JYU DISSERTATIONS 107

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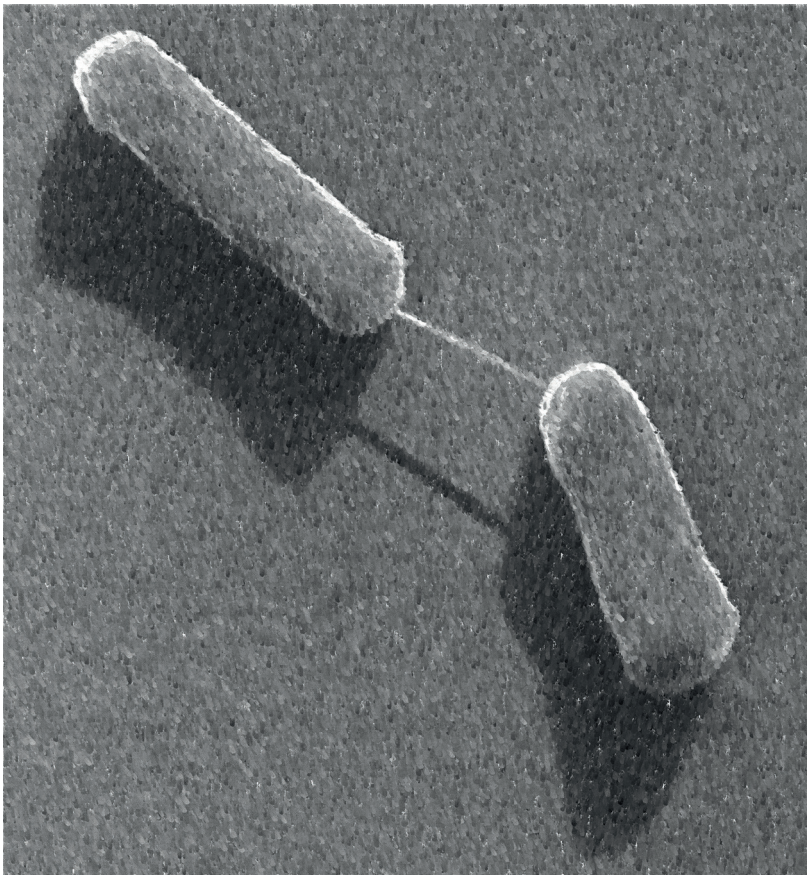
Risks during Antibiotic Treatment and Potential Solutions to Cure Carriage



UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

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Extended-Spectrum β-Lactamase-Producing *Enterobacteriaceae*

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
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Cover picture: Image of two bacterial cells connected with conjugation channel.
Image: Pilvi Ruotsalainen

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ABSTRACT

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Extended-spectrum β-lactamase-producing *Enterobacteriaceae*: risks during antibiotic treatment and potential solutions to cure carriage
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Yhteenveto: Laajakirjoista β-laktamaasia tuottavien enterobakteerien aiheuttamat riskit antibioottihoidon aikana ja uusien menetelmien kehittäminen näiden enterobakteerien kantajuuden poistamiseksi
Diss.

The abundant consumption and negligent use of antibiotics have resulted in the global emergence of antibiotic-resistant bacteria. This is largely due to the rapid spread of multi-resistance plasmids in bacterial communities via conjugation. The increased carriage of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* in the human gut increases the probability of conjugative ESBL plasmids spreading to new bacterial hosts. Therefore, identifying factors that affect the dispersal of plasmids is essential to control their spread. In this thesis, I demonstrate that bacteria-harbouring ESBL plasmids can evolutionarily rescue antibiotic-susceptible cells in a bacterial community via conjugation even under lethal β-lactam concentrations. Thus, antibiotic-sensitive pathogens may also become resistant after an apparently efficient treatment is initiated. In this thesis, a conjugative clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 plasmid system (i.e., midbiotics) was developed to eradicate sequence-specifically different ESBL-bacteria from bacterial community, such as gut microflora. Several genes can be targeted simultaneously with a single midbiotic plasmid. The dispersal of the midbiotic plasmids results in efficient re-sensitisation of the exposed strains to β-lactams. However, before introducing this system *in vivo*, the following concerns need to be resolved: the dissemination of unwanted genes in the flora, mutations that nullify CRISPR activity, and the spread of the conjugative plasmid without its ESBL-targeting plasmid partner. In addition to midbiotics, lytic phages, which infect and kill resistant bacterial pathogens, may provide a potential option to decrease ESBL carriage. In this thesis, it was demonstrated that phages can be isolated on-demand from environmental reservoirs to carry out personalised phage therapy against *Enterobacteriaceae*, which are frequently associated with ESBL infections.

Keywords: Antibiotic resistance; horizontal gene transfer; conjugative plasmids; bacteriophages; phage therapy; CRISPR-Cas9.

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TIIVISTELMÄ

Ruotsalainen, Pilvi
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Runsas ja huolimaton antibioottien kulutus on johtanut antibioottiresistenttien bakteerikantojen globaaliin kasvuun. Pääosin tämä johtuu moniresistenssi plasmidien nopeasta leviämisestä bakteeriyhteisössä konjugaation avulla. Lisäksi laajakirjoisia β-laktamaaseja (ESBL) tuottavien enterobakteerien yleistyminen ihmisten suoliston normaalifloorassa mahdollistaa konjugatiivisten ESBL-plasmidien leviämisen uusiin bakteeri-isäntiin. Kontrolloidaksemme näiden plasmidien leviämistä, on tärkeää selvittää tekijöitä, jotka vaikuttavat leviämiseen. Tässä väitöskirjassa esitän, kuinka bakteerit voivat evolutiivisesti pelastaa bakteeriyhteisön antibiooteille alttiit bakteerit jopa letaalissa β-laktaami konsentraatiossa konjugoimalla ESBL-plasmidin näihin soluihin. Näin ollen jopa tehokas antibioottihoito ei välttämättä estä antibioottisensitiivisten patogeenien muuntumista resistenteiksi. Lisäksi näytän, kuinka kehittämäni geenisaksia (CRISPR-Cas9) koodaava konjugatiivinen plasmidisysteemi (nim. midbiotic) tuhoaa sekvenssispesifisesti erilaisia ESBL-plasmideja bakteeriyhteisöstä. Yhdellä plasmidilla pystytään kohdentamaan useita eri resistenssigeenejä samanaikaisesti ja lopputuloksena bakteerit muuntuvat jälleen alttiiksi β-laktaameille. Tätä menetelmää voisi hyödyntää esimerkiksi hävittämään resistenssigeenejä suolistofloorasta. Ennen midbioticin käyttöönottoa *in vivo*, menetelmää tulee kehittää niin, että epäsuotuisten geenien leviäminen ja midbiotic-plasmidien erillinen leviäminen estetään. Myös geenisaksien tehokkuutta alentavien mutaatioiden vaikutusta tulee vähentää. Vaihtoehtoisesti ESBL-kantajuuden poistamiseksi voitaisiin käyttää bakteereita infektoivia ja tappavia lyyttisiä faageja. Tässä väitöskirjassa osoitan, että tarvittaessa faageja voidaan eristää ympäristöstä ESBL-infektioihin liitetyille enterobakteereille ja valmistaa yksilöllinen faagikoktaili faagiterapiaa varten.

Avainsanat: antibioottiresistenssi; horisontaalinen geenin siirto; konjugatiiviset plasmidit; bakteriofaagit; faagiterapia; CRISPR-Cas9.

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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

I Mattila S., Ruotsalainen P., Ojala V., Tuononen T., Hiltunen T. & Jalasvuori M. 2017. Conjugative ESBL-plasmids differ in their potential to rescue susceptible bacteria via horizontal gene transfer. *The Journal of Antibiotics.* 70: 805-808.

II Ruotsalainen P., Penttinen R. & Jalasvuori M. 2019. Evolutionary rescue by ESBL plasmid-conjugation in lethal penam and cephem concentrations. *Manuscript.*

III Ruotsalainen P., Penttinen R., Mattila S. & Jalasvuori M. 2019. Midbiotics: Conjugative plasmids for genetic engineering of natural gut flora. *Gut Microbes.* 5:1-11.

IV Mattila S., Ruotsalainen P. & Jalasvuori M. 2015. On-demand isolation of bacteriophages against drug-resistant bacteria for personalized phage therapy. *Frontiers in Microbiology.* 6:1271.

RESPONSIBILITIES OF PILVI RUOTSALAINEN IN THIS THESIS ARTICLES:

I I participiated equally in designing the experiment and performed my equal share of the conjugation experiments with the other authors. I isolated over half of the plasmids from transconjugants and performed the majority of PCR analysis for the transconjugants. I collected the data with SM and wrote the article with the co-authors. This article was part of SM's thesis. In this thesis, I am focusing on the examination of the evolutionary rescue potential of plasmid sets originating from nosocomial *Escherichia coli* and carrying differing ESBL-genes.

II I designed and performed the experiments. MJ and RP assisted with the plating in the conjugation studies. In addition, I collected the data and performed the data and bioinformatic analyses. I prepared all the figures and wrote the article with the co-authors.

III I designed and cloned all the plasmids in this article. For deleting genes from the conjugative plasmid, RP4, I developed a cloning method which combined deletion-PCR and homologous recombination. Also, I designed and performed all the experiments; conjugation experiment, colony-forming assay and sequencing. RP assisted with the plating in two colony-

forming assays. I analysed the data. In addition, I prepared majority of the figures and tables in the article and wrote the article with the co-authors.

IV I have participitated equally in the design of the experiments with SM and performed half of the experiments, including phage isolation, phage stock preparation and stock stability testing as well as cross-infection assays. Also, I collected all the data from my own experiments for the analysis and analyzed the results of cross-infection tests. In addition, I made the supplementary tables and the first draft of the cross-infection test figure. This article was part of SM's thesis. Perspective in this thesis is to estimate the probability for isolating phages for personalized prophylactic phage therapy in order to eradicate *Enterbacteriaceae* associated with ESBL-carriage. Therefore, all the data that I have acquired is not presented in this thesis.

The initials refer to MJ= Matti Jalasvuori, RP= Reetta Penttinen and SM=Sari Mattila.

ABBREVIATIONS

Cas9	CRISPR-associated protein 9
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
ESBL	extended-spectrum β-lactamase
HGT	horizontal gene transfer
Inc	incompatibility
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
MGE	mobile genetic element
MRSA	methicillin-resistant *Staphylococcus aureus*
MPF	mating pair formation
oriT	origin-of-transfer
PAM	protospacer adjacent motif
PBP	penicillin-binding protein
TA	toxin-antitoxin
tracrRNA	trans-activating crRNA
T4SS	type IV secretion system

1 INTRODUCTION

Currently, there is concern regarding a global increase in the numbers of multidrug-resistant bacteria. The World Health Organization (WHO) recently stated that this emergence is a substantial threat to modern medicine and society because it substantially limits the options for treating bacterial infections (WHO 2018). A joint report of the European Centre for Disease Prevention and Control (ECDC) and European Medicines Agency (EMEA) published in 2009 states that 25,000 people die annually in Europe due to multi-resistance bacterial infections (ECDC and EMEA 2009). This problem burdens society on many levels, including healthcare, agriculture, and fisheries, as well as the economy. When the increasing prevalence of multi-resistance is combined with the nearly non-existent development of new antibiotics over the past 30 years (Silver 2011), the development of alternative methods that treat bacterial infections is needed.

Before antibiotics were introduced in healthcare in the 1940s, the mortality rate associated with bacterial infections was high; for example, it was was 50 % for tuberculosis (Dineen *et al.* 1976). Therefore, the discovery of the first antibiotic, penicillin, by Alexander Fleming in 1928 (Fleming 1929) was a significant advancement in medicine. Antibiotics lower infection morbidity and mortality and enabled the development of other medical treatments, such as organ transplantation and aggressive cancer therapies (Laxminarayan *et al.* 2013). These treatments weaken the immune system; hence, antibiotics are needed to prevent infections. Unfortunately, resistance emerged soon after the first administration of antibiotics in hospital care. Penicillin was introduced in 1940, and the first resistant strains of *Staphylococcus aureus* were isolated in 1942 (Kirby 1944).

