot5-ER75_Gib	
AAGCTTATGCAACAATTGAATCTTAAC	sID56-FW_backbone-pZE4Dp7	to amplify backbone p7
GGGTGGTTCCTCATGTTGTTTT	sID57-RV_backbone-pZE4Dp7	
CATGAGGAACCCATGAAAACGCAATTTAACCAATCA	sID58-FW_prot7_LiB	to amplify ϕ LiB gene 7
ATTGTTGCATAAGCTTTCAAACGATTCCAGAGTCAAAAC	sID59-RV_prot7_LiB	
AAGAACTCTGTAGCACCGCC	ER528	to screen via colony PCR the obtained cloned plasmid
GCCCTCCTACATCGAAGCTG	ER469	

Table A-1: Primers designed for the bacteriophage for killing chapter.

A2. Synthetic Strands and Primers for the Actuator Machinery

Synthetic strand and primers 5'-3' sequences	Features/Name	Usage
gtcaggAAGCTTGTCTTCTCGAATTCGCGGCCGCTTCTAGAG ttgacagctagctcagtcctaggtataatgctagc TACTAGTAGCGGCCG	ds DNA cloned into pSGAb-km	to insert the EcoRI, XbaI, SpeI and PstI recognition sites
caagaagatcatcttattaagaa GATCCTTTGATCTTTTCTACGGGGTCTG	FW pSGAb backbone	to amplify the backbone pSGAb except the ORI
agccagtatacactccgctaAACGCCAGCAACGCGGCCTT aaggccgcgttgctggcgtTAGCGGAGTGATACTGGCT	RV pSGAb backbone	
gaaaagatcaaagatcttcTTAATAAGATGATCTTCTGAGATCG	FW pSB3K3 ori	amplifying the p15a origin of replication
	RV pSB3K3 ori	
tgtcaatctctatcactgat	sgRNA_pTet	sgRNA targeting the promoter pTet
ataacaattgacattgtgag	sgRNA_pLac	sgRNA targeting the promoter pLac
aataaatcgcttctaactc	sg175_OmpA	sgRNA design on OmpA in <i>A. baumannii</i>
tcagcttcgaacctaacca	sg211_OmpA	sgRNA design on OmpA in <i>A. baumannii</i>
tgccacctgacgtctaaga	FW_sg_157_OmpA_ACBA	sg157 OmpA amplifying primers
gctcactcaaaggcggttaa	RV_sg157_OmpA_ACBA	
tgccacctgacgtctaaga	FW_sg211_OmpA_ACBA	sg211 OmpA amplifying primers
gctcactcaaaggcggttaa	RV_sg211_OmpA_ACBA	
CGGCCGCTTCTAGAGgcatgcaagctttccctatag	FW_prefix_insertion	mutagenesis to add EcoRI and XbaI sites
CGAATTCGAAGAAACactggccgtcgtttaca	RV_prefix_insertion	
CTGCAGGAAGAAACgcatgcaagctttccctatag	FW_suffix_insertion	mutagenesis to add SpeI and PstI sites
CGGCCGCTACTAGTactctagaagcgccgcgaat	RV_suffix_insertion	
cctaggactgtgctagctaCTAGA	Mut-J116-109 FW	mutagenesis to replace J23116 promoter upstream dCas9 with J23109
actgagctagctgtaaactctagaag	Mut-J116-109 RV	
gcaactggtctatttcctc	FW_seq_pSGAb	to sequence cloning site of pSGAb backbone
gcaacgcaattaatgtgagt	RV_seq_pSGAb	
caattgttatattcactataacaaaccattttctt	RV_mut_sgpLac	to replace the sgpTet with the sgpLac
acattgtgagGTTTGTAGAGCTAGAAATAGCAAGTAAAA	FW_mut_sgpLac	
caatgcgcaaatgaaactc	FW_seq_pLacmut	to sequence the pSGAb with the replaced sgpLac
gatgatgatgatgatggtcg	RV_seq_pLacmut	
TCTTGGTGGTCACTGAAGC	OmpA Forward	to evaluate the expression of <i>ompA</i> with real time PCR
ACTCTTGTGGTTGTGGAGCA	OmpA Reverse	
GTTGCTGAAGAAGAAGCTGCTG	FW_rpoB_RT_ACBA	to evaluate the expression of <i>rpoB</i> with real time PCR
ACTGTACCCATTTACGCATGTA	RV_rpoB_RT_ACBA	
aaacaaataggggtccgcgtcggaacgataggccgctctaga	pME6032 FWD	to amplify the backbone pME6032 with removing the <i>tetR</i>
cctcactgattaagcattggcaggaccgctgccggagcg	pME6032 REV	

