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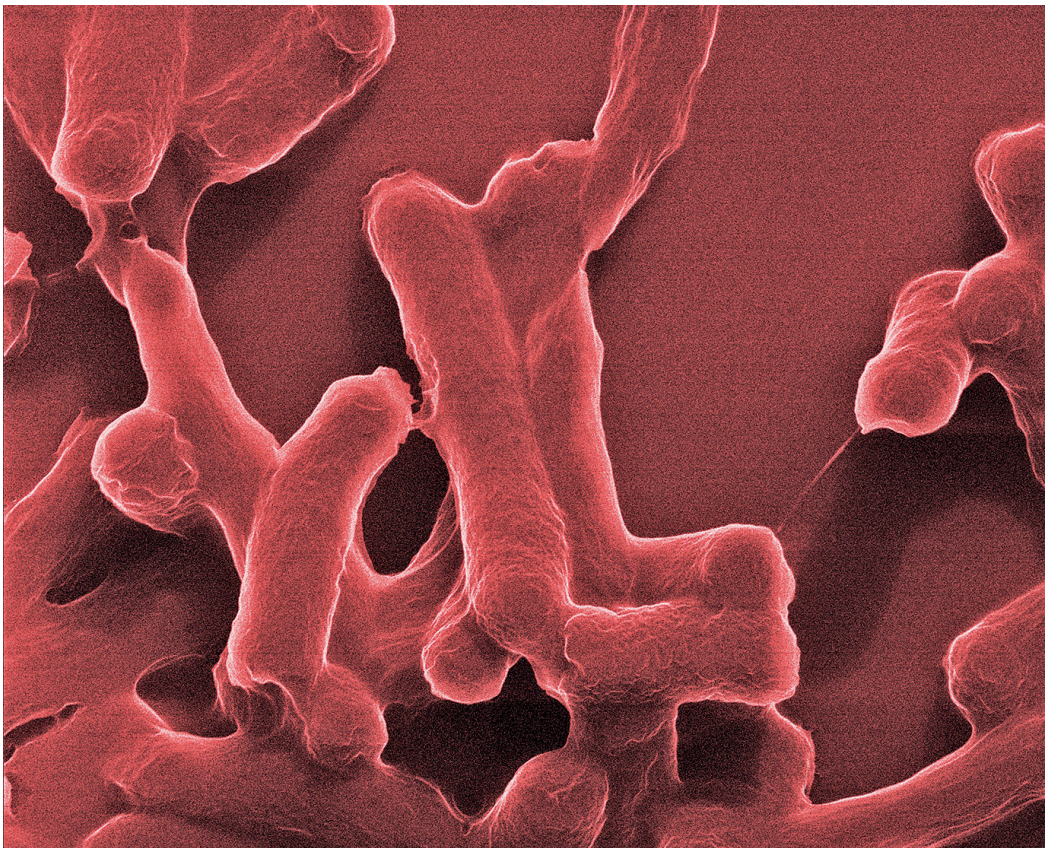
317

Ville Ojala

Counteracting the Horizontal Spread of Bacterial Antibiotic Resistance with Conjugative Plasmid-Dependent Bacteriophages



JYVÄSKYLÄN YLIOPISTO

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ABSTRACT

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Counteracting the horizontal spread of bacterial antibiotic resistance with conjugative plasmid-dependent bacteriophages
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Yhteenveto: Bakteerien antibioottivastustuskyvyn horisontaalisen leviämisen estäminen konjugatiivisista plasmideista riippuvaisilla bakteriofageilla
Diss.

For over half a century, antibiotics have played an integral role in modern medicine's quest to combat bacterium-caused illnesses. However, the extensive use and misuse of these vital drugs has led to the widespread emergence of highly resistant infections with few, or even no, available treatment options. The horizontal transfer of resistance genes via conjugative plasmids is considered the main culprit for the accumulation of multiple resistance determinants to pathogenic bacterial strains. Conjugative plasmids are extrachromosomal selfish genetic entities capable of independent replication and transfer to novel bacterial hosts, and they also routinely code for various host-beneficial traits, such as antibiotic resistance. Given the importance of conjugative plasmids in the global resistance epidemic, we would greatly benefit from having a deeper understanding of the eco-evolutionary factors affecting their spread and maintenance in bacterial communities and also from developing novel anti-conjugation therapeutic strategies, especially since the pipeline of new antibiotics has been virtually dry for decades. In this thesis, it is demonstrated that the conjugative transfer of resistance genes from the surrounding bacterial community can evolutionarily rescue antibiotic-susceptible bacteria exposed to an otherwise highly lethal dose of antibiotics. This suggests that the mere establishment of a high therapeutic concentration of antibiotics may not be enough to clear an infection when the targeted pathogens can rapidly acquire resistance from other bacteria via conjugation. However, it was also found that the so-called plasmid-dependent bacteriophages, which are natural enemies of both the conjugative plasmids and their bacterial hosts, can effectively counter the spread of resistance genes between bacteria even in the presence of conjugation-favouring antibiotic selection. Thus, these specialised bacteriophages show potential as future anti-conjugation therapeutic agents.

Keywords: antibiotic resistance; conjugative plasmids; horizontal gene transfer; phage therapy; plasmid-dependent phages.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-III.
The contributions of myself and other authors in each paper are listed in the table at the bottom of the page.

I Ojala V., Mattila S., Hoikkala V., Bamford J.K.H. & Jalasvuori M. 2014. Evolutionary rescue of bacteria via horizontal gene transfer under a lethal β-lactam concentration. *Journal of Global Antimicrobial Resistance* 2(3): 198–200.

II Ojala V., Laitalainen J. & Jalasvuori M. 2013. Fight evolution with evolution: plasmid-dependent phages with a wide host range prevent the spread of antibiotic resistance. *Evolutionary Applications* 6(6): 925–932.

III Ojala V., Mattila S., Hoikkala V., Bamford J.K.H., Hiltunen T. & Jalasvuori M. Scoping the effectiveness and evolutionary obstacles in utilizing plasmid-dependent phages to fight antibiotic resistance. Submitted manuscript.

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	I	**II**	**III**
Original idea	MJ, **VO,** SM	MJ	**VO**, MJ
Experimental work	**VO**, SM, VH, MJ	**VO**, JL, MJ	**VO**, SM, MJ, VH
Data analysis	**VO**	**VO**	**VO**, MJ, SM
Writing	MJ, **VO,** SM, JB	**VO**, MJ	**VO**, MJ, SM, JB, TH

ABBREVIATIONS

ATP	adenosine triphosphate
CFU	colony-forming unit
dsDNA	double-stranded DNA
ESBL	extended-spectrum β-lactamase
HGT	horizontal gene transfer
Inc	incompatibility
MIC	minimum inhibitory concentration
Mpf	mating pair formation
MRSA	methicillin-resistant *Staphylococcus aureus*
NDM-1	New Delhi metallo-β-lactamase-1
PBP2A	penicillin-binding protein 2A
rRNA	ribosomal RNA
T4SS	type IV secretion system

1 INTRODUCTION

The discovery of antibiotics some 80 years ago marked a pivotal turning point in the history of medicine. With antibiotics in our arsenal, many common bacterial infections that had been major sources of morbidity and mortality throughout much of human existence were now readily treatable. Scottish biologist Alexander Fleming, who discovered penicillin in 1928 (Fleming 1929), is commonly credited as the father of the modern antibiotic era; however, it was not until 1945 that penicillin became ready for mass production and distribution, after the publication of a proper purification method by Oxford pathologist Howard Florey and biochemist Ernst Chain (Chain *et al*. 1940), both of whom consequently shared the Nobel Prize in Physiology or Medicine with Fleming. The research and discovery of antibiotics subsequently went through its golden period between the 1950s and 1970s, during which time most of the major classes of antibiotics in use today were introduced (Aminov 2010).

Following the initial antibiotic success story, many held an optimistic view that bacterial diseases would eventually be completely eradicated. However, the rapid emergence of bacterial resistance to almost all of the introduced antibiotics and the drying up of the pipeline of new drugs soon suggested that these predictions might have been premature. For example, clinically relevant penicillin resistance was observed already in the late 1940s (Barber and Rozwadowska-Dowzenko 1948). This development did not catch everybody (at least in the scientific community) by surprise, though; indeed, in his Nobel Prize acceptance speech, Fleming himself warned about the possibility of resistance (Fleming 1945). In essence, the resistance problem stems from simple Darwinian evolution (Darwin 1859): The presence of antibiotics exerts a selective pressure on a bacterial population by killing off the susceptible cells, thus giving competitive advantage to initially rare antibiotic-resistant mutants whose frequency subsequently increases. To make matters worse, resistance in bacterial populations does not only arise *de novo* through random mutations, but bacteria of different strains or species also routinely engage in swapping of resistance genes (among others) through various methods of horizontal gene transfer (HGT) (Davies and Davies 2010). HGT thus greatly increases bacterial

evolution potential and is considered one of the main contributors to the escalating global antibiotic resistance crisis (Bennett 2008).

Although the basic Darwinian principles behind resistance development may be simple enough to grasp, on a societal and global level, where various types of human behaviour contribute to the problem, the causes and consequences of antibiotic resistance are considerably more difficult to qualify and quantify. Hospitals are the main hotspots for drug resistance because antibiotics are used the most there and resistance genes are commonly carried from patient to patient by health care professionals (Struelens 1998). The problem is further exacerbated in developing countries, where limited resources often result in poor levels of hygiene and inadequacies in other health care standards and practices. Moreover, predominantly in these poorer parts of the world, antibiotics are often widely available and used without prescription, leading to the consumption of wrong, unnecessary and/or incomplete antibiotic regimens (Hart and Kariuki 1998, Laxminarayan *et al.* 2013). In addition, substantial amounts of antibiotics are used in commercial agriculture and aquaculture for disease prevention and growth promotion, and the implications of this practice on human health remains a controversial topic (Laxminarayan *et al.* 2013). As mentioned above, an accurate numerical measurement of the resistance burden is challenging, but even cautious estimates suggest that annually there are hundreds of thousands of deaths and billions of dollars of economic losses directly or indirectly attributable to antimicrobial resistance (World Health Organization 2014). What is clear, though, is that the supply of effective antibiotics against serious bacterial pathogens such as *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* is rapidly dwindling, and the pharmaceutical industry is sorely lagging behind in terms of compensating with the development of new drugs (Livermore 2004, Levy and Marshall 2004, Coates *et al.* 2011). As a result, there is growing concern that without quick and decisive measures, the coming decades may see the return of the pre-antibiotic era – with dire global consequences (Cohen 1992, Appelbaum 2012).

Fighting the resistance epidemic requires a global, multifaceted response, and unfortunately most of the necessary measures face considerable technical, economic or political challenges. The foremost objective is to reduce the overall use of antibiotics by, for example, implementing effective antibiotic stewardship programmes in hospitals, communities and commercial farming, developing faster and more accurate methods of disease diagnostics, and improving hygiene and sanitation practices to prevent infections in the first place (Smith and Coast 2002). Nevertheless, bacterial infections meriting the use of antibiotics will continue to exist for the foreseeable future, and thus the resistance problem cannot be solved by merely eliminating antibiotic misuse, since appropriate and necessary use also exerts a selective pressure for resistance development. This means that there is an urgent need for research and development of novel antibiotics and alternative or complementary methods of treatment. The latter include antibiotic adjuvants and various

biological therapeutics, such as vaccines, immune system modulators, monoclonal antibodies and bacteriophages (Laxminarayan *et al.* 2013).

In this thesis, I explore the importance of HGT in rapid resistance development during antibiotic treatment and look into the potential of bacteriophages to interfere with this horizontal transfer of resistance genes between bacteria.

1.1 Antibiotic targets and bacterial resistance mechanisms

Antibiotics are small molecules which, in sufficient concentrations, either kill (bactericidal antibiotics) or prevent the growth (bacteriostatic antibiotics) of bacteria (and fungi). Antibiotics are further classified into narrow- and broad-spectrum drugs based on the range of pathogens they are effective against. The majority of antibiotics are naturally produced by the bacteria themselves to inhibit the growth of their competitors, and in low concentrations they also act as signalling molecules that inducing adaptive changes in the surrounding microbial community (Fajardo and Martínez 2008). In addition, there are also a few classes of synthetic (i.e. man-made) antibiotics, namely sulfonamides, quinolones and oxazolidinones. The clinical application of antibiotics is based on using molecules affecting such bacterial cellular targets that are either absent in higher eukaryotes or represent sufficient structural dissimilarities to their human and animal counterparts to allow the selective elimination of pathogens. The five main antibiotic targets in bacteria include: (1) cell wall synthesis, (2) protein synthesis, (3) DNA and RNA synthesis, (4) folate synthesis and (5) cell membrane structure and function (Fig. 1) (Walsh 2003).

Cell walls protect bacteria from external osmotic pressure and are thus vital for cell integrity. They are made of peptidoglycan, which in turn consists of a meshwork of polysaccharides and peptide chains, the latter of which are extensively covalently cross-linked and consequently provide cell walls with much of their mechanical rigidity. Various steps in bacterial peptidoglycan synthesis present potential targets for antibiotic intervention. β-lactams, which inhibit transpeptidase enzymes required for peptide cross-linking, are the most important and commonly used of these antibiotics (Van Boeckel *et al.* 2014). The four main classes of β-lactams include penicillins, cephalosporins, carbapenems and monobactams. Outside the β-lactam family, another cell wall-targeting antibiotic worth mentioning is vancomycin, which is used as a last-resort treatment against infections by methicillin-resistant *Staphylococcus aureus* (MRSA) (Schentag *et al.* 1998). Also of interest is the recently discovered teixobactin (not yet in clinical trials), which inhibits the cell wall synthesis of Gram-positive bacteria by binding the lipid precursors of peptidoglycan and teichoic acid, seemingly with very little resistance development (Ling *et al.* 2015). However, past experiences with, for example, vancomycin, suggest that caution should be exercised in long-term predictions about the likelihood of resistance development (Arias and Murray 2015).

Antibiotic treatment that inhibits bacterial protein synthesis exploits the differences between prokaryotic and eukaryotic translation systems. Some antibiotics, such as aminoglycosides and tetracyclines, bind the 30S subunit of a bacterial ribosome; while others, like macrolides and oxazolidones, target the 50S subunit. Inhibition can take place at any of the main steps of the translation process – that is, initiation, elongation or termination - depending on the antibiotic in question. In contrast to mostly bactericidal β-lactams, antibiotics targeting protein synthesis tend to be bacteriostatic, with the notable exception of aminoglycosides (Davis 1987), and thus require co-operation from the immune system in clearing the infection.

Topoisomerases are enzymatic regulators of DNA topology whose function is crucial during replication, transcription and recombination. Synthetic broad-spectrum antibiotics of the quinolone family disrupt topoisomerase activity with bactericidal consequences. Quinolones target topoisomerase types not found in animal cells (i.e. DNA gyrase and topoisomerase IV), which enables the selective killing of bacteria (Fàbrega *et al.* 2009). Another class of antibiotics, rifamycins, bind to bacterial DNA-dependent RNA polymerase and subsequently halt the transcription process. Rifamycins are vital for treating tuberculosis (caused by *Mycobacterium* spp.); however, resistance to these drugs develops easily and combination therapy with other antibiotics is thus required (Walsh 2003).