Most antibiotics were originally metabolites of soil microbes, primarily actinomycetes and fungi (Demain 1998). Their primary purposes vary from being pigments to growth promoters, but they also function as toxins and effectors in ecological competition for resources in the community (Demain 1998). These metabolites, as well as their resistance genes, are ancient and, therefore, developed before the clinical use of antibiotics. The discoveries of genes homologous to antibiotic resistance genes from pristine environments, such as permafrost and isolated caves, support this hypothesis (D'Costa *et al.* 2006,

Bhullar *et al.* 2012). The evolution of resistance is a natural outcome of selection pressure and provides a competitive growth advantage for the resistant mutants over their susceptible cohorts. The tolerance for therapeutic levels of antibiotics is simply a fortunate by-product of their indigenous function, providing intrinsic resistance to its original host against these antimicrobials (Macinga and Rather 1999). The mobilisation of these genes through horizontal gene exchange enabled human pathogens to acquire resistance against therapeutic antimicrobials (Martínez 2008). In addition to horizontal gene transfer (HGT), bacteria can acquire resistance through chromosomal *de novo* mutations (Davies and Davies 2010). Nevertheless, many of the clinically-relevant antibiotic resistance genes are mainly localised in various mobile genetic elements (MGEs), such as conjugative plasmids, transposons, and integrons, which can sometimes spread even between distantly-related bacterial species (van Hoek *et al.* 2011). The ability of conjugative plasmids to encode several resistance genes has resulted in the emergence of multi-resistant bacteria that can tolerate a wide spectrum of antibiotics. Currently, they are the primary cause of multi-resistance genes' dispersal, such as extended-spectrum β-lactamases (ESBLs) within *Enterobacteriaceae* pathogens (Carattoli 2009, Iredell *et al.* 2016), and only a few plasmid types account for a notable portion of both nosocomial- and community-acquired multi-resistant infections (Boyd *et al.* 2004, Lavollay *et al.* 2006, Chowdhury *et al.* 2011).

Reports by the WHO (2014) and ECDC (2017) have both stated that the major concerns are especially the clinically-relevant gram-negative bacteria from the *Enterobacteriaceae* family (*Escherichia coli* and *Klebsiella pneumoniae*), as well as *Acinetobacter* species (e.g., *Acinetobacter baumannii*) and *Pseudomonas aeruginosa*. These species often encode resistance genes against third-generation cephalosporins, fluoroquinolones, and aminoglycosides, and recently, they have acquired resistance to carbapenems, the last-resort β-lactam antibiotic used to treat multi-resistant bacteria (ECDC 2017). Considering *Enterobacteriaceae*, the trend of increasing resistance against third-generation cephalosporins are seen in *E. coli* isolates in Europe (ECDC 2017). Combined resistance against third-generation cephalosporins, fluoroquinolones, and aminoglycosides has increased in *K. pneumoniae* isolates. Elevated resistance against carbapenems has also been detected (ECDC 2017). Altogether, more than half of *E. coli* (58.2 %) and more than a third of *K. pneumoniae* (34.1 %) isolates have been found to be resistant to at least one of the following surveilled antibiotics: aminopenicillin, fluoroquinolones, third-generation cephalosporins, and aminoglycosides (ECDC 2017).

A special concern is the global spread of ESBL-producing *Enterobacteriaceae* (WHO 2014, ECDC 2017). These opportunistic pathogens, which encode ESBL, have been isolated from farms, wilderness, and food, as well as in companion animals and from healthy humans (Mesa *et al.* 2006, Guenther *et al.* 2011, Wieler *et al.* 2011). ESBL-producing bacteria cause both hospital- and community-acquired infections. *K. pneumoniae* spreads rapidly in healthcare settings between patients and medical-care professionals, sometimes causing hospital outbreaks (ECDC 2017). It has been associated with nosocomial bloodstream (Marra *et al.*

2006), skin, and lung infections (ECDC 2017), as well as both nosocomial- and community-acquired intra-abdominal infections (Hawser *et al.* 2010). Previously, ESBL infections were predominantly hospital-acquired (Chong *et al.* 2011). However, the increased prevalence of ESBL carriers, referring to the asymptomatic carriage of ESBL-producing bacteria in the gut of a healthy individual, has led to an increasing number of community-acquired infections (Valverde *et al.* 2004, Oteo *et al.* 2010, Woerther *et al.* 2010). Currently, the main cause of community-acquired infections is ESBL-producing *E. coli* (Tenaillon *et al.* 2010, Woerther *et al.* 2013). Furthermore, community-acquired ESBL-infections have been shown to increase the colonisation of ESBL-producing pathogens in the gastrointestinal tract of family members who live in the same household (Valverde *et al.* 2008, Haverkate *et al.* 2017). ESBL carriage is linked to an increased risk of *Enterobacteriaceae* infections in patients (Goulenok *et al.* 2013, Biehl *et al.* 2016), and bacteremia caused by *Enterobacteriacecae* is further associated with increased mortality and delays in the effective treatment of bacterial infection (Schwaber and Carmeli 2007). Altogether, the resistant and pathogenic *E. coli,* which expresses various virulence and adhesion factors, causes intestinal and extra-intestinal infections in humans in community and hospital settings. These include gastrointestinal (Seiffert *et al.* 2013), urinary tract, abdominal, and bloodstream infections (Fluit *et al.* 2001, Chen, Y. *et al.* 2011, Lu *et al.* 2012).

The major cause of this largely self-inflicted antibiotic crisis is the imprudent anthropogenic overuse of antibiotics in healthcare, agriculture, animal husbandry, and fishery settings. It has been estimated that already by 2002, more than one million tonnes of antibiotics were consumed since their clinical introduction in 1940 (Wise 2002). Unfortunately, antibiotic consumption is increasing globally, and between 2000 and 2015, it increased by 65 % (Klein *et al.* 2018). In developing countries, the combination of poor hygiene, the high consumption and prescription-free use of antibiotics, and inadequate access to healthcare has led to unnecessary antibiotic consumption and, possibly, with the incomplete execution of the entire antimicrobial course, an increase in the risk of resistance emergence (Morgan *et al.* 2011, Ayukekbong *et al.* 2017, Klein *et al.* 2018). Furthermore, substantial amounts of antibiotics are administered to livestock. For example, approximately 80 % of the annual consumption of antibiotics in the United States (US) is used for animal husbandry purposes (Hollis and Ahmed 2013). This abundant consumption of antibiotics has led to the accumulation of resistance elements in the gut microflora of both humans and animals.

Human commensal microflora harbour up to 10^{12} bacteria with a great diversity of symbionts, as well as commensal and opportunistic pathogens (Sekirov *et al.* 2010). The gut microflora of healthy and hospitalised individuals serves as a reservoir of resistance genes, a gut resistome (Jernberg *et al.* 2007, Sommer *et al.* 2009, Forslund *et al.* 2013, Hu *et al.* 2013, Pérez-Cobas *et al.* 2013). Studies of neonatal gut microbiomes show that the gut resistome begins to form during the neonatal period, even in the absence of antibiotics exposure (Moore *et al.* 2013, Fouhy *et al.* 2014). However, according to metagenomic studies, there are

geographical differences in the abundance of resistance genes in gut microflora, which is linked to the level of antibiotic consumption in the individual's respective country of residence (Forslund *et al.* 2013, Hu *et al.* 2013). The high level of antibiotic consumption is correlated with the greater abundance of resistant genes (Forslund *et al.* 2013, Hu *et al.* 2013). In addition to human gut flora, the commensal flora of livestock, companion animals, and wild animals act as a reservoir of resistance elements (Costa *et al.* 2008, Poeta *et al.* 2009). One of the reasons for the gut resistome's emergence is the accumulation of sublethal antibiotic concentrations in the gut. It enhances mutagenesis in the bacterial community by inducing the SOS response (Cirz *et al.* 2005, Baharoglu and Mazel 2011, Thi *et al.* 2011) and increasing the accumulation of reactive oxygen species (Kohanski *et al.* 2007, Kohanski *et al.* 2010a). Thus, the frequency of protein synthesis errors increases (Ren *et al.* 1999, Balashov and Humayun 2002), eventually leading to the gradual accumulation of mutations and the emergence of resistance, sometimes even cross-resistance to other antibiotics (Kohanski *et al.* 2010a, Wistrand-Yuen *et al.* 2018). The gradual accumulation of mutations and ESBL-gene amplification in commensal flora has been noted to occur during the antibiotic treatment of human bacterial infections (Fantin *et al.* 2009, Meletiadis *et al.* 2017). Sublethal concentrations also induce recombination in bacteria, further expediting the evolution of resistance (López *et al.* 2007). The abundant misuse of antibiotics as growth promoters and in disease prevention in animal husbandry has also increased the frequency of resistant bacteria in the gut flora of many food-producing animals, such as chickens and swine (Witte 1998, Aarestrup 1999). Therefore, food-producing animals are considered a source of gram-negative resistant bacteria (Marshall and Levy 2011), and the use of the same antibiotics in both animal husbandry and human medicine is concerning as the risk of bacterial cross-resistance increases (Hammerum *et al.* 2010, Shryock and Richwine 2010).

The gut resistome not only acts as a reservoir of resistance genes but also as a platform for the dissemination of both resistant pathogens and mobile resistance elements (Casals-Pascual *et al.* 2016). Studies have shown that the transfer of conjugative resistance elements occurs with and without antibiotic selection in commensal microflora (Bidet *et al.* 2005, Karami *et al.* 2007, Gumpert *et al.* 2017). However, these studies were performed on neonatal gut microflora, so the results might differ when performed on stabilised and fully-formed adult gut microflora. Sublethal antibiotic concentrations, as well as close ecological proximity, are known to accelerate the rate of HGT of MGEs (Beaber *et al.* 2004, Smillie *et al.* 2011, Headd and Bradford 2018). From livestock, the transmission of pathogens and resistance elements to humans can occur through several routes, including direct contact with livestock and the ingestion of contaminated food (Lipsitch *et al.* 2002). Another setting where resistant pathogens or MGEs are transferred into human gut flora is hospitals, where transmission occurs between patients and healthcare professionals (Struelens 1998). Long-term hospitilisation, stays in the intensive-care-unit of a hematology or oncology ward, and antibiotic consumption increase the risk of ESBL-producing bacteria colonising in commensal microflora (Han *et al.* 2012, Goulenok *et al.* 2013, Biehl *et al.* 2016). The

colonisation period lasts approximately six months or slightly longer but varies significantly between individuals (Birgand *et al.* 2013). Humans can also become ESBL carriers while travelling, and travellers can spread these elements around the globe (Pitout *et al.* 2009, Tängdén *et al.* 2010, Dhanji *et al.* 2011). For example, high acquisition rates of ESBL-resistance elements were observed after travel to India, Asia, and the Middle East (Tham *et al.* 2010, Tängdén *et al.* 2010). Antibiotic treatment also assists the colonisation of resistant pathogens into the gut by causing taxonomic changes in the gut microflora, leading dysbiosis, an imbalance of bacterial genera in the gut's natural microflora. Dysbiosis lowers colonisation resistance, the joint defence of the indirect activation of mucosal-innate immune and direct resource competition among microflora (Lawley and Walker 2013, Pamer 2016). These factors expose microflora to the colonisation of antibiotic-resistant mutants and gastrointestinal infections (Francino 2015, Becattini *et al.* 2016). From the gut flora of animals and humans, the resistance elements continue their dispersal into wastewater treatment plants (Rizzo *et al.* 2013) and aquatic and terrestrial environments (Pal *et al.* 2016), encompassing the dissemination cycle. The accumulation of sublethal antibiotic concentrations in various environmental sites accelerates mutagenesis and HGT in a similar manner as in the gut (Andersson and Hughes 2012, Forsberg *et al.* 2012). From the environment, pathogens carrying resistance elements are disseminated to animals and humans. Unsurprisingly, resistant pathogens also disseminate via food-borne outbreaks through contaminated meat, berries, and vegetables (Rolain 2013).

Global and local actions are needed to control the multi-facet problem of antibiotic resistance and lower the overall consumption of antibiotics. Regulation and new antibiotic prescription practices in human healthcare and agriculture and improved diagnostics could prevent the unnecessary use of antibiotics, for example, in treating viral infections (Smith and Coast 2002). The European Union (EU) banned the nonmedical use of antibiotics in livestock in 2006, and the US has restricted antibiotic use in animal husbandry (Chang *et al.* 2015). However, once antibiotic resistance gene-encoding plasmids are acquired, limiting antibiotic consumption does not necessarily abolish the resistance genes (Yates *et al.* 2006, Brolund *et al.* 2010, Sundqvist *et al.* 2010) as the replacement of resistant individuals with sensitive ones may take a long time (De Gelder *et al.* 2004). If an antibiotic is introduced into the environment again, even a small population of resistant mutants can regain prominence over the habitat (Levin *et al.* 1997, Austin *et al.* 1999, Heinemann *et al.* 2000). Decreased susceptibility to antibiotics has been observed even in the absence of antibiotics when bacteria naturally adapt to a changing environment (Knöppel *et al.* 2017). However, because farms and hospitals are hot spots for the emergence and transmission of multi-resistance, new safety and hygiene protocols are needed (Smith and Coast 2002). Routine surveillance is also essential; the characterisation of multi-resistant bacteria should be consistent, containing identification of both the plasmid type and the resistance genes, so more reliable interpretations can be made from epidemiological data (Seiffert *et al.* 2013). EpicPCR with next-generation sequencing would be an efficient tool for detecting and connecting multi-

resistance genes into specific bacterial species (Spencer *et al.* 2016, Karkman *et al.* 2018). The resistome of heterogeneous bacterial communities could be monitored using this method. Promisingly, alternatives to antibiotics (e.g., phages, vaccination, and probiotics), biosecurity, and overall intensive hygiene practices have been shown to effectively reduce the prevalance of and infections by multi-resistant bacteria in livestock (Doyle and Erickson 2006, Doyle and Erickson 2012).

