Removal of antimicrobial resistance genes from bacterial strains and communities using CRISPR-Cas9

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Abstract

Antimicrobial resistance (AMR) is one of the largest threats facing modern-day healthcare and society in the coming decades. AMR genes are widely disseminated on genetic vehicles called plasmids, leading to resistant bacteria in many environments. Development of new antibiotics is inefficient, and stewardship of existing antibiotics is often ineffective. One promising novel approach to reduce AMR in bacteria is the delivery of genes coding for CRISPR-Cas9, which can specifically cleave a target sequence of choice – and in this way can be utilised to kill bacteria or remove their resistance plasmids.

The general concept of such CRISPR delivery tools has been proven to be effective under laboratory conditions, however antibiotic resensitisation is more complex when targeting natural plasmids in mixed microbial communities. In this thesis, I aimed to develop a CRISPR delivery tool that can reach various species of bacteria embedded in microbial communities and resensitise these to antibiotics, allowing successful treatment using existing antibiotic drugs.

In the first chapter, I reviewed the role which plasmids play in the AMR crisis by horizontal transfer of resistance genes. I summarised various approaches of counteracting this, with a focus on CRISPR-mediated AMR plasmid removal. In the second chapter, I engineered a broad host-range plasmid pKJK5 to encode CRISPR-Cas9 (pKJK5::Cas). I showed that this plasmid can be used to block target AMR plasmid uptake in *Escherichia* and *Pseudomonas* isolates. In the third chapter, I utilised pKJK5::Cas' conjugative ability to remove a target AMR plasmid from recipient bacteria, which depended on pKJK5::Cas conjugation efficiency and CRISPR targeting efficiency.

In the fourth chapter, I investigated removal of the broad host-range conjugative plasmid RP4 by pKJK5::Cas. I found that presence of toxin-antitoxin systems and target plasmid incompatibility can interfere with the use of pKJK5::Cas. In the fifth chapter, I assayed pKJK5::Cas transfer and maintenance in a synthetic bacterial community. Surprisingly, pKJK5::Cas maintenance and fitness of its host was dependent on community context where the plasmid became lost from a *Variovorax* host strain in presence of *Stenotrophomonas* growth partners. Finally, I offer concluding remarks on my data where I speculated under which conditions target plasmid removal may be successful in such a community context.

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Author's Declaration

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I am the lead author and carried out all experiments, data analysis, and took the lead on study design, but do not claim sole authorship over relevant sections of this chapter.

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Abbreviations

AMP – antimicrobial peptide

AMR – antimicrobial resistance

Ap – 100 μg/mL Ampicillin

Ara – 0.5% (w/v) Arabinose

BEVA – bacterial expression vector archive

BHI – Brain Heart infusion (high-nutrient growth medium)

BLAST – Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi

Carb – 250 μg/mL Carbenicillin

Cas – CRISPR-associated

CFU – colony forming units

Cp – 25 μg/mL Chloramphenicol

CPE – carbapenemase-producing *Enterobacterales*

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

crRNA – CRISPR RNA

DAP – diaminopimelic acid

ESBL – extended-spectrum beta lactamase

GLM – generalised linear model

Gluc – 0.5% (w/v) Glucose

Gm – 50 μg/mL Gentamicin

GFP – Green Fluorescent Protein

GLM – Generalised Linear Model

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HGT – horizontal gene transfer

IBD – inflammatory bowel disease

Inc group – Incompatibility group (of a plasmid)

IS – Insertion Sequence

KB – King's Medium B (high-nutrient growth medium)

Km – 50 μg/mL Kanamycin

LB – Lysogeny Broth (high-nutrient growth medium)

MCS – multiple cloning site

MDR – multi-drug resistance

MGE – mobile genetic element

MIC – minimal inhibitory concentration (of an antibiotic)

nt - nucleotide

PAM – protospacer adjacent motif

Phage – Bacteriophage

PLE – pathogenicity island-like element

qPCR – quantitative PCR

QS – quorum sensing

rpm – rotations per minute

sgRNA – single guide RNA

Sm – 50 µg/mL Streptomycin

SOB – Super Optimal Broth (high-nutrient growth medium)

SpyCas9 – *Streptococcus pyogenes* Cas9

TA system – toxin-antitoxin system

Tc – 12 µg/mL Tetracyline

Tmp – 10 µg/mL Trimethoprim

TSB – Tryptone Soy Broth

w/v – weight/volume

WT – wild type

WWTP – waste-water treatment plant

Chapter 1: Literature Review

Introduction

Routine healthcare is heavily dependent on antibiotics (Aminov, 2010). Alarmingly, many bacterial pathogens that cause infections are becoming increasingly resistant to antibiotics due to the acquisition of resistance through mutation and mobile resistance genes coding for antimicrobial resistance (AMR). In 2019, over one million deaths were directly attributable to bacterial AMR (Murray *et al.*, 2022), and the problem is getting worse: it is predicted that AMR will surpass cancer to be the leading cause of death worldwide by 2050 (O'Neill, 2016). The issue is now so urgent that it has been argued that a paradigm shift is needed which changes the value society attaches to antibiotics in order to incentivise development of new antibiotic pharmaceuticals (Årdal *et al.*, 2017).

AMR encompasses resistance of all microbes against their drugs, but as this thesis focuses only on AMR in bacteria, I use the term AMR throughout this thesis as a shorthand for bacterial antibiotic resistance.

Antibiotics and Antimicrobial Resistance

With the discovery of Penicillin in 1928, antibiotics initiated a new era in healthcare. The following decades saw rapid development of a multitude of classes of antibiotics, with aminoglycosides, cephalosporins, tetracyclines, and macrolides all being discovered in the 1940s. More recent additions to this list are glycopeptides and (fluoro-)quinolones, discovered in the 1950s and 1970s respectively. In recent decades, the rate of discovery of new antibiotics has however come to a near-standstill. Resistance to hallmark drugs of all antibiotic classes was first identified within, at most, 11 years after their discovery (Medical Research Council, 2014). Drug-resistant infections are now the norm in most countries across the world: for instance, the vast majority of bloodstream and urinary tract infections worldwide are resistant to Ampicillin, with resistance against other antibiotics varying. While such resistance may, in part, be intrinsic to certain pathogens, examples of multidrug-resistant pathogens are also common (World Health Organisation, 2021). Antibiotic resistance is hence a global problem that transcends all bacterial pathogens, and can be found in pathogenic as well as non-pathogenic bacteria where many of the resistance genes first evolved (e.g. β-lactamases; (Humeniuk *et al.*, 2002)).

Mechanistically, antibiotic resistance can be grouped into three main mechanisms of action (reviewed in (Cag *et al.*, 2016; Iskandar *et al.*, 2022)): efflux pumps reduce drug accumulation and induce their expulsion, target modification prevents drugs acting upon their target enzymes, and drug inactivation modifies the antibiotic into an inactive form. All these different resistance mechanisms are encoded by AMR genes.

Acquisition of Antimicrobial Resistance

Broadly seen, bacteria can become resistant to antibiotics by two distinct processes. Firstly, bacteria can become resistant by mutations to existing genes. This emergence of resistance is higher in mutator strains (Cag *et al.*, 2016), readily occurs in the laboratory (Toprak *et al.*, 2012), and for instance has been shown to be the cause of resistance in clinical isolates of *Pseudomonas aeruginosa* (Yee *et al.*, 1996). Secondly, bacteria can acquire AMR genes through horizontal gene transfer (HGT). HGT is the process of exchange of genetic material between bacteria of the same or different species, and is considered a major factor in the spread of AMR genes (Partridge *et al.*, 2018). Acquisition of AMR genes by HGT is the most frequent cause of resistant clinical infections (Yelin and Kishony, 2018).

Horizontal Gene Transfer and Mobile Genetic Elements

HGT is a common process in bacterial evolution that is thought to be essential for survival of bacterial populations by countering gene loss through mutation (Koonin, 2016). Although HGT can occur between distantly related bacteria, this process is still restricted by phylogenetics due to preferential transfer to and gene uptake from closely related bacteria. For instance, acquired AMR genes cluster phylogenetically across *E. coli* genomes, and inter-phylum transfer does occur but is very rare (Petitjean *et al.*, 2021).

Most commonly, HGT can occur by natural transformation (Griffith, 1928), by transduction (Zinder and Lederberg, 1952), or by conjugation (Lederberg and Tatum, 1956) (Figure 1.1). Of these mechanisms, HGT by conjugation is thought to be by far the most prevalent and most effective means of rapidly dispersing genes through bacterial communities, such as in the gut or soil (Ogilvie *et al.*, 2012). In 2018, a study estimated gene transfer rates arising from conjugation, transduction, and natural transformation as well as vesicle-mediated gene transfer. Using data from past studies and other properties intrinsic to each mode

of HGT, such as the ability to mobilise genes across species, the author's models predicted that genes transferred by conjugation alone or by all three mechanisms together can reach fixation in bacterial communities in under a month, while this process takes 7-8 years without conjugative transfer (Nazarian *et al.*, 2018). However, recent work argues that the relative importance of transduction may have been underestimated with the discovery that small plasmids may also be transduced (Humphrey *et al.*, 2021).

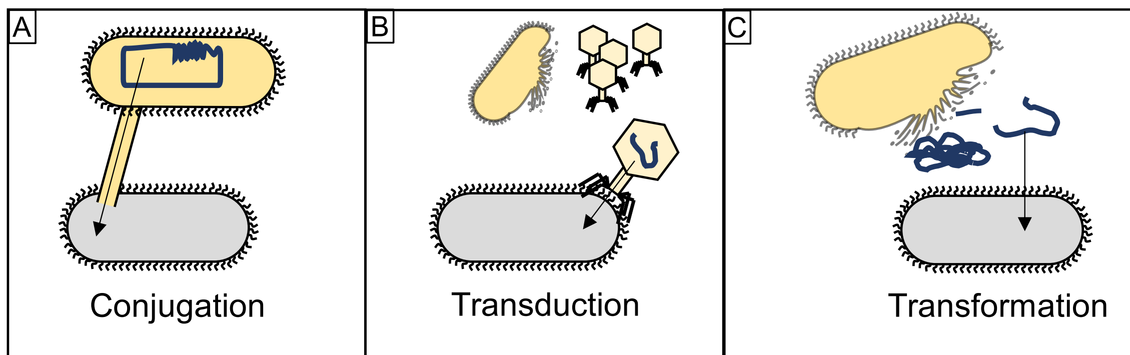


Figure 1.1 **Prominent mechanisms of Horizontal Gene Transfer.**
A Conjugation. A donor cell (yellow) contains a plasmid (blue), which encodes genes necessary to form a conjugative pilus, connecting it to a recipient cell (grey). The plasmid is copied and transferred into the recipient cell, generating a transconjugant. **B Transduction.** The donor cell (yellow) is lysed by bacteriophage (pale yellow), which infected and replicated within this host. Some phage particles mistakenly packaged bacterial DNA (blue), which is transferred into the next cell infected by such a virion. **C Transformation.** The recipient cell (grey) takes up DNA from the environment, e.g. released by a dead bacterium (yellow).

Transformation is a general method of uptake of environmental DNA fragments and their incorporation into the genome. This process is highly relevant to the evolution of a small subset of bacterial species, such as *Acinetobacter baylyi* or *Staphylococcus aureus* (Ray *et al.*, 2009; Ambur *et al.*, 2016). HGT through conjugation and transduction is mediated by particular mobile genetic elements (MGEs; see Box 1.1), specifically plasmids and bacteriophage (phage) respectively. Plasmids are usually circular pieces of DNA, and during conjugation a pilus forms to connect two bacterial cells, after which the plasmid is linearised, copied, and transferred into the recipient cell, generating a transconjugant (Garcillán-Barcia *et al.*, 2009). Phage are viruses that infect and replicate inside bacterial hosts. During transduction, bacterial DNA is packaged into phage particles and mobilised into other cells (Clokie *et al.*, 2011). In specialised transduction, DNA sequences flanking an integrated prophage are packaged into virions once the phage excises (Kwoh and Kemper, 1978). Generalised

Box 1.1 Mobile Genetic Elements (MGEs)

MGEs are autonomous genetic elements that, while needing the replicative machinery of their bacterial host, divide and spread independently of the bacterial chromosome. MGEs often impose a growth cost on their host, but depending on their genetic payload can also give them a fitness benefit – for instance, by carrying AMR genes. The most common MGEs, plasmid and phage, are mediators of HGT by conjugation and transduction respectively.

Plasmids are usually circular pieces of DNA with a broad range in size. Conjugative plasmids encode all elements necessary to transfer (conjugate) to a different bacterial cell, while mobilisable plasmids encode no transfer machinery and instead hitchhike with co-residing conjugative plasmids. Smaller plasmids are often not mobilisable by conjugation, but recent evidence suggests that they may be transferred via phage transduction instead (Rodríguez-Rubio *et al.*, 2020). Plasmids of the same incompatibility group, defined by their replicative machinery, cannot be distinguished as separate elements by the host cell and thus cannot be co-maintained (reviewed in (Shintani *et al.*, 2015)).

Bacteriophage (phage) are bacterial viruses which encase their genetic material in a protein capsid. Specialised tail fibre proteins inject the phage DNA (or RNA) into the bacterial host, and the bacterial machinery gets hijacked to produce progeny phage. Phage can broadly be categorised into lytic, where progeny virion particles form immediately upon entering a new host cell and lyse the cell, and lysogenic, where phage DNA can temporarily integrate into the bacterial genome as a temperate part of the phage's life cycle (prophage; (reviewed in (Clokie *et al.*, 2011)). Transduction describes the erroneous transfer of bacterial DNA between unrelated bacterial cells by phage particles.

Many more MGEs beyond plasmids and phage form a complex network of genetic material and move between different locations within and between bacterial cells. Genomic islands, plasmids, gene cassettes, integrons, transposons, insertion sequences and their derivatives are all different types of MGEs which act in parallel and often associate with each other in complex mosaics. In this way AMR and other payload genes can be moved between various bacteria.

Some MGEs are related to plasmid or phage, but don't fully fit either definition. Integrative conjugative elements transfer like conjugative plasmids but integrate into their host's genome. Phage-plasmids possess hallmarks of both phage and plasmids, and it is unknown whether they form virions as well as transfer conjugatively (Pfeifer *et al.*, 2020). Phage satellites (Christie and Dokland, 2012) and phage-induced chromosomal islands (Fillol-Salom *et al.*, 2018) are dependent on phage infection to disseminate by parasitising on an infecting phage. Well known such elements include *Staphylococcus aureus* pathogenicity islands which are heavily implicated in *S. aureus* toxicity and sometimes carry resistance genes (reviewed in (Novick *et al.*, 2010), and *Vibrio cholera* PLEs, which protect their host cells from phage infection (Barth *et al.*, 2020).

A plethora of smaller MGEs can capture genetic material or move it between different compartments in bacterial cells; clinically relevant AMR plasmids frequently play host to these (Partridge *et al.*, 2018). Mobile integrons, residing on plasmids or on the chromosome, capture and integrate genes from their host or shuffle the order of genes within their operon to change their expression levels (Cambray *et al.*, 2010; Souque *et al.*, 2021). Integrons are often associated with transposons, which can copy themselves between different genetic compartments. Insertion sequences (IS) are very short sequences often only containing a single transposase gene which insert themselves into different areas of genomes. A simple composite transposon carries cargo genes and is bound by two ISs. Typically, larger transposons carry multiple transposases, inverted repeats, and several cargo genes – and can thus carry multiple AMR genes between genomic regions (Partridge *et al.*, 2018).

1

transduction sees genetic material located elsewhere on the chromosome packaged into the phage capsid. During lateral transduction, large segments of the chromosome are erroneously transferred between bacteria – and recent data indicates that this large-scale transfer of genetic material may result in the chromosome being more mobile than “classical” MGEs in species such as *Staphylococcus* and *Salmonella* (Hall, 2021; Humphrey *et al.*, 2021).

Plasmids are majorly implicated in the spread of AMR (Carattoli, 2013), and despite phage sometimes transducing AMR genes and some claims of high relative importance of phage transduction (e.g. (Debroas and Siguret, 2019; Jian *et al.*, 2021) it is generally accepted that AMR gene transfer by transduction is rare compared to conjugation (Volkova *et al.*, 2014), although these processes remain poorly quantified in the natural environment.

In addition to the three main mechanisms of HGT, less well-described means of DNA uptake can be mediated by microvesicles, nanotubes, or phage-like gene transfer agents (Arnold *et al.*, 2021). Of these, especially DNA transfer by microvesicles – which is not specific towards particular DNA sequences (Tran and Boedicker, 2017) – has been implicated in AMR gene transfer, for instance in *Acinetobacter* (Rumbo *et al.*, 2011).

Antimicrobial Resistance in the Environment

HGT between different bacterial species leads to a situation where AMR genes from harmless environmental bacteria can find their way into pathogens which then proceed to cause infections in humans (Andersson and Hughes, 2014). Bacteria resistant to clinically relevant antibiotics can be found in many environments, for instance in rivers (Amos *et al.*, 2015), coastal waters (Leonard *et al.*, 2018), and in soils (Knapp *et al.*, 2010). In general, the open environment is strongly linked to the emergence of antibiotic resistant strains of bacteria (e.g. reviewed in (Wellington *et al.*, 2013)) and is now recognized as a key reservoir of AMR in addition to the more traditional reservoirs of resistant bacteria in humans or animals (POST, 2019).

To further compound this issue, antibiotics are also used beyond healthcare in livestock farms (often to a greater extent than in humans; (O’Neill, 2016)), in crop agriculture (McKenna, 2019), in aquaculture (Lulijwa *et al.*, 2020), and until recently in apiaries (Bulson *et al.*, 2021). Run-off from these environments and from wastewater after human consumption of antibiotics release low

1 concentrations of antibiotics in the environment, which can select for transfer of
2 resistance genes by providing slight fitness benefits (Andersson and Hughes,
3 2014). Additionally, heavy metal-contaminated environments can co-select for
4 antibiotic resistance (reviewed in (Seiler and Berendonk, 2012)), either because
5 resistance mechanisms against these two contaminants are similar (e.g. efflux
6 pumps), or because the genes are linked (e.g. both residing on the same
7 plasmids). Together, this led to AMR being identified as an emerging issue of
8 environmental concern by the United Nations in 2017 (United Nations, 2017).

9 Current Strategies and Concepts to Mitigate Resistance

10 Tackling AMR requires a multilateral approach, whereby responsible use of
11 existing antibiotics, development of new antibiotics, and development of
12 alternative treatments are all crucial.

13 Responsible use of antibiotics

14 Antibiotic stewardship aims to enable us to keep using existing antibiotics by
15 reducing the rate of AMR evolution. While some antibiotic use in agriculture is
16 necessary to prevent animal illness, many countries are now banning clear mis-
17 and overuse of antibiotics in these settings, such as for a prophylaxis,
18 metaphylaxis or growth promotion. For instance, aquaculture antibiotic use is now
19 strictly regulated in most high-income countries across the world (Lulijwa *et al.*,
20 2020). Beyond this, new initiatives aim to curb even the more traditional use of
21 antibiotics, for example by teaching farmers to identify sheep illnesses early
22 enough to make them treatable without antibiotics (Jones *et al.*, 2020).

23 Regarding healthcare, broad-spectrum antibiotics are often prescribed as a 'one-
24 size-fits-all' treatment, and as such are sometimes unnecessarily used or are
25 used when a more appropriate narrow spectrum antibiotic would be preferable.
26 A public information campaign attempted to bring this issue closer to the general
27 public by imploring them to not unnecessarily demand antibiotics, always finish a
28 course of antibiotics, and to use antibiotics for pets responsibly (Public Health
29 England, 2014). This was effective in re-enforcing previous positive behaviours,
30 but had a limited effect in engaging unresponsive members of the public (Kesten
31 *et al.*, 2017).

Novel antibiotics

Alongside these preventative strategies, discovery of novel antibiotics is crucial to allow treatment of pathogens resistant to even our last-resort antibiotics.

In general, antibiotic development is not a profitable investment for the pharmaceutical industry due to novel antibiotics being reserved for last-resort treatments, antibiotics' short-lived treatment and functional time-span, and regulations often changing (Renwick *et al.*, 2016; Iskandar *et al.*, 2022). One problem with discovery of novel antibiotics is rediscovery. Identifying potential bactericidal activity in environmental isolates is straightforward, but identification of the proteins and genes responsible for this activity can be laborious. Substantial amount of research money may be wasted if the compound in question turns out to be previously known, or very closely related to a previously known antibiotic. To counteract this, one approach is to search through the microbiome of traditionally under-analysed environments, e.g. of deep-sea sponges, which are more likely to contain novel microbes, genes, and gene products (Williams *et al.*, 2020). Functional metagenomics (dos Santos *et al.*, 2017) and enhanced screens of functional metagenomics libraries (Stocker *et al.*, 2020) additionally allow a screen of antibiotic activity without the need for identification of the microbe producing these novel compounds. Taking this one step further, in *in situ* cultivation approaches enable to grow and analyse a larger breadth of microbes (Berdy *et al.*, 2017) and led to discovery of teixobactin, produced by a previously unculturable soil microbe (Ling *et al.*, 2015). While this antibiotic shows promising activity and low resistance development *in vitro* and *in vivo*, challenges such as drug delivery, activity against Gram negative pathogens, and larger scale production remain (reviewed in (Gunjal *et al.*, 2020)).

On balance, the development of new antibiotics is inefficient and expensive (Årdal *et al.*, 2017), so there is a need to look further than stewardship of existing and discovery of new antibiotics to tackle this mounting problem of AMR.

Alternative means of tackling antimicrobial resistance

A broad variety of alternative approaches to reduce AMR aim to find new means of killing bacteria, or to reduce the need for antibiotics by decreasing the prevalence of infection, or to resensitise bacteria to antibiotics, or a combination of these (reviewed in (Kumar *et al.*, 2021)).

Most prominently, phage therapy involves the use of bacteriophage to target and kill pathogenic bacterial species. Phage are highly effective at killing bacteria, and have been used to treat bacterial infections in some eastern European countries throughout the latter half of the 20th century (Międzybrodzki *et al.*, 2018). Now this approach is being revisited around the world (Roach and Debarbieux, 2017). For example, their efficacy was tested to treat infected wounds of burn patients in a recently completed clinical trial (Jault *et al.*, 2019).

One of the advantages of phage therapy is the relative ease by which phage can be isolated: bacteriophage are the world's most abundant biological entity and ubiquitous in nature. Furthermore, phage epidemics spread rapidly through large populations as lysis can occur within minutes after first contact and results in hundreds of progeny phage (Clokie *et al.*, 2011). Phage treatment can sometimes select for collateral antibiotic sensitivity, making this approach attractive for use alongside traditional antibiotic treatments (Chan *et al.*, 2016), but in other cases phage may also select for antibiotic resistance (Tariq *et al.*, 2019). Finally, phage are notoriously specific to their bacterial hosts, allowing targeting of a single pathogenic strain while leaving other bacteria unharmed. However, when treating a more undefined infection, narrow phage host-range becomes a drawback – phage host-range engineering, directed evolution, and application of mixtures of different phages (phage cocktails) can alleviate target specificity to some extent (Pires *et al.*, 2016). Furthermore, bacteria have evolved several defense mechanisms against phage lysis, for example abortive infection, receptor mutation, restriction-modification, and CRISPR-Cas (reviewed in (Westra *et al.*, 2012; Tal and Sorek, 2022)). To circumvent some of these issues, the application of phage lysins may help to directly lyse pathogenic bacteria (Vázquez *et al.*, 2018).

Antimicrobial peptides (AMPs) can also be used to kill bacteria. Unlike antibiotics, these small molecules are peptide-based and naturally produced by prokaryotes and eukaryotes (including humans) as part of the innate immune response, showing broad activity against microbes. Many AMPs simply function to enhance other parts of the innate immune system, while others have active bactericidal properties (Jenssen *et al.*, 2006). More recent work has shown that resistance to AMPs is less likely to occur than resistance to antibiotics due to their fundamental multi-target nature, and even more encouragingly that antibiotic resistant bacteria

generally show sensitivity to AMPs. Unlike for antibiotic resistance genes, HGT doesn't play an important role in AMP resistance (Lázár *et al.*, 2018). This makes AMPs a promising area for clinical investigation (Mahlapuu *et al.*, 2016).

Other bactericidal approaches include the use of predatory *Bdellovibrio* bacteria to clear pathogenic bacterial infections (Negus *et al.*, 2017) or the use of non-coding RNAs which can, for instance, control gene expression or induce mRNA degradation (reviewed in (Parmeciano Di Noto *et al.*, 2019)). Nanomaterials can be used to deliver novel drugs with antimicrobial properties, and sometimes possess bactericidal properties themselves (reviewed in (Baptista *et al.*, 2018)).

Manipulation of the microbiome can both reduce infection prevalence and, in some cases, directly kill problematic bacteria. For instance, faecal microbiome transplants aim to augment the gut flora of a patient with dysbiosis with a healthy gut microbiome. This has long been in use for livestock and experimentally in ancient and modern human medicine, but the clinical efficacy remains unclear (Kumar *et al.*, 2021). As a less extreme approach, the use of probiotics sees the application of one or several strains of bacteria to revert dysbiosis of a microbiome. However, recent trials suggest that there is considerable variation in how susceptible a patient is to microbiome manipulation, with some individuals naturally being resistant to probiotic colonisation (Zmora *et al.*, 2018).

With the aims of reducing infection, quorum sensing (QS) inhibitors disrupt bacterial communication which is key to biofilm formation and bacterial virulence. Disrupting this process with QS inhibitors (reviewed in (Saeki *et al.*, 2020)) could help prevent persistent bacterial infections – but applying these to clinical settings may not be straightforward (Kumar *et al.*, 2021). Additionally, several different areas of research aim to boost our immune system, for instance by application of monoclonal antibodies – some of which also have direct bactericidal properties (reviewed in (Streicher, 2021).

Beyond this, plasmid curing approaches aim to remove plasmids and resensitise bacterial hosts to allow successful antibiotic treatment (reviewed in (Buckner *et al.*, 2018; Vrancianu *et al.*, 2020)). The earliest efforts involved the use of plasmid-curing compounds (e.g bile, (García-Quintanilla *et al.*, 2006), Ethidium Bromide (Bouanchaud *et al.*, 1969), sodium dodecyl sulfate (SDS; (Tomoeda *et al.*, 1968)), apramycin (DeNap *et al.*, 2004)). These generally only affect specific bacteria-plasmid combinations, need high local dosage, and show toxicity *in vivo*.

Alternatively, phage can produce plasmid-loss-inducing compounds, or create a selective pressure against uptake of certain plasmids (e.g. PRD1 (Jalasvuori *et al.*, 2011), M13KE (Lin *et al.*, 2011), SBW252ϕ (Harrison *et al.*, 2015)).

Most prominently, plasmid incompatibility – defined by their replicative machinery – can be exploited for plasmid curing. Different plasmids of the same incompatibility group can temporarily coexist, but not be co-maintained in the same bacterial cell. This was first exploited with the discovery that high-copy vectors can replace resident plasmids of the same incompatibility (Inc) group (Bringel *et al.*, 1989). In this way, problematic AMR plasmids might be replaced by harmless incompatible plasmids. This approach shows less toxicity than other compound-based curing, but it can fail when target plasmids encode toxin-antitoxin systems which cause post-segregational killing upon plasmid loss. To circumvent this, a series of "pCURE" plasmids was engineered to encode various origins of replication, origins of transfer, as well as antitoxin genes to rescue cells cured of persistent plasmids (Hale *et al.*, 2010). Further tweaking of this approach resulted in small, high-copy number pCURE derivatives which can cure resident AMR plasmids from the mouse gut, and can subsequently be selected against (Lazdins *et al.*, 2020). Other similar displacement plasmids could be constructed by deleting unwanted antibiotic resistance and toxin genes from natural AMR target plasmids (Kamruzzaman *et al.*, 2017).

Finally, as a relatively new approach which has found increasing attention in recent years, CRISPR-Cas9 and related nucleases may be used either as an antimicrobial or to remove AMR genes (reviewed in (Pursey *et al.*, 2018)). CRISPR-based antimicrobials are the focus of this thesis and reviewed in detail below.

CRISPR-based antimicrobials

In nature, CRISPR-Cas is a microbial immune system which bacteria and archaea use in the battle against their viruses, bacteriophage. There are several types of CRISPR-Cas systems, which use related enzyme complexes for the same overall process (reviewed in (Makarova *et al.*, 2020)) . An immune memory is formed by integration of short sequences from phage genomes into a "spacer" region on the bacterium's chromosome, separated by repeat sequences – this is where the acronym CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) comes from. Later, when infected by a phage of the same genotype,

the bacterium uses these spacers, transcribed as crRNA (CRISPR RNA), to specifically guide its effector Cas (CRISPR-associated) proteins to the complementary target sequence on the phage genome. Cas nucleases then cleave the phage DNA, conferring immunity upon the host to this phage (reviewed e.g. in (Faure *et al.*, 2019); Figure 1.2A).

The high sequence specificity and versatility of CRISPR-Cas, especially of the relatively simple *Streptococcus pyogenes'* Type II CRISPR-Cas9 system, has led to a revolution in genome editing. What makes the use of CRISPR-Cas9 attractive is that rather than using a complex made up of multiple Cas proteins this CRISPR system boasts a single nuclease, Cas9. In 2012, Jinek *et al.* fused tracRNA and crRNA, the short RNAs necessary for target recognition, into a single guide RNA (sgRNA). This transformed CRISPR-Cas9 into a simple, two component system, ready to a target sequence of choice depending on the sequence of the short sgRNA supplied together with the protein ((Jinek *et al.*, 2012); Figure 1.2B). Minor restrictions regarding target sequence remain, where only a 20-nucleotide sequence followed by the sequence "NGG" can be targeted.

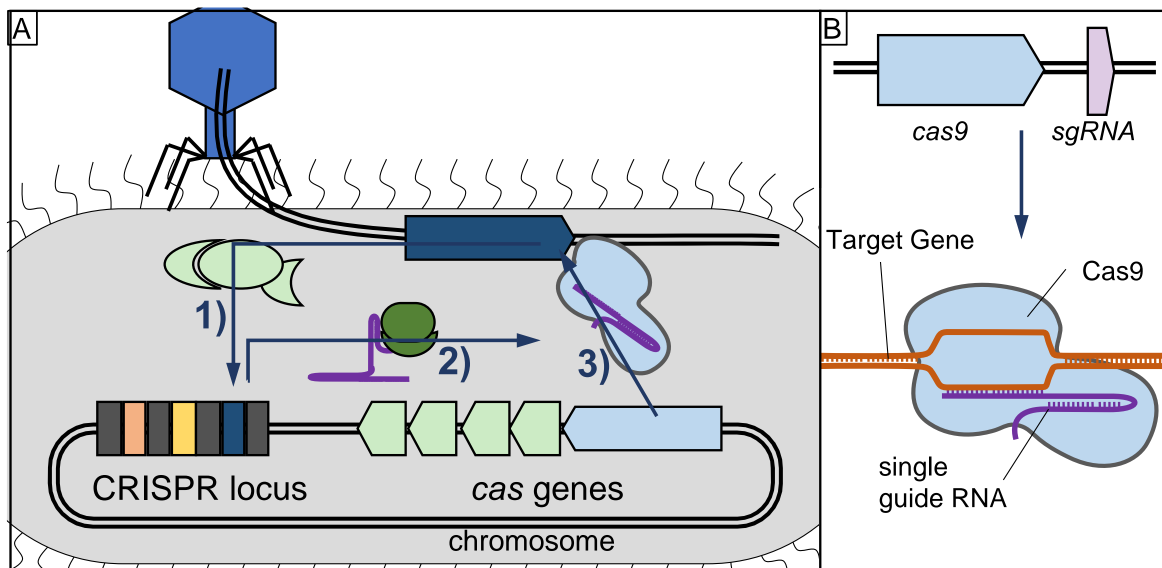


Figure 1.2 **CRISPR-Cas in nature and in biotechnology applications.**
A: As an immune response, CRISPR-Cas functions by integrating incoming bacteriophage DNA into its CRISPR locus as a spacer (1). This spacer is transcribed and processed to CRISPR RNA (crRNA), which associates with effector Cas nucleases (e.g. Cas9) (2). Upon a subsequent infection by a phage of the same genotype the crRNA specifically guides the Cas nuclease to the complementary sequence on the phage genome. The Cas nuclease cleaves the phage DNA, aborting the infection (3). **B: For biotechnology applications**, crRNA processing steps are skipped by use of a single guide RNA (sgRNA). In this way, only two genes, *cas9* and *sgRNA*, are needed to mediate specific target sequence cleavage.

This is called the proto-spacer adjacent motif (PAM) restriction, hardwired into the Cas9 protein to ensure bacteria don't cleave spacers in the CRISPR locus on their own genome. Some applications surpass even this issue by engineering Cas9 to change its PAM requirements (Leenay and Beisel, 2017).

Since then, CRISPR-Cas9 and related CRISPR systems have found numerous biotechnology applications in most areas of biology. These are very diverse and include eukaryotic gene editing and gene therapy (reviewed in (Williams and Warman, 2017), microbial genetic and population engineering (Choi and Lee, 2016; Rubin *et al.*, 2020), gene expression modulation (Vigouroux *et al.*, 2018), cellular event recording (Tang and Liu, 2018), and even population-level engineering of insect populations using gene drives (Hammond *et al.*, 2016). In 2008, Marraffini and Sontheimer suggested for the first time that CRISPR could be used as an alternative to antibiotics in the face of rising resistance (Marraffini and Sontheimer, 2008). In 2010, other ground-breaking work showed how natural CRISPR systems retargeted towards lysogenised phage, therefore targeting the host cell's own genome, led to cell death (Edgar and Qimron, 2010). When used in bacteria, double-strand DNA breaks can cause cell death if the bacterial chromosome is targeted, or plasmid removal if such an accessory genetic element is the nuclease's target.

Therefore, CRISPR can be applied as an alternative to antibiotics with two different approaches: bacteria can be directly killed, or they can be resensitised to antibiotics by removal of AMR plasmids. Approaches which aim to directly kill bacteria are pathogen-targeted, as they are specific to the target pathogens. In contrast, approaches which resensitise bacteria to antibiotics can be seen as gene-targeted, as they are specific to the nucleotide sequence of the target gene. This perspective might allow appropriate choice of delivery vehicle for CRISPR-Cas genes, where a pathogen-specific delivery vehicle such as phage may be more suited to pathogen-targeted bactericidal approaches (Sünderhauf *et al.*, 2018).

Bactericidal CRISPR treatments

Most earlier studies regarding CRISPR-based antimicrobials focussed on direct killing of target strains. In 2014, several groups independently carried out proof-of-concept experiments in which CRISPR genes were delivered to target bacteria to selectively kill a specific, pathogenic strain while keeping other closely related

strains alive. Gomaa *et al.* (2014) delivered the genes necessary for CRISPR-killing by transformation, a technique which is unlikely to work outside a laboratory setting. The other studies, in which pathogenic *Escherichia coli* (Citorik *et al.*, 2014) or *Staphylococcus aureus* (Bikard *et al.*, 2014) strains were specifically killed utilised engineered bacterial viruses, termed phagemids, as CRISPR delivery tools. The use of phagemids has the advantage of being more suitable to *in vivo* applications than direct transformation, as indicated by the use of *Galleria mellonella* or mouse skin animal models in these studies.

As such, many later studies essentially combined phage therapy with CRISPR by generating engineered bacteriophage. For instance, Park *et al.* engineered *S. aureus* phage to carry CRISPR-Cas9 and broadened their host range by modifying their tail fibres (Park *et al.*, 2017). In a combined plasmid-delivery and phage-capsid-delivery study, Kiga *et al.* deployed Cas13a to *E. coli* and *S. aureus*, targeted towards antibiotic resistance genes. Cas13a has pleiotropic RNAase activity when a target sequence is present, therefore target strain growth inhibition occured regardless of the genomic position of the target gene (Kiga *et al.*, 2020). In the same year, Selle *et al.* engineered a *Clostridioides difficile* phage to carry spacers matching the bacterial host genome. The host's own CRISPR system could utilise these spacers, and this made target strain killing more effective, both *in vitro* and in a mouse model (Selle *et al.*, 2020). Similarly, use of engineered Cas9-expressing M13 phage allowed depletion of specific *E. coli* strains from the mouse gut (Lam *et al.*, 2021).

Beyond phage, researchers have experimented with other MGEs as delivery vehicles. For example, Ram *et al.* engineered a Staphylococcal pathogenicity island to encode CRISPR-Cas9. This treatment cleared *S. aureus* infections and improved disease outcome in mice (Ram *et al.*, 2018). In a study with the aim to kill *Salmonella enterica*, conjugative Cas9 plasmids were developed and delivered to the target from an *E. coli* donor. Cas9 and the conjugation machinery being expressed from the same rather than from separate plasmids was shown to increase killing efficiency (Hamilton *et al.*, 2019). In a different study, conjugative delivery of CRISPR-Cas9 on a plasmid that had undergone accelerated evolution to be a more effective spreader allowed manipulation of target strain frequencies in the mouse gut (Neil, Kevin *et al.*, 2021).

A single study investigated a delivery method not linked to MGEs: A Cas9-sgRNA nanocomplex could directly be delivered to methicillin-resistant *S. aureus* despite their thick cell wall to induce cell killing (Kang *et al.*, 2017).

Resensitisation of Target Strains

Rather than targeting bacterial chromosomes, several studies have used CRISPR to target and remove accessory AMR genes, therefore resensitising bacteria to the relevant antibiotics. Gene delivery methods also varied throughout these studies. One of the earliest studies used direct transformation of bacterial cells to deliver the necessary CRISPR genes to target β-lactamase plasmids (Kim *et al.*, 2015). With the same delivery method, Sun *et al.* resensitised a clinical *E. coli* isolate to colistin by removing its *mcr-1*-plasmid, and addition of an antimicrobial peptide enhanced Cas9 activity in this system (Sun *et al.*, 2017). Using another transformation-based approach, Tagliaferri *et al.* transformed *E. coli* with a Cas9-plasmid and found that this technique could remove high copy-number plasmids. When resensitising clinical isolates, this method was more effective in *E. coli* than *Klebsiella* (Tagliaferri *et al.*, 2020). In an alternative approach to more effectively clear high-copy number plasmids, Valderrama *et al.* developed a gene-drive-like methodology where target *E. coli* were transformed with a Cas9 plasmid and a second plasmid, which encoded lambda-red recombination systems and sgRNA flanked by arms homologous to the target sequence. This enabled the target gene to be knocked out by insertion of the targeting sgRNA and was considerably more effective at resensitising *E. coli* than standard Cas9 targeting (Valderrama *et al.*, 2019). As a slightly different approach which did not target MGEs, catalytically inactive dCas9, which is now widely used as a gene expression inhibition tool (Vigouroux *et al.*, 2018), was delivered to *S. aureus* to abrogate chromosomal *mecA* AMR gene expression. While qPCR showed a significant reduction in gene expression, this wasn't sufficient to resensitise the bacteria to methicillin (Wang and Nicholaou, 2017).

Phage-based Cas9 delivery has also been experimented with for plasmid clearance: Yosef *et al.* used a system with two different engineered phagemids to first remove antibiotic resistance genes from bacteria, whilst at the same time giving resensitised bacteria a selective advantage by immunising them against the second engineered phage, applied afterwards (Yosef *et al.*, 2015).

The most promising CRISPR-directed plasmid clearance applications deliver Cas9 by conjugation on a conjugative or mobilizable plasmid. This theoretically allows delivery of Cas9 genes to, and plasmid clearance from, bacterial strain and species not typically tractable in the laboratory. However, most past studies developed these CRISPR delivery tools as proof-of-concept and applied them to *E. coli* only. For instance, Dong *et al.* removed accessory *mcr-1* genes from *E. coli* by constructing a conjugative CRISPR plasmid with a relatively narrow host-range (Dong *et al.*, 2019). An alternative approach of curing *mcr-1* plasmids from *E. coli* clinical isolates applied a mobilisable Cas9 plasmid in an engineered *E. coli* donor which encoded its own conjugative machinery (Wang *et al.*, 2019). A different mobilisable Cas9 approach used broad host-range conjugative plasmid RP4 to help deliver the CRISPR plasmid between different *E. coli* strains. Target plasmids could be removed using this approach, but after 72 hours of growth RP4 spread further without mobilising the CRISPR plasmid (Ruotsalainen *et al.*, 2019).

A common theme throughout the different studies using conjugative or mobilizable plasmids for Cas9 delivery is that low conjugation rates can limit the efficacy of target plasmid removal. The same was true in an experimental system established by Wongpayak *et al.*, where a mobilizable Cas9 plasmid was ineffective in clearing *E. coli* target plasmids when applying an *E. coli* donor engineered to encode conjugative machinery. This was improved when the Cas9 plasmid was instead mobilised by means of a second conjugative plasmid. However, this also caused target plasmid mobilisation, counteracting its removal. Addition of a third incompatible displacement plasmid to stop re-infection by the target plasmid addressed this and led to effective resensitisation (Wongpayak *et al.*, 2021).

Targeting of AMR plasmids can sometimes have knock-on effects. In *Enterococcus faecalis*, an inactive CRISPR-system could be reactivated to protect the host from incoming plasmids. However, transconjugants could still be generated and plasmids could temporarily co-exist with the active CRISPR system. If both were forced to be maintained over longer periods this resulted in a fitness cost to the host strain, and thus allowed manipulation of *E. faecalis* strain proportions in mixed populations (Hullahalli *et al.*, 2017). Further work developed a conjugative Cas9 plasmid which removed erythromycin-encoding *E. faecalis*

plasmids *in vitro* and in a mouse model *in vivo*, albeit with poor efficiency due to low conjugation efficiency. Interestingly, targeting plasmids also led to recipient depletion due to slow growth of this strain, and surviving recipients were slightly protected against uptake of AMR plasmids (Rodrigues *et al.*, 2019).

Issues with current research

While these previous studies form a solid basis for future work and provide a valuable proof-of-concept for the use of CRISPR against AMR, there are still several challenges associated with developing this technology. These can be summarised as challenges of gene delivery, evolution of resistance, community complexity, and legislation (reviewed in (Pursey *et al.*, 2018)).

CRISPR delivery

Perhaps experimentally most pressing, the challenge of gene delivery highlights the need for development of delivery methods which can be applied *in vivo* or *in situ* and are an effective means of reaching target bacteria. It is essential to tailor this to the final application: If CRISPR is being used to target and kill specific pathogenic strains, phage-based delivery might be best. If instead a resistance gene should be removed from an entire microbial community, conjugative plasmid-based delivery may be more appropriate (Sünderhauf *et al.*, 2018). While previous studies have already trialled both delivery methods (see previous section), further work needs to be carried out to make these more feasible. Disadvantages of phage include the work needed to engineer them to encode CRISPR-Cas as well as having to find an appropriate phage cocktail. This negates the utility of CRISPR-Cas and the ease with which its target sequence can be programmed.

Conjugative delivery needs to be made more broad-host range without dependence on a specially engineered donor strain, which would likely be outcompeted quickly in the microbial communities it is introduced to. Development of a truly broad host-range delivery plasmid is needed. Such plasmids are often large and difficult to engineer, which is why most past studies relied on a natural conjugative plasmid and engineered a smaller mobilizable CRISPR plasmid. However, data show that systems like these are not an effective means of reaching an entire target population (Hamilton *et al.*, 2019; Ruotsalainen *et al.*, 2019).

1 **Target Community Complexity**

2 The challenge of gene delivery ties in nicely with community complexity, which
3 increases when moving from controlled lab experiments to *in vivo* and *in situ*
4 applications. Upon removal of a bacterial target species, other more virulent
5 community bacteria may fill this ecological niche: microbiomes with altered
6 species composition (for instance after antibiotic application) are prone to
7 invasion by bacterial pathogens (Theriot *et al.*, 2014). The same could be
8 possible for plasmid removal, upon which recolonization by other plasmids with
9 alternate resistance genes or virulence determinants could follow. More
10 generally, community composition can be tightly linked to community functioning,
11 with perturbations having unwanted consequences (Sierocinski *et al.*, 2018). For
12 these reasons, careful consideration and risk assessment tailored to target
13 communities is necessary when applying CRISPR delivery tools to natural
14 communities.

15 **Evolution of Resistance**

16 From the studies outlined above, we know that resistance against CRISPR-Cas
17 (target avoidance) can and will evolve. The main mechanisms of resistance are
18 loss of CRISPR-Cas activity and point mutations in the target sequence (Bikard
19 *et al.*, 2014; Citorik *et al.*, 2014). The former can perhaps be addressed by
20 expressing multiple or engineered Cas variants; and the latter might be
21 circumvented by multiplexing, which involves expression of multiple sgRNAs
22 targeting different regions of the same gene. Furthermore, depending on the gene
23 delivery method, natural bacterial defences (including CRISPR-Cas itself as well
24 as other defenses such as Restriction-Modification) might interfere with CRISPR-
25 Cas delivery, too.

26 **Legislative and Public Opinion Issues**

27 Finally, apart from optimising the technology itself, there are significant legislative
28 and public acceptance barriers to novel environmental gene-editing technologies
29 such as this (reviewed in (Kofler *et al.*, 2018)). A similar technology further in
30 development are CRISPR-Cas gene drives, used for manipulation and genetic
31 engineering of insect populations. Several scientific working groups have
32 published guidelines and frameworks around safe, ethical deployment of this
33 CRISPR-Cas technology in the laboratory and in the field (Akbari *et al.*, 2015;
34 National Academies of Sciences, Engineering, and Medicine, 2016; James *et al.*,

2018). However, while these are thorough resources, such self-governance by researchers is not sufficient, as large-scale consequences of rolling out genetic engineering technologies like these are often not considered (De Graeff *et al.*, 2019).

As both are environmental gene editing technologies, the main concepts, frameworks, and ethical and regulatory shortcomings are transferrable from CRISPR-Cas gene drives to CRISPR-Cas antimicrobials. In summary, most current approval frameworks do not consider ethical and societal perspectives and legislation is sorely lacking. Overall, local stakeholder support is crucial to work towards legislative approval. (Kofler *et al.*, 2018)

Thesis Objectives

This thesis aims to understand the efficacy of CRISPR-Cas delivery and AMR target removal in a microbial community context.

In Chapter 2, I engineer Cas9-encoding broad host-range plasmid pKJK5::Cas and use this to protect *Escherichia* and *Pseudomonas* laboratory strains and environmental isolates from AMR plasmid uptake. In Chapter 3, I use pKJK5::Cas to cure resident AMR plasmids from a target *E. coli* strain by conjugative delivery of the CRISPR plasmid. I assess the impact of CRISPR targeting efficiency and of CRISPR plasmid conjugation efficiency on target plasmid removal. In Chapter 4, I turn towards the impact of target-plasmid-specific properties on their removal by pKJK5::Cas. Specifically, I assess the impact of toxin/antitoxin system presence and of target plasmid incompatibility. In Chapter 5, I assess pKJK5::Cas transfer and maintenance in a synthetic soil microbial community.

Finally, in the General Discussion, I apply the data generated throughout these chapters by analysing how effective target plasmid removal may be from the synthetic soil microbial community, and finish by reviewing in which environments and under which conditions pKJK5::Cas application would be most effective, and how this CRISPR delivery tool may be improved in future.

Chapter 2: pKJK5::Cas as a broad host-range barrier to plasmid uptake

Abstract

Antimicrobial resistance (AMR) is a key challenge facing healthcare. AMR genes are often horizontally transferred between bacteria via plasmids; therefore, blocking AMR plasmid uptake could reduce the prevalence of resistance genes. Previous work has used CRISPR-Cas9 to target and cleave AMR plasmids for this purpose, but Cas9 delivery has typically been achieved using narrow host-range genetic elements, requiring re-engineering for application in a different host background.

In this chapter, I engineered the broad host-range plasmid pKJK5 to encode Cas9 (pKJK5::Cas) and a sgRNA programmed to remove cloning plasmid pHERD30T, encoding gentamicin resistance. After testing which sgRNA target sequence led to the most effective target plasmid removal, I demonstrated pKJK5::Cas target removal by measuring transformation efficiency of a targeted plasmid in *Escherichia coli*. Finally, I utilised the broad host-range feature of pKJK5::Cas and showed that AMR plasmid uptake can be blocked in human, pig-gut, and environmental coliform isolates as well as two *Pseudomonas* species.

This study shows that pKJK5::Cas can block AMR uptake in a range of species, crucially without the need for re-engineering. This is a promising approach of curbing resistance gene transfer to problematic species of bacteria.

Introduction

The adaptive immune system CRISPR-Cas provides bacterial cells with protection against not only their natural predators, bacteriophage, but also against other genetic elements including plasmids (Kamruzzaman and Iredell, 2020) which often carry antimicrobial resistance (AMR) genes, and can therefore act as a barrier to horizontal gene transfer (HGT). In fact, when analysing thousands of genomes of pathogenic *Enterococcus, Staphylococcus, Acinetobacter,* and *Pseudomonas*, it was found that presence of a CRISPR-Cas system is associated with AMR genes (Pursey *et al.*, 2021). This was shown experimentally in *Enterococcus faecalis*, where a CRISPR-Cas system could be reactivated to protect its host from incoming plasmids (Hullahalli *et al.*, 2017).

1 CRISPR-Cas systems, particularly CRISPR-Cas9, have found a large range of
2 applications in biotechnology. It was first postulated over a decade ago that
3 CRISPR may provide a barrier against plasmid uptake (Marraffini and
4 Sontheimer, 2008). Previous approaches have used non-replicating phage
5 plasmids (phagemids) (Bikard *et al.*, 2014; Citorik *et al.*, 2014), expression
6 vectors (Gomaa *et al.*, 2014), or synthetic conjugative or mobilisable plasmids
7 (Dong *et al.*, 2019; Wongpayak *et al.*, 2021) to deliver minimal CRISPR-Cas9
8 systems. These may be effective at blocking transfer of resistance genes into
9 specific strains, but would need to be re-engineered and re-tested for application
10 in a new target species due to their narrow host range. Therefore, we sought to
11 design a broad host-range CRISPR-Cas9 expression system which can block
12 AMR gene uptake in multiple species.

13 Such a CRISPR delivery tool may find application in healthcare or in the
14 environment: In healthcare, applying this treatment before exposure to antibiotic
15 resistant bacteria could prevent the resident microbiome from becoming
16 colonised by AMR plasmids. In the environment, this treatment would prevent
17 colonisation of soil or waste-water treatment plant microbiomes from colonisation
18 by AMR plasmids when exposed to contaminated slurry. In both scenarios, such
19 a CRISPR delivery tool would prevent local microbiomes from becoming
20 reservoirs of mobile AMR genes.

21 As a backbone for this CRISPR delivery tool we chose IncP-1ε plasmid pKJK5,
22 which has been shown to have a particularly broad host-range and can effectively
23 spread through soil, rat microbiome, pig gut, and waste-water treatment plant
24 communities using *Escherichia coli*, *Pseudomonas putida*, or *Kluyvera* spp. as
25 donors (Bahl *et al.*, 2007a, 2007b; Klümper *et al.*, 2015; Li *et al.*, 2020). pKJK5
26 was found to be taken up by multiple species of at least 10 phyla of mostly Gram
27 negative, but also Gram positive bacteria. These phyla included Acidobacteria,
28 Actinobacteria, Bacteroidetes,Firmicutes, Fusobacter, Gemmatimonadetes,
29 Planctomycetes, Pseudomonodota, Spirochatetes, and Verrucomicrobia
30 (Klümper *et al.*, 2015). Classically, plasmids were considered broad host-range if
31 they could replicate in *Enterobacteria* and *Pseudomonas* species, but pKJK5
32 further meets the modern requirments of broad host-range plasmids to have the
33 ability to transfer between bacteria of different phylogenetic groups (Jain and
34 Srivastava, 2013). Generally, Inc-P1ε plasmids are commonly found in

environments such as soils, rhizospheres (Jechalke *et al.*, 2013b), and organic digestates in biogas plants (Wolters *et al.*, 2014).

The gentamicin resistance-encoding cloning vector pHERD30T was chosen as a target plasmid for these first experiments, as it can be maintained by *Escherichia* and *Pseudomonas spp.*, is compatible with pKJK5, and encodes no payload genes which may interfere with CRISPR targeting.

In this chapter, I aimed to (1) engineer pKJK5 to encode a CRISPR-Cas9 cassette programmed to target AMR, and (2) test the ability of this recombinant plasmid pKJK5::Cas to act as a barrier to AMR plasmid acquisition in different bacterial species.

Methods

Strains, growth conditions, and molecular cloning

All bacterial strains used throughout the thesis are listed in Thesis Supplement Table S1. Unless otherwise specified, all strains were cultured in LB at 37°C whilst shaking at 180 rpm. Where necessary for plasmid selection, antibiotics were added to achieve the following final concentrations: Ap – 100 µg/mL Ampicillin; Carb – 250 µg/mL Carbenicillin; Cp – 25 µg/mL Chloramphenicol; Gm – 50 µg/mL Gentamicin; Km – 50 µg/mL Kanamycin; Sm – 50 µg/mL Streptomycin; Tc – 12 µg/mL Tetracycline; Tmp – 10 µg/mL Trimethoprim. Where necessary, the following additives were added to growth media after preparation of stock solutions and filter-sterilisation: Ara – 0.5% (w/v) Arabinose; Gluc – 0.5% (w/v) Glucose.