cgctccggcagcggtcctgaccaatgcttaatcagtgaggc	pTZ19R iGEMized FWD	to amplify the <i>blar</i> gene from the backbone pTZ19R
gagcggcctatcggtttccacgacgcggaacccctattgttt	pTZ19R iGEMized RV	
tgtttcctgtgtgaaattgttatccgc	FW_Ramp_A	to amplify the of pME6032_blaR backbone
gctccaaggatcgggccttgatgtta	RV_Ramp_A	
caaggcccgatccttggagcaagtgccacctgacgtctaagaaac	I13521_FW_Gibson_A	to amplify the <i>rfp</i> driven by pTet
acaatttcacacaggaacaattaccgcctttgagtgagctgata	I13521_RV_Gibson_A	
cacgatgatcgtgccgtgat	FW_seq_pME6032_bla_pTet	to sequence the antibiotic selection marker in pME6032
gtaaatgcatgccgcttcgc	RV_seq_pME6032_bla_pTet	
atgaaaaagcccgtagcggg	FW_seq_pME6032_bla	to sequence the cloning site in pME6032
gcgacatgtcgttttcagaa	RV_seq_pME6032_bla	
agtcctaggtattatgtagctaCTAGAGAAAGAGG	Mut_J101_dcas_FW	to replace the J23116 promoter upstream the <i>dCas9</i> with J23101
gagctagctgtaaaactctagTAAGTTCACCGACAAAC	Mut_J101_dcas_RV	
CCGACGCTATTGTGCCG	Seq_dcas_promoter_RV	to sequence the replacement of J23116 with J23101 upstream <i>dCas9</i>
agtgagcgtactgtcact	Seq_dcas_promoter_FW	

Table A-2: Synthetic DNA fragment and primers designed for the actuator machinery.

A3. Synthetic Strand and Primers for the Delivery Platform

Synthetic strand and primers 5'-3' sequences	Features/Name	Usage
taacgcttacaaattccattc	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
acaggtttcccgactgga	RV_seq_pTZ19_NEb	
gtaaaaaggccgctgtgctggc	pTZ19R_rt-PCR_FWD 1	qPCR on pTZ19RFP
tcgggtttgccacctctgact	pTZ19R_rt-PCR_REV 2	
ccccctgacgagcatcacaaaa	pTZ19R_rt-PCR_PROBE2	FAM-TAMRA probe
cactaagggttagttagttttacagctagctcagtcctaggga	FW_DFdcas264	to amplify the <i>dcas9</i> from the backbone pTZ19_J109dCas9
ttacgccaagctctaatacagctcactatagggaagcttgcac	RV_DFdcas264	
gtcacgacgttgtaaacgataTACTAGAGTTGACGGCTAGCTC	FW_DFnewarray	to amplify the double array (ndm1-mcr1) from T10-ORIT-AedCas9-ARRAYndm1mcr1 and the single (ndm1) from T10-ORIT-AedCas9-ARRAYndm1
ggactgagctagctgtaaaaactaactaaccttagtgactcct	RV_DFnewarray	
gtattagagcttggcgtaacatgg	FW_DFnewback264	to amplify the pTZ19 backbone from pTZ19_J109dCas9
cgtttacaacgtcgtgactg	RV_DFback264	
gctcactcaaggcggtaac	FW_backbone3K3	to amplify the backbone pSB3K3
cagaaatcatccttagcgaaag	RV_backbone3K3	
Taaggatgattctgtatcggtgattcattctgctaac	FW_MCR-1	to amplify the <i>mcr-1</i> from pGDP2-mcr-1
cgcccttgagtgagccatgagcggatacatatttgaatg	RV_MCR-1	
taaggatgattctgattaacgcttacaatttagtgcc	FW_NDM-1	to amplify from the <i>bla ndm1</i> from pGDP1-ndm-1
cgcccttgagtgagcgtcgtgagtttttctaagaattaat	RV_NDM-1_new1_P39	
atccagtcattccatgggtgc	FW_seq_3K3back	to sequence the cloning site of the pSB3K3 backbone
gcagtttcatttgatgctcg	RV_seq_3K3back	
gaattctactagagttgacggctagctcagtcctaggtacag tgctagcggaaaccattcaaaacagcatagcaagttaaaa taaggctagtcggtatcaactgaaaaagtggcaccgaggt cggtgcttttttcgaaaaaacccgcttcggcgggttttttc gcactagtaattgtgagcggataacaattgacattgtgagcg gataacaagatactgagcacagtttttagagctatgctgtttg aatgggtcccaaacgccacaagaacaacggactgacc gagcgtgttttagagctatgctgtttgaatgggtcccaaac gatctaaaaaaaaccccgccctgacagggcgggggttt tttttctaga	synthesized mcr-1 array	to clone cutting EcoRI-XbaI the single crRNA mcr-1 in the pTZ19_J109dCas9