In this thesis, I explore the evolutionary rescue potential of several nosocomial ESBL-encoding plasmids via conjugation under lethal antibiotic concentrations (I and II). In addition, I investigate the ability of conjugative CRISPR-Cas9 plasmid system to eradicate these resistance genes from the bacterial population (III), and the success of isolating new bacteriophages against common ESBL-bacteria (IV).

1.1 The bacterial target of β-lactam and resistance mechanisms against it

Antibiotics are antimicrobial molecules that either inhibit the proliferation of bacteria (i.e., bacteriostatic) or kill them (i.e., bacteriocidic). The target spectrum of antibiotics, which ranges from narrow to broad, is defined by the range of pathogens they are effective against. They can be natural products of soil microbes, such as penicillin, or semisynthetic derivates of natural antimicrobials, such as the second- and third-generation β-lactams, or fully-synthetic, like quinolones (Walsh 2000). Antibiotics primarily hinder the essential and conserved metabolic pathways of prokaryotes, such as cell membrane maintenance, as well as protein and DNA synthesis (Walsh 2000). β-lactams are the most clinically-relevant antibiotics due to their milder side-effects compared to many other antimicrobials and their ability to penetrate many tissues in different parts of the body (Seiffert *et al.* 2013). They are bacteriocidic and prevent the synthesis of the cell wall by disturbing the normal cross-linking of the important component of a cell wall, peptidoglycan (Walsh 2000). Except for vancomycin, all the other β-lactams bind and acylate the active site of the transpeptidase domain of penicillin-binding proteins (PBPs) (Kohanski *et al.* 2010b). Without a cross-linking peptidoglycan, the mechanical strength of a cell wall weakens, eventually leading to cell lysis as the osmotic pressure increases (Walsh 2000).

Prokaryotes protect themselves from antibiotics via various resistance mechanisms. In many cases, the development of clinically-relevant resistance is not a straightforward or simple event, but a complex adaptation process involving a combination of different resistance mechanisms (Hughes and Andersson 2017). Bacterial resistance to antibiotics can be categorised into three main types: intrinsic, acquired, and adaptive. Intrinsic resistance is an inherited phenotypic trait, whereas acquired resistance is obtained through mutations or

gaining foreign DNA via HGT (Fernández and Hancock 2012). With gram-negative bacteria, such as *P. aeruginosa* and *A. baumannii*, high intrinsic resistance is based on the LPS-rich outer membrane (OM) structure's low permeability, in concert with membrane proteins, porins, and efflux pumps (Bonomo and Szabo 2006). Porins, residing in the OM, sieve through hydrophobic antibiotics with a certain size exclusion limit and are assorted into so-called general-porins and porins with a more defined substrate spectrum (Fernández and Hancock 2012). Various efflux pumps also take part by exporting several molecules in an energy-dependent manner (Nikaido and Pagès 2012). The down-regulation of porins and up-regulation efflux through pumps can either prevent the entry of antibiotics or decrease the inhibitory concentration of antibiotics in the cell, respectively (Fernández and Hancock 2012). As a result, the negative effects of various antibiotics are inhibited.

β-lactam resistance is mainly acquired and can be gained via the following mechanisms: inactivating the antimicrobial drug enzymatically or bypassing the target by altering the target via mutations, chemical modifications, or replacing the target (Munita and Arias 2016). The most common resistance mechanism against antibiotics of natural origin, such as penicillin, is the enzymatic inactivation of the drugs (Nikaido 2009). β-lactamase hydrolyses the characteristic β-lactam ring, causing the ring-structure to open, thus inactivating the antibiotic, either in the periplasmic space in gram-negative bacteria or outside the cell in gram-positive ones (Munita and Arias 2016). These enzymes are encoded from genes located either in the chromosome or various MGEs, such as plasmids, transposons, or integrons, and their expression is either constitutive or induced by the introduction of β-lactams (Munita and Arias 2016). Resistance via bypassing the target can be achieved by altering the chemical structure of the target; for example, vancomycin-resistant *Enterococci* modify the peptidoglycan precursor (Courvalin 2006). In addition to modifying the target, it can be replaced by acquiring a less susceptible target protein via HGT, such as *mecA,* that encodes a low-affinity PBP2A, which provides resistance to all β-lactams in methicillin-resistant *Staphylococcus aureus* (MRSA) (Chambers 1997, Wielders *et al.* 2002). Alternatively, bacteria can acquire mosaic genes through recombination, parts of which originate from other bacteria, for example, penicillin-resistant *Streptococcus pneumoniae* (Spratt 1994).

In addition to intrinsic resistance, porins and efflux pumps participate in constructing acquired resistance. For antibiotics to be effective, they need to reach their target and an effective concentration level within a reasonable period of time (Walsh 2000). Therefore, resistance can be acquired by preventing the drugs from accessing the target or through active efflux without altering the antibiotic itself (Nikaido 2009, Fernández and Hancock 2012). Some pumps can transport several compounds, including β-lactams, and are associated with multi-drug resistance (Piddock 2006). Although multi-drug efflux pumps are generally chromosomally encoded, the antibiotic-specific ones are plasmid-mediated (Poole 2007). Both have been shown to provide wide-spectrum resistance to several antibiotics in both gram-positive and -negative bacteria (Poole 2007, Fernández and Hancock 2012). The mutational changes occur mainly in

regulators by either activating or down-regulating the expression of efflux pumps and, occasionally, in the genes encoding the pump, leading to the increased efflux of antibiotics (Fernández and Hancock 2012). Unfortunately, clinical strains have been noted to have acquired several mutational changes that increase the efflux pumps' efficacy (Tomás *et al.* 2010). For example, the accumulation of mutations that affect the function of porins and decrease their expression are associated with increased resistance against carbapenems in several clinically-relevant *Enterobacteriaceae* (Novais *et al.* 2012, Wozniak *et al.* 2012, Tängdén *et al.* 2013), as well as in *P. aeruginosa* (Tamber and Hancock 2003).

Recently, the role of adaptive resistance has been acknowledged in the acquisition and evolution of antibiotic tolerance (Fernández *et al.* 2011). Adaptive resistance is a nonheritable, transient, non-mutational resistance mechanism against one or more antimicrobial agents that emerges in response to a specific environmental stimulus or results passively from bacterial persistence (Fernández *et al.* 2011). After the removal of a triggering agent, such as an antibiotic, the increased resistance reverts but, in many cases, not to its original level (Mawer and Greenwood 1978). This resistance mechanism might explain the phenomenon of 'baseline creep' wherein the average minimum inhibitory concentration (MIC) of antibiotics steadily increases over time for clinically-important pathogens (Baquero 2001). Eventually, the 'creep' may enable the emergence of greater resistance via HGT or the accumulation of successive low-effect mutations (Baquero 2001). Such evolutionary paths have been identified among clinical isolates of multi-resistant *P. aeruginosa* and *S. aureus* (Fernández *et al.* 2011). A good example of adaptive resistance is chromosomally-encoded *ampC* (Fernández *et al.* 2011). Various gram-negative bacterial species carry this gene, which encodes inducible class C β-lactamase (Fernández *et al.* 2011). In the absence of certain β-lactams, the expression of this gene is low, whereas the presence of β-lactams increases the production of β-lactamase enzymes (Lindberg and Normark 1986, Livermore 1987), leading to drug inactivation and therapeutic failure (Pai *et al.* 2004).

From the evolutionary ecology perspective, β-lactam resistance is an altruistic resistance mechanism. The β-lactamase-producing bacteria of a bacterial community can provide cooperative resistance by degrading β-lactam antibiotic molecules (Brook 1984, Brook 2004, Perlin *et al.* 2009, Yurtsev *et al.* 2013), which lowers the antibiotic's concentration and generates local resistance to non-resistant bacteria in the community. Furthermore, via cooperative resistance, susceptible bacteria (i.e., 'cheaters') in the bacterial community can survive long enough to acquire plasmid-mediated β-lactam resistance from resistant bacteria in the surrounding bacterial community (Ojala *et al.* 2014). Thus, a new generation of resistant cells (i.e., novel cooperators) is created (Ojala *et al.* 2014) that can further disseminate β-lactam resistance in the bacterial community. For example, a high frequency of ESBL-producing *Enterobacteriaceae* in the gut microflora (Woerther *et al.* 2013) may disseminate the resistance further to the surrounding bacterial community and compromise antibiotic treatment by rescuing susceptible bacteria in the community (I and II). Therefore, maintaining an environmental antibiotic concentration above the so-called MIC would be

insufficient in preventing the emergence of resistance (Levison and Levison 2009).

1.1.1 The emergence and dissemination of extended-spectrum β-lactamases (ESBLs)

ESBL-producing clinical isolates are continuously being discovered, and they can inactivate extended-spectrum β-lactams: penicillins and first-, second-, and third-generation cephalosporins and monobactams but not cephamycins or carbapenems. They are usually sensitive to β-lactamase inhibitors, such as clavulanic acid and tazobactam (Paterson and Bonomo 2005, Bush and Jacoby 2010), although resistance to β-lactamase inhibitors has emerged (Drawz and Bonomo 2010).

The classification of ESBL enzymes is based on their homology (Ambler *et al.* 1991) or functional similarities, taking into account their substrate spectrum and β-lactam inhibitor sensitivity profile. Functional similarity is a more practical categorisation approach for diagnostics as the possible substrates and effective inhibitors correlate with the phenotype (Bush *et al.* 1995, Bush and Jacoby 2010). Many of the clinically-relevant ESBL enzymes belong to the TEM, SHV, and CTX-M classes (Bush and Jacoby 2010). All the currently known β-lactamase genes (*bla*-genes) of classes *blaTEM* and *blaSHV* are derivates of their ancestors: all *blaTEM* genes spawn from *blaTEM-1* and *blaTEM-2*, and all *blaSHV*s derive from *blaSHV-1* (Paterson and Bonomo 2005). After the introduction of extended-spectrum β-lactams in the early 1980s (Salverda *et al.* 2010), the first ESBL enzyme SHV-2 was discovered within a couple of years in Germany (Knothe *et al.* 1983). TEM-12 and TEM-3 encoding pathogens were isolated in the late 1980s in England and France, respectively (Sirot *et al.* 1987, Sougakoff *et al.* 1988, Du Bois *et al.* 1995). All enzymes were identified in different species of *Klebsiella*. Compared to ancestor genes *blaSHV-1* and *blaTEM-2*, only one nucleotide substitution in *blaSHV-2*, and two nucleotide substitutions in *blaTEM-3,* respectively, were needed for SHV-2 and TEM-3 to acquire an enhanced ability to hydrolyse cefotaxime (Chong *et al.* 2011). A sequence comparison of β-lactamases from clinical-isolates shows that a varying number of different nucleotide substitutions has led to an enlarged active site cavity, which eases the binding of third-generation cephalosporins with bulky oxyimino side-chains (Wang *et al.* 2002). Decreased stability due to an enlarged binding site is compensated by mutations further away from the binding site, ensuring the stable function of the enzyme (Wang *et al.* 2002). Some of these mutations have slightly, but not completely, decreased enzyme activity against penicillin (Wang *et al.* 2002).

In contrast to *blaTEM* and *blaSHV*, *blaCTX-M* genes are more divergent and only share a 40 % homology with *blaTEM* and *blaSHV* genes (Bonnet 2004, D'Andrea *et al.* 2013). The first *blaCTX-M* was identified in 1989 (Bauernfeind *et al.* 1990) and they presumably originate from chromosomal β-lactamases of *Kluyvera spp*. (Decousser *et al.* 2001, Humeniuk *et al.* 2002, Poirel *et al.* 2002). The high allelic diversification of *blaCTX-M* genes is a result of their evolution

through random mutations and recombination of different *blaCTX-M* genes, especially *blaCTX-M-1* and *blaCTX-M-9* (Novais *et al.* 2010, Cantón *et al.* 2012). Compared to other ESBL gene-types, they have especially high activity against cefotaxime (He *et al.* 2016). The recombination of the most prevalent ESBL types world-wide, *blaCTX-M-14* and *blaCTX-M-15*, has resulted in the emergence of hybrid CTX-M β-lactamases with increased hydrolytic activity against cephalosporins, leading to increased MIC (Nagano *et al.* 2009, Tian *et al.* 2014, He *et al.* 2015, He *et al.* 2016).

Until the end of the 1990s, *K. pneumoniae*-encoding TEM or SHV was the main cause of nosocomial outbreaks of ESBL-producing bacteria (Cantón and Coque 2006). Since then, CTX-M-encoding *E. coli* has become the most wide-spread ESBL-producing gram-negative *Enterobacteriaceae,* which is associated with community-acquired infections (Pitout and Laupland 2008). In addition to being isolated from highly virulent *E. coli* and *K. pneumoniae* pathogens (Naseer and Sundsfjord 2011, Rogers *et al.* 2011, Woodford *et al.* 2011), the global dispersal of CTX-M enzymes has enabled their spread into other clinically-relevant pathogens, such as *Salmonella* and *P. aeruginosa* (al Naiemi *et al.* 2006, Cantón *et al.* 2012). Although CTX-M is widespread, nosocomial infections caused by TEM and SHV-producing bacteria are still identified in hospitals worldwide, especially in Europe (Cantón *et al.* 2008, Valverde *et al.* 2008).