Pig gut isolate bhiF2 was isolated from a microbial pig gut community: Briefly, faecal pig samples, collected from four Cornish black pigs, were suspended in 10% Glycerol and 0.9% (w/v) NaCl, and subsequently blended and strained. The resulting pig gut slurry was plated onto BHI (brain heart infusion) agar plates without selection, and bhiF2 was one of several visually distinct bacterial isolates picked from these plates. Genus identity was confirmed as *Escherichia/Shigella* by 16S colony PCR (amplified using primers Forward 27F/Reverse 1492R; Table S3), Sanger sequencing, and BLAST homology search.

Where *E. coli* MFDpir was used, cultures were supplemented with 300mM DAP (diaminopimelic acid) to ensure growth of this auxotrophic strain. By omitting DAP the strain could be selected against.

Unless otherwise stated, all molecular cloning steps were carried out with high-fidelity restriction enzymes (NEB) and according to manufacturer protocols, using commercially chemically competent *E. coli* DH5α cells (NEB).

in silico cassette construction and specificity swap

A CRISPR-Cas9 gene cassette was constructed and restriction sites were identified using Benchling (Benchling, 2015); an overview of the workflow is shown in Figure 2.1. Sources of nucleotide (nt) sequences for each module are summarised in Table 2.1.

Table 2.1: Sequence sources of CRISPR-Cas9 cassette coding and non-coding elements.

Element	Source
Cas9	Addgene plasmid # 39312 (Jinek *et al.*, 2012) Coding sequence only.
sgRNA	Addgene plasmid # 44251 (Qi *et al.*, 2013); N20 replaced to target *aacC1*. Promoter and terminator as in source. Upper stem edited as described in text.
GFPmut3b	(Cormack *et al.*, 1996)
Multiple cloning site	pBAM1 (Martínez-García *et al.*, 2011). The final version is heavily edited to exclude restriction sites used elsewhere.
Cas9 promoter / terminator	As found on pBAM1 (Martínez-García *et al.*, 2011): *bla* Ampicillin resistance upstream region (70 nts) as promoter with two final nts changed to CC (to create NcoI restriction site for promoter exchange), downstream region (54 nts) as terminator.
GFP promoter	Pa1/04/03 as found on Genbank acc. no. DQ493878. Constitutive, LacI-repressible promoter with strong ribosome binding site.
GFP terminator	*neo* Kanamycin resistance downstream region (29 nts) as found on pBAM1 (Martínez-García *et al.*, 2011)
Homology arms	Upper homology: nts 450-550; lower homology: nts 551-651 of *dfrA* on pKJK5 (GenBank accession AM261282.1). This insert was chosen by sgRNA identification for potential Cas9-assissted recombineering.

Genes were codon optimised using OPTIMIZER (Puigbò *et al.*, 2007) with pKJK5 codon usage database tables (Nakamura *et al.*, 2000). To enable a modular cassette build, common restriction sites were removed from coding sequences. In these instances, the codons were changed to the second most common on pKJK5. When creating or altering multiple cloning sites, random nts were added to increase spacing and allow double digestions with multiple restriction enzymes at the same time. Terminator presence (and absence from unwanted regions) was checked using Arnold (Gautheret and Lambert, 2001). The initial single guide RNA (sgRNA) carried the specificity to [aacC1-164] and targets Gentamicin resistance gene *aacC1* on pHERD30T (Table 2.2).

1 The sgRNA gene was placed under control of synthetic, strong constitutive
2 promoter J23119 (which contains a SpeI restriction site; as on pgRNA (Qi *et al.*,
3 2013)) and was edited to encode a SacI restriction site in its upper stem region,
4 the function of which is generally resilient to mutations (Briner *et al.*, 2014). These
5 two restriction sites allow simple exchange of the specificity-defining N20 stretch
6 on the sgRNA (Figure 2.1C).

7 The CRISPR-Cas9 gene cassette was commercially synthesised
8 (ThermoScientific). A fully annotated sequence of pMA-RQ_Cas, an expression
9 vector which carries the final version of this CRISPR cassette, is available in the
10 Thesis supplement.

11 To exchange sgRNA target specificity of pMA-RQ_Cas, I designed DNA
12 oligonucleotides containing a 20-nt specificity region with SpeI and SacI
13 compatible overhangs (N20_xx_top/btm; Table S3). These were annealed by
14 mixing 10µL of each 100µM oligo with 80µL of annealing buffer (100 mM
15 potassium acetate, 30 mM HEPES; pH=7.5) and heating to 95°C followed by slow
16 overnight cooling to room temperature. Subsequently, the annealed oligos were
17 phosphorylated using T4 polynucleotide kinase (NEB) according to
18 manufacturer's instructions. The annealed and phosphorylated oligos were
19 inserted between pMA-RQ_Cas's SpeI and SacI restriction sites following
20 standard molecular cloning protocols, resulting in pMA-RQ_Cas[new specificity]
21 (Figure 2.1C).

22 **Evaluation of Cas9 guide RNA efficiency**

23 Possible Cas9 guide targets (spacers) for targeting Gentamicin plasmid
24 pHERD30T were identified using CRISPOR (Haeussler *et al.*, 2016). Several
25 random guides with high and low off-target scores were chosen and are
26 presented in Table 2.2. Off-target hits were identified against *Pseudomonas*
27 *aeruginosa* PA01 and *E. coli* K12 genomes.

28 To test efficacy of these guides, I constructed a series of pHERD20T_sgRNA
29 expression vectors. First, I constructed a template pCDF1b_sgRNA vector by
30 amplifying a sgRNA-coding region using Taq polymerase (PCRbiosystems) and
31 pgRNA (bacteria) as a template with primers sgRNA_amp_fwd and
32 sgRNA_amp_rev (Table S2-3). This amplicon was inserted into pCDF1b's NcoI
33 restriction site following standard molecular cloning protocols. To exchange
34 sgRNA specificity, a stretch containing the new N20 specificity was amplified

1 using pCDF1b_sgRNA as a template and primers sgRNA_amp_rev and
2 sgRNAxp_[specificity]_fwd with corresponding N20 specificity stretches (all
3 guides listed in Table 2.2 except 'nt'). The amplicon was re-inserted into
4 pCDF1b_sgRNA using SpeI and HindIII restriction sites to generate
5 pCDF1b_sgRNA[new specificity]. To allow expression in streptomycin-resistant
6 *P. aeruginosa* PA01::cas9, these sgRNAs were cut out of vector pCDF1b_sgRNA
7 and inserted into pHERD20T using NcoI and HindIII restriction sites.

8 **Table 2.2**: sgRNA guide sequences and details: "off-target" indicates number of
9 off-target hits in genome identified for *n* number of mismatches in guide
10 sequence. A visualisation of pHERD30T-targeting guides can be found in Figure
11 2.2A.

Guide name	Target (gene / plasmid)	Guide sequence (5' → 3')	Off-target *(P. aeruginosa* PA01)	Off-target *(E. coli* K12)
dfrA	*dfrA* (pKJK5)	ACGACCGCATACTTTCGGTT	None identified	None identified
aacC1 60/fw	*aacC1* (pHERD30T)	CGCCCTAAAACAAAGTTAGG	None identified	None identified
aacC1 72/fw	*aacC1* (pHERD30T)	AAGTTAGGTGGCTCAAGTAT	None identified	None identified
aacC1 164/rev	*aacC1* (pHERD30T)	CGGCTGATGTTGGGAGTAGG	1 hit (4 mis-matches)	None identified
aacC1 185/fw	*aacC1* (pHERD30T)	CACCTACTCCCAACATCAGC	None identified	None identified
aacC1 341/rev	*aacC1* (pHERD30T)	GCCCTGCCTCCGGTGCTCGC	1 hit (3 mis-matches); 18 hits (4 mis-matches)	None identified
MCS18	multiple cloning site (pHERD30T)	GGTCGACTCTAGAGGATCCC	None identified	None identified
nt	n/a	GGTAAGACCATTAGAAGTAG	None identified	None identified

12

13 To prepare electrocompetent PA01::cas9, 1mL aliquots of an overnight culture
14 were washed twice with 300mM sucrose solution and resuspended in 100µL 300
15 mM sucrose. PA01::cas9 was electroporated in 2mm gap cuvettes at 2.5 kV with
16 500ng plasmid DNA of each pHERD20T_sgRNA variant, and electroporated cells
17 were recovered by adding 1mL LB and incubation at 37°C, 250rpm for 1 hour.
18 The sgRNA plasmid was selected for and maintained using Carb.

19 For the guide test experiment, PA01::cas9 carrying each pHERD20T_sgRNA
20 variant were incubated overnight in presence of Carb (for sgRNA maintenance)

and Ara (for Cas9 expression) and transformed with 500 ng pHERD30T plasmid DNA as described above, using either 3 or 4 replicates. Each transformant was plated onto LB + Gm + Ara as well as LB + Gm + 0.2% Glucose. No differences were detected between these treatments, so averages between the plates were used to calculate the transformation efficiency for each replicate. A no-plasmid control transformation of PA01_Cas9 yielded no colonies.

pKJK5::Cas recombineering

The CRISPR-Cas9 cassette was introduced to pKJK5 (original size approximately 54 kb) using homologous recombineering. Initial attempts using pKD46 (Datsenko and Wanner, 2000) proved unsuccessful. Successful recombineering was carried out with an altered version of pDOC and pACBSCE plasmids as described in (Lee *et al.*, 2009). To construct pDOC_Cas as a template vector containing the CRISPR cassette, the kanamycin resistance gene was removed from pDOC-K using AvrII and NheI restriction sites. The following steps were carried out in parallel with pMA-RQ_Cas[aacC1-72] and pMA-RQ_Cas[nt]. The CRISPR cassette was inserted from pMA-RQ_Cas using restriction sites EcoRI and HindIII to create pDOC_Cas.

The recombineering workflow is summarised in Figure 2.3A. *E. coli* DH5α + pKJK5 was transformed with pACBSCE and pDOC_Cas following standard procedures for electrotranformation in *E. coli*. After electrotransformation, cells were cultured in the presence of Tc + Tmp (pKJK5) + Ap (pDOC_Cas), + Cp (pACBSCE) to maintain plasmids, and in the presence of Gluc to prevent leaky λ-red expression.

10μL of an overnight culture of this recombineering-ready strain were grown in 1mL LB + Tc + Tmp + Cp + Ap + Gluc at 37°C, 250 rpm for 2 hours in triplicate. The cultures were spun and resuspended in 1 mL LB + Tc + Ara and incubated at 37°C until turbid (4-5 hours) to allow recombination. Finally, the cultures were plated onto LB + Tc + 5% sucrose in several dilutions and incubated at 28°C for 48 hours (to allow counterselection of bacteria with intact pDOC_Cas plasmids). To isolate recombinants, bacterial lawns were investigated for GFP expression under a fluorescence microscope. Green colonies were restreaked onto LB + tetracycline several times until all colonies appeared GFP+, indicating successful recombination events. Next, GFP+ colonies were checked for correct CRISPR cassette insertion by colony PCR using primer combinations dfrA_fw / Cas9_bw

1 and GFPend_fw / dfrA_bw (Figure 2.3B). A ~300 / 500 bp DNA band from each
2 PCR reaction respectively indicated successful recombination. Additionally,
3 primers dfrA_fw / dfrA_rv were used to check for presence of WT pKJK5: a ~500
4 bp band indicated presence of the WT dfrA gene (with cassette insertion, the
5 band would be >6kb long and did not amplify under standard PCR conditions).
6 Three colonies which were positive for the first two PCR reactions and negative
7 for the third were chosen and investigated for Cas9 activity and conjugative
8 ability.

9 **pKJK5::Cas conjugation and Cas9 targeting**

10 Three independent *E. coli* DH5α + pKJK5::Cas[aacC1] / [nt] recombinants each
11 (see above) were chosen as replicate donors to verify pKJK5::Cas conjugative
12 ability. 50µL of overnight cultures of donors were mixed with 50µL of overnight
13 culture of recipients (*E. coi* DH10β or *E. coli* K12::mCherry) in 5 mL LB without
14 antibiotic selection and incubated overnight at 37˚C, 45 rpm. After overnight
15 incubation, the cultures were stored at 4˚C for one day before selective plating.
16 All matings were plated onto LB, those with DH10β as recipients were additionally
17 plated onto LB + Sm and LB + Sm + Tc (to select for recipients and
18 transconjugants respectively); those with K12::mCherry as recipients onto LB +
19 Km and LB + Km + Tc. Additional ‘no-recipient’ and ‘no-donor’ controls yielded
20 colonies as expected. CFU on different selective plates were assessed and
21 proportion of transconjugants per recipients calculated independently for each
22 replicate. Additionally, GFP expression was randomly checked by fluorescence
23 microscopy for colonies on selective plates and found to be as expected (all
24 transconjugants assessed were GFP+, most recipients were GFP-).

25 For Cas9 activity verification, *E. coli* DH5α + pKJK5::Cas[aacC1]/[nt] were
26 electrotransformed using standard protocols, using 500ng of plasmid DNA
27 (pHERD30T / pHERD20T) in 6 replicates. Briefly, an overnight culture of *E. coli*
28 carrying each plasmid was diluted 1:100 in 25 mL LB supplemented with
29 appropriate antibiotics and grown at 37°C, 250rpm to a culture density of
30 OD600=0.6. Cells were immediately chilled and washed twice with ice-cold 10%
31 (w/v) Glycerol. Cells were concentrated approximately 25-fold and immediately
32 electroporated in 100 µL aliquots using 1mm gap cuvettes and 1.8kV, and
33 recovered in 1 mL LB at 37°C, 250rpm for 1 hour.

50µL of transformed cells were plated onto either single (LB + Gm / Amp) or double selective plates (LB + Tc + Gm/Amp), but pKJK5::Cas was maintained in the presence and absence of Tc, so no differences between plates were found. Therefore, counts on both plates were averaged for each replicate. Colony counts allowed to calculate transformation efficiency of each strain (cfu/mL/µg DNA). Where no colonies could be recovered, transformation efficiency was set to ½ of the limit of detection (transformation efficiency if a single colony were recovered).

pKJK5::Cas as a tool to block plasmid uptake in multiple species

Using *E. coli* DH5α or *E. coli* MFDpir as a donor, pKJK5::Cas[aacC1-72] / [nt] transconjugants of bhiF2, C743E1, TV1-2, 6TB-1, *P. aeruginosa* PA14, and of *Pseudomonas fluorescens* SBW25 were generated and pKJK5::Cas[nt]/[aacC1] was maintained with Tc. Next, each strain was made electrocompetent and transformed with 600 ng pHERD30T plasmid DNA following protocols described above for *E. coli* (bhiF2, C743E1, TV1-2, 6TB-1) or PA01 (PA14). To prepare electrocompetent *P. fluorescens* cells, the protocol for PA01 was followed with the exception that SBW25 was grown at 28°C, cultures were grown until log phase (estimated OD600: 0.5-0.6) and then the protocol was started. Cultures were electroporated at 1.8kV in 2mm gap cuvettes, and recovered in 1mL SOB at 28°C, 250 rpm for 1 hour.

800µL of all strains were plated onto LB + Gm, and transformation efficiency calculated as described above. For *P. fluorescens* SBW25 transformations, only 50µL of transformed cells were plated resulting in a higher limit of detection.

Statistical analyses

Data processing, data visualisation, and statistical analyses were carried out using R software version 4.1.0 and RStudio version 1.4.1717 with the following packages: tidyverse version 1.3.1, janitor version 2.1.0.

For statistical analyses of data presented in Figures 2.2, 2.4, and 2.5 linear or generalised linear models were fitted, see below for model details. For all models, other model types were tested and the best fitting model was chosen. Model assumptions were tested and found to be upheld. For data in Figures 2.2, 2.4B, and 2.5, a Tukey's post-hoc test was carried out to assess statistical difference between treatment categories.

1 Guide Test (Figure 2.2) Linear model describing log(transformation efficiency) as
2 a function of guide identity. F=169.4; d.f.=6&17; $p=3.2x10^{-14}$; adjusted R^2=0.978.

3 pKJK5::Cas mating (Figure 2.4A) Gaussian generalised linear model (GLM) with
4 identity link function describing log(conjugation efficiency) as a function of
5 recipient strain and pKJK5::Cas variant. F=57.2; d.f.=2&9; $p=7.643x10^{-6}$;
6 adjusted R^2=0.9109.

7 E. coli transformation efficiency (Figure 2.4B) Gamma GLM with log link function
8 describing log(transformation efficiency) as a function of transformed plasmid,
9 pKJK5::Cas variant, and their interaction. $F=3.757x10^5$; d.f.=3&20; $p<2.2x10^{-16}$;
10 adjusted R^2=1.

11 Transformation efficiency of other host strains (Figure 2.5) Inverse Gaussian
12 GLM with log link function describing log(transformation efficiency) as a function
13 of the interaction of host strain and pKJK5::Cas variant. F=374.6; d.f.=11&48;
14 $p<2.2x10^{-16}$; adjusted R^2=0.9858.

15 Results

16 I aimed to engineer the broad host-range plasmid pKJK5 to carry a CRISPR-
17 Cas9 cassette which would block uptake of pHERD30T, a plasmid encoding
18 Gentamicin resistance gene *aacC1*.

19 *in silico* CRISPR-Cas9 cassette construction

20 The initial step in engineering a pKJK5-based CRISPR-Cas9 expression system
21 was to design a CRISPR entry cassette *in silico* which could later be
22 recombineered in pKJK5. The gene cassette was designed to include the
23 nuclease Cas9, a sgRNA for targeting specificity, and a GFP (green fluorescent
24 protein) gene to track plasmid transfer (Figure 2.1A). Strategic restriction sites
25 were incorporated in the gene cassette design to ensure full modularity (Figure
26 2.1B), and the sgRNA gene was edited to allow simple exchange of the
27 specificity-defining N20 stretch (Figure 2.1C). As a final main module, GFP was
28 added under control of lacI-repressible promoter $P_{a1/04/03}$ (Lanzer and Bujard,
29 1988) to allow optional repression of GFP expression. Next, the entire CRISPR
30 entry cassette was flanked by homology arms to allow homologous
31 recombination with *dfrA* on pKJK5, the trimethoprim resistance gene which the
32 cassette would be inserted into.

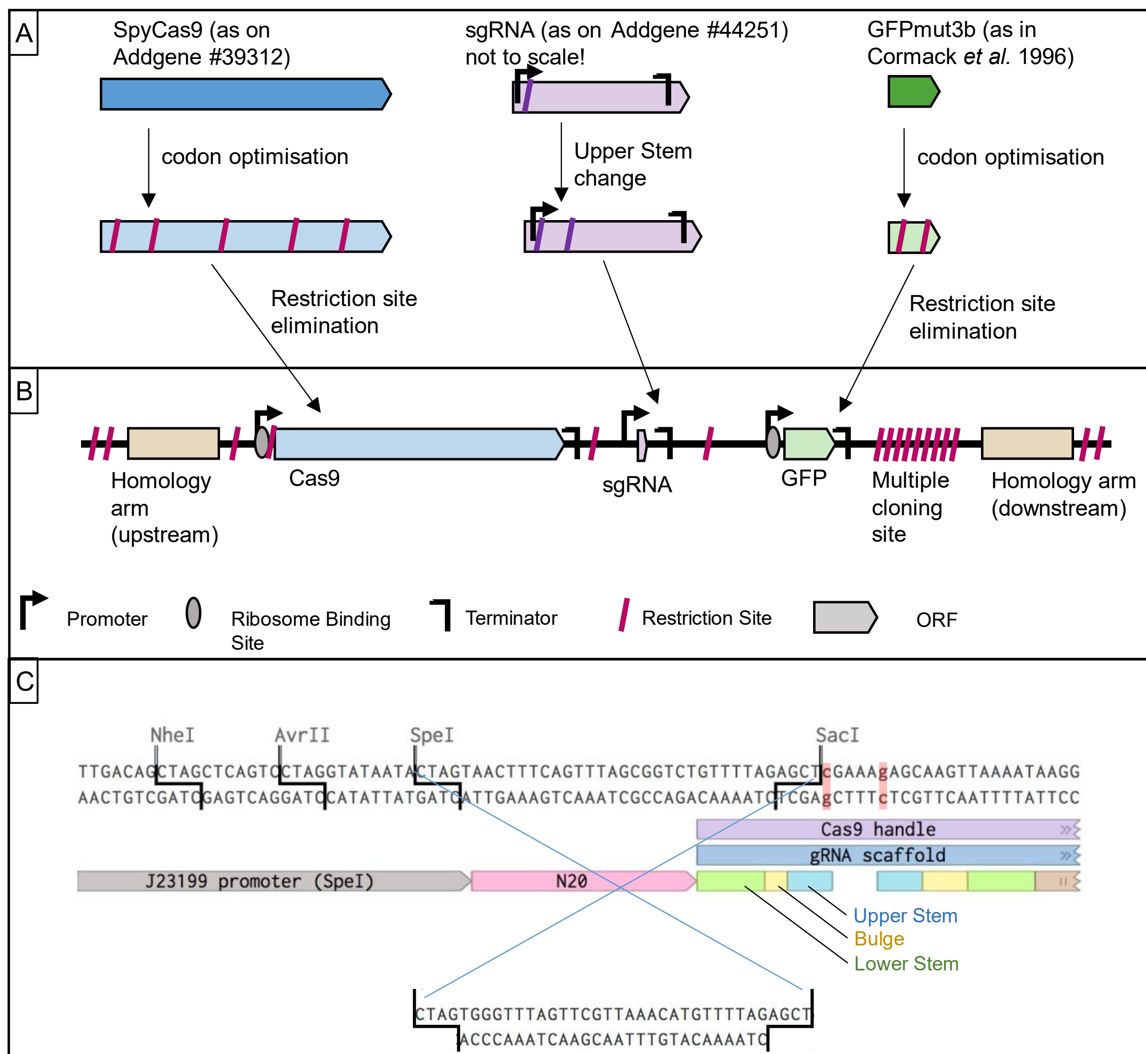


Figure 2.1: ***in silico* CRISPR-Cas9 cassette construction.**
A Source of genes included in cassette (*cas9, sgRNA, GFPmut3b*) and alterations undertaken. See C for sgRNA details. **B Final cassette layout.** Gene lengths are to scale. Spacings, restriction sites, ribosome binding sites, promoters, terminators, and homology arms are not. **C sgRNA region in detail.** Highlighted in red: nucleotide mutations undertaken in upper stem region to form SacI restriction site. The region to be exchanged for N20 specificity exchange is indicated with blue crossover lines.

1 The final CRISPR cassette is 5722 nts in length, and its protein-coding
2 sequences have an average GC content of 59.45%, which matches that of pKJK5
3 backbone genes (ranging from 55-70% GC (Bahl *et al.*, 2007a)). This means that
4 the codon-optimised genes can be predicted to be compatible with the pKJK5
5 backbone. The cassette was synthesised by Thermo Fisher's Gene Art service
6 and delivered as vector pMA-RQ_Cas, placing the CRISPR cassette onto a pUC-
7 based vector. A fully annotated sequence of vector pMA-RQ_Cas can be found
8 in the supplementary material.

sgRNA target determines CRISPR targeting efficiency

First, I determined which sgRNA sequence efficiently targets Gentamicin resistance-encoding vector pHERD30T, which was chosen as an AMR target plasmid for initial experiments. Crucially, pHERD30T is a shuttle vector and can be maintained by *Eschericha* as well as *Pseudomonas* species to allow testing of pKJK5::Cas' broad host range. The efficiency of several sgRNAs was tested by electroporating Cas9-expressing *P. aeruginosa* PA01::cas9 carrying various sgRNA plasmids with pHERD30T. I tested five sgRNAs targeting gentamicin resistance gene *aacC1*, and one which targets pHERD30T in an intergenic region [MCS18]. As a control, I used a sgRNA targeting *dfrA*, a gene sequence absent from pHERD30T (Figure 2.2A).

Presence of all guides significantly impaired pHERD30T transformation efficiency compared to the control guide, and decreased transformation efficiency by at least 1 order of magnitude (p<0.05; Figure 2.2B). Three guides (185/fw, 60/fw, and 72/fw) showed very stringent transformation blocking, reducing transformation efficiency by nearly 3 orders of magnitude compared to the *dfrA*

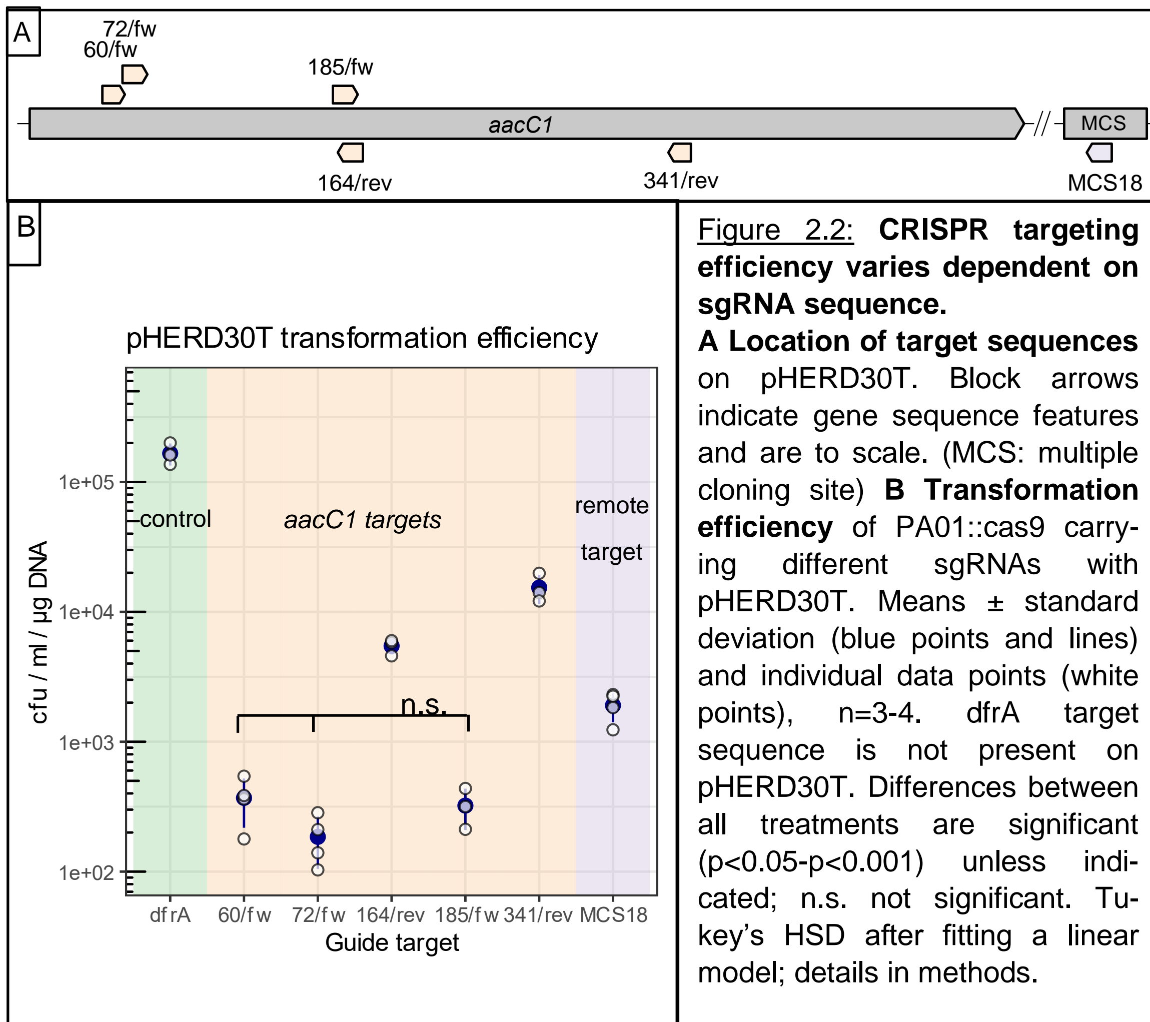


Figure 2.2: **CRISPR targeting efficiency varies dependent on sgRNA sequence.** **A Location of target sequences** on pHERD30T. Block arrows indicate gene sequence features and are to scale. (MCS: multiple cloning site) **B Transformation efficiency** of PA01::cas9 carrying different sgRNAs with pHERD30T. Means ± standard deviation (blue points and lines) and individual data points (white points), n=3-4. dfrA target sequence is not present on pHERD30T. Differences between all treatments are significant (p<0.05-p<0.001) unless indicated; n.s. not significant. Tukey's HSD after fitting a linear model; details in methods.

control. Out of the remaining guides, 341/rev and 164/rev showed comparatively low transformation inhibition (1-1.5 orders of magnitude). Interestingly, the guide targeting a non-essential region of the plasmid backbone (MCS 18/rev) showed a greater inhibition of transformation than the two aforementioned guides (~ ½ of the transformation efficiency of 164/rev; $p<0.05$, see methods for model details).

As the guide *aacC1* 72/fw was most effective at blocking transformation by pHERD30T (albeit non-significantly in comparison with 60/fw and 185/fw), its specificity was used in all further experiments.

pKJK5::Cas can conjugate and target plasmids in *E. coli*

Next, I constructed pKJK5::Cas by carrying out homologous recombineering of the CRISPR-Cas9 cassette with pKJK5 using established methods and *E. coli* DH5α (adapted from (Lee *et al.*, 2009); see methods; Figure 2.3). I generated two pKJK5::Cas variants with different sgRNA specificities: pKJK5::Cas[aacC1-72] targets gentamicin resistance gene *aacC1* on pHERD30T and is the most efficient guide to do so, as identified in Figure 2.1. As a non-targeting (nt) control, pKJK5::Cas[nt]'s sgRNA carries a random nucleotide sequence with no full matches in the BLAST database (two matches exist, but these lack a protospacer adjacent motif (PAM) adjacent to the target sequence, which is essential to CRISPR-Cas9 targeting). After verifying successful insertion of the CRISPR-Cas9 cassette into pKJK5 using GFP expression and PCR (see methods), I investigated the conjugation and Cas9-mediated targeting properties of pKJK5::Cas.

First, to understand whether sgRNA variant matters to pKJK5::Cas conjugation, I set up a series of co-inoculations of donors and different recipients. Three independently generated recombinants of *E. coli* DH5α + pKJK5::Cas[aacC1-72]/[nt] were used as donors, and either *E. coli* DH10β or *E. coli* K12::mCherry were used as recipients. While nearly 50% of recipient *E. coli* K12::mCherry formed transconjugants of both pKJK5::Cas variants, this proportion was ~2 orders of magnitude lower for *E. coli* DH10β (Figure 2.4A). pKJK5::Cas variant did not significantly influence conjugation efficiency ($p=0.40$), whereas recipient strain identity was a significant predictor for these data ($p<0.01$; see methods for model details). These results show that in the absence of a Cas9 target, the

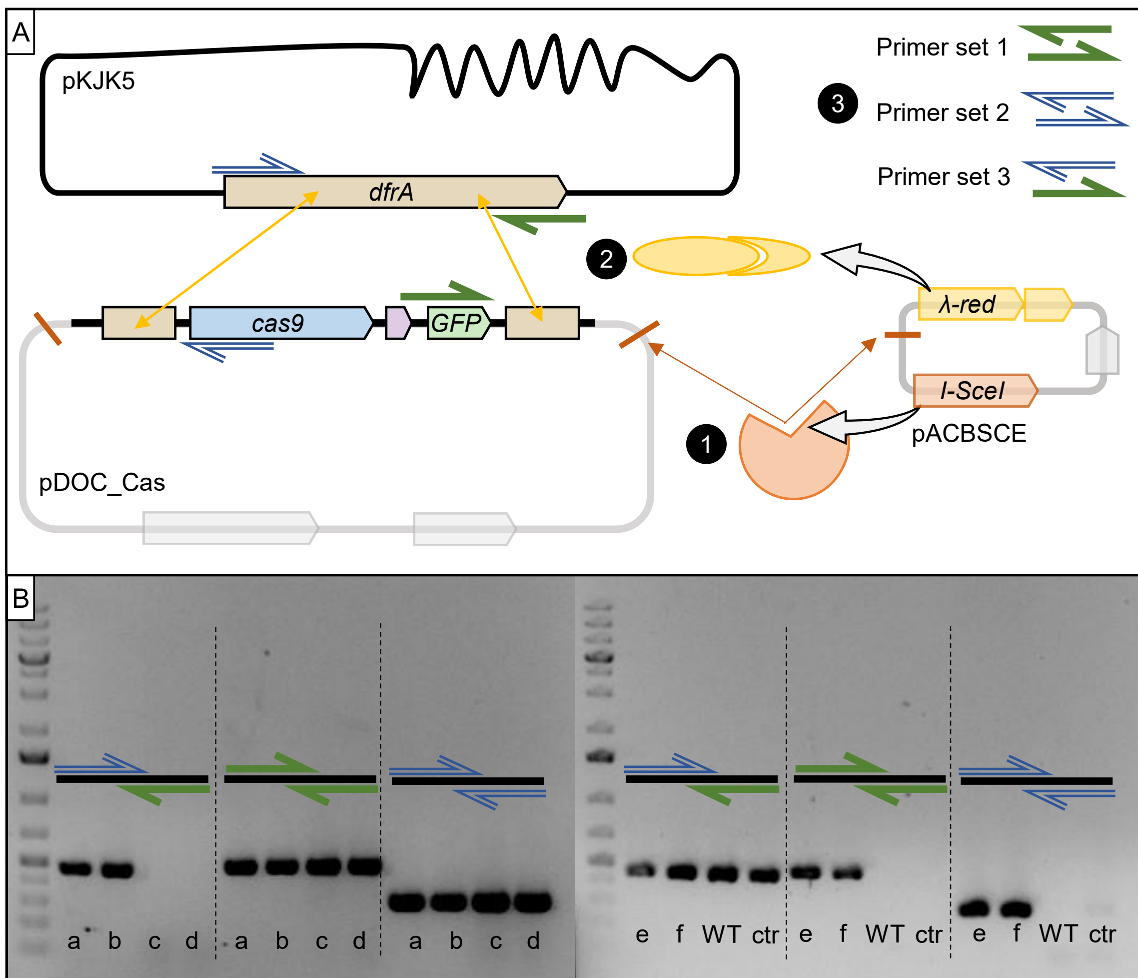


Figure 2.3 **pKJK5::Cas recombineering.**
A Gene doctoring recombineering method. Plasmids pKJK5, pDOC_Cas, and pACBSCE are transferred into *E. coli* DH5α. Upon Arabinose induction, meganuclease I-SceI is expressed by pACBSCE (1). It linearises the CRISPR cassette on pDOC_Cas and cleaves pACBSCE to destroy the vector (I-SceI recognition sites shown in red). Simultaneously, λ-red machinery is expressed by pACBSCE (2). It recombines pKJK5 and the CRISPR cassette using homologous regions (beige). Additionally, *sacB* expression on pDOC_Cas at 30ºC (not shown) ensures complete template plasmid removal in the presence of sucrose. Successful recombinants are isolated based on GFP expression and verified by PCR using several primer combinations (3). **B Representative gel** of isolates after recombineering with Cas[aacC1]: all samples a-f show successful recombination, evidenced by primer sets 1 and 2. However, a combination of both primers shows that only samples c and d are free of WT pKJK5. a-f: individual pKJK5::Cas[aacC1] recombinants. WT: non-recombined pKJK5. ctr: unsuccessful recombination using pKD46. A very faint band is visible for primer set 2.

sgRNA specificity does not interfere with pKJK5::Cas conjugation, but also indicate that conjugation efficiency depends on recipient strain identity.

As each independent pKJK5::Cas[aacC1-72] and pKJK5::Cas[nt] recombinant displayed comparable conjugation abilities (Figure 2.4A), one recombinant of

each was chosen randomly as the clone to work with for future experiments. To test Cas9 targeting activity, I measured the transformation efficiency of a targeted plasmid (pHERD30T) or an untargeted control plasmid (pHERD20T) in *E. coli* carrying pKJK5::Cas[aacC1-72]/[nt]. Instead of pHERD30T's *aacC1* Gentamicin resistance gene, pHERD20T encodes ampicillin resistance gene *bla*. Therefore, pHERD20T is not targeted by either guide and accordingly transformation efficiency was high regardless of sgRNA specificity (~10^6 cfu/mL/µg DNA; Figure 2.4B). Instead, for the targeted plasmid no successful transformants of DH5α + pKJK5::Cas[aacC1] could be recovered, while the same plasmid showed transformation efficiencies of ~10^4 cfu/mL/µg DNA in DH5α + pKJK5::Cas[nt]. This means that transformation efficiency of a target plasmid was reduced to at

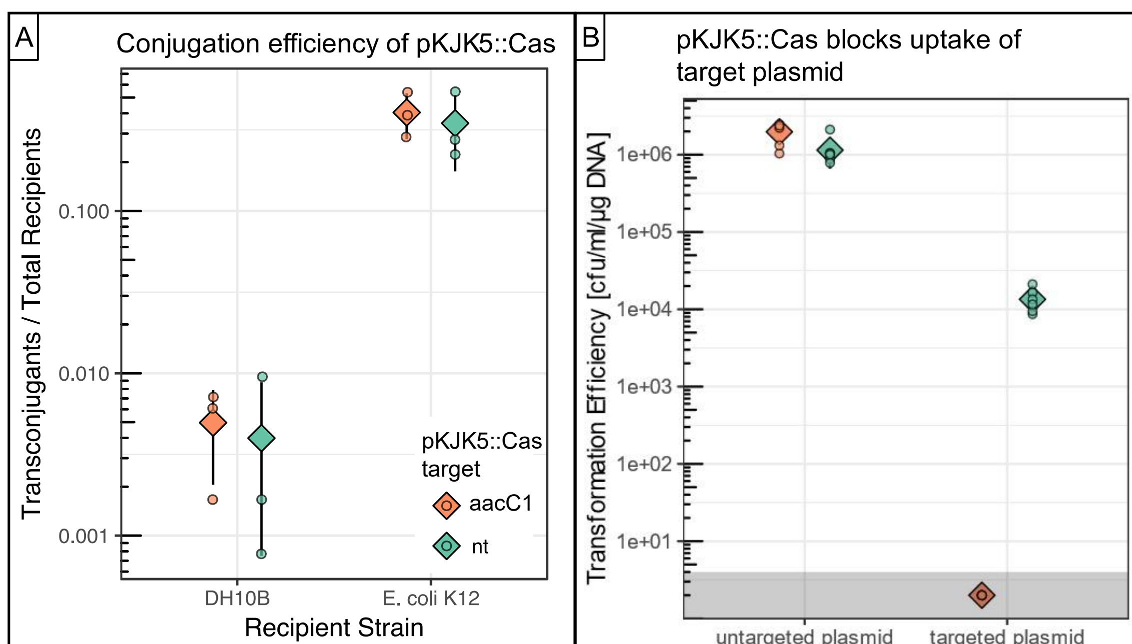


Figure 2.4 **pKJK5::Cas verification.**
A Conjugation efficiency of pKJK5::Cas[aacC1]/[nt] using *E. coli* DH5α as a donor and either *E. coli* DH10β or *E. coli* K12::mCherry as a recipient. Data is recorded as proportion of transconjugants out of total recipients, n=3. diamond/line=mean/standard deviation, circles=individual datapoints. Recipient strain ($p<0.01$), but not pKJK5::Cas variant ($p=0.399$) are significant data predictors when fitting a gaussian GLM. F=57.2; d.f.=2&9; $p=7.643 \times 10^{-6}$, adjusted $R^2=0.9109$. **B Transformation efficiency** of transformation of *E. coli* DH5α + pKJK5::Cas[aacC1]/[nt] with pHERD20T (untargeted plasmid) or pHERD30T (targeted plasmid). [aacC1] transformation with pHERD30T did not yield any transformants, datapoints are displayed as ½ of the limit of detection. Grey box: datapoints underneath the limit of detection. n=6, diamonds=mean, circles=individual datapoints. All treatments are significantly different from each other ($p<0.05$) when fitting a Gamma GLM. $F=3.757 \times 10^5$; d.f.=3&20; $p<2.2 \times 10^{-16}$; adjusted $R^2=1$.

1 least the limit of detection (4 cfu/mL/µg DNA) and was nearly four orders of
2 magnitude lower than the non-targeting control.

3 pKJK5::Cas is a barrier to plasmid acquisition in various species

4 Finally, after constructing and verifying pKJK5::Cas[aacC1] and its non-targeting
5 control pKJK5::Cas[nt], I aimed to test their ability to act as a barrier to plasmid
6 acquisition in a broader range of bacterial species. Therefore, I transformed
7 pKJK5::Cas transconjugants of several environmental, animal, and human-
8 associated coliform isolates as well as two species of *Pseudomonas* (*P.*
9 *aeruginosa* PA14 and *P. fluorescens* SBW25; Table 2.3) with pHERD30T. The
10 coliform isolates included pig-gut *Escherichia/Shigella spp.* isolate bhiF2 (this
11 study), human isolate C743E1, and environmental isolates TV1-2 and 6TB-1
12 (Leonard *et al.*, 2018). These isolates were chosen for their ability to carry target
13 plasmid pHERD30T.

14 **Table 2.3**: Isolates used for pKJK5::Cas transformation assay.

Name	Source	Species	Other information
bhiF2	Pig faeces samples	*Escherichia / Shigella*	Strain characterised by Sanger sequencing of 16S PCR only (this study)
C743E1	Human rectal swab	*Escherichia coli*	ST131; O16:H5. Strain characterised by PCR testing and Illumina sequencing (Leonard *et al.*, 2018; Leonard, in prep)
TV1-2	Sewage water	*Escherichia coli*	ST196; O8:H7. Strain characterised by PCR testing and Illumina sequencing (Leonard *et al.*, 2018; Leonard, in prep)
6TB-1	Bathing water	*Escherichia coli*	ST527; O139:H9. Strain characterised by PCR testing and Illumina sequencing (Leonard *et al.*, 2018; Leonard, in prep)
PA14	Laboratory strain	*Pseudomonas aeruginosa*	Originally isolated from burns patient in the 1970s (Schroth *et al.*, 2018)
SBW25	Laboratory strain	*Pseudomonas fluorescens*	Originally isolated from sugar beet in the 1990s (De Leij *et al.*, 1995)

15

16 Transformation of these isolates carrying pKJK5::Cas[nt] was successful, but
17 efficiency remained slightly below of that of *E. coli* DH5α from the previous
18 experiment (~666-4320 cfu/mL/µg DNA; Figure 2.5). In contrast, transformation
19 efficiency of all isolates when carrying pKJK5::Cas[aacC1-72] was close to or

below their limits of detection and remained at least 2-3 orders of magnitude below transformation efficiency when carrying pKJK5::Cas[nt] ($p<0.001$; see methods for details).

Together, these data show that pKJK5::Cas is an effective barrier to uptake of a plasmid containing a targeted AMR gene by transformation. Most likely, blocked uptake is achieved by Cas9-mediated cleavage of the target plasmid after it has entered the cell, which prevents plasmid replication. Blocking plasmid uptake was effective in a range of species of laboratory strains as well as environmental, animal-, and human-associated isolates without the need for re-engineering of pKJK5::Cas.

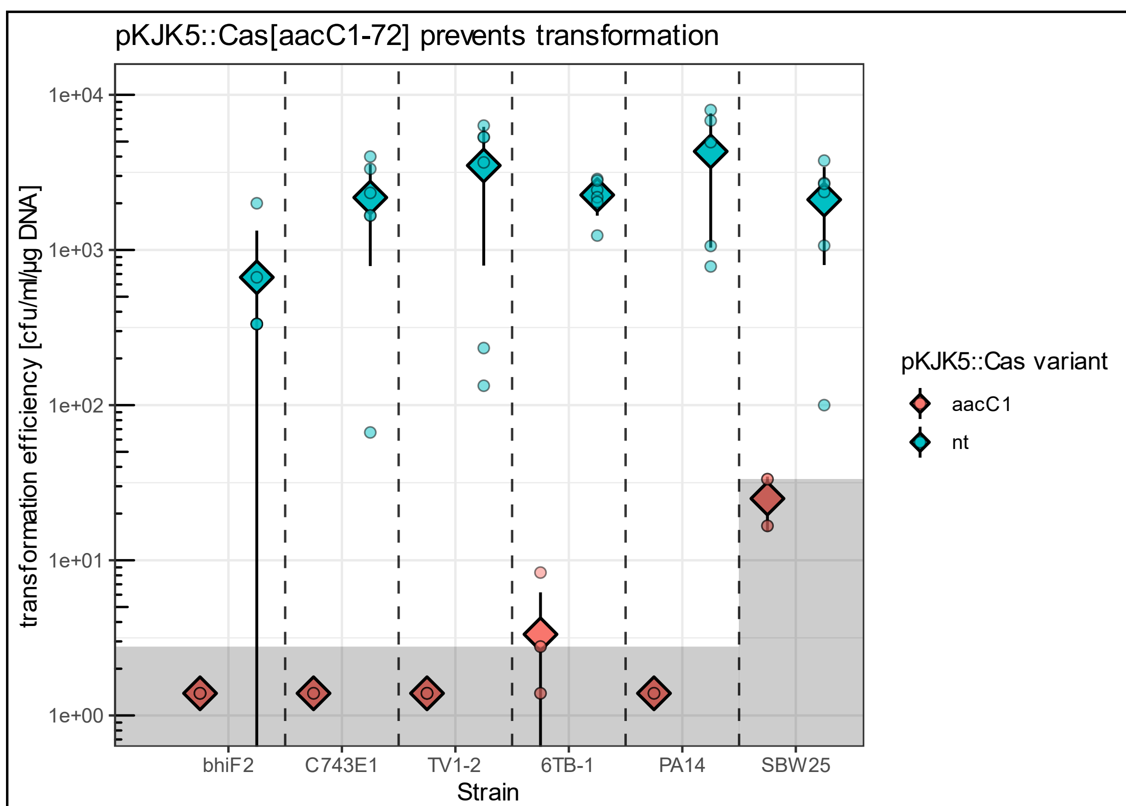


Figure 2.5 **pKJK5::Cas prevents transformation in various host backgrounds.**
Transformation efficiency of various isolates carrying pKJK5::Cas[aacC1] or pKJK5::Cas[nt] with target plasmid pHERD30T. Diamonds and lines indicate mean ± standard deviation, points indicate individual replicates; N=3-6 (samples arcing during electroporation were discarded). Shaded areas indicate the limit of detection; counts of 0 were manually set to ½ of the limit of detection. bhiF2, C743E1, TV1-2, 6TB-1: coliform environmental and human isolates. PA14: *Pseudomonas aeruginosa* PA14. SBW25: *Pseudomonas fluorescens* SBW25. aacC1 and nt transformation efficiency are significantly different for all strains; $p<0.001$ as assessed by Tukey's HSD after fitting an inverse Gaussian GLM; details in methods.

Discussion

In this work, I engineered pKJK5::Cas as a broad host-range platform to prevent plasmid uptake by transformation and validated this process in a series of *Escherichia* and *Pseudomonas* strains. Species belonging to these genera can be found together in, for instance, wastewater treatment plants (Li *et al.*, 2018) or human microbiomes (Martinson and Walk, 2020; Wheatley *et al.*, 2022). Therefore, this work provides a foundation for pKJK5::Cas to protect multiple species of the same microbiome from AMR plasmid invasion.

Other studies also investigated the use of CRISPR-Cas9 to prevent uptake of targeted plasmids, and delivered these genes to *E. coli* or *Staphylococcus aureus* using engineered phage (Yosef *et al.*, 2015201), phagemids (Bikard *et al.*, 2014), or narrow-host range plasmids (Dong *et al.*, 2019). In these approaches the host range of the Cas9 delivery vehicle typically only extends to a single species or strains. In contrast, this work shows that plasmid targeting is possible in a range of bacterial species and natural isolates, crucially without the need for re-engineering (Figure 2.5). Nevertheless, suitability of this approach may vary between species: Interestingly, *P. fluorescens* cultures grew very poorly when carrying pKJK5::Cas (data not shown). Despite this apparent fitness cost of a lower growth rate, a significant proportion of the cultures maintained pKJK5::Cas as evidenced by the reduction in transformants of the SBW25 + pKJK5::Cas[aacC1-72] culture (Figure 2.5). Nevertheless, this points towards fitness and maintenance dynamics of pKJK5::Cas being dependent on their bacterial host, and could lead to failure of target plasmid removal in species which struggle to maintain pKJK5::Cas. This plasmid maintenance is further investigated for different soil isolates in Chapter 5.

Further to possible fitness costs arising from pKJK5::Cas maintenance, protection from plasmid uptake may also be impacted by the presence of off-target hits in some species' genomes: To test sgRNA and Cas9 targeting efficiency, I generated a series of different sgRNAs targeting different areas of *aacC1* (Figure 2.2A) which were randomly chosen out of a total of 59 different possible guides targeting this sequence, with a range of off-target hits predicted by CRISPOR ((Haeussler *et al.*, 2016); Table 2.2). Off-target hits seem to be an important determinant of target plasmid removal efficiency: Compared with other sgRNAs targeting *aacC1*, both guides with predicted off-target activity in *P.*

aeruginosa PA01 (164/rev & 341/rev) resulted in ~1-2 orders of magnitude higher transformation efficiency with the target plasmid ($p<0.05$; Figure 2.2). While both these sgRNAs also target *aacC1* on the reverse rather than forward strand, strand directionality has been shown to have no effect on CRISPR targeting efficiency (Guo *et al.*, 2018). This indicates that utility of pKJK5::Cas may be species-specific, where plasmid removal from species containing similar sequences to the target in their chromosome could be less effective.

Beyond this, other host-specific factors may also impact plasmid targeting by pKJK5::Cas: For instance, *Enterococcus faecalis*'s native CRISPR system was reactivated to target resident plasmids, but maintenance of plasmids remained possible despite a fitness cost to their host (Hullahalli *et al.*, 2018). Additionally, some bacteria's immune mechanisms may prohibit plasmid entry (Westra *et al.*, 2012) and resident prophage or plasmids could encode anti-CRISPRs which might attenuate the actions of such a treatment (Pawluk *et al.*, 2018).

It is promising that pKJK5::Cas can block uptake of plasmids carrying AMR genes in a range of different bacterial species, despite efficacy probably varying between species. Multiple studies investigate plasmid targeting with similar engineered plasmids, phage, or phagemids, but also go beyond prevention of plasmid uptake by analysing how well such a CRISPR treatment can cure resident AMR plasmids (Bikard *et al.*, 2014; Yosef *et al.*, 2015; Dong *et al.*, 2019). Both entry exclusion and curing of resident plasmids are likely needed for a CRISPR treatment to be effective to reduce AMR gene prevalence in natural populations. Therefore, in the next chapter, I investigate the ability of pKJK5::Cas to conjugatively remove AMR plasmids from target bacteria.

Conclusion

In this chapter, I aimed to develop a CRISPR-Cas9 plasmid which can protect a range of bacterial isolates from AMR plasmid uptake.

I generated broad host-range conjugative plasmid pKJK5::Cas, which was able to block AMR plasmid uptake in a range of *Pseudomonas* species and *Escherichia* isolates without the need for re-engineering. These experiments showed that CRISPR-Cas9 mediated plasmid removal was possible in multiple species using the same expression plasmid, and is thus a promising approach for protecting mixed bacterial communities from AMR plasmid uptake.

1 Effectivity of this approach may vary between species dependent on sequence
2 similarity between genome sequences and the target gene, and on host-
3 dependent fitness costs of pKJK5::Cas. Further experiments are needed to
4 consider how pKJK5::Cas may be feasibly applied to remove resident plasmids.

Chapter 3: pKJK5::Cas-dependent properties determine efficiency of target plasmid removal

Abstract

Antimicrobial Resistance (AMR) is on the rise, a problem exacerbated by AMR genes spreading between different bacterial species via plasmids. To curb the spread of resistance, CRISPR-Cas9 has found application in removal of AMR plasmids. In the previous chapter, I designed the recombinant conjugative plasmid pKJK5::Cas and used this as a barrier to AMR plasmid uptake in a range of different bacterial species. Here, I utilised its conjugative ability to remove resident AMR plasmids from a target strain.

I discovered that two variables, conjugation efficiency and CRISPR targeting efficiency, affected target plasmid removal and that an optimal conjugation efficiency was most important for effective target plasmid clearance. Additionally, experiments revealed that maintenance of pKJK5::Cas together with a target plasmid was costly, even in the absence of CRISPR targeting.

This work provides a solid basis for the use of pKJK5::Cas as a conjugative tool to remove AMR plasmids from bacterial communities. Further work is needed to assess how target plasmid properties affect their removal by pKJK5::Cas, and to assess how pKJK5::Cas spreads through bacterial communities. These questions are addressed in the following chapters.

Introduction

CRISPR-Cas delivery tools are a novel means of resensitising bacteria to antibiotics by removal of antimicrobial resistance (AMR) plasmids (reviewed in (Vrancianu *et al.*, 2020)). Due to the relative ease by which conjugation could reach bacteria embedded in natural communities, those CRISPR delivery tools which deliver genes necessary for target plasmid removal on conjugative or mobilizable plasmids are particularly promising, but a low efficiency of conjugation often led to only modest target plasmid removal (Ruotsalainen *et al.*, 2019; Valderrama *et al.*, 2019; Wang *et al.*, 2019; Wongpayak *et al.*, 2021).