Table A-3: Synthetic DNA fragment and primers designed for the delivery platform.

A4. Plasmids for the Actuator Machinery

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</i> <i>E.coli</i> origin of replication replaced with the p15a origin of replication
pSB3K3	iGem Registry	origin of replication
<i>pSB1A2_I13521</i>	iGem Registry	expression of Red Fluorescent Protein under the pTet promoter
pSGAb_pTet	this work	expression of RFP under pTet promoter in the shuttle vector
pSGAb-LuxR	this work	to assess the functionality of the inducible- HSL system in <i>A. baumannii</i>
pE47	iGem Registry	to clone the insert (HSL-inducible system in pSGAb
pSB3K3_dCas9	iGem Registry	to mutagenize the EcoRI site in the dCas9 sequence
pSB3K3_dCas9mut	this work	to express a compatible BioBrick standard dCas9
pAEgpTtet	iGem Registry	to clone the insert (HSL-inducible <i>sgpTet</i> in the pSGAb vector)
pSGAb_CRISPRi	pAEgpTet;	to characterize the CRISPRi system in <i>A. baumannii</i>
pAEgpLac	iGem Registry	to replace the <i>sgpTet</i> with the <i>sgpLac</i> in the pSGAb vector
pSGAb_CRISPRi_asp	pSGAb_CRISPRi;	to characterize the CRISPRi system in <i>A. baumannii</i>
pSGAb_sg157	this work	to silence the <i>OmpA</i> gene in <i>A. baumannii</i> with the guide
pSGAb_sg211	this work	to silence the <i>OmpA</i> gene in <i>A. baumannii</i> with the guide
pSGAb_2X	this work	to silence the <i>OmpA</i> gene in <i>A. baumannii</i> with the guide
pSBJ23114_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23114_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23103_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23103_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23105_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23105_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23113_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23113_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23101_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23101_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23110_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23110_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23116_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23116_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23100_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23100_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23106_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23106_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23112_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23112_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23113_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23113_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23117_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23117_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>
pSBJ23118_RFP	iGem Registry	to extract the RFP cassette
pSGAb_J23118_RFP	this work	to evaluate the promoter strength in <i>A. baumannii</i>