Folate is an essential cofactor for nucleic acid synthesis in both prokaryotes and eukaryotes. Whereas many eukaryotes (e.g. mammals) are able to obtain folate directly from their diet, most prokaryotes (e.g. bacteria) must synthesise folate *de novo*, thus making the folate biosynthesis pathway a potential target for antibiotics. For example, combination therapy with sulfamethoxazole and trimethoprim is commonly used to treat urinary tract infections; the two antibiotics target different enzymatic steps in the folate biosynthesis pathway, providing a synergistic effect (Bushby and Hitchings 1968).

Finally, antibiotics targeting the structure and function of bacterial cell membranes include polymyxins (colistin and polymyxin B) and daptomycin. The advantage of these antibiotics is their effectivity against non-dividing bacteria (often present in chronic infections) and the relatively low probability of resistance development (Bush 2012); however, the alarming recent discovery of conjugative plasmid-mediated colistin resistance threatens to change this situation drastically (Liu *et al.* 2015). Nonetheless, polymyxins especially show some neuronal and renal toxicity and are thus mainly used as last-resort drugs against multi-resistant infections (Falagas and Kasiakou 2006).

In natural microbial communities, a vast number of antibiotic resistance genes are found both in antibiotic producing and non-antibiotic producing bacteria, and these genes long predate the era of human antibiotic use (so-called intrinsic resistance) (D'Costa *et al.* 2006). Consequently, in their original environment, some of these genes are known to have functions beyond bacterial (self-)protection against antibiotics, having to do with, for example, signalling, virulence and peptidoglycan synthesis (Mártinez 2009). In contrast,

the acquired resistance via random mutations and HGT observed in pathogens in clinical settings arises solely as an evolutionary response to the recent therapeutic use of antibiotics. HGT can potentially facilitate the dissemination of resistance genes from the environmental microbiota to the pathogens, in the process of which the original function of a gene may change completely (Mártinez 2008, Mártinez 2009).

On a molecular level, antibiotic resistance mechanisms can be divided into three main categories: (1) enzymatic inactivation of the antibiotic, (2) replacement or modification of the antibiotic target, and (3) low permeability to the antibiotic or its active transport out of the bacterial cell (Fig. 1) (Walsh 2003).

The most clinically important antibiotic deactivators are the β-lactamase enzymes, which hydrolyse the four-atom ring that is the chemical warhead of a β-lactam antibiotic. Genes coding for β-lactamases are found both in chromosomes and in mobile genetic elements (transposons and plasmids), where they can be expressed either constitutively or as a response to the presence of β-lactams in the environment (Zeng and Lin 2013). In gram-positive bacteria, β-lactamase molecules are secreted outside the cell, whereas in gram-negative bacteria, β-lactamases are translocated into the periplasmic space. In both cases, enzymatic inactivation by β-lactamases prevents β-lactams from disrupting transpeptidase activity during peptidoglycan cross-linking. The so-called extended-spectrum β-lactamases (ESBLs) are significant contributors to the resistance problem, since they confer resistance not only to penicillins, but also to widely used later-generation cephalosporins (Paterson and Bonomo 2005). Even more troublesome are the carbapenem-hydrolysing carbapenemases, such as New Delhi metallo-β-lactamase-1 (NDM-1) (Kumarasamy *et al.* 2010); carbapenems are usually non-susceptible to β-lactamases, thus rendering them important last-resort therapeutic agents. Another example of resistance arising through enzymatic inactivation is the modification of aminoglycoside antibiotics to reduce their binding to 16S rRNA in the 30S ribosomal subunit. The ribosome binding specificity of aminoglycosides is provided by their OH and NH_2 groups, and resistance emerges through covalent modification of these groups either by acetylation, phosphorylation or adenylation (Mingeot-Leclercq *et al.* 1999).

The second main bacterial resistance strategy is altering the cellular target of an antibiotic while still preserving its original function. This can be accomplished either through mutations in the original target-coding genes or by acquisition of new genes via HGT. The resistance development process to rifampicin (a rifamycin derivative) is a good example of the first strategy; during antibiotic treatment, mutations accumulate rapidly in the gene coding for the β-subunit of bacterial DNA-dependent RNA polymerase, resulting in drastically lowered binding affinity for rifampicin (Telenti *et al.* 1993). The latter strategy is well exemplified by the horizontal acquisition of the *mecA* gene by MRSA, which renders it resistant to all β-lactams (Wielders *et al.* 2002). The *mecA* gene encodes for a transpeptidase called penicillin-binding protein 2A (PBP2A), which shows low affinity to penicillins, cephalosporins and

carbapenems and thus allows the peptidoglycan cross-linking to proceed despite the presence of β-lactams (Chambers 1997).

The last major resistance strategy is to maintain a low intracellular level of antibiotics either by preventing the entry of antibiotic molecules in the first place or by actively pumping them out of the bacterial cell. The gram-negative opportunistic pathogen *Pseudomonas aeruginosa* illustrates the effective deployment of both of these strategies: First, the outer membrane of *P. aeruginosa* has low permeability to many antibiotics due to ineffective porin function (Hancock and Tamber 2003). Second, *P. aeruginosa* has an array of efflux pumps in its cytoplasmic and outer membranes, which use either proton motive force or ATP to pump molecules against their concentration gradients. In general, some pumps are highly specific, while others, the so-called multidrug efflux pumps, can transport a wide variety of antibiotics out of the cell (Webber and Piddock 2003). Chromosomes usually code for multidrug efflux pumps, whereas the antibiotic-specific pumps are mostly located in mobile genetic elements (Poole 2007).

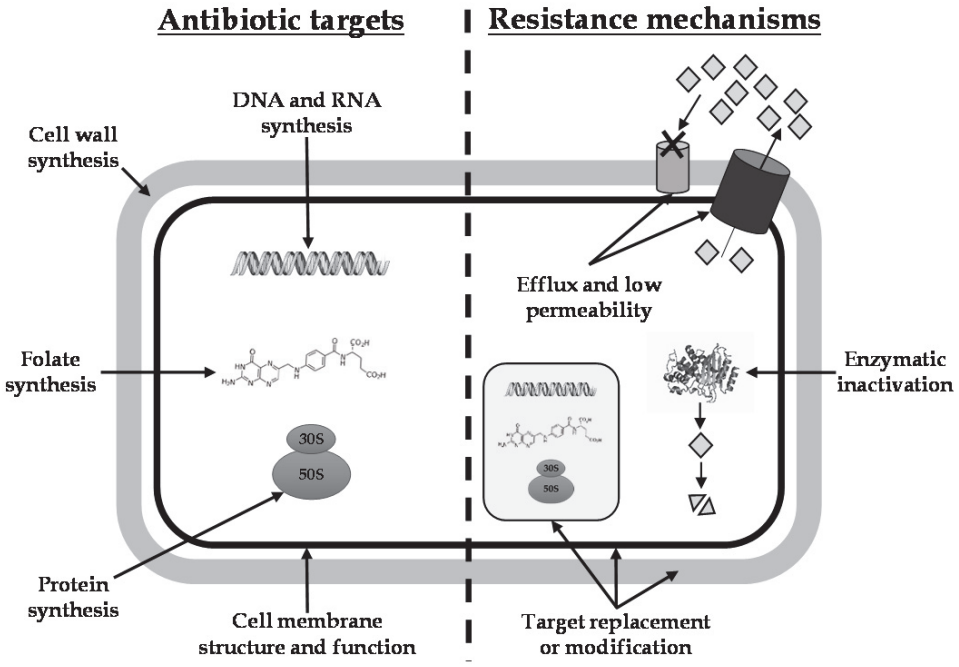

FIGURE 1 Antibiotic cellular targets and bacterial resistance mechanisms.

1.2 Horizontal gene transfer

In the 1950s, the rapid emergence of similar antibiotic resistance patterns in different bacterial strains and species was among the first indicators of the importance of HGT in bacterial evolution (Ochman *et al.* 2000). The shared spectrum of resistance suggested that different bacterial lineages had not independently developed resistance *de novo*, but rather had engaged in horizontal exchange of resistance determinants. However, a full appreciation of the significance of HGT has developed only during the past few decades, and it is now clear that Darwin's metaphorical tree of life paints an incomplete picture of prokaryotic evolutionary history, which resembles a complex network rather than a branching tree (Woese 2000, Jain *et al.* 2003, Gogarten and Townsend 2005, Smets and Barkay 2005).

The three mechanisms of HGT are transformation, transduction and conjugation. Transformation is the bacterial uptake and incorporation of naked DNA from the environment. The extracellular DNA originates from disintegrated cells and virus particles and can sometimes be secreted by viable cells as well (Draghi and Turner 2006). Theoretically, transformation is the mechanism that allows for HGT between the most distantly related taxa (Thomas and Nielsen 2005). Transduction is the phage-mediated transfer of DNA from one bacterium to another, which results from accidental packaging of bacterial DNA into phage particles during the latter part of the life cycle of a virus. The taxonomic range in which this method of HGT operates is determined by the host range of the transducing phage. Lastly, conjugation involves the formation of a channel between two bacteria (cases of inter-kingdom transfer are also known [Ferguson and Heinemann 2002]) through which DNA can be copied from a donor to a recipient. The transferred genetic elements include conjugative transposons and mobilisable or conjugative plasmids.

1.2.1 Conjugative plasmids

The process of bacterial conjugation was first discovered by Edward Lawrie Tatum and Joshua Lederberg (the latter of whom was also the originator of the term 'plasmid') years before the basic structural properties of plasmids had been determined (Lederberg and Tatum 1946, Lederberg 1952). Today, conjugative plasmids are known to be extrachromosomal, usually circular, double-stranded DNA (dsDNA) molecules characterised by their independent replication from the host chromosome and the ability to facilitate their own transfer to novel bacterial hosts (Norman *et al.* 2009). The capacity to move between different hosts provides clear evolutionary advantages to a conjugative plasmid, such as the lowered probability of extinction when the current host faces adverse environmental conditions. In addition, another defining feature of conjugative plasmids is their dispensability to the host bacterium, which means that they do not code for any functions essential to the basic host cell's viability.

In contrast, conjugative plasmids are selfish genetic entities whose carriage is energetically costly to their bacterial hosts, and thus, in theory, they should be lost via purifying selection (which clearly is not the case) (Eberhard 1990, Slater *et al.* 2008, Harrison and Brockhurst 2012). The fitness cost to the host can be compensated for by the plasmid-encoded traits that are host-beneficial in particular selective contexts (e.g. plasmid-mediated resistance in the presence of antibiotics). When carrying a plasmid gives the original host bacterium a selective advantage over its competitors in the microbial community, any subsequent horizontal spread of host-beneficial traits to unrelated bacteria is a clear demonstration of the genetically selfish nature of the conjugative plasmid (Jalasvuori 2012). Again, from a theoretical perspective, positive selection for plasmid-encoded traits should lead to incorporation of beneficial genes into the bacterial chromosome and the loss of the rest of the plasmid (Bergstrom *et al.* 2000). The apparent persistence of (conjugative) plasmids in contrast to theoretical predictions has been described as the 'plasmid paradox' (Harrison and Brockhurst 2012). Based on data from various co-culture experiments, amelioration of the cost of plasmid carriage through plasmid–host coevolution and effective re-infection of plasmid-cured cells through high conjugation rates have been put forward as possible explanations for this puzzle (Lundquist and Levin 1986, Dahlberg and Chao 2003, Dionisio *et al.* 2005, Harrison and Brockhurst 2012). However, in natural systems, additional factors such as predation by lytic phages may further limit the existence conditions for conjugative plasmids (Harrison *et al.* 2015) and, consequently, conclusive answers to the plasmid paradox remain elusive.

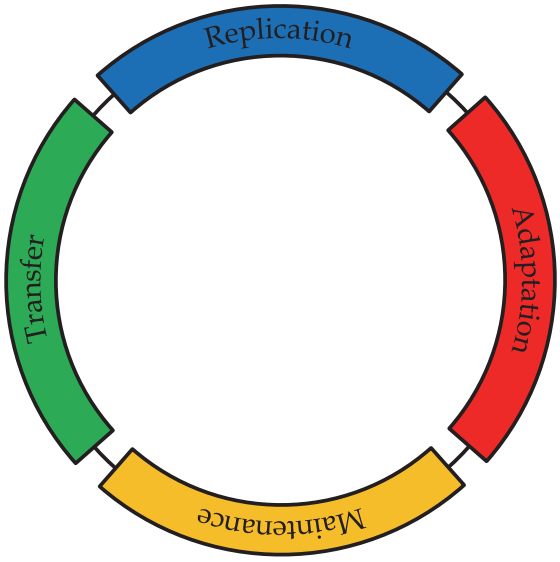

FIGURE 2 Schematic representation of the modular genetic structure of conjugative plasmids. Adapted from Norman *et al.* (2009).

The basic structure of a conjugative plasmid consists of distinct genetic modules which can be broadly categorised into plasmid-selfish 'backbone modules', essential for the replication, maintenance and transfer of the plasmid, and 'adaptation modules', which code for host-beneficial traits like antibiotic resistance (Fig. 2) (Thomas 2000, Norman *et al.* 2009).

The genes located in a replication module are responsible for synchronising plasmid replication with the cell division cycle and controlling the copy number of the plasmid inside a bacterial host. Keeping the copy number stable and at a suitable level is vital for the long-term persistence of a plasmid: a copy number that is too high imposes an unmanageable metabolic burden on the bacterial host, whereas having too few copies can lead to the segregational loss of the plasmid. The copy number is almost always regulated at the stage of the initiation of replication by various activators or inhibitors coded for by the plasmid (Del Solar *et al.* 1998). As a result, too much similarity between the replication modules of two given plasmids will prevent their coexistence inside the same bacterial host because the homologous copy number control systems will begin to interfere with each other. This phenomenon is the basis for classifying conjugative plasmids into so-called incompatibility (Inc) groups (Novick 1987, Couturier *et al.* 1988). The properties of the replication module of a given conjugative plasmid are also the primary determinants of the range of different bacteria it can propagate in, since successful replication is required within every host through which the plasmid passes. Consequently, some conjugative plasmids have a narrow host range, whereas others are capable of disseminating themselves into a wide variety of bacterial species (Jain and Srivastava 2013). An example of the latter are the conjugative plasmids belonging to incompatibility group IncP, which can propagate in most gram-negative bacteria (Popowska and Krawczyk-Balska 2013, Yano *et al.* 2013).

The genes of plasmid maintenance modules act as further safeguards for the segregational fidelity of a plasmid. For example, multimer resolution systems extricate plasmid multimers that can emerge as a result of homologous recombination, which can compromise the proper partitioning of plasmids during cell division (Summers *et al.* 1993). In addition, conjugative plasmids employ so-called active partitioning mechanisms because, due to their comparatively big size, their copy number is usually relatively low, and thus they cannot depend on random diffusion to ensure similar copy distribution between daughter cells. During active partitioning, copies of conjugative plasmids are transported to proper intracellular positions in preparation for cell division (analogously to eukaryotic mitosis) (Hayes and Barillà 2006). Finally, some plasmids engage in post-segregational killing of bacteria that have been cured of the plasmid. For example, plasmids can code for toxin–antitoxin systems wherein both the toxin and its antitoxin are constitutively expressed but the latter is much less stable, a result of which is that any bacteria which have lost the plasmid will succumb to the effects of the toxin (Yamaguchi *et al.* 2011).