One study directly compared a *trans*-acting conjugative plasmid system (conjugation and CRISPR genes encoded on separate plasmids) with a *cis*-acting system (conjugation and CRISPR genes encoded on the same plasmid) and

found that target plasmid removal is more effective in the latter case, primarily due to increased conjugation (Hamilton *et al.*, 2019).

In the previous chapter, I inserted *cas9, sgRNA,* and *gfp* onto IncP-1ε plasmid pKJK5. This broad host-range conjugative plasmid can conjugate to bacteria across the prokaryotic tree of life (Klümper *et al.*, 2015). The engineered plasmid pKJK5::Cas can block transformation of its host strain with targeted gentamicin plasmid pHERD30T, and is effective in *Escherichia coli*, *Pseudomonas aeruginosa, Pseudomonas fluorescens,* and in a range of coliform environmental isolates (Chapter 2).

In this chapter, I test how effective target plasmid removal is from a recipient *E. coli* strain when pKJK5::Cas is conjugatively delivered. I test the impact of two key variables – conjugation efficiency and CRISPR targeting efficiency – on target plasmid removal. For this study, I define conjugation efficiency as the fraction of transconjugants within the recipient population at the end of each experiment. This descriptor therefore explains the ultimate outcome of plasmid transfer, taking into account conjugation, plasmid loss, and vertical transconjugant expansion. However, conjugation efficiency does not provide temporal information and therefore is not equivalent to conjugation rate, for instance classically described as the conjugational transfer rate parameter γ (Stewart and Levin, 1977). Similarly, I define CRISPR targeting efficiency as the singular outcome of stringency in CRISPR-Cas9 plasmid removal or target escape once pKJK5::Cas has entered the target cell. Therefore, this variable takes into account sgRNA targeting stringency, cleavage efficiency, and plasmid escape by mutation or DNA repair.

Lastly, I analyse transfer dynamics of non-targeting pKJK5::Cas controls and uncover costs to the co-existence of pKJK5::Cas and the target plasmid in the absence of CRISPR-Cas targeting.

Methods

Strains, plasmids, and growth conditions

All bacterial strains and plasmids used throughout the thesis are listed in Thesis Supplement Table S1-2. Unless otherwise specified, all strains were grown in LB at 37°C and 180 rpm. For selective plating and where necessary in liquid culture,

antibiotics were added at the following concentrations: Gm – 50 µg/mL Gentamicin; Km – 50 µg/mL Kanamycin; Tc – 12 µg/mL Tetracycline.

Where *E. coli* MFDpir was used, cultures were supplemented with 300mM DAP to ensure growth of this auxotrophic strain. By omitting DAP, the strain could be selected against.

Unless otherwise stated, all molecular cloning steps were carried out using high-fidelity restriction enzymes (NEB) using commercially competent *E. coli* DH5α cells (NEB) and manufacturer protocols. PCR reactions were carried out using PCRbio Taq master-mix according to manufacturer's instructions.

pKJK5::Cas construction

To construct pKJK5::Cas[aphA99] and [nt2], initially the new specificities (aphA99: AAATGGGCGCGTGATAAGGT; nt2: GTTTTCTGCCTGTCGATCCA) were inserted into pMA-RQ_Cas as described in Chapter 2. Next, the homology arms flanking the CRISPR cassette were exchanged to match *intI1* on pKJK5. The upper and lower homology tracts were amplified using primers uphom_intI1_fw/uphom_intI1_rv or lohom_intI1_fw/lohom_intI1_rv respectively (Table S3) and pKJK5 as a template, and the PCR products were inserted into pMARQ_Cas using HindIII/XhoI or BamHI/EcoRI restriction sites respectively. Subsequently, using HindIII and EcoRI restriction sites, CRISPR cassettes were cut out of pMARQ_Cas[aphA99]/[nt2] and inserted into pDOC, which was used as a template for homologous recombineering (Chapter 2) with *intI1* on pKJK5. Recombined pKJK5::Cas[aphA99] and [nt2] and correct insertion of the CRISPR cassette into *intI1* were verified using PCR as described in Chapter 2. Primer intI1_fw was used in a primer pair with either intI1_rv or GFPend_fw, and intI1_rv was also used in a pair with Cas9_bw.

MFDpir +pKJK5::Cas transconjugants were generated by co-incubating *E.* coli DH5α +pKJK5::Cas with MFDpir and selecting for transconjugants in the presence of Tc and 250µg/mL Erythromycin. *K12*+pKJK5::Cas strains were prepared by co-incubation of MFD*pir* + pKJK5::Cas and K12 followed by plating on LB+Tc in the absence of DAP to select against the donor strain.

pHERD99 construction

To construct pHERD99, oligos aphA99PAM_HK_top and aphA99PAM_HK_btm were annealed as described previously (Chapter 2) and inserted into

pHERD30T's multiple cloning site using HindIII and KpnI. This yielded pHERD99, a plasmid 12bp shorter than pHERD30Tand targetable by pKJK5::Cas[aphA99] due to insertion of the aphA99 target sequence.

Mating experiments

Liquid mating

For liquid mating, single colonies of donors (*E. coli* DH5α + pKJK5::Cas[aacC1-72]/[nt]) and recipients (*E. coli* K12::mCherry + pHERD30T) were suspended and grown overnight in 5 mL each LB + Tc or LB + Gm respectively. Cultures were washed twice with 0.9% (w/v) NaCl, 50 µL of donors and recipients were co-incubated in fresh 5 mL LB microcosms in 6 replicates, and incubated overnight at 37°C, 50rpm. The next day, all cultures were frozen in 20% (w/v) Glycerol at -80°C and plated onto various selective media: LB without selection allowed donors and recipients to grow, LB+Km selected for all recipients, LB+Km+Tc selected for recipients which had taken up pKJK5::Cas (transconjugants), LB+Km+Gm selected for recipients with target plasmid pHERD30T, and LB+Km+Gm+Tc selected for recipients containing both plasmids. Additional controls (not shown) included donor-only and recipient-only controls, and yielded colonies as expected. Enumerating colonies on selective plates allowed to calculate proportions of recipients carrying various plasmids.

Filter mating

For the filter mating experiments, 6-12 colonies each of donors (*E. coli* K12 + pKJK5::Cas[aacC1-72] / [nt] for the first experiment; *E. coli* DH5α + pKJK5::Cas[aphA99] / [nt2] for the second experiment) and recipients (*E. coli* K12 + pHERD30T / pHERD99) were suspended in 15mL LB+Tc or LB+Gm respectively and grown overnight. Then, all strains were washed twice with 15mL 0.9% (w/v) NaCl and resuspended to OD600=0.5.

Filter matings were carried out using a 12-stream Millipore vacuum pump, sterilised with 70% Ethanol and UV light before and after each filter mating and assembled in a Cat2 biosafety cabinet. For each mating, a 0.22µM glass microfibre filter (Whatman) was placed onto a vacuum pump position, dampened with 200µL sterile 0.9% NaCl, and topped with a 0.22µM cyclopore membrane (Whatman). Fully assembled filter positions were equilibrated by pumping through 2mL 0.9% NaCl by applying a vacuum. Next, 1 mL of OD-adjusted

donors, 1 mL of OD-adjusted recipients, and 1 mL of 0.9% NaCl were added to each sample position and pumped through by applying a vacuum. Matings were carried out with 12 (first experiment) or 6 biological replicates (second experiment). Cyclopore membranes were placed onto an LB plate (cell-side-up) and incubated at 37°C overnight, after which cells were recovered by placing each filter into 3mL of 0.9% NaCl and vortexing for 15 seconds. This cell suspension was then frozen in 20% glycerol at -80°C and plated onto selective plates as for the liquid mating experiment above. Additional controls (not shown) included donor-only and recipient-only controls, and yielded colonies as expected.

For both mating methods, only an endpoint measure of donors, recipients, and their subpopulations was taken by selective plating. Bacterial growth was not tracked during the experiments.

Growth curves

Frozen samples from the experiments above were thawed and re-plated onto selective media to extract recipients with various plasmid content (LB+Km+Tc: Transconjugants. LB+Km+Tc+Gm: Recipients containing both plasmids). For recipients containing pHERD30T, T0 recipients (those strains used to start the experiment) were plated onto LB+Gm. One colony of each recipient from each experiment was picked of each biological replicate (n=6 for liquid mating and filter mating (low efficiency), n=12 for the first filter mating). Four replicate colonies were picked for T0 recipients, donor strains, and of the empty *E. coli* K12::mCherry recipient. Some samples did not yield any colonies on certain selective media when replating and were omitted from the downstream growth curve analysis. Consequently, the filter-mating experiment only had 4 replicates for recipients + pKJK5::Cas[nt], and 5 replicates for recipients + pKJK5::Cas[nt] + pHERD30T.

Colonies were suspended in 200 µL LB broth in a 96-well plate, supplemented with appropriate antibiotics and grown overnight statically at 37°C. During growth in all 96-well plates, a single row or column around each edge was left with blank media to allow for a barrier to evaporation.

After overnight growth, cultures were thoroughly resuspended and 20 µL of each culture was transferred into a fresh 96-well plate filled with 180 µL LB. The freshly inoculated 96-well plates were placed into an absorbance reader (Biotek Synergy

2) and run on a cycle which incubated the sample at 37°C, slowly shook the plate, and read the optical density (OD600) every 10 minutes for 16 hours. Analysing OD readings of blank wells showed that out of >180 blank controls, only two were contaminated.

Finally, growth parameters were modelled for each replicate individually. To extract growth rates, a rolling regression was performed on log-transformed OD readings by calculating the linear growth rate for each interval spanning 7 measurements (70 minutes) throughout the entire measurement time and selecting the maximum linear growth rate. To estimate lag time, the corresponding time point of the maximum growth rate interval was selected. Finally, endpoint OD was estimated by calculating the median of the 10 highest measurements of OD600 throughout the time-series.

Statistical analyses

Data processing, data visualisation, and statistical analyses were carried out using R software version 4.1.0 and RStudio version 1.4.1717 with the following packages: tidyverse version 1.3.1, arm version 1.11-2, MuMIn version 1.43.17, bbmle version 1.0.24, ggpubr version 0.4.0, lemon version 0.4.5, purrr version 0.3.4, lubridate version 1.7.10, lme4 version 1.1-27.1, LMERConvenience Functions version 3.0

For all models, assumptions were tested and found to be upheld. Other model types, link functions, and model structures were tested and the overall best models were chosen.

Target plasmid retention (Figure 3.1A) A Gaussian Generalised Linear Model (GLM) was fitted with an identity link function; explaining log-transformed pHERD30T/99 proportion with Experiment, pKJK5::Cas target, and their interaction as explanatory variables. Datapoints with infinite, NA, or values of 0 were removed; to compare between categories, Tukey's post-hoc honest significance differences were carried out, the relevant results of which are listed in the figure legend. F=127.5; d.f.=5 & 39; $p<2.2 \times 10^{-16}$; adjusted R^2=0.935

Conjugation efficiency (Figure 3.1B) A Gaussian GLM was fitted with an identity link function; explaining log-transformed transconjugant proportion (conjugation efficiency) with the Experiment, pKJK5::Cas target, and their interaction as explanatory variables. Datapoints with infinite, NA, or values of 0 were removed;

to compare between categories, Tukey's post-hoc honest significance differences were carried out, the relevant results of which are listed in the figure legend. F=14.92; d.f.=5 & 33; $p<1.41\times10^{-7}$; adjusted R^2=0.6469

Correlation of the two (Figure 3.1C) For each pKJK5::Cas variant, a correlation was carried out between proportion of recipients with the target plasmid and proportion of recipients with pKJK5::Cas by fitting a linear model using log-transformed data. Datapoints with infinite, NA, or values of 0 were removed. Targeting: F=22.11; d.f.=1&16; p=0.0002399; R^2=0.5801.

Non-targeting: F=0.2709; d.f.=1&7; p=0.6188; R^2=0.03725.

CRISPR targeting efficiency (Figure 3.2A) A Gaussian GLM was fitted with an identity link function; explaining log-transformed proportion of transconjugants with the target plasmid (proxy for CRISPR targeting efficiency) with Experiment, pKJK5::Cas target, and their interaction as explanatory variables. Datapoints with infinite, NA, or values of 0 were removed; to compare between categories, Tukey's post-hoc honest significance differences were carried out, the relevant results of which are listed in the figure legend. F=146.5; d.f.=5 & 32; $p<2.2\times10^{-16}$; adjusted R^2=0.9516

Correlation with target plasmid retention (Figure 3.2B) For each pKJK5::Cas variant, a correlation was carried out between proportion of recipients with the target plasmid and proportion of transconjugants with the target plasmid by fitting a linear model using log-transformed data. Datapoints with infinite, NA, or values of 0 were removed.

Targeting: F=114.3; d.f.=1&16; $p=1.076\times10^{-8}$; R^2=0.8772.

Non-targeting: F=0.6959; d.f.=1&6; p=0.4361; R^2=0.1039.

Recipient prevalence (Figure 3.3) A Gaussian GLM was fitted with an identity link function; explaining log-transformed overall proportion of recipients with Experiment, pKJK5::Cas target, and their interaction as explanatory variables. Datapoints with infinite, NA, or values of 0 were removed; to compare between categories, Tukey's post-hoc honest significance differences were carried out, the relevant results of which are listed in the figure legend. F=149.8; d.f.=5 & 39; $p<2.2\times10^{-16}$; adjusted R^2=0.9442

Recipient growth (Figure 3.4) GLMs (Gaussian, identity link function) were fitted to these data. The 3 models described each growth parameter (culture capacity,

lag time, growth rate (not shown)) as a function of Experiment, pKJK5::Cas target, pHERD30T presence and their two- and three-way interactions. Model statistics are summarised below. Statistical differences between individual treatments were assessed by a Tukey's post-hoc test; see Figure legend for details.

Culture capacity: $F=8.288$; d.f.=15 & 80; $p=5.568 \times 10^{-11}$; adjusted $R^2=0.5351$

Lag time: $F=14.2$; d.f.=15 & 80; $p=2.2 \times 10^{-16}$; adjusted $R^2=0.6757$

Growth Rate (not shown): $F=1.006$; d.f.=15 & 80; $p=0.4576$; adjusted $R^2=0.0009708$

Recipient prevalence correlations (Figure 3.5) For each panel, a correlation was carried out between recipient prevalence and proportion of recipients with both plasmids by fitting a linear model using log-transformed data. Datapoints with infinite, NA, or values of 0 were removed.

Liquid mating: $F=153.2$; d.f.=1 & 22; $p=2.185 \times 10^{-11}$; $R^2=0.8744$

Filter mating: $F=405.8$; d.f.=1 & 26; $p<2.2 \times 10^{-16}$; $R^2=0.9398$

Filter mating (low efficiency): $F=32.11$; d.f.=1 & 22; $p=7.565 \times 10^{-8}$; $R^2=0.7394$

Donor growth correlations (Figure 3.S2) For each panel, a correlation was carried out between recipient prevalence and each growth metric by fitting a linear model using log-transformed means.

Non-targeting; lag time: $F=0.6347$; d.f.=1 & 1;$p=0.5717$; $R^2=0.3883$

Targeting; lag time: $F=1$; d.f.=1 & 1; $p=0.5$; $R^2=0.5$

Non-targeting; culture capacity: $F=1.327$; d.f.=1 & 1; $p=0.4551$; $R^2=0.5702$

Targeting; culture capacity: $F=312.3$; d.f.=1 & 1; $p=0.03598$; $R^2=0.9968$

Non-targeting; growth rate: $F=5.084$; d.f.=1 & 1; $p=0.2657$; $R^2=0.8356$

Targeting; growth rate: $F=0.5919$; d.f.=1 & 1; $p=0.5825$; $R^2=0.3718$

Results

In this chapter, I explored pKJK5::Cas's ability to conjugatively remove targeted AMR plasmids from recipient bacterial strains. I hypothesised that the efficiency of target plasmid removal would depend on conjugation efficiency and CRISPR targeting efficiency. Both variables are defined as singular outcomes and break down the process of conjugative AMR plasmid removal into two distinct steps;

delivery of pKJK5::Cas to target bacteria (conjugation efficiency) and subsequent removal of the target plasmid (CRISPR targeting efficiency). Conjugation efficiency could easily be measured by determining the proportion of recipients which formed transconjugants. To find a proxy for CRISPR targeting efficiency, I measured the proportion of transconjugants which still contained a target plasmid at the end of each experiment. If this proportion is low, there is little escape from CRISPR-Cas9 targeting, and therefore CRISPR targeting efficiency is high.

To test how these variables determine target plasmid removal, I carried out a series of three mating experiments where a donor *E. coli* strain delivers pKJK5::Cas to *E. coli* recipients carrying target plasmid pHERD30T or pHERD99 under different conditions (experimental design overview in Figure 3.S1). In the first experiment, donors and recipients were co-incubated in liquid medium ("Liquid Mating"). The second experiment utilised filter mating for higher predicted conjugation efficiency ((Bradley, 1983); "Filter Mating"). These experiments were both carried out using pKJK5::Cas[aacC1-72], which carried a guide that targets plasmid pHERD30T with high efficiency (Chapter 2). Its corresponding non-targeting control is pKJK5::Cas[nt] and targets a random nucleotide sequence not present in the study system (Figure 3.S1A-B). For the third experiment, filter mating was carried out using pKJK5::Cas[aphA99]. Initially, pKJK5::Cas[aphA99] was designed to target kanamycin resistance plasmids, but here it was used as a low-efficiency means of targeting pHERD99 in its multiple cloning site (comparable to targeting of pHERD30T within and outside *aacC1* in Chapter 2). The corresponding non-targeting control plasmid, pKJK5::Cas[nt2], is targeted towards a different random nucleotide sequence not present in the study system (Figure 3.S1C). With this set-up, the impact of conjugation efficiency could be assessed when comparing experiments 1 and 2, where CRISPR targeting efficiency remained constant. The impact of CRISPR targeting efficiency could be assessed when comparing experiments 2 and 3, where conjugation efficiency remained constant.

Target plasmid removal was dependent on conjugation efficiency

First, I aimed to determine to which extent target plasmids were removed from recipients. Selective plating revealed that the proportion of recipients carrying the target plasmid varied after each experiment (Figure 3.1A). In both filter mating experiments, target plasmid prevalence was reduced significantly by ~1-3 orders

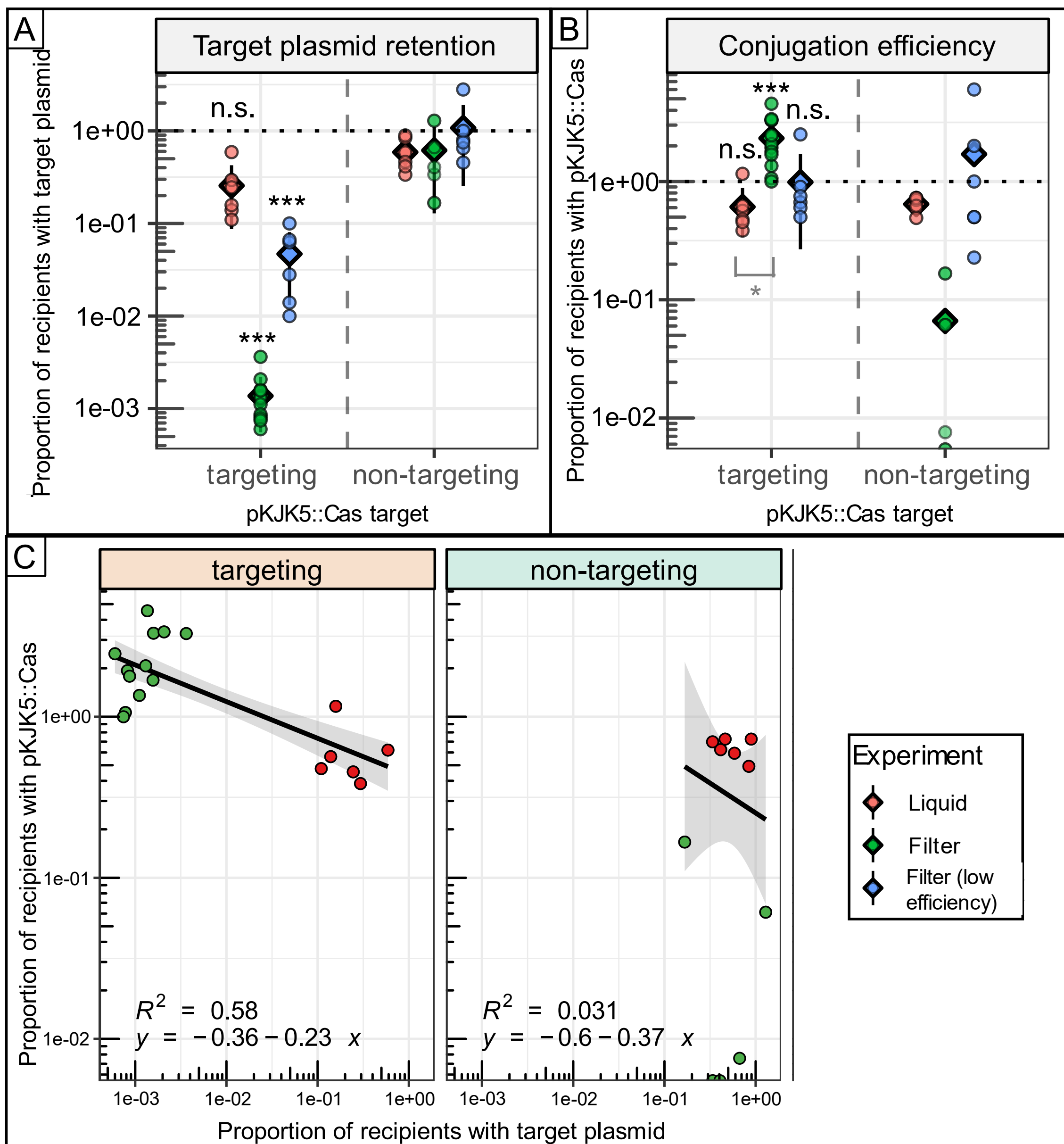


Figure 3.1: **Target plasmid removal is dependent on conjugation efficiency.** A-B: Means (diamonds) and standard deviation (lines) of various colony proportions after three different mating experiments (circles; n=6-12) using pKJK5::Cas[aacC1-72]/[aphA99] (targeting) or pKJK5:: Cas[nt]/[nt2] (non-targeting) in donors and pHERD30T/pHERD99 in recipients. The dotted line indicates a relative proportion of 1. **A: Targeting versions of pKJK5::Cas reduce the proportion of recipients retaining the target plasmid. B: Conjugation efficiency of pKJK5::Cas varies throughout experiments**. Black significance identifiers indicate significant differences from corresponding non-targeting treatments; grey brackets indicate additional relevant comparisons. *p<0.05, **p<0.01, ***p<0.001, n.s.- non-significant. as revealed by Tukey's HSD after fitting GLMs. **C: Plasmid removal efficiency is dependent on conjugation efficiency.** Correlation of data in A and B for experiments with similar CRISPR-Cas9 targeting efficiency (using pKJK5::Cas[aacC1-72]). For targeting guides, higher conjugation rates are associated with lower target plasmid proportions (p<0.001). This association is non-significant for non-targeting guides (p=0.62). Statistical details in methods.

1 of magnitude compared to the non-targeting controls (p<0.001; see Methods for
2 details of statistical models). In contrast, plasmid removal was modest during

liquid mating and did not reach statistical significance (~25% of recipients retained the target plasmid, compared with ~59% for the non-targeting control; $p=0.2$).

As predicted, transconjugant proportions, which indicate conjugation efficiency, were highest during filter mating (Figure 3.1B), with ~100% of recipients receiving targeting pKJK5::Cas compared to only ~60% during liquid mating ($p<0.05$ when comparing liquid and filter experiments). Unexpectedly, the transconjugant proportion of the non-targeting controls was highly variable and reached as low as 1%; this is discussed later.

To determine the significance of conjugation efficiency to plasmid removal, I correlated these two metrics in Figure 3.1C for experiments using the same sgRNA target sequence (liquid, filter). When pKJK5::Cas carried the targeting sgRNA guide, a higher conjugation efficiency was significantly associated with a lower proportion of recipients carrying target plasmid pHERD30T ($p<0.001$). This relationship was not upheld for the non-targeting controls ($p=0.62$). While investigating this relationship for the filter mating experiment in isolation led to a qualitatively opposite relationship, these subtle within-experiment differences in conjugation efficiency are unreliable for these data, as comparing colony counts on plates selecting for recipients and plates selecting for transconjugants led to numeric conjugation efficiencies exceeding 100% in nearly all filter mating samples, which is not biologically possible. Instead, it is therefore more robust to carry out between-experiment comparisons with a larger range in conjugation efficiency. This experimental shortcoming is further examined in the General Discussion.

Together, these data show that the target plasmid pHERD30T/pHERD99 could be removed by conjugatively applying pKJK5::Cas, and that removal efficiency was dependent on conjugation efficiency.

Target plasmid removal was dependent on CRISPR targeting efficiency

Next, I hypothesised that CRISPR targeting efficiency also influenced target plasmid removal. As a proxy of CRISPR targeting efficiency, I calculated the proportion of transconjugants which retained the target plasmid throughout the experiment (Figure 3.2A). Where pKJK5::Cas[aacC1-72] was used, this proportion remained very low during both experimental setups (~$2\text{-}4 \times 10^{-4}$), which

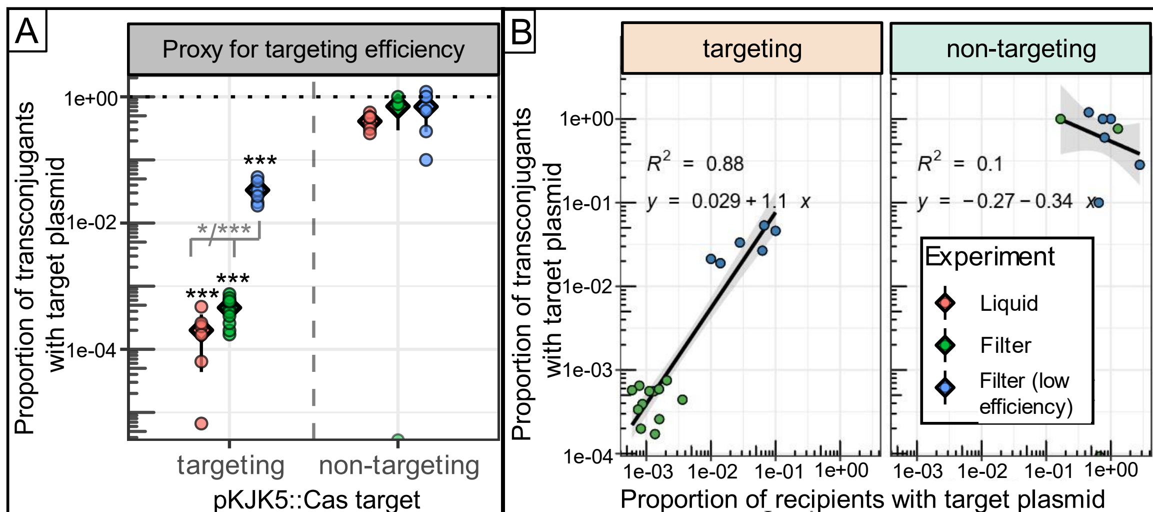


Figure 3.2: **Target plasmid removal is dependent on CRISPR targeting efficiency.**
A Transconjugants with target plasmids as a proxy for CRISPR targeting efficiency: Means (diamonds) and standard deviation (lines) of the proportions of transconjugants retaining the target plasmid after three different mating experiments (circles; n=6-12) using pKJK5::Cas[aacC1-72]/[aphA99] (targeting) or pKJK5::Cas[nt]/[nt2] (non-targeting) in donors and pHERD30T/pHERD99 in recipients. The dotted line indicates 100%. A higher proportion indicates lower CRISPR targeting efficiency. *$p<0.05$, **$p<0.01$, ***$p<0.001$, n.s.- non-significantly different from corresponding non-targeting treatments, as revealed by Tukey's HSD after fitting a GLM. **B Correlation with target plasmid removal.** Proportion of transconjugants which carry the target plasmid (targeting efficiency) correlated with proportion of all recipients, including transconjugants, with target plasmids (removal efficiency; Figure 3.1A) for filter matings using pKJK5::Cas[aacC1-72] or pKJK5::Cas[aphA99]. Higher targeting efficiency (low proportion of pHERD30T+ transconjugants) is associated with lower target plasmid carriage ($p<0.001$). This association is non-significant for non-targeting guides ($p=0.44$). See Methods for details of all statistical models.

indicates that only in ~1 in 5000 cases CRISPR targeting of the plasmid failed. On the other hand, where pKJK5::Cas[aphA99] was used, the pHERD99+ proportion of transconjugants was significantly higher (~3.3×10^{-2}; $p<0.05$) which reflects a lower efficiency of CRISPR targeting (targeting failed in ~1 in 30 cases). This difference in CRISPR targeting efficiency is likely due to the varying location of CRISPR-Cas9 target on each target plasmid (within antibiotic resistance gene vs within multiple cloning site), presumably leading to alternating frequencies of plasmid escape from CRISPR-Cas9 targeting by point mutation (Chapter 2).

Correlating this metric with the proportion of recipients carrying target plasmids for experiments with the same conjugation efficiency (filter, filter (low efficiency)) confirmed that there was a significant association between these variables for

targeting treatments (Figure 3.2B; $p<0.001$) and showed that more efficient CRISPR targeting correlated with lower proportions of recipients with target plasmids.

Non-targeting controls varied in conjugation efficiency and recipient density

The non-targeting control pKJK5::Cas[nt] had a significantly lower proportion of transconjugants than its corresponding targeting pKJK5::Cas[aacC1-72] during filter mating (>1 order of magnitude; $p<0.001$). The same was not true when using pKJK5::Cas[nt2] or for pKJK5::Cas[nt] during a liquid mating experimental setup (Figure 3.1B). As conjugation rates can depend on relative donor and recipient frequencies, I investigated the prevalence of donors and recipients after each experiment. Interestingly, the recipient prevalence varied for each experiment and treatment (Figure 3.3). Most drastically, recipient proportion of the non-targeting control dropped to a miniscule 6×10^{-6} during filter mating, and was partially restored to ~3% when pKJK5::Cas[nt2] was used instead.

Overall, this shows that unexpected dynamics governed transfer of non-targeting pKJK5::Cas. This coincided with unexpected overall recipient prevalence in the same experiments.

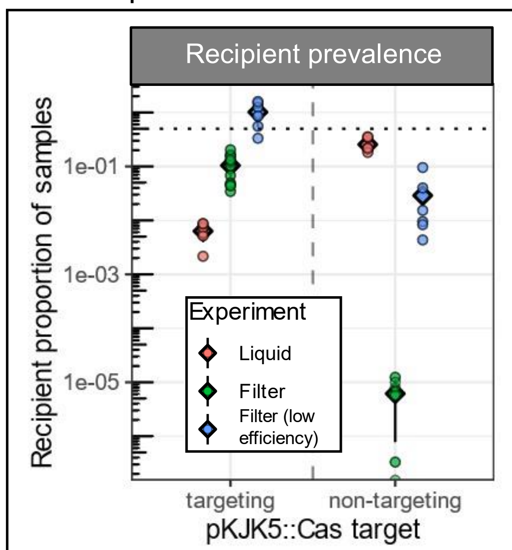


Figure 3.3: **Recipient prevalence after mating experiments.** Means (diamonds) and standard deviation (lines) of the proportion of recipients out of overall bacteria for each treatment. The dotted line indicates 50% (starting proportion). All relevant categories are significantly different from each other (**/***) **$p<0.01$, ***$p<0.001$ as revealed by Tukey's HSD after fitting a GLM. $F=149.8$; d.f.=5&39; $p<2.2 \times 10^{-16}$; adjusted $R^2=0.9442$.

Maintenance of both plasmids is costly and led to low recipient prevalence

A possible explanation for the observed differences in recipient prevalence is a variation of donor or recipient growth rates. I therefore assessed culture capacity, lag time, and growth rate of each donor strain, but no meaningful correlation with recipient prevalence was detected (Figure 3.S2). Therefore, I expanded the

growth analysis to recipients with all possible plasmid contents arising throughout the experiments. Recipients carrying any combination of plasmids had a generally lower culture capacity and higher lag time than plasmid-free recipients ($p<0.05$ for most recipients isolated from the "Filter" experiment, and some recipients isolated from other experiments; see Figure 3.4 for full breakdown). Furthermore, the lag time of recipients containing pKJK5::Cas in addition to the target plasmid was higher than the lag time of recipients containing pKJK5::Cas only – intriguingly, this was especially the case for recipients isolated from experiments carried out on filters ($p<0.05$ for both filter mating experiments; see Figure 3.4 for full breakdown). Growth rates had a high standard error across all samples and did not vary significantly (not shown).

Throughout these experiments, non-targeting controls had higher proportions of recipients which carry both plasmids than their targeting counterparts (Figure 3.2A), which led to more incidence of costly plasmid co-maintenance in non-

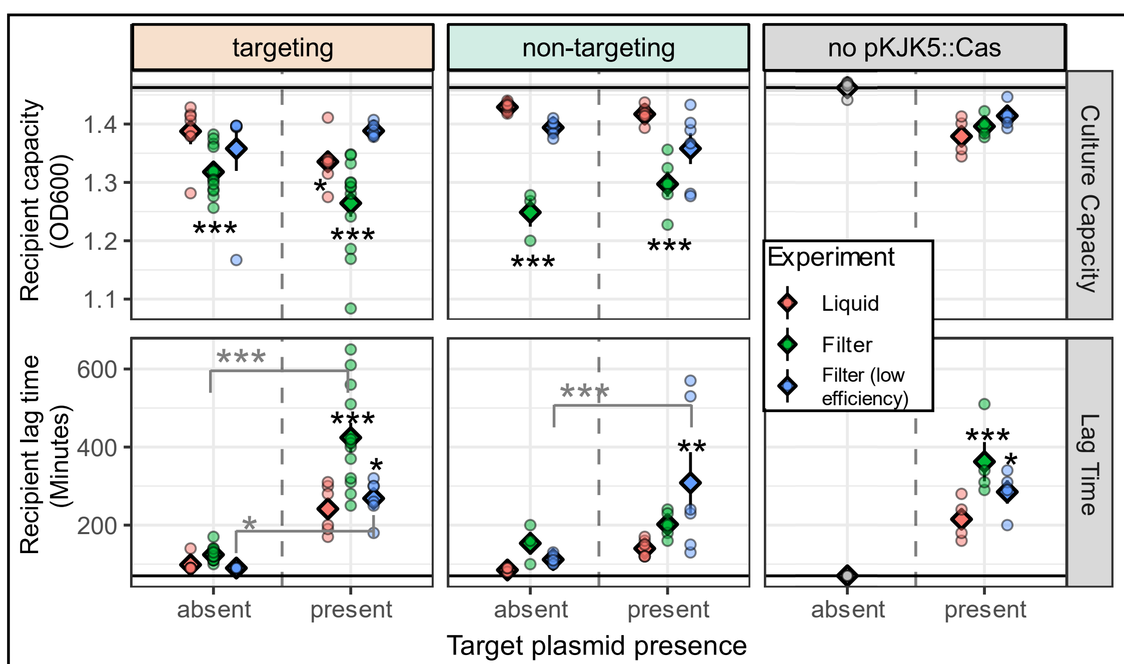


Figure 3.4: **Plasmid maintenance is costly.** Growth metrics of all recipients, including transconjugants, with various plasmid content isolated after or before all three experiments (red, green, blue) in comparison with recipients with no resident plasmids (not from experiments; grey). Circles show individual data points, diamonds + bars show means ± standard error. For comparison across all data, black horizontal bar and grey boxes show mean ± standard error of the corresponding growth metric of recipients without plasmids. Asterisks highlight treatments which are significantly different from recipients without plasmids. Grey brackets highlight treatments which are significantly different when the target plasmid is present or absent respectively. ***$p<0.001$, **$p<0.01$, *$p<0.05$, as assessed by Tukey's post-hoc test after fitting GLMs;

1 targeting controls. I hypothesised that this might be an explanation for the
2 unexpected recipient prevalence and transconjugant proportions.

3 Therefore, I visualised the relationship between these variables. This showed that
4 recipient prevalence was significantly associated with proportion of recipients
5 carrying both plasmids in each experiment ($p<0.001$; Figure 3.5). Interestingly,
6 this relationship was dependent on experimental background and during liquid
7 mating, high proportions of recipients with both plasmids were associated with
8 high recipient prevalence, while the opposite was true for both filter mating
9 experiments.

10 In summary, co-maintenance of pKJK5::Cas and its target plasmid led to a growth
11 detriment in the recipient strain, and these dynamics became clear when matings
12 were carried out on filters. Plasmid co-maintenance primarily occurred using the
13 non-targeting control, which could be the reason why some non-targeting controls
14 had extremely low recipient prevalence.

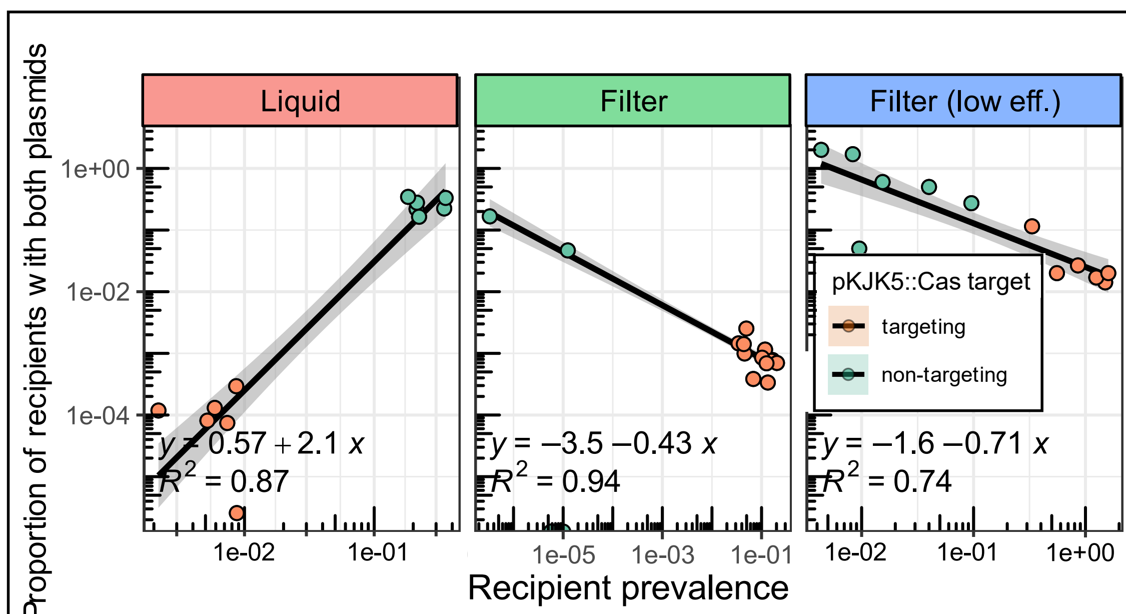


Figure 3.5: **Recipient prevalence is associated with proportion of recipients containing both plasmids**, but this relationship is dependent on experimental background. Correlation of transconjugant+pHERD30T proportion and recipient prevalence for all three conjugation experiments. All correlations are significant; $p<0.001$. See Methods for model details.

15 ## Discussion

16 In this chapter, I assayed the efficiency of target plasmid removal from a bacterial
17 strain through conjugation of pKJK5::Cas from a donor strain. I found that target
18 plasmid removal was dependent on both the conjugation efficiency and on the
19 efficiency of Cas9 targeting. As I chose the endpoint measures of conjugation

efficiency and CRISPR targeting efficiency rather than tracking donor and recipient growth and plasmid dynamics throughout the experiment, it is unclear which underpinning dynamics contribute the most to the measured outcome. For instance, an increased conjugation efficiency during filter mating may be due to an increase in mating pair formation, or an increase in vertical growth of early transconjugants. Nevertheless, the key metrics of conjugation efficiency and CRISPR targeting efficiency allowed me to break the process of AMR plasmid removal down into two stages: initially, how well does pKJK5::Cas transfer, and secondly, how well does it remove target plasmids once in target cells?

Overall, it seems that conjugation efficiency is the more important factor: the combination of low-efficiency CRISPR targeting and high-efficiency conjugation (filter mating (low efficiency)) led to ~10 times as much target plasmid removal as high-efficiency CRISPR targeting and low-efficiency conjugation (liquid mating; Figure 3.1A). Therefore, optimising conjugation and delivery of pKJK5::Cas will be key to clearing plasmids from natural populations. This adds to a bulk of past research which already identified conjugation efficiency as a crucial factor (e.g. (Hamilton *et al.*, 2019; Wongpayak *et al.*, 2021)), and which proposed low conjugation efficiency as a key reason for failure when applying CRISPR delivery tools in animal models (Rodrigues *et al.*, 2019) or to complex communities (Ruotsalainen *et al.*, 2019). Interestingly, my data indicate that even relatively modest optimisations of conjugation efficiency will lead to considerable improvement of target plasmid removal, as an increase of recipients forming transconjugants from ~60% to 100% led to a large drop in recipients retaining the target plasmid from ~25% to 0.1%.

Previous work which removed *mcr-1* plasmids from *E. coli* showed that removal of plasmids is more effective when replicon-associated or toxin/antitoxin system genes are targeted rather than antibiotic resistance genes (Wang *et al.*, 2019). While this indicates that efficiency of targeting pHERD30T could further be improved by targeting replicon-associated sequences, my data indicate that targeting antibiotic resistance genes provides a clear advantage over targeting non-essential plasmid sequences. Escape mutations that allow pHERD30T to evade CRISPR are expected to occur more readily in non-essential regions of the plasmid (e.g. target sequence on pHERD99), compared to its antibiotic resistance gene which is essential under selective pressure, and is targeted by

pKJK5::Cas[aacC1-72]. This is analogous to bacteriophage mutations to escape from natural CRISPR targeting, the frequency of which is dependent on essentiality of the bacteriophage gene targeted by CRISPR (Watson *et al.*, 2019). Additionally, targeting antibiotic resistance genes has the advantage that multiple plasmids with different AMR genes could be removed using the same CRISPR delivery tool, for instance by targeting conserved sequences in divergent β-lactamase genes (Kim *et al.*, 2015; Ruotsalainen *et al.*, 2019).

Recipient prevalence drastically decreased for non-targeting controls during filter mating due to costs of plasmid co-maintenance (Figure 3.3-3.4). Intriguingly, this was not the case for liquid mating. This suggests that these costs may arise as a result of cell density, cell-to-cell contact, or from being attached to a substrate – all of which are low or absent during liquid mating. For instance, high local cell densities during filter-mating might lead to low local nutrient concentrations, which increases the importance of competition for nutrients between donors and recipients and allows donors to rapidly outcompete recipients struggling to grow. On the other hand, a better-mixed environment during liquid mating ensures higher relative nutrient availability and decreases the importance of moderate differences in bacterial growth. Beyond this, phenomena such as lethal zygosis (where high relative donor frequencies lead to recipient cell death; (Skurray and Reeves, 1973)) may lead to runaway decreases after an initial drop in recipient prevalence under conditions with high conjugation rates (i.e. filter mating), further inflating the importance of moderate growth detriments.

Conjugation efficiency might be linked to recipient prevalence through the growth detriment of co-maintaining plasmids: Rather than being reflective of lower overall conjugation rates, low final transconjugant proportions are probably a result of pKJK5::Cas loss after initial transconjugant generation. However, transconjugant proportions also varied between non-targeting pKJK5::Cas variants (Figure 3.1B), which target different random nucleotide sequences (neither of which are present in the model system). Therefore, this difference in conjugation efficiency suggests unexpected effects due to Cas9 off-target binding. For instance, Cas9 plays a role in gene regulation using a smaller-than-usual CRISPR RNA which has an imperfect match to a chromosomal target (Ratner *et al.*, 2019). Possible off-target hits of each non-targeting sgRNA with PAM in this model system are summarised in Table 3.1.

1 **Table 3.1**: Off-target hits for non-targeting guides: possible off-target hits of each
2 guide + PAM identified by a BLAST homology search. “Genome” indicates
3 genetic compartment targeted. Additional off-target hits on all genomes with <10
4 matching base pairs are not shown. “Matching bases” lists the number of base
5 pairs matched between guide + PAM and target. **E. coli* K12 matches are also
6 present in corresponding areas of the *E. coli* DH5α genome.

Genome	Accession number	Matching bases	Target area	Genetic context
[nt] guide off-target hits (including PAM)				
E. coli K12*	NZ_CP010444.1	12	685180-685191	*yjiM* (putative 2-hydroxyacyl-CoA dehydratase – enzyme used in fermentation)
		11	3202156-3202146	*sixA* (phosphohistidine phosphatase – involved in nitrogen-related phosphotransferase system)
		10	Various	18 different targets, mostly metabolic genes. Two with unknown function, two involved in transcription (*pcnB*, *ydcI*), and *tetB* tetracycline efflux.
pKJK5	AM261282.1	9	31570-31562	*tetA* tetracycline efflux pump.
pHERD30T	EU603326.1	/	/	No hits identified
[nt2] guide off-target hits (including PAM)				
E. coli K12*	NZ_CP010444.1	13	4298110-4298122	*asd* (aspartate synthesis)
		12	882073-882062	*ampD* (peptidoglycan recycling)
		12	3809958-3809969	*fbaA* (fructose metabolism)
		11	313972-313982	*thiC* (phosphomethylpyrimidine synthase)
		11	2069577-2069567	*yciV* (5’-3’ exoribonuclease)
		10	Various	15 different targets, mostly metabolic genes. One gene with unknown function, two efflux pumps (*mdtD, yebQ), gspA* component of Type 2 secretion system, and *rarA* which is involved in replication.
pKJK5	AM261282.1	10	22178-22196	*intI1* (Integrase; inactive on pKJK5 and disrupted by CRISPR cassette)
pHERD30T	EU603326.1	/	/	No hits identified

7

Overall, this comparison shows that both non-targeting guides may bind to separate genetic regions, which could hint towards such non-specific binding playing a role in conjugation rates. These predictions would have to be tested experimentally. To assess why conjugation efficiency of the non-targeting control is low, strains with varying plasmid contents should be directly competed against each other, and a CRISPR-Cas9-free pKJK5 control would indicate whether off-target Cas9 effects play a role.

Conclusion

In this chapter, I assessed removal of a target plasmid from *E. coli* by application of a donor *E. coli* strain containing pKJK5::Cas. Plasmid removal was effective but dependent on several factors. Specifically, I found that CRISPR targeting efficiency and especially conjugation efficiency were key determinants of target plasmid removal efficiency. Intriguingly, non-targeting pKJK5::Cas controls showed lower conjugation efficiency than their targeting counterparts, and non-targeting treatments had a lower overall recipient prevalence. This could be due to fitness costs of maintaining pKJK5::Cas together with the target plasmid, and perhaps due to unexpected off-target effects of Cas9.

Together, these experiments provide a robust basis for application of pKJK5::Cas to remove resident plasmids from target bacteria. Several barriers remain before applying pKJK5::Cas in healthcare or environmental settings: Firstly, the target plasmid used is a synthetic cloning vector. How does pKJK5::Cas fare against more natural target plasmids? Secondly, transfer and maintenance of pKJK5::Cas are expected to be altered in mixed bacterial communities and environmental isolates compared to a standard laboratory *E. coli* strain. How effective is pKJK5::Cas spread through a community made up of natural isolates? These questions are addressed in Chapters 4 and 5 respectively.

1

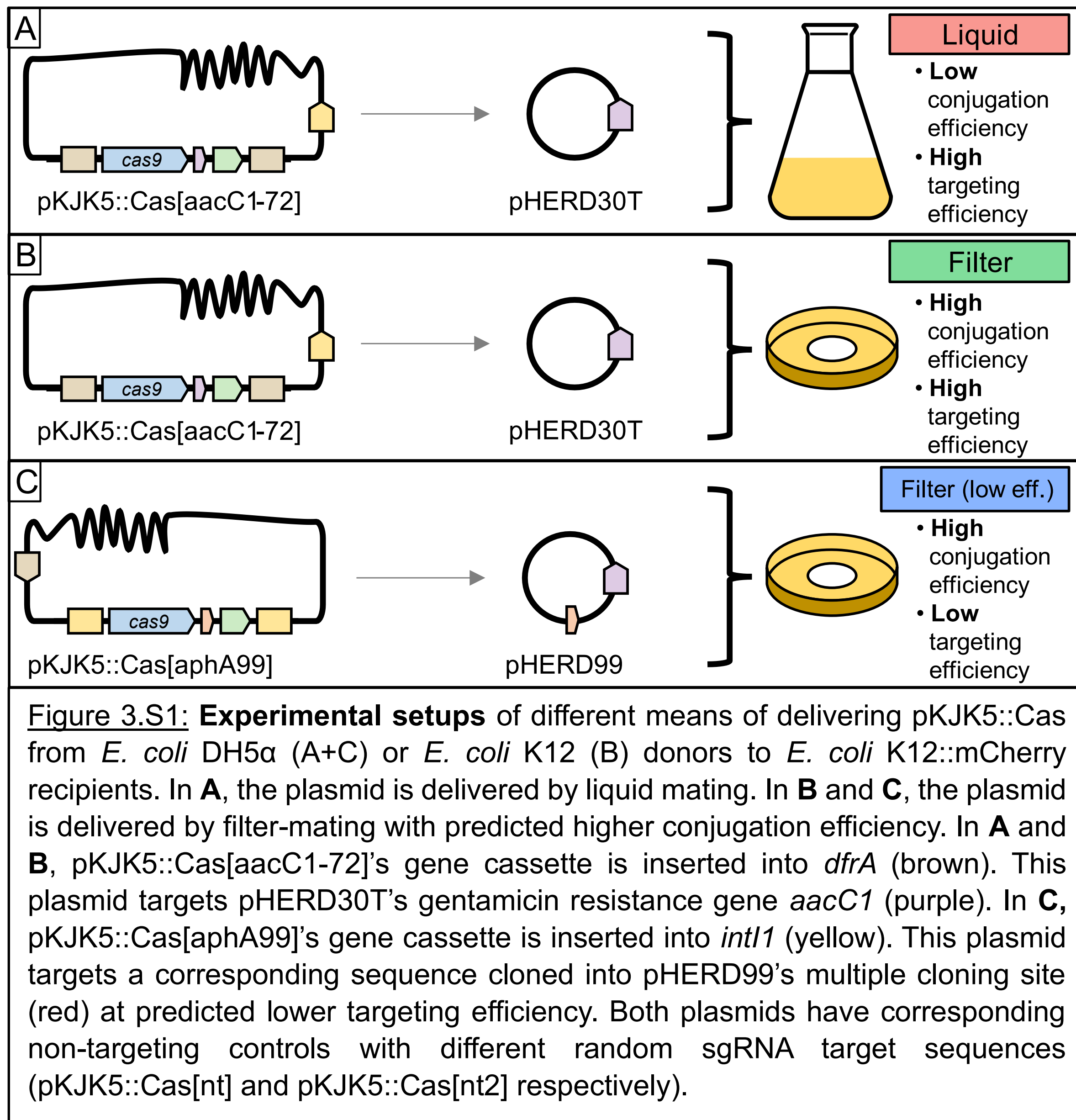
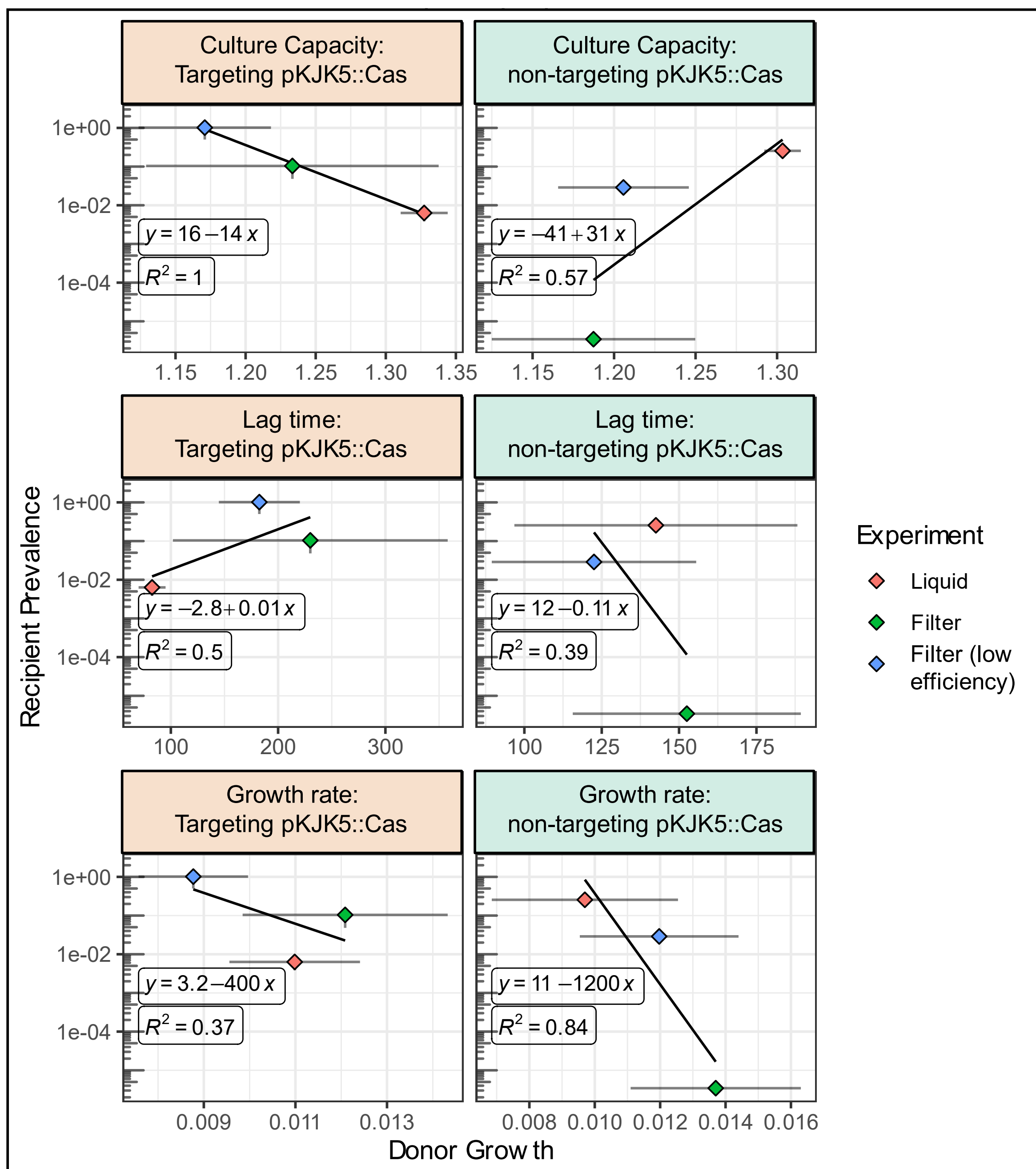


Figure 3.S1: **Experimental setups** of different means of delivering pKJK5::Cas from *E. coli* DH5α (A+C) or *E. coli* K12 (B) donors to *E. coli* K12::mCherry recipients. In **A**, the plasmid is delivered by liquid mating. In **B** and **C**, the plasmid is delivered by filter-mating with predicted higher conjugation efficiency. In **A** and **B**, pKJK5::Cas[aacC1-72]'s gene cassette is inserted into *dfrA* (brown). This plasmid targets pHERD30T's gentamicin resistance gene *aacC1* (purple). In **C,** pKJK5::Cas[aphA99]'s gene cassette is inserted into *intI1* (yellow). This plasmid targets a corresponding sequence cloned into pHERD99's multiple cloning site (red) at predicted lower targeting efficiency. Both plasmids have corresponding non-targeting controls with different random sgRNA target sequences (pKJK5::Cas[nt] and pKJK5::Cas[nt2] respectively).