All the known *blaCTX-M* genes are localised in insertion sequences or transposons, which enables their mobilisation (Poirel *et al.* 2005, Novais *et al.* 2006). They are carried by both narrow- and broad-host-range plasmids (Cantón and Coque 2006, Carattoli 2009). The localisation of *blaCTX-M-15* in *Tn2* transposon, carried by incompatibility group (Inc) FII plasmid, is assumed to partly explain the pandemic spread (Branger *et al.* 2018). The adaptation of IncFII plasmid into *Enterobacteriaceae* is suggested to have happened prior to the use of antibiotics (Datta *et al.* 1980), and, in addition to a plasmid-encoded addiction system (Mnif *et al.* 2010), could explain the persistence of this plasmid in *Enterobacteriaceae*. Other plasmid types associated with the global dissemination of ESBL genes in *Enterobacteriaceae* are from the IncA/C, IncL/M, IncN, and IncI1 groups (Carattoli 2011). Plasmid types IncFI, IncI2, IncHI2, and IncK are represented in isolates detected both in humans and food animals (Seiffert *et al.* 2013). In contrast to globally-dispersed CTX-M-15 and CTX-M-14, other CTX-M types show a geographical distribution in different areas (Cantón and Coque 2006, Suzuki *et al.* 2009, Chong *et al.* 2011).

Further, co-selection by several antibiotics enhances the persistence of the multi-resistance plasmids as many ESBL-producing bacteria are co-resistant to aminoglycosides, tetracyclines, and sulphonamides, and some *blaCTX-M*-encoding (CTX-M-9, CTX-M-14 or CTX-M-15) are even resistant to fluoroquinoloes (Cantón and Coque 2006). This co-resistance reduces the antibiotic options for treating multi-resistant infections (Paterson *et al.* 2001, Giamarellou and Poulakou 2009). Consequently, carbapenems are used increasingly more often, which has caused the emergence of carbapenem resistance (Nordmann *et al.* 2009, Perez *et al.* 2010) that can co-exist in the same plasmid with CTX-M (Miró *et al.* 2010, Chen *et al.* 2011). This has led to the

increased use of polymyxin antibiotics, such as colistin, which is the last-resort antibiotic for carbapenem-resistant infections (Trimble *et al.* 2016). The first colistin resistance gene (*mrc-1*) was identified in 2015, and since then, it has been observed to have already spread globally (McGann *et al.* 2016, Ye *et al.* 2016). It has been discovered in plasmids with ESBL genes ($bla_{CTX\text{-}M}$), carbapenem resistance genes (bla_{KPC}), and the New Delhi metallo-β-lactamase gene (bla_{NDM}) (Falgenhauer *et al.* 2016, Haenni *et al.* 2016, Yao *et al.* 2016).

Clonal dispersion is an interesting characteristic of the spread of CTX-M, which is linked to the highly virulent *E. coli* strain ST131 (Coque *et al.* 2008, Woodford *et al.* 2011). This strain might be transmitted even to family members from colonised or infected patients and usually carries an IncFII plasmid that encodes two widely spread CTX-M types, CTX-M-14 or CTX-M-15 (Cao *et al.* 2011, Woodford *et al.* 2011, Cantón *et al.* 2012). CTX-M-15-producing ST131 *E. coli* cause community-acquired ESBL infections, primarily urinary tract infections and bacteremia (Ben-Ami *et al.* 2006, Pitout *et al.* 2009), in addition to hospital-acquired infections (Peirano and Pitout 2010). Other virulent extraintestinal *E. coli* strains also spread CTX-M (Cantón *et al.* 2012). Their dissemination in *K. pneumoniae* is similarly linked to certain clones on different continents (Damjanova *et al.* 2008, Lee *et al.* 2011, Nielsen *et al.* 2011).

In addition to humans, CTX-M enzymes are found in food and companion animals, as well as in wild animals (Bonnedahl *et al.* 2010, Literak *et al.* 2010, Silva *et al.* 2011, Gonçalves *et al.* 2012) and in the environment, such as in rivers (Chen *et al.* 2010, Dhanji *et al.* 2011). In all food-producing animals, CTX-M-1 is the most prevalent ESBL enzyme among *E. coli* isolates (Seiffert *et al.* 2013). Other ESBL enzymes associated with *E. coli* from livestock are CTX-M-9, -14, -15, -32, and -55, followed by SHV-12 and TEM-52 (Seiffert *et al.* 2013). However, CTX-M-15 is encountered in food-producing animals more rarely than in humans (Seiffert *et al.* 2013). The risk of acquiring ESBL-producing *Enterobacteriaceae* was 5.8–33 % higher in professions (i.e., farmers and veterinarians) where direct contact with food-producing animals is common (Geser *et al.* 2012, Dierikx *et al.* 2013). The food-borne ESBL-outbreaks of several types of gram-negative *Enterobacteriaceae* and *Salmonella* in Europe and the US suggest ESBL-producing *Enterobacteriaceae* can be transferred from food-producing animals to humans (Gupta *et al.* 2003, Weill *et al.* 2004, Calbo *et al.* 2011, Leverstein-van Hall *et al.* 2011).

1.2 The dissemination of antibiotic-resistance via horizontal gene transfer

As the name implies, HGT is transfer of genetic material which is independent of cell division and can occur between organisms without parental-offspring relationship (Soucy *et al.* 2015). Through HGT, bacteria can acquire new genes from other bacteria and, hence, enrich their gene pool (Frost *et al.* 2005, Soucy *et al.* 2015). Along with relatively rapid evolution, HGT assists bacteria's adaptation

to fluctuating or hostile environments (Groisman and Ochman 1996). The importance of HGT in the bacterial adaptation was acknowledged when HGT was discovered to play a major role in the global emergence of antibiotic multiresistance (Ochman *et al.* 2000). The emergence of resistance to the same antibiotics in several bacterial lineages indicated that the resistance could not have arisen through *de novo* mutations and vertical heritage (Ochman *et al.* 2000). In addition to antibiotic resistance, HGT has also been shown to increase the cooperation and virulence of bacteria (Nogueira *et al.* 2009).

Genetic elements can disseminate by HGT via (at least) three pathways: transformation, transduction, and conjugation. In transformation, competent bacteria can engulf naked DNA originating from dead cells or viruses in their environment (Chen and Dubnau 2004). Transformation is the form of HGT that can happen between the most distant bacterial species, and even between other domains of life (Ochman *et al.* 2000). Phages can also accidentally acquire part of a host's genome or plasmid, sometimes containing an antibiotic resistance gene, add it to their genome, and encapsulate it into capsids (Haaber *et al.* 2016). The phage's progeny can then disseminate these genes to new bacterial hosts, where the genes are recombined into the host's genome if appropriate homologous sites are available (Torres-Barceló 2018). This type of phage-mediated transfer is called transduction. However, transduction is limited by the host range of phages, which contains only certain bacterial species or strains (Smillie *et al.* 2010). The size of the capsid also limits the amount of transferred genetic material (Ochman *et al.* 2000). Contact-dependent conjugation is considered to have the most significant impact on the dispersal of multi-resistance genes in clinically relevant bacteria (de la Cruz and Davies 2000). In conjugation, various MGEs, such as conjugative transposons or integrative and conjugative elements (ICEs), as well as plasmids, are transferred from one bacterium to another via a mating-pair channel (Smillie *et al.* 2010). Compared to phages, conjugative plasmids play a more substantial role in enriching the diversity of the prokaryotic pangenome because they have a more flexible host range and can transfer more genetic material (Bates *et al.* 1998, Smillie *et al.* 2010).

1.2.1 Conjugative plasmids—molecular vehicles for resistance dissemination

Conjugation, also called bacterial 'sex', is mediated by the plasmid-encoded type IV secretion system (T4SS) (Ilangovan *et al.* 2015) and was first discovered by Tatum and Lederberg in 1946 (Lederberg and Tatum 1953). Plasmids are extrachromosomal, primarily circular DNA, and capable of self-replication within the host cell (Norman et al. 2009). Although non-essential for their host's viability in most environments, plasmids often deliver host-benefiting traits that help the host survive in and adapt to hostile environments, such as those containing antibiotics (Barlow 2009).

Based on their mobility, plasmids can be classified into conjugative, mobilisable, and non-transferrable (Smillie *et al.* 2010). Half of the sequenced plasmids isolated from proteobacteria are non-transferrable and one-fourth of the remaining plasmids are conjugative and the same number are mobilisable

(Smillie *et al.* 2010). Mobilisable plasmids can be transferred via conjugation with the aid of a conjugative helper plasmid, which encodes the conjugative machinery (Smillie *et al.* 2010). Conjugative plasmids are organised into genetic modules formulating the conserved core of genes, or the backbone, and the rapidly changing accessory modules (Norman *et al.* 2009). The functional modules of the backbone of conjugative plasmids take part in replication, maintaining the stability and establishment of the plasmid in the host and transfer via conjugation (Thomas 2000, Norman *et al.* 2009).

The replication of a conjugative plasmid is steadily controlled, usually at the initiation step by either plasmid-encoded activators or initiators that vary depending on the plasmid type (del Solar *et al.* 1998). The plasmid's copy number is often selected to remain low as this minimises the fitness cost to the host (Watve *et al.* 2010). The segregational loss of plasmids during cell division is prevented with efficient plasmid-mediated maintenance systems (Watve *et al.* 2010). When colonising a new host, the concentration of plasmid-mediated replication inhibitors is low, which enables the plasmid to reach a necessary copy number for successful establishment and maintenance (del Solar *et al.* 1998). Stable maintenance requires successful propagation in the new host; therefore, the replication module also determines plasmids' host range (Garcillán-Barcia *et al.* 2011). The host range varies from narrow to broad; for example, IncF plasmids' host range is limited to *Enterobacteriaceae* (Pukall *et al.* 1996), whereas IncP plasmids can replicate in various gram-negative bacteria (Schmidhauser and Helinski 1985, Popowska and Krawczyk-Balska 2013), enabling broad interspecific gene exchange.

Although plasmids are maintained in certain copy numbers, various persistence mechanisms encoded by the stability module assist in the maintenance process. The multimer resolution system prevents multimer plasmids' segregational loss by converting them back to monomers (Summers *et al.* 1993, Hodgman *et al.* 1998), whereas the toxin-antitoxin (TA) system kills the plasmid-free progeny cells by stable toxins (Yang and Walsh 2017). Compared to toxins, antitoxins are labile and, therefore, unable to prevent cell death caused by toxins in the absence of a plasmid encoding the antitoxin gene (Yang and Walsh 2017). The partition system organises plasmid copies in a similar fashion as chromosomes in mitosis so that they can be disseminated evenly in progeny cells during cell division (Baxter and Funnell 2014). Furthermore, various plasmids encode for entry exclusion systems that inhibit the conjugation of similar plasmid types in the host cell (Garcillán-Barcia and de la Cruz 2008). IncF-type plasmids manifest both surface- and entry exclusion; the former prevents the formation of stable mating pairs, whereas the latter inhibits conjugation in a later stage (Garcillán-Barcia and de la Cruz 2008).

The highly variable accessory module encodes for clinically-relevant genes, such as virulence or antibiotic resistance (Norman *et al.* 2009, Garcillán-Barcia *et al.* 2011). Many antibiotic resistance genes are often co-localised into translocative elements, such as integrons and transposons. These further aid the dispersal of resistance genes by transferring them to new plasmids or bacterial chromosomes (Partridge *et al.* 2009). In addition to providing beneficial functions for the whole

bacterial community (as in β-lactam resistance), this module can encode bacteriocins, which are harmful to other bacteria (Rankin *et al.* 2011). Moreover, genes that bestow resistance to heavy metals can lead to the co-selection of multi-resistance plasmids even in the absence of antibiotic selection (Gullberg *et al.* 2014).