Successful bacterial conjugation requires the establishment of physical contact between a plasmid donor and a compatible recipient, followed by the copying of the plasmid DNA into the new host. The transfer modules of conjugative plasmids code for both of these functions: The so-called mating pair formation (Mpf) is accomplished through expression of type IV secretion systems (T4SS), which consist of a dozen or so proteins encoded from a single operon of a transfer module (Lawley *et al.* 2003, Cabezón *et al.* 2014). Conjugation-associated T4SSs are membrane-spanning complexes in which some of the protein components are cytoplasmic, while others are located in the periplasmic space or on the outer membrane. T4SSs form protruding filamentous structures called conjugative pili (sing., pilus) which bind to suitable recipient bacteria and then, by retracting, bring the cells into close proximity. The ubiquity of the bacterial cell-surface structure a given pilus-type recognises and the favoured medium for conjugation (liquid vs. solid surfaces) are important factors (in addition to replication system compatibility) in determining the host range of a plasmid (Norman *et al.* 2009). After the Mpf is completed, one of the plasmid DNA strands is cut at the origin of transfer and processed to form a so-called relaxosome: a nucleoprotein complex containing single-stranded plasmid DNA, enzyme relaxase and various other proteins (Pansegrau *et al.* 1990). The relaxosome is subsequently transferred to the recipient bacterium; whether the transfer happens through the pilus itself, or via formation of some separate conjugation channel, remains a debated topic (Srivastava 2013, Cabezón *et al.* 2014). The conjugation process is completed by the synthesis of a new complementary strand in both the donor and the recipient. Finally, in addition to the functions described above, the transfer modules of conjugative plasmids code for mechanisms preventing either Mpf (surface exclusion) or DNA transfer (entry exclusion) with bacteria carrying incompatible plasmids (Garcillán-Barcia and de la Cruz 2008). The genes for surface and entry exclusion are usually part of the same operon that codes for the conjugation-associated T4SS.

In addition to their prominent role in providing resistance to antibiotics, the adaptation modules of conjugative plasmids also code for many other traits beneficial to their host bacteria, such as tolerance to environmental heavy metals, secretion of (and self-resistance to) toxins targeting bacterial competitors, the metabolisation capability of new carbon sources for energy, and the production of virulence factors that help in the colonisation of eukaryotic hosts (Eberhard 1990, Rankin *et al.* 2011). In contrast to backbone modules, which usually are comparatively genetically conserved, adaptation modules show a high degree of genetic mosaicism, with evidence of multiple insertion events of transposons, integrons and insertion sequence elements from different sources (Bennett 2008, Norman *et al.* 2009). The presence of multiple insertion sites inside adaptation modules allows for the incorporation of additional genetic material into the plasmid without disrupting the essential functions of the backbone modules.

Overall, understanding the structural and evolutionary idiosyncrasies of conjugative plasmids forms a basis for finding ways to depress their role in the global antibiotic resistance crisis.

1.3 Bacteriophages and their therapeutic potential

Bacteriophages (in short, phages) are obligate, intracellular viral parasites of bacteria. Phages are tiny, ubiquitous and abundant: their size is measured in nanometers and, whereas the estimated number of bacteria in the biosphere is ca. 10^{30} (Weinbauer 2004), phages are thought to outnumber their bacterial hosts by at least an order of magnitude (Chibani-Chennoufi *et al.* 2004). In addition, phages show substantial structural and genetic diversity: At its most basic level, a phage particle (or virion) consists of proteins encapsulating the viral genome, which can be either DNA or RNA. Further classification can be done based on, for example, virion morphology (tailed, polyhedral, filamentous or pleomorphic), genome topology (linear or circular), genome strand number (single-stranded or double-stranded) and the presence of lipid envelopes (Ackermann 2009). In general, phages use one of the two main life cycle types for propagation: the lytic cycle or the lysogenic cycle. Both cycles begin with a virion binding to a specific bacterial cell-surface receptor, followed by the injection of the viral genome into the cell. In the lytic cycle, the cell machinery of the host is hijacked for the production of new viral particles, which are eventually released into the environment, resulting in the death of the bacterium. Contrastingly, in the lysogenic cycle, the nucleic acid of the phage is (in most cases) incorporated into the bacterial chromosome, where it is replicated in concert with the host genome and transmitted to daughter cells as a so-called prophage. A prophage can either continue the lysogenic cycle indefinitely or, as a result of some environmental cue, switch to the lytic cycle, where the host bacterium is again killed. Overall, phages are known to be a major contributor to bacterial mortality in natural microbial communities (Suttle 1994, Chibani-Chennoufi *et al.* 2004), and the idea of harnessing this killing potential for therapeutic purposes has been around for a long time (Summers 2001).

1.3.1 Anti-conjugation phage therapy

After the discovery of phages in the early 20th century, their potential in treating bacterial infections was extensively studied during the 1920s and 1930s. However, following the introduction of antibiotics, the pursuit of phage therapy was largely abandoned in the Western world. Conversely, in Eastern Europe and the Soviet Union, the research and application of phage therapy continued in full force, with reports of great efficacy against many important bacterial pathogens – although the methodological rigor applied in assessing the effectiveness of phages was often inadequate (Alisky *et al.* 1998). In addition

to some universal technical challenges, the clinical application of phages in Western societies also faces various regulatory, economic and public perception issues (Loc-Carrillo and Abedon 2011, Henein 2013). Nonetheless, as a result of the worsening resistance epidemic, interest in phage therapy has recently been somewhat revived, and there is some optimism that with proper resource allocation and enough political will it should be possible to overcome the obstacles currently preventing the wider use of phages as potential antibacterials (Lu and Koeris 2011, Reardon 2014).

The pivotal clinical importance of bacterial conjugation in the dissemination of antibiotic resistance genes also makes it an attractive target for the development of novel therapeutic approaches (Smith and Romesberg 2007, Williams and Hergenrother 2008). For example, some studies have explored the possibility of using unsaturated fatty acids (Fernandez-Lopez *et al.* 2005) or various relaxase-targeting molecules (Garcillán-Barcia *et al.* 2007, Lujan *et al.* 2007) to interfere with the conjugation process. The so-called plasmid-dependent phages are another promising candidate for anti-conjugation therapeutic applications. These phages specifically target conjugative plasmid-carrying bacteria by recognising and binding parts of the plasmid-encoded T4SS to gain entry into the host cell (Loeb 1960, Caro and Schnös 1966). A distinctive feature of plasmid-dependent phages is their display of extremely broad host ranges compared to phages targeting regular bacterial cell-surface receptors; many plasmid-dependent phages use such T4SS structures as their receptors, which are shared by conjugative plasmids belonging to multiple different incompatibility groups, and susceptibility is generally not affected by the strain or species of the bacterium within which a conjugative plasmid resides at a given moment (Frost 1993).

A few *in vitro* studies have shown that the process of bacterial conjugation can be physically inhibited using either replicative lytic or non-lytic plasmid-dependent phages or only their coat proteins (Novotny *et al.* 1968, Ou 1973, Lin *et al.* 2011, Wan and Goddard 2012). Furthermore, another study demonstrated that this phage-induced inhibition of conjugation can also disrupt biofilm formation, which is a major virulence determinant in many bacterial infections (May *et al.* 2011). Finally, in a longer-term co-evolution experiment, Jalasvuori *et al.* (2011) found that in the absence of antibiotic selection and other bacteria, the presence of the plasmid-dependent phage PRD1 either causes bacteria to lose their resistance plasmids or, in rare cases, selects for bacteria carrying plasmids with lost conjugation ability.

1.3.2 Plasmid-dependent phage PRD1

PRD1 is a lytic dsDNA plasmid-dependent phage belonging to the family *Tectiviridae* (Olsen *et al.* 1974). Its linear genome is 14.9 kb in length and codes for at least 27 proteins (Bamford *et al.* 1995). The protein capsid of the PRD1 virion has an icosahedral symmetry with a diameter of approximately 65 nanometers (Abrescia *et al.* 2004). Furthermore, it has an inner lipid membrane containing additional proteins that play an important role in the injection of

viral DNA into a bacterial cell (Grahn *et al.* 2002). The so-called spike complexes located in the capsid vertices contain the receptor-binding protein of PRD1, which can recognise T4SSs encoded by the transfer modules of conjugative plasmids belonging to incompatibility groups P, W and N (Olsen *et al.* 1974, Kotilainen *et al.* 1993, Haase *et al.* 1995). Consequently, PRD1 has a very broad host range, since the plasmids of the aforementioned incompatibility groups can generally propagate in both *Enterobacteriaceae* and *Pseudomonas aeruginosa* (Popowska and Krawczyk-Balska 2013), wherein they also regularly code for clinically important antibiotic resistance determinants like, for example, metallo-β-lactamases which confer resistance to carbapenems (Carattoli 2009). As such, PRD1-like plasmid-dependent phages represent an interesting target for further anti-conjugation research.

2 AIMS OF THE STUDY

This thesis focuses on a series of *in vitro* evolution experiments, which had the following objectives:

I. To study whether the horizontal transfer of resistance genes from the surrounding microbial community can evolutionarily rescue bacteria exposed to a lethal concentration of antibiotics;

II. To examine the potential of plasmid-dependent phage PRD1 in preventing the conjugative spread of resistance genes between bacteria in the presence of sub-lethal antibiotic selection favouring conjugation;

III. To assess the long-term effectiveness of and potential evolutionary obstacles to applying plasmid-dependent phages as anti-conjugation/anti-resistance agents.

3 OVERVIEW OF THE METHODS

An overview of the methods used in this thesis is presented in Table 1. A detailed description of the materials and methods used can be found in the original publications indicated by Roman numerals.

TABLE 1 Methods used in the original publications included in the thesis.

Method	Publication
In vitro evolution experiments	I, II, III
Selective plating methods	I, II, III
Conjugation assays	II, III
Phage resistance tests	III
MIC testing	I
Spectrophotometric determination of non-lethal antibiotic concentrations	II, III
Plasmid sequencing	III
Statistical analysis	II, III

4 RESULTS AND DISCUSSION

4.1 Evolutionary rescue of bacteria via conjugation under lethal β-lactam selection (I)

A minimum inhibitory concentration (MIC) of an antibiotic is the lowest concentration capable of preventing the growth of a particular bacterial agent. Concentrations below and above MIC have different implications for potential resistance development: The presence of sub-MIC antibiotic levels in various natural environments, or *in vivo* during therapeutic applications, is considered to select for a gradual emergence of low-fitness-cost resistant genotypes via enrichment of existing or *de novo* mutations and an accelerated rate of HGT between bacteria (Andersson and Hughes 2012, Andersson and Hughes 2014). Establishing a systemic and sustained above-MIC antibiotic concentration is essential in eradicating human and animal bacterial infections; however, pre-existing, rare high-fitness-cost resistant mutants can still be selected for in a specific above-MIC concentration range called the 'mutation selection window' (Drlica and Zhao 2007). In addition, susceptible bacteria can also be rescued from lethal antibiotic exposure by the surrounding microbial community. For example, β-lactamase-producing bacteria (co-operators) can provide existence conditions for susceptible bacteria (cheaters) through the inactivation of β-lactam molecules present in the environment (Hackman and Wilkins 1975, Dugatkin *et al.* 2005, Perlin *et al.* 2009, Brook 2009, Yurtsev *et al.* 2013). Recently, Yurtsev and colleagues (2013) demonstrated that the relative frequencies of co-operators and cheaters in a population eventually reach an equilibrium, which depends on initial cell density and the concentration of β-lactam. However, in clinical settings, the horizontal transfer of β-lactamase genes between co-operators and cheaters could potentially also contribute to the above-MIC rescue dynamics. To address this issue, we performed a 24-hour *in vitro* co-culture experiment, where two *E. coli* K-12 strains, JE2571(RP4) (Bradley 1980) and HMS174 (Campbell *et al.* 1978), were exposed to a wide range (15–600 mg l^{-1}) of above-MIC β-lactam (ampicillin) concentrations. JE2571 carries RP4 (Datta *et al.* 1971): a broad host-range IncP conjugative plasmid encoding a TEM-1 type

β-lactamase which confers resistance to high levels of ampicillin (MIC > 256 mg l^{-1}). The MIC of ampicillin for HMS174 is only 0.75 mg l^{-1}.

We found that the ampicillin-sensitive HMS174 bacteria were commonly rescued by the JE2571(RP4) co-operators, not only via ampicillin inactivation, but also through the conjugative transfer of the β-lactamase-producing RP4 plasmid (Fig. 3). Upon receiving the plasmid, the HMS174(RP4) transconjugants cease to be cheaters and instead become so-called novel co-operators, since they themselves start inactivating ampicillin and thus further enable cheating behaviour by the remaining ancestral HMS174 bacteria. Naturally, HMS174(RP4) can also further conjugate the RP4 plasmid to HMS174, thus creating more novel co-operators.

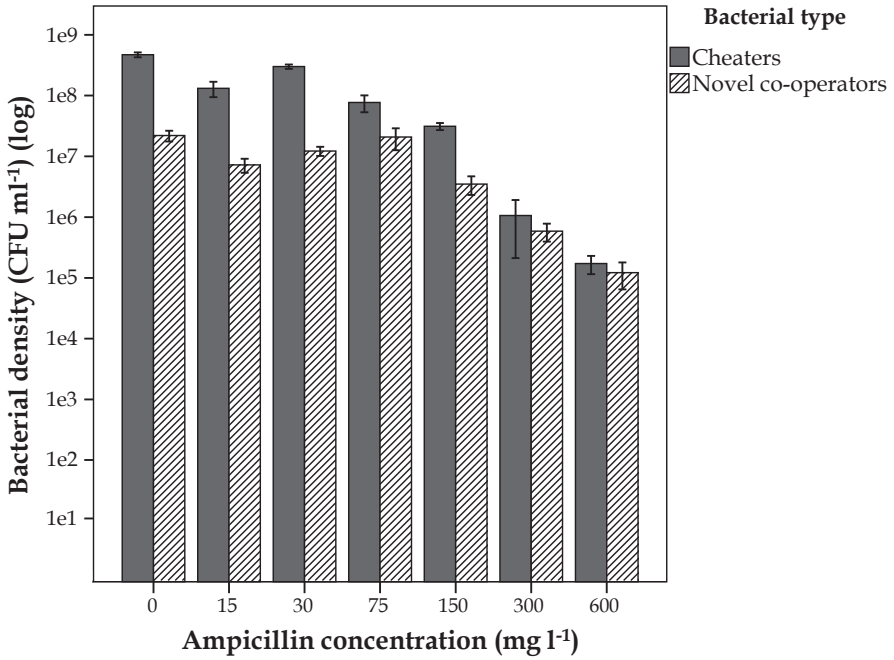

FIGURE 3 The mean (± SE) densities (CFU ml^{-1}) of *E. coli* HMS174 (cheaters) and HMS174(RP4) (novel co-operators) after a 24-hour co-culture with JE2571(RP4) (co-operators) at different above-MIC (15–600 mg l^{-1}) ampicillin concentrations. N = 4 for all antibiotic treatments.