1 Figure 3.S2: **There is no clear association between recipient prevalence and donor growth metrics.** Growth metrics of donor strains from all experiments were assessed (n=4, see methods for details). Mean±standard deviation of modelled culture capacity, lag time, and growth rate of donors used for each experiment, plotted against mean±standard deviation of recipient prevalence (Figure 3.3). None of the correlations, except culture capacity of targeting pKJK5::Cas (p=0.036) are statistically significant.

Chapter 4: pKJK5::Cas-mediated AMR plasmid removal is dependent on target plasmid properties

Abstract

Plasmids encoding antimicrobial resistance (AMR) genes are a major contributing factor to drug-resistant infections. Such plasmids can be transferred between bacterial species, but technologies based on CRISPR-Cas9 provide a means to stop this transfer.

Past studies have generated effective CRISPR delivery tools which can clear targeted resident plasmids from bacterial species; I developed broad host-range plasmid pKJK5::Cas to achieve this. Generally, removal of synthetic target plasmids is easily achieved. However, it is unclear if CRISPR-Cas9 conjugative delivery can effectively target natural plasmids, which are typically larger than synthetic cloning vectors and often encode multiple AMR genes, stability systems, and other payload genes.

In this chapter, I contrasted removal of a synthetic target plasmid with that of clinical multi-drug resistance plasmid RP4. I identified target plasmid properties which can protect from CRISPR mediated removal, specifically their incompatibility group and toxin-antitoxin system presence. Both factors considerably reduced plasmid removal efficiency by pKJK5::Cas in isolation, and in combination entirely stopped effectivity of this approach. Despite removal not being effective, pKJK5::Cas was used to prevent uptake of RP4 in a natural isolate.

This study identified target plasmid properties which limit the efficacy of this CRISPR delivery tool, and this knowledge may be utilised by employing alternative pKJK5::Cas delivery strategies or by tailoring its use to applications which avoid its shortcomings.

Introduction

AMR of bacterial pathogens is becoming highly problematic to modern healthcare, and AMR-associated infections are predicted to be the leading cause of death within the next few decades (O'Neill, 2016). Thanks to horizontal transfer of antibiotic resistance genes and low concentrations of antibiotics present which are sufficient to select for resistance (e.g. sub-inhibitory concentrations (Murray *et al.*, 2021)), environments such as livestock farms or waste water see frequent

exchange of resistance genes between different bacterial species (United Nations, 2017). In this way, these so-called reservoirs of AMR are hotspots for transfer of resistance genes, primarily driven by plasmids (Partridge *et al.*, 2018). There is clear evidence that pathogenicity and resistance of bacteria arises through horizontal gene transfer in the environment (e.g. of *Pseudomonas aeruginosa;* (Laborda *et al.*, 2021)).

CRISPR-Cas9 may provide a novel avenue to block transfer of resistance plasmids in microbial communities. For example, a *sgRNA* (single guide RNA) - directed nuclease Cas9 (or a closely related enzyme) can be delivered to bacterial cells and communities to specifically remove AMR-carrying plasmids, thereby leading to resensitisation of bacteria to antibiotics (Pursey *et al.*, 2018).

In the previous chapters, I developed pKJK5::Cas, a broad host-range plasmid encoding *cas9* and an AMR gene-targeting *sgRNA*, showed how it can protect host cells from uptake of AMR plasmids (Chapter 2), and that it can conjugatively remove target plasmids from a recipient strain by application of an unrelated donor. The efficiency of target plasmid removal is dependent on pKJK5::Cas targeting efficiency and conjugation efficiency (Chapter 3).

Beyond this, target plasmid properties and which cargo genes they carry are hypothesised to have an impact on their removal by CRISPR delivery tools (Lauritsen *et al.*, 2017; Yuen *et al.*, 2017). Therefore, in this chapter, I aim to assess which target plasmid properties can prohibit removal by pKJK5::Cas. Specifically, I compare removal of synthetic cloning vector pHERD99 (Chapter 3) with removal of conjugative, multi-drug-resistance plasmid RP4. RP4, originally isolated from multiple burns patients in a clinical setting, is a broad host-range IncP-1α plasmid and, as it belongs to the same incompatibility group, cannot be maintained together with pKJK5 (Pansegrau *et al.*, 1994). Despite this, both plasmids can transiently co-exist in the same cells (Bahl *et al.*, 2007b), which allows pKJK5::Cas to theoretically target this plasmid. Additionally, RP4 encodes several stability and post-segregational killing systems, one of which is the *par* operon. *parABC* form a multimer resolution system and ensure stable inheritance of RP4 by preventing catenation of daughter plasmids. *parDE* are a post-segregational killing component, with *parE* being a toxin that stops bacterial replication and *parD* the antitoxin (Adamczyk and Jagura-Burdzy, 2003). This means that RP4 loss can be expected to lead to cell death. I hypothesised that

both RP4's incompatibility and presence of its *par* toxin-antitoxin (TA) system might contribute to impaired CRISPR mediated removal of this plasmid.

Therefore, I initially contrast removal of pHERD99 with RP4 and find that RP4 cannot be removed. To analyse the cause of this effect, I construct a series of synthetic target plasmids with and without *par* TA genes, and of different or the same incompatibility group as pKJK5::Cas. Finally, I speculate under which conditions pKJK5::Cas may be successfully used against RP4 and trial this application in a soil bacterial isolate.

Methods

Strains, plasmids, and growth conditions

All bacterial strains, plasmids, and primers used throughout the thesis are listed in Thesis Supplement Table S1-3. Unless otherwise specified, all strains were cultured in LB at 37°C whilst shaking at 180 rpm. Where necessary for plasmid selection, antibiotics were added at the following concentrations: Ap – 100 µg/mL Ampicillin; Gm – 50 µg/mL Gentamicin; Km – 50 µg/mL Kanamycin; Tc – 12 µg/mL Tetracycline; Cp – 25 µg/mL Chloramphenicol. Where *Escherichia coli* MFDpir was used, cultures were supplemented with 0.3 mM DAP (diamino pimelic acid) to ensure growth of this auxotrophic strain. By omitting DAP the strain could be selected against.

Unless otherwise stated, all molecular cloning steps were carried out using high-fidelity restriction enzymes (NEB) in *E. coli* DH5α using commercially competent cells (NEB) and manufacturer's protocols. PCR reactions were carried out using PCRbio Taq master-mix according to manufacturer's instructions.

<u>pACYC_Cas construction:</u> To test Cas9 + sgRNA[aphA99] targeting capability, the CRISPR cassette (Cas9+sgRNA) with specificity [aphA99] and the non-targeting [nt2] was cut from pMA-RQ_Cas (Chapter 2-3) and inserted in vector pACYCduet using EcoRI and HindIII restriction sites.

<u>DH5α::CpR construction:</u> Recipient strain *E. coli* DH5α::CpR was constructed by pBAM_Cp delivery. The Chloramphenicol-resistance-encoding Tn5 transposon was delivered to DH5α using MFDpir as described previously (Dimitriu *et al.*, 2021) and chloramphenicol resistance in *E. coli* DH5α::CpR was phenotypically confirmed by selective plating.

Target plasmid construction: Natural target plasmid RP4 encodes resistance against tetracycline, ampicillin, and kanamycin, and possess several addiction systems including the *parABCDE* TA operon (Pansegrau *et al.*, 1994). I constructed a series of synthetic target plasmids of different incompatibility groups and with or without inclusion of *par* TA genes. Their gene contents are summarised in Figure 4.S1 and their construction process is outlined below.

pOGG99_par was constructed by deleting genes from pOPS0378 (Table S2; (Mendoza-Suárez *et al.*, 2020)) to generate a minimal vector. As such, pOGG99_par is a reverse-engineered vector constructed from BEVA parts (bacterial expression vector archive; (Geddes *et al.*, 2019)). First, *mCherry* was removed from pOPS0378 by digestion with EcoRI, extraction, and re-ligation of the 6043 bp band. Next, site-directed mutagenesis of *aphA* was carried out using primers pOGG0_mut_fw and pOGG0_mut_rv and Thermo Scientific's site-directed mutagenesis kit according to manufacturer's instructions. In this way, the nucleotide at position 96 within *aphA* was silently mutated from C to G to bring the gene sequence in line with that found on our laboratory's version of RP4 and to allow targeting by pKJK5::Cas[aphA99]. Successful mutation was confirmed by Sanger sequencing of the finished plasmid using primer pOGG0_sequence. The final plasmid pOGG99_par includes the following components with corresponding BEVA module names: *aphA* Kanamycin resistance gene originally sourced from RP4 (pOGG008), *oriV, oriT* and *trfA* originally sourced from RP4 (pOGG010), *parABCDE* stability module originally sourced from RP4 (pOGG012). Compared with RP4, this *par* module is 99.96% identical with a single nucleotide mis-match in the non-coding area between *parA* and *parB.*

pOGG99 was constructed by excising *parABCDE* from pOGG99_par: pOGG99_par was digested with BlpI and BglII, the 3664 bp fragment was extracted and religated with linker oligos (pOGG099_parRemoval_top & _btm; annealed and phosphorylated as described previously; Chapter 2). This yielded pOGG99, a version of pOGG99_par where *parABCDE* are replaced with a multiple cloning site module based on BEVA component pOGG004.

pHERD99 was constructed as described in Chapter 3.

pHERD99_par was constructed by amplifying *parABCDE* using pOGG99_par as a template and primers par_fw/rv. The PCR was carried out using high-fidelity Phusion polymerase (Thermo Scientific) according to manufacturer's

instructions, and the amplicon was inserted into pHERD99's KpnI site. The final plasmid was digested with BspHI to verify correct insertion and to test directionality of *parABCDE*: the operon was inserted in an orientation where *parA* and *parB* have the same directionality as pHERD30T's *aacC1*.

Verification of RP4 targeting

To verify the ability of Cas9+sgRNA[aphA99] to target RP4, *E. coli* K12 containing RP4 was made electrocompetent as described previously (Chapter 2), ensuring selection for RP4 using Km throughout. Competent cells were transformed with 500ng plasmid DNA each of pACYC_Cas[aphA99] or pACYC_Cas[nt2] and plated on LB containing Cp, Km, Amp, and Tc to select for both plasmids, and colony counts after an overnight incubation at 37°C were used to calculate transformation efficiency.

Removal of various target plasmids with different *par* status and incompatibility

Single colonies of each donor (*E. coli* DH5α+pKJK5::Cas[aphA99]/[nt2]) were suspended in 25mL LB+Tc and grown overnight. Single colonies of each recipient (*E. coli* DH5α::CpR containing RP4, pHERD99, pHERD99_par, pOGG99, or pOGG99_par) were suspended in 15mL LB and grown overnight, supplemented with Km for RP4 and pOGG-based plasmids, or supplemented with Gm for pHERD-based plasmids. These T0 cultures were washed twice in 0.9% (w/v) NaCl and adjusted to OD600=0.5. Next, cultures were filter-mated in a 1:1 ratio in 5 replicates as described previously (Chapter 3). Filters were placed onto 10% LB plates (diluted in 0.9% NaCl) and incubated at 37°C for 48 hours. To recover cells, filters were placed into 3mL 0.9% NaCl and vortexed for 15 seconds. Recovered cells were plated onto different selective media which allowed growth of recipients with varying plasmid content: LB for all donors and recipients, LB+Cp to select for all recipients, LB+Cp+Tc to select for transconjugants, LB+Cp+Km to select for recipients with RP4/pOGG-based target plasmids, LB+Cp+Gm to select for recipients with pHERD-based target plasmids, and onto LB+Cp+Tc+Km/Gm to select for recipients with both plasmids respectively. Additional controls included donor-only and recipient-only matings for each strain and yielded colonies as expected.

Blocking RP4 entry in *Stenotrophomonas*

To construct recipient strains, pKJK5::Cas[aphA99]/[nt2] was delivered to *Stenotrophomonas sp.* using *E. coli* DH5α as a donor and filter-mating protocols as described previously, with incubation steps carried out at 28˚C (Chapter 3). *Stenotrophomonas* +pKJK5::Cas transconjugants were selected for using Ap (selects for *Stenotrophomonas*) and Tc (selects for pKJK5::Cas), and visually confirmed by GFP fluorescence using a NightSea fluorescence lamp.

A single donor colony (*E. coli* MFDpir+RP4) was suspended in 15mL LB+Km+DAP and grown overnight. Single colonies of each recipient (WT *Stenotrophomonas spp.*; *Stenotrophomonas* + pKJK5::Cas[aphA99]; *Stenotrophomonas* + pKJK5::Cas[nt2]) were suspended in 5mL LB and grown overnight at 28°C, supplemented with Tc for pKJK5::Cas plasmids. These T0 cultures were washed twice in M9 buffer (for 1L: 60g Na_2HPO_4; 30g KH_2PO_4; 5g NaCl; 10g NH_4Cl) and resuspended to OD600=0.5. Filter matings were carried out in a 1:1 ratio with three biological replicates as described previously (Chapter 3), with the exception that a single-channel filterpump (Millipore) was used and sterilised in 70% ethanol between each sample. Filters were placed onto 10% King's Medium B (KB) + DAP plates and incubated at 28°C for 48 hours. Cells were recovered by placing each filter into 3mL LB and vortexing for 15 seconds. Finally, cells were plated onto KB (selection against donors due to absence of DAP) and KB+Tc+Km (selection for RP4) and incubated at 28°C for 48 hours, after which colonies were counted and conjugation efficiency calculated.

Statistical Analyses

Data processing, data visualisation, and statistical analyses were carried out using R software version 4.1.0 and RStudio version 1.4.1717 with the following packages: tidyverse version 1.3.1, arm version 1.11-2, MuMIn version 1.43.17, ggpubr version 0.4.0, lemon version 0.4.5, MASS version 7.3-54. For all statistical models, other model structures and types were tested and the best models selected. Assumptions were tested and found to be upheld.

<u>Target plasmid removal (Figures 4.1 and 4.2)</u> A gaussian GLM was fitted with identity link function, describing the log of target plasmid proportion as a function of pKJK5::Cas target, target plasmid, and their interaction. F=37.42; d.f.=9&40; adjusted R^2=0.8699; $p<2.2\times10^{-16}$.

A Tukey's HSD test revealed significant differences between targeting and non-targeting control of the same target plasmid treatment: pHERD99 $p<1\times10^{-7}$; pHERD99_par $p=0.000187$; pOGG99 $p=0.0518$; pOGG99_par $p=0.949$; RP4 $p=0.977$.

Transconjugant proportion (Figure 4.3A) A gaussian GLM was fitted with identity link function, describing the log of transconjugant proportion as a function of pKJK5::Cas target, recipient plasmid, and their interaction. Counts of 0 were removed to allow fitting of the model. $F=56.73$; d.f.=6 & 17; adjusted $R^2=0.9356$; $p=2.615\times10^{-10}$.

A Tukey's HSD test revealed significant differences between targeting and non-targeting control of the same target plasmid treatment: pHERD99 $p=0.177$; pHERD99_par $p=0.999$; pOGG99 $p=0.000$; pOGG99_par NA (only one datapoint remains after removal of counts of 0).

Transconjugant proportion correlation (Figure 4.3B) Linear models were fitted describing log of target plasmid proportion as a function of the log of transconjugant rate for each pKJK5::Cas target. Transconjugant proportion values of 0 were manually set to ½ of the detection limit.

Targeting: $F=15.5$; d.f.=1&18; multiple $R^2=0.4627$; $p=0.000965$.

Non-targeting: $F=0.1297$; d.f.=1&18; multiple $R^2=0.007155$; $p=0.7229$.

Sample density (Figure 4.4A) A gaussian GLM was fitted with identity link function, describing the log of sample density (CFU/mL) as a function of pKJK5::Cas target, recipient plasmid, and their interaction. $F=95.8$; d.f.=9&40; adjusted $R^2=0.9457$; $p<2.2\times10^{-16}$.

A Tukey's HSD test revealed significant differences between targeting and non-targeting control of the same target plasmid treatment: pHERD99 $p=0.0840$; pHERD99_par $p=0.00434$; pOGG99 $p=0.992$; pOGG99_par $p=0.570$; RP4 $p=0.997$.

Recipient proportion (Figure 4.4B) A gaussian GLM was fitted with identity link function, describing the log of recipient proportion as a function of pKJK5::Cas target, recipient plasmid, and their interaction. $F=16.96$; d.f.=9&40; adjusted $R^2=0.7456$; $p=4.58\times10^{-11}$.

A Tukey's HSD test revealed significant differences between targeting and non-targeting control of the same target plasmid treatment: pHERD99 p=0.000410; pHERD99_par p=0.000485; pOGG99 p=0.999; pOGG99_par p=0.693; RP4 p=0.999.

Additional relevant treatment comparisons include: Targeting treatments: pHERD99_par-pHERD99 p=0.227; pOGG99_par-pOGG99 p=0.0000051. Non-targeting treatments: pHERD99_par-pHERD99 p=0.246; pOGG99_par-pOGG99 p=0.00550.

RP4 delivery to Stenotrophomonas (Figure 4.5) A linear model was fitted describing the log of conjugation frequency as a function of recipient plasmid content. F=82.49; d.f.=2&6; multiple R^2=0.9649; p=4.321x10^{-5}.

A Tukey's HSD test revealed significant differences between individual treatment categories: aphA99-nt2 p=0.837; WT-nt2 p=0.0000908; WT-aphA99 p=0.0000674.

RP4 targeting (Figure 4.S2) A two-tailed t test was carried out to test for a significant difference between the treatment categories. t = -5.787, df = 3, p-value = 0.01026.

Results

Conjugative removal of RP4 is not possible using pKJK5::Cas

First, I aimed to compare the ability of pKJK5::Cas to conjugatively remove the naturally occurring and multi-drug resistant conjugative plasmid RP4 with removal of the cloning vector pHERD99 (Chapter 3). Both these plasmids are targeted by pKJK5::Cas[aphA99]; pHERD99 has a target cloned into its multiple cloning site and RP4 encodes kanamycin resistance gene *aphA* (Figure 4.S1-2). After conjugative delivery of pKJK5::Cas to *E. coli* recipients, pKJK5::Cas [aphA99] reduced pHERD99 target plasmid proportion by ~2 orders of magnitude compared to the non-targeting control (Figure 4.1A). In stark contrast, all recipients retained RP4 after both treatments (Figure 4.1B). This demonstrates that conjugative removal of RP4 cannot be achieved with pKJK5::Cas using established experimental set-ups.

Due to the multi-drug resistant nature of RP4, it was not possible to further investigate the failure of RP4 removal by e.g. determining pKJK5::Cas conjugation efficiency through selective plating. Therefore, I investigated two

properties of target plasmid RP4 in isolation to determine to what extent they can explain my results. These are the presence of TA systems and plasmid incompatibility.

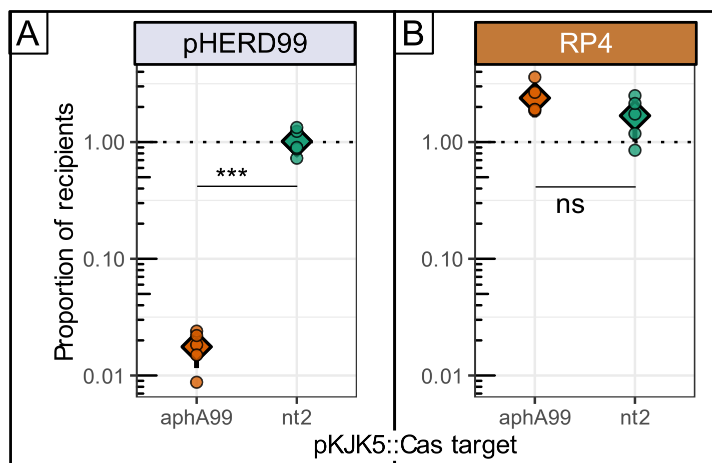


Figure 4.1: **Conjugative removal of pHERD99 and RP4.** Conjugative delivery of pKJK5::Cas[aphA99]/[nt2] to recipients containing pHERD99 (A) or RP4 (B). Diamonds and lines show means ± standard deviation; circles show individual datapoints of proportions of recipients carrying the target plasmid after the experiment; n=5. Dotted line indicates 100%. ***p<0.001, ns – not significant as assessed by Tukey's HSD after fitting a GLM (full model fitted to data in Figure 4.2).

Toxin-antitoxin operon *parABCDE* and replicative incompatibility prevent target plasmid removal

To investigate the impact of a toxin-antitoxin system found on RP4 on target plasmid removal, I cloned *parABCDE* onto pHERD99 (pHERD99_par; Figure 4.S1C). To investigate the impact of plasmid income-patibility, I used pOGG99. This minimal (3.7kB) mobilizable IncP-1 plasmid consists of RP4's origin of replication, origin of transfer, *trfA*, and *aphA* Kanamycin resistance genes (Figure 4.S1B). Finally, to investigate the impact of both TA system presence and plasmid incompatibility, I used pOGG99_par, which in addition to the above encodes *parABCDE* (Figure 4.S1A). To allow comparisons with pHERD99 (Figure 4.S1D) and RP4 (Figure 4.S1E), their data are presented alongside each other:

After conjugative delivery of pKJK5::Cas to *E. coli* recipients carrying each target plasmid, plasmid maintenance remained at ~100% for all treatments when using the non-targeting control (Figure 4.2). In contrast, when delivering pKJK5::Cas[aphA99], target plasmid maintenance depended on plasmid identity: pHERD99 removal was efficient (~1.8% of recipients retain pHERD99; significantly lower than non-targeting control, $p<1x10^{-7}$), but this efficiency decreased by >1 order of magnitude on addition of *par* genes (~27% of recipients retained pHERD99_par; significantly lower than non-targeting control, $p=1.87x10^{-4}$). Removal of IncP-1-backbone plasmids was even more inefficient:

1 50% of recipients retained pOGG99 (not significantly lower than non-targeting
2 control; p=0.052; see Methods for model details). For pOGG99_par, plasmid
3 maintenance was restored to ~100%.

4 These data show that the presence of a *par* TA system is an important factor
5 preventing target plasmid removal by pKJK5::Cas[aphA99]. In addition, the data
6 indicate that plasmid incompatibility is likely another factor that can interfere with
7 removal.

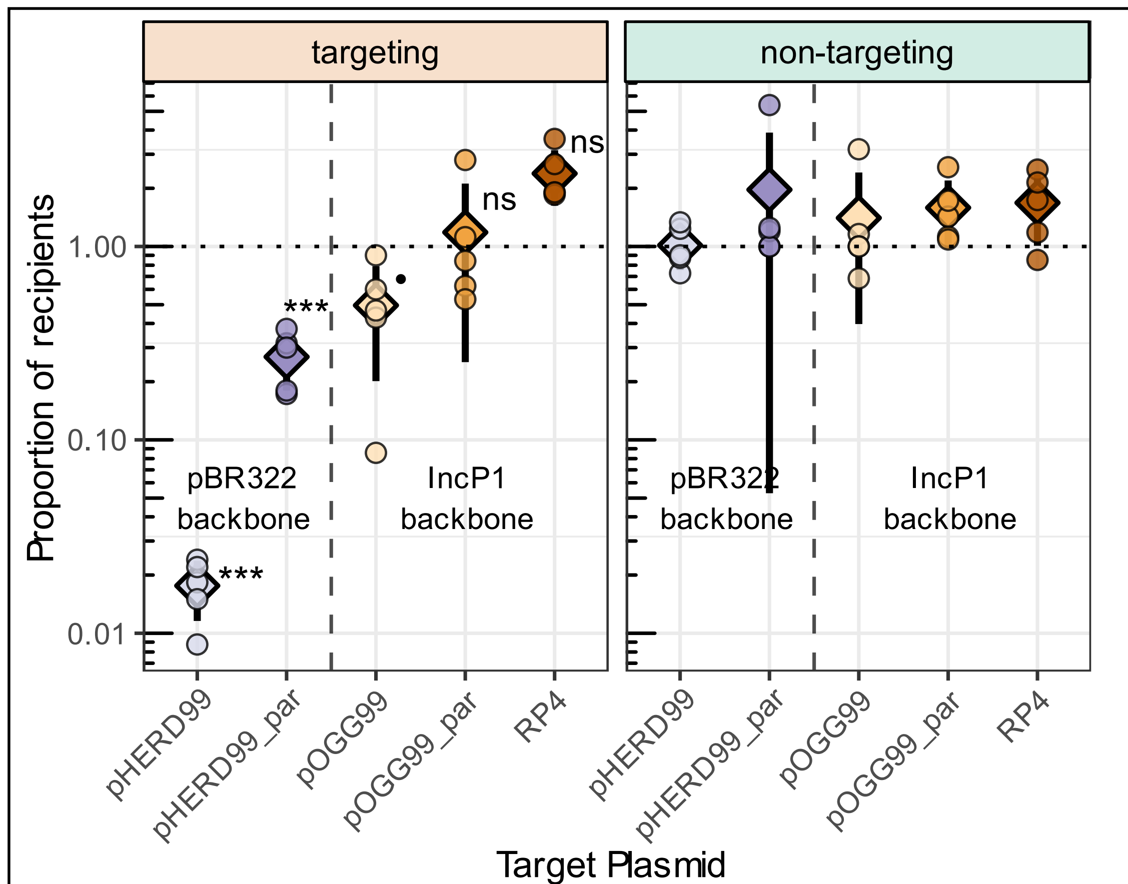


Figure 4.2: **Proportion of recipients carrying different target plasmids.** pKJK5::Cas[aphA99]/[nt2] was delivered to *E. coli* carrying one of five target plasmids. Means (diamonds) ± standard deviation (lines) and individual replicates (circles) of proportions of recipients carrying each target plasmid; n=5. Dotted line indicates 100%, panels are split by targeting pKJK5::Cas[aphA99] and non-targeting control pKJK5::Cas[nt2]. Stars indicate significant differences from corresponding non-targeting controls as assessed by Tukey's HSD after fitting a GLM; ***p<0.001, •p=0.052, n.s. not significant; F=37.42; d.f.=9&40; adjusted R^2=0.8699; $p<2.2\times10^{-16}$.

8 **CRISPR targeting can overcome incompatibility exclusion and leads**
9 **to target plasmid removal**

10 I hypothesised that differences in target plasmid removal were due to differences
11 in uptake and maintenance of pKJK5::Cas. To test this, I assessed the proportion

of recipients with pKJK5::Cas after each experiment as an indication of conjugation efficiency. This was done for samples with all target plasmids excluding RP4 due to its multi-drug-resistant nature.

Interestingly, this revealed that when pOGG99 was the target plasmid, Cas9 targeting was essential for pKJK5::Cas to become established (Figure 4.3A): when delivering the non-targeting control, a very small proportion of recipients (0.00026%) formed transconjugants. This number was >4 orders of magnitude higher when pKJK5::Cas[aphA99] was delivered (7.1%; significantly higher, $p<1x10^{-7}$; see Methods for model details). These dynamics were not seen for compatible target plasmids pHERD99 and pHERD99_par, where conjugation efficiency was not dependent on pKJK5::Cas target.

Furthermore, I confirmed that plasmid removal effects were CRISPR-dependent by plotting transconjugant proportions against target plasmid proportion (Figure 4.3B). This revealed that these two variables were significantly correlated for the targeting pKJK5::Cas[aphA99] treatments where high conjugation rates were associated with low target plasmid proportions ($p<0.001$). No such correlation existed for non-targeting control treatments as target plasmid proportions remained constant ($p=0.72$; see Methods for model details). These associations remained when grouping target plasmids either by incompatibility group or by *par* presence (not shown).

Together, these data show that when the target plasmid matched pKJK5::Cas' Inc group, incompatibility exclusion could be overcome by CRISPR targeting – but only in the absence of *par* genes. Throughout all treatments, high pKJK5::Cas[aphA99] conjugation efficiency was associated with low target plasmid maintenance. In the most extreme case, full pOGG99_par retention correlated with undetectable pKJK5::Cas uptake.

par target plasmids reduce recipient prevalence

I hypothesised that the apparent persistence of target plasmids encoding *par* was due to fitness costs associated with recipients losing these plasmids.

To test this, I first analysed the overall cell density of all experiments. This revealed that overall cell densities of treatments using pHERD-based target plasmids was ~2 orders of magnitude lower than those bearing any other target plasmid (Figure 4.4A). Interestingly, density of pHERD99_par samples was

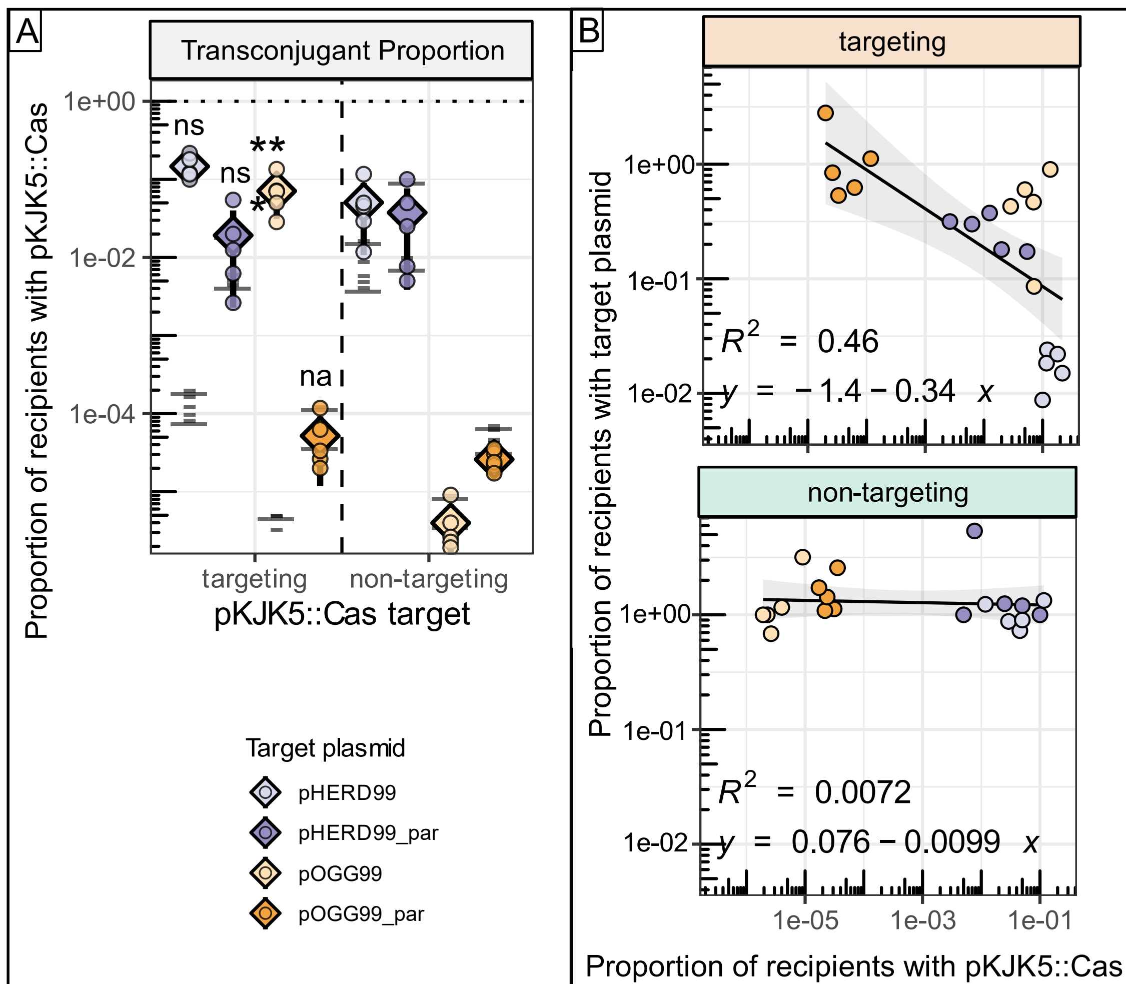


Figure 4.3: **Transconjugant proportion determines extent of target plasmid removal.**
A: Transconjugant proportion. Means (diamonds) ± standard deviation (lines) and individual datapoints (circles) of proportion of recipients which carry pKJK5::Cas, as assessed by selective plating after filter-mating (Figure 4.2). n=5. Horizontal lines indicate the limit of detection for each sample, which varies with overall recipient prevalence and sample density. Transconjugant proportions of 0 and standard deviation which reaches <0 were set to ½ of the limit of detection to allow visualisation on a log scale, ***p<0.001; ns-not significant; na-not analysed (insufficient data points). Significance identifiers indicate statistical differences to corresponding non-targeting treatments; identified by Tukey's HSD after fitting a GLM; F=56.73; d.f.=6 & 17; adjusted R^2=0.9356; p=2.615x10^{-10}. **B: Transconjugant proportion is significantly associated with target plasmid proportion** throughout all target plasmids when delivering targeting pKJK5::Cas (p<0.001). The association is not significant for the non-targeting controls (p=0.72). Transconjugant proportions of 0 were set to ½ of the limit of detection. See methods for linear model details.

1 significantly lower (~20%) when targeting pKJK5::Cas[aphA99] was used as
2 compared to the non-targeting control (p=0.0043; see Methods for model details),
3 potentially due to bacterial cell death caused by the addiction system after target
4 plasmid removal in DH5α::CpR + pHERD99_par + pKJK5::Cas[aphA99]

transconjugants. No difference in cell density was observed between targeting and non-targeting treatments for any IncP-1 plasmid, probably due to the lower efficiency of pKJK5::Cas uptake in these samples (Figure 4.3).

Next, to further investigate population dynamics when *par*-plasmids were targeted, I determined the recipient proportion of all samples (Figure 4.4B). For both pHERD99 and pHERD99_par, recipient proportion was dependent on CRISPR target, and reduced by ~1 order of magnitude in the non-targeting treatment as compared to the targeting treatment (p= 0.00041 & 0.00049 respectively; see Methods for model details). This effect was previously described and is likely due to increased incidence of costly co-maintenance of non-targeting pKJK5::Cas and pHERD99 (Chapter 3). Interestingly, this effect was not observed for plasmids pOGG99 and pOGG99_par. These treatments had higher overall cell densities (Figure 4.4A), which could indicate overall lower costs of maintenance of these plasmids. However, recipient prevalence for the pOGG99_par treatment was >1 order of magnitude lower than for pOGG99 (p<0.001) which indicates a cost imposed by *parABCDE* expression when recipients transiently formed transconjugants.

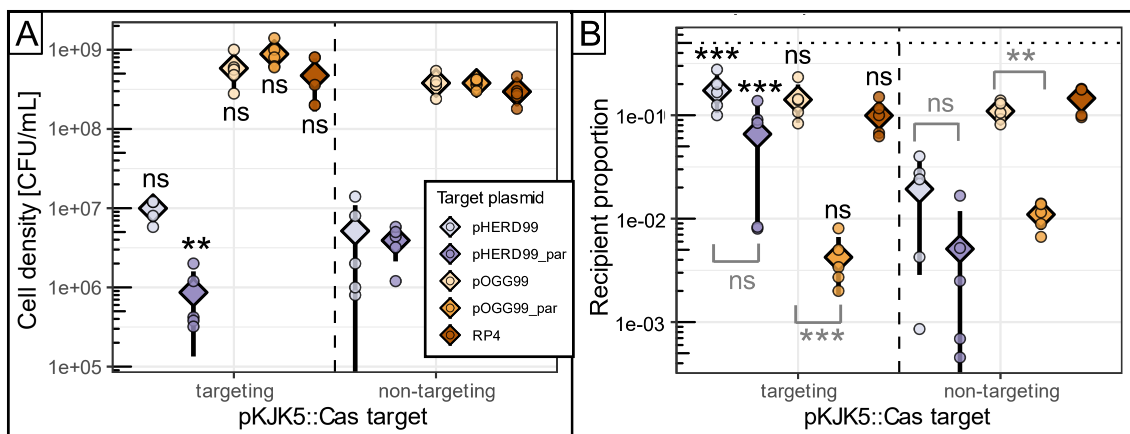


Figure 4.4: **Cell density and recipient proportion reveal costs of targeting *par*.** Means (diamonds) ± standard deviation (lines) and individual datapoints (circles) of **overall cell density in CFU/ml (A)** and **proportion of recipients within each sample (B)** as assessed by selective plating after filter mating. Note that initial recipient proportion was 0.5 (dotted line). n=5. ***p<0.001; **p<0.01; *p<0.05; ns-not significant; significance identifiers indicate statistical differences to corresponding non-targeting treatments, grey lines include significant differences between other relevant treatment categories; identified by Tukey's HSD after fitting GLMs. See Methods for model details.

In summary, these data show *par*-plasmids that are targeted confer a cost upon their hosts, as evident through their population dynamics. pHERD99_par cell

density drops when targeting pKJK5::Cas is used, and pOGG99_par recipient proportion is significantly lower than for its non-*par*-encoding counterpart.

pKJK5::Cas prevents RP4 entry

Finally, I hypothesised that while pKJK5::Cas conjugative removal of RP4 was ineffective, this technology may still find its use by "vaccinating" a target strain to prevent uptake of conjugative AMR plasmids such as RP4. This may be particularly helpful in a setting such as a soil microbiome, which could become a reservoir of AMR genes after being exposed to contaminated slurry. To test the ability of pKJK5::*cas* to prevent AMR plasmid uptake, I therefore conjugatively delivered RP4 to soil isolate *Stenotrophomonas spp.* carrying either pKJK5::Cas[aphA99], pKJK5::Cas[nt2], or neither plasmid.

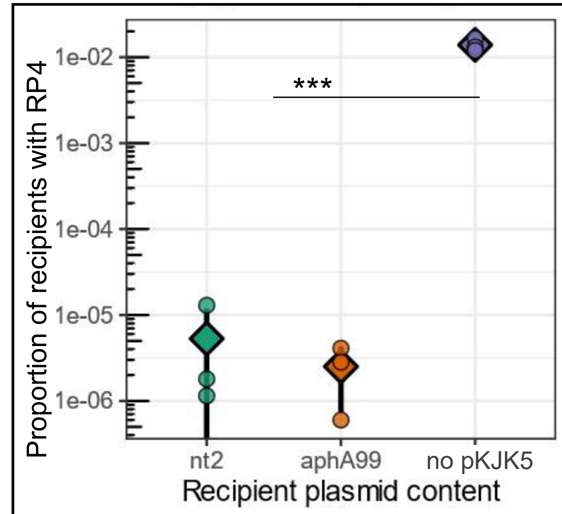


Figure 4.5: **pKJK5::Cas prevents RP4 entry.** Delivery of RP4 by *E. coli* to *Stenotrophomonas sp.* with varying plasmid content, n=3. nt2- pKJK5::Cas[nt2]. aphA99- pKJK5::Cas[aphA99]. Diamonds and lines show means ± standard deviation, circles show individual datapoints of conjugation efficiency (proportion of recipients which took up RP4). ***$p<0.001$, assessed by Tukey's HSD after fitting a linear model. See methods for model details.

When not carrying pKJK5, *Stenotrophomonas* took up RP4 at >3 orders of magnitude higher frequencies than when it carried pKJK5::Cas, independent of its *sgRNA* specificity (Figure 4.5; $p=6.7\text{-}9.1\times10^{-5}$; see Methods for model details). This indicates that RP4 uptake was blocked and not dependent on CRISPR, but likely mediated by incompatibility exclusion of pKJK5.

Discussion

In this chapter, I assayed the impact of target plasmid properties on their removal using pKJK5::Cas and found that TA (*par*) gene presence and plasmid incompatibility both decrease removal efficiency and work additively (Figure 4.2).

In order to ascertain how TA system presence impacts target plasmid removal, I compared removal of plasmids with and without the *par* operon. *parABCDE* encodes two separate components; the stable inheritance system *parABC* and the TA system *parDE* (Adamczyk and Jagura-Burdzy, 2003). The protective

effect of *par*-encoding plasmids is likely due to a combination of each component, but lower recipient prevalence and overall lower cell densities when *par*-encoding plasmids are used (Figure 4.4) suggests that this effect is primarily due to selective pressure imposed by cell death, mediated by the TA component.

parDE is a well-studied TA system, and the NCBI nucleotide database records presence of these genes in genomes of Enterobacteriaceae such as *Escherichia, Salmonella, Klebsiella,* and *Shigella* as well as at least 15 other species belonging to *Proteobacteria* (Clark *et al.*, 2016). The prevalence of these specific genes on plasmids beyond RP4 is unclear, while closely related TA loci on plasmids are more widespread (e.g. (Kamruzzaman and Iredell, 2019)). Overall, TA systems are highly prevalent throughout chromosomes and mobile genetic elements and highly diverse (Jurėnas *et al.*, 2022). Particularly Type II TA systems, to which *parDE* belongs, are well-distributed: Searching publicly available sequences revealed that Type II TA systems are represented in about two thirds of bacterial genomes and carried by about one third of plasmids, both of which often carry multiple type II TA systems (Xie *et al.*, 2018). Generally, TA systems are highly relevant to plasmids carried by pathogenic bacteria: together with multimer resolution systems and partition systems, TA systems are ubiquitous amongst virulence plasmids in *Enterobacteriaceae* (which often encode multiple TA systems) and predicted to be essential to virtually all large plasmids (Sengupta and Austin, 2011). Therefore, understanding the impact of TA systems on target plasmid removal is crucial in the roll-out of CRISPR-based plasmid removal tools. In comparison with other TA systems, *parABCDE* was found to be particularly adept at killing plasmid-free cells and causing stable plasmid maintenance (Jensen *et al.*, 1995), so perhaps other TA systems would mediate less persistence during CRISPR-mediated plasmid removal. To avoid these protective effects imposed by TA systems, pKJK5::Cas could be improved by addition of antitoxin genes (e.g. *parD*). This strategy was successfully employed in the development of pCURE, which removed resident plasmids via incompatibility exclusion and by blocking target plasmid TA systems (Lazdins *et al.*, 2020).

Beyond stability systems and plasmid incompatibility, other target plasmid properties might also play a role in persistence after pKJK5::Cas delivery in this study system.

Firstly, Cas9 could target pOGG99 and pHERD99 with differing efficiency: pOGG99's target sequence is within its antibiotic resistance gene, while pHERD99's target sequence is in an intergenic region inserted into its multiple cloning site, meaning CRISPR targeting and therefore plasmid removal efficiency is predicted to be lower for pHERD99 (Chapter 2; Chapter 3). In Chapter 3, a higher proportion of plasmid co-incidence indicated a lower CRISPR targeting efficiency. In this chapter no transconjugants with pOGG99 could be recovered, in contrast to pHERD99 (Figure 4.S3). While this might support a higher CRISPR targeting efficiency for pOGG99, this comparison is not straightforward due to pOGG99 incompatibility. Unfortunately, the limit of detection for non-targeting pKJK5::Cas and pOGG99 was too high (≧100%) to see if this effect was present in the absence of CRISPR targeting. Therefore, to conclusively determine targeting efficiency of this plasmid, these experiments would have to be repeated with a higher conjugation efficiency to lower the limit of detection.

Secondly, all plasmids used in this chapter are mobilizable by IncP-1 plasmids such as pKJK5::Cas. This leaves the model system open to re-infection of naïve recipient cells by plasmids which have a mutated target site to escape CRISPR targeting. This phenomenon was observed when applying a similar CRISPR delivery tool, where it led to only moderate plasmid removal (~50%; (Wongpayak *et al.*, 2021)). Therefore, this analysis should be further expanded to target plasmids not mobilizable by pKJK5::Cas; perhaps the impact of TA system presence is less severe in such cases as hosts cannot be re-infected with target plasmids and encounter TA-mediated toxicity a second time.

Put together, *par* presence and presumably incompatibility exclusion of pOGG99_par were sufficient to entirely stop removal of this target plasmid, similar to the persistence observed for RP4 (Figure 4.2). With an overall more efficient model system, it is likely that further differences in removal efficiency would be revealed between these plasmids: RP4 is nearly 10x the size of pOGG99_par (Figure 4.S1) and, amongst other cargo genes, encodes at least three stability loci (*par, kil/kor,* and *Tn1*), multiple AMR genes and transposons (Pansegrau *et al.*, 1994), an entry exclusion system (Haase *et al.*, 1996), and a putative anti-CRISPR (Acr) operon (7 low-confidence putative Acr genes identified using AcrFinder (Yi *et al.*, 2020)). Recipient prevalence during removal of pOGG99_par was very low (Figure 4.4B), likely due to the cost of target

plasmid removal. In contrast, recipient prevalence for RP4 treatments was maintained at control levels, which could be a result of virtually non-existent formation of pKJK5::Cas transconjugants. Together, this indicates that RP4 is a highly persistent target plasmid, even in comparison with pOGG99_par.

More broadly, the persistence of incompatible target plasmids indicates that spread of pKJK5::Cas in communities with prevalent IncP-1 plasmids would lead to less AMR plasmid removal than spread in communities lacking these plasmids. This could inform effective application of this CRISPR delivery tool. For instance, a waste-water treatment plant study found an increased IncP-1 plasmid prevalence after treatment (Pallares-Vega *et al.*, 2019), suggesting that pKJK5::Cas may be more effective as a pre-treatment step to reduce AMR plasmid prevalence rather than when it is applied afterwards.

Alternatively, timing of application may allow pKJK5::Cas to be used effectively by preventing AMR plasmid uptake rather than removal of resident plasmids. The final experiment simulates such a situation: a field may be exposed to *E. coli* carrying AMR plasmids from contaminated slurry, and AMR plasmids may in turn become established in soil microbiomes (including species such as *Stenotrophomonas spp.*) to act as a future reservoir of resistance. pKJK5::Cas can prevent this by stopping transfer into this strain (Figure 4.5). Despite this effect not being CRISPR-dependent, the use of pKJK5::Cas rather than purely incompatibility-based plasmid removal systems (e.g. (Lazdins *et al.*, 2020)) has some advantages: prevention of RP4 uptake may become CRISPR-dependent when there is weak selection for RP4, e.g. when low concentrations of antibiotics are present – a situation often observed in environments such as rivers, coastal waters, or soils (Knapp *et al.*, 2010; Amos *et al.*, 2015; Leonard *et al.*, 2018). In such a case, pKJK5::Cas[aphA99] may prevent RP4 entry, while pKJK5::Cas[nt2] would become displaced by RP4 due to its selective pressure. These predictions need to be tested experimentally to determine in which situations CRISPR targeting provides an advantage in protection from highly persistent plasmids.

The limitations of pKJK5::Cas uncovered in this work may help inform the most appropriate applications of this CRISPR treatment – to protect a microbiome from exposure to AMR plasmids, for instance in waste-water treatment plants or in the human gut microbiome. This is further reviewed in the General Discussion.

Generally, the presumptive failure of pKJK5::Cas to become established in RP4+ hosts (Figure 4.1B) and vice-versa (Figure 4.5) indicates that there may be a priority effect, where the first plasmid to invade a host has an advantage over a subsequently infecting plasmid of the same incompatibility group. Interestingly, my data indicate that the presence of a competitive immune system on an invading plasmid (i.e. CRISPR-Cas9) may not be sufficient to overcome this priority effect, but more experiments are needed to ascertain the dynamics underpinning this competition between RP4 and pKJK5::Cas.

In the previous chapters and in this chapter, I determined which delivery-vehicle-specific properties and which target-plasmid-specific properties contribute to effective AMR plasmid removal by pKJK5::Cas. For applications in the environment or in healthcare, pKJK5::Cas would have to spread through communities of mixed bacterial species. Therefore, in the next chapter, I investigate transfer and maintenance of pKJK5::Cas in a soil bacterial community.

Conclusion

In this chapter, I aimed to ascertain which target plasmid properties impact their removal by pKJK5::Cas. I showed that removal of multi-drug-resistance plasmid RP4 cannot be achieved with established protocols and break these dynamics down further using a series of synthetic target plasmids: pKJK5::Cas could remove plasmids carrying a single TA system (*par*), albeit at greatly reduced efficiency. Plasmids of the same incompatibility group as pKJK5::Cas could perhaps be removed with very low efficiency. In combination, an incompatible *par*-encoding target plasmid could not be removed in this model system. Despite this, pKJK5::Cas could be used to protect a soil isolate from RP4 uptake, although this effect was not CRISPR-dependent and therefore likely mediated by incompatibility exclusion.

Overall, this study allows us to understand target plasmid properties which might prevent their removal by pKJK5::Cas or related CRISPR tools and adds to the established basis of knowledge on CRISPR delivery vehicle properties influencing target plasmid removal. This will allow tailoring of applications to situations in which target plasmid removal can be predicted to be very effective,

or to further engineer pKJK5::Cas to circumvent these barriers imposed by persistent target plasmids.