pSB1A2_I13507	iGem Registry	to clone the I13507 (RPF gene) downstream different promoters
pSGAb_J23119	pSB1A2_I13507	to clone the I13507 (RPF gene) downstream
pME6032	Heeb et al. (79)	shuttle vector for <i>A. baumannii</i> and <i>E. coli</i>
pME6032_blaR	this work	pME6032 edited to change the original TetR resistance gene with a
pME6032_pTet	this work	pME6032_blaR edited to insert BBa_I13521 with flanking RFC[10]
pSB4C5_ATC	UniPV	to extract the aTc inducible system for mRFP expression and insert
pSGAB_aTc	this work	To clone aTc inducible system inside <i>A. baumannii</i> .
Y32_pLac	UniPV	Backbone used to construct the system Y32_pLac_RFP by cloning
Y32_pLac_RFP	this work	To create an IPTG-inducible system for mRFP expression under
pSGAbi_Y32_pLac_RFP	this work	To clone the IPTG-inducible system inside <i>A. baumannii</i>

Table A-4: Plasmids used in the actuator machinery section.

A5. Plasmids for the Delivery Platform

Plasmid	Source	Usage
pTZ19R	Thermo Fisher (SD0141)	phagemid
pTZ19	this work	phagemid following the BioBrick standard
pSB1A2_I13521	iGem Registry	to express <i>rfp</i> under pTet
m13cp	LANL	helper plasmid to generate recombinant phages
pTZ19_pTet	this work	to express <i>rfp</i> under pTet in the phagemid
pSB3K3_dCas9	iGem Registry	to mutagenize the EcoRI site in the <i>dCas9</i> sequence
pSB3K3_dCas9mut	this work	to express a compatible BioBrick standard <i>dCas9</i>
pSB3K3_J109dCas9	this work	to express a compatible BioBrick standard <i>dCas9</i> driven by J23109
pTZ19_J109dCas9	this work	to express <i>dcas9</i> driven by J23109 in the phagemid
pGDP2-mcr-1	UniPV	to express <i>mcr-1</i>
mcr-1_3K3	this work	to express <i>mcr-1</i> in a backbone compatible with the phagemid backbone
pGDP1-ndm-1	UniPV	to express <i>bla ndm-1</i>
ndm-1_3K3	this work	to express <i>bla ndm-1</i> in a backbone compatible with the phagemid backbone
T10-ORIT-AedCas9-ARRAYndm1	UniPV	plasmid carrying the CRISPRi targeting <i>bla ndm-1</i>
pTZ19_ndm-1	this work	phagemid carrying the CRISPRi targeting <i>bla ndm-1</i>
T10-ORIT-AedCas9-ARRAYndm1mcr1	UniPV	plasmid carrying the CRISPRi targeting <i>bla ndm-1</i> and <i>mcr-1</i>
pTZ19_ndm-1_mcr-1	this work	phagemid carrying the CRISPRi targeting <i>bla ndm-1</i> and <i>mcr-1</i>
mcr-1-array_pUC57	Genscript	plasmid harboring the crRNA:tracrRNA targeting <i>mcr-1</i>
pTZ19_mcr-1	this work	phagemid carrying the CRISPRi targeting <i>mcr-1</i>
pTZ19_CRISPRi_HSL	this work	phagemid carrying the inducible HSL CRISPRi circuit targeting the <i>rfp</i>

Table A-5: Plasmids used in the delivery platform section.

A6. Bacteriophages

Bacteriophages	Generator plasmid	Usage
M13KO7	none (mutated version of the wt)	helper phage to generate recombinant phages
ΦM13::RFP	pTZ19_pTet	recombinant M13 phage carrying <i>rfp</i> as payload
ΦM13::CRISPRi_mcr-1	pTZ19_mcr-1	recombinant M13 phage carrying the CRISPRi targeting <i>mcr-1</i>
ΦM13::CRISPRi_ndm-1	pTZ19_ndm-1	recombinant M13 phage carrying the CRISPRi targeting <i>bla_ndm-1</i>
ΦM13::CRISPRi_ndm-1_mcr-1	pTZ19_ndm-1_mcr-1	recombinant M13 phage carrying the CRISPRi targeting <i>bla_ndm-1</i> and <i>mcr-1</i>
ΦM13::CRISPRi_HSL	pTZ19_CRISPRi_HSL	recombinant M13 phage carrying the CRISPRi targeting <i>rfp</i> with a HSL-inducible sgRNA

Table A-6: Bacteriophages used in the delivery platform section.