The conjugation module enables the mobilisation of resistance plasmids. It contains two transfer modules. The first module encodes for the mobility (MOB) genes required for DNA processing (i.e., origin-of-transfer [*oriT*], relaxase, and type IV coupling proteins), while the second one has T4SS genes needed to construct the mating-pair-formation (MPF) complex, a membrane-expanding channel through which DNA is transferred between bacteria cells (Smillie *et al.* 2010). Mobilisable plasmids often contain genes for DNA processing, except for some that encode type IV coupling proteins (Smillie *et al.* 2010). However, some of them, such as Inc-P plasmid RP4, can mobilise a plasmid containing only an *oriT* site (Pansegrau *et al.* 1988). During conjugation, the *oriT* site of a plasmid is first recognised and processed by the relaxosome, and with the help of coupling proteins, the relaxosome complex recruits the plasmid to an MPF channel (de la Cruz *et al.* 2010). During the process, the relaxosome complex replicates the plasmid so both the donor and the recipient will receive their own copies (de la Cruz *et al.* 2010). In addition to DNA, T4SS can transfer effector proteins and toxins to bacteria and eukaryotes (de Jong *et al.* 2008, Hood *et al.* 2010). A plasmid's stability in the new host is maintained by the establishment module (Garcillán-Barcia *et al.* 2011). This module is part of the leading region, which reaches the new host first. It carries various genes, such as single-strand DNA binding proteins (Golub and Low 1985, Golub and Low 1986) and anti-restriction systems (Delver *et al.* 1991, Read *et al.* 1992) that prevent the plasmid from being destroyed.

Informative classification systems for conjugative plasmids help clarify their evolution and dissemination routes within bacterial communities. The current classification is primarily based on replicon and MOB typing (Carattoli *et al.* 2005, Garcillán-Barcia *et al.* 2011). The classical classification of conjugative plasmids is based on incompatibility groups, determined by the discordance of two plasmids with the same replication modules occupying the same cell (Novick 1987), leading to the eradication of one of the plasmids (Datta and Hedges 1971, Datta and Hughes 1983, Couturier *et al.* 1988). Currently, PCR-based replicon typing (Carattoli *et al.* 2005) is used to identify replicons, although this method is unreliable because, sometimes, only one single point mutation is needed to change the Inc group (Tomizawa and Itoh 1981). The main defect of this method is its inability to identify new replicons and define single replicons for plasmids with mosaic replicons or multiple replication sites (Garcillán-Barcia *et al.* 2011). Plasmid classification based on MOB and MPF genes has accompanied replicon typing to discover phylogenetic relationships and follow the evolution of conjugative plasmids (Smillie *et al.* 2010, Garcillán-Barcia *et al.* 2011). The former method categorises mobilisable and conjugative plasmids into six MOB families (i.e., MOB_C, MOB_F, MOB_H, MOB_P, MOB_Q, and MOB_V) based on the homology of amino acid sequences of relaxase, while the latter classifies the

conjugative plasmid based on the homology of T4SS MPF components into four families (i.e., MPF_F, MPF_G, MPF_I, and MPF_T) (Smillie *et al.* 2010). Comprehending multi-resistance plasmid dispersal is essential to not only controlling the spread of these agents among pathogens but also for using conjugative plasmids in a controlled manner as therapeutic vectors, for example, to eradicate antibiotic-resistance genes from bacterial communities (III).

Plasmids are often considered to be selfish, antagonistic genetic elements, because they can survive and spread without inducing notable benefits to their host and, occasionally, even work against the host (Jalasvuori 2012). Plasmid maintenance is energy-demanding and induces fitness costs for the host's reproduction; therefore, without positive selection, the cost of plasmids should outweigh the benefits, leading to plasmid disappearance through purifying selection (Harrison *et al.* 2015). However, even high fitness-cost plasmids can persist in bacterial communities, a phenomenon called plasmid paradox (Harrison and Brockhurst 2012). Naturally, beneficial traits encoded by accessory genes, such as antibiotic resistance, compensates the fitness cost by giving an advantage over competitors under antibiotic selection. In addition to the plasmid-mediated persistence mechanisms mentioned above (Bahl *et al.* 2009), compensatory evolution between the plasmid and its host (Harrison *et al.* 2015) and continued conjugation promote plasmid persistence in the bacterial population even in the absence of selection (Lopatkin *et al.* 2017, Stevenson *et al.* 2017). Ecological interactions, such as predation by lytic phages or protozoa, also affect the maintenance of conjugative plasmids. Plasmid-dependent lytic phages reduce or remove plasmids' conjugation ability and may drive them to extinction (Ojala *et al.* 2013, Harrison *et al.* 2015), whereas the protozoan predation of the plasmid host increases the persistence and spread of conjugative plasmids (Cairns *et al.* 2016). However, under the combined selective pressure of the lytic plasmid-dependent phage and antibiotic, the sociality of an antibiotic resistance mechanism (i.e., selfish versus altruistic) has a major influence on whether the plasmid prevails (Cairns *et al.* 2018). Antibiotic-susceptible bacteria benefit from altruistic resistance mechanisms, such as with β-lactamases, without having to bear the fitness cost of plasmids (Cairns *et al.* 2018). Therefore, only a subpopulation of bacteria retains the plasmid (Cairns *et al.* 2018). In contrast, the selfish resistance mechanism favours bacteria harboring the resistance-plasmid, causing the whole population to maintain the plasmid (Cairns *et al.* 2018).

1.3 Bacteriophages—modifiers of bacterial communities

Bacteriophages, or phages, are intracellular bacterial viruses. These 'bacteria-eaters' were discovered independently by two scientists, William Tort and Felix d'Herelle, in the early 20th century (Clokie *et al.* 2011). The latter conducted the first clinical trials for phage therapy by attempting to treat dysentery (Abedon 2011). The estimated number of phages in the biosphere is 10^{30}, making them the most abundant entity, even surpassing the number of their bacterial host by one

order of magnitude (Hendrix *et al.* 1999, Chibani-Chennoufi *et al.* 2004). Phages can be considered as obligate viral parasites because they are dependent on their host for propagation and survival (Hendrix 2003). For this reason, phages and their host are also found in the same environments (Hendrix 2003).

Phages, or more precisely virions (i.e., infective extracellular virus particles), are composed of a proteinaceous capsid encapsulating the genome (Ackermann and Prangishvili 2012). Although the overall structure of a phage seems simple, phage genomes and structures are diverse (Hatfull and Hendrix 2011). They lack a universal genetic marker, which would allow their uniform categorization into phylogenetically related families (Lawrence *et al.* 2002, Lima-Mendez *et al.* 2008). Commonly, phages are categorised into families and smaller taxonomic groups based on genomic similarity, the homological structure of their capsid proteins or overall morphology of the capsid (Tolstoy *et al.* 2018). Most of the identified phages belong to the *Caudovirales* family, characterised by a double-stranded DNA genome with an icosahedral capsid head and a tail (Krupovic *et al.* 2011). This family is further sub-divided into three phylogenetically-related families based on their tail morphology: long contractile tails (*Myoviridae)*, long non-contractile tails (*Siphoviridae*), and short tails (*Podoviridae*) (Krupovic *et al.* 2011). Along with these three morphologies, phages are categorised as tail-less icosahedral with either an inner or outer membrane, without a membrane, filamentous, or pleomorphic (Krupovic *et al.* 2011). Due to their abundance, phages in the *Caudovirales* family are most commonly used in phage-based applications, such as phage therapy (Drulis-Kawa *et al.* 2012).

A phage's genome can be comprised of either DNA or RNA, and its configuration can vary from single- to double-stranded (Hatfull and Hendrix 2011). Genomes can also be segmented, especially in RNA phages (Hatfull and Hendrix 2011). A phage's genome is compact, and in *Caudovirales* family, it is organised in functional modules that encode structural proteins (e.g., capsid and tail fibre) or enzymes needed for the lysogenic or lytic life cycle (Weinbauer and Rassoulzadegan 2004). In order to establish lysogeny, temperate phages encode enzymes (e.g., transcription repressor and integrase) needed for integration and maintenance in the host's genome (Fogg *et al.* 2011).

In addition to morphology, phages can be categorised as virulent or temperate based on their lifecycles. The initial step in the lifecycle of both types of phages begins with the absorption of the phage to the entry receptor on the host cell via specific receptor-binding protein(s). Phages typically use surface proteins, polysaccharides, or LPSs as receptors (Rakhuba *et al.* 2010), and the specificity of this interaction often determines a phage's host range (Weinbauer 2004). Next, a phage creates a hole in the rigid cell wall using peptidoglycan-hydrolysing enzymes (Loessner 2005) and injects its genome into the cell through the tail structure (Bhardwaj *et al.* 2014). After this stage, the life cycle of virulent and temperate phages differs: virulent phages enter the lytic life cycle, whereas temperate phages continue to either a lytic or lysogenic life cycle.

In the lytic cycle, phages take over the host's cellular machinery for phage production. During this life cycle, the phage's genome is replicated, and the capsid proteins are translated and assembled. Genomes are packed into the

capsids either during or after assembly (Russel 1991). Finally, the expression of holins and other lytic enzymes release the progeny phages by inducing the host cell to enter lysis. Holins create pores in the cytoplasmic membrane, enabling the lytic enzymes to reach and hydrolyse the peptidoglycan layer. This weakens the cell wall structure and causes a rise in osmotic pressure, which eventually erupts the host cell.

In the lysogenic cycle, a temperate phage's genome is incorporated into the host's genome as a prophage via site-specific recombination (Casjens and Hendrix 2015) or random transposition (Harshey 2014). The host is now called a lysogen, and it may contain several prophages simultaneously. An average of 2.6 prophages are estimated to be in one bacterial genome (Lawrence *et al.* 2002). Transcriptional repressors inhibit the expression of phage genes, thus preventing the production of phage particles. Therefore, as part of the host's genome, the phage genome is replicated along with the host's genome and can persist there indefinitely. Environmental stimuli or the host's stress response can prompt a prophage to enter the lytic cycle (Casjens and Hendrix 2015). Other phage life cycles are also known, such as another dormant state called pseudolysogeny, where the phage is not integrated into the host's genome but resides in the cytoplasm as a circular or linear episome (Cenens *et al.* 2013), and chronic infection, where phages are continously released from the cell by budding or extrusion without the host undergoing lysis (Weinbauer 2004).

During their evolution, bacteria have developed numerous antiviral mechanisms. Entry can be prevented by absorption inhibition in various ways: entry receptors may be modified through mutations (Levin and Bull 2004), receptors may change conformation (Nordström and Forsgren 1974, Riede and Eschbach 1986), and cell-covering capsule may mask the receptor (Letarov and Kulikov 2009). Bacteria also prevent access to the receptor by altering its expression, a phenomenon called phase variation (Liu, M. *et al.* 2002). After entry, phage propagation can be prevented by degrading the injected phage's genome by restriction enzymes or by CRISPR-associated protein 9 (Cas9). Cas9 is guided to the target site by CRISPR RNA (crRNA), which is encoded from adaptive clustered regularly interspaced short palindromic repeats (CRISPR) (Gasiunas *et al.* 2014). In a recently discovered defence mechanism, anthracycline molecules, encoded by *Streptomyces*, block viral infection at an early stage by becoming embedded in the injected viral DNA (Kronheim *et al.* 2018). This blockage is assumed to prevent the circularisation of the phage's genome, thus exposing the linear DNA to restriction enzymes (Kronheim *et al.* 2018). As a last resort response, the death of infected cells can be triggered by an abortive infection (abi) system before any phage progeny are released (Molineux 1991, Fineran *et al.* 2009). This altruistic behaviour allows remaining bacterial cells in the population to avoid phage infection.

As a countermeasure, phages have evolved to circumvent hosts' resistance mechanisms. To overcome absorption inhibition, phages can acquire novel receptor tropism through point mutations in their receptor binding proteins (RBPs) (Samson *et al.* 2013) or uncover the masked receptor by enzymatic hydrolysis (Leiman *et al.* 2007) or cleavage (Drulis-Kawa *et al.* 2012) of the

capsule. These changes in RBPs occasionally lead to expanding the phage's host range (Stummeyer *et al.* 2006) or to developing the ability to use several entry receptors or a new receptor (Meyer *et al.* 2012). The variability of RBPs increase the probability of successful infection when the expression of an entry receptor is stochastic, as in phase variation (Samson *et al.* 2013). A phage can prevent its genome from being restricted via chemical epigenetic modifications, such as methylation (Samson *et al.* 2013). The genomes may also contain fewer restriction sites (Krüger and Bickle 1983, Bickle and Krüger 1993) or, alternatively, the orientation of and distance between restriction recognition sites are changed (Krüger *et al.* 1988, Meisel *et al.* 1992). Phages may also co-inject proteins that bind either to the phage's genome to mask the restriction sites (Iida *et al.* 1987) or to restriction enzymes, thereby inhibiting their function (Atanasiu *et al.* 2002, Walkinshaw *et al.* 2002). The CRISPR-Cas defence system can be inhibited by cooperative infections by multiple phages expressing anti-CRISPR (Arc) proteins (Borges *et al.* 2018, Landsberger *et al.* 2018). Several phages infect and produce Arc proteins in a single cell to overcome the critical threshold needed for immunosuppression (Borges *et al.* 2018, Landsberger *et al.* 2018). After these failed infections, the following Arc-encoding phage can successfully infect the immunosuppressed cell (Borges *et al.* 2018, Landsberger *et al.* 2018). As for absorption inhibition, abi systems are circumvented by mutating genes coding the targeted proteins, such as transcriptional co-activator (*motA*) in coliphage T4rII (Shinedling *et al.* 1987, Hinton 2010).