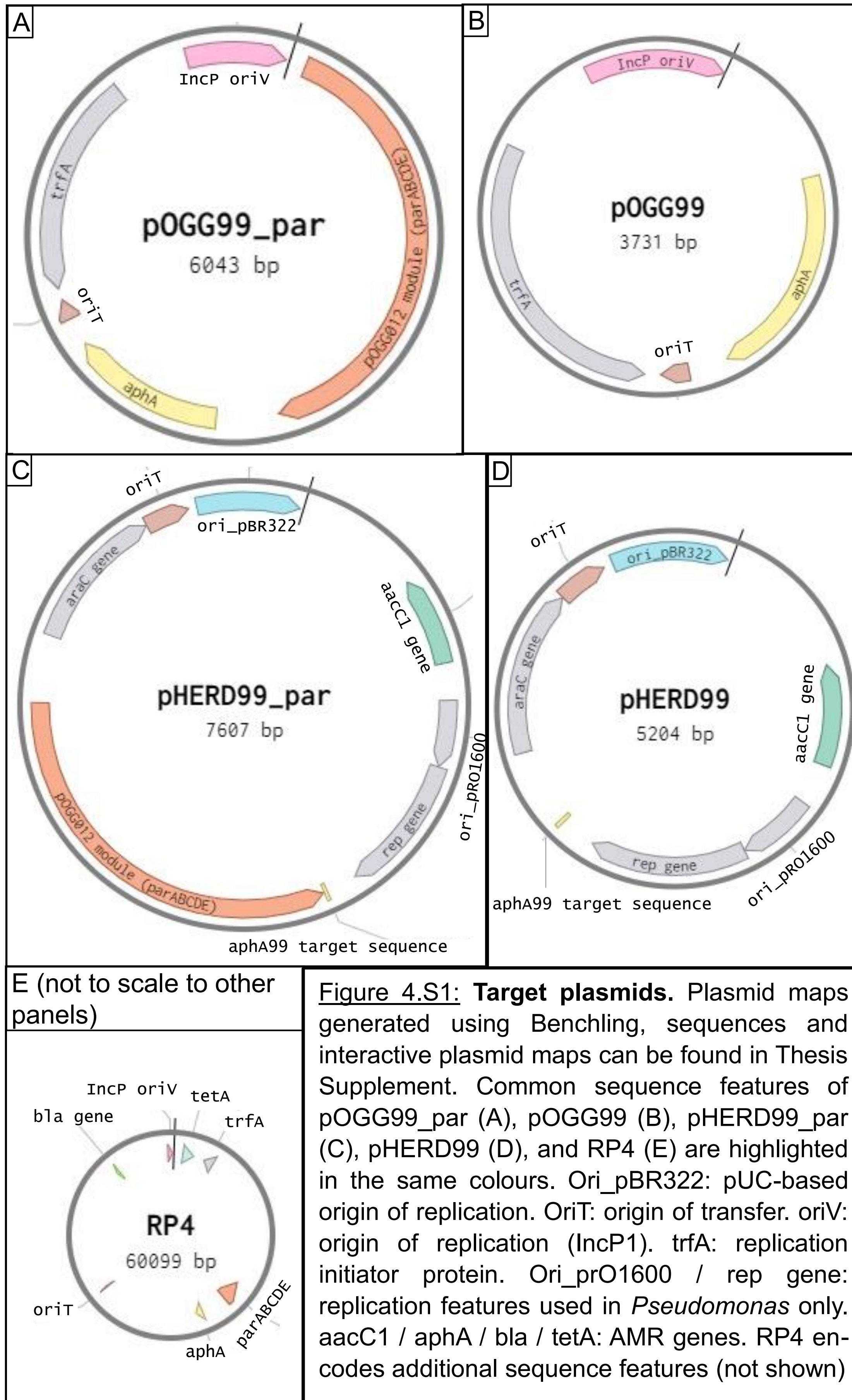


Figure 4.S1: **Target plasmids.** Plasmid maps generated using Benchling, sequences and interactive plasmid maps can be found in Thesis Supplement. Common sequence features of pOGG99_par (A), pOGG99 (B), pHERD99_par (C), pHERD99 (D), and RP4 (E) are highlighted in the same colours. Ori_pBR322: pUC-based origin of replication. OriT: origin of transfer. oriV: origin of replication (IncP1). trfA: replication initiator protein. Ori_prO1600 / rep gene: replication features used in *Pseudomonas* only. aacC1 / aphA / bla / tetA: AMR genes. RP4 encodes additional sequence features (not shown)

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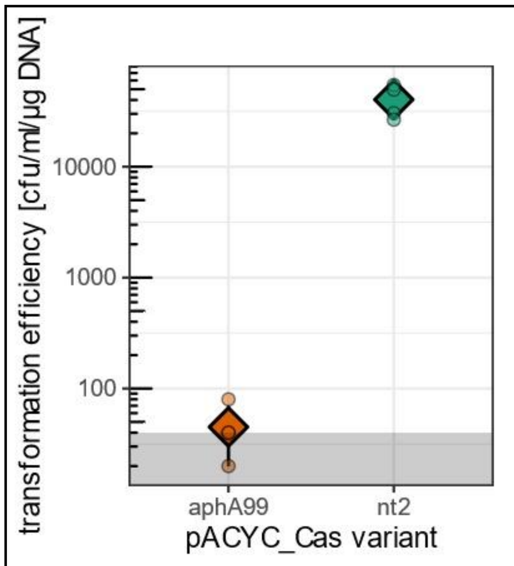

Figure 4.S2: **RP4 prevents transformation with pACYC_Cas [aphA99].** DH5α carrying RP4 was transformed with pACYC_Cas [aphA99]/[nt2], template plasmids carrying the gene cassette that was inserted into pKJK5 for the targeting and non-targeting control respect-tively. Means ± standard deviation (diamonds/lines) together with individual datapoints (circles) of transformation efficiency, as-sessed by selective plating for both plasmids. Grey box indicates the limit of detection, lower datapoints were manually set to ½ of the limit. N=3, p=0.01026 as assessed by two-tailed T test. (t=-5.787, d.f.=3).

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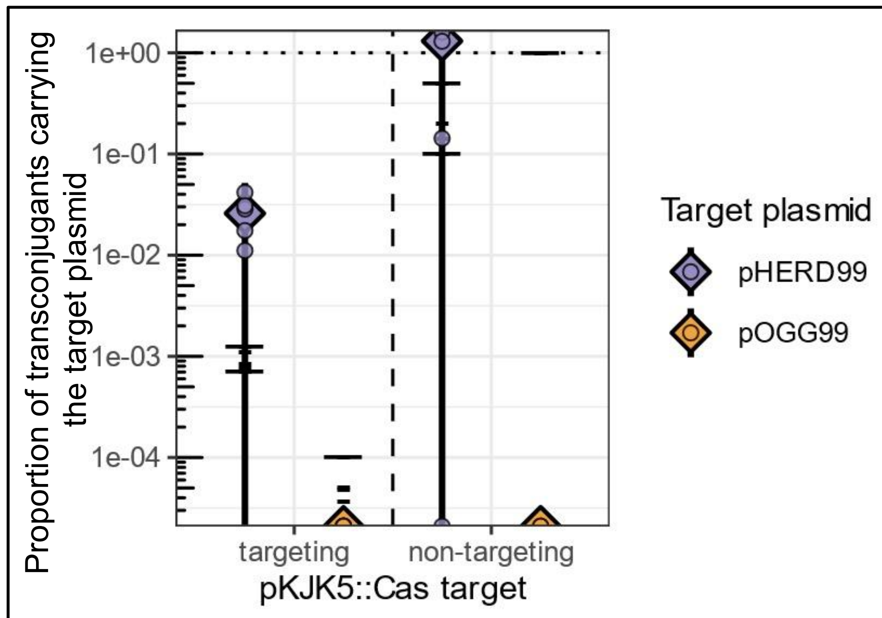

Figure 4.S3: **Proportion of transconjugants still carrying the target plasmids** might indicate CRISPR targeting efficiency. Means (diamonds) ± standard deviation (lines) and individual datapoints (circles) of the proportions of transconjugants which also carry the target plasmid. Horizontal lines indicate the limit of detection for each sample. In Chapter 3, this could be used as a proxy for CRISPR targeting efficiency. However, due to pOGG99 incompatibility this may be more complicated for these data (see text).

Chapter 5: pKJK5::Cas transfer and maintenance in a community context

Abstract

Plasmids are key disseminators of antimicrobial resistance (AMR) genes and virulence factors, and it is therefore critical to predict and manipulate their spread in microbial communities. The development of CRISPR delivery tools such as pKJK5::Cas may enable removal of AMR plasmids from natural communities, provided they can be transferred and maintained in such settings. The cost of plasmid carriage is a key metric which can be used to predict plasmid ecological fate.

In this chapter, I assessed transfer of pKJK5::Cas between individual compost isolates which can form a stable 5-species community, and found that plasmid transfer was not dependent on donor and recipient relatedness. Using the synthetic 5-species community and broad host-range plasmid pKJK5::Cas as a model, I report that both the cost of plasmid carriage and its long-term maintenance in a focal strain depended on the presence of competitors and their species identities.

Together with previous work, these data allow predictions of how target plasmid removal from a microbial community might be achieved using pKJK5::Cas. Further, I propose that the destabilising effect of interspecific competition on plasmid maintenance may be leveraged in clinical and natural environments to cure plasmids from focal species.

Introduction

Plasmids are important vehicles of horizontal gene transfer (HGT) and crucial components of microbial ecosystems. They shape microbial evolution (Koonin, 2016; Brockhurst *et al.*, 2019) and are of profound clinical relevance as disseminators of antimicrobial resistance (AMR) genes (Partridge *et al.*, 2018) and virulence factors (Elwell and Shipley, 1980; Dewar *et al.*, 2021). Many plasmids, particularly those with a broad host range, have the potential to transfer between bacterial species and mobilise resistance genes from environmental strains into clinically relevant pathogens. Hence, being able to predict and manipulate the spread of plasmids and the genes they carry is critical to limit the spread of AMR.

The development of CRISPR delivery tools (reviewed in (Pursey *et al.*, 2018)) is being investigated as a means of removing AMR plasmids from bacterial communities, particularly to counteract the role the environment currently plays in spread of AMR genes (United Nations, 2017). In the previous chapters, I developed broad host-range conjugative plasmid pKJK5::Cas, showed how it can be used as a barrier to plasmid uptake, and assessed which factors underpin plasmid removal efficiency when conjugatively applying it to a target strain. Before application of pKJK5::Cas in natural communities can be considered, its transfer and maintenance in mixed populations of natural bacterial isolates needs to be investigated.

A large number of studies have considered the factors that underpin plasmid spread and maintenance in bacterial populations and communities (reviewed in (Brockhurst *et al.*, 2019)), a key determinant of which is the fitness effect plasmids have on their bacterial host. Costs can arise at different steps of the plasmid lifecycle and can result, amongst others, from the expression of genes carried on the plasmid and their interference with host processes (reviewed in (San Millan and MacLean, 2017)). As a consequence, the costs of plasmid carriage vary not only between plasmids (Hall *et al.*, 2015) but also between hosts (Alonso-del Valle *et al.*, 2021). Moreover, these costs are strongly dependent on the environment; plasmids that are costly in the absence of antibiotics or heavy metals can become highly beneficial in their presence if they encode resistance genes (Hall *et al.*, 2015).

Theory and data suggest that costly plasmids can be lost readily from bacterial populations or communities due to purifying selection, unless conjugation rates, either within or between species, are sufficiently high to support their maintenance (Stewart and Levin, 1977; Bergstrom *et al.*, 2000; Hall *et al.*, 2016). For example, bacteria that lose a plasmid when cultured on their own may still associate with this plasmid when co-cultured with another species, due to high rates of interspecific plasmid transfer (Hall *et al.*, 2016). Hence, even bacteria that are unable to maintain plasmids in monoculture may show increased plasmid persistence in a microbial community.

Initially, I measured transfer of pKJK5::Cas[nt2] between compost isolates which can form a stable microbial community *in vitro*. Further, I hypothesised that the maintenance of plasmids may be negatively affected by the microbial community

context, through amplification of the costs of carrying the plasmid. This would be analogous to the amplification of fitness costs of bacterial genome mutations (Alseth *et al.*, 2019) or of chromosomal AMR genes (Klümper *et al.*, 2019) in the presence of competitor species.

To test this hypothesis, I measured how the fitness costs and maintenance of broad host-range plasmid pKJK5::Cas in compost isolate *Variovorax sp.* depend on the presence of additional soil bacteria. *Variovorax* is a β-proteobacterium, and members of this genus are often found in microbial soil communities. *Variovorax* forms a stable, long-term community with species that were isolated from the same sample, belonging to *Pseudomonas sp.*, *Stenotrophomonas sp.*, *Achromobacter sp.*, and *Ochrobactrum sp.* (Castledine *et al.*, 2020; Padfield *et al.*, 2020).

pKJK5 is a 54 kb IncP-1ε plasmid originally isolated from a manure-associated microbial soil community that carries resistance genes to tetracycline, trimethoprim, aminoglycosides, and sulfonamides within an *intI1* integron cassette (Bahl *et al.*, 2007b), and which can transfer readily into soil and waste-water treatment plant communities (Klümper *et al.*, 2015; Li *et al.*, 2020). Transfer and maintenance dynamics were primarily investigated using CRISPR delivery tool pKJK5::Cas[nt2], which encodes *gfp, cas9* and a non-targeting *sgRNA* coding for a random nucleotide sequence as a target.

Methods

Strains and Growth conditions

Focal strain *Variovorax sp.* (V) forms a synthetic community with bacterial compost isolates *Pseudomonas sp*. (P), *Stenotrophomonas sp*. (S), *Achromobacter sp.*(A), and *Ochrobactrum sp.* (O). These species form a stable community over very long timescales (>1 year) when cultured in low-nutrient 1/64 Tryptone Soy Broth (TSB; diluted in water) and form visually distinct colonies on King's B medium (KB) agar, allowing to enumerate species frequencies without the need for selective plating (Castledine *et al.*, 2020). All communities and monocultures were incubated in 6mL TSB statically at 28°C unless otherwise stated. For analysis, samples were plated onto KB agar plates at appropriate dilutions and incubated at 28°C for 2-3 days. Community composition was assessed by counting each colony phenotype, and plasmid carriage was

1 assessed by screening GFP expression using a fluorescence lamp (NightSea
2 lamp with RB bandpass filter).

3 Chromosomally tagged soil isolates were constructed using mini-Tn5-transposon
4 vectors pBAM1-Gm and pBAM1-Sm (Martínez-García *et al.*, 2011), and
5 derivative pBAM1-Cp which contains Chloramphenicol resistance gene *catR*.
6 These suicide transposon vectors were delivered to P, S, O, and V using
7 auxotrophic donor strain *E. coli* MFD*pir* following established protocols (Dimitriu
8 *et al.*, 2021). Successful insertion of *aacC1, aadB* and *catR* genes was confirmed
9 by their resistant phenotype and by PCR (*aacC1* and *catR* only, using primers
10 aacC1_fw&rv / Cm_F/R respectively). The tagged soil isolates are P(SmR),
11 S(GmR), O(GmR), V(GmR) and V(CpR).

12 Soil isolate transconjugants of pKJK5::Cas[nt2] were generated using *E. coli*
13 MFDpir + pKJK5::Cas[nt2] as a donor, and by selecting for pKJK5::Cas[nt2] (P,
14 S, O, V) or by selecting GFP+ colonies (A) while selecting against the donor strain
15 due to absence of DAP.

16 **Mating pair experiment**

17 Single GFP+ colonies of each donor (P, S, A, O, and V each carrying
18 pKJK5::Cas[nt2], and *E. coli* K12::mCherry carrying pKJK5::Cas[nt2] or
19 pKJK5::Cas[aphA99]) were suspended in 15mL LB+12µg/mL Tetracycline (Tc)
20 and incubated for two nights at 28°C; 180rpm. For the community treatment, a
21 GFP+ colony each of P, S, A, O and V carrying pKJK5::Cas[nt2] was suspended
22 in a single 6mL 1/64 TSB+Tc microcosm and incubated static at 28°C for 6 days.
23 After this, 100µL of the mixed community were transferred into 15mL LB+Tc12
24 and incubated for two nights at 28°C; 180rpm. Single colonies of each recipient
25 (P(SmR), S(GmR), O(GmR), and V(GmR) were suspended in 20mL of LB and
26 incubated for two nights at 28°C; 180rpm.

27 Donors and recipients were washed twice and adjusted to OD600=0.25 using
28 0.9% (w/v) NaCl dissolved in H_2O, and 1mL of each mating pair were used to set
29 up filter-matings as described previously (Chapter 3). Filters were incubated on
30 10% LB (diluted in 0.9% NaCl) plates at 28°C for 72 hours, and cells were
31 recovered by placing each filter in 3mL 0.9% NaCl and vortexing for 15 seconds.
32 To assess transconjugant proportions, each sample was plated onto KB,
33 KB+50µg/mL Gentamicin (Gm), and KB+ Gm + Tc (for mating pairs using
34 P(SmR) as a recipient, Gentamicin was replaced with Streptomycin at the same

1 concentration). Mating pairs with *Achromobacter* as a recipient could not be
2 formed due to *Achromobacter*'s phenotypic resistance to Tetracycline. Mating
3 pairs with *Pseudomonas* as a recipient could only be formed using *E. coli*
4 K12::mCherry as a donor due to all other strains' phenotypic resistance to
5 Streptomycin.

6 To assess likely composition of community donors, five replicate communities
7 were set up and cultured in the same way as donors above, and samples were
8 plated onto KB to assess species frequencies. Data presented in Figure 5.1A are
9 estimated average species frequencies, which had a similar distribution
10 throughout each replicate.

11 Phylogenetic analysis

12 16S rRNA sequences of P (14 replicates), S (2 replicates), O (10 replicates), and
13 V (4 replicates) were amplified using primers Forward 27F and Reverse 1492R
14 (Table S3) and manufacturer's Taq PCR protocols (PCRbio) using colonies as a
15 template. Sanger sequencing was carried out using primer Forward 27F. All
16 replicate 16S sequences were aligned using CLUSTAL or pairwise aligned (for
17 S) using NEEDLE, and consensus 16S sequences were generated using
18 EMBOSS (Madeira *et al.*, 2019). For *Achromobacter* and *E. coli*, 16S sequences
19 of the most closely related strain deposited in Genbank were used
20 (*Achromobacter agilis,* NR_152013.1; *E. coli* K12 CP012CP012868.1:431955-
21 433510868.1).

22 16S consensus and Genbank sequences were aligned using CLUSTAL (Madeira
23 *et al.*, 2019), and a phylogenetic tree was generated using iqtree (Trifinopoulos
24 *et al.*, 2016).

25 *Variovorax* fitness experiment

26 Five replicate V(GmR) + pKJK5::Cas[nt2] transconjugants (generated as
27 described above) were suspended in 1/64 TSB + Tc, and five replicate colonies
28 each of plasmid-free V(GmR), V(CpR), P, S, A, and O were suspended in 1/64
29 TSB. After an initial two-day incubation, antibiotic-including cultures were washed
30 twice with 0.9% (w/v) NaCl solution before being used to start the experiment.
31 Communities were established by using 20µL of each *Variovorax* strain (adjusted
32 to OD600=0.065) mixed with 50µL of P, S, A, or O. For the plasmid-bearing
33 treatment, V(GmR)+pKJK5::Cas[nt2] and V(CpR) competed against each other
34 either alone or together with P, S, A, or O. In the plasmid-free control, V(GmR)

and V(CpR) competed against each other in the same contexts. All competitions were carried out in the absence of selection.

The communities were cultured for three days, vortexed and transferred into fresh microcosms and incubated for another two days. Then, communities were vortexed and each sample was plated onto KB, KB+Gm, and KB+25µg/mL Chloramphenicol (Cp) plates.

Robustness of chromosomal tags was confirmed by colony PCR of *aacC1* and *catR* of 66 random *Variovorax* colonies across treatments. Colony identities of all species were assessed on each plate. The relative fitness of V(GmR) and pKJK5 selection coefficient *s* for each treatment were calculated using the following equations:

$$relative\ fitness\ of\ V(GmR) = \frac{\text{Variovorax}\ colonies\ on\ KB + 50\frac{\mu g}{mL}Gentamicin\ plate}{\text{Variovorax}\ colonies\ on\ KB + 25\frac{\mu g}{mL}Chloramphenicol\ plate}$$

$$s = (relative\ fitness\ of\ V\ (GmR)\ with\ plasmid) - (relative\ fitness\ of\ V\ (GmR)\ without\ plasmid).$$

Plasmid maintenance experiments

To set up monocultures and communities for the plasmid maintenance experiments (Figure 5.3 and 5.6), five colonies of each community constituent were individually suspended in 1/64 TSB, supplemented with Tc where the community constituent carried pKJK5::Cas[nt2] or pKJK5::GFP. Adding Tetracycline at this step ensured pKJK5 maintenance in all strains except *Achromobacter*.

After 2 days incubation, 1mL of these cultures was pelleted and washed twice with 0.9% NaCl to remove all traces of antibiotics. For monocultures, 20µL of each of the five separately cultured colonies per isolate were transferred into fresh microcosms, giving rise to five biological replicate monocultures per treatment. Additionally, 15 community combinations were established by mixing 20µL of each five replicates of P, S, A, and/or O and V, giving rise to five replicate communities per treatment (Figure 5.S2B). All experiments were carried out in the absence of selection.

Monocultures and communities were cultured for three days, vortexed, and 100uL of each culture transferred into a fresh microcosm and incubated for another two days. Communities were then further passaged into fresh microcosms the same way every two days until 17 total days of co-culture

(Community Maintenance experiment only). To assess community composition, samples were plated onto KB agar plates at T0, T5, and T17 (Figure 5.S2C).

Statistical analyses

Data processing, data visualisation, and statistical analyses were carried out using R version 4.0.5 and RStudio version 1.4.1103 with the following packages: dplyr v1.07, tidyr v1.13, readr v2.0.0, ggplot2 v3.3.5, ggpubr v0.4.0, lme4 v1.1-27.1, MASS v7.3-54, betareg v3.1-4, treeio v1.16.1 and ggtree v3.0.2.

For all analyses, other model types, link functions, and the inclusion of additional variables were tested. The final models were found to be the most robust. Model assumptions were checked and found to be upheld. For comparison of specific treatment categories, Tukey's post-hoc test of honest significance differences was carried out. The model details are as follows:

Mating pair experiment (Figure 5.1) Binomial generalised linear model with cloglog link function: Transconjugant frequency as a function of Recipient species and the interaction of plasmid type and donor species. F=0.1448, df=13 & 116, R^2=0.816. Inclusion of phylogenetic distance was tested as an additional variable but found not to be significant, and was therefore removed from the final model (Recipient species: p=0.00626; Phylogenetic distance: p=0.553; Donor species:Plasmid type: p=0.00660).

To test for differences between individual treatment categories, a separate linear model was constructed. This described log-transformed transconjugant frequency as a function of treatment. F=45.59, df=25&104, R^2=0.8963, $p<2.2\times10^{-16}$. A Tukey's HSD revealed significant differences between individual categories as mentioned in text.

Variovorax fitness (Figure 5.2) Gaussian generalised linear model with logit link function: Plasmid selection coefficient as a function of the interaction of Treatment and Growth Partner, Treatment, and Growth Partner. F=20.71, df=9 & 140, adjusted R^2=0.5435.

Growth partner Experiment (Figure 5.3) Binomial generalised linear model with logit link function: Plasmid-bearing *Variovorax* fraction as a function of Treatment (community), *Stenotrophomonas* proportion, and *Achromobacter* proportion. F =0.4635, df=17 & 62, pseudo R^2=0.848. A Tukey's HSD revealed significant differences between individual treatment categories, see Table 5.S1. See Table

5.S2 for a summary of which additional model variables were tested but dropped due to insignificance.

Table 5.S1. Growth partner experiment model values. P value refers to probability of treatment average being significantly different from average of treatment 1.1 as assessed by the statistical model in Figure 5.3.

Treatment (n=5)	**Mean plasmid-bearing *Variovorax* fraction**	**Standard deviation**	**p value**
1.1 V	0.96	0.01	NA
2.1 PV	0.71	0.06	0.12
2.2 SV	0.53	0.06	0.00011
2.3 AV	0.93	0.06	1.00
2.4 OV	0.87	0.04	1.00
3.1 PSV	0.60	0.08	0.0023
3.2 PAV	0.77	0.07	0.77
3.3 POV	0.71	0.16	0.11
3.4 SAV	0.47	0.17	0.0000010
3.5 SOV	0.64	0.11	0.0092
3.6 AOV	0.97	0.05	1.00
4.1 PSAV	0.67	0.23	0.043
4.2 PSOV	0.48	0.22	0.00000080
4.3 PAOV	0.84	0.09	0.84
4.4 SAOV	0.71	0.11	0.031
5.1 PSAOV	0.83	0.19	0.66

Table 5.S2. Growth partner experiment statistical model details. This table outlines the constituents of a statistical model fitted to the data of the experiment testing *Variovorax* plasmid maintenance in the presence of various growth partners. Significance values of individual model constituents are derived by Chi test. This model is a binomial model with logit link function and was further reduced by removal of non-significant (Pr>0.05) constituents for final data analysis for Figure 5.3.

Model function: V_fraction ~ Treatment + replicate + comp_P + comp_S + comp_A + comp_O

Variable	Description	Probability of significance (Pr |>Chi|)
V_fraction	GFP+ fraction of *Variovorax* colonies	NA (response variable)
Treatment	Treatments from 1.1-5.1 as in Table 5.S1	2.86×10^{-11}
replicate	Replicate 1-5 for each treatment	0.79
comp_P	Proportion of *Pseudomonas* within community.	0.45
comp_S	Proportion of *Stenotrophomonas* within community.	0.0032
comp_A	Proportion of *Achromobacter* within community.	0.0016
comp_O	Proportion of *Ochrobactrum* within community.	0.20

Plasmid maintenance-fitness correlation (Figure 5.5) The dataset combines selection coefficient data (Figure 5.2) with plasmid maintenance data (Figure 5.3). To synthesise matching treatments, I built a general linear mixed-effects model using replicates from each experiment as random effects. This was done to statistically investigate all datapoints, rather than just arithmetic means. Only arithmetic means and standard deviation are displayed in Figure 5.5 because aligning these metrics for each replicate across the two experiment is not meaningful.

General linear mixed-effects model: Plasmid-bearing *Variovorax* fraction as a function of plasmid selection coefficient, with random intercept effects of fitness experiment replicate and maintenance experiment replicate. F=5.2581. 25 observations with 2x 5 random-effect groups, conditional R^2 = marginal R^2 = 0.1797. Chi-test of inclusion of selection coefficient confirms this variable is a significant constituent of the model; p = 0.023.

Community maintenance experiment (Figure 5.6) A single extremely influential datapoint was removed for statistical analyses (P+pKJK5::GFP community treatment at T5; the only replicate where *Pseudomonas* was detected with one GFP- colony). A small amount of monoculture samples was contaminated with colonies of other species (2 samples at T5, 4 samples at T17). These replicates were entirely removed for all data visualisation and analyses, so N=4-5 for all treatments. Binomial GLM with logit link function: Plasmid-bearing colony fraction as a function of the interaction of Timepoint, Species, culture conditions (monoculture/community), and plasmid type (pKJK5::GFP / pKJK5::Cas[nt2]). F=0.471, df=48 & 185, pseudo R^2=0.987.

Results

pKJK5::Cas transfer between soil isolates is independent of phylogeny

I aimed to understand pKJK5::Cas transfer between soil isolates which can form a stable microbial community (Castledine *et al.*, 2020). To assess this, I set up individual mating pairs where each of *Pseudomonas* (P), *Stenotrophomonas* (S), *Achromobacter* (A), *Ochrobactrum* (O), and *Variovorax* (V) were donors of and recipients to non-targeting pKJK5::Cas[nt2]. Additionally, I included treatments where a mixed community consisting of these five species was used as a donor, and where *E. coli* K12::mCherry (E) was used to deliver either pKJK5::Cas[nt2] or pKJK5::Cas[aphA99] (which targets *aphA*; not present in the model system) to the soil isolates. Due to the soil isolates' antibiotic resistance profiles, mating pairs with *Pseudomonas* or *Achromobacter* as recipients could not be assessed, except for *E. coli* donors and *Pseudomonas* recipients.

The conjugation efficiency (proportion of recipients which took up pKJK5::Cas) varied for each mating pair (Figure 5.1C). Fitting a generalised linear model revealed that there is strong evidence that donor identity ($p=0.0066$) and recipient identity ($p=0.0063$) are predictors of conjugation efficiency, but there was no evidence that phylogenetic distance (Figure 5.1B) can predict mating outcome in this model system ($p=0.55$; see methods for model details). In line with this, intraspecific plasmid transfer was not associated with the highest conjugation efficiency. For all species, either *E. coli* or *Variovorax* were the most effective donors. Intraspecific *Variovorax* conjugation is highly effective (82.9%), but not significantly different compared to use of *E. coli* as a donor (24.2%; $p=0.953$). Interestingly, transfer of pKJK5::Cas[aphA99] by *E. coli* to *Stenotrophomonas* and *Variovorax* was impaired in comparison to pKJK5::Cas[nt2] transfer ($p=1.37 \times 10^{-11}$ and $p=1.18 \times 10^{-11}$ respectively, see methods for model details), despite no target sequences being present in any strain to the best of my knowledge.

Overall, analysing pKJK5::Cas transfer frequency in individual mating pairs revealed that *E. coli* K12::mCherry was the best donor to transfer pKJK5::Cas to these isolates. *Variovorax* was the only donor native to the community that could successfully deliver the plasmid to each member (Figure 5.1A). These dynamics were largely driven by *Variovorax*' non-permissive nature as a recipient, where

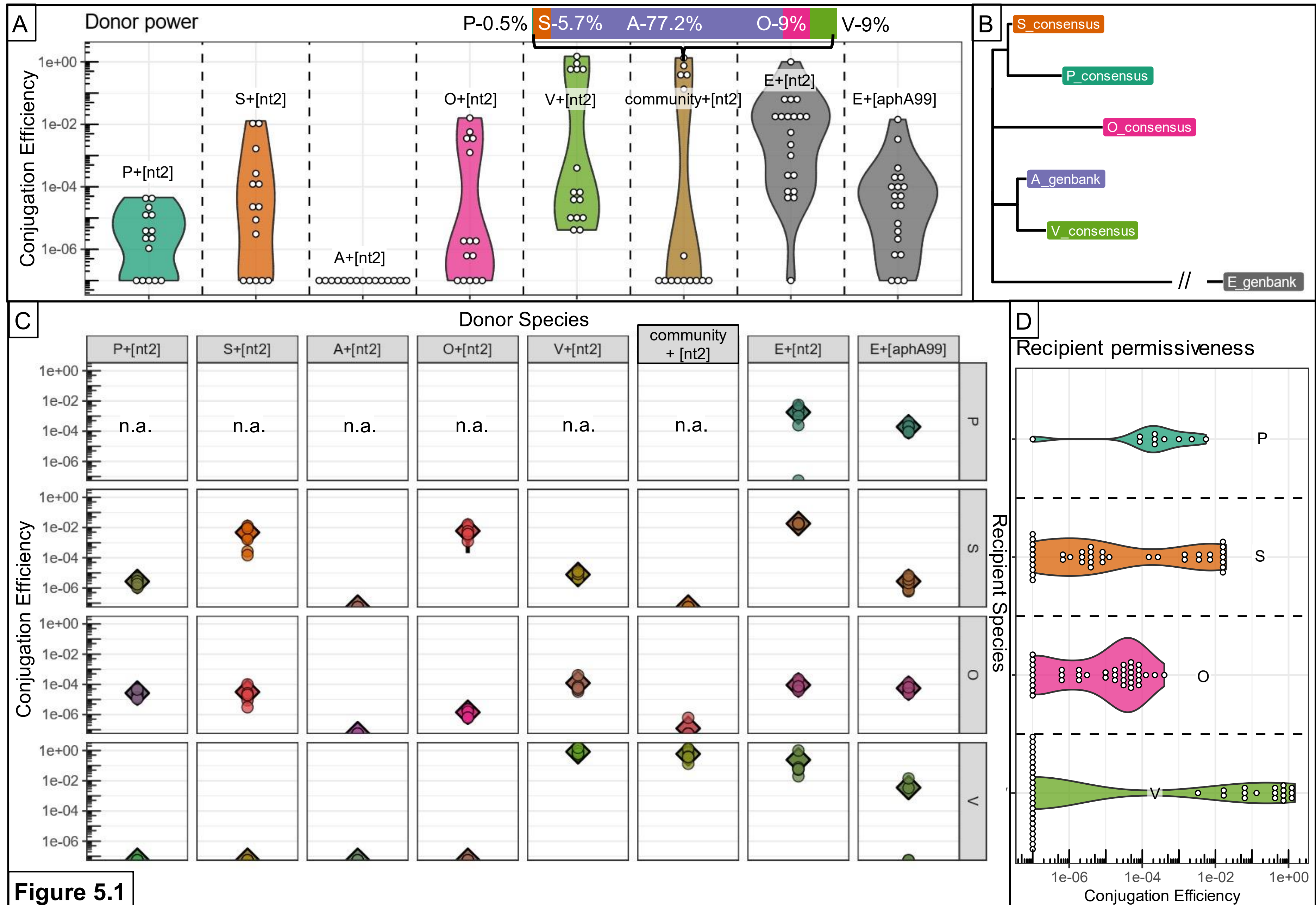

Figure 5.1

Figure 5.1 (previous page) **Transfer dynamics of pKJK5::Cas** between individual mating pairs of *Pseudomonas sp.* (P), *Stenotrophomonas sp.* (S), *Achromobacter sp.* (A), *Ochrobactrum sp.* (O), *Variovorax sp.* (V), and *E. coli* K12::mCherry (E). An additional 'community' donor treatment consists of a mixture of P, S, A, O, and V. Conjugation Efficiency (transconjugants per recipients) was assessed by selective plating after filter-mating, n=5. Donors either carry pKJK5::Cas[nt2] ([nt2]) or pKJK5::Cas[aphA99] ([aphA99]). Values of 0 were manually set to 10^{-7} to allow plotting on a logarithmic axis. **A Donor power**: Dotplot of conjugation efficiency of all mating samples using each donor, describes how well each donor can mobilise pKJK5::Cas to various species. Bar and values above 'community' treatment indicate average community composition at T0. **B Species relatedness:** Phylogenetic tree based on either the consensus of several 16S sanger sequencing reactions ('consensus'), or the 16S sequence of the most closely related strain found on Genbank ('genbank'). All branch lengths are to scale except *E. coli*'s, which is ~10x longer than all other branches. **C Individual mating pairs:** Mean ± standard deviation (diamonds, lines) and individual datapoints (circles) of transconjugant frequencies for each individual mating pair. n.a.-not assessed; mating pair could not be formed. **D Recipient permissiveness:** Dotplot of conjugation efficiency of all mating samples using each recipient, describes how permissive each recipient is to pKJK5::Cas uptake from various species.
Donor identity (A; $p<0.01$) and Recipient identity (D; $p<0.01$) are significant predictors of conjugation efficiency. There is no evidence that phylogenetic distance (B; $p=0.55$) plays a role. See Methods for model details.

no transconjugants could be recovered for any other donor (Figure 5.1C-D). Interestingly, relatedness did not determine transfer between these species, and there may be difficulties associated with transfer of pKJK5::Cas[aphA99] through natural communities.

pKJK5::Cas[nt2] is costly to *Variovorax*

Due to the unique transfer dynamics observed for *Variovorax sp.*, I chose this strain as a focal species to further investigate the fitness dynamics pKJK5::Cas[nt2] provides to its host. I hypothesised that the cost of carrying pKJK5::Cas to *Variovorax* depends on the microbial community context.

To test this, I measured the fitness costs of carrying the plasmid by competing plasmid-bearing *Variovorax* that was chromosomally tagged with Gentamicin resistance gene *aacC1* (V(GmR)) with pKJK5-free *Variovorax* that was chromosomally tagged with Chloramphenicol resistance gene *catR* (V(CpR)). These clones were competed either on their own, or in the presence of each growth partner (P, S, A, or O). To enable visualisation of plasmid transfer, and to ensure that the plasmid carries a fitness cost for the host, I used pKJK5::Cas[nt2]

(Chapter 3), which encodes green fluorescent protein (GFP) and *cas9* and non-targeting *sgRNA* as a genetic payload (Figure 5.S1B). As a control, I also competed pKJK5-free V(GmR) with pKJK5-free V(CpR) in each of these contexts.

This revealed that pKJK5::Cas[nt2] carriage was associated with a fitness cost to *Variovorax* in monoculture (Figure 5.2). The plasmid selection coefficient did not alter in the presence of *Achromobacter* or *Ochrobactrum*. However, the presence of either *Pseudomonas* or *Stenotrophomonas* significantly decreased the selection coefficient of pKJK5::Cas[nt2] ($p<0.001$ and $p<0.05$ respectively; see methods for model details). This demonstrates that carrying pKJK5::Cas[nt2] was more costly to *Variovorax* in the presence of these species compared to monoculture.

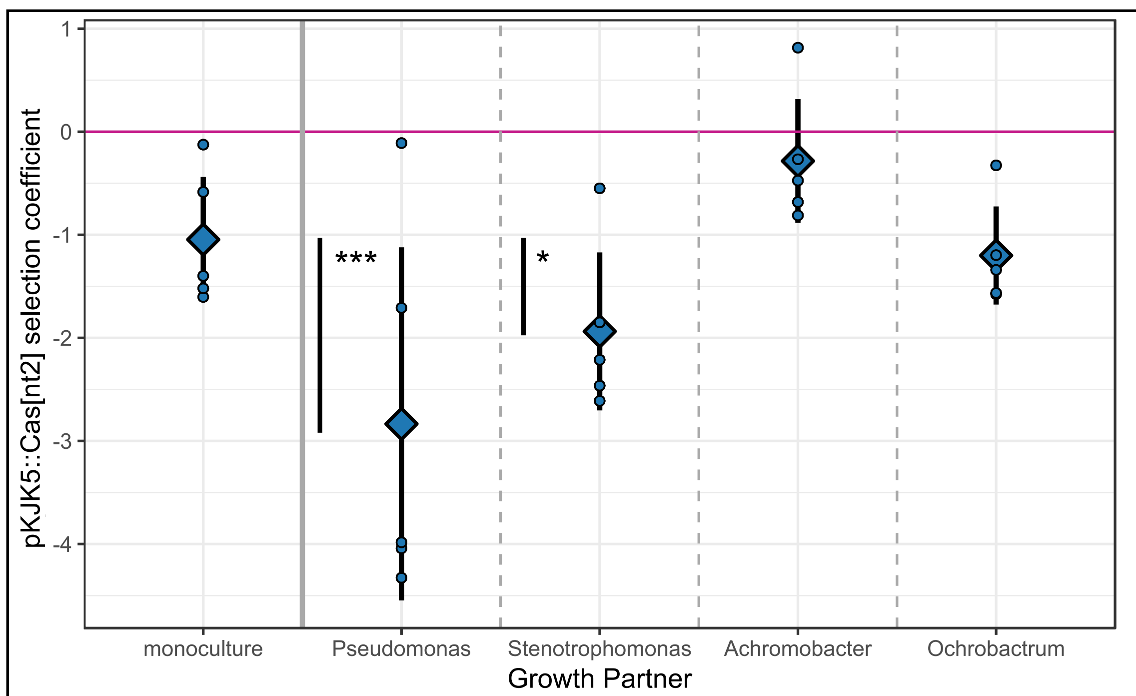

Figure 5.2 **Addition of growth partners alters pKJK5::Cas[nt2]'s selection coefficient.** Mean ± standard deviation (including individual data points) of pKJK5::Cas[nt2] selection coefficient for *Variovorax* in monoculture and with different growth partners after 5 days of co-culture. Values >0 indicate a fitness benefit and values <0 indicate a fitness cost of carrying pKJK5::Cas[nt2] in each context. ***$p<0.001$, *$p<0.05$ as calculated by Tukey's HSD after fitting a Gaussian GLM; $F=20.71$; df=9 & 140; adjusted $R^2= 0.5435$; N=5.

pKJK5::Cas[nt2] maintenance is linked to its costs and is community-dependent

Next, I hypothesised that the increased fitness cost of pKJK5::Cas[nt2] to *Variovorax* in the presence of *Pseudomonas* and *Stenotrophomonas* would lead to decreased plasmid maintenance in their presence.

To test this, I generated 16 different synthetic microbial communities composed of all possible combinations of one-, two-, three-, four-, and five-species of *Variovorax* carrying pKJK5::Cas[nt2] with plasmid-free *Pseudomonas*, *Stenotrophomonas*, *Achromobacter,* and/or *Ochrobactrum* (Figure 5.S2). I measured plasmid maintenance after 5 days of co-culture. This revealed that in monoculture nearly all *Variovorax* clones in the population retained pKJK5::Cas[nt2] (Figure 5.3). In contrast, plasmid maintenance in *Variovorax* was significantly decreased in several of the synthetic communities. Interestingly, these corresponded to the communities that contain *Stenotrophomonas*, with the sole exception of the full 5-species community where the reduction in *Variovorax* plasmid maintenance was not significant ($p=0.65$; see methods for model details).

To quantify the relative contribution of the different species to the observed plasmid loss in *Variovorax*, I measured the proportion of each constituent species and plotted this against the proportion of plasmid-bearing *Variovorax* for each community (Figure 5.4).

This reveals that the proportion of *Pseudomonas*, *Ochrobactrum*, and *Variovorax* did not show a significant association with the proportion of *Variovorax* that carried pKJK5::Cas[nt2]. However, both the proportions of *Stenotrophomonas* ($p = 0.0023$) and of *Achromobacter* ($p = 0.0036$) were statistically significant when fitting a model to these data (Table 5.S2). There was a clear negative association between the proportion of *Stenotrophomonas* and the plasmid-bearing *Variovorax* fraction. Interestingly, *Achromobacter* had the opposite effect: its presence was associated with higher plasmid maintenance, although this effect was found to be comparatively small ($R^2 = 0.18$ for *Achromobacter* proportion; $R^2 = 0.39$ for *Stenotrophomonas* proportion, Figure 5.4).

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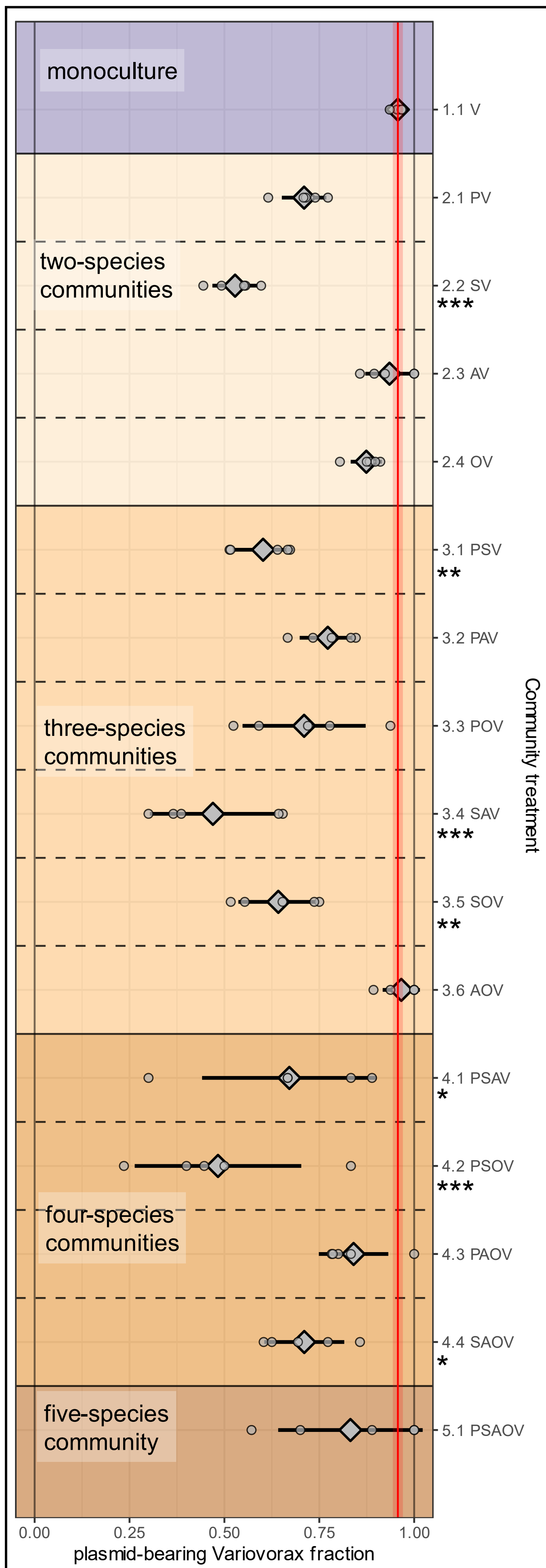

Figure 5.3 **Different combinations of growth partners elicit different *Variovorax* plasmid loss effects.** Mean ± standard deviation (including individual data points) of GFP+ *Variovorax* (V) fraction as a proxy of plasmid-bearing *Variovorax* in presence of various growth partners (*Pseudomonas* (P), *Stenotrophomonas* (S), *Achromobacter* (A), or *Ochrobactrum* (O)) after 5 days of co-culture. For comparison, the vertical red line and shaded areas indicate the mean ± standard deviation of the monoculture treatment. Stars indicate treatments with significantly lower GFP+ fraction than in monoculture. See Table 5.S1 for values of these summary statistics.
*p<0.05, **p<0.01, ***p<0.001 as calculated by Tukey's HSD after fitting a binomial GLM; F=0.4635; df=17 & 62; pseudo R^2= 0.848; N=5.

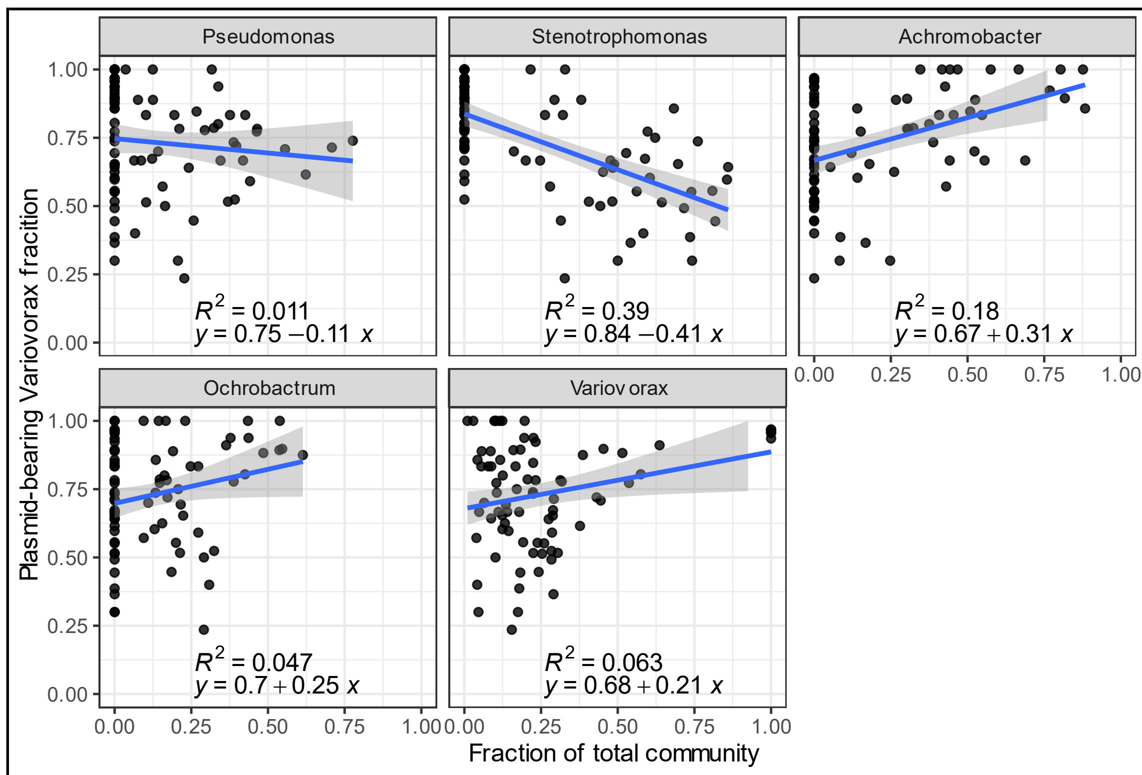

Figure 5.4 **The relationship of community composition and *Variovorax* plasmid maintenance.** Community composition of all samples of various communities as in Figure 2 plotted against the GFP+ fraction of *Variovorax* colonies as a proxy of plasmid-bearing *Variovorax*. Community composition is broken up into individual panels describing *Pseudomonas*, *Stenotrophomonas, Achromobacter, Ochrobactrum,* and *Variovorax* proportions of the whole community respectively. As the statistical model (Figure 5.3, Table 5.S2) is fitted to the full dataset, all datapoints including counts of 0 for each species are plotted. The relationships plotted in these panels are for visualization only and not used to determined statistical significance.

Blue lines and shaded areas indicate fitted linear models with equations and R^2 displayed in each panel. Of these five metrics, only *Stenotrophomonas* and *Achromobacter* fraction constitute significant terms of the statistical model fitted to the data; see Table 5.S2.

I hypothesised that the differences in plasmid maintenance across the synthetic communities were caused by differences in the cost of plasmid carriage for *Variovorax.* To explore this, I performed a correlational analysis between the selection coefficient of pKJK5::Cas[nt2] to *Variovorax* (Figure 5.2) and plasmid maintenance (Figure 5.3).

This revealed a clear association: treatments in which the *Variovorax* plasmid selection coefficient was higher also showed higher levels of *Variovorax* plasmid maintenance (Figure 5.5). The monoculture treatment and community treatments consisting of either *Achromobacter* or *Ochrobactrum* as a growth partner all cluster in the top-right quadrant, representing treatments where plasmid cost was

low and plasmid maintenance high. In contrast, community treatments that contained either *Pseudomonas* or *Stenotrophomonas* as a growth partner were associated with low plasmid maintenance and high costs of plasmid carriage for *Variovorax*.

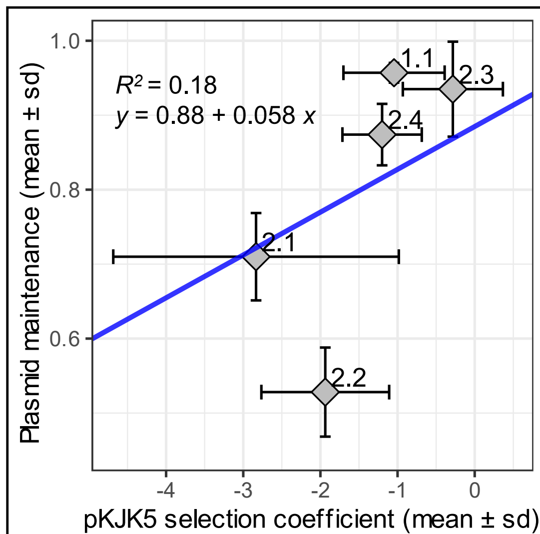

Figure 5.5 ***Variovorax* pKJK5::Cas[nt2] selection coefficient correlates with plasmid maintenance.**
Mean ± standard deviation of pKJK5::Cas[nt2] selection coefficient as in Figure 1 and plasmid-bearing *Variovorax* fraction in corresponding treatments as in Figure 2. Treatment designations indicate growth partners: 1.1 monoculture, 2.1 *Pseudomonas*, 2.2 *Stenotrophomonas*, 2.3 *Achromobacter*, 2.4 *Ochrobactrum.* Blue line indicates fitted mixed-effects linear model with equation and R^2 as displayed; details in methods. Selection coefficient is a significant determinant of plasmid-bearing *Variovorax* fraction, p=0.023.

pKJK5 plasmid maintenance is community-dependent in multiple species

Finally, to generalise the community-dependent effects on plasmid maintenance, I explored whether similar effects were observed in different host species and when the plasmid lacked *cas9* and *sgRNA* payload genes. The frequencies of pKJK5::Cas[nt2] and of pKJK5::GFP (Klümper *et al.*, 2015) (Figure 5.S1C) were measured over 17 days in five different hosts (*Pseudomonas, Stenotrophomonas, Achromobacter, Ochrobactrum, Variovorax*) that were either cultured individually or that were cultured together to form a stable microbial community. For both pKJK5 variants, maintenance of the plasmid strongly depended on host identity; *Ochrobactrum* retained pKJK5::GFP and pKJK5::Cas[nt2] to high levels (Figure 5.6), while *Pseudomonas* and *Achromobacter* lost these plasmids during the course of the experiment. Notably, these differences in plasmid maintenance were independent of the community context in which the hosts were cultured.