A.7 Supplementary Material

The two images in the *Figure A-1* were captured using a Leica fluorescence microscope to verify the presence of fixed bacteria on the slides and to confirm the expression of red fluorescent protein. The scale bar at the bottom right represents 0.50 μm , consistent with the typical size of the coccobacillus *Acinetobacter baumannii*. In panel a, the cells are shown under bright-field conditions, revealing a clear background and confirming the presence of bacteria. In panel b, the same cells are visualized under green light excitation, highlighting the red fluorescence of the plasmid. These images are included to complement this work; however, the images presented in the 2.4.1.2.3 *CRISPRi Characterization in A. baumannii* (Results Section) were acquired with a confocal microscope, providing higher-resolution visualization.

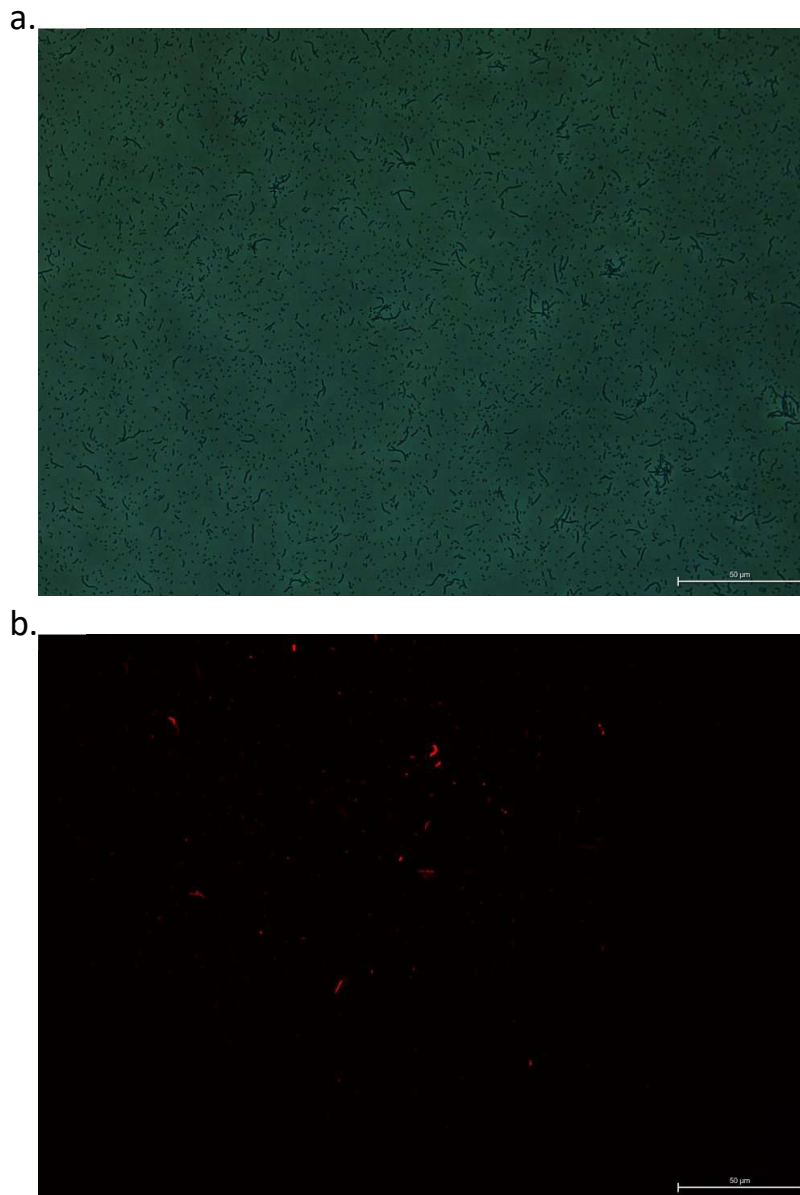


Figure A-1: Visualization of *A. baumannii* carrying the pSGAb_pTet plasmid.