Altogether, phages have a huge impact on both the ecology and evolution of prokaryotic communities in terrestrial (Ashelford *et al.* 1999) and aquatic environments (Wilhelm and Suttle 1999). As one of the major causes of bacterial mortality (Suttle 1994, Chibani-Chennoufi *et al.* 2004), they influence the dynamics and diversity of the bacterial community by altering the number and abundance of different bacterial species or strains by controlling the competitive dominants (Weinbauer and Rassoulzadegan 2004). At the single-cell level, phages enrich the host's genome by introducing new genes, such as resistance or virulence genes, via transduction or as prophages. This increases genetic variability, which can, in time, lead to evolutionary diversification and even speciation (Weinbauer and Rassoulzadegan 2004).

1.3.1 Phage therapy as a prophylactic treatment

After the discovery of antibiotics and their ease of administration, phages were discarded as therapeutic agents in the treatment of bacterial infections in the West (Summers 2012). However, their use as antimicrobials continued in parts of the former Soviet Union, such as Georgia (Summers 2012). Phages are used to treat, amongst other conditions, pathogenic gastrointestinal infections and purulent wound or skin infections (Abedon 2011). In contrast, phage research conducted in the West has mainly focused on only a few phages used in molecular and eco-evolutionary studies (Clokie *et al.* 2011). The emerging failure of antibiotics to

cure multi-resistant bacterial infections has increased researchers' interest in phage therapy as an alternative antimicrobial treatment.

The diversity and abundance of phages in the biosphere make them an almost endless resource for treating bacterial infections (Chibani-Chennoufi *et al.* 2004, Örmälä and Jalasvuori 2013). Alternatively, phages could be used as a prophylactic treatment (Abedon 2011) to modify bacterial communities and remove colonised multi-resistant opportunistic pathogens, such as ESBL-producing *Enterobacteriaceae*, from the gut microflora (IV). The latter would help prolong the lifetime of current antibiotics. Phages could be converted to prophylactic use as probiotics, defined by the WHO as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" (Abedon 2011). The regulation of probiotics is also simpler in comparison to therapeutic drugs. Indeed, there is evidence suggesting that phages can accurately target specific bacteria in a community. Cieplak *et al.* (2018) compared the effects of broad-spectrum ciproflaxin and phage cocktail infecting *E. coli* to the cell densities of different bacterial species of gut microflora (Cieplak *et al.* 2018). Ciproflaxin is an antibiotic commonly used to treat *E. coli* infections in the gut (Cieplak *et al.* 2018). Both ciproflaxin and the phage cocktail reduced the cell density of *E. coli* by 2.5 log in an experiment conducted under simulated gut conditions (Cieplak *et al.* 2018). However, the reduction in the cell density of other bacterial species of gut microflora was insignificant when the phage cocktail was compared to ciproflaxin (Cieplak *et al.* 2018). Although the eradication of a targeted bacteria community would be incomplete, in mice, the remaining resistant bacteria have shown a decreased ability to colonise the gut (Myhal *et al.* 1983, Atterbury *et al.* 2005). In contrast, in chickens, the resistant mutants have shown to revert back to parental phenotype and regain their ability to colonise (Carvalho *et al.* 2010). However, the outcome might depend on the targeted bacterial species, and thus, further research is required on this topic. Altogether, using phages, the bacterial load could be reduced to sufficient levels for the immune system to eradicate the remaining pathogens (Smith and Huggins 1982, Debarbieux *et al.* 2010). Additionally, other contemporary bacteria species could outcompete the targeted strain through resource competition.

As natural enemies of bacteria, phages and their hosts are in a constant evolutionary arms-race (Levin and Bull 2004). Due to their antagonistic co-evolution, phages can be considered 'self-renewable': they can overcome resistance, probably even during treatment (Levin and Bull 2004). Besides *de novo* mutations, phages can evolve during co-infection when the exchange of genome segments can occur with prophages or different phages infecting the same cell (Bouchard and Moineau 2000, McDonald *et al.* 2016). This generates chimeric phages with new traits if the superinfection exclusion by the primary phage or prophage does not prevent the secondary infection of a homologous phage (Abedon 2015, Bondy-Denomy *et al.* 2016). To prevent therapeutic phages from acquiring and disseminating undesirable genes, such as antibiotic resistance or virulence genes, only lytic phages are recommended for phage therapy (Skurnik *et al.* 2007). Furthermore, the use of virulent phages in high titers could diminish the probability of emerging resistant mutants (Levin and Bull 2004).

Other beneficial features of phages, in comparison to antibiotics, are their self-replication, self-limitation, and host-specificity characteristics. As phages proliferate in the presence of a host, as little as one dose could be adequate to clear an infection (Abedon and Thomas-Abedon 2010). Unlike unspecific and broad-spectrum antibiotics, phages' host-specificity enables the eradication of a single strain or subtype of a specific bacterial species (Hyman and Abedon 2010), leaving the rest of the community, such as beneficial gut microflora, intact (Skurnik *et al.* 2007). This would prevent dysbiosis and potential secondary infections, such as invasion by *Clostridium difficile*, which is associated with antibiotic treatment (Rea *et al.* 2011). Resistance would only develop in the bacterium being targeted, unlike with antibiotics, which cause resistant mutants to develop in the whole bacterial community. However, phage specificity can also be a disadvantage because, in many cases, the specific pathogen causing the disease is unknown (Clark 2015). This problem can be overcome using multiple phages or phages with a broad host-range (Kelly *et al.* 2011). By selecting phages that are virulent and bind to different receptors, the emergence of resistant mutants is mostly prevented, and the prospective synergistic effects further improve the efficacy of the phage cocktail (Rhoads *et al.* 2009, Drulis-Kawa *et al.* 2012). To acquire resistance against the phage cocktail, a bacterium would have to acquire several costly mutations simultaneously.

The downside of phage therapy is its complicated pharmacology. The dilution of phage titers due to the slow diffusion of huge phage particles, especially with intravenous delivery, and their multiplication inside the host complicates pharmacokinetics and pharmacodynamics, respectively (Abedon 2014). A productive infection is density-dependent and relies on an adequate number of phages reaching the target site so that the bacterial population is successfully eradicated (Drulis-Kawa *et al.* 2012). A productive infection is essential to keeping the phage titer high throughout the treatment, which further prevents resistant mutants from emerging. Also, the infectivity of phages is most efficient when the bacterial population is in the exponential growth phase (compared to the stationary or adaptation phase); therefore, the timing of phage administration is important (Ryan *et al.* 2011, Drulis-Kawa *et al.* 2012). Direct delivery to the center of the infection, for example, by injection, has produced promising results *in vivo* (Saussereau and Debarbieux 2012). However, the administration of large numbers of phages can cause phages to absorb simultaneously, and the concurrent enzymatic activity can lead to cell death by lysis without phage propagation (Abedon 2011), hence requiring the repeated administration of phages. The simultaneous release of large quantities of endotoxins from the lysed target cells or endotoxins released into the medium during the preparation of a phage cocktail might also cause cytotoxic side effects (Abedon 2011). Phages can also induce a humoral or cellular immune response (Clark *et al.* 2002, Gabig *et al.* 2002, Łusiak-Szelachowska *et al.* 2014). Although not harmful to the patient, the immune response can inhibit the phage infection or clear the phages from circulation before they have a therapeutic effect, decreasing the treatment's effectiveness (Łusiak-Szelachowska *et al.* 2014). By using a topical application method, some of these problems could be avoided.

Phages could be genetically modified several ways, such as mutating capsid proteins to increase their intravenous circulation time (Vitiello *et al.* 2005), widening their host range (Mahichi *et al.* 2009), and preventing the release of endotoxins from bacteria by using lysis-deficient phages (Matsuda *et al.* 2005, Paul *et al.* 2011).

Several safety studies (Bruttin and Brüssow 2005, Sarker *et al.* 2012) and phase I/II clinical trials with placebo controls indicate that phages are safe therapeutic agents for use in human treatment (Wright *et al.* 2009, Kutter *et al.* 2010). Phage therapy has successfully cured various multi-resistant *Enterobacteriaceae* infections in many animal models (e.g., mice, lambs, calves, piglets) with various types of internal infections (Wittebole *et al.* 2014). Unfortunately, many of the clinical trials for human infections at the beginning of the twentieth century were non-randomised and uncontrolled (Wittebole *et al.* 2014). Only a few mid-scale human trials have been performed according to strict scientific protocols. In two studies, *P. aeruginosa* causing either burn infection or otitis was targeted with variable success in phase I/II trials (Wright *et al.* 2009, Jault *et al.* 2019). There are also a few examples where phage therapy was used as the last-resort treatment of patients with incurable multi-resistant *A. baumannii* infections, with one successful recovery (Schooley *et al.* 2017, LaVergne *et al.* 2018). More large-scale clinical trials are still needed to evaluate the safety and efficacy of phage therapy. Standardised methods for large-scale production and storage conditions, as well as the establishment of quality standards for therapeutic phages, including sterility and stability, are required (Pirnay *et al.* 2011). Currently, the main hurdles hindering the development of phage therapy are the lack of specific regulatory guidelines for personalised therapies and difficulties encountered when pharmaceutical companies attempt to register intellectual patents for their phage cocktails (Nobrega *et al.* 2015).

Fortunately, due to more flexible regulation, satisfying progress has been made in phage applications designed to replace antibiotics in food safety. The US Food and Drug Administration (FDA) has approved the use of commercial phages against pathogens contaminating food, such as *Listeria monocytogenes* and *Salmonella* (Carlton *et al.* 2005, Oliveira *et al.* 2015). Phages that degrade exopolysaccrarides (EPSs), such as biofilms and slime, have attracted considerable interest due to their potential use in preventing biofilm formation on medical devices, such as catheters and intubation tubes, which are major sources of pathogenic infections in intensive care units (Fu *et al.* 2010). Phages could also be used as preventative surface disinfectants (Viazis *et al.* 2011) or non-infective phages as vectors for delivering genetic material encoding addiction toxins against bacteria (Westwater *et al.* 2003). With broad-spectrum cell-wall degrading endolysins, several gram-positive bacterial infections, such as antibiotic-resistant *Enterococcus faecalis* and *faecium* and MRSA, could be cleared (Yoong *et al.* 2004, Proença *et al.* 2012, Gilmer *et al.* 2013).

In addition to synthetic fatty acids (Getino *et al.* 2015), lytic plasmid-dependent phages could also be used to hinder the dissemination of multi-resistance plasmids (Lin *et al.* 2011). *In vitro* studies of the plasmid-dependent phage PRD1 have shown that it can reduce the spread of plasmid RP4 in bacterial

populations, even under sublethal antibiotic selection that favours conjugation (Ojala *et al.* 2013, Ojala *et al.* 2016). Phage predation has led to either the loss of plasmid-mediated resistance or, occasionally, the loss conjugation ability (Jalasvuori *et al.* 2011, Ojala *et al.* 2016). Alternatively, phages delivering genetic material encoding CRISPR-Cas system could be used to clear multi-resistant bacteria (Citorik *et al.* 2014, Bikard *et al.* 2014).

1.4 CRISPR-Cas9

CRISPR was first described in *E. coli* by Japanese researchers in 1987 (Ishino *et al.* 1987) and, later, in archaea (Mojica *et al.* 2000). However, it was not until almost twenty years later that its original function as a prokaryotic adaptive immune system against invading phages (Barrangou *et al.* 2007) or conjugative plasmids (Marraffini and Sontheimer 2008) was understood (Makarova *et al.* 2006). Because they evolve rapidly, CRISPR-Cas systems are highly diverse. The classification of CRISPR-Cas systems is multifaceted: the two classes are divided into six types (I-VI) and, further, into sub-types based on various factors (Koonin *et al.* 2017). These include the specific signature *cas* genes of each type and subtype and the protein sequence similarity of shared Cas proteins, the phylogeny of the best-conserved protein Cas1, the structure of CRISPR loci, as well as the organisation of the whole CRISPR-Cas loci (Koonin *et al.* 2017). Every CRISPR-Cas system has two distinctive genetic modules: genes encoding for proteins involved in adaptation and genes needed for effector functions, including precursor-DNA processing and target recognition and cleavage (Koonin *et al.* 2017). The genes of the adaptation module are largely uniform across CRISPR-Cas systems (Amitai and Sorek 2016).

The genomic arrangement of the type II-A CRISPR-Cas9 system consists of trans-activating crRNA (tracrRNA), which is trans-encoded upstream from the operon of *cas* genes (Jansen *et al.* 2002). This operon encodes a set of Cas proteins with DNAase and endonuclease activity, operating in spacer acquisition (Brouns *et al.* 2008, Carte *et al.* 2008, Wiedenheft *et al.* 2009) and CRISPR interference (Jinek *et al.* 2012), respectively. Downstream from the *cas* operon is the AT-rich leader sequence (Jansen *et al.* 2002), preceding the CRISPR locus of identical palindromic repeats with spacer sequences from invading foreign DNA having been incorporated in between repeats (Bolotin *et al.* 2005, Mojica *et al.* 2005).