As expected, *Variovorax* plasmid maintenance was dependent on the community context it was cultured in. In monoculture, 93% of colonies retained pKJK5::Cas[nt2] after 5 days. In contrast, significantly fewer (57%) *Variovorax*

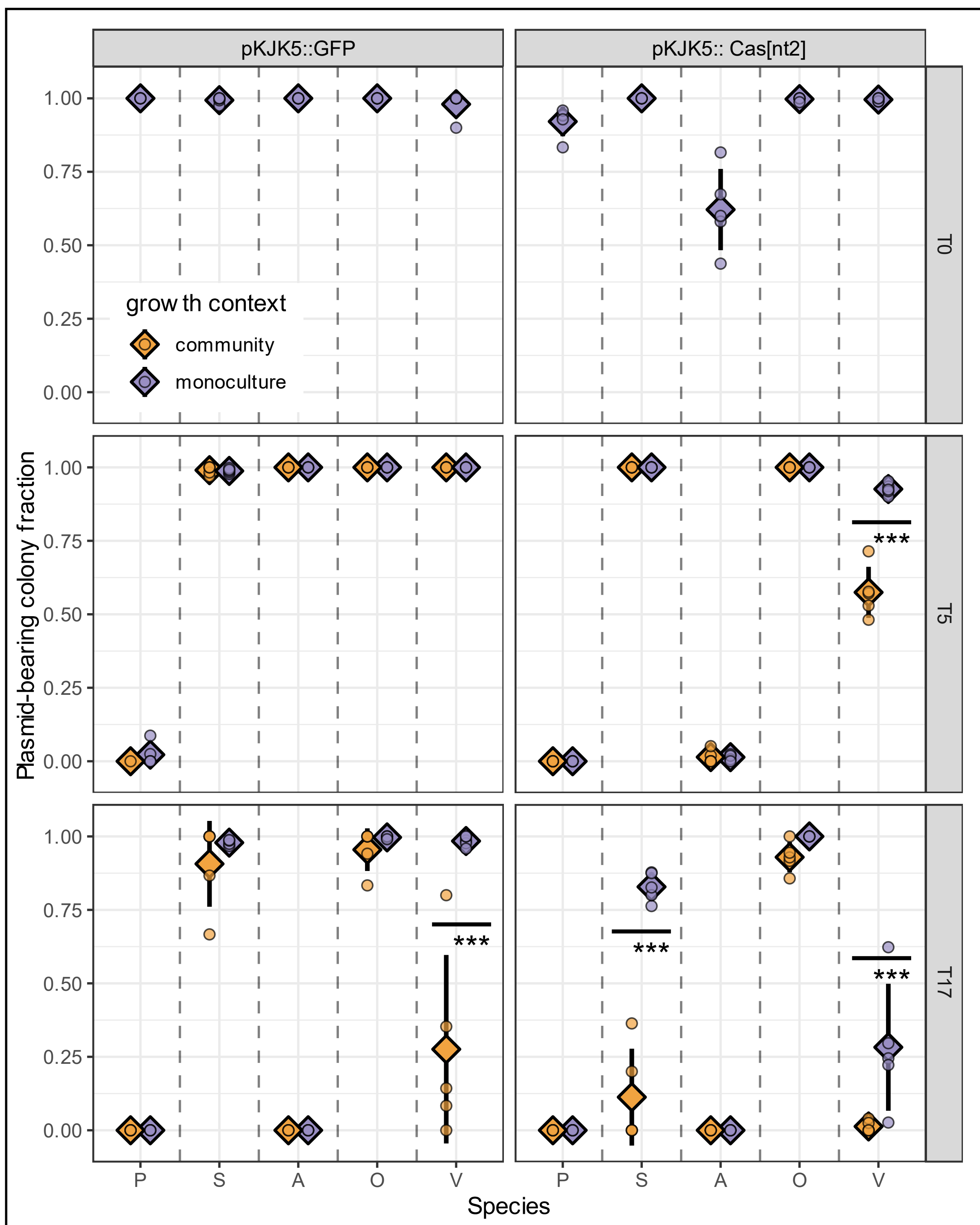

Figure 5.6 ***Variovorax* and *Stenotrophomonas* pKJK5 maintenance is dependent on community context.** Mean ± standard deviation (including individual data points) of GFP+ fraction of colonies as a proxy of pKJK5::GFP or pKJK5::Cas[nt2] maintenance. Data is split into species *Pseudomonas* (P), *Stenotrophomonas* (S), *Achromobacter* (A), *Ochrobactrum* (O), and *Variovorax* (V) after 5 and 17 days of growth in monoculture or in a community context. T0 indicates plasmid-maintaining proportion of strains used to start the experiment. Left-hand panels indicate bacteria containing pKJK5::GFP, right-hand panels indicate bacteria containing pKJK5::Cas[nt2].
***$p < 0.001$ as calculated by Tukey's HSD after fitting a binomial GLM; F=0.471; df=48 & 185; pseudo R^2= 0.987; N=5. No other treatment combinations are significantly different from each other.

1

colonies within the community retained pKJK5::Cas[nt2] ($p<0.001$; see methods for model details). This effect remained evident after 17 days of culture (28% *Variovorax* plasmid maintenance in monoculture, 1.3% maintenance in community context). Similar dynamics could be observed for *Variovorax* carrying pKJK5::GFP after 17 days, where 98% of colonies retained pKJK5::GFP in monoculture compared to 28% of colonies in a community context.

Plasmid maintenance in *Stenotrophomonas* remained very high for both pKJK5 variants after 5 days, independent of community context. However, after 17 days, *Stenotrophomonas* pKJK5::Cas[nt2] maintenance remained significantly higher in monoculture (83%) than in a community context (11%; $p<0.001$). While no statistical difference of *Stenotrophomonas* pKJK5::GFP maintenance was observed, average maintenance of pKJK5::GFP was slightly lower in community context after 17 days of growth (91% maintenance in community vs. 98% maintenance in monoculture).

Overall, both pKJK5 variants followed similar maintenance dynamics in all host species. Community-dependent pKJK5::Cas[nt2] plasmid loss occurred in *Variovorax* and *Stenotrophomonas* after a shorter time period than loss of pKJK5::GFP (Figure 5.6), probably due to pKJK5::Cas[nt2]'s larger genetic payload (Figure 5.S1).

Discussion

In this chapter, I aimed to assess pKJK5::Cas transfer and maintenance in a microbial community context. The initial experiment aimed to assess efficiency of pKJK5::Cas[nt2] transfer between individual natural isolates. While conjugation efficiency can be dependent on relatedness (Dimitriu *et al.*, 2019), this is not the case in this model system. Instead, transfer dynamics are dominated by non-permissive *Variovorax* recipients and poor *Achromobacter* donors. The failure of transconjugants to be recovered when *Achromobacter* is a donor (Figure 5.1A) is probably due to very poor maintenance of pKJK5::Cas[nt2] in this species (Figure 5.6). Similarly, relatively poor transfer when using the mixed community as a donor can also be attributed to this, as the community is dominated by *Achromobacter* when growing under these conditions (Figure 5.1A). Interestingly, community-mediated conjugation to *Variovorax* remained effective (Figure 5.1B). This is especially unexpected in the light of *Variovorax* pKJK5::Cas[nt2] plasmid loss when growth partners are present (Figures 5.3-6), and suggests that

Variovorax can mediate intraspecific plasmid transfer even when donors are present at low frequencies.

The remaining part of this work aimed to understand how costs and maintenance of conjugative plasmid pKJK5::Cas[nt2] can be determined by the microbial community context of its host. pKJK5 is not known to encode functional toxin-antitoxin systems (Li *et al.*, 2020), and its re-infection rate is negligible in these experiments: throughout the competition experiment, 14 out of a total of 2240 colonies of the initially plasmid-free tag variant were found to be GFP+ (~0.6% transconjugants/recipients). Therefore, plasmid selection coefficient is the most important metric to predict maintenance in this model system. Despite the presence of *Pseudomonas* increasing the relative cost of the plasmid (Figure 5.2), *Pseudomonas* community proportion did not significantly influence *Variovorax* plasmid maintenance throughout the subsequent growth partner experiment (p=0.45, Table 5.S2). This is probably because total *Pseudomonas* proportion was typically low in mixed communities, not giving it much opportunity to elicit fitness-altering effects.

In pKJK5-free conditions, the five model species form a locally mal-adapted community in which the most common form of community interactions is resource competition. No bacterial warfare in form of direct growth inhibition or killing of growth partners takes place in this system (Castledine *et al.*, 2020; Padfield *et al.*, 2020). In pairwise interactions, *Variovorax* benefits from the presence of all other community members (Padfield *et al.*, 2020). Therefore, differences in fitness are likely to be a result of resource limitation during growth together with growth partners. In line with this, an increase in fitness costs of chromosomal mutations (Alseth *et al.*, 2019) and of chromosomal AMR genes (Klümper *et al.*, 2019) to focal species when embedded within a microbial community has previously been observed.

I found that due to increased costs of plasmid carriage, pKJK5::Cas[nt2] maintenance was decreased when focal strain *Variovorax sp.* was placed in a community context. Interestingly, Hall *et al.* (2016) found the opposite effect: in monoculture, a mercury resistance plasmid was rapidly lost from its host species. Together with growth partners, the plasmid was maintained in the focal strain due

to reinfection by conjugation (Hall *et al.*, 2016; Kottara *et al.*, 2021a). This phenomenon of plasmid persistence through conjugation can be observed for multiple types of plasmids, and in communities consisting of several *E. coli* strains and plasmids (Lopatkin *et al.*, 2017). Together, these studies show that in an experimental system where conjugation is highly relevant, a community context can increase plasmid maintenance in a focal species by providing a reservoir of plasmids in other hosts for reinfection. In contrast, our data show that in conditions where conjugation only occurs at very low levels, community context can decrease plasmid maintenance. This suggests an intricate interplay of abiotic and biotic conditions underpinning plasmid maintenance. Further, recent work indicates that a community context may limit conjugation to focal species due to the dilution effect (Kottara *et al.*, 2021b), which suggests a higher importance of plasmid loss due to increased costs in more complex communities.

Plasmid fitness costs are not fixed; they depend on several variables. Firstly, costs evolve over time. For instance, plasmid costs can be completely eliminated by rapid compensatory mutations in host and plasmid genomes (Stalder *et al.*, 2017; Hall *et al.*, 2019). These can be pleiotropic and allow evolved hosts to maintain other plasmids, too (Jordt *et al.*, 2020). Therefore, plasmid costs lose importance for long-term maintenance dynamics when they arise as a result of a specific genetic conflict between the plasmid and host chromosome (Hall *et al.*, 2021). Secondly, interactions between co-infecting plasmids may alter plasmid cost and affect maintenance (Gama *et al.*, 2020), and plasmid co-existence is usually selected against when they encode redundant traits (Carrilero *et al.*, 2021). More generally, work with other mobile genetic elements (MGEs) such as bacteriophage (phage) has shown that a microbial community context can have a range of ecological and evolutionary effects on bacteria-MGE interactions of focal species. Community presence tends to decrease focal species and phage densities, while evolutionary effects can be more diverse (Blazanin and Turner, 2021). It is unknown whether *Variovorax* or the other isolates carry their own plasmids or other MGEs, but a fitness conflict between pKJK5::Cas[nt2] and a resident plasmid which is essential only in a community context could explain the observed maintenance dynamics. Such a fitness conflict was observed between pKJK5::Cas[nt] and cloning vector pHERD30T in Chapter 3. Together, this

highlights that different biotic and abiotic conditions could lead to evolution of different plasmid maintenance outcomes in our model system, where compensation of plasmid costs to *Variovorax* was not observed during the 17-day period.

I found that plasmid maintenance was community-dependent in not only *Variovorax* but also *Stenotrophomonas* (Figure 5.6). Therefore, it is very likely that other species beyond this model system maintain plasmids in a community-dependent manner. For instance, an *E. coli* pathogenicity plasmid was found to become depleted from its host strain during the course of an infection in the human gut, while it was stably maintained in laboratory settings or vegetable-associated communities (Zhang *et al.*, 2013). Perhaps this was due to a different microbial community context in the human gut, especially as a large range of abiotic factors could not recapitulate loss of this virulence plasmid *in vitro* in preliminary results (Whelan and McVicker, 2021). Bearing this in mind, community-dependent plasmid loss could have wider implications: In healthcare, virulent species could lose a plasmid containing virulence factors or antibiotic resistance genes in a new community context. This would mean risk assessments carried out for environmental hotspots of horizontal gene transfer (Andersson and Hughes, 2014) may have to be reassessed, where plasmid transfer in e.g. a livestock farm would not necessarily guarantee plasmid persistence in a human microbiome. Furthermore, community-level plasmid maintenance is typically dependent on dozens of highly permissive member species which readily take up and maintain plasmids (Klümper *et al.*, 2015; Li *et al.*, 2020). If further experiments reveal that previously characterised highly permissive plasmid hosts lose these properties when placed into a different community context, this would have impacts on plasmid prevalence within an entire microbial community.

I observed an increased cost of plasmid carriage when pKJK5 carries payload genes *cas9* and *sgRNA*, even when not targeting a relevant sequence (Figure 5.6; pKJK5::Cas[nt2] becomes lost more rapidly than pKJK5::GFP). *Cas9* on pKJK5::Cas[nt2] is under control of a standard *bla* cloning vector promoter and expressed at relatively high levels. Generally, heterologous expression of Cas9

can be toxic to some bacterial species (Jiang *et al.*, 2017; Zhang *et al.*, 2018) and even catalytically inactive Cas9 can be toxic to *E. coli* when overexpressed (Cho *et al.*, 2018). Furthermore, transfer of pKJK5::Cas[aphA99] to *Stenotrophomonas* and *Variovorax* was impaired in comparison to pKJK5::Cas[nt2] transfer (Figure 5.1, $p=1.37 \times 10^{-11}$ and $p=1.18 \times 10^{-11}$ respectively; see methods for model details). A BLAST search of the aphA99 and nt2 target sequences revealed no hits against partially assembled genomes for these isolates. This suggests that when natural sequences rather than randomly generated nucleotide sequences are the sgRNA target, conjugation to some natural isolates may be impaired due to unexpected off-target effects. Therefore, Cas9 expression and targeting of natural sequences may be associated with fitness costs in many bacterial species – which has implications on the utility of pKJK5::Cas and other CRISPR delivery tools. Even if a CRISPR-plasmid is effective at AMR gene removal in a simple, two-strain setup (Chapters 3-4), it may not be effective in a microbial community due to reduced plasmid maintenance.

Finally, this work opens exciting research avenues for manipulation of plasmid content of focal species. For example, removal of virulence and resistance plasmids within pathogens might be achieved by addition of certain plasmid loss-inducing growth partners such as *Stenotrophomonas* as a plasmid-targeted probiotic treatment.

Conclusion

In this chapter, I assessed pKJK5::Cas transfer and maintenance in a model community context. I found that plasmid transfer of pKJK5::Cas between soil isolates was not driven by their phylogeny, but that dynamics were dominated by non-permissive *Variovorax* recipients. Furthermore, I discovered that the fitness costs of plasmid carriage are influenced by growth partner presence. Accordingly, plasmids were depleted from focal species in a community-dependent manner. These data highlight the importance of variable plasmid costs when considering plasmid maintenance in a community context. In the context of previous literature, this work highlights an alternative outcome of embedding a plasmid host into a microbial community: in model systems with high conjugation rates, this leads to increased plasmid maintenance. In model systems with low conjugation rates such as mine, this leads to plasmid loss due to increased fitness costs.

1 While further work needs to address how general community-dependent plasmid
2 maintenance is, these findings may have important implications for maintenance
3 of virulence plasmids in different community contexts, for community-level
4 plasmid maintenance if similar effects are observed in highly permissive plasmid
5 hosts, and for the utility of conjugative plasmids for CRISPR-based
6 antimicrobials. The phenomenon of community-dependent plasmid loss could
7 find application by manipulating the prevalence of virulence and resistance
8 plasmids in target strains upon addition of certain community members.

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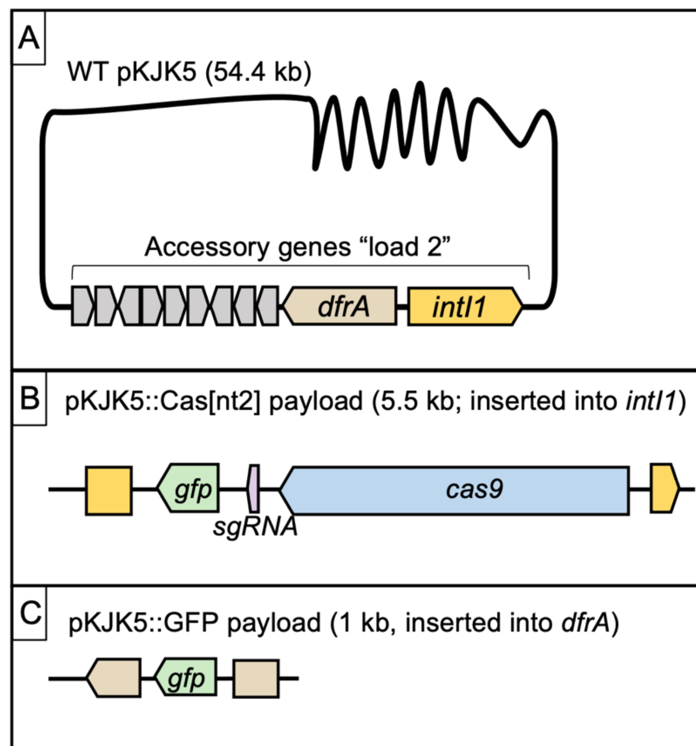

Figure 5.S1 **pKJK5 variants.** Block arrows indicate ORFs, length not to scale. **A WT pKJK5**. Published by Bahl *et al.*, 2007, genbank acc. AM261282.1. Indicated section is accessory gene load 2 spanning from nts 21682-33379. **B pKJK5::Cas[nt2]** payload is inserted at position 22540 within *intI1* and consists of SpyCas9 and non-targeting sgRNA with constitutive promoters as well as GFPmut3b as in c. **C pKJK5::GFP** payload is inserted at position 23107 and consists of GFPmut3b with lacI-repressible promoter. Published by Klümper *et al.* 2015.

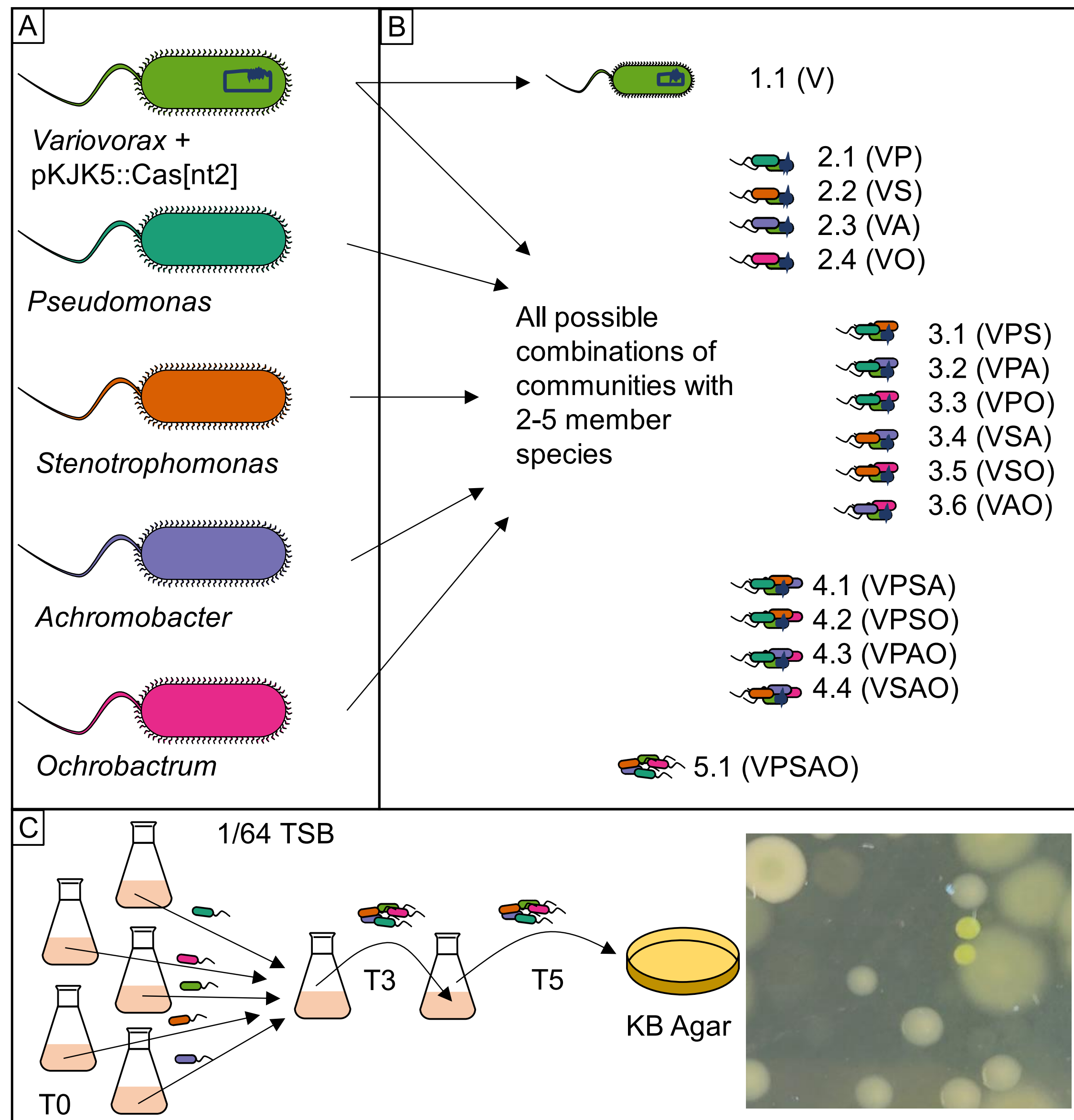

Figure 5.S2 **Layout of Growth Partner Experiment.**
A Strains at T0. *Variovorax* carries pKJK5::Cas[nt2], all other strains do not. **B Various community treatments.** *Variovorax* was passaged in monoculture (1.1) or with 1-4 growth partners. **C Experimental setup.** T0 strains were co-incubated (in absence of selection) and transferred at T3. At T5, communities were plated onto KB agar and community composition was determined by counting colony morphologies. Plasmid-bearing *Variovorax* fraction was determined by analysing plates underneath a fluorescence lamp (not shown).

General Discussion

In this thesis, I developed pKJK5::Cas, a broad host-range plasmid which encodes *cas9* and an *sgRNA* to protect its hosts from AMR plasmid uptake (Chapter 2). Applied conjugatively, pKJK5::Cas was able to remove resident AMR plasmids from recipient bacteria. The extent of this removal was dependent on conjugation efficiency and CRISPR targeting efficiency (Chapter 3). Additionally, TA system presence and target plasmid incompatibility protected from their removal (Chapter 4). When allowed to spread in a synthetic bacterial community, pKJK5::Cas became lost from a focal species in the presence of certain bacterial community members (Chapter 5).

In this discussion, I begin by discussing shortcomings of experiments carried out throughout this thesis, followed by pKJK5::Cas plasmid removal in the context of its application to a synthetic microbial community. Finally, I consider which real-world applications may be best suited to pKJK5::Cas and suggest means of making this CRISPR delivery tool more effective.

Development of pKJK5::Cas and experimental shortcomings

When designing the CRISPR-Cas9 cassette *in silico* (which would be used for recombination with pKJK5; Chapter 2), I decided to use the nuclease Cas9 from *Streptococcus pyogenes'* Type II CRISPR system (SpyCas9). Cas nucleases from other CRISPR systems, such as Cpf1, are smaller and therefore more pliable for genetic engineering. However, at the time of study conception literature was peppered with conflicting information on protospacer adjacent motif (PAM) requirements and stringency (e.g. (Kim *et al.*, 2017; Yamada *et al.*, 2017)), while SpyCas9 had been thoroughly studied with a well-defined PAM and cleavage efficiency.

To assess how well pKJK5::Cas can remove target plasmids, I used selective plating throughout my experiments. Despite its ease and relative high throughput, this technique has some drawbacks:

Firstly, colony counts based on selective plating can be inaccurate. For instance, conjugation efficiency after filter mating (Figure 6.1A) and target plasmid proportions of *par*-encoding and incompatible target plasmids (Figure 6.1C) exceeded 100%. Despite this, selective plating was sensitive enough to detect clear differences between treatments by showing that target plasmid removal was

1 dependent on conjugation efficiency for targeting treatments only (Figure 6.1B,
2 Figure 6.1D).

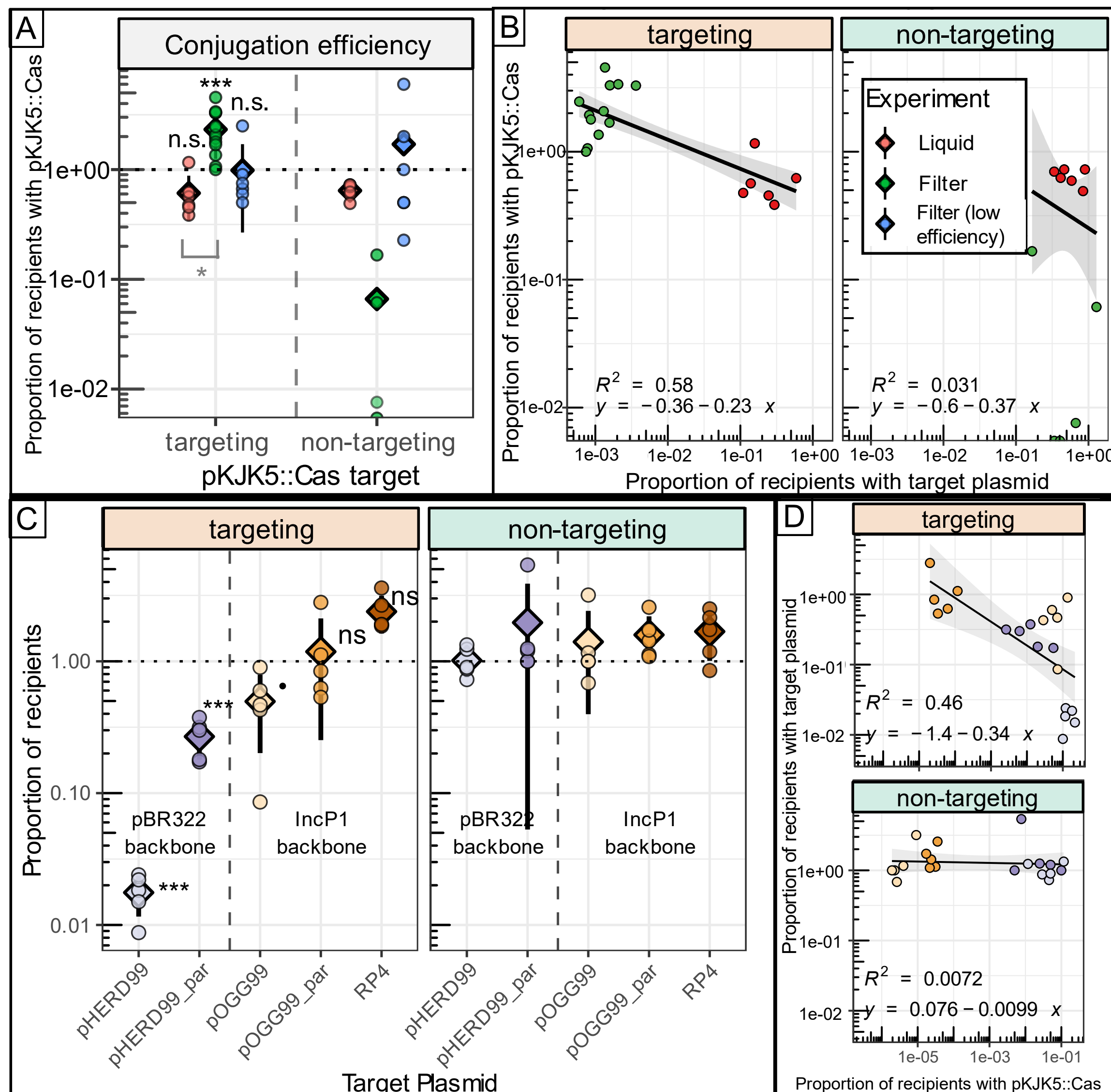

Figure 6.1: **Selective plating can lead to inaccurate proportions.**
Panels reproduced from Figures throughout thesis. A-Figure 3.1B; B-Figure 3.1C; C-Figure 4.2; D-Figure 4.3B.
A-B After filter mating, selective plating gives conjugation exceeding 100% (A). Despite this, correlating these data with corresponding target plasmid proportions is significant for targeting pKJK5::Cas only (B), indicating that selective plating is sufficient to detect removal effects.
C-D After filter mating, target plasmid proportions of incompatible and *par*-encoding plasmids can exceed 100% (C). Despite this, correlating these data with corresponding conjugation efficiencies is significant for targeting pKJK5::Cas only (D), indicating that selective plating is sufficient to detect removal effects.
*p<0.05; **p<0.01; ***p<0.001; ns not significant. Significance identifiers indicate treatments significantly different from corresponding non-targeting controls; see methods of respective chapters for model details.

The inaccuracy due to selective plating may have led me to miss more subtle difference between treatments, for example between removal of pOGG99_par, which encodes a single toxin-antitoxin (TA)-system, and removal of RP4, which encodes multiple stability systems and additional payload genes (discussed in Chapter 4).

Secondly, selective plating did not allow me to assess whether plasmids escaped CRISPR targeting by mutation of the target sequence and inactivation of their AMR genes. Past data show that, for CRISPR delivery tools, escape from CRISPR targeting is more likely to occur through delivery of defective CRISPR machinery than target mutation (Bikard *et al.*, 2014; Wan *et al.*, 2020). However, the differences in targeting efficiency I observed when the plasmid's target sequence was in or outside its AMR gene seem to speak against this consensus (Figure 3.2A; filter and filter (low eff.)), as this is analogous to bacteriophage mutations to escape from natural CRISPR targeting, the frequency of which is dependent on essentiality of the bacteriophage gene targeted by CRISPR (Watson *et al.*, 2019). This might indicate that escape by target plasmid mutation gains relevance when non-essential plasmid sequences are targeted.

Together, these points highlight that selective plating can be inaccurate for assaying plasmid maintenance. In addition, selective plating might not be possible when working with natural isolates or plasmids which can encode multiple AMR genes. Instead, the use of other means to determine plasmid presence, such as qPCR or fluorescent plasmid tagging coupled with flow cytometry should be considered for more complex model systems. Beyond this, methods such as sequencing would be needed to investigate the reason for plasmid escape of CRISPR targeting.

Application of pKJK5::Cas in a synthetic bacterial community

This project was aimed towards application of pKJK5::Cas to remove AMR genes from mixed microbial communities, but due to time and methodological constraints I could not thoroughly test target plasmid removal in my model community. Pilot trials of RP4 removal from this mixed community (consisting of *Pseudomonas, Stenotrophomonas, Achromobacter, Ochrobactrum,* and *Variovorax spp.*; PSAOV) showed no decreases of RP4 abundance in any treatments, and pKJK5::Cas conjugation efficiency was negligible when culturing these species in low-nutrient 1/64 tryptone soy broth at 28˚C (which are the

optimal growth conditions to promote long-term maintenance of community diversity).

However, thorough examination of the impact of pKJK5::Cas properties (conjugation efficiency, CRISPR targeting efficiency; Chapter 3) and target plasmid properties (*par* toxin-antitoxin (TA) presence, incompatibility; Chapter 4) which impact their removal, as well as investigation of pKJK5::Cas transfer and maintenance in the model community (Chapter 5) allow me to make predictions how target plasmids might be successfully removed from this synthetic bacterial community.

In the following, I consider which factors need to be taken into account when removing target plasmids from the PSAOV community, specifically (i) donor selection; (ii) pKJK5::Cas stability in the community; (iii) target plasmid properties; and (iv) pKJK5::Cas delivery strategy.

(i) Donor selection

Mating experiments revealed which donor may be best suited to deliver pKJK5::Cas to the entire PSAOV community (Figure 5.1). In isolation, *Escherichia coli* and *Variovorax* are the only species which could deliver this plasmid to all target bacteria. Use of *Variovorax* would optimise delivery to *Variovorax* recipients, but for all other species *E. coli* was the most proficient donor.

The stability of *E. coli* in this synthetic community context needs to be experimentally assessed. If it is rapidly outcompeted by the stably coexisting PSAOV, then donors native to the community, for instance *Stenotrophomonas* and *Variovorax* in combination, would be more appropriate. Furthermore, the relative success of mixed PSAOV donors – in which *Variovorax* is present at low frequency (~9%; Figure 5.1A) – suggests that either low-frequency use of *Variovorax*, or early low-frequency *Variovorax* transconjugants generated by a different donor are sufficient for effective spread into the *Variovorax* fraction of the recipient community.

In summary, pilot experiments would have to determine whether an individual *E. coli* donor or a donor cocktail consisting of *Stenotrophomonas* and low-frequency *Variovorax* would be more effective for pKJK5::Cas spread through this community.

(ii) pKJK5::Cas stability in the community

Even on filters – which led to very high (~100%) conjugation efficiency between *E. coli* strains (Figure 3.1B) – conjugation efficiency over 48 hours between PSAOV community members remained, in most cases, several orders of magnitude below this (Figure 5.1C). Therefore, to effectively reach various community members, pKJK5::Cas spread would have to take place over longer periods of time.

However, over time scales of 5-17 days, pKJK5::Cas was lost from most community members when grown in a community context (all except *Ochrobactrum*, Figure 5.6). Therefore, pKJK5::Cas maintenance would become an issue over longer incubation periods. Specifically, *Variovorax* (and to a lesser extent *Stenotrophomonas*) lost pKJK5::Cas in a community- and fitness-dependent manner where growth in the presence of a community led to more plasmid loss (Figure 5.6) and an increased plasmid cost to the host (Figure 5.2) compared to growth in monoculture. Interestingly, this effect was very context-dependent: Presence of all four plasmid-free growth partners in a previous experiment did not reduce *Variovorax* plasmid maintenance significantly, as opposed to their effect on *Variovorax* when all species carried pKJK5::Cas (Figure 6.2A & D). Presumably, whether or not community members carry pKJK5::Cas[nt2] has an effect on their fitness, too – and this results in a different community structure if all species carry the plasmid. For instance, 5-species communities in which only *Variovorax* carries pKJK5::Cas[nt2] tended to have lower proportions of *Pseudomonas* and *Variovorax* than communities in which all 5 species carried the plasmid (Figure 6.2). Ideally, these treatments from independent experiments would need to be tested side-by-side to draw definite conclusions.

In summary, depending on experimental setup, the community composition may not favour pKJK5::Cas maintenance. To counteract this, I propose amongst others to equip pKJK5::Cas with a toxin-antitoxin (TA) system (e.g. *parABCDE*) or to utilise sub-minimal inhibitory concentration (sub-MIC) tetracycline and trimethoprim antibiotic concentrations (discussed below).

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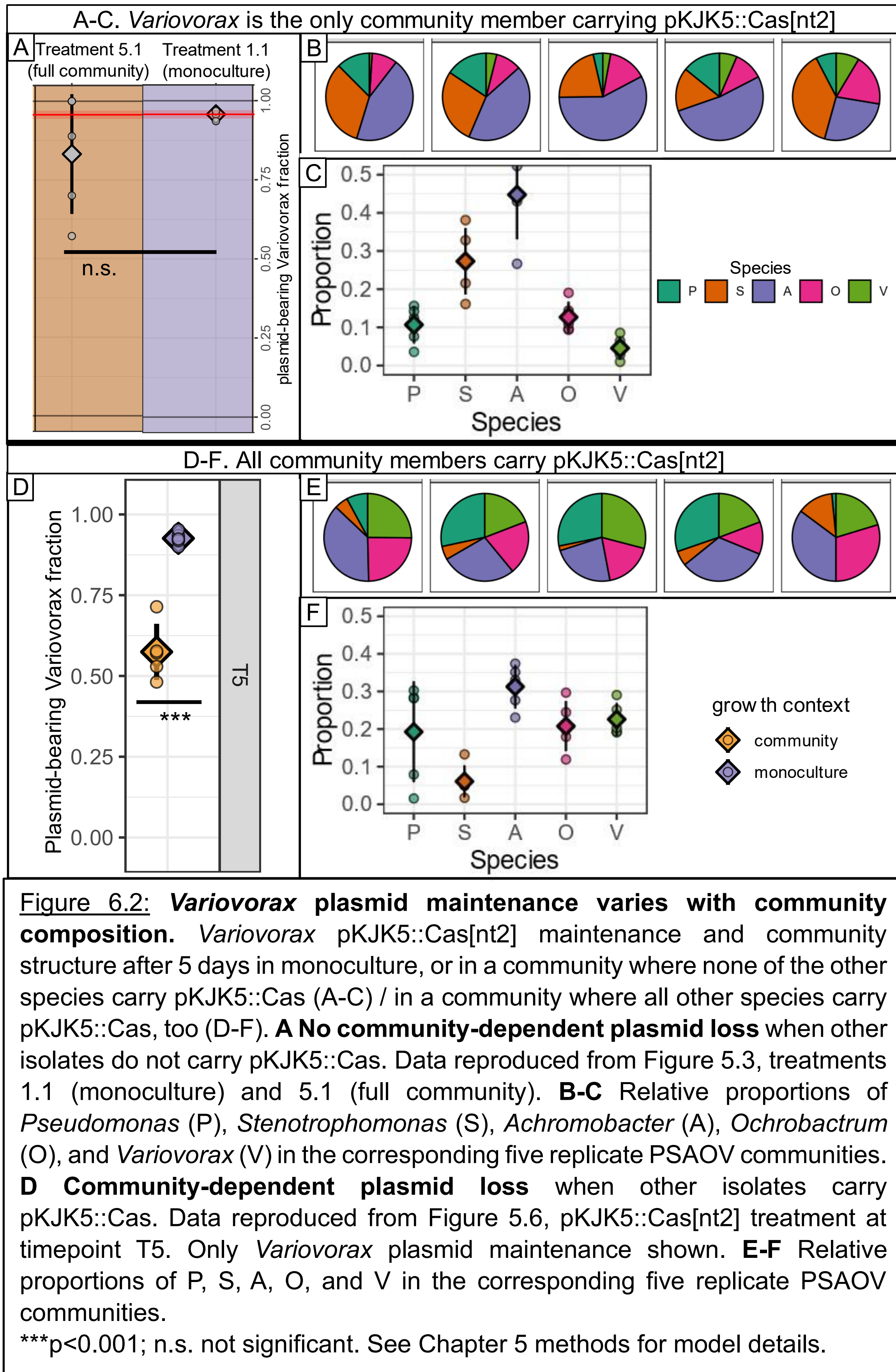

Figure 6.2: ***Variovorax* plasmid maintenance varies with community composition.** *Variovorax* pKJK5::Cas[nt2] maintenance and community structure after 5 days in monoculture, or in a community where none of the other species carry pKJK5::Cas (A-C) / in a community where all other species carry pKJK5::Cas, too (D-F). **A No community-dependent plasmid loss** when other isolates do not carry pKJK5::Cas. Data reproduced from Figure 5.3, treatments 1.1 (monoculture) and 5.1 (full community). **B-C** Relative proportions of *Pseudomonas* (P), *Stenotrophomonas* (S), *Achromobacter* (A), *Ochrobactrum* (O), and *Variovorax* (V) in the corresponding five replicate PSAOV communities. **D Community-dependent plasmid loss** when other isolates carry pKJK5::Cas. Data reproduced from Figure 5.6, pKJK5::Cas[nt2] treatment at timepoint T5. Only *Variovorax* plasmid maintenance shown. **E-F** Relative proportions of P, S, A, O, and V in the corresponding five replicate PSAOV communities.
***p<0.001; n.s. not significant. See Chapter 5 methods for model details.

(iii) Target plasmid properties

Target plasmid TA systems and incompatibility hinder their removal (Chapter 4). Accordingly, target plasmid pOGG99_par persisted during pKJK5::Cas application in *E. coli* (Figure 4.2), and pilot data based on flow cytometry methods indicate that the same effect is also seen when pOGG99_par is carried by the PSAOV community (Figure 6.3; Supplemental Methods).

Removal of an incompatible target plasmid would be difficult in the PSAOV community without finding a means of increasing conjugation efficiency or pKJK5::Cas persistence to allow for longer experiments, even if the target plasmid does not encode *par*: In *E. coli* with ~10% transconjugant generation, pOGG99 prevalence was reduced by ~50% (Figure 4.2; this was just non-significant; p=0.052). Accordingly, pOGG99 prevalence might be reduced in *Variovorax* only (~50-100% uptake using *E. coli* or *Variovorax* as donors respectively).

In contrast, removal of a compatible target plasmid might be observed in other species, too (especially *Stenotrophomonas*, which also shows high uptake from *Stenotrophomonas* or *E. coli* donors). Beyond this, it is unlikely that significant target plasmid removal could be achieved from the other community members: even with highly proficient *E. coli* donors, their plasmid uptake remained at or below ~10^{-4} transconjugants per recipient (Figure 5.1). This is ~2-3 orders of magnitude lower than liquid mating conjugation efficiencies in *E. coli*, which led to only modest pHERD30T removal, if any (Figure 3.1).

Obviously, these predictions would need to be confirmed experimentally. Unfortunately, pHERD03T cannot be maintained by any PSAOV strain except *Pseudomonas*, so a different compatible target plasmid would have to be found. Generally, IncQ or IncW plasmids are considered to be broad host-range, and cloning vectors utilising these backbones are available (Lale *et al.*, 2011). IncQ plasmids can be mobilised by IncP-1 plasmids (Meyer, 2009) such as pKJK5::Cas. Nevertheless, removal of IncQ plasmids could still be effective – pHERD30T is also mobilizable by IncP-1 plasmids (Qiu *et al.*, 2008).

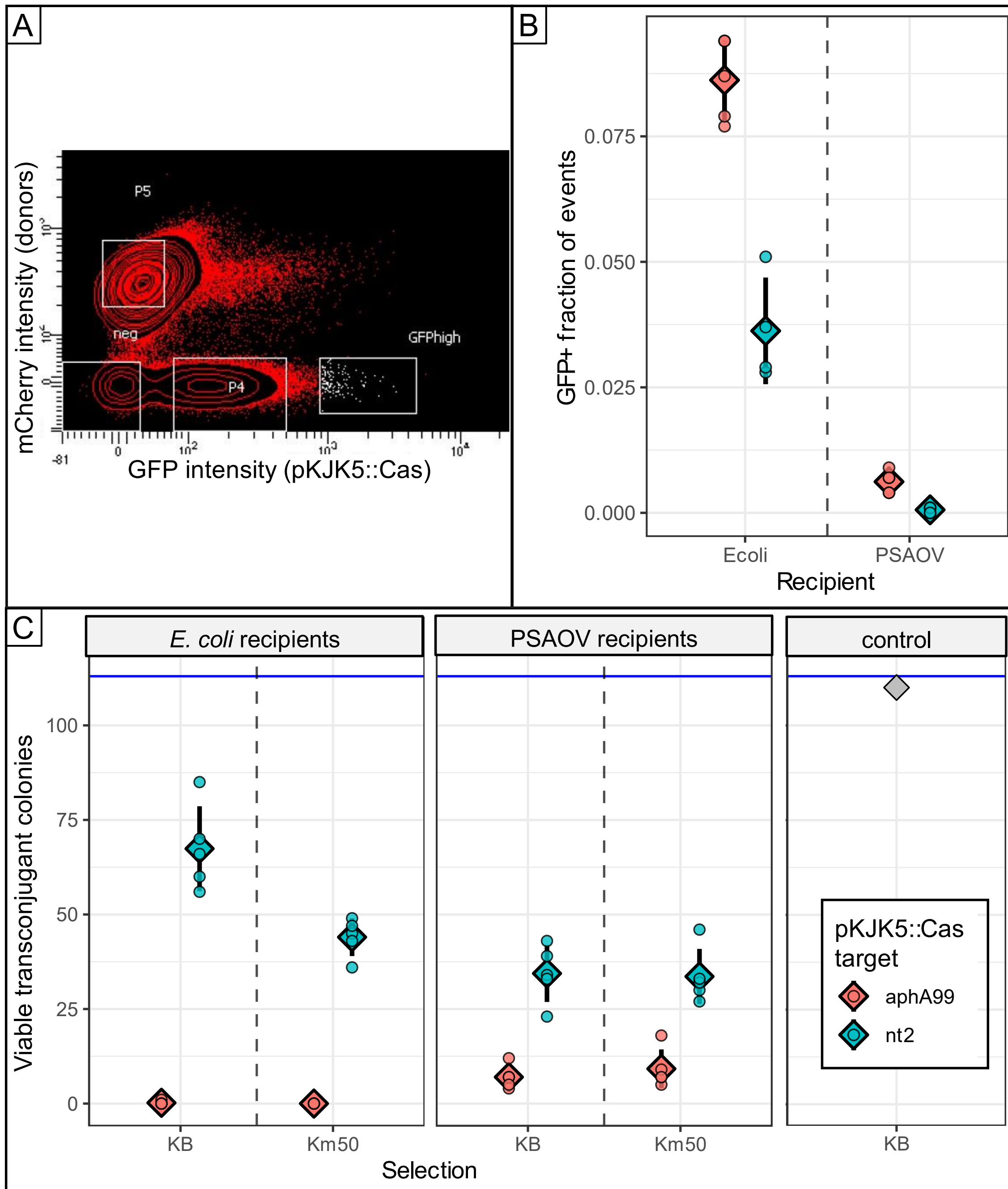

Figure 6.3: **Preliminary FACS data indicate pOGG99_par plasmid costs in multiple organisms.** pKJK5::Cas[nt2]/[aphA99] was delivered to *E. coli* DH5α / the PSAOV community carrying pOGG99_par using *E. coli* K12::mCherry as a donor and filter-mating, n=5.
A: Representative flow cytometry plot of fluorescence intensity of pKJK5::Cas[aphA99] *E. coli* mating experiment. Events are displayed according to their GFP intensity and mCherry intensity. Sorted events for B and C are sorted from a gate not shown (encompassing P4 and GFPhigh, excluding mCherry). **B: GFP+ fraction of samples.** This indicates the pKJK5::Cas transconjugant proportion of the entire population. **C: Viability of GFP+ events** (cells and debris). 113 events (blue line) of each sample were sorted onto plates with no selection, or plates containing 50µg/mL kanamycin (selecting for the target plasmid), mean±sd of viable colonies out of these is shown. "Control" indicates viability of mCherry+ fraction of donor-only control.

The use of multiple target plasmids of different incompatibility groups would have a different advantage, too: It is very difficult to test the impact of plasmid incompatibility in isolation, as I attempted in Chapter 4 using pUC-based pHERD99 and IncP-1 vector pOGG99 (Figure 4.2). Most prominently, copy number also varied between these plasmids: RP4 and synthetic plasmids with its origin of replication (i.e. pOGG99) typically have a copy number of 4-7 plasmids per cell ((Figurski and Helinski, 1979); but has been reported to reach up to 25 (Fang and Helinski, 1991)), and pHERD99 has a pUC-based *E. coli* origin of replication (Qiu *et al.*, 2008) and therefore a high copy number in this species (~60 when growing at 37°C (Lin-Chao *et al.*, 1992)). Previous studies found no clear link between plasmid copy number and CRISPR-mediated removal efficiency (Lauritsen *et al.*, 2017; Tagliaferri *et al.*, 2020; Wan *et al.*, 2020), although removal of high-copy vectors has been described as challenging due to their high target gene dosage and, for instance, gene-drive-like systems have been designed to achieve their effective removal (Valderrama *et al.*, 2019). Furthermore, CRISPR targeting efficiency might differ between these plasmids (Chapter 4). Therefore, a better experiment to test the isolated impact of plasmid incompatibility on pKJK5::Cas-mediated removal would trial removal of multiple minimal target plasmids, all of different incompatibility groups. In this way, failure of removal only of IncP-1 plasmids would strongly indicate that incompatibility to pKJK5::Cas protects from plasmid removal.

Finally, pKJK5::Cas[aphA99] showed reduced transfer into *Stenotrophomonas* and *Variovorax*, despite no fully matching target sequence being present in these strains to the best of my knowledge (Figure 5.1). Therefore, should the target plasmid encode the same *aphA* kanamycin gene, a different guide for plasmid removal which does not impair its transfer should be designed.

In summary, it is unlikely that removal of IncP-1 target plasmids from the PSAOV community would be successful in species other than *Variovorax*. IncW or IncQ target plasmids might allow their removal from *Stenotrophomonas*, too – and would provide a more robust means of assaying the impact of plasmid incompatibility on CRISPR-mediated removal.

(iv) pKJK5::Cas delivery strategy

As removal of plasmids such as RP4 was not effective using pKJK5::Cas in *E. coli* (Figure 4.1), and generally plasmid removal is predicted to be more difficult

in the PSAOV community due to lower pKJK5::Cas conjugation efficiency, an alternative pKJK5::Cas delivery strategy might be more effective. In this thesis, I predominantly tested how pKJK5::Cas removes resident plasmids, which is applicable to situations when it is applied after a target plasmid becomes established in a community. While this strategy was not effective against RP4, pKJK5::Cas could nevertheless protect from RP4 invasion when the CRISPR plasmid was already established in a *Stenotrophomonas* host (Figure 4.5). Similarly, pKJK5::Cas could protect a range of isolates from plasmid uptake by transformation (Figure 2.5).

Therefore, 'vaccinating' the PSAOV community using pKJK5::Cas[aphA99] would probably protect from RP4 uptake. It would be very interesting to test different scenarios in which either pKJK5::Cas or a target plasmid are allowed to spread in the community before applying the other, or in which they are applied simultaneously; this would reveal which levels of plasmid co-invasion, community vaccination, or resident plasmid removal lead to effective reduction of AMR plasmid prevalence in the PSAOV community.

In summary, using either *E. coli* or *Stenotrophomonas* and *Variovorax* donors, pKJK5::Cas might be used to remove target plasmids from PSAOV community members. To reach sufficient conjugation efficiency, mating experiments would need to be carried out over longer periods of time and pKJK5::Cas community maintenance would need to be addressed (for instance by addition of TA genes). Compatible IncQ or IncW target plasmids might be removed from *Stenotrophomonas* and *Variovorax* hosts; it is unlikely that *Pseudomonas* or *Ochrobactrum* plasmid prevalence can be reduced using this model system due to low uptake of pKJK5::Cas. Incompatible IncP-1 target plasmids (e.g. pOGG99) are predicted to be more difficult to tackle if resident, but their prevalence might be reduced in *Variovorax.* Finally, the delivery strategy and timing of pKJK5::Cas and target plasmid application should be trialled: different amounts of community vaccination, community co-invasion, and resident plasmid removal may lead to different outcomes in members of the PSAOV community.

Application of pKJK5::Cas in natural communities

Many of the findings, predictions, and considerations of pKJK5::Cas application in the synthetic community setting are applicable to natural communities. It is

important to carefully choose applications which favour pKJK5::Cas' plasmid removal features.

pKJK5::Cas delivery optimisation by choice of donor and environment

Generally speaking, conjugation efficiency is expected to be lower in nature than in the laboratory: IncP-1 plasmids are best suited to transfer on filters, on which cells form biofilms (Bradley, 1983). The gut or sludge-like environments have more shear forces acting on mating pairs which are predicted to negatively affect plasmid transfer (Lazdins *et al.*, 2020). Accordingly, pKJK5::Cas might be best applied in environments where biofilms are abundant or cells are attached to surfaces, e.g. on medical devices (reviewed in (Percival *et al.*, 2015), in biofilm-associated infections such as cystic fibrosis (Singh *et al.*, 2000), or perhaps in structured soil environments.

When considering which donor species to apply, mating experiments with dominant members of a target bacterial community (as carried out in Chapter 5) might indicate which donor species is suitable. Highly permissive pKJK5 hosts might be a good starting point for this, as they are crucial to overall plasmid maintenance within their communities (e.g. identified for waste-water treatment plant communities in (Li *et al.*, 2020)).

Plasmid incompatibility is an important consideration for target plasmids

Previous work has shown that, generally speaking, CRISPR targeting is effective when removing high copy-number plasmids (Tagliaferri *et al.*, 2020), and that it can be used to target multiple plasmids with closely related AMR gene sequences (Dong *et al.*, 2019; Wang *et al.*, 2019).

pKJK5::Cas was most effective when used against compatible target plasmids, even if they encoded a single TA system. On the other hand, incompatible target plasmids appeared to be more difficult to remove (Figure 4.2). Therefore, it might be ideal to apply this plasmid to communities in which IncP-1 plasmid prevalence is generally low, but through which pKJK5::Cas can nevertheless spread well. This is a difficult balance, as one can expect the most permissive pKJK5::Cas hosts to be found in communities with prevalent IncP-1 plasmids.

Generally, presence of IncP-1 plasmids in grasslands, watershed and river sediment is associated with anthropogenic pollution (Dealtry *et al.*, 2014; Cyriaque *et al.*, 2020; Dungan and Bjorneberg, 2020; Willms *et al.*, 2020). Temporal variation in farmland microbiomes indicates that IncP-1 plasmid prevalence increases with pesticide, sludge, or waste-water application (Jechalke *et al.*, 2013a, 2015; Rahube *et al.*, 2016; Nour *et al.*, 2017). Waste water is an important reservoir of IncP-1 plasmids (Bahl *et al.*, 2009), and its treatment does not help: IncP-1 plasmid prevalence is higher in waste water treatment plant (WWTP) effluent than in influent (Pallares-Vega *et al.*, 2019).

Together, this suggests that if pKJK5::Cas is allowed to spread through more pristine, unpolluted communities (e.g. on a field before fertilisation by slurry) or in untreated waste water, it could subsequently protect these communities from invasion by other IncP-1 AMR plasmids. More recent evidence suggests that in unpolluted environments, IncP-1 plasmids are present too - albeit without resistance genes (Shintani *et al.*, 2020). Therefore, this prediction would have to be tested by assaying spread of pKJK5::Cas in various such communities.

Concerning human-associated microbial communities, *Enterobacteriaceae* are a large family of harmless commensals as well as especially food-borne disease-causing pathogens (Rock and Donnenberg, 2014). In the healthy human microbiome, *Enterobacteriaceae* are usually present at very low densities (Stecher *et al.*, 2012), but conditions such as obesity or inflammatory bowel disease (IBD) can increase their prevalence (Santacruz *et al.*, 2010; Walters *et al.*, 2014). In recent years, epidemics of multidrug resistant *Enterobacteriaceae* have spread around the world. These are associated with AMR plasmids, which encode resistance to β-lactams, carbapenems, quinolones, and aminoglycosides amongst others (reviewed in (Mathers *et al.*, 2015)). Interestingly, only the minority of these plasmids are of the IncP-1 group, which indicates that this plasmid group might be present at low frequencies in this family (Carattoli, 2009). Despite this, previous data has shown that pKJK5 spreads well through *Enterobacteriaceae* (Klümper *et al.*, 2015), which makes these a promising focus for pKJK5::Cas applications. Otherwise, little is known about IncP-1 plasmid prevalence in the human microbiome.