The densities of both cheaters and novel co-operators generally decreased as the antibiotic concentration increased; however, even at the highest ampicillin concentration used (600 mg l^{-1}), both types of rescued bacteria were still present at a relatively high density (approximately 10^5 CFU ml^{-1}). At lower, yet still above-MIC ampicillin concentrations of 15–150 mg l^{-1}, the cheaters were more abundant than the novel co-operators (except at 75 mg l^{-1}) (independent samples *t*-test; 15 mg l^{-1}: $t(6) = 3.325$, $p = 0.016$; 30 mg l^{-1}: $t(6) = 11.635$, $p < 0.001$; 75 mg l^{-1}: $t(6) = 2.234$, $p = 0.067$; 150 mg l^{-1}: $t(3.4781) = 6.370$, $p = 0.005$). In

contrast, at very high ampicillin concentrations of 300–600 mg l^{-1}, there was no difference in the densities of cheaters and novel co-operators (300 mg l^{-1}: $t(6) = 0.546$, $p = 0.605$; 600 mg l^{-1}: $t(6) = 0.623$, $p = 0.556$). In the control treatment with no antibiotics, ancestral HMS174 bacteria were more common than HMS174(RP4) transconjugants ($t(6) = 10.357$, $p < 0.001$). Finally, in the absence of resistance genes (i.e. the RP4 plasmid), the co-culture of JE2571 and HMS174 was completely inhibited, even at the lowest ampicillin concentration used (15 mg l^{-1}).

The expression of TEM-1 β-lactamase by the conjugative plasmid RP4 and its horizontal self-transmission to new bacterial hosts can be construed as plasmid-selfish behaviour which ensures the survival and propagation of the plasmid in a bacterial community facing lethal β-lactam selection. In gram-negative bacteria, β-lactamase molecules are translocated to the periplasmic space, where they not only provide a high level of resistance to the β-lactamase producer but, as discussed above, also allow the nearby susceptible bacteria to survive. Supposing that the resistant plasmid-carrier and any unrelated antibiotic-susceptible bacteria present in the community compete over limited resources, the plasmid-carrier would clearly benefit from exclusive access to β-lactamase resistance action and the absence of any horizontal transfer of resistance genes to the competing bacteria. In contrast, for the conjugative plasmid, the production of an outside-host-protective β-lactamase could potentially be evolutionarily beneficial by ensuring the continued availability of novel bacterial hosts to which the plasmid could subsequently conjugate. Consequently, as the conjugative plasmid evolutionarily rescues its original host, it also simultaneously forces the host to co-operate with its competitors. Overall, this highlights the importance of considering conjugative plasmids as selfish genetic entities, both when studying their basic biology and when assessing their clinical importance – for example, in the global antibiotic resistance crisis.

Given that the antibiotics of the β-lactam family play an integral role in the control of a wide variety of bacterial infections, and in light of the fact that the most clinically troublesome resistance determinants (i.e. β-lactamases), such as TEM, SHV, CTX-M or NDM-1, are frequently found in conjugative plasmids (Paterson and Bonomo 2005, Hudson *et al.* 2014), it is vital that we develop a better understanding of the eco-evolutionary factors which affect the spread and maintenance of these plasmids. Our results showed that the relative importance of the conjugative transfer of resistance genes as the mechanism of evolutionary rescue of β-lactam-susceptible bacteria increased at very high ampicillin concentrations. These concentrations (300–600 mg l^{-1}) were well above the typically observed, effective *in vivo* ampicillin concentrations (Martin *et al.* 1998), which suggests that a therapeutic strategy based on increasing the antibiotic dosage to avoid the 'mutation selection window' (Drlica and Zhao 2007) could be ineffective when resistance genes can be acquired 'on the fly' from the surrounding microbial community via conjugative plasmids or some other mobile genetic elements. However, one potential solution to this problem

could be the coupling of traditional antibiotic treatments with novel anti-conjugation therapeutic strategies, such as the use of plasmid-dependent phages.

4.2 Plasmid-dependent phage PRD1 as a selective force against conjugative spread of antibiotic resistance (II)

In another co-culture experiment, we studied the potential of the plasmid-dependent phage PRD1 to select against the conjugative transfer of the resistance plasmid RP4 – again, from JE2571(RP4) to HMS174. In addition to carrying a gene for TEM-1 β-lactamase, the RP4 plasmid also provides its host with resistance to kanamycin through the expression of type I aminoglycoside 3'-phosphotransferase (Pansegrau *et al.* 1987) and tetracycline through the production of active efflux pumps (Hedstrom *et al.* 1982); HMS174, in turn, harbours a chromosomally based resistance to rifampicin. Utilising these resistance patterns, we added sub-MIC, yet growth reducing, antibiotic selections into our experimental system, which should have favoured conjugation between the two bacterial strains. In other words, by using a combination of antibiotics and plasmid-dependent phages, we aimed to create conflicting selection pressures, both for and against the spread and maintenance of the resistance plasmid, in order to see whether the dissemination of resistance genes could still be prevented. The sub-MIC antibiotic selections were done with either (1) kanamycin, (2) rifampicin or (3) kanamycin and rifampicin; in addition, an antibiotic-free control treatment was included. Kanamycin selects for the spread and maintenance of the RP4 plasmid in the bacterial community, whereas the presence of rifampicin favours HMS174; on the other hand, it could also select for the movement of RP4 from JE2571 to a more competitive host. Naturally, the selection with both kanamycin and rifampicin most strongly favours conjugation, since the transconjugants, namely HMS174(RP4), have a selective advantage over both of the ancestral bacterial strains. Each antibiotic treatment was done both in the presence and absence of the plasmid-dependent phage PRD1. The length of the experiment was approximately 72 hours and the transconjugant frequencies were monitored daily.

We found that the anti-conjugation selective pressure posed by PRD1 generally caused a multiple orders of magnitude reduction in the formation of transconjugant HMS174(RP4) bacteria, even in the presence of conjugation-favouring counterselection with antibiotics. In the absence of phages, the RP4 plasmid was efficiently conjugated from JE2571 to HMS174, with the transconjugants reaching a high frequency in all treatments in the course of the 72-hour experiment. In contrast, both in the absence of antibiotics and in the single-antibiotic (kanamycin or rifampicin) treatments, phages were capable of keeping the transconjugant frequencies low throughout the whole experiment.

In the double-antibiotic treatment, where conjugation was most strongly selected for, the anti-conjugation effect of phages still held back the spread of resistance for 24 hours; however, after 48 hours the observed transconjugant frequencies were comparable to those of the phage-free control. The fact that infective PRD1 particles were abundantly present at the end of the experiment in every phage-containing treatment begs the question of how the plasmid-carrying bacteria survive at such high densities despite the threat of plasmid-dependent phages. Previous studies have shown that PRD1 can select for mutant RP4 plasmids that are phage-resistant but also have either lost or markedly reduced conjugation ability (Kornstein *et al.* 1992, Kotilainen *et al.* 1993, Grahn *et al.* 1997, Jalasvuori *et al.* 2011). Consequently, we isolated random, individual HMS174(RP4) transconjugant clones from both the phage-containing and phage-free double-antibiotic treatments and tested them for their conjugation rate to a third *E. coli* K-12 strain. We found that 65% (N = 72) of the clones that had co-evolved with PRD1 were completely conjugation defective, whereas all of the HMS174(RP4) clones with no phage-history retained full conjugation ability. PRD1 did not noticeably affect the overall bacterial numbers in any culture, all of which reached the saturated density of approximately 10^8 CFU ml^{-1}. Instead, the presence of plasmid-dependent phages had a substantial effect on the relative frequencies of different bacterial strains and conjugation phenotypes in the population and, promisingly, the formation of a new multi-antibiotic resistant strain via conjugation was frequently selected against. In order to further assess the efficacy and possible limitations of applying plasmid-dependent phages for anti-conjugation purposes, we next took a closer look into the phenotypic and genotypic changes in phage resistance and conjugation ability for which PRD1 selects.

4.3 PRD1-induced changes in the conjugation ability and phage susceptibility of plasmid-harbouring bacteria (II, III)

Bacterial resistance to plasmid-dependent phage PRD1 can arise through the loss of the conjugative plasmid (Jalasvuori *et al.* 2011) or, as discussed above, come associated with either completely lost or drastically diminished plasmid conjugation rate. Naturally, the phage-induced eradication of the plasmid from the population eliminates any possibility for further horizontal transfer of antibiotic resistance genes to novel bacterial hosts. However, in cases where the so-called 'non-conjugative' or 'semi-conjugative' plasmid-carrying phenotypes are selected for, the long-term implications of phages on the spread and maintenance of resistance plasmids in the bacterial community are less obvious. Consequently, we performed additional *in vitro* experiments to address the following questions: How effectively can the evolved, phage-resistant non-conjugative and semi-conjugative phenotypes transfer the RP4 plasmid to a new bacterial host when the PRD1 selection is removed and a prolonged sub-

MIC conjugation-favouring antibiotic selection is introduced? Under these circumstances, can the ancestral, fully-conjugative phenotype return, and if so, is susceptibility to PRD1 also restored? Lastly, is the likelihood of reversion dependent on the type of genetic change that led to the formation of a particular conjugation phenotype?

First, we exposed JE2571(RP4) to PRD1 and subsequently isolated individual clones showing phage resistance and either lost or reduced conjugation ability. The semi-conjugative clones had a conjugation rate around 2.5 orders of magnitude lower than the ancestral, fully-conjugative phenotype. Next, in a 5-day co-culture experiment, the non-conjugative and semi-conjugative clones were tested for their ability to transfer the RP4 plasmid to the recipient strain HMS174(pSU18), either in the absence of antibiotics or in the presence of a sub-MIC double-antibiotic selection (kanamycin + chloramphenicol) favouring transconjugants (pSU18 is a non-conjugative plasmid coding for chloramphenicol resistance [Bartolomé *et al.* 1991]). We found that, regardless of the presence of antibiotic selection, HMS174(pSU18)(RP4) transconjugants remained either absent or very rare in cultures where the potential plasmid donor clones were originally non-conjugative. The same observation was also made when five non-conjugative HMS174(RP4) clones isolated at the end of the previous phage selection experiment (described in section 4.2) were tested for their conjugation rate to *E. coli* JM109(pSU19) (Bartolomé *et al.* 1991) under a similar transconjugant-favouring antibiotic selection (kanamycin + chloramphenicol). These results suggest that once the conjugation ability is completely lost as a result of exposure to the plasmid-dependent phage PRD1, reversion to the fully-conjugative phenotype is improbable. With originally semi-conjugative JE2571(RP4) clones as plasmid donors, and in the absence of antibiotics, the efficacy of the conjugative spread of RP4 strongly depended on the particular donor clone in question: for some donors, virtually no transconjugants were formed; whereas for others, the observed transconjugant frequencies at the end of the 5-day experiment were comparable to those of the control treatment in which fully-conjugative JE2571(RP4) acted as the plasmid donor. Contrastingly, in the presence of conjugation-favouring antibiotic selection, the measured transconjugant frequencies for different semi-conjugative donor clones were uniformly similar to those observed in the control treatment after 5 days of co-culturing. However, this result does not by itself imply that reversion from a semi-conjugative to fully-conjugative phenotype has taken place, because despite the lowered conjugation rate, 5 days may be a sufficiently long time period for transconjugants to approach fixation in the population, especially when conjugation-favouring antibiotic selection is present in the system.

In order to test whether reversion to full conjugation ability had occurred, we isolated random HMS174(pSU18)(RP4) transconjugant clones from the end-points of the 5-day experiment, both from treatments where RP4 had an evolutionary history with PRD1 and from the control treatment with fully-conjugative phage-naïve plasmids, and then compared the conjugation rates

back to a plasmid-free JE2571 strain. Moreover, we also tested all of the isolated clones for their resistance/susceptibility to PRD1 and correlated the results with the conjugation rate data. We found that phage resistance was always coupled with significantly reduced conjugation ability; in other words, no phage-resistant clones with a conjugation rate comparable to that of the fully-conjugative control were discovered. Reversion to full conjugation ability was observed in only one of the cultures where the plasmid donor was originally semi-conjugative; however, all of the tested transconjugants were also susceptible to infection by PRD1. Overall, these findings suggest that the selective pressure exerted by PRD1 on a plasmid-carrying bacterial population can have a sustained preventive or limiting effect on the plasmid's conjugation ability (and thus on the spread of resistance genes), lasting even after the phage selection is lifted and a sub-MIC conjugation-favouring antibiotic selection is added. Moreover, the observed phenotypic linkage between full conjugation ability and susceptibility to PRD1 suggests that plasmid-dependent phages potentially have a sustained usability, since upon possible reversion to full conjugation ability, the therapeutic re-administration of phages is again effective.

Finally, we sequenced the RP4 plasmids of the two non-conjugative and two semi-conjugative phage-resistant JE2571(RP4) clones used in the 5-day conjugation experiment (discussed above). One of the semi-conjugative clones was the original plasmid donor in the only treatment where we observed the return of the ancestral phenotype (fully-conjugative and phage-susceptible). Consequently, from the same treatment, we also sequenced the plasmids of three end-point HMS174(pSU18)(RP4) transconjugants which showed the ancestral phenotype to see whether the ancestral genotype had also returned.

All observed mutations were located in the conjugation-associated Tra2 operon of RP4. Tra2 contains 11 genes (TrbB to TrbL) which code for a corresponding number of proteins, 10 of which are known to be essential for both plasmid transfer and successful PRD1 infection (Lessl *et al.* 1993, Haase *et al.* 1995, Grahn *et al.* 1997); only TrbK, which functions in entry exclusion (Haase *et al.* 1996), is disposable in this regard. For the majority of these Trb genes, the incorporation of mutations has been shown to confer resistance to PRD1; however, this also seems to invariably lead to some type of disruption in the structure and function of the plasmid T4SS, thus causing the conjugation ability to be lost or become drastically compromised (Grahn *et al.* 1997). Our sequencing data showed that both of the clones with a non-conjugative phage-resistant phenotype had an identical nine-nucleotide deletion in the gene TrbJ, which codes for a structural protein located in the periplasmic fraction of the T4SS of RP4 (Grahn *et al.* 2000). In the case of the irreversible semi-conjugative phage-resistant clone, a transposon had integrated between genes TrbJ and TrbK. Finally, the RP4 plasmid of the reversible semi-conjugative phage-resistant clone contained a tandem repeat addition in the gene TrbI (the protein product of which is again part of the T4SS), in this case embedded in the cytoplasmic membrane (Grahn *et al.* 2000). However, the mutation in question

had disappeared in all of the reversed HMS174(pSU18)(RP4) transconjugants we tested. These types of readily reversible phenotypic and genotypic changes through the addition and removal of short tandem repeats are often referred to as 'dynamic mutations', since they allow for a rapid evolutionary response to changing selective environments (Treangen *et al.* 2009, Zhou *et al.* 2014).

Conjugative plasmids are known to have elaborate transfer regulation systems in order to mitigate the cost of plasmid replication and gene expression on the host bacterium (Zatyka and Thomas 1998). However, our results, together with those of Jalasvuori *et al.* (2011), suggest that selection by plasmid-dependent phages can lead to adaptive changes in bacterial conjugation ability through mutations and plasmid loss, instead of merely inducing phenotypic changes through gene regulation. Switching genes on and off may not be a fast enough response to such stress factors, which are lethal in a short period of time (in this case phage infection) (Treangen *et al.* 2009). Dynamic mutations represent sort of a 'best of both worlds' strategy where the bacteria both evolve rapidly and are also capable of reverting to their previous geno- and phenotype when the selection pressures in the system change. In our study, following the withdrawal of the selection pressure exerted by PRD1, the return to an ancestral conjugation phenotype was possible only when phage resistance had originally emerged as a result of a dynamic mutation.

Overall, it is clear that conjugative plasmids and/or their host bacteria can be evolutionarily rescued from plasmid-dependent phages through the emergence of a variety of different geno- and phenotypes. Furthermore, these different rescue trajectories have different implications on the subsequent conjugative spread of plasmid-carried antibiotic resistance genes, since either plasmid-free (Jalasvuori *et al.* 2011), non-conjugative or semi-conjugative (Kornstein *et al.* 1992) phenotypes can be selected for, and the permanency of these phenotypes depends on the nature of the underlying genetic mutations. Consequently, from the standpoint of potential anti-conjugation applications of plasmid-dependent phages, it is important to acknowledge the factors which affect the likelihood of these different rescue scenarios. For example, exposure to phages alone tends to select for plasmid-free bacteria (Jalasvuori *et al.* 2011), whereas the simultaneous use of antibiotics (against which the plasmid provides resistance) and phages is more likely to favour non-conjugative and semi-conjugative phage-resistant plasmid-carrying phenotypes. Therefore, if the objective were to prevent the horizontal transfer of resistance genes from commensal bacteria to a susceptible pathogenic strain during antibiotic treatment, a sequential administration of phages and antibiotics, respectively, would (circumstances allowing) be the preferable therapeutic strategy. In other words, plasmid-dependent phages could potentially be used prophylactically to purge the commensal bacteria of their resistance plasmids, thus lowering the probability of pathogenic bacteria getting evolutionarily rescued from the antibiotic treatment at some later time point. However, toward this end, even the simultaneous administration of phages and antibiotics could still be successful, since we saw that plasmid-dependent phages effectively selected

against the formation of transconjugants in the presence of conjugation-favouring sub-MIC antibiotic selection.

4.4 Future perspectives

It is evident that the horizontal transfer of resistance genes via conjugation is increasingly compromising our ability to control bacterial infections with antibiotics and undermining our efforts to put breaks on the escalating global resistance epidemic. Recently, this fact was alarmingly brought home with the first-time discovery of conjugative plasmid-mediated colistin resistance in *E. coli* in China (Liu *et al.* 2015). Colistin is mainly a last-resort drug used to treat infections unresponsive to all other antibiotics, which means that the potential horizontal transfer of colistin resistance to some already highly resistant bacterial strains (i.e. carbapenemase-producing *Enterobacteriaceae*) can lead to a significant rise in the occurrence of untreatable 'superbug' infections, where the patients can only be provided with palliative care (Göttig *et al.* 2014, Zowawi *et al.* 2015). The reported fact that the conjugative plasmid carrying the novel colistin resistance gene (named *mcr-1*) is stably maintained, has a very high conjugation rate, and moves readily between many important gram-negative human pathogens (Liu *et al.* 2015) suggests that global dissemination of *mcr-1* is essentially inevitable: a prediction also supported by past experiences with, for example, NDM-1 (Kumarasamy *et al.* 2010). Indeed, the spread of plasmid-carried *mcr-1* into Europe has already been confirmed (Hasman *et al.* 2015, Arcilla *et al.* 2015, Webb *et al.* 2015). These recent developments again demonstrate the importance of furthering our knowledge of how conjugative plasmids persist and spread in bacterial communities under different selective conditions, and also highlight the urgent need for effective anti-conjugation strategies to mitigate the role of HGT in introducing resistance genes to new pathogenic strains. The research presented in this thesis suggests that the conjugative transfer of resistance genes from the surrounding microbial community can potentially lead to the evolutionary rescue of antibiotic-treated susceptible bacteria even at very high above-MIC antibiotic concentrations. Promisingly, however, we also found that plasmid-dependent bacteriophages, as natural enemies of conjugative plasmids and the bacteria that harbour them, show substantial anti-conjugation potential, which perhaps could be harnessed for our use in the struggle to stop the HGT-mediated accumulation of resistance genes into pathogenic bacterial strains. Naturally, as is the case with all *in vitro* studies, these results are to be interpreted with caution, and they should act as a stepping stone to research in actual *in vivo* systems, which is of course a self-evident requirement should there be a future pursuit of using plasmid-dependent phages as anti-conjugation therapeutic agents.

The pros and cons of applying phages therapeutically have been extensively discussed in the literature (see e.g. Alisky *et al.* 1998, Sulakvelidze *et al.* 2001, Summers 2001, Loc-Carrillo and Abedon 2011, Lu and Koeris 2011,

Henein 2013). The main pros include the strict infection specificity of phages to prokaryotic cells, the lack of reported serious side effects in patients, and phage self-replication at the site of the infection; some of the cons include the generally narrow host range of phages, the risk of the presence of lysogenic (or any other) phages encoding toxins and other bacterial virulence factors in insufficiently characterised phage preparations, and the technical challenges involved in the large-scale manufacturing and storing of therapeutic-grade phage antibacterials. However, many of these commonly raised points of concern regarding the feasibility of phage therapy seem, to a certain extent, not to apply to phage PRD1, since it is well characterised, strictly lytic, relatively readily producible in large quantities, and stably storable for long time periods (Olsen *et al.* 1974, Ackermann *et al.* 2004, Mesquita *et al.* 2010). Also, the attractive feature of plasmid-dependent phages in general is their comparatively broad host range (especially so with PRD1), which means that a wide range of resistance plasmids could potentially be targeted with a relatively simple cocktail of phages specific to different T4SSs. In addition, the fact that no fully-conjugative phage-resistant phenotypes were observed in our experiments gives some indication that these types of cocktails could have a sustained anti-conjugation therapeutic efficacy. However, in Western countries, various laws and regulations restrict the *in vivo* application of phages and, moreover, the idea of purposefully putting 'viruses' into the human body can also cause a backlash from a general public ignorant of the specifics of the subject matter (Loc-Carrillo and Abedon 2011, Henein 2013). For the pharmaceutical industry, these non-technical points of contention have further added to the unattractiveness of pursuing phage therapy, which is deemed economically unprofitable under current circumstances. Still, it is possible that the waning efficacy of antibiotics and the resulting loss of life and money will eventually provide society as a whole with the necessary incentive to look into all conceivable alternatives to prevent us from returning to the pre-antibiotic era in controlling bacterial diseases.

5 CONCLUSIONS

The primary conclusions of this thesis are as follows:

I. The conjugative transfer of β-lactamase-coding genes from the surrounding bacterial community can evolutionarily rescue antibiotic-susceptible bacteria exposed to β-lactam levels significantly above the minimum inhibitory concentration. This suggests that establishing a high therapeutic concentration of antibiotics can potentially still be ineffective in clearing an infection when the targeted pathogens are capable of rapid acquisition of resistance genes from other bacteria via conjugation.

II. Plasmid-dependent phage PRD1 can effectively select against the horizontal movement of the resistance-conferring conjugative plasmid RP4 between bacteria even in the presence of conjugation-favouring sub-lethal antibiotic selection. PRD1 selection favours phage-resistant bacterial phenotypes, which have either discarded the plasmid or carry a mutated version of the RP4 with either lost or drastically reduced conjugation ability.

III. The PRD1-induced lost or lowered conjugation ability of plasmid RP4 does not readily revert even when the phage selection is lifted and a conjugation-favouring antibiotic selection is added; in cases where the fully-conjugative phenotype does return, the susceptibility to phages is also always restored. The likelihood of reversion depends on the type of mutation that originally led to phage resistance and compromised conjugation ability.

The horizontal transfer of resistance-coding conjugative plasmids to pathogenic bacterial strains is among the main culprits of the worsening global antibiotic resistance crisis. The lack of new antibiotics in the pipeline calls for the development of alternative and complementary methods of treatment. The

findings of this thesis suggest that plasmid-dependent phages, as naturally occurring enemies of both conjugative plasmids and their host bacteria, show potential for novel anti-conjugation therapeutic applications.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Bakteerien antibioottivastustuskyvyn horisontaalisen leviämisen estäminen konjugatiivisista plasmideista riippuvaisilla bakteriofageilla

Antibiootit ovat elintärkeä osa modernia lääketiedettä; antibioottien löytyminen 1900-luvun keskivaiheilla mahdollisti monien sellaisten bakteeriperäisten tautien tehokkaan hoitamisen, jotka aiemmin ihmiskunnan historiassa olivat olleet merkittäviä sairastavuuden ja kuolleisuuden aiheuttajia. Vuosikymmeniä jatkunut antibioottien laajamittainen tarpeellinen ja tarpeeton käyttö on kuitenkin johtanut antibioottivastustuskykyisten bakteerikantojen räjähdysmäiseen yleistymiseen, ja pelkona onkin, että ilman nopeita ja tehokkaita maailmanlaajuisia toimia edessä on paluu antibiootteja edeltävään aikaan bakteeritautien hoitamisessa. Tämä tarkoittaisi valtavia menetyksiä sekä taloudellisesti että ihmishengissä mitattuna.

Eräs suurimmista tekijöistä useille antibiooteille vastustuskykyisten bakteerikantojen syntymisessä on vastustuskykyä koodaavien geenien horisontaalinen siirtyminen bakteerikannasta ja lajista toiseen, jota tapahtuu erityisesti niin kutsuttujen konjugatiivisten plasmidien välityksellä. Konjugatiiviset plasmidit ovat isäntäbakteerin kromosomista (useimmiten) erillisiä itsekkäitä DNA-molekyyleja, jotka monistuvat omatoimisesti ja joilla on kyky siirtyä uusiin bakteeri-isäntiin niin kutsutun konjugaatiokanavan lävitse, jonka muodostumista bakteereiden välille plasmidi itse koodaa. Tämän lisäksi konjugatiiviset plasmidit koodaavat yleisesti monenlaisia isäntäbakteerille hyödyllisiä ominaisuuksia, kuten esimerkiksi juuri vastustuskykyä eri antibiooteille. Ottaen huomioon konjugatiivisten plasmidien keskeinen rooli maailmanlaajuisessa antibioottivastustuskykyepidemiassa, on tärkeää ymmärtää, millaiset eko-evolutiiviset tekijät vaikuttavat näiden plasmidien leviämiseen ja säilymiseen bakteeriyhteisöissä. Lisäksi tarvittaisiin innovatiivisia tapoja estää vastustuskykygeenien horisontaalinen siirtyminen taudinaiheuttajakantoihin, etenkin kun uusien antibioottien kehitys lääketeollisuuden toimesta on jo pitkään ollut lähes pysähdyksissä.

Tämän väitöskirjatyön ensimmäisessä osassa osoitettiin, että ympäröivästä bakteeriyhteisöstä konjugatiivisen plasmidin välityksellä siirtyvä β-laktamaasi-antibioottivastustuskykygeeni voi pelastaa antibiootille alttiit bakteerit muutoin kuolettavalta hoitoannokselta β-laktaami-antibioottia. Tulos antaa viitteitä siitä, että edes erittäin korkea hoitoannos antibioottia ei välttämättä aina riitä bakteeri-infektion parantamiseen, mikäli vastustuskykygeenejä on nopeasti saatavilla muilta bakteereilta konjugaation välityksellä. Tällaisessakin tilanteessa olisi siis hyödyllistä pystyä luomaan evolutiivinen valintapaine konjugaation tapahtumista vastaan; väitöskirjatyön kahdessa jälkimmäisessä osassa tutkittiin ns. plasmidi-riippuvaisten bakteriofagien (lyh. faagi) käyttöä kyseiseen tarkoitukseen. Plasmidi-riippuvaiset faagit ovat viruksia, jotka ovat erikoistuneet infektoimaan ja tappamaan ainoastaan konjugatiivisia plasmideja kantavia bakteereita. Kokeissa havaittiin, että plasmidi-riippuvainen faagi PRD1 rajoittaa tehokkaasti antibioottivastustuskykyä koodaavan konjugatiivisen plasmidin,

RP4:n, leviämistä bakteerien välillä, jopa silloin kun ympäristössä on läsnä konjugaatiota suosiva ei-kuolettava antibioottiselektio. PRD1:n aiheuttama valintapaine suosii populaatiossa sellaisia faagille vastustuskykyisiä bakteereita, jotka ovat joko menettäneet plasmidin kokonaan tai jotka kantavat mutatoituneita RP4-plasmideita, joilla konjugaatiokyky on joko huomattavasti alentunut tai täysin kadonnut. Jatkokokeet osoittivat, että plasmidin menetetty tai alentunut konjugaatiokyky ei palaudu helposti edes silloin, kun faagiselektio poistetaan ja tilalle tuodaan konjugaatiota suosiva antibioottiselektio; lisäksi tapauksissa, joissa RP4 mutatoituu takaisin täysin konjugaatiokykyiseksi, myös alttius PRD1-infektiolle palautuu poikkeuksetta. Palautumisen todennäköisyys riippuu siitä, millainen mutaatiotyyppi alun perin johti faagivastustuskykyyn ja heikentyneeseen tai menetettyyn konjugaatiokykyyn. Kokonaisuutena tulokset antavat lupaavia viitteitä siitä, että plasmidi-riippuvaisia faageja voitaisiin tulevaisuudessa hyödyntää antibioottivastustuskyvyn horisontaalisen leviämisen estämisessä.

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ORIGINAL PAPERS

I

EVOLUTIONARY RESCUE OF BACTERIA VIA HORIZONTAL GENE TRANSFER UNDER A LETHAL B-LACTAM CONCENTRATION

by

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Short Communication

Evolutionary rescue of bacteria via horizontal gene transfer under a lethal β-lactam concentration

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ABSTRACT

β-Lactams are a commonly used class of bactericidal antibiotics. The number of β-lactam-resistant pathogens is constantly increasing in hospitals around the world. Interestingly, most of the β-lactam-resistant bacteria carry mobile genetic elements, such as conjugative plasmids, that render the pathogen resistant. These elements mediate their own transfer from one bacterium to another, producing new resistant strains via horizontal gene transfer. Here we investigated whether it is possible that transfer of the resistance element from another bacterium may evolutionarily rescue a susceptible bacterium exposed to a lethal concentration of the β-lactam ampicillin. Indeed, the rescuing occurs even at very high, clinically significant antibiotic levels, suggesting that pathogens may acquire the resistance 'on the fly' from commensal bacteria during treatment.