The number of spacers can vary from two to a few hundred. They encode for the immune memory of previous infections. The newest spacers are at the proximal end of the leader, and the older and more conserved ones are at the distal end (Marraffini and Sontheimer 2010). Spacers are acquired during the first stage of adaptive CRISPR immunity, adaptation (Marraffini and Sontheimer 2010). When the invading DNA of a phage or plasmid enters the cell, a small sequence is cut off and incorporated as a spacer into the CRISPR locus (Tyson and Banfield 2008). The mechanisms of spacer acquisition and incorporation are mostly unknown, but the universal Cas1, with DNase activity, and Cas2 proteins

have been shown to be essential (Makarova *et al.* 2015). The long precursor RNA is transcribed from the CRISPR locus, followed by maturation when the host-encoded RNase III splices it into smaller individual crRNAs (Deltcheva *et al.* 2011), composed of a partial repeat and a spacer (Brouns *et al.* 2008). TracrRNA acts as a guide for the Cas protein to recognize the target (Deltcheva *et al.* 2011, Jinek *et al.* 2012). Finally, the crRNA-guided double-strand cleavage of foreign DNA is performed by a Cas protein at the site complementary to the crRNA spacer (see Fig. 1) (Doudna and Charpentier 2014). The mechanism behind the recognition and cleavage of target sites varies between CRISPR-Cas types, but type II Cas9 protein identifies the target site by interacting with the protospacer adjacent motif (PAM), a triple-nucleotide sequence immediately downstream from the crRNA-targeted sequence (Doudna and Charpentier 2014, Sternberg *et al.* 2014). TracrRNA is needed to trigger the cleavage that occurs three nucleotides downstream from the PAM sequence (NGG) (Deltcheva *et al.* 2011). Thus, in principle, any sequence next to PAM could be targeted by Cas9 (Jinek *et al.* 2012), although nucleotides at both the PAM-distal and PAM-proximal ends of crRNA, the genomic context of the target sequence, the GC percentage, and the secondary structure of crRNA affect Cas9's targeting efficiency (Liu *et al.* 2016, Jiang *et al.* 2013).

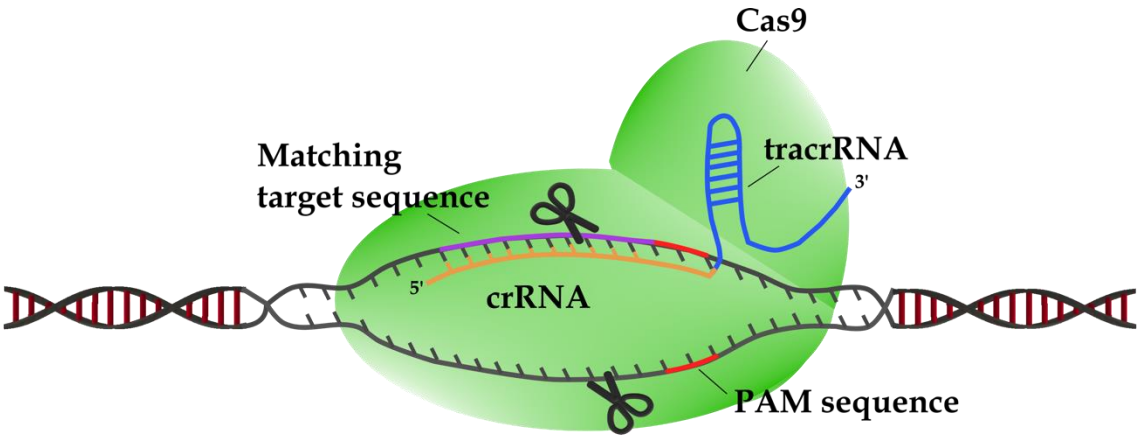

FIGURE 1 Sequence-specific double-stranded cleavage of target DNA by Cas9. Adapted from Charpentier and Doudna (2013).

From the CRISPR-Cas systems, type II CRISPR-Cas9 is convenient for genetic engineering because only one Cas protein is needed for the recognition and cleavage of the target site (Gasiunas *et al.* 2012, Jinek *et al.* 2012). It has been used for several genetic applications, such as generating cancer models (Xue *et al.* 2014) and studying gene functions (Zhou *et al.* 2014) in species ranging from plants (Zhang *et al.* 2014) to eukaryotes (Wu *et al.* 2013, Mali *et al.* 2013). For genetic engineering purposes, the original CRISPR-Cas9 expression system is modified to contain only the essential components: tracrRNA, *cas9,* and the leader following the CRISPR locus that contains the targeting spacers. For some applications, tracrRNA and crRNA are fused together to form a single-guide RNA (sgRNA) (Jinek *et al.* 2012). Compared to zinc-finger nucleases (ZFNs) and

TAL effector nucleases (TALENs), genetic engineering with CRISPR-Cas9 does not involve laborious protein engineering; merely changing the spacer sequence(s) is enough (Doudna and Charpentier 2014).

One of the most recent applications uses CRISPR-Cas9 to eradicate multi-resistance plasmids from heterogenous communities (Bikard *et al.* 2014, Citorik *et al.* 2014). Unlike plasmid-dependent phages, CRISPR-Cas9 is independent of plasmid Inc types. In addition, similar to both synthetic fatty acids and plasmid-dependent phages, inhibition can result in plasmid curing. Indeed, both Bikard *et al.* (2014) and Citorik *et al.* (2014) have shown that the sequence-specific targeting of ESBL-resistance genes by CRISPR-Cas9 leads to plasmid loss, thus re-sensitising bacteria to antibiotics. Alternatively, the target bacteria are killed by the toxins encoded by the targeted plasmid's TA-system. Double-stranded cleavage by Cas9 leads to the linearisation and destabilisation of TA-encoding plasmids and, thus, prevents the expression of the antitoxin. Consequently, the bacteria are exposed to the toxins, which leads to a cytotoxic response and cell death (Citorik *et al.* 2014). Due to the Cas9-guiding crRNA, the system is programmable to target any resistance gene, and therefore, it can be exploited to target only the strains carrying these resistance genes, even within a heterogeneous population (Citorik *et al.* 2014).

2 AIMS OF THE STUDY

The rapid dispersal of multi-resistance plasmids and the increasing prevalence of ESBL carriers in the community pose a major threat to healthcare as it could increase infection-related morbidity and mortality rates. Little is known about the transmission of plasmids during antibiotic treatment. It is entirely possible that genetic elements disseminating antibiotic resistances gain the necessary leverage, essentially, and perhaps, even only, in the presence of antibiotics. Bacteria are extremely adaptive, so we must also adapt. It is essential to discover new methods to eradicate resistant bacterial reservoirs to inhibit the dissemination of resistance genes and to prolong the lifetime of existing antibiotics. These methods could also, or primarily, be applied as a prophylactic treatment. The specified aims of this PhD thesis are as follows:

i. To examine the interbacterial transfer of different ESBL plasmids after the bacteria are exposed to different (lethal) antibiotic concentrations

ii. To study evolutionary rescue of antibiotic sensitive bacteria by horizontal transfer of ESBL plasmid: the influence of different classes of β-lactam antibiotics, the exposure time to antibiotic, and the potential to rescue by interspecies transfer

iii. To develop a conjugative CRISPR-Cas9 plasmid system for targeting and eradicating resistance genes

iv. To study the efficiency of the CRISPR-Cas9 system in targeting several ESBL genes simultaneously, as well as the system's stability and dispersal in a bacterial community

v. To evaluate genetic changes within the CRISPR locus by which the targeted ESBL bacteria has escaped the CRISPR-Cas9 treatment

vi. To determine the probability of isolating new phages for personalised phage therapy to selectively eradicate ESBL-bacteria from their potential reservoirs, such as the gut

3 SUMMARY OF THE MATERIAL AND METHODS

The methods used is this thesis are summarized in the Table 1. More detailed description of the methods and, also all the bacterial strains, plasmids and phages are found from the original publications (I-IV).

TABLE 1 The methods used in this thesis

Method	Publication
Agarose gel electrophoresis	I, III
Traditional cloning and cloning by homological recombination	III
Comparative genomics	I, II
Colony forming assay	I, II, III
Colony-PCR	I, III
Conjugation assay	I, II, III
Deletion-PCR	III
Electroporation	II, III
Heat shock transformation	III
In vitro evolution experiment	I, II
Nucleotide sequencing and annotation	I, III
Phage isolation	IV
Phage resistance test	IV
Plaque assay	IV
Propagation of phages	IV
Statistical analysis	I, II, III, IV

4 RESULTS AND DISCUSSION

4.1 Evolutionary rescue via conjugation of ESBL-plasmids under lethal antibiotic concentrations varies between Inc-types and the selective β-lactam antibiotic (I and II)

Resistance plasmids are known to disseminate via conjugation under sub-lethal antibiotic concentrations (Andersson and Hughes 2012). However in case of β-lactams, these plasmids may spread into antibiotic-susceptible bacteria even under lethal antibiotic levels. This is due to the altruistic nature of β-lactam resistance generated by β-lactamase-producing bacteria that degrade β-lactam antibiotic molecules, thus saving the antibiotic-sensitive community members (Brook 1984, Brook 2004, Perlin *et al.* 2009, Yurtsev *et al.* 2013). Evolutionary rescue via HGT is practically an unexplored mechanism that may accelerate the dissemination of antibiotic resistance genes during antibiotic treatment. Via evolutionary rescue, the growth of originally susceptible bacteria is restored, preventing the extinction of bacteria. As such, we investigate its effect by using various ESBL plasmids of clinical origin.

The selected *E. coli* strains carried ESBL plasmids, which were isolated from patients with multi-resistant infections admitted to Turku University Hospital in Finland. The examined plasmid sets comprised one to three plasmids (Table 2). Each plasmid within a set was marked with a Roman numeral. In this thesis, plasmid set names are used to refer to all the plasmids belonging to the same set (e.g., pEC15 refers to both pEC15I and pEC15II).

TABLE 2 pEC plasmid features. Alterations to plasmid size due to the shufflon area are indicated in parentheses. *A non-conjugative mobilisable plasmid

pEC plasmid sets	Plasmid size (bp)	Inc type	β-lactamase identified	Other resistance genes
pEC3I	91 885	*IncB/O/K/Z*	*blaTEM-1C*	*strA, strB, sul2*
pEC3II	59 192 (59 192)	*IncI2*	-	-
pEC13	71 656	*IncFII*	*blaCTX-M-14*	-
pEC14I	143 590	*IncFII, IncQ1, IncP, IncFIB(AP001918)*	*blaTEM-1B*	*strA, strB, aadA1, mph(B), sul1, sul2, tet(A), dfrA1*
pEC14II	87 848 (87 666)	*IncI1*	-	-
pEC14III	80 057	*IncFII*	-	-
pEC15I	87 811 (87 767)	*IncI1*	-	-
pEC15II	38 611	*IncX1*	*blaTEM-52B*	-
pEC16I	94 325 (95 380)	*IncI1*	*blaSHV-12*	-
pEC16II*	7 939	*ColRNAI*	-	-

All the plasmid sets encode one ESBL enzyme. The ESBL-resistance genes (i.e., *blaTEM, blaCTX-M,* and *blaSHV),* as well as the plasmid Inc types (i.e., IncF, IncX, and IncI) are representative of the types currently causing nosocomial and community-acquired infections (Bielak *et al.* 2011, Kim *et al.* 2011, Rohde *et al.* 2011, Yamaichi *et al.* 2015). IncX1 plasmids are commonly found in opportunistic pathogens (Norman *et al.* 2008), and the IncI1 plasmid was identified in the outbreak of enterohemorrhagic *E. coli* 0104 in Germany in 2011 (Yamaichi *et al.* 2015). The IncF plasmid type, such as pEC13, is especially associated with not only the dissemination of ESBL-resistance genes in *Enterobacteriaceae* (Carattoli 2009) but also virulence factors (Herrero *et al.* 2008). Also, the pEC13-encoded *blaCTX-M-14* gene is currently one of the most prevalent ESBL genes globally and abundant in food-producing animals (Cottell *et al.* 2011, Liao *et al.* 2015, Bevan *et al.* 2017). According to blastN search (NCBI), highly similar plasmids as our IncFII plasmid (pEC13) have been isolated from various enterobacterial species, such as *K. pneumoniae, Salmonella enterica, Citrobacter sp.* as well as *Shigella sonnei* and *flexneri*. pA1705-NDM plasmid isolated from *K. pneumoniae* (GenBank acc: MH909349) shared the highest coverage percentage (99,85%), although, in addition to CTX-M-14, it encoded two other β-lactamases, OXA-1 and NDM-1, from which the latter is also capable of hydrolysing carbapenems. Additionally, the plasmid provides resistance to several other antibiotics, such as fluoroquinolone, aminoglycoside, phenicol, rifampicin, sulphonamide and tetracycline. Therefore, evolutionary rescue via conjugation enables the spread of antibiotic multiresistance as well. The progressive Mauve alingment (Geneious 11.1.5) showed that in addition to size homogeny, the plasmids of these enterobacterial species shared gene and operon synteny (see. Fig. 2). Although pA1705-NDM makes an exception in size for being three times bigger plasmid due to the accumulation of several mobile genetic elements and resistance cassettes.