In summary, due to incompatibility exclusion, pKJK5::Cas might be most effective when applied before a pollution event in environmental applications. For human

treatment, application of pKJK5::Cas in communities where *Enterobacteriaceae* are prevalent, e.g. in individuals affected by IBD or in foodborne pathogen communities might be especially promising.

Other target plasmid considerations

Other factors encoded by target plasmids, beyond their incompatibility and TA systems, might affect their removal. This is particularly pertinent when applying pKJK5::Cas to natural microbial communities, where a broad diversity of plasmids might be present. Most directly, plasmid-encoded anti-CRISPR proteins (e.g. on *Enterococcus* plasmids (Mahendra *et al.*, 2020)) can inhibit Cas9 activity. Additionally, plasmid-plasmid interactions beyond incompatibility could impair pKJK5::Cas spread or maintenance. These include entry exclusion (reviewed in (Garcillán-Barcia and de la Cruz, 2008)) or plasmid-encoded defense systems such as CRISPR-Cas (Pinilla-Redondo *et al.*, 2021). On the other hand, phenomena such as facilitation (reviewed in (Dionisio *et al.*, 2019)) might increase pKJK5::Cas transfer rates, or positive epistatic interactions might increase pKJK5::Cas maintenance (Gama *et al.*, 2020) if appropriate co-residing plasmids are present.

Overall, complexity rapidly increases when applying pKJK5::Cas to a microbial community – not only do microbes need to be permissive to pKJK5::Cas spread, but resident mobile genetic elements need to favour spread of this CRISPR plasmid, too. These dynamics are difficult to predict, but could be specifically investigated if pKJK5::Cas application to a certain community fails.

pKJK5::Cas used for specific applications

In the following, I offer two specific opposing scenarios for pKJK5::Cas application against mobile AMR genes and discuss which might be the most promising.

<u>(A) AMR plasmid removal from the gut environment.</u>

Small populations of *Salmonella typhimurium* pathogens which survive antibiotic treatment can later migrate back into the gut lumen and act as donors of extended-spectrum beta lactamase (ESBL) plasmids, which eventually leads to spread of AMR plasmids and gut colonisation by resistant bacteria (Bakkeren *et al.*, 2019). Crucially, this is a scenario where AMR colonisation and plasmid dissemination is caused by a small fraction of persistent pathogens and low

frequency of initial AMR plasmid transfer events, and where no antibiotics are present.

(B) AMR plasmid removal in waste-water treatment plants.

After human consumption, antibiotics are excreted into wastewater where they are often found at low concentrations and can select for AMR (Andersson and Hughes, 2014), which makes WWTPs hotspots for AMR gene transfer. Carbapenemase-producing *Enterobacteriaceae* (CPE) are frequently found in wastewater (e.g. CPE were found in 89% of WWTP influent samples in a Dutch survey (Blaak *et al.*, 2021)), and while wastewater treatment does reduce their prevalence (~2 orders of magnitude), these carbapenem resistant bacteria are often found in WWTP effluent. Their resistance is plasmid-associated (Yang *et al.*, 2016; Blaak *et al.*, 2021). Crucially, this is a scenario where AMR plasmids are prevalent throughout a target species, and selection for plasmids can be expected to be present.

Biofilms generally form in WWTPs, and low concentrations of antibiotics may even promote conjugation and select for pKJK5, therefore general environmental conditions might favour pKJK5::Cas transfer in scenario (B) over (A). General expected community structure would predict less IncP-1 plasmids in the gut in scenario (A), which might make plasmid removal in this setting more effective. Furthermore, low concentrations of antibiotics will probably select for target plasmid maintenance in (B). Finally, with overall low environmental delivery rates, target plasmid prevalence and timing of pKJK5::Cas application would be quite important. Therefore, I would predict that scenario (A), where pKJK5::Cas could be used to prevent initial low-frequency AMR plasmid transfer events in the absence of a selective pressure for the target gene, is the more promising approach for this CRISPR delivery tool.

Outlook: improved pKJK5::Cas designs

This discussion has revealed several limitations of pKJK5::Cas, particularly in its spread in a community and in its ability to target incompatible plasmids.

I hypothesise that some of these shortcomings can be addressed by equipping pKJK5::Cas with additional genes that can increase its stability and transfer, which I will discuss below. To reduce total plasmid size after addition of such

genes, accessory pKJK5 regions such as 'load 1' (2 kB, consists of an insertion sequence) or 'load 2' (12 kB, consists of an integron) (Bahl *et al.*, 2007b) could be deleted.

sgRNA multiplexing to clear multiple target plasmids

Overall, CRISPR targeting efficiency is not predicted to be a major driver of efficient target plasmid removal but might still provide moderate improvements in plasmid removal efficiency (Chapter 3). Especially highly variable plasmids or plasmids prone to CRISPR targeting escape might be targeted by multiple guides in the same target gene, which reduces the likelihood of escape mutations in all target sequences or allows to target divergent plasmid sequences. The use of several sgRNAs in parallel is called multiplexing.

Moreover, CRISPR delivery tools can be used to clear multiple resident plasmids from target cells when multiple sgRNAs are supplied (Wang *et al.*, 2019). Therefore, an improved pKJK5::Cas might target several resident plasmids in addition to targeting an incompatible plasmid to protect a community from its invasion.

Toxin-antitoxin genes to improve pKJK5::Cas stability

For plasmid spread over extended time periods or longer-term applications, pKJK5::Cas stability needs to be addressed. This could be most effectively done by addition of TA genes and would further improve resident target plasmid removal where they encode the same toxin genes. For instance, inclusion of the *parABCDE* operon on pKJK5::Cas would promote pKJK5::Cas maintenance and likely negate the effect *parABCDE* has on target plasmid removal – as the antitoxin gene would remain present in resensitised cells. Specifically, this should make pHERD99_par removal as effective as that of pHERD99.

Taking this approach further, multiple TA operons or simply antitoxin genes could be encoded on pKJK5::Cas to negate a protective effect from multiple common TA systems. This is an approach successfully trialled in the development of pCURE, an incompatibility-based plasmid curing agent which encodes multiple replications of origin and antitoxin genes (Lazdins *et al.*, 2020). Diversity of TA systems on different plasmids could make this more challenging and narrow applicability of this approach, where antitoxin-encoding pKJK5::Cas would have to be re-designed for specific applications.

Use of multiple CRISPR delivery tools or origins of replication to address target incompatibility

To remove incompatible target plasmids, pKJK5::Cas could be delivered together with a second CRISPR delivery tool of a different incompatibility group, ensuring that target plasmids can be reached by at least one CRISPR delivery tool. Alternatively, additional origins of replication could be inserted onto pKJK5::Cas which might have a similar result. Instead of additional cloning steps, a naturally occurring plasmid with multiple origins of replication might be chosen as a delivery vehicle for the CRISPR-Cas9 cassette (e.g. large plasmids in *Bacillus cereus* often encode multiple replicons (Zheng *et al.*, 2013)), but additional work would be needed to assess their utility for spread through communities. A pKJK5::Cas variant with multiple origins of replication might also be more proficient at removing various target plasmids, due to a joint effect of CRISPR targeting and incompatibility exclusion.

Alternatively, more widespread dissemination and removal of incompatible target plasmids could also be achieved by designing a gene-drive-like pKJK5::Cas variant, discussed in more detail below.

pKJK5::Cas transfer improvement by optimising conjugation or gene drive-like designs

Particularly when pKJK5::Cas can only be applied in a donor with low prevalence, or in environments where low overall conjugation rates are predicted, enhancing pKJK5::Cas' conjugative ability would be paramount. This could be achieved by using conjugation-promoting compounds, such as sub-MIC antibiotic concentrations - specifically, fluoroquinolones like ciprofloxacin and levoloxacin enhance conjugation by upregulating plasmid genes (Zeng *et al.*, 2017). However, this would inevitably select for resistance to these antibiotics – and using antibiotics to resensitise a community to different antibiotics is counter-productive. Therefore, it would be interesting to test transfer of pKJK5::Cas in environments where sub-MIC ciprofloxacin is already present, e.g. in wastewater (Larsson *et al.*, 2007).

Alternatively, rather than optimising pKJK5::Cas plasmid spread, spread of the CRISPR-Cas9 cassette itself could be optimised. This could be achieved by insertion of the CRISPR-Cas9 cassette into an active transposon on a different CRISPR delivery plasmid, and might lead to Cas9 and sgRNA spreading to other

plasmids as cargo genes. Alternatively, homology-directed recombination using λ-red (e.g. as in (Datsenko and Wanner, 2000; Lee *et al.*, 2009)) could be utilised for a gene drive-like design of pKJK5::Cas (Figure 6.4). This could work as follows: in pKJK5::Cas[aphA99] the CRISPR-Cas9 cassette is inserted into *intI1*. The CRISPR-Cas9 cassette would be supplemented with λ-red genes alongside an sgRNA targeting intact *intI1* sequences. In this way, alongside removal of AMR plasmids, other plasmids with similar *intI1* sequence could act as propagators of the CRISPR-Cas9 cassette due to sequence homology between the two plasmids. Further, this design could be improved by addition of multiple homology arms and "conserved-sequence sgRNAs", which target plasmid regions corresponding to the additional homology arms. Especially targeting plasmids of different incompatibility groups in this way could allow the CRISPR-Cas9 cassette to reach different microbial hosts, and perhaps reach almost all members of different microbiomes.

A different CRISPR delivery tool used λ-red machinery to propagate only the *sgRNA* component, which led to efficient removal of high copy-number AMR plasmids or their target gene disruption by homology-directed insertion of *sgRNA.* The nuclease component remained stationary on the delivery vector within each cell (Valderrama *et al.*, 2019). In comparison, pKJK5::Cas' entire CRISPR-Cas9 cassette is very large, especially if expanded with λ-red genes. Therefore, this technology likely needs optimisation before implementation.

Alternatively, the recent discovery of transposon-associated CRISPR systems (Klompe *et al.*, 2019) may allow to achieve CRISPR-Cas9 cassette transposition without the need for homology sequences, but instead guided by sgRNAs only. These were applied in the development of single-vector DNA-editing All-in-one RNA-guided CRISPR-Cas Transposase (DART) systems, where genetic cargo, guided by sgRNAs, could be inserted into microorganisms embedded in their communities (Rubin *et al.*, 2020). This method might allow for more leniency in sequence divergence by abrogating the need for homology-directed insertion, but would mean a larger overall pKJK5::Cas payload size.

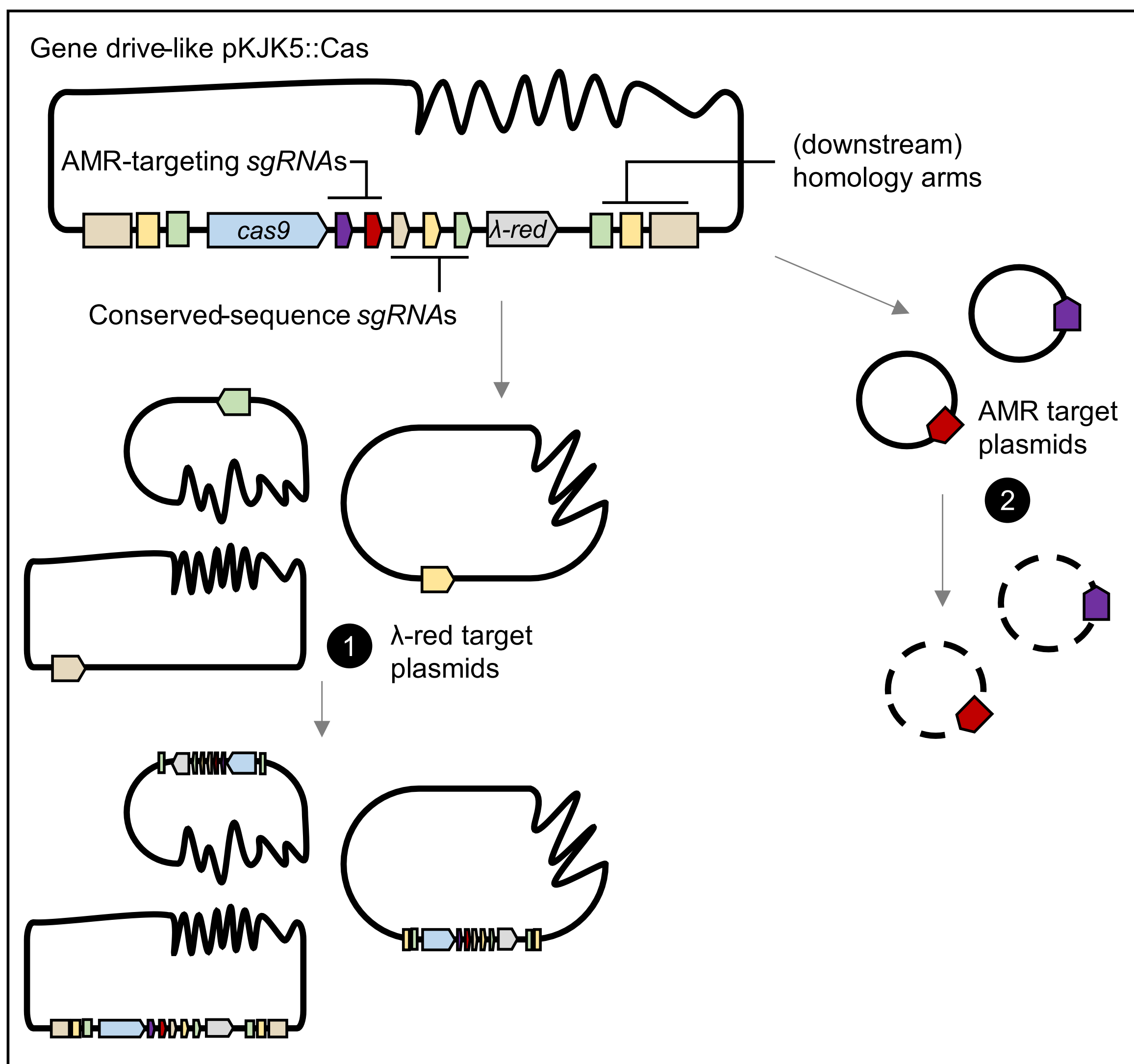

Figure 6.4: **Gene drive-like pKJK5::Cas design**
pKJK5::Cas is engineered to encode *cas9,* λ-red genes, and a series of conserved-sequence *sgRNAs*. These target and cleave conserved sequences of plasmids of different incompatibility groups (1), after which λ-red mediates homology-directed insertion of the entire CRISPR-Cas9 cassette, utilising homology arms upstream and downstream of the cassette on pKJK5::Cas which border the target sequence of the respective conserved-sequence sgRNA. This generates a series new CRISPR delivery plasmids.
Beyond this, pKJK5::Cas (and new CRISPR delivery plasmids) retain their ability of removing AMR plasmids targeted by one or several AMR-targeting *sgRNAs* (2).

1

2 **Optimising pKJK5::Cas transfer and maintenance by application of**
3 **selective pressure**

4 Finally, a selective pressure could be applied to encourage pKJK5::Cas transfer
5 as well as its maintenance.

With current pKJK5::Cas design, this could most easily be achieved by application of low concentrations of tetracycline or trimethoprim, against which this CRISPR delivery tool encodes resistance. While constant antibiotic application defeats the point of an antibiotic resensitisation treatment, this might be a useful approach in the laboratory to quickly ascertain whether, in general, target plasmid removal from a certain isolate could be achieved if a pKJK5::Cas selective pressure was present.

For environmental and in-patient applications different selective pressures would need to be applied, for instance lytic bacteriophage (phage). Such a setup was trialled by Yosef *et al.*, who employed an engineered non-replicating temperate phage as a CRISPR delivery tool. Alongside target plasmid clearance, the phage would lysogenise and provide its *E. coli* host with immunity against a lytic bacteriophage, applied afterwards. In this way, only target bacteria that had received the CRISPR treatment and were therefore sensitised to antibiotics survived the lytic phage infection (Yosef *et al.*, 2015).

Similarly, additional sgRNAs which target one or several lytic phage could be inserted onto pKJK5::Cas. Compared with pKJK5, phage have a very narrow host-range, which means that a broad cocktail of lytic phage would have to be applied. Pilot experiments could determine which community constituents struggle to take up and maintain pKJK5::Cas, or which community constituents are key pKJK5::Cas disseminators, and tailor the phage cocktail towards these. Alternatively, the entire CRISPR treatment could be targeted to specific cases of AMR plasmid uptake (e.g. *Salmonella* in the human gut, see above), in which case a lytic bacteriophage cocktail could be targeted towards certain strains.

Compared with phage therapy alone, using pKJK5::Cas has the advantage that this treatment can reside in bacterial hosts for longer and protect from plasmid re-infection. Additionally, use of lytic phage in isolation is likely to change overall community structure by decreasing prevalence of a target strain, whereas pKJK5::Cas application and decrease of plasmid prevalence within the target bacteria does not necessarily change community structure.

In summary, pKJK5::Cas design could be further improved by a number of modifications: multiplexing sgRNAs could see simultaneous removal of multiple target plasmids, or allow clearance of a family of divergent plasmids. The addition

of TA systems would improve pKJK5::Cas stability and removal of TA-encoding target plasmids. Incompatible plasmid removal could be achieved by simultaneous use of a second CRISPR delivery tool. Transfer of pKJK5::Cas might be improved by application in environments with conjugation-stimulating compounds, or by designing a gene-drive like pKJK5::Cas variant with a mobile CRISPR-Cas9 cassette (Figure 6.3). Application of a selective pressure, for instance by application of lytic bacteriophage against which pKJK5::Cas provides immunity, would also improve pKJK5::Cas transfer as well as its maintenance.

Concluding Remarks

CRISPR delivery tools are a new addition to the arsenal against increasing antibiotic resistance and can be applied to clear target AMR plasmids from recipient bacteria. Here, I developed the broad host-range CRISPR delivery tool pKJK5::Cas and interrogated its ability to protect from AMR plasmid uptake, remove resident plasmids, and its transfer and maintenance in a model bacterial community.

Data generated throughout this thesis allow predictions of pKJK5::Cas effectivity in different environments. Its successful application depends on appropriate donor selection and improved pKJK5::Cas stability; and different delivery strategies would have to be trialled to achieve protection from incompatible target plasmids.

In real-life applications, pKJK5::Cas utility would depend on conjugation efficiency within the target environment, and on prevalence of incompatible plasmids and other target plasmids in the recipient community. In future research, pKJK5::Cas design could be improved by addition of toxin-antitoxin systems to promote stability, by the use of several sgRNAs to target multiple plasmids, by design of a mobile CRISPR-Cas9 cassette for dissemination beyond pKJK5, or by application of a selective pressure, e.g. using lytic bacteriophage.

Appendix 1: Supplementary Tables

Table S1: Bacterial strains used throughout this thesis.

Strain	Shorthand Name	Description	Chap-ters	Reference
E. coli DH5α	DH5α		2, 3, 4.	laboratory strain
Pseudomonas aeruginosa PA01::Cas9	PA01::Cas9	Cas9 inserted downstream of *glmS*	2	(Peters, unpublished)
E. coli K12 MG1655::mCherry	K12::mCherry	Chromosomal *lacI*, *mCherry*, Kanamycin resistance tags	2, 3, 4, 5.	(Klümper *et al.*, 2015)
E. coli MFDpir	MFDpir	Auxotrophic (requires DAP), *Erm* resistance	2,	(Ferrières *et al.*, 2010)
E. coli DH10β	DH10β	Chromosomal Streptomycin resistance tag	2	laboratory strain
Escherichia/Shigella pig gut isolate bhiF2	bhiF2	Pig gut isolate, 16S-typed as *Escherichia/Shigella*	2	this study
Coliform isolate C743E1	C743E1	Human-associated isolate	2	(Leonard *et al.*, 2018)
Coliform isolate TV1-2	TV1-2	Environmental isolate	2	(Leonard *et al.*, 2018)
Coliform isolate 6TB-1	6TB-1	Environmental isolate	2	(Leonard *et al.*, 2018)
Pseudomonas aeruginosa PA14	PA14		2	laboratory strain
Pseudomonas fluorescens SBW25	SBW25		2	laboratory strain
E. coli K12 MG1655	K12		3	laboratory strain
E. coli DH5α::CpR		Chromosomal chloramphenicol resistance (*catR*) tag	4	This study
Stenotrophomonas spp.	S	Soil isolate	4, 5	(Castledine *et al.*, 2020)
Pseudomonas spp.	P	Soil isolate	5	
Achromobacter spp.	A	Soil isolate	5	
Ochrobactrum spp.	O	Soil isolate	5	
Variovorax spp.	V	Soil isolate	5	
Pseudomonas (SmR)	P(SmR)	Chromosomal streptomycin resistance (*aadB*) tag	5	This study
Stenotrophomonas (GmR)	S(GmR)	Chromosomal gentamicin resistance (*aacC1*) tag	5	This study
Ochrobactrum (GmR)	O(GmR)	Chromosomal gentamicin resistance (*aacC1*) tag	5	This study
Variovorax (GmR)	V(GmR)	Chromosomal gentamicin resistance (*aacC1*) tag	5	This study
Variovorax (CpR)	V(CpR)	Chromosomal chloramphenicol resistance (*catR*) tag	5	This study

1 Table S2: Plasmids used throughout this thesis.

2 [target] indicates several variants with different sgRNA payloads. See
3 appropriate chapters for target variants.

Plasmid	Relevant resistance	Payload	Chap-ter	Reference
pMA-RQ_Cas[aacC1-72] / [nt]	Ampicillin	Cas9, sgRNA, GFP.	2	This study.
pCDF1b	Streptomycin	/	2	Novagen (EMD Millipore)
pCDF1b_sgRNA	Streptomycin	sgRNA	2	This study.
pgRNA (bacteria)	Ampicillin			(Qi *et al.*, 2013)
pHERD20T	Ampicillin / Carbenicillin	/	2	(Qiu *et al.*, 2008)
pHERD20T_sgRNA[target]	Ampicillin / Carbenicillin	sgRNA	2	This study
pDOC-K	Ampicillin, Kanamycin	*sacB*	2, 3	(Lee *et al.*, 2009)
pDOC	Ampicillin	*sacB*	2, 3	This study
pDOC_Cas[target]	Ampicillin	*sacB,* CRISPR-Cas9 cassette	2, 3	This study
pACBSCE	Chloramphenicol	I-SceI, λ-red proteins	2, 3	(Lee *et al.*, 2009)
pKJK5	Tetracycline, Trimethoprim	Natural plasmid	2	(Bahl *et al.*, 2007b)
pKJK5::Cas[aacC1-72] / [nt]	Tetracycline	Cas9, sgRNA, GFP in *dfrA*	2, 3	This study
pHERD30T	Gentamicin	/	2, 3	(Qiu *et al.*, 2008)
pKJK5::Cas[aphA99] / [nt2]	Tetracycline, Trimethoprim	Cas9, sgRNA, GFP in *intI1*	3,	This study
pHERD99	Gentamicin	[aphA99] target sequence in MCS	3, 4	This study
pHERD99_par	Gentamicin	[aphA99] target sequence and *parABCDE* in MCS	4	This study
pOGG99	Kanamycin	/	4	This study
pOGG99_par	Kanamycin	*parABCDE*	4	This study
RP4	Ampicillin, Kanamycin, Tetracycline	Natural plasmid	4	(Pansegrau *et al.*, 1994)
pOPS0378	Kanamycin	*parABCDE*, mCherry	4	Addgene 133229 (Mendoza-Suárez *et al.*, 2020)
pACYCduet	Chloramphenicol	/	4	Novagen
pACYC_Cas[aphA99]/[nt2]	Chloramphenicol	Cas9, sgRNA, GFP	4, 5	This study

pBAM_Cp	Ampicillin	Tn5 transposon with *catR*	4	(Dimitriu *et al.*, 2021)
pBAM1-Gm	Ampicillin	Tn5 transposon with *aacC1*	5	(Martínez-García *et al.*, 2011)
pBAM1-Sm	Ampicillin	Tn5 transposon with *aadB*	5	(Martínez-García *et al.*, 2011)

Table S3: Primers and oligonucleotides used throughout this thesis.

Name	Sequence (5’ → 3’)	Purpose	Chap-ter
Forward_27F	AGAGTTTGATCMTGGCTCAG	Amplification of 16S rRNA gene	2
Reverse_1492R	ACCTTGTTACGACTT		2
sgRNA_amp_fw	TAACCATGGTTGACAGCTAGCTCAGTCCTAGGT	Amplification of sgRNA and insertion into an NcoI site.	2
sgRNA_amp_rv	TTCCCATGGAAGCTTCAAAAAAAGCACCG		
sgRNAxp_xx_fw	TATAATACTAGTNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAA	Exchange of sgRNA specificity on pCDF1b_sgRNA. The 20nt-‘N’ stretch is replaced with each specificity from Table 2.2.	2
dfrA_fw	GTGAAACTATCACTAATGGTAG	Checking of recombined pKJK5::Cas	2
dfrA_rv	TTAACCCTTTTGCCAGATTT		2
Cas9_bw	ATGCTGTACTTCTTGTCCAT		2, 3
GFPend_fw	CATGGACGAACTGTATAAGT		2, 3
N20_xx_top	CTAGTNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCT	Exchange of sgRNA specificity on pMA-RQ_Cas. The 20nt-‘N’ stretch is replaced with specificities listed in each chapter	2, 3
N20_xx_btm	CTAAAACNNNNNNNNNNNNNNNNNNNNA		
intI1_fw	GCATTACAGCTTACGAACCG	Checking of recombined pKJK5::Cas[nt2]/[aphA99]	3
intI1_rv	TAACATCAAGGCCCGATCCT		3
Uphom_intI1_fw	CATAAGCTTACCTGAGCACCTCAGCAGTTGCAAACCCTCACTGATCCGCA	Amplify upper *intI1* homology region	3
Uphom_intI1_rv	GCTACTCGAGATGAAGTGGTTCGCATCCTCGGTTT		3
Lohom_intI1_fw	ATCGGATCCCGGGGTCAGCACCACCGGC	Amplify lower *intI1* homology region	3
Lohom_intI1_rv	CCAGGAATTCCAATGACTTCGAACTGTTCTTCTACGGCAAGGTGCTGTGCAC		3
aphA99PAM_HK_top	AGCTTAAATGGGCGCGTGATAAGGTTGGTAC	Insert [aphA99] target sequence + PAM into pHERD30T	3
aphA99PAM_HK_btm	CAACCTTATCACGCGCCCATTTA		3
par_fw	TCGGTACCTGCATGAGCTTGTGGAAGTG	*parABCDE* amplification and KpnI insertion	4
par_rv	CAGGTACCTGCTCAACAGGTTCGCA		4
pOGG0_mut_fw	TGGGCGCGTGATAAGGTGGGTCAGAGCGGC	Site-directed mutagenesis to generate pOGG99	4
pOGG0_mut_rv	TTTATAGCCATACAGATCCGCATCCATGTTGCTGTTCAGACGC		4
pOGG0_sequence	TATTGGTGAGAATCCAGGCA	Sequence pOGG99 mutation	4
pOGG099_parRemoval_top	TGAGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCACTA	Remove *par* genes from pOGG99_par	4

pOGG099_parRemoval_btm	GATCTAGTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGC		4
Forward 27F	AGA GTT TGA TCM TGG CTC AG	Amplify 16S rRNA for genus typing	2, 5
Reverse 1492R	ACC TTG TTA CGA CTT		2, 5
aacC1_fw	ATGTTACGCAGCAGCAACGA	Amplify *aacC1*	5
aacC1_rv	TTAGGTGGCGGTACTTGGGT		5
Cm_F	AGACGGCATGATGAACCTGA	Amplify *cat*	5
Cm_R	CGGTGAGCTGGTGATATGGG		5

1

Appendix 2: Supplementary Methods

Flow Cytometry-assessed pOGG99_par removal (Figure 6.3)

I delivered pKJK5::Cas[aphA99]/[nt2] using an *E. coli* K12::mCherry donor to either *E. coli* DH5α or mixed PSAOV recipients by filter mating (as in Chapter 5; n=5). All recipients carried pOGG99_par, which is incompatible with pKJK5::Cas and encodes a *parABCDE* toxin-antitoxin (TA)-system (see Chapter 4 methods). After mating, recovered cells were analysed by flow cytometry, utilising the chromosomal mCherry fluorophore in donors and GFP on pKJK5::Cas (repressed in K12::mCherry donors; Figure 6.3A). Approximate GFP positivity rates of 5 replicates reveal that overall dynamics when delivering pKJK5::Cas to PSAOV recipients were similar to delivering pKJK5::Cas to *E. coli* (Figure 6.3B**;** more transconjugants generated for targeting than for non-targeting variant; due to incompatibility exclusion (Chapter 4)). Sorting GFP+ events (transient transconjugants) onto non-selective (KB agar) and selective plates (KB+50µg/mL Kanamycin) revealed that almost all pKJK5::Cas[aphA99] transconjugants are non-viable, which is likely due to cell death upon pOGG99_par loss (Figure 6.3C). Overall, this indicates that pOGG99_par incompatibility and TA presence protect the target plasmid from pKJK5::Cas[aphA99]-mediated removal in PSAOV the same way as in *E. coli*, and that this effect was mediated by toxicity after pKJK5::Cas uptake.

Appendix 3: Plasmid sequences

All plasmid sequences are available below in Genbank format, or in fully annotated and interactive form in a folder on Benchling here:

https://benchling.com/davvi36/f_/6jFvhQBU-david-walker-sunderhauf-phd-thesis-supplement/

pMA-RQ_Cas[aacC1-164]

The CRISPR-Cas9 cassette was generated *in silico* and delivered by ThermoFisher's Gene Art service on this plasmid. Before use in any experiments, sgRNA specificity was exchanged to [aacC1-72] (Chapter 2).

pMA-RQ_Cas[aacC1-164] can be directly accessed here:

https://benchling.com/s/seq-yGpYnXprPBGtzIl3tru1?m=slm-yuOrrt2tJ0c3qyZ7Zjmk

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LOCUS       pMA-RQ_Cas[aacC1-164]   8063 bp ds-DNA     circular     14-DEC-2021
DEFINITION  .
KEYWORDS    "Name:pMA-RQ_Cas[GmR164]" "Name of Depositor" "Type"
            "Incompatibility (origin backbone)" "Resistance:Ampicillin"
            "Confirmed by Sequencing:Yes" "Parent plasmid" "Harboured in
            strain:E. coli dh5α" "Category" "Box location" "Box"
FEATURES             Location/Qualifiers
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                        /label="upstream homology"
     feature            497..529
                        /label="upstream mini-MCS"
     misc_feature       530..4793
                        /label="Cas9 module"
     regulatory         536..564
                        /label="AmpR_promoter"
     misc_feature       536..605
                        /label="Ampicillin upstream region [pBAM1]"
     misc_feature       606..4712
                        /label="SpyCas9 (pKJK5 optimised; RS removed)"
     CDS                606..4712
                        /label="Translation 606-4712"
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                        /label="AmpR downstream (pBAM1)"
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                        /label="N20b"
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                        /label="J23199 promoter (SpeI)"
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     misc_feature       4835..4854
                        /label="gRNA spacer [aacC1-164]"
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     regulatory         4983..5061
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                        /label="GFPmut3b (pKJK5 optimised, RS removed)"
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     regulatory      complement(7977..8005)
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      181 GGGAAGGGCG TTTCGGTGCG GGCCTCTTCG CTATTACGCC AGCTGGCGAA AGGGGGATGT
      241 GCTGCAAGGC GATTAAGTTG GGTAACGCCA GGGTTTTCCC AGTCACGACG TTGTAAAACG
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      481 CATTACCCAA CTCGAGTAGC CAGATGCATT GCCTTAGATC TTGGTCATAA CGCGTTTCAA
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pHERD99

pHERD99 is based on pHERD30T (Qiu *et al.*, 2008) and was constructed and used throughout Chapters 3-4.

pHERD99 can be directly accessed here: https://benchling.com/s/seq-wBMQaLaKpQpX5keVW4Te?m=slm-rMmmdh8N4gUGCjMA40sP

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pHERD99_par

pHERD99_par is based on pHERD99 and was constructed and used throughout Chapter 4.

pHERD99_par can be directly accessed here: https://benchling.com/s/seq-hT7baVvn3aNWsbWWzPix?m=slm-PbURlhYIdYrWyosVYqJw

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```

pOGG99

pOGG99 was constructed and used throughout Chapters 4.

poGG99 can be directly accessed here: https://benchling.com/s/seq-zf78siekTtdzv0RHeV0Z?m=slm-KVtaK6aSnxWENSdaOtMh

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LOCUS       pOGG99                  3731 bp ds-DNA     circular     14-DEC-2021
DEFINITION  .
KEYWORDS    "Name:pOGG99" "Name of Depositor" "Type" "Incompatibility (origin
            backbone):IncP1" "Resistance:Kanamycin" "Confirmed by Sequencing"
            "Parent plasmid" "Harboured in strain:E. coli dh5α" "Category" "Box
            location" "Box"
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     CDS             3558..3619
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pOGG99_par

pOGG99_par is based on pOGG99 and was constructed and used throughout Chapters 4.

poGG99_par can be directly accessed here: https://benchling.com/s/seq-Qe4Yij8EAFTMo5ugOMfu?m=slm-5Uhz8m53hsBTex4QeLeG

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LOCUS       pOGG99_par             6043 bp ds-DNA     circular     14-DEC-2021
DEFINITION  .
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KEYWORDS    "Name" "Name of Depositor" "Type" "Incompatibility (origin
            backbone)" "Resistance" "Confirmed by Sequencing" "Parent plasmid"
            "Harboured in strain" "Category" "Box location" "Box"
FEATURES             Location/Qualifiers
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                     /label="lambda t0 terminator"
     CDS             218..1033
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     oriT            1184..1292
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     CDS             complement(1353..2501)
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     rep_origin      2894..3425
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     misc_feature    3530..5929
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     mRNA            complement(3589..4219)
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     CDS             complement(3637..3948)
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44       5581 gatcggcgcg agcgtcaacg tcagggtgtc cagttggcga aggccgccgg ccgctacacc
45       5641 ggccgcaaac gtgacgccgg catgcacgac cgcatcatca cgcttcgctc cggcggatcg
46       5701 agcattgcca agacggccaa gctggtcgga tgcagcccga gccaggtcaa acgagtgtgg
47       5761 gcggcctgga acgcgcagca gcaaaaataa agccgggcag tgcccggctt ttctcacctt
48       5821 ttcgcgtccc gcagggccgc tgcgagcgcc ctacctagat cctcgctttc cccctcggtg
49       5881 tagtccggcc agggcacgaa gggcgcggat gcgaacctgt tgagcaggtc agatctaggg
50       5941 cggcggattt gtcctactca ggagagcgtt caccgacaaa caacagataa aacgaaaggc
51       6001 ccagtctttc gactgagcct ttcgttttat ttgatgcctg ccg
52   //
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Bibliography

Adamczyk, M. and Jagura-Burdzy, G. 2003. Spread and survival of promiscuous IncP-1 plasmids. *Acta Biochimica Polonica* 50: 425–453.

Akbari, O.S., Bellen, H.J., Bier, E., Bullock, S.L., Burt, A., Church, G.M., Cook, K.R., Duchek, P., Edwards, O.R., Esvelt, K.M., Gantz, V.M., Golic, K.G., Gratz, S.J., Harrison, M.M., Hayes, K.R., James, A.A., Kaufman, T.C., Knoblich, J., Malik, H.S., Matthews, K.A., O'Connor-Giles, K.M., Parks, A.L., Perrimon, N., Port, F., Russell, S., Ueda, R. and Wildonger, J. 2015. Safeguarding gene drive experiments in the laboratory: Multiple stringent confinement strategies should be used whenever possible. 349: 927–929.

Alonso-del Valle, A., León-Sampedro, R., Rodríguez-Beltrán, J., DelaFuente, J., Hernández-García, M., Ruiz-Garbajosa, P., Cantón, R., Peña-Miller, R. and San Millán, A. 2021. Variability of plasmid fitness effects contributes to plasmid persistence in bacterial communities. *Nature Communications* 12: 2653.

Alseth, E.O., Pursey, E., Luján, A.M., McLeod, I., Rollie, C. and Westra, E.R. 2019. Bacterial biodiversity drives the evolution of CRISPR-based phage resistance. *Nature* 574: 549–552.

Ambur, O.H., Engelstädter, J., Johnsen, P.J., Miller, E.L. and Rozen, D.E. 2016. Steady at the wheel: Conservative sex and the benefits of bacterial transformation. *Philosophical Transactions of the Royal Society B: Biological Sciences* 371.

Aminov, R.I. 2010. A brief history of the antibiotic era: Lessons learned and challenges for the future. *Frontiers in Microbiology* 1.

Amos, G.C., Gozzard, E., Carter, C.E., Mead, A., Bowes, M.J., Hawkey, P.M., Zhang, L., Singer, A.C., Gaze, W.H. and Wellington, E.M.H. 2015. Validated predictive modelling of the environmental resistome. *The ISME Journal* 9: 1467–1476.

Andersson, D.I. and Hughes, D. 2014. Microbiological effects of sublethal levels of antibiotics. *Nature Reviews Microbiology* 12: 465–478.

Årdal, C., Baraldi, E., Ciabuschi, F., Outterson, K., Rex, J.H., Piddock, L.J.V. and Findlay, D. 2017. To the G20: incentivising antibacterial research and development. *The Lancet Infectious Diseases* 17: 799–801.

Arnold, B.J., Huang, I.-T. and Hanage, W.P. 2021. Horizontal gene transfer and adaptive evolution in bacteria. *Nature Reviews Microbiology* 1–13.

Bahl, M.I., Hansen, L.H., Licht, T.R. and Sørensen, S.J. 2007a. Conjugative transfer facilitates stable maintenance of IncP-1 plasmid pKJK5 in Escherichia coli cells colonizing the gastrointestinal tract of the germfree rat. *Applied and Environmental Microbiology* 73: 341–343.

Bahl, M.I., Hansen, L.H., Goesmann, A. and Sørensen, S.J. 2007b. The multiple antibiotic resistance IncP-1 plasmid pKJK5 isolated from a soil environment is phylogenetically divergent from members of the previously established α, β and δ sub-groups. *Plasmid* 58: 31–43.

Bahl, M.I., Burmølle, M., Meisner, A., Hansen, L.H. and Sørensen, S.J. 2009. All IncP-1 plasmid subgroups, including the novel ε subgroup, are prevalent in the influent of a Danish wastewater treatment plant. *Plasmid* 62: 134–139.

Bakkeren, E., Huisman, J.S., Fattinger, S.A., Hausmann, A., Furter, M., Egli, A., Slack, E., Sellin, M.E., Bonhoeffer, S., Regoes, R.R., Diard, M. and Hardt, W.D. 2019. Salmonella persisters promote the spread of antibiotic resistance plasmids in the gut. *Nature* 573: 276–280.

Baptista, P.V., McCusker, M.P., Carvalho, A., Ferreira, D.A., Mohan, N.M., Martins, M. and Fernandes, A.R. 2018. Nano-Strategies to Fight Multidrug Resistant Bacteria—"A Battle of the Titans". *Frontiers in Microbiology* 9: 1441.

Barth, Z.K., Silvas, T.V., Angermeyer, A. and Seed, K.D. 2020. Genome replication dynamics of a bacteriophage and its satellite reveal strategies for parasitism and viral restriction. *Nucleic Acids Research* 48: 249–263.

Benchling 2015. Benchling · Better tools, faster research. Available at https://benchling.com/. Accessed September 11, 2017.

Berdy, B., Spoering, A.L., Ling, L.L. and Epstein, S.S. 2017. In situ cultivation of previously uncultivable microorganisms using the ichip. *Nature Protocols* 12: 2232–2242.

Bergstrom, C.T., Lipsitch, M. and Levin, B.R. 2000. Natural selection, infectious transfer and the existence conditions for bacterial plasmids. *Genetics* 155: 1505–1519.

Bikard, D., Euler, C.W., Jiang, W., Nussenzweig, P.M., Goldberg, G.W., Duportet, X., Fischetti, V.A. and Marraffini, L.A. 2014. Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nature Biotechnology* 32: 1146–50.

Blaak, H., Kemper, M.A., de Man, H., van Leuken, J.P.G., Schijven, J.F., van Passel, M.W.J., Schmitt, H. and de Roda Husman, A.M. 2021. Nationwide surveillance reveals frequent detection of carbapenemase-producing Enterobacterales in Dutch municipal wastewater. *Science of The Total Environment* 776: 145925.

Blazanin, M. and Turner, P.E. 2021. Community context matters for bacteria-phage ecology and evolution. *The ISME Journal* 1–10.

Bouanchaud, D.H., Scavizzi, M.R. and Chabbert, Y.A. 1969. Elimination by ethidium bromide of antibiotic resistance in enterobacteria and staphylococci. *Journal of General Microbiology* 54: 417–425.

Bradley, D.E. 1983. Specification of the conjugative pili and surface mating systems of Pseudomonas plasmids. *Microbiology* 129: 2545–2556.

Briner, A.E., Donohoue, P.D., Gomaa, A.A., Selle, K., Slorach, E.M., Nye, C.H., Haurwitz, R.E., Beisel, C.L., May, A.P. and Barrangou, R. 2014. Guide RNA functional modules direct Cas9 activity and orthogonality. *Molecular Cell* 56: 333–339.

Bringel, F., Frey, L. and Hubert, J.-C. 1989. Characterization, cloning, curing, and distribution in lactic acid bacteria of pLP1, a plasmid from Lactobacillus plantarum CCM 1904 and its use in shuttle vector construction. *Plasmid* 22: 193–202.

Brockhurst, M.A., Harrison, E., Hall, J.P.J., Richards, T., McNally, A. and MacLean, C. 2019. The Ecology and Evolution of Pangenomes. *Current Biology* 29: R1094–R1103.

Buckner, M.M.C., Ciusa, M.L. and Piddock, L.J.V. 2018. Strategies to combat antimicrobial resistance: anti-plasmid and plasmid curing. *FEMS Microbiology Reviews* 42: 781–804.

Bulson, L., Becher, M.A., McKinley, T.J. and Wilfert, L. 2021. Long-term effects of antibiotic treatments on honeybee colony fitness: A modelling approach. *Journal of Applied Ecology* 58: 70–79.

Cag, Y., Caskurlu, H., Fan, Y., Cao, B. and Vahaboglu, H. 2016. Resistance mechanisms. *Annals of Translational Medicine* 4: 326–326.

Cambray, G., Guerout, A.-M. and Mazel, D. 2010. Integrons. 44: 141–166.

Carattoli, A. 2009. Resistance plasmid families in Enterobacteriaceae. 53: 2227–2238.

Carattoli, A. 2013. Plasmids and the spread of resistance. *International Journal of Medical Microbiology* 303: 298–304.

Carrilero, L., Kottara, A., Guymer, D., Harrison, E., Hall, J.P.J. and Brockhurst, M.A. 2021. Positive Selection Inhibits Plasmid Coexistence in Bacterial Genomes. *MBio* 12: e00558-21.

Castledine, M., Padfield, D. and Buckling, A. 2020. Experimental (co)evolution in a multi-species microbial community results in local maladaptation. *Ecology Letters* 23: 1673–1681.

Chan, B.K., Sistrom, M., Wertz, J.E., Kortright, K.E., Narayan, D. and Turner, P.E. 2016. Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. *Scientific Reports* 6: 26717.

Cho, S., Choe, D., Lee, E., Kim, S.C., Palsson, B. and Cho, B.-K. 2018. High-Level dCas9 Expression Induces Abnormal Cell Morphology in Escherichia coli. *ACS Synthetic Biology* 7: 1085–1094.

Choi, K.R. and Lee, S.Y. 2016. CRISPR technologies for bacterial systems: Current achievements and future directions. *Biotechnology Advances* 34: 1180–1209.

Christie, G.E. and Dokland, T. 2012. Pirates of the Caudovirales. *Virology* 434: 210–221.

Citorik, R.J., Mimee, M. and Lu, T.K. 2014. Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. *Nature Biotechnology* 32: 1141–1145.

Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. and Sayers, E.W. 2016. GenBank. 44: D67-72.

Clokie, M.R., Millard, A.D., Letarov, A.V. and Heaphy, S. 2011. Phages in nature. *Bacteriophage* 1: 31–45.

Cormack, B.P., Valdivia, R.H. and Falkow, S. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173: 33–38.

Cyriaque, V., Géron, A., Billon, G., Nesme, J., Werner, J., Gillan, D.C., Sørensen, S.J. and Wattiez, R. 2020. Metal-induced bacterial interactions promote diversity in river-sediment microbiomes. *FEMS Microbiology Ecology* 96.

Datsenko, K.A. and Wanner, B.L. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proceedings of the National Academy of Sciences* 97: 6640–5.

De Graeff, N., Jongsma, K.R., Johnston, J., Hartley, S. and Bredenoord, A.L. 2019. The ethics of genome editing in non-human animals: A systematic review of reasons reported in the academic literature. 374.

De Leij, F., Sutton, E.J., Whipps, J.M., Fenlon, J.S. and Lynch, J.M. 1995. Impact of Field Release of Genetically Modified Pseudomonas fluorescens on Indigenous Microbial Populations of Wheat. *Applied and Environmental Microbiology* 61: 3443–3453.

Dealtry, S., Ding, G.-C., Weichelt, V., Dunon, V., Schlüter, A., Martini, M.C., Papa, M.F.D., Lagares, A., Amos, G.C.A., Wellington, E.M.H., Gaze, W.H., Sipkema, D., Sjöling, S., Springael, D., Heuer, H., Elsas, J.D. van, Thomas, C. and Smalla, K. 2014. Cultivation-Independent Screening Revealed Hot Spots of IncP-1, IncP-7 and IncP-9 Plasmid Occurrence in Different Environmental Habitats. *PLOS ONE* 9: e89922.

Debroas, D. and Siguret, C. 2019. Viruses as key reservoirs of antibiotic resistance genes in the environment. *The ISME Journal* 13: 1–12.

DeNap, J.C.B., Thomas, J.R., Musk, D.J. and Hergenrother, P.J. 2004. Combating Drug-Resistant Bacteria: Small Molecule Mimics of Plasmid Incompatibility as Antiplasmid Compounds. *Journal of the American Chemical Society* 126: 15402–15404.

Dewar, A.E., Thomas, J.L., Scott, T.W., Wild, G., Griffin, A.S., West, S.A. and Ghoul, M. 2021. Plasmids facilitate pathogenicity, not cooperation, in bacteria. *ResearchSquare Preprint*.

Dimitriu, T., Marchant, L., Buckling, A. and Raymond, B. 2019. Bacteria from natural populations transfer plasmids mostly towards their kin. *Proceedings of the Royal Society B: Biological Sciences* 286: 20191110.

Dimitriu, T., Kurilovich, E., Lapinska, U., Severinov, K., Pagliara, S., Szczelkun, M.D. and Westra, E.R. 2021. Bacteriostatic antibiotics promote the evolution of CRISPR-Cas immunity. *BioRxiv* 2021.05.04.442568.

Dionisio, F., Zilhão, R. and Gama, J.A. 2019. Interactions between plasmids and other mobile genetic elements affect their transmission and persistence. *Plasmid* 102: 29–36.

Dong, H., Xiang, H., Mu, D., Wang, D. and Wang, T. 2019. Exploiting a conjugative CRISPR/Cas9 system to eliminate plasmid harboring mcr-1 gene from Escherichia coli. *International Journal of Antimicrobial Agents* 53.

Dungan, R.S. and Bjorneberg, D.L. 2020. Antibiotic resistance genes, class 1 integrons, and IncP-1/IncQ-1 plasmids in irrigation return flows. *Environmental Pollution* 257: 113568.

Edgar, R. and Qimron, U. 2010. The Escherichia coli CRISPR System Protects from λ Lysogenization, Lysogens, and Prophage Induction. *Journal of Bacteriology* 192: 6291–6294.

Elwell, L.P. and Shipley, P.L. 1980. Plasmid-Mediated Factors Associated with Virulence of Bacteria to Animals. *Annual Review of Microbiology* 34: 465–496.

Fang, F.C. and Helinski, D.R. 1991. Broad-host-range properties of plasmid RK2: importance of overlapping genes encoding the plasmid replication initiation protein TrfA. *Journal of Bacteriology* 173: 5861–5868.

Faure, G., Makarova, K.S. and Koonin, E.V. 2019. CRISPR–Cas: Complex Functional Networks and Multiple Roles beyond Adaptive Immunity. *Journal of Molecular Biology* 431: 3–20.

Ferrières, L., Hémery, G., Nham, T., Guérout, A.M., Mazel, D., Beloin, C. and Ghigo, J.M. 2010. Silent mischief: Bacteriophage Mu insertions contaminate products of Escherichia coli random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery. *Journal of Bacteriology* 192: 6418–6427.

Figurski, D.H. and Helinski, D.R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proceedings of the National Academy of Sciences* 76: 1648–1652.