1. Introduction

Antibiotic resistance is a major problem for modern health care [1]. The costs to treat resistant hospital-acquired infections have been steadily increasing over the past few decades, reaching in the USA alone annual costs of tens of billions of dollars [2]. The crisis has been declared as one of the utmost challenges for science to overcome. Whilst drug-resistant bacteria are the cause of much human tragedy, they are also a significant evolutionary puzzle. Indeed, extensive use of antibiotics over the past 60 years has generated one particularly interesting evolutionary consequence: the majority of pathogenic bacteria have acquired their resistance genes horizontally from other bacteria [3]. In many cases, bacteria did not adapt to the selection pressure by favouring a few resistant individuals within the population. Instead, they received a mobile genetic element that carried the resistance gene from another bacterium. In other words, bacteria genetically networked their way around lethal antibiotics [4].

In addition to use in hospitals and other healthcare facilities, antibiotics are being applied during various production processes where bacteria may cause problems [5]. The amount of antibiotics being used is less controlled when humans are not the direct target of the treatment, and thus substantial quantities of antibiotics have been released to various environments other than in the direct vicinity of hospitals [6]. For example, in the farming industry antibiotics are applied not only to cure diseases but also to pre-emptively destroy disease-causing agents and to increase the growth and well-being of animals. There have been serious attempts to study and control the release of genetically engineered antibiotic-resistant bacteria to the environment [7,8], but much less effort has been put into analysing the potential effects that such environmental 'engineering' due to antibiotic use has on commensal bacterial populations or on the dynamics of naturally and unnaturally occurring antibiotic resistances [9].

Given the extent to which antibiotics are being used in animal husbandry, plant production and aquaculture (i.e. fish and shrimp farming), bacteria face significant selection pressure to become resistant [10]. Continuous use of antibacterial agents creates concentration gradients both at the site of their application as well as in the surrounding environment, resulting in areas where bacteria are subjected to either lethal or sublethal levels of antibiotics. Exposure to below inhibitory concentrations of antibiotics can lead to the gradual emergence of resistance. As a result, the community evolves to become increasingly more resistant to even higher and higher levels of antimicrobials. Indeed, prolonged exposure to sublethal antibiotic concentrations has been argued to play an important role in the emergence of resistant bacterial pathogens and in the transmission of resistance genes between various bacterial species [10].

In contrast to sublethal concentrations, the evolutionary situation is different in areas where antibiotics still remain above the minimum inhibitory concentration (MIC). Given the surplus

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use of antibiotics as routine pre-emptive treatment during animal production and plant farming, the above-MIC areas can be substantially large. Moreover, antibiotic treatment of bacterial infections also aims to maintain an above-MIC pharmacokinetic concentration in order to remove the pathogenic agent. In such conditions, bacteria are unable to gradually adapt to the selection pressure, leaving generally only three possible fates for the susceptible bacterial strains. The first possibility is that the strain dies altogether, leaving no new antibiotic-resistant strains for consideration as future resistant pathogens. In the second alternative, the susceptible bacterial population may be inherently variable so that the strain survives the selection via a few naturally resistant mutants. This range has been referred to in some discussions by the term 'mutation selection window' [11]. Evolutionary rescue by a few bacterial cells leads the population through a bottleneck that enriches the rescuing phenotypes and thus establishes a new resistant strain. In the third potential scenario, the surrounding bacterial community may save the susceptible strain in one way or another. Here we focus on this third possibility and especially on the potential for susceptible bacteria to acquire antibiotic resistance 'on the fly' after they have been subjected to a lethal antibiotic concentration.

β-Lactams are a major class of antibiotics that disturb cell wall generation in bacteria. Extended-spectrum β-lactamase-producing Gram-negative bacteria are one of the most common and problematic drug-resistant hospital pathogens [1]. Resistance genes often reside on mobile genetic elements that are readily transferred between different and sometimes distantly related bacterial strains [3]. In this study, two *Escherichia coli* strains were used, one of which harboured a conjugative plasmid with a TEM-1 type β-lactamase-encoding gene and one that was susceptible to β-lactams. Different variations of TEM-1 β-lactamases are common in hospitals. To the best of our knowledge, here we demonstrate for the first time that the conjugative element and therefore the resistance-inducing gene can be transferred from the resistant strain to the susceptible bacteria even after subjection to clinically relevant lethal antibiotic concentrations, thus rescuing the recipient bacterium and generating a novel drug-resistant strain.

2. Materials and methods

β-Lactamase (TEM-1)-producing *E. coli* K-12 strain JE2571(RP4) was cultured with β-lactam-susceptible but rifampicin-resistant *E. coli* K-12 HMS174 in the presence of various β-lactam (ampicillin; Sigma–Aldrich, St. Louis, MO) concentrations. The resistance-conferring gene TEM-1 in JE2571 cells is expressed by an incompatibility group β conjugative plasmid RP4 that also provides resistance to aminoglycosides (kanamycin) and tetracycline [12]. The plasmid encodes for a conjugation channel through which it can spread to other (even distantly related) bacterial cells. HMS174 cells carry rifampicin resistance on the chromosome and are thus unable to transfer the gene to other cells [13]. The natural ampicillin resistance levels both for HMS174 and JE2571(RP4) were determined with an ampicillin MIC Test Strip (Liofilchem, Roseto degli Abruzzi, Italy). The MIC for HMS174 was 0.75 mg/L, whereas JE2571(RP4) was completely resistant even to the highest ampicillin concentration (256 mg/L) on the MIC test strip.

Approximately 2.0×10^6 susceptible HMS174 cells and the same amount of resistant JE2571(RP4) cells (added in this order) were used to initiate 5 mL cultures in Luria–Bertani medium. Antibiotic was introduced to the medium before bacterial cells. Cultures were grown at 37 °C with shaking at 200 rpm. The amount of different cell types was measured after ca. 24 h of growth by plating the cultures on different antibiotic-containing plates. Given that HMS174 cells (cheaters) are resistant to rifampicin, transconjugant HMS174(RP4) cells (novel co-operators) are resistant to rifampicin, ampicillin, kanamycin and tetracycline, and JE2571(RP4) cells (co-operators) are resistant to ampicillin, kanamycin and tetracycline but susceptible to rifampicin, with these differences in the resistance profiles we can infer the amount of each bacterial cell type in the culture after 24 h of cultivation. Various dilutions of all of the cultures were plated on all of the different plates in order to accurately determine bacterial densities. Only plates with sufficient amount of colonies (ca. 40–300) were used for calculations. All antibiotics were purchased from Sigma–Aldrich.

All concentrations of ampicillin were determined to be lethal to the same amount of HMS174 and JE2571 cells as used in the evolutionary rescue experiments. In other words, the controls differed only by the absence of resistance plasmid in JE2571 cells, which then resulted in the demise of the whole culture.

3. Results and discussion

We observed that horizontal transfer of the resistance gene evolutionarily rescues a significant portion of susceptible bacteria that were subjected to a lethal antibiotic concentration (Fig. 1). In the absence of resistance genes, bacteria were unable to survive. Although the overall number of evolutionary rescued bacteria decreases as the antibiotic concentration increases, the fraction of susceptible bacteria receiving the plasmid remains relatively high. At the most extreme, evolutionary rescue took place at an ampicillin concentration of 600 mg/L. This is significantly above the previously documented effective concentrations (ca. 1–150 mg/L) [14], suggesting that increasing antibiotic dosage is not sufficient to nullify the observed dynamics. Therefore, emergence of resistant mutants by avoiding the mutation selection window [11] is not easily achieved when resistant mutants emerge via horizontal gene transfer. Naturally, caution needs to be exercised when in vitro results are compared with actual patient cases.

Recently, Yurtsev et al. demonstrated the co-operative nature of antibiotic-resistant bacteria in the presence of β-lactam antibiotics [15]. Susceptible bacteria can survive clinically relevant, inhibitory antibiotic concentrations when 'co-operating' bacteria in the system produce β-lactam-hydrolysing β-lactamase (TEM-1 type). The fraction of resistant bacteria and the susceptible cheaters reaches an equilibrium, which depends on the initial number of antibiotic-resistant cells as well as on the antibiotic concentration. This study suggests that social dynamics can play an important role in the evolution of antibiotic resistance. However, the cheaters are still susceptible to antibiotics if the resistant bacterium disappears from the system. Therefore, to truly generate new antibiotic-resistant strains that can survive antibiotics even in the absence of co-operators, the cheaters must somehow evolve inherent resistance. As we have shown here, and given that antibiotic resistance genes often reside on mobile genetic elements such as conjugative plasmids [3], it is a reasonable possibility that in real systems cheaters routinely acquire resistance elements from co-operators. This may be a significant yet previously undemonstrated evolutionary mechanism in the development of new antibiotic-resistant strains under high concentrations of β-lactams.

Combining the co-operative effect of resistant bacteria [15] with the mobile nature of most resistance genes helps us to better understand how pathogens may develop antibiotic resistance 'on the fly' during antibiotic treatment. In hospitals where mobile antibiotic resistance elements, infectious bacterial pathogens and antibiotics are commonplace, the ecological conditions for the transfer of resistances from competitively inferior but co-operative commensal bacteria to pathogenic cheaters may be especially

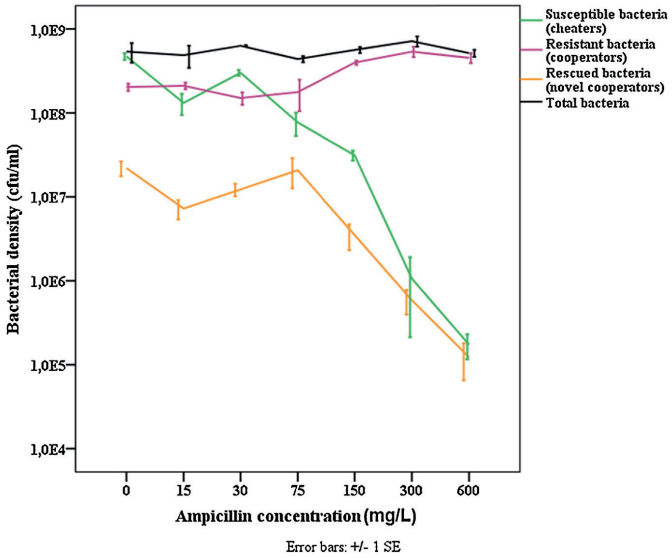

Fig. 1. Bacterial densities (CFU/mL) of the original resistant bacterium (co-operator) JE2571(RP4), the susceptible bacterium (cheater) HMS174 and the evolutionarily rescued bacteria (novel co-operator) HMS174(RP4) as a function of β-lactam (ampicillin) concentration after ca. 24 h of cultivation at 37 °C. $N = 4$ for all data points. All used antibiotic concentrations were determined to be lethal to the same bacteria in the absence of resistance genes.

selected for. Indeed, antibiotic resistance genes in soil bacteria are often perfectly similar to the genes in pathogenic bacteria, suggesting recent horizontal gene transfer to have occurred between these bacterial types [16]. Given that resistant strains may emerge via horizontal gene transfer during treatment, it may partly explain the prevalence of mobile elements in antibiotic-resistant hospital-acquired infectious pathogens along with the sequence similarities of resistance genes between pathogenic and commensal bacteria.

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Competing interests

None declared.

Ethical approval

Not required.

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II

FIGHT EVOLUTION WITH EVOLUTION: PLASMID-DEPENDENT PHAGES WITH A WIDE HOST RANGE PREVENT THE SPREAD OF ANTIBIOTIC RESISTANCE

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Fight evolution with evolution: plasmid-dependent phages with a wide host range prevent the spread of antibiotic resistance

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Abstract

The emergence of pathogenic bacteria resistant to multiple antibiotics is a serious worldwide public health concern. Whenever antibiotics are applied, the genes encoding for antibiotic resistance are selected for within bacterial populations. This has led to the prevalence of conjugative plasmids that carry resistance genes and can transfer themselves between diverse bacterial groups. In this study, we investigated whether it is feasible to attempt to prevent the spread of antibiotic resistances with a lytic bacteriophage, which can replicate in a wide range of gram-negative bacteria harbouring conjugative drug resistance–conferring plasmids. The counter-selection against the plasmid was shown to be effective, reducing the frequency of multidrug-resistant bacteria that formed via horizontal transfer by several orders of magnitude. This was true also in the presence of an antibiotic against which the plasmid provided resistance. Majority of the multiresistant bacteria subjected to phage selection also lost their conjugation capability. Overall this study suggests that, while we are obligated to maintain the selection for the spread of the drug resistances, the 'fight evolution with evolution' approach could help us even out the outcome to our favour.

Introduction

The rapidly increasing number of antibiotic-resistant bacterial infections is of a major concern to modern health care worldwide, causing both substantial financial loss and numerous deaths (Taubes 2008; Bush et al. 2011). From an evolutionary standpoint, the problem is hardly a surprising one, because the constant application (both appropriate and inappropriate) of antibiotics has exerted a strong pressure on bacteria to develop resistance (Cohen 1992; Levin et al. 1997; Austin et al. 1999; Levy and Marshall 2007). Nevertheless, the predictability of the issue has not made it any easier to deal with, and in the case of many serious bacterial pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) or certain strains of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, the number of viable treatment options is dangerously declining (MacKenzie et al. 1997; Livermore 2002; Taubes 2008; Wise et al. 2011). The situation is further complicated by the fact that only a few novel classes of antibiotics have been introduced during the past 50 years (Walsh 2003; Coates et al. 2011).

Resistant bacteria commonly harbour mobile genetic elements, such as conjugative plasmids that contain genes conferring resistance to several classes of antibiotics (Bennett 2008). Conjugative plasmids replicate independently of the host genome and they can facilitate their own transfer from one bacterial strain or species to another by coding for a channel through which a copy of the plasmid is transferred from donor to recipient cell (Brinton 1965). This horizontal gene transfer (HGT) allows for a highly efficient spread of resistances in bacterial communities (Davies 1994; Grohmann et al. 2003). Autonomous replication and the ability to move between (sometimes distantly related) bacteria mean that conjugative plasmids are independently evolving genetic elements (Norman et al. 2009). Consequently, their presence and horizontal movement can, depending on the circumstances, be an advantage, a disad-

vantage or neutral in terms of fitness of both the host they reside in and the other bacteria in the microbial community (Eberhard 1990; Kado 1998; Dionisio et al. 2005; Slater et al. 2008; Norman et al. 2009). For example, the transfer of a conjugative plasmid from a bacterial donor to a (unrelated) recipient could potentially lower the fitness of the donor and increase the fitness of the recipient if the two bacteria compete over resources, and the possession of the plasmid provides some competitive advantage, such as resistance to the antibiotics present in the system (Jalasvuori 2012).

Interfering with the process of bacterial conjugation has been proposed as one potential way of combating the spread of plasmid-mediated antibiotic resistances (Smith and Romesberg 2007; Williams and Hergenrother 2008). Certain bacteriophages (phages) specifically infect and kill conjugative plasmid–harbouring bacteria (Caro and Schnös 1966). These phages use conjugative plasmid–encoded proteins as their receptor to gain entrance to a host cell. The host range of a given conjugative plasmid–dependent (or male-specific) phage is therefore mainly determined by the host range of suitable conjugative plasmids (Olsen et al. 1974). In practice, conjugative plasmid–dependent phages are natural enemies of both the conjugative plasmids and the bacteria that harbour them. A previous study suggests that in the absence of antibiotic selection and other bacteria, the presence of a lytic conjugative plasmid–dependent phage can efficiently select for bacteria that either have lost their conjugative plasmids or harbour a conjugation-deficient version of the plasmid (Jalasvuori et al. 2011). However, the capability of these phages to limit the rate of horizontal transfer of plasmids between bacteria was not investigated. Other studies have shown that nonlytic filamentous phages are capable of preventing the spread of conjugative plasmids by physically inhibiting conjugation (Novotny et al. 1968; Lin et al. 2011).

Elaborating from these previous studies, we here investigated whether a lytic conjugative plasmid–dependent phage can prevent the emergence of new multiresistant strains by selecting against the plasmid or, more specifically, the plasmid-encoded sex apparatus facilitating the transfer of the plasmid to other bacteria. Moreover, we measure how much the presence of nonlethal antibiotic selection favouring different plasmid and bacterium combinations alters the counter-selective effect of phages (Fig. 1). In our experiments, two antibiotic-resistant bacterial strains of *Escherichia coli* K-12 were cultivated in daily replenished cultures together for 3 days. One of the used strains, JE2571(RP4), contains a conjugative plasmid RP4 conferring resistance to several antibiotics of different classes (ampicillin, kanamycin and tetracycline), whereas the other strain HMS174 is plasmid free but resistant to rifampicin due to a chromosomal mutation. In this experimental setup, the potential

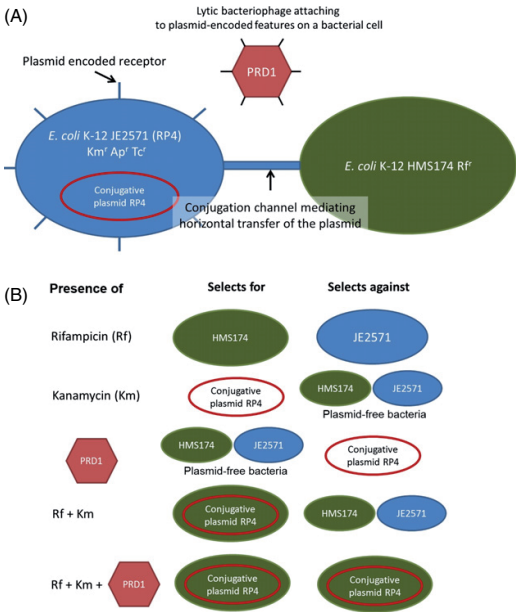

Figure 1 Schematic presentation of the experimental setup and the selection pressures.

conjugative transfer of the RP4 plasmid from JE2571(RP4) to HMS174 would create a new multiresistant strain HMS174(RP4). The presence of the conjugative plasmid–dependent phage PRD1 selects against all bacteria representing plasmid-encoded receptors on the cell surface. Bacteria are resistant to phage infections if they are free of the plasmid or they harbour a conjugation-defective mutant (Jalasvuori et al. 2011).

Altogether, we here demonstrate that conjugative plasmid–dependent phage PRD1 effectively restricts the emergence of the multiresistant HMS174(RP4) strain even in the presence of nonlethal antibiotic selection. While growth-reducing antibiotic concentrations may play an important role in the evolution of bacterial antibiotic resistance (Andersson and Hughes 2012), these results suggest that is possible to combat this evolution with counter-selective attempts.

Materials and methods

Bacterial strains, bacteriophages and culture conditions

Escherichia coli K-12 strains JE2571(RP4) (Bradley 1980), HMS174 (Campbell et al. 1978) and JM109(pSU19) were used in this study. JE2571 harbours a conjugative incompatibility group P plasmid RP4 (Datta et al. 1971), which induces antibiotic resistance to kanamycin, ampicillin and

tetracycline. HMS174 contains chromosomal rifampicin resistance. JM109(pSU19) contains a nonconjugative plasmid pSU19 (Bartolomé et al. 1991) that induces chloramphenicol resistance. All strains were cultivated in Luria–Bertani (LB) medium (Sambrook et al. 1989) at 37°C. Shaking at 200 revolutions per minute (rpm) was used, with the exception of the evolution experiments where the cultures were unshaken. For general antibiotic selection, kanamycin, rifampicin and chloramphenicol were used in final concentrations of 32 μg/mL, 55 μg/mL and 25 μg/mL, respectively. The bacteriophage used in this study was PRD1; a lytic conjugative plasmid–dependent phage infecting a wide range of gram-negative bacteria that contain conjugative plasmids belonging to incompatibility groups P, N and W (Olsen et al. 1974).

Evolution experiments

5 μL of JE2571(RP4) and HMS174 overnight cultures were inoculated into the same tube containing 5 mL of fresh LB medium. The mixed cultures were treated with (i) no antibiotics, (ii) kanamycin, (iii) rifampicin or (iv) kanamycin and rifampicin. When appropriate, kanamycin and rifampicin were added in nonlethal but growth-reducing concentrations of 3.2 μg/mL and 3.7 μg/mL, respectively (Fig S1A,B). Each antibiotic treatment was performed both in the presence and in the absence of conjugative plasmid–dependent phage PRD1. Immediately after the transfer of the bacteria, 5 μL of phage stock containing approximately 10^{11} plaque-forming units per millilitre (pfu/mL) was added to the appropriate treatments. Cultures were grown at 37°C without shaking. The length of the experiment was approximately 72 hours, and the cultures were renewed at 24- and 48-hour time points by transferring 5 μL of culture to 5 ml of fresh LB medium (containing the appropriate antibiotics; no new phage was added during the refreshments). Each treatment was sampled during the culture renewals and at the end of the experiment. These samples were diluted and plated on either regular or antibiotic-containing (kanamycin and rifampicin) 1% LB agar plates to obtain the total bacterial densities and the number of bacteria resistant to both antibiotics. Also, from all treatments, a random sample of clones ($n_{total} = 210$) growing on kanamycin- and rifampicin-containing plates were transferred to kanamycin-, tetracycline-, ampicillin- and rifampicin-containing plates to further confirm that the formed multiresistant clones harbour a plasmid (i.e. controlling the frequency of spontaneous antibiotic-resistant mutants). In addition, final phage densities were determined at the end of the experiment by plating diluted samples (on 1% LB agar plates with a 0.7% soft agar overlay) from phage-containing treatments with the ancestral form of JE2571(RP4) bacteria.

Conjugation assay

To study the ability of evolved multiresistant bacteria to further transfer their resistance-conferring plasmid through conjugation, random individual bacterial clones (both from phage-containing and from phage-free treatments) were transferred from the kanamycin–rifampicin plates to 5 mL of fresh LB medium and then grown overnight at 37°C and 200 rpm. Similar culture was made of strain JM109(pSU19). Next day, the cultures of the multiresistant clones were mixed in 1:1 ratio with JM109(pSU19), and fresh LB medium was added (12.5% of the combined volume of the two bacteria). These cultures were then grown for 24 hours at 37°C without shaking. A sample from each culture was plated on 1% LB agar plate containing chloramphenicol, kanamycin, ampicillin and tetracycline to see whether the RP4 plasmid had transferred itself to JM109(pSU19) and again formed a new multiresistant strain: JM109(pSU19)(RP4). Clones were scored conjugation defective if no colonies formed on chloramphenicol–kanamycin–tetracycline–ampicillin plates. Five randomly selected clones that turned out to be conjugation deficient were further grown in LB medium with JM109(pSU19), now in the presence of chloramphenicol and kanamycin in nonlethal but growth-reducing concentrations (in final concentrations of 0.625 μg/mL and 1.25 μg/mL, respectively (Fig S1C–D)), to see whether the selective pressure posed by the antibiotics would revert the conjugation ability. Five clones that had been capable of conjugation in the first experiment were used as a control. This experiment lasted for 72 hours with the initiation, culture renewing, sampling and plating carried out similarly to the main experiment with the exception of using different antibiotics. The number of potential JM109(pSU19)(RP4) bacteria was measured every day.

Data analysis

The frequencies of multiresistant bacteria in different treatments were calculated by dividing the density of multiresistant bacteria by the total bacterial density. For statistical tests, arcsine transformation was performed on the obtained frequencies, and the transformed frequencies were compared between phage-free and phage-containing treatments using one-way ANOVA. The level of statistical significance was adjusted with Bonferroni correction to control the effects of multiple comparisons.

Results

The presence of the conjugative plasmid–dependent phage PRD1 significantly reduced the formation of multidrug-resistant *E. coli* HMS714(RP4) bacteria by infecting all bac-

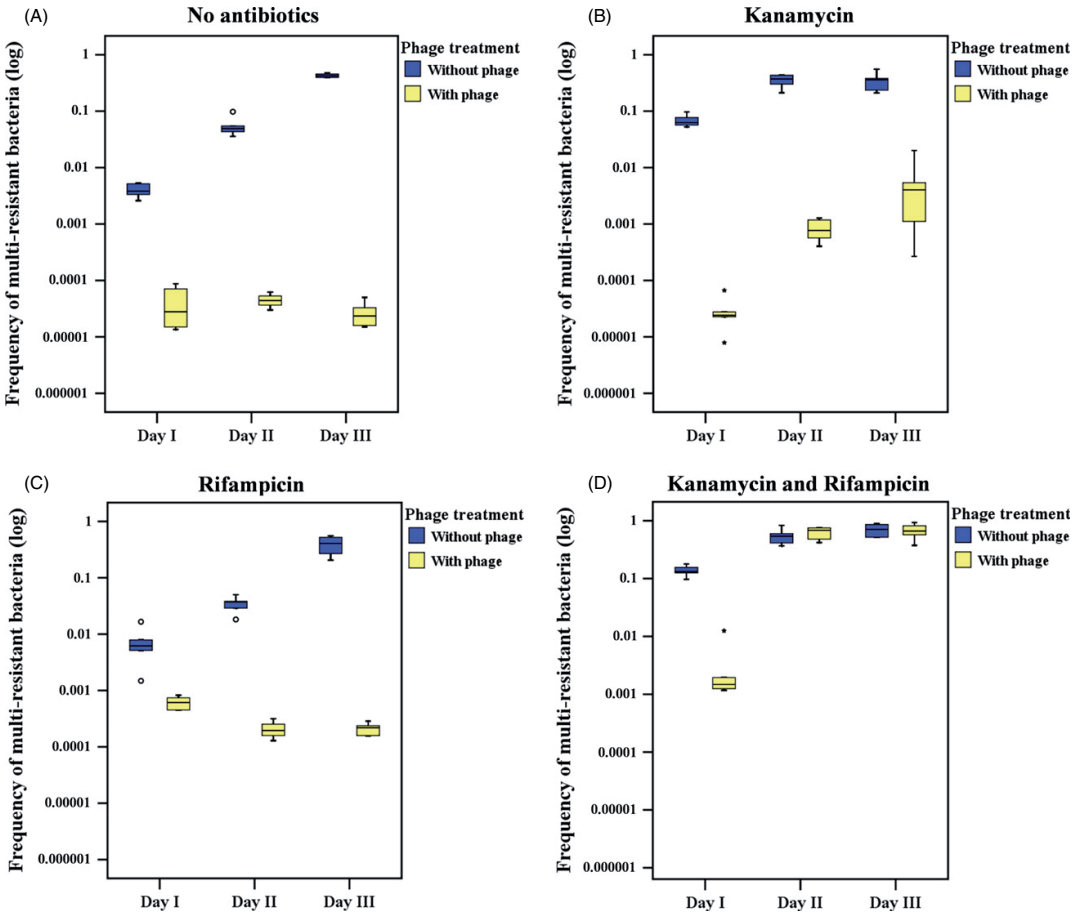

Figure 2 Frequencies of multiresistant (kanamycin + rifampicin) bacteria in the presence and absence of phages. In antibiotic treatments (A) 'no antibiotics', (B) 'kanamycin' and (C) 'rifampicin', there were significantly less multiresistant bacteria in phage-containing treatments throughout the experiment; in treatment (D) 'kanamycin and rifampicin', the difference was significant only after the first experimental day (Table S1).

teria harbouring actively conjugating resistance–conferring plasmids (Fig. 2). In the absence of phages, multiresistant bacteria quickly became common in both antibiotic-free and all antibiotic-containing treatments. The addition of phages resulted in several orders of magnitude lower levels of multiresistant bacteria. In treatments where neither or only one of the two antibiotics (kanamycin or rifampicin) was present in nonlethal but growth-reducing concentration, the phages reduced the prevalence of multiresistance for the entire length of the three-day experiment (Fig. 2A–C). With the double-antibiotic selection, there were still significantly fewer multiresistant bacteria in the phage-containing treatment after the first experimental day, but by the second day, the difference to the phage-free treatment had mostly disappeared (Fig. 2D). Descriptive statistics for the frequencies of multiresistant bacteria in different treatments and the statistical comparisons thereof (one-way ANOVA) are given in Table S1. The addition of phages did not considerably affect the total number of bacteria (Table S2) in any treatment (all cultivations grew to a saturated density of approximately 10^8 colony-forming units per millilitre; cfu/mL), but rather had an effect on the relative numbers of different bacterial types in a population, often selecting against the multiresistant ones. Furthermore, infective PRD1 particles were still abundant at the end of the experiment in all phage-containing treatments (Table S3).

Given that multiresistant bacteria were capable of taking over the double-antibiotic system (Fig. 2D) despite the presence of phages, we decided to further investigate the

properties of these particular bacteria. They may be harbouring RP4 plasmids with mutations in the cell surface complex that PRD1 uses as a receptor and which is also needed for successful conjugation (Kornstein et al. 1992; Kotilainen et al. 1993). The kanamycin- and rifampicin-resistant clones in all experiments were resistant also to ampicillin and tetracycline, confirming that it was the plasmid and not spontaneous resistance that produced the observed resistance pattern. Nevertheless, such mutated plasmids would provide the host bacteria with simultaneous antibiotic and phage resistance but, due to disturbed conjugation machinery, be unable to transfer the plasmid. Following this line of reasoning, we tested whether the multiresistant bacteria emerged under the simultaneous double-antibiotic and phage selection were still capable of conjugation and, indeed, found that only 35% of randomly selected kanamycin–rifampicin-resistant clones ($n = 72$) were capable of transferring the RP4 plasmid to a third chloramphenicol-resistant *E. coli* strain, JM109(pSU19). Moreover, in three of the total five independent selection experiments, the lost conjugation capability did not revert even after 3 days of subsequent cultivation under antibiotic selection that would have favoured the reversion. In two selection experiments, few multiresistant clones of total $\sim 5 \times 10^8$ bacteria appeared, but they remained at very low quantities ($\sim 10^2$) throughout the three-day experiment. This suggests that after phage exposure, a prolonged selective condition would be required for the potential conjugative multiresistant strains to become abundant in the population. In contrast, all multiresistant bacterial clones isolated from the phage-free treatment retained the conjugation ability.

Discussion

Our results demonstrate that conjugative plasmid–dependent bacteriophage PRD1 can significantly reduce the horizontal spread of antibiotic resistance genes in a bacterial community even when the bacteria are exposed to antibiotic selection that should favour the evolution of multidrug-resistant strains via conjugation. The addition of conjugative plasmid–dependent phages to any of the antibiotic treatments acted as counter-selection against the spread of multiresistance commonly reducing it by several orders of magnitude. Only the selection specifically for the formation of HMS174(RP4) transconjugants coupled with 48 hours of evolution was a strong enough selective pressure to cancel the differences between the phage-containing and phage-free treatments. However, most bacteria in this phage-containing treatment had also lost their conjugation ability, whereas all bacteria in the phage-free treatment were still capable of conjugation.

It is known that conjugative plasmids can regulate their rate of transfer in several ways (Gasson and Willetts 1975). More specifically to this study, previous empirical work has shown that the presence of PRD1 can select for plasmid-harbouring bacteria that are phage resistant but conjugation deficient (Kotilainen et al. 1993; Jalasvuori et al. 2011). Theoretical models have suggested that heterogeneity in the rate of transfer is essential for the stable maintenance of conjugative plasmids in bacterial communities when conjugative plasmid–dependent phages are present (Dionisio 2005). From this heterogeneity, it follows that phages may be unlikely to be able to completely eradicate conjugative plasmids from a bacterial community but they can, nevertheless, potentially hinder the further spread of plasmid-mediated antibiotic resistances to other bacterial species that may already possess some other resistances (thus being candidates for new multiresistant agents). In our experiments, the lost conjugation ability of a given multiresistant bacterial clone did not seem to revert easily even when the phage selection was lifted and a three-day antibiotic selection favouring the reversion was added.

Recently Zhang and Buckling (2012) demonstrated that the combined bacteriosidic effect of antibiotic kanamycin and a lytic bacteriophage significantly decreased the rate at which bacteria developed resistance against the antibiotic. Therefore, these studies, along with the present results, suggest that it is reasonable to presume the combination of both plasmid-dependent phages with other lytic phages will induce significant constraints for bacteria to maintain resistances, acquire them horizontally or develop resistances *in situ*. However, Escobar-Páramo et al. (2012) showed that application of antibiotic rifampicin against the host bacteria of a phage decreased the survival of phages in the system and would therefore potentially hinder the efficacy of combined phage and antibiotic treatments. We noticed similar effects when rifampicin alone was used in the system. In these experiments, the phage densities at the end of the three-day serial culture were more than 10-fold lower in comparison with other selection pressures (Table S3). This, nevertheless, is what was expected given that rifampicin selects against the initial plasmid-harbouring bacterium JE2571. Yet, the frequency of multiresistant bacteria in the end of the three-day experiment was relatively high in the presence of rifampicin, suggesting that the lower number of phages eased the selection pressure on the formed HMS174(RP4) transconjugants. However, and in contrast to rifampicin experiments, presence of kanamycin alone or both kanamycin and rifampicin elevated the phage densities above those of antibiotic-free experiments. This was also as predicted as kanamycin selects for the plasmid and thus the hosts of phage PRD1.

The concept of preventing the horizontal transfer of antibiotic resistance genes has been explored by a handful of

earlier *in vitro* studies that have successfully used different nonphage molecules, phage coat proteins or replicative nonlytic and lytic conjugative plasmid–dependent phages to interfere with the bacterial conjugation (Novotny et al. 1968; Ou 1973; Fernandez-Lopez et al. 2005; Garcillán-Barcia et al. 2007; Lujan et al. 2007; Jalasvuori et al. 2011; Lin et al. 2011). Our study, to our knowledge, is the first one to demonstrate that lytic conjugative plasmid–dependent phages can, in principle, be effective selective agents against conjugative elements and thus the spread of drug resistances even when the bacteria are under sublethal antibiotic selection favouring the horizontal spread of resistance. Such growth-reducing concentrations of antibiotics have been thought to generate new multiresistant strains (Andersson and Hughes 2012). There are, however, important caveats to keep in mind when assessing these promising results. For example, it is unclear whether the evolutionary trajectories observed in this one particular experimental system are also common in other systems with different sets of bacteria, antibiotics, conjugative plasmids and conjugative plasmid–dependent phages. Also more generally, the relevance of results of *in vitro* experiments to the situation in natural environments is always uncertain.

As it currently seems inevitable that the development of new antibiotics will not be able to keep up with the worldwide emergence of resistance in pathogenic bacteria, it is increasingly important that we come up with alternative and complementary methods of treatment. Phage therapy has traditionally been overlooked by the Western medicine, whereas in Eastern Europe and Soviet Union, it was extensively studied and applied, although not always accordingly to the standards and rigour expected in Western science (Alisky et al. 1998; Chanishvili 2012). The reluctance in the West has largely been due to various technical, financial and safety challenges associated with developing and applying phage therapy. However, the worsening resistance epidemic has led to a revived interest in looking into phages as potential antibacterial agents (Lu and Koeris 2011). We suggest that, along with direct attempts to eliminate pathogenic bacteria via phages, the use of conjugative plasmid–dependent viruses could be one interesting avenue to explore. Characteristics of PRD1-like viruses are particularly promising for the development of phage applications. While phages are usually very host specific infecting only some strains of a given species, PRD1 has an extremely wide host range for a phage and it can exploit plasmids from various incompatibility groups (Olsen et al. 1974). PRD1 can also be produced easily in sufficient quantities (Mesquita et al. 2010) and stored stably over long times (Ackermann et al. 2004). Therefore, it may be possible to develop a wide host range cocktail of phages recognizing a wide variety of conjugation apparatuses and be thus usable in different contexts where antibiotic resistances cause problems. For instance, in hospitals, antibiotics are often administrated both before and after a surgical operation to reduce the risk of complications caused by bacterial infections. The number of postoperative hospitalization days under antibiotic treatment correlates positively with the probability of the emergence of life-threatening multiresistant infections (Schentag et al. 1998). Given that antibiotic resistances rise via horizontal gene transfer in various bacterial groups, including both opportunistic pathogens such as *Actinobacter baumannii* (Joshi et al. 2003) and common nosocomial pathogens like *Escherichia coli*, *Klebsiella pneumoniae* (Harajly et al. 2010) and *Staphylococcus aureus* (Lyon and Skurray 1987; Chang et al. 2003; Weigel et al. 2003), the presence of plasmid-dependent phages could hypothetically give antibiotics and the immune system more time to clear the infection before the emergence of highly resistant strains and also restrict the spread of resistances within the hospital in general. Yet, while this concept appears promising, future research in actual *in vivo* systems that are inevitably much more complex in all respects is essential to evaluate the real potential of plasmid-dependent phages.

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Data archiving statement

Data deposited in the Dryad repository: doi:10.5061/dryad.4194c.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Bacterial growth curves illustrating the effects of lethal and non-lethal antibiotic selection on bacterial growth.

Table S1. Arcsine transformed mean frequencies (± standard deviation) of multi-resistant (kanamycin and rifampicin) bacteria) in different antibiotic treatments divided into experimental days, and statistical comparisons (1-way ANOVA) between phage-containing and phage-free treatments.

Table S2. Mean total number (± standard deviation) of bacteria (colony-forming units per milliliter; cfu/mL) in different antibiotic treatments divided into experimental days.

Table S3. Mean number (± standard deviation) of PRD1 phage particles (particle-forming units per millilitre; pfu/mL) present in different antibiotic treatments at the end of the experiment.

Supplementary materials

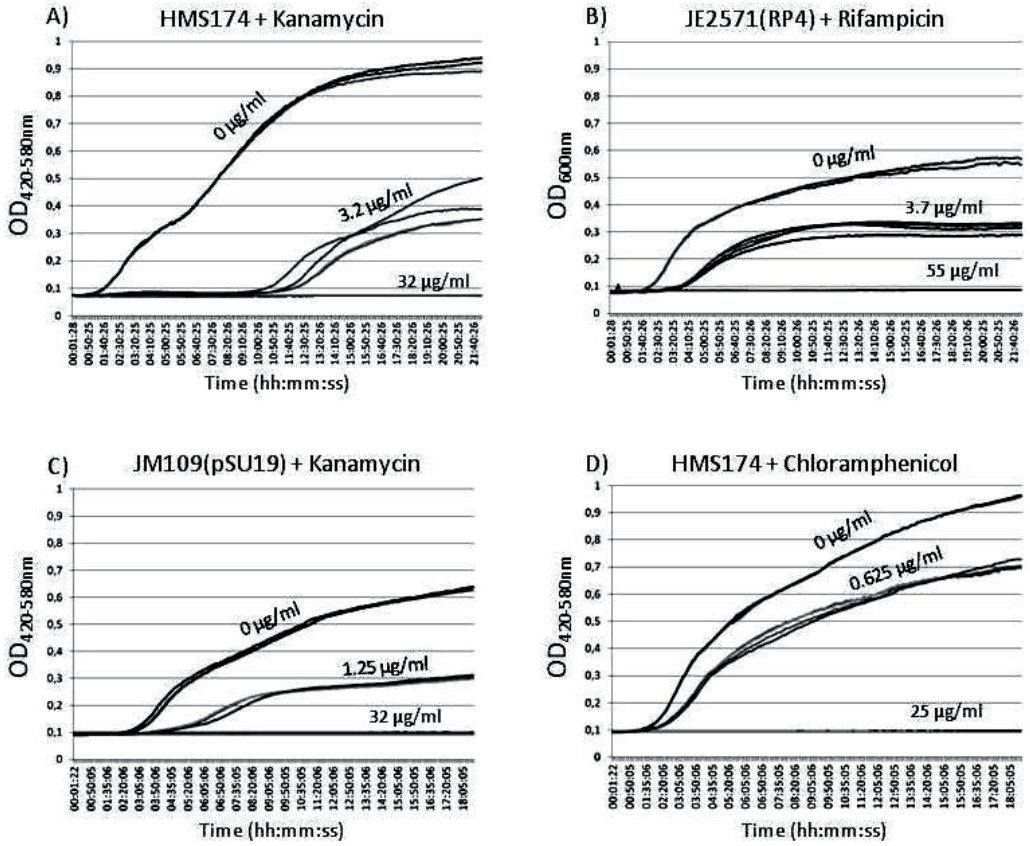

Figure S1 Bacterial growth curves illustrating the effects of lethal and non-lethal antibiotic selection on bacterial growth. 10 µl of overnight cultures (in LB-medium) of E. coli K-12 strains HMS174, JE2571(RP4) and JM109(pSU19) were inoculated into the wells of "Honeycomb 2" plates (Oy Growth Curves Ab Ltd) containing fresh LB-medium and either: 1) no antibiotics, 2) antibiotics in lethal concentration, 3) antibiotics in presumably non-lethal but growth-reducing concentration. Each "bacterium + antibiotic" combination (A-D) had three replicates for treatments 1) and 2), and four replicates for treatment 3). Growth curves were obtained by measuring the optical densities (OD) of the cultures with Bioscreen C spectrophotometer (Oy Growth Curves Ab Ltd). Measurements were done overnight in +37 ºC with no shaking and using either a 420-580 nm wideband filter or a 600 nm brown filter. (A) The effects of lethal (32 µg/ml) and non-lethal (3.2 µg/ml) kanamycin selection on

the growth of HMS174. (B) The effects of lethal (55 μg/ml) and non-lethal (3.7 μg/ml) rifampicin selection on the growth of JE2571(RP4). (C) The effects of lethal (32 μg/ml) and non-lethal (1.25 μg/ml) kanamycin selection on the growth of JM109(pSU19). (D) The effects of lethal (25 μg/ml) and non-lethal (0.625 μg/ml) chloramphenicol selection on the growth of HMS174.

Table S2 Arcsine transformed mean frequencies (±standard deviation) of multi-resistant (kanamycin and rifampicin) bacteria) in different antibiotic treatments divided into experimental days, and statistical comparisons (1-way ANOVA) between phage-containing and phage-free treatments. Each treatment consists of six replicates. Following a Bonferroni correction, the differences between phage-free and phage-containing treatments are considered statistically significant when $p < 0.01667$.

Antibiotic treatment	Day	Phage treatment	Mean±SD (arsin_freq.)	$F_{1, 10}$	*p*-value
No antibiotics	I	Without phage	0.0040±0.0011	84.86	< 0.001
		With phage	0.000040±0.000031		
	II	Without phage	0.055±0.022	37.64	< 0.001
		With phage	0.000045±0.000012		
	III	Without phage	0.44±0.036	896.34	< 0.001
		With phage	0.000027±0.000013		
Kanamycin	I	Without phage	0.068±0.017	99.12	< 0.001
		With phage	0.000029±0.000020		
	II	Without phage	0.37±0.10	80.76	< 0.001
		With phage	0.00083±0.00034		
	III	Without phage	0.36±0.13	41.56	< 0.001
		With phage	0.0058±0.0072		
Rifampicin	I	Without phage	0.0072±0.0051	10.29	0.009
		With phage	0.00060±0.00015		
	II	Without phage	0.0349±0.011	64.10	< 0.001
		With phage	0.00020±0.000070		
	III	Without phage	0.4132±0.16105	39.46	< 0.001
		With phage	0.00021±0.000050		
Kanamycin and Rifampicin	I	Without phage	0.1374±0.028	131.35	< 0.001
		With phage	0.0033±0.0045		
	II	Without phage	0.59±0.21	0.77	0.401
		With phage	0.69±0.19		
	III	Without phage	0.80±0.24	0.05	0.828
		With phage	0.77±0.28		

Table S3 Mean total number (±standard deviation) of bacteria (colony-forming units per milliliter; cfu/ml) in different antibiotic treatments divided into experimental days. Each treatment consists of six replicates.

Antibiotic treatment	Day	Phage treatment	Mean±SD (cfu/ml)
No antibiotics	I	Without phage	3.77e8±8.73e7
		With phage	2.82e8±9.50e7
	II	Without phage	6.30e8±1.50e8
		With phage	5.98e8±6.65e7
	III	Without phage	4.42e8±1.21e8
		With phage	5.18e8±1.21e8
Kanamycin	I	Without phage	4.00e8±5.18e7
		With phage	2.92e8±6.02e7
	II	Without phage	6.47e8±1.94e8
		With phage	2.47e8±6.86e7
	III	Without phage	2.62e8±5.74e7
		With phage	1.40e8±6.75e7
Rifampicin	I	Without phage	4.13e8±7.28e7
		With phage	3.78e8±5.78e7
	II	Without phage	5.85e8±1.33e8
		With phage	5.07e8±8.26e7
	III	Without phage	3.42e8±1.25e8
		With phage	4.53e8±9.37e7
Kanamycin and Rifampicin	I	Without phage	3.03e8±4.03e7
		With phage	3.10e8±7.56e7
	II	Without phage	6.02e8±1.30e8
		With phage	3.18e8±1.14e8
	III	Without phage	5.80e8±5.83e7
		With phage	5.10e8±1.39e8

Table S4 Mean number (±standard deviation) of PRD1 phage particles (particle-forming units per millilitre; pfu/ml) present in different antibiotic treatments at the end of the experiment. Each treatment consists of six replicates.

Antibiotic treatment	Mean±SD (pfu/ml)
No antibiotics	7.08e7±2.82e7
Kanamycin	2.16e9±1.57e9
Rifampicin	2.83e7±7.53e6
Kanamycin and Rifampicin	2.23e8±2.10e8

III

SCOPING THE EFFECTIVENESS AND EVOLUTIONARY OBSTACLES IN UTILIZING PLASMID-DEPENDENT PHAGES TO FIGHT ANTIBIOTIC RESISTANCE

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