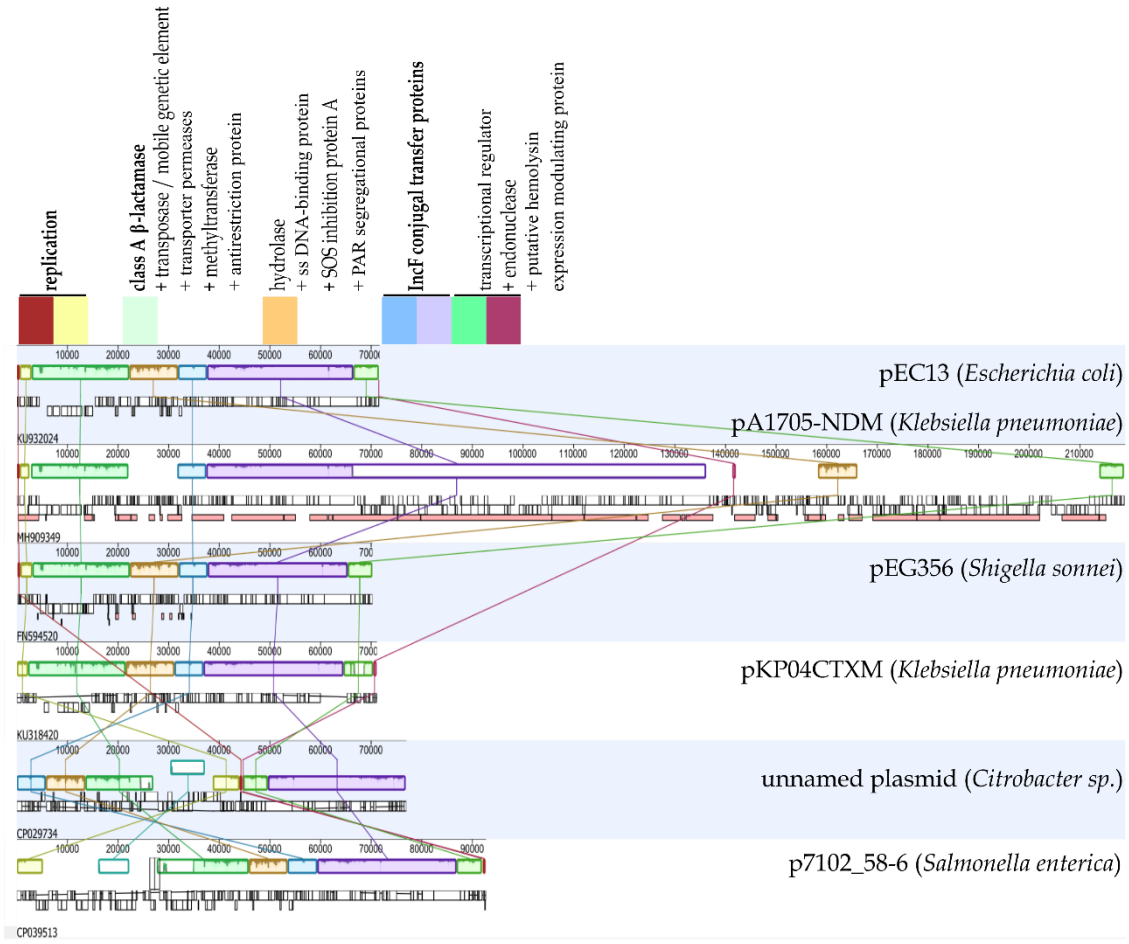

FIGURE 2 The progressie Mauve alignment of pEC13 and highly similar plasmids isolated from various *Enterobacteriaceae* species. The bars above the alignment present the operon synteny and the genes encoded from these operons. However, the exact gene content vary slightly between the plasmids.

In all conjugation experiments a sensitive recipient was added first to the culture and, depending on the experiment, after certain exposure time, the donor carrying the ESBL-plasmid was introduced. *E. coli* K-12 JM109(pSU19) carrying ESBL-plasmid (Table 2) was used as a donor in each experiment and as recipients *E. coli* HMS174, *K. pneumoniae* DSM681 and *Salmonella typhimurium* serovar Enterica SL5676. All the assays were performed in triplicates. After the 24 h incubation period, the density of β-lactamase-resistant recipient cells (colony forming units/milliliter; cfu/ml) were determined by colony-forming assay with appropriate antibiotic selection. All tested antibiotic concentrations used in the conjugation assays were lethal for the antibiotic-susceptible recipients.

First, we examined the evolutionary rescue potential of five ESBL plasmid sets (Table 2) by measuring their transfer frequency under full resource (100% L-broth) conditions with three different, but lethal, ampicillin concentrations (15 μgml^{-1}, 75 μgml^{-1} and 150 μgml^{-1}), as well as in the absence of antibiotics. The evolutionary rescue potential was also tested under lower resources (5 % L-broth) with 150 μgml^{-1} ampicillin. The donor in this experiment was introduced to the culture after five minute exposure to the ampicillin. The mean frequency of

transconjugants, proportioned to the mean cell density of all bacteria, was calculated to evaluate the evolutionary rescue potential of different plasmid sets. The results indicated that the evolutionary rescue potential via HGT varied greatly among the five different ESBL plasmids (see Fig. 3a). pEC14 had the lowest evolutionary rescue potential in every antibiotic concentration, as well as without antibiotic selection, although in the absence of antibiotics, the assay measures only the transfer rate and not the rescue potential *per se*. Its rescue potential was completely lost in low resources (5% L-broth) with 150 μgml^{-1} ampicillin selection. pEC16 had the highest rescue potential, followed by pEC13 and pEC15, respectively. For all the plasmids, the frequency of rescued transconjugants dropped gradually with increasing antibiotic concentrations, and pEC15 had a steep drop in the frequency of transconjugants in the higher antibiotic concentrations. This phenomenon can be partly explained by differences in the β-lactamase gene-types' ability to maintain the varying frequency of antibiotic-susceptible cheaters. However, this does not solely explain the results because, compared to pEC15, the other plasmids maintained approximately the same frequency of cheaters in the different ampicillin concentrations (see Fig. 3b). For example, pEC14 maintained a higher frequency of cheaters but possessed the lowest rescue potential. The differing plasmid profiles could affect the ability to save susceptible recipients. Compared to plasmid sets with one or two plasmids, the three large plasmids of pEC14 might transfer slower (Smillie *et al.* 2010) and decrease the its ability to rescue antibiotic-sensitive cheaters. The replication of pEC14I and pEC14III plasmids belonging to same IncFII group might exhaust the cell, thus lowering the transfer rate. The complete transfer of all plasmids of the set to the recipient cell was determined by colony PCR and agarose gel electrophoresis. The total plasmid content was isolated in case we could not determine the presence of plasmid by colony-PCR. Surprisingly, all three plasmids of pEC14 were transferred in every ampicillin concentration. In contrast, only half of the pEC3 transconjugants contained the second plasmid, pEC3II, which did not contain the resistance gene. With the other plasmid sets, all the plasmids were always transferred. In general, the rescue potential decreased in the lower nutrient resources; however, the rescue pattern remained similar, meaning that the same plasmids had the highest rescue potential in both nutrient levels.

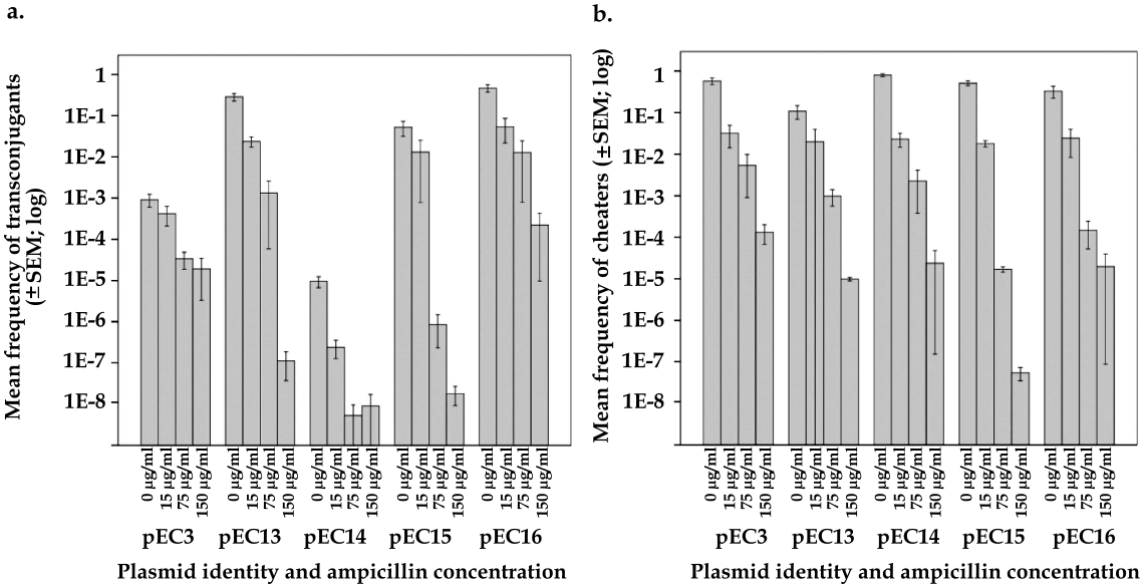

FIGURE 3 The mean frequency (±SEM) of HMS174(pEC) transconjugants (**a.**) and the HMS174 cheaters (**b.**) (n=3) after 24 h incubation in different but lethal ampicillin concentrations or without antibiotc selection.

According to our results, different plasmids have varying potential to save sensitive recipients, but neither the MOB nor the MPF type of the plasmid backbone, as well as the number of cheaters, was correlated with it. The low transfer rates of pEC14, containing both IncFII and IncI1 plasmids, was surprising as both MPF_F (containing IncF) and MPF_I (containing IncI) are shown to allow conjugation in high frequencies in a liquid culture (Achtman 1975, Bradley 1984). Some MOB_F plasmids (containing IncF) even encode a mating-pair-channel stabilisation system, ameliorating the transfer in liquid culture (Garcillán-Barcia *et al.* 2011).

We further investigated the dissemination of pEC13 to three β-lactam sensitive and clinically-relevant enterobacterial recipients; *E. coli, K. pneumoniae* and *Salmonella typhimurium*. Both *E. coli* and *S. typhimurium* were sensitive to ampicillin and cephalothin, whereas *K. pneumoniae* was only sensitive to cephalothin. The recipients were exposed to two β-lactams, ampicillin and cephalothin (both 50 µg/ml), for different time periods (1 h, 6 h and 16 h) before introduction of the donor carrying ESBL-plasmid pEC13. We discovered that *Escherichia coli* carrying conjugative ESBL-resistance encoding plasmid can disseminate into antibiotic-susceptible bacteria even after sixteen hours exposure to two different β-lactams (see. Fig. 4a and b). The exposure of *E. coli* to these β-lactams showed that the survival of the recipient was more likely under cephalothin than ampicillin selection (see. Fig. 4a). A worrying result was that the cell density of both *E. coli* and *Klebsiella* transconjugants remained approximately the same independent of the duration of cephalothin exposure. In contrast to cephalothin selection, the cell density of *E. coli* transconjugants dropped over two orders of magnitude after six hours exposure to ampicillin. The conjugation assay was also performed without antibiotic exposure, when donor was introduced after one hour incubation. The cell densities of both

enterobacterial transconjugants were the almost the same without β-lactam selection (see Fig. 4a and b). These results indicate that the targeted susceptible bacteria can survive several hours under antibiotic treatment and become resistant by via HGT when encoutered with a donor carrying conjugative ESBL-plasmid. In addition to antibiotic-resistance, bacteria can acquire virulence genes with the plasmid. All this could lead to treatment failure, recurrent antibiotic courses and chronic infections. Unfortunately, the density of *Salmonella* transconjugants was undetectable in both antibiotic treatments. Also the evolutionary rescue potential of pEC15 to all recipients was examined, but preliminary tests suggested that it is unable to conjugate to *K. pneumoniae* and *Salmonella.*

The effect of temperature (37°C, room temperature 22°C (RT) and 4°C) to density of *E. coli* transconjugants after sixteen-hour exposure to both β-lactams was also examined. The different temperatures had no effect on the cell density under cephalothin selection, whereas under ampicillin concentration the cell densities were two to one order of magnitude lower in 37°C and 4°C, and were undetectable in room temperature (see Fig. 4c). However, the cell densities in 37°C and 4°C under ampicillin exposure were approximately the same. As a conclusion, conjugative IncFII ESBL-plasmids can disseminate even in the lower temperatures and the impact of temperature on the dissemination depends on the β-lactam antibiotic in question. This result suggests that the dissemination of ESBL-plasmids