1 Fillol-Salom, A., Martínez-Rubio, R., Abdulrahman, R.F., Chen, J., Davies, R. and
2 Penadés, J.R. 2018. Phage-inducible chromosomal islands are ubiquitous within
3 the bacterial universe. *The ISME Journal* 12: 2114–2128.
4 Gama, J.A., Zilhão, R. and Dionisio, F. 2020. Plasmid Interactions Can Improve Plasmid
5 Persistence in Bacterial Populations. *Frontiers in Microbiology* 11: 2033.
6 García-Quintanilla, M., Prieto, A.I., Barnes, L., Ramos-Morales, F. and Casadesús, J. 2006.
7 Bile-induced curing of the virulence plasmid in Salmonella enterica serovar
8 typhimurium. *Journal of Bacteriology* 188: 7963–7965.
9 Garcillán-Barcia, M.P. and de la Cruz, F. 2008. Why is entry exclusion an essential feature
10 of conjugative plasmids? *Plasmid* 60: 1–18.
11 Garcillán-Barcia, M.P., Francia, M.V. and de La Cruz, F. 2009. The diversity of conjugative
12 relaxases and its application in plasmid classification. *FEMS Microbiology Reviews*
13 33: 657–687.
14 Gautheret, D. and Lambert, A. 2001. Direct RNA motif definition and identification from
15 multiple sequence alignments using secondary structure profiles. *Journal of*
16 *Molecular Biology* 313: 1003–1011.
17 Geddes, B.A., Mendoza-Suárez, M.A. and Poole, P.S. 2019. A Bacterial Expression Vector
18 Archive (BEVA) for flexible modular assembly of golden gate-compatible vectors.
19 *Frontiers in Microbiology* 10.
20 Gomaa, A. a, Klumpe, H.E., Luo, M.L., Selle, K., Barrangou, R. and Beisel, L. 2014.
21 Programmable Removal of Bacterial Strains by Use of Genome-Targeting
22 CRISPR/Cas Systems. *MBio* 5: e00928-13.
23 Griffith, F. 1928. The Significance of Pneumococcal Types. *Journal of Hygiene* 27: 113–
24 159.
25 Gunjal, V.B., Thakare, R., Chopra, S. and Reddy, D.S. 2020. Teixobactin: A Paving Stone
26 toward a New Class of Antibiotics? *Journal of Medicinal Chemistry* 63: 12171–
27 12195.
28 Guo, J., Wang, T., Guan, C., Liu, B., Luo, C., Xie, Z., Zhang, C. and Xing, X.-H. 2018.
29 Improved sgRNA design in bacteria via genome-wide activity profiling. *Nucleic*
30 *Acids Research* 46: 7052–7069.
31 Haase, J., Kalkum, M. and Lanka, E. 1996. TrbK, a small cytoplasmic membrane
32 lipoprotein, functions in entry exclusion of the IncP alpha plasmid RP4. *Journal of*
33 *Bacteriology* 178: 6720–6729.
34 Haeussler, M., Schönig, K., Eckert, H., Eschstruth, A., Mianné, J., Renaud, J.-B.,
35 Schneider-Maunoury, S., Shkumatava, A., Teboul, L., Kent, J., Joly, J.-S. and
36 Concordet, J.-P. 2016. Evaluation of off-target and on-target scoring algorithms and
37 integration into the guide RNA selection tool CRISPOR. *Genome Biology* 17: 148.
38 Hale, L., Lazos, O., Haines, A.S. and Thomas, C.M. 2010. An efficient stress-free strategy
39 to displace stable bacterial plasmids. *Biotechniques* 48: 223–228.
40 Hall, J.P.J. 2021. Is the bacterial chromosome a mobile genetic element? *Nature*
41 *Communications* 12: 6400.
42 Hall, J.P.J., Wood, A.J., Harrison, E. and Brockhurst, M.A. 2016. Source–sink plasmid
43 transfer dynamics maintain gene mobility in soil bacterial communities. *Proceedings*
44 *of the National Academy of Sciences* 113: 8260–8265.
45 Hall, J.P.J., Wright, R.C.T., Guymer, D., Harrison, E. and Brockhurst, M.A. 2019. Extremely
46 fast amelioration of plasmid fitness costs by multiple functionally diverse pathways.
47 *Microbiology* 166.
48 Hall, J.P.J., Harrison, E., Lilley, A.K., Paterson, S., Spiers, A.J. and Brockhurst, M.A. 2015.
49 Environmentally co-occurring mercury resistance plasmids are genetically and
50 phenotypically diverse and confer variable context-dependent fitness effects.
51 *Environmental Microbiology* 17: 5008–5022.
52 Hall, J.P.J., Wright, R.C.T., Harrison, E., Muddiman, K.J., Wood, A.J., Paterson, S. and
53 Brockhurst, M.A. 2021. Plasmid fitness costs are caused by specific genetic
54 conflicts enabling resolution by compensatory mutation. *PLOS Biology* 19:
55 e3001225.
56 Hamilton, T.A., Pellegrino, G.M., Therrien, J.A., Ham, D.T., Bartlett, P.C., Karas, B.J.,
57 Gloor, G.B. and Edgell, D.R. 2019. Efficient inter-species conjugative transfer of a
58 CRISPR nuclease for targeted bacterial killing. *Nature Communications* 10.
59 Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C., Katsanos, D., Gribble, M.,
60 Baker, D., Marois, E., Russell, S., Burt, A., Windbichler, N., Crisanti, A. and Nolan,

1 T. 2016. A CRISPR-Cas9 gene drive system targeting female reproduction in the
2 malaria mosquito vector *Anopheles gambiae*. *Nature Biotechnology* 34: 78–83.
3 Harrison, E., Wood, A.J., Dytham, C., Pitchford, J.W., Truman, J., Spiers, A., Paterson, S.
4 and Brockhurst, M.A. 2015. Bacteriophages Limit the Existence Conditions for
5 Conjugative Plasmids. *MBio* 6: e00586-15.
6 Hullahalli, K., Rodrigues, M. and Palmer, K.L. 2017. Exploiting CRISPR-Cas to manipulate
7 Enterococcus faecalis populations. *ELife* 6: e26664.
8 Hullahalli, K., Rodrigues, M., Nguyen, U.T. and Palmer, K. 2018. An attenuated CRISPR-
9 cas system in enterococcus faecalis permits DNA acquisition. *MBio* 9.
10 Humeniuk, C., Arlet, G., Gautier, V., Grimont, P., Labia, R. and Philippon, A. 2002. β-
11 Lactamases of Kluyvera ascorbata, Probable Progenitors of Some Plasmid-
12 Encoded CTX-M Types. *Antimicrobial Agents and Chemotherapy* 46: 3045–3049.
13 Humphrey, S., Fillol-Salom, A., Quiles-Puchalt, N., Ibarra-Chávez, R., Haag, A.F., Chen, J.
14 and Penadés, J.R. 2021. Bacterial chromosomal mobility via lateral transduction
15 exceeds that of classical mobile genetic elements. *Nature Communications* 12:
16 6509.
17 Iskandar, K., Murugaiyan, J., Hammoudi Halat, D., Hage, S.E., Chibabhai, V.,
18 Adukkadukkam, S., Roques, C., Molinier, L., Salameh, P. and Van Dongen, M.
19 2022. Antibiotic Discovery and Resistance: The Chase and the Race. *Antibiotics*
20 11: 182.
21 Jain, A. and Srivastava, P. 2013. Broad host range plasmids. *FEMS Microbiology Letters*
22 348: 87–96.
23 Jalasvuori, M., Friman, V.-P., Nieminen, A., Bamford, J.K.H. and Buckling, A. 2011.
24 Bacteriophage selection against a plasmid-encoded sex apparatus leads to the loss
25 of antibiotic-resistance plasmids. *Biology Letters* 7: 902–905.
26 James, S., Collins, F.H., Welkhoff, P.A., Emerson, C., J Godfray, H.C., Gottlieb, M.,
27 Greenwood, B., Lindsay, S.W., Mbogo, C.M., Okumu, F.O., Quemada, H.,
28 Savadogo, M., Singh, J.A., Tountas, K.H. and Toure, Y.T. 2018. Pathway to
29 deployment of gene drive mosquitoes as a potential biocontrol tool for elimination of
30 malaria in sub-Saharan Africa: Recommendations of a scientific working group. 98:
31 1–49.
32 Jault, P., Leclerc, T., Jennes, S., Pirnay, J.P., Que, Y.-A., Resch, G., Rousseau, A.F.,
33 Ravat, F., Carsin, H., Le Floch, R., Schaal, J.V., Soler, C., Fevre, C., Arnaud, I.,
34 Bretaudeau, L. and Gabard, J. 2019. Efficacy and tolerability of a cocktail of
35 bacteriophages to treat burn wounds infected by Pseudomonas aeruginosa
36 (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *The Lancet*
37 *Infectious Diseases* 19: 35–45.
38 Jechalke, S., Dealtry, S., Smalla, K. and Heuer, H. 2013a. Quantification of IncP-1 Plasmid
39 Prevalence in Environmental Samples. *Applied and Environmental Microbiology* 79:
40 1410–1413.
41 Jechalke, S., Schreiter, S., Wolters, B., Dealtry, S., Heuer, H. and Smalla, K. 2013b.
42 Widespread dissemination of class 1 integron components in soils and related
43 ecosystems as revealed by cultivation-independent analysis. *Frontiers in*
44 *Microbiology* 4: 1–8.
45 Jechalke, S., Broszat, M., Lang, F., Siebe, C., Smalla, K. and Grohmann, E. 2015. Effects
46 of 100 years wastewater irrigation on resistance genes, class 1 integrons and IncP-
47 1 plasmids in Mexican soil. *Frontiers in Microbiology* 6: 163.
48 Jensen, R.B., Grohmann, E., Schwab, H., Díaz-Orejas, R. and Gerdes, K. 1995.
49 Comparison of ccd of F, parDE of RP4, and parD of R1 using a novel conditional
50 replication control system of plasmid R1. *Molecular Microbiology* 17: 211–220.
51 Jenssen, H., Hamill, P. and Hancock, R.E.W. 2006. Peptide Antimicrobial Agents. *Clinical*
52 *Microbiology Reviews* 19: 491–511.
53 Jian, Z., Zeng, L., Xu, T., Sun, S., Yan, S., Yang, L., Huang, Y., Jia, J. and Dou, T. 2021.
54 Antibiotic resistance genes in bacteria: Occurrence, spread, and control. *Journal of*
55 *Basic Microbiology*.
56 Jiang, Y., Qian, F., Yang, J., Liu, Y., Dong, F., Xu, C., Sun, B., Chen, B., Xu, X., Li, Y.,
57 Wang, R. and Yang, S. 2017. CRISPR-Cpf1 assisted genome editing of
58 Corynebacterium glutamicum. *Nature Communications* 8.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A. and Charpentier, E. 2012. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* 337: 816–821.

Jones, M., Hughes, R., Murray, A.K., Verdezoto, N. and Barnish, M. 2020. Exploring antibiotic use practices in livestock production through a novel, game-based approach. *GW4 Crucible 2020 Seed Funding Projects*.

Jordt, H., Stalder, T., Kosterlitz, O., Ponciano, J.M., Top, E.M. and Kerr, B. 2020. Coevolution of host–plasmid pairs facilitates the emergence of novel multidrug resistance. *Nature Ecology & Evolution* 4: 863–869.

Jurėnas, D., Fraikin, N., Goormaghtigh, F. and Van Melderen, L. 2022. Biology and evolution of bacterial toxin–antitoxin systems. *Nature Reviews Microbiology* 20: 335–350.

Kamruzzaman, M. and Iredell, J. 2019. A ParDE-family toxin antitoxin system in major resistance plasmids of Enterobacteriaceae confers antibiotic and heat tolerance. *Scientific Reports* 9: 9872.

Kamruzzaman, M. and Iredell, J.R. 2020. CRISPR-Cas System in Antibiotic Resistance Plasmids in Klebsiella pneumoniae. *Frontiers in Microbiology* 10.

Kamruzzaman, M., Shoma, S., Thomas, C.M., Partridge, S.R. and Iredell, J.R. 2017. Plasmid interference for curing antibiotic resistance plasmids in vivo. *PLOS ONE* 12: 1–20.

Kang, Y.K., Kwon, K., Ryu, J.S., Lee, H.N., Park, C. and Chung, H.J. 2017. Nonviral Genome Editing Based on a Polymer-Derivatized CRISPR Nanocomplex for Targeting Bacterial Pathogens and Antibiotic Resistance. *Bioconjugate Chemistry* 28: 957–967.

Kesten, J.M., Bhattacharya, A., Ashiru-Oredope, D., Gobin, M. and Audrey, S. 2017. The Antibiotic Guardian campaign: a qualitative evaluation of an online pledge-based system focused on making better use of antibiotics. *BMC Public Health* 18: 5.

Kiga, K., Tan, X.E., Ibarra-Chávez, R., Watanabe, S., Aiba, Y., Sato'o, Y., Li, F.Y., Sasahara, T., Cui, B., Kawauchi, M., Boonsiri, T., Thitiananpakorn, K., Taki, Y., Azam, A.H., Suzuki, M., Penadés, J.R. and Cui, L. 2020. Development of CRISPR-Cas13a-based antimicrobials capable of sequence-specific killing of target bacteria. *Nature Communications* 11: 1–11.

Kim, E., Koo, T., Park, S.W., Kim, D., Kim, K., Cho, H.-Y., Song, D.W., Lee, K.J., Jung, M.H., Kim, S., Kim, J.H., Kim, J.H. and Kim, J.-S. 2017. In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni. *Nature Communications* 8: 14500.

Kim, J.S., Cho, D.H., Park, M., Chung, W.J., Shin, D., Ko, K.S. and Kweon, D.H. 2015. Crispr/cas9-mediated re-sensitization of antibiotic-resistant escherichia coli harboring extended-spectrum β-lactamases. *Journal of Microbiology and Biotechnology* 26: 394–401.

Klompe, S.E., Vo, P.L.H., Halpin-Healy, T.S. and Sternberg, S.H. 2019. Transposon-encoded CRISPR–Cas systems direct RNA-guided DNA integration. *Nature* 571: 219–225.

Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J. and Smets, B.F. 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. *The ISME Journal* 9: 934–945.

Klümper, U., Recker, M., Zhang, L., Yin, X., Zhang, T., Buckling, A. and Gaze, W.H. 2019. Selection for antimicrobial resistance is reduced when embedded in a natural microbial community. *The ISME Journal* 13: 2927–2937.

Knapp, C.W., Dolfing, J., Ehlert, P.A.I. and Graham, D.W. 2010. Evidence of Increasing Antibiotic Resistance Gene Abundances in Archived Soils since 1940. *Environmental Science & Technology* 44: 580–587.

Kofler, N., Collins, J.P., Kuzma, J., Marris, E., Esvelt, K., Nelson, M.P., Newhouse, A., Rothschild, L.J., Vigliotti, V.S., Semenov, M., Jacobsen, R., Dahlman, J.E., Prince, S., Caccone, A., Brown, T. and Schmitz, O.J. 2018. Editing nature: Local roots of global governance. Environmental gene editing demands collective oversight. *Science* 362: 527–529.

Koonin, E.V. 2016. Horizontal gene transfer: essentiality and evolvability in prokaryotes, and roles in evolutionary transitions. *F1000Research* 5: 1805.

1 Kottara, A., Hall, J.P.J. and Brockhurst, M.A. 2021a. The proficiency of the original host
2 species determines community-level plasmid dynamics. *FEMS Microbiology*
3 *Ecology* 97: fiab026.
4 Kottara, A., Carrilero, L., Harrison, E., Hall, J.P.J. and Brockhurst, M.A. 2021b. The dilution
5 effect limits plasmid horizontal transmission in multispecies bacterial communities.
6 *BioRxiv* 2021.05.18.444624.
7 Kumar, M., Sarma, D.K., Shubham, S., Kumawat, M., Verma, V., Nina, P.B., JP, D., Kumar,
8 S., Singh, B. and Tiwari, R.R. 2021. Futuristic Non-antibiotic Therapies to Combat
9 Antibiotic Resistance: A Review. *Frontiers in Microbiology* 12: 16.
10 Kwoh, D.Y. and Kemper, J. 1978. Bacteriophage P22-mediated specialized transduction in
11 Salmonella typhimurium: high frequency of aberrant prophage excision. *Journal of*
12 *Virology* 27: 519–534.
13 Laborda, P., Sanz-García, F., Hernando-Amado, S. and Martínez, J.L. 2021. Pseudomonas
14 aeruginosa: an antibiotic resilient pathogen with environmental origin. *Current*
15 *Opinion in Microbiology* 64: 125–132.
16 Lale, R., Brautaset, T. and Valla, S. 2011. Broad-Host-Range Plasmid Vectors for Gene
17 Expression in Bacteria. In: Williams, J.A. (Ed.) , *Strain Engineering: Methods and*
18 *Protocols,* Humana Press, Totowa, NJ, Methods in Molecular Biology. pp. 327–343.
19 Lam, K.N., Spanogiannopoulos, P., Soto-Perez, P., Alexander, M., Nalley, M.J., Bisanz,
20 J.E., Nayak, R.R., Weakley, A.M., Yu, F.B. and Turnbaugh, P.J. 2021. Phage-
21 delivered CRISPR-Cas9 for strain-specific depletion and genomic deletions in the
22 gut microbiome. *BioRxiv* 2020.07.09.193847.
23 Lanzer, M. and Bujard, H. 1988. Promoters largely determine the efficiency of repressor
24 action. *Proceedings of the National Academy of Sciences of the United States of*
25 *America* 85: 8973–7.
26 Larsson, D.G.J., de Pedro, C. and Paxeus, N. 2007. Effluent from drug manufactures
27 contains extremely high levels of pharmaceuticals. *Journal of Hazardous Materials*
28 148: 751–755.
29 Lauritsen, I., Porse, A., Sommer, M.O.A. and Nørholm, M.H.H. 2017. A versatile one-step
30 CRISPR-Cas9 based approach to plasmid-curing. *Microbial Cell Factories* 16: 135.
31 Lázár, V., Martins, A., Spohn, R., Daruka, L., Grézal, G., Fekete, G., Számel, M., Jangir,
32 P.K., Kintses, B., Csörgő, B., Nyerges, Á., Györkei, Á., Kincses, A., Dér, A., Walter,
33 F.R., Deli, M.A., Urbán, E., Hegedűs, Z., Olajos, G., Méhi, O., Bálint, B., Nagy, I.,
34 Martinek, T.A., Papp, B. and Pál, C. 2018. Antibiotic-resistant bacteria show
35 widespread collateral sensitivity to antimicrobial peptides. *Nature Microbiology* 3:
36 718–731.
37 Lazdins, A., Maurya, A.P., Miller, C.E., Kamruzzaman, M., Liu, S., Stephens, E.R., Lloyd,
38 G.S., Haratianfar, M., Chamberlain, M., Haines, A.S., Kreft, J.U., Webber, M.A.,
39 Iredell, J. and Thomas, C.M. 2020. Potentiation of curing by a broad-host-range
40 self-transmissible vector for displacing resistance plasmids to tackle AMR. *PLOS*
41 *ONE* 15: 1–23.
42 Lederberg, J. and Tatum, E.L. 1956. Gene Recombination in Escherichia Coli. *Nature* 158:
43 558.
44 Lee, D.J., Bingle, L.E., Heurlier, K., Pallen, M.J., Penn, C.W., Busby, S.J. and Hobman, J.L.
45 2009. Gene doctoring: A method for recombineering in laboratory and pathogenic
46 Escherichia coli strains. *BMC Microbiology* 9.
47 Leenay, R.T. and Beisel, C.L. 2017. Deciphering, Communicating, and Engineering the
48 CRISPR PAM. *Journal of Molecular Biology* 429: 177–191.
49 Leonard, A. in prep.
50 Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Hawkey, P.M., Murray, A.K.,
51 Ukoumunne, O.C. and Gaze, W.H. 2018. Exposure to and colonisation by
52 antibiotic-resistant E. coli in UK coastal water users: Environmental surveillance,
53 exposure assessment, and epidemiological study (Beach Bum Survey).
54 *Environment International* 1–8.
55 Li, L., Dechesne, A., He, Z., Madsen, J.S., Nesme, J., Sorensen, S.J. and Smets, B.F.
56 2018. Estimating the Transfer Range of Plasmids Encoding Antimicrobial
57 Resistance in a Wastewater Treatment Plant Microbial Community.
58 acs.estlett.8b00105.

1 Li, L., Dechesne, A., Madsen, J.S., Nesme, J., Sørensen, S.J., Smets, B.F., Barth, • and
2 Smets, F. 2020. Plasmids persist in a microbial community by providing fitness
3 benefit to multiple phylotypes. *The ISME Journal* 14: 1170–1181.
4 Lin, A., Jimenez, J., Derr, J., Vera, P., Manapat, M.L., Esvelt, K.M., Villanueva, L., Liu, D.R.
5 and Chen, I.A. 2011. Inhibition of Bacterial Conjugation by Phage M13 and Its
6 Protein g3p: Quantitative Analysis and Model. *PLOS ONE* 6: e19991.
7 Lin-Chao, S., Chen, W.-T. and Wong, T.-T. 1992. High copy number of the pUC plasmid
8 results from a Rom/Rop-suppressible point mutation in RNA II. *Molecular*
9 *Microbiology* 6: 3385–3393.
10 Ling, L.L., Schneider, T., Peoples, A.J., Spoering, A.L., Engels, I., Conlon, B.P., Mueller, A.,
11 Schäberle, T.F., Hughes, D.E., Epstein, S., Jones, M., Lazarides, L., Steadman,
12 V.A., Cohen, D.R., Felix, C.R., Fetterman, K.A., Millett, W.P., Nitti, A.G., Zullo, A.M.,
13 Chen, C. and Lewis, K. 2015. A new antibiotic kills pathogens without detectable
14 resistance. *Nature* 517: 455–459.
15 Lopatkin, A.J., Meredith, H.R., Srimani, J.K., Pfeiffer, C., Durrett, R. and You, L. 2017.
16 Persistence and reversal of plasmid-mediated antibiotic resistance. *Nature*
17 *Communications* 8: 1689.
18 Lulijwa, R., Rupia, E.J. and Alfaro, A.C. 2020. Antibiotic use in aquaculture, policies and
19 regulation, health and environmental risks: a review of the top 15 major producers.
20 *Reviews in Aquaculture* 12: 640–663.
21 Madeira, F., Park, Y.M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P.,
22 Tivey, A.R.N., Potter, S.C., Finn, R.D. and Lopez, R. 2019. The EMBL-EBI search
23 and sequence analysis tools APIs in 2019. *Nucleic Acids Research* 47: W636–
24 W641.
25 Mahendra, C., Christie, K.A., Osuna, B.A., Pinilla-Redondo, R., Kleinstiver, B.P. and
26 Bondy-Denomy, J. 2020. Broad-spectrum anti-CRISPR proteins facilitate horizontal
27 gene transfer. *Nature Microbiology* 5: 620–629.
28 Mahlapuu, M., Håkansson, J., Ringstad, L. and Björn, C. 2016. Antimicrobial Peptides: An
29 Emerging Category of Therapeutic Agents. *Frontiers in Cellular and Infection*
30 *Microbiology* 6: 194.
31 Makarova, K.S., Wolf, Y.I., Iranzo, J., Shmakov, S.A., Alkhnbashi, O.S., Brouns, S.J.J.,
32 Charpentier, E., Cheng, D., Haft, D.H., Horvath, P., Moineau, S., Mojica, F.J.M.,
33 Scott, D., Shah, S.A., Siksnys, V., Terns, M.P., Venclovas, Č., White, M.F.,
34 Yakunin, A.F., Yan, W., Zhang, F., Garrett, R.A., Backofen, R., van der Oost, J.,
35 Barrangou, R. and Koonin, E.V. 2020. Evolutionary classification of CRISPR–Cas
36 systems: a burst of class 2 and derived variants. *Nature Reviews Microbiology* 18:
37 67–83.
38 Marraffini, L.A. and Sontheimer, E.J. 2008. CRISPR Interference Limits Horizontal Gene
39 Transfer in Staphylococci by Targeting DNA. *Science* 322: 1843–1845.
40 Martínez-García, E., Calles, B., Arévalo-Rodríguez, M. and de Lorenzo, V. 2011. pBAM1:
41 an all-synthetic genetic tool for analysis and construction of complex bacterial
42 phenotypes. *BMC Microbiology* 11: 38.
43 Martinson, J.N.V. and Walk, S.T. 2020. Escherichia coli Residency in the Gut of Healthy
44 Human Adults. *EcoSal Plus* 9.
45 Mathers, A.J., Peirano, G. and Pitout, J.D.D. 2015. The Role of Epidemic Resistance
46 Plasmids and International High-Risk Clones in the Spread of Multidrug-Resistant
47 Enterobacteriaceae. *Clinical Microbiology Reviews*.
48 McKenna, M. 2019. Antibiotics set to flood Florida's troubled orange orchards. *Nature* 567:
49 302–303.
50 Medical Research Council 2014. *Antimicrobial Resistance*.
51 Mendoza-Suárez, M.A., Geddes, B.A., Sánchez-Cañizares, C., Ramírez-González, R.H.,
52 Kirchhelle, C., Jorrin, B. and Poole, P.S. 2020. Optimizing Rhizobium-legume
53 symbioses by simultaneous measurement of rhizobial competitiveness and N2
54 fixation in nodules. *Proceedings of the National Academy of Sciences* 117: 9822–
55 9831.
56 Meyer, R. 2009. Replication and conjugative mobilization of broad host-range IncQ
57 plasmids. *Plasmid* 62: 57–70.
58 Międzybrodzki, R., Hoyle, N., Zhvaniya, F., Łusiak-Szelachowska, M., Weber-Dąbrowska,
59 B., Łobocka, M., Borysowski, J., Alavidze, Z., Kutter, E., Górski, A. and Gogokhia,
60 L. 2018. Current Updates from the Long-Standing Phage Research Centers in

1 Georgia, Poland, and Russia. In: Harper, D.R., S.T. Abedon, B.H. Burrowes and
2 M.L. McConville (Eds.) , *Bacteriophages: Biology, Technology, Therapy,* Springer
3 International Publishing, Cham, pp. 1–31.
4 Murray, A.K., Stanton, I., Gaze, W.H. and Snape, J. 2021. Dawning of a new ERA:
5 Environmental Risk Assessment of antibiotics and their potential to select for
6 antimicrobial resistance. *Water Research* 200: 117233.
7 Murray, C.J., Ikuta, K.S., Sharara, F., Swetschinski, L., Aguilar, G.R., Gray, A., Han, C.,
8 Bisignano, C., Rao, P., Wool, E., Johnson, S.C., Browne, A.J., Chipeta, M.G., Fell,
9 F., Hackett, S., Haines-Woodhouse, G., Hamadani, B.H.K., Kumaran, E.A.P.,
10 McManigal, B., Agarwal, R., Akech, S., Albertson, S., Amuasi, J., Andrews, J.,
11 Aravkin, A., Ashley, E., Bailey, F., Baker, S., Basnyat, B., Bekker, A., Bender, R.,
12 Bethou, A., Bielicki, J., Boonkasidecha, S., Bukosia, J., Carvalheiro, C., Castañeda-
13 Orjuela, C., Chansamouth, V., Chaurasia, S., Chiurchiù, S., Chowdhury, F., Cook,
14 A.J., Cooper, B., Cressey, T.R., Criollo-Mora, E., Cunningham, M., Darboe, S.,
15 Day, N.P.J., Luca, M.D., Dokova, K., Dramowski, A., Dunachie, S.J., Eckmanns, T.,
16 Eibach, D., Emami, A., Feasey, N., Fisher-Pearson, N., Forrest, K., Garrett, D.,
17 Gastmeier, P., Giref, A.Z., Greer, R.C., Gupta, V., Haller, S., Haselbeck, A., Hay,
18 S.I., Holm, M., Hopkins, S., Iregbu, K.C., Jacobs, J., Jarovsky, D., Javanmardi, F.,
19 Khorana, M., Kissoon, N., Kobeissi, E., Kostyanev, T., Krapp, F., Krumkamp, R.,
20 Kumar, A., Kyu, H.H., Lim, C., Limmathurotsakul, D., Loftus, M.J., Lunn, M., Ma, J.,
21 Mturi, N., Munera-Huertas, T., Musicha, P., Mussi-Pinhata, M.M., Nakamura, T.,
22 Nanavati, R., Nangia, S., Newton, P., Ngoun, C., Novotney, A., Nwakanma, D.,
23 Obiero, C.W., Olivas-Martinez, A., Olliaro, P., Ooko, E., Ortiz-Brizuela, E., Peleg,
24 A.Y., Perrone, C., Plakkal, N., Ponce-de-Leon, A., Raad, M., Ramdin, T., Riddell,
25 A., Roberts, T., Robotham, J.V., Roca, A., Rudd, K.E., Russell, N., Schnall, J.,
26 Scott, J.A.G., Shivamallappa, M., Sifuentes-Osornio, J., Steenkeste, N.,
27 Stewardson, A.J., Stoeva, T., Tasak, N., Thaiprakong, A., Thwaites, G., Turner, C.,
28 Turner, P., Doorn, H.R. van, Velaphi, S., Vongpradith, A., Vu, H., Walsh, T., Waner,
29 S., Wangrangsimakul, T., Wozniak, T., Zheng, P., Sartorius, B., Lopez, A.D.,
30 Stergachis, A., Moore, C., Dolecek, C. and Naghavi, M. 2022. Global burden of
31 bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet*.
32 Nakamura, Y., Gojobori, T. and Ikemura, T. 2000. Codon usage tabulated from
33 international DNA sequence databases: status for the year 2000. *Nucleic Acids*
34 *Research* 28.
35 National Academies of Sciences, Engineering, and Medicine 2016. *Gene Drives on the*
36 *Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with*
37 *Public Values*. The National Academies Press, Washington, DC, .
38 Nazarian, P., Tran, F. and Boedicker, J.Q. 2018. Modeling Multispecies Gene Flow
39 Dynamics Reveals the Unique Roles of Different Horizontal Gene Transfer
40 Mechanisms. *Frontiers in Microbiology* 9: 2978.
41 Negus, D., Moore, C., Baker, M., Raghunathan, D., Tyson, J. and Sockett, R.E. 2017.
42 Predator Versus Pathogen: How Does Predatory Bdellovibrio bacteriovorus
43 Inferface with the Challenges of Killing Gram-Negative Pathogens in a Host
44 Setting? *Annual Review of Microbiology* 71: 441–57.
45 Neil, Kevin, Allard, Nancy, Roy, Patricia, Grenier, Frederic, Menendez, Alfredo, Burrus,
46 Vincent, and Rodrigue, Sebastien 2021. High-efficiency delivery of CRISPR-Cas9
47 by engineered probiotics enables precise microbiome editing. *Molecular Systems*
48 *Biology* 17: e10335.
49 Nour, E.H., Elsayed, T.R., Springael, D. and Smalla, K. 2017. Comparable dynamics of
50 linuron catabolic genes and IncP-1 plasmids in biopurification systems (BPSs) as a
51 response to linuron spiking. *Applied Microbiology and Biotechnology* 101: 4815–
52 4825.
53 Novick, R.P., Christie, G.E. and Penadés, J.R. 2010. The phage-related chromosomal
54 islands of Gram-positive bacteria. *Nature Reviews Microbiology* 8: 541–551.
55 Ogilvie, L.A., Firouzmand, S. and Jones, B.V. 2012. Evolutionary, ecological and
56 biotechnological perspectives on plasmids resident in the human gut mobile
57 metagenome. *Bioengineered* 3: 13–31.
58 O'Neill, J. 2016. *Tackling Drug-Resistant Infections Globally: Final Report and*
59 *Recommendations*. The Review on Antimicrobial Resistance, .

1 Padfield, D., Castledine, M., Pennycook, J., Hesse, E. and Buckling, A. 2020. Short-term
2 relative invader growth rate predicts long-term equilibrium proportion in a stable,
3 coexisting microbial community. *BioRxiv*.
4 Pallares-Vega, R., Blaak, H., van der Plaats, R., de Roda Husman, A.M., Hernandez Leal,
5 L., van Loosdrecht, M.C.M., Weissbrodt, D.G. and Schmitt, H. 2019. Determinants
6 of presence and removal of antibiotic resistance genes during WWTP treatment: A
7 cross-sectional study. *Water Research* 161: 319–328.
8 Pansegrau, W., Lanka, E., Barth, P.T., Figurski, D.H., Guiney, D.G., Haas, D., Helinski,
9 D.R., Schwab, H., Stanisich, V.A. and Thomas, C.M. 1994. Complete Nucleotide
10 Sequence of Birmingham IncPa Plasmids. *Journal of Molecular Biology* 623–663.
11 Park, J.Y., Moon, B.Y., Park, J.W., Thornton, J.A., Park, Y.H. and Seo, K.S. 2017. Genetic
12 engineering of a temperate phage-based delivery system for CRISPR/Cas9
13 antimicrobials against Staphylococcus aureus. *Scientific Reports* 7: 44929.
14 Parmeciano Di Noto, G., Molina, M.C. and Quiroga, C. 2019. Insights Into Non-coding
15 RNAs as Novel Antimicrobial Drugs. *Frontiers in Genetics* 10: 57.
16 Partridge, S.R., Kwong, S.M., Firth, N. and Jensen, S.O. 2018. Mobile genetic elements
17 associated with antimicrobial resistance. *Clinical Microbiology Reviews* 31.
18 Pawluk, A., Davidson, A.R. and Maxwell, K.L. 2018. Anti-CRISPR: discovery, mechanism
19 and function. *Nature Reviews Microbiology* 16: 12–17.
20 Percival, S.L., Suleman, L., Vuotto, C. and Donelli, G. 2015 2015. Healthcare-associated
21 infections, medical devices and biofilms: risk, tolerance and control. *Journal of*
22 *Medical Microbiology* 64: 323–334.
23 Peters, J. unpublished. PA01::cas9 (Gross Lab, unpublished). .
24 Petitjean, M., Condamine, B., Burdet, C., Denamur, E. and Ruppé, E. 2021. Phylum barrier
25 and Escherichia coli intra-species phylogeny drive the acquisition of antibiotic-
26 resistance genes. *Microbial Genomics* 7: 000489.
27 Pfeifer, E., de Sousa, J.A.M., Touchon, M. and Rocha, E.P.C. 2020. Bacteria have
28 numerous phage-plasmid families with conserved phage and variable plasmid gene
29 repertoires. *BioRxiv*.
30 Pinilla-Redondo, R., Russel, J., Mayo-Muñoz, D., Shah, S.A., Garrett, R.A., Nesme, J.,
31 Madsen, J.S., Fineran, P.C. and Sørensen, S.J. 2021. CRISPR-Cas systems are
32 widespread accessory elements across bacterial and archaeal plasmids. *BioRxiv*
33 2021.06.04.447074.
34 Pires, D.P., Cleto, S., Sillankorva, S., Azeredo, J. and Lu, T.K. 2016. Genetically
35 Engineered Phages: a Review of Advances over the Last Decade. *Microbiology*
36 *and Molecular Biology Reviews* 80: 523–543.
37 POST 2019. *Reservoirs of Antimicrobial Resistance*. 1–6 pp.
38 Public Health England 2014. Antibiotic Guardian. *Antibiot. Guard.* Available at
39 https://antibioticguardian.com. Accessed September 22, 2021.
40 Puigbò, P., Guzmán, E., Romeu, A. and Garcia-Vallvé, S. 2007. OPTIMIZER: A web server
41 for optimizing the codon usage of DNA sequences. *Nucleic Acids Research* 35:
42 126–131.
43 Pursey, E., Sünderhauf, D., Gaze, W.H., Westra, E.R. and van Houte, S. 2018. CRISPR-
44 Cas antimicrobials: Challenges and future prospects. *PLOS Pathogens* 14:
45 e1006990.
46 Pursey, E., Dimitriu, T., Paganelli, F.L., Westra, E.R. and van Houte, S. 2021. CRISPR-Cas
47 is associated with fewer antibiotic resistance genes in bacterial pathogens.
48 *Proceedings of the Royal Society B: Biological Sciences* 377.
49 Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P. and Lim,
50 W.A. 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific
51 control of gene expression. *Cell* 152: 1173–1183.
52 Qiu, D., Damron, F.H., Mima, T., Schweizer, H.P. and Yu, H.D. 2008. PBAD-based shuttle
53 vectors for functional analysis of toxic and highly regulated genes in Pseudomonas
54 and Burkholderia spp. and other bacteria. *Applied and Environmental Microbiology*
55 74: 7422–7426.
56 Rahube, T.O., Marti, R., Scott, A., Tien, Y.-C., Murray, R., Sabourin, L., Duenk, P., Lapen,
57 D.R. and Topp, E. 2016. Persistence of antibiotic resistance and plasmid-
58 associated genes in soil following application of sewage sludge and abundance on
59 vegetables at harvest. *Canadian Journal of Microbiology* 62: 600–607.

Ram, G., Ross, H.F., Novick, R.P., Rodriguez-Pagan, I. and Jiang, D. 2018. Conversion of staphylococcal pathogenicity islands to CRISPR-carrying antibacterial agents that cure infections in mice. *Nature Biotechnology* 36: 971–976.

Ratner, H.K., Escalera-Maurer, A., Le Rhun, A., Jaggavarapu, S., Wozniak, J.E., Crispell, E.K., Charpentier, E. and Weiss, D.S. 2019. Catalytically Active Cas9 Mediates Transcriptional Interference to Facilitate Bacterial Virulence. *Molecular Cell* 75: 1–13.

Ray, J.L., Harms, K., Wikmark, O.G., Starikova, I., Johnsen, P.J. and Nielsen, K.M. 2009. Sexual isolation in Acinetobacter baylyi is locus-specific and varies 10,000-fold over the genome. *Genetics* 182: 1165–1181.

Renwick, M.J., Brogan, D.M. and Mossialos, E. 2016. A systematic review and critical assessment of incentive strategies for discovery and development of novel antibiotics. *The Journal of Antibiotics* 69: 73–88.

Roach, D.R. and Debarbieux, L. 2017. Phage therapy: awakening a sleeping giant. *Emerging Topics in Life Sciences* 1: 93–103.

Rock, C. and Donnenberg, M.S. 2014. Human Pathogenic Enterobacteriaceae. In: *Reference Module in Biomedical Sciences,* Elsevier, .

Rodrigues, M., McBride, S.W., Hullahalli, K., Palmer, K.L. and Duerkop, B.A. 2019. Conjugative Delivery of CRISPR-Cas9 for the Selective Depletion of Antibiotic-Resistant Enterococci. *Antimicrobial Agents and Chemotherapy* 63: e01454-19.

Rodríguez-Rubio, L., Serna, C., Ares-Arroyo, M., Matamoros, B.R., Delgado-Blas, J.F., Montero, N., Bernabe-Balas, C., Wedel, E.F., Mendez, I.S., Muniesa, M. and Gonzalez-Zorn, B. 2020. Extensive antimicrobial resistance mobilization via multicopy plasmid encapsidation mediated by temperate phages. *Journal of Antimicrobial Chemotherapy* 75: 3173–3180.

Rubin, B.E., Diamond, S., Cress, B.F., Crits-christoph, A., He, C., Xu, M., Zhou, Z., Smock, D.C., Tang, K., Trenton, K., Krishnappa, N., Jillian, F. and Doudna, J.A. 2020. Targeted Genome Editing of Bacteria Within Microbial Communities. *BioRxiv* 1–49.

Rumbo, C., Fernández-Moreira, E., Merino, M., Poza, M., Mendez, J.A., Soares, N.C., Mosquera, A., Chaves, F. and Bou, G. 2011. Horizontal Transfer of the OXA-24 Carbapenemase Gene via Outer Membrane Vesicles: a New Mechanism of Dissemination of Carbapenem Resistance Genes in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy* 55: 3084–3090.

Ruotsalainen, P., Penttinen, R., Mattila, S. and Jalasvuori, M. 2019. Midbiotics: conjugative plasmids for genetic engineering of natural gut flora. *Gut Microbes* 10.

Saeki, E.K., Kobayashi, R.K.T. and Nakazato, G. 2020. Quorum sensing system: Target to control the spread of bacterial infections. *Microbial Pathogenesis* 142: 104068.

San Millan, A. and MacLean, R.C. 2017. Fitness Costs of Plasmids: a Limit to Plasmid Transmission. *Microbiology Spectrum* 5: 5.5.02.

Santacruz, A., Collado, M.C., García-Valdés, L., Segura, M.T., Martín-Lagos, J.A., Anjos, T., Martí-Romero, M., Lopez, R.M., Florido, J., Campoy, C. and Sanz, Y. 2010. Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women. *British Journal of Nutrition* 104: 83–92.

dos Santos, D.F.K., Istvan, P., Quirino, B.F. and Kruger, R.H. 2017. Functional Metagenomics as a Tool for Identification of New Antibiotic Resistance Genes from Natural Environments. *Microbial Ecology* 73: 479–491.

Schroth, M.N., Cho, J.J., Green, S.K., Kominos, S.D. and Microbiology Society PublishingYR 2018 2018. Epidemiology of Pseudomonas aeruginosa in agricultural areas*. *Journal of Medical Microbiology* 67: 1191–1201.

Seiler, C. and Berendonk, T. 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Frontiers in Microbiology* 3: 399.

Selle, K., Fletcher, J.R., Tuson, H., Schmitt, D.S., McMillan, L., Vridhambal, G.S., Rivera, A.J., Montgomery, S.A., Fortier, L.-C., Barrangou, R., Theriot, C.M. and Ousterout, D.G. 2020. In Vivo Targeting of Clostridioides difficile Using Phage-Delivered CRISPR-Cas3 Antimicrobials. *MBio* 11: e00019-20.

Sengupta, M. and Austin, S. 2011. Prevalence and Significance of Plasmid Maintenance Functions in the Virulence Plasmids of Pathogenic Bacteria. *Infection and Immunity* 79: 2502–2509.

1 Shintani, M., Sanchez, Z.K. and Kimbara, K. 2015. Genomics of microbial plasmids:
2 classification and identification based on replication and transfer systems and host
3 taxonomy. *Frontiers in Microbiology* 6: 242.
4 Shintani, M., Nour, E., Elsayed, T., Blau, K., Wall, I., Jechalke, S., Spröer, C., Bunk, B.,
5 Overmann, J. and Smalla, K. 2020. Plant Species-Dependent Increased
6 Abundance and Diversity of IncP-1 Plasmids in the Rhizosphere: New Insights Into
7 Their Role and Ecology. *Frontiers in Microbiology* 11: 2784.
8 Sierocinski, P., Bayer, F., Yvon-Durocher, G., Burdon, M., Großkopf, T., Alston, M.,
9 Swarbreck, D., Hobbs, P.J., Soyer, O.S. and Buckling, A. 2018. Biodiversity–
10 function relationships in methanogenic communities. *Molecular Ecology* 27: 4641–
11 4651.
12 Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J. and Greenberg,
13 E.P. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected
14 with bacterial biofilms. *Nature* 407: 762–764.
15 Skurray, R.A. and Reeves, P. 1973. Characterization of Lethal Zygosis Associated with
16 Conjugation in Escherichia coli K-12. *Journal of Bacteriology* 113: 58–70.
17 Souque, C., Escudero, J.A. and MacLean, R.C. 2021. Integron activity accelerates the
18 evolution of antibiotic resistance. *ELife* 10: e62474.
19 Stalder, T., Rogers, L.M., Renfrow, C., Yano, H., Smith, Z. and Top, E.M. 2017. Emerging
20 patterns of plasmid-host coevolution that stabilize antibiotic resistance. *Scientific*
21 *Reports* 7: 4853.
22 Stecher, B., Denzler, R., Maier, L., Bernet, F., Sanders, M.J., Pickard, D.J., Barthel, M.,
23 Westendorf, A.M., Krogfelt, K.A., Walker, A.W., Ackermann, M., Dobrindt, U.,
24 Thomson, N.R. and Hardt, W.-D. 2012. Gut inflammation can boost horizontal gene
25 transfer between pathogenic and commensal Enterobacteriaceae. *Proceedings of*
26 *the National Academy of Sciences* 109: 1269–1274.
27 Stewart, F.M. and Levin, B.R. 1977. The Population Biology of Bacterial Plasmids: A
28 PRIORI Conditions for the Existence of Conjugationally Transmitted Factors.
29 *Genetics* 87: 209–228.
30 Stocker, F., Obermeier, M.M., Resch, K., Berg, G. and Müller Bogotá, C.A. 2020. A New
31 High-Throughput Screening Method to Detect Antimicrobial Volatiles from
32 Metagenomic Clone Libraries. *Antibiotics* 9: 726.
33 Streicher, L.M. 2021. Exploring the future of infectious disease treatment in a post-antibiotic
34 era: A comparative review of alternative therapeutics. *Journal of Global*
35 *Antimicrobial Resistance* 24: 285–295.
36 Sun, L., He, T., Zhang, L., Pang, M., Zhang, Q., Zhou, Y., Bao, H. and Wang, R. 2017.
37 Generation of Newly Discovered Resistance Gene mcr-1 Knockout in Escherichia
38 coli Using the CRISPR/Cas9 System. *Journal of Microbiology and Biotechnology*
39 27: 1276–1280.
40 Sünderhauf, D., Pursey, E. and van Houte, S. 2018. CRISPR-based antimicrobials: gene-
41 and pathogen-focused applications. Available at https://www.id-
42 hub.com/2018/11/01/crispr-based-antimicrobials-gene-pathogen-focused-
43 applications-2/. Accessed November 1, 2018.
44 Tagliaferri, T.L., Guimarães, N.R., Pereira, M. de P.M., Vilela, L.F.F., Horz, H.P., dos
45 Santos, S.G. and Mendes, T.A. de O. 2020. Exploring the Potential of CRISPR-
46 Cas9 Under Challenging Conditions: Facing High-Copy Plasmids and
47 Counteracting Beta-Lactam Resistance in Clinical Strains of Enterobacteriaceae.
48 *Frontiers in Microbiology* 11: 1–11.
49 Tal, N. and Sorek, R. 2022. SnapShot: Bacterial immunity. *Cell* 185: 578-578.e1.
50 Tang, W. and Liu, D.R. 2018. Rewritable multi-event analog recording in bacterial and
51 mammalian cells. *Science* 360: eaap8992.
52 Tariq, M.A., Everest, F.L.C., Cowley, L.A., Wright, R., Holt, G.S., Ingram, H., Duignan,
53 L.A.M., Nelson, A., Lanyon, C.V., Perry, A., Perry, J.D., Bourke, S., Brockhurst,
54 M.A., Bridge, S.H., De Soyza, A. and Smith, D.L. 2019. Temperate Bacteriophages
55 from Chronic Pseudomonas aeruginosa Lung Infections Show Disease-Specific
56 Changes in Host Range and Modulate Antimicrobial Susceptibility. *MSystems* 4:
57 e00191-18.
58 Theriot, C.M., Koenigsknecht, M.J., Carlson, P.E., Hatton, G.E., Nelson, A.M., Li, B.,
59 Huffnagle, G.B., Z. Li, J. and Young, V.B. 2014. Antibiotic-induced shifts in the

mouse gut microbiome and metabolome increase susceptibility to Clostridium difficile infection. *Nature Communications* 5: 3114.

Tomoeda, M., Inuzuka, M., Kubo, N. and Nakamura, S. 1968. Effective elimination of drug resistance and sex factors in Escherichia coli by sodium dodecyl sulfate. *Journal of Bacteriology* 95: 1078–1089.

Toprak, E., Veres, A., Michel, J.-B., Chait, R., Hartl, D.L. and Kishony, R. 2012. Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nature Genetics* 44: 101–105.

Tran, F. and Boedicker, J.Q. 2017. Genetic cargo and bacterial species set the rate of vesicle-mediated horizontal gene transfer. *Scientific Reports* 7: 1–10.

Trifinopoulos, J., Nguyen, L.-T., von Haeseler, A. and Minh, B.Q. 2016. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research* 44: W232–W235.

United Nations 2017. Frontiers 2017: Emerging Issues of Environmental Concern. *UNEP - UN Environ. Programme*. Available at http://www.unep.org/resources/frontiers-2017-emerging-issues-environmental-concern. Accessed September 10, 2021.

Valderrama, J.A., Kulkarni, S.S., Nizet, V. and Bier, E. 2019. A bacterial gene-drive system efficiently edits and inactivates a high copy number antibiotic resistance locus. *Nature Communications* 10: 1–8.

Vázquez, R., García, E. and García, P. 2018. Phage Lysins for Fighting Bacterial Respiratory Infections: A New Generation of Antimicrobials. *Frontiers in Immunology* 9: 2252.

Vigouroux, A., Oldewurtel, E., Cui, L., Bikard, D. and van Teeffelen, S. 2018. Tuning dCas9's ability to block transcription enables robust, noiseless knockdown of bacterial genes. *Molecular Systems Biology* 14: e7899.

Volkova, V.V., Lu, Z., Besser, T. and Gröhn, Y.T. 2014. Modeling the Infection Dynamics of Bacteriophages in Enteric Escherichia coli: Estimating the Contribution of Transduction to Antimicrobial Gene Spread. *Applied and Environmental Microbiology* 80: 4350–4362.

Vrancianu, C.O., Popa, L.I., Bleotu, C. and Chifiriuc, M.C. 2020. Targeting Plasmids to Limit Acquisition and Transmission of Antimicrobial Resistance. *Frontiers in Microbiology* 11.

Walters, W.A., Xu, Z. and Knight, R. 2014. Meta-analyses of human gut microbes associated with obesity and IBD. *FEBS Letters* 588: 4223–4233.

Wan, P., Cui, S., Ma, Z., Chen, L., Li, X., Zhao, R., Xiong, W. and Zeng, Z. 2020. Reversal of mcr-1-mediated colistin resistance in Escherichia coli by CRISPR-Cas9 system. 13: 1171–1178.

Wang, K. and Nicholaou, M. 2017. Suppression of Antimicrobial Resistance in MRSA Using CRISPR-dCas9. *American Society for Clinical Laboratory Science* 30: 207–213.

Wang, P., He, D., Li, B., Guo, Y., Wang, W., Luo, X., Zhao, X. and Wang, X. 2019. Eliminating mcr-1-harbouring plasmids in clinical isolates using the CRISPR/Cas9 system. *Journal of Antimicrobial Chemotherapy* 74: 2559–2565.

Watson, B.N.J., Easingwood, R.A., Tong, B., Wolf, M., Salmond, G.P.C., Staals, R.H.J., Bostina, M. and Fineran, P.C. 2019. Different genetic and morphological outcomes for phages targeted by single or multiple CRISPR-Cas spacers. *Philosophical Transactions of the Royal Society B: Biological Sciences* 374: 20180090.

Wellington, E.M., Boxall, A.B., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D.L., Lee, N.M., Otten, W., Thomas, C.M. and Williams, A.P. 2013. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *The Lancet Infectious Diseases* 13: 155–165.

Westra, E.R., Swarts, D.C., Staals, R.H., Jore, M.M., Brouns, S.J. and van der Oost, J. 2012. The CRISPRs, they are a-changin': how prokaryotes generate adaptive immunity. *Annual Review of Genetics* 46: 311–339.

Wheatley, R.M., Caballero, J.D., Schalk, T.E. van der, Winter, F.H.D., Kapel, N., Recanatini, C., Timbermont, L., Kluytmans, J., Esser, M., Lacoma, A., Prat-Aymerich, C., Oliver, A., Kumar-Singh, S., Malhotra-Kumar, S., MacLean, R.C. and Group, W. working 2022. Assessing the contribution of gut-to-lung translocation to bacterial colonization and antibiotic resistance in an ICU patient. .

Whelan, R. and McVicker, G. 2021. Molecular genetics of the virulence plasmid of pathogenic Escherichia coli O104:H4. *Poster 211, Microbiology Society Annual Conference*.

Williams, B.O. and Warman, M.L. 2017. CRISPR/CAS9 Technologies. *Journal of Bone and Mineral Research* 32: 883–888.

Williams, S.E., Stennett, H.L., Back, C.R., Tiwari, K., Gomez, J.O., Challand, M.R., Hendry, K.R., Spencer, J., Essex-Lopresti, A.E., Willis, C.L., Curnow, P. and Race, P.R. 2020. The bristol sponge microbiome collection: A unique repository of deep-sea microorganisms and associated natural products. *Antibiotics* 9: 1–18.

Willms, I.M., Yuan, J., Penone, C., Goldmann, K., Vogt, J., Wubet, T., Schöning, I., Schrumpf, M., Buscot, F. and Nacke, H. 2020. Distribution of Medically Relevant Antibiotic Resistance Genes and Mobile Genetic Elements in Soils of Temperate Forests and Grasslands Varying in Land Use. *Genes* 11: 150.

Wolters, B., Kyselková, M., Krögerrecklenfort, E., Kreuzig, R. and Smalla, K. 2014. Transferable antibiotic resistance plasmids 1 from biogas plant digestates often belong to the IncP-1ε subgroup. *Frontiers in Microbiology* 5: 1–11.

Wongpayak, P., Meesungnoen, O., Saejang, S. and Subsoontorn, P. 2021. A highly effective and self-transmissible CRISPR antimicrobial for elimination of target plasmids without antibiotic selection. *PeerJ* 9: e11996.

World Health Organisation 2021. Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report: 2021. Available at https://www.who.int/publications-detail-redirect/9789240027336. Accessed September 21, 2021.

Xie, Y., Wei, Y., Shen, Y., Li, X., Zhou, H., Tai, C., Deng, Z. and Ou, H.-Y. 2018. TADB 2.0: an updated database of bacterial type II toxin–antitoxin loci. *Nucleic Acids Research* 46: D749–D753.

Yamada, M., Watanabe, Y., Gootenberg, J.S., Hirano, H., Ran, F.A., Nakane, T., Ishitani, R., Zhang, F., Nishimasu, H. and Nureki, O. 2017. Crystal Structure of the Minimal Cas9 from Campylobacter jejuni Reveals the Molecular Diversity in the CRISPR-Cas9 Systems. *Molecular Cell* 65: 1109-1121.e3.

Yang, F., Mao, D., Zhou, H. and Luo, Y. 2016. Prevalence and Fate of Carbapenemase Genes in a Wastewater Treatment Plant in Northern China. *PLOS ONE* 11: e0156383.

Yee, Y.C., Kisslinger, B., Yu, V.L. and Jin, D.J. 1996. A mechanism of rifamycin inhibition and resistance in Pseudomonas aeruginosa. *Journal of Antimicrobial Chemotherapy* 38: 133–137.

Yelin, I. and Kishony, R. 2018. Antibiotic Resistance. *Cell* 172: 1136-1136.e1.

Yi, H., Huang, L., Yang, B., Gomez, J., Zhang, H. and Yin, Y. 2020. AcrFinder: genome mining anti-CRISPR operons in prokaryotes and their viruses. *Nucleic Acids Research* 48: W358–W365.

Yosef, I., Manor, M., Kiro, R. and Qimron, U. 2015. Temperate and lytic bacteriophages programmed to sensitize and kill antibiotic-resistant bacteria. *Proceedings of the National Academy of Sciences* 112: 201500107.

Yuen, G., Khan, F.J., Gao, S., Stommel, J.M., Batchelor, E., Wu, X. and Luo, J. 2017. CRISPR/Cas9-mediated gene knockout is insensitive to target copy number but is dependent on guide RNA potency and Cas9/sgRNA threshold expression level. *Nucleic Acids Research* 45: 12039–12053.

Zeng, J.-M., Yuan, H., Lu, Y., Cai, R.-X. and Chen, C. 2017. Sub-inhibitory concentrations of fluoroquinolones increase conjugation frequency. *Microbial Pathogenesis* 114: 57–62.

Zhang, J., Zong, W., Hong, W., Zhang, Z.-T. and Wang, Y. 2018. Exploiting endogenous CRISPR-Cas system for multiplex genome editing in Clostridium tyrobutyricum and engineer the strain for high-level butanol production. *Metabolic Engineering* 47: 49–59.

Zhang, W., Bielaszewska, M., Kunsmann, L., Mellmann, A., Bauwens, A., Köck, R., Kossow, A., Anders, A., Gatermann, S. and Karch, H. 2013. Lability of the pAA Virulence Plasmid in Escherichia coli O104:H4: Implications for Virulence in Humans. *PLOS ONE* 8.

Zheng, J., Peng, D., Ruan, L. and Sun, M. 2013. Evolution and dynamics of megaplasmids with genome sizes larger than 100 kb in the Bacillus cereus group. *BMC Evolutionary Biology* 13: 262.

1 Zinder, N.D. and Lederberg, J. 1952. Genetic exchange in Salmonella. *Journal of*
2 *Bacteriology* 64: 679–699.
3 Zmora, N., Zilberman-Schapira, G., Suez, J., Mor, U., Dori-Bachash, M., Bashiardes, S.,
4 Kotler, E., Zur, M., Regev-Lehavi, D., Brik, R.B.-Z., Federici, S., Cohen, Y.,
5 Linevsky, R., Rothschild, D., Moor, A.E., Ben-Moshe, S., Harmelin, A., Itzkovitz, S.,
6 Maharshak, N., Shibolet, O., Shapiro, H., Pevsner-Fischer, M., Sharon, I., Halpern,
7 Z., Segal, E. and Elinav, E. 2018. Personalized Gut Mucosal Colonization
8 Resistance to Empiric Probiotics Is Associated with Unique Host and Microbiome
9 Features. *Cell* 174: 1388-1405.e21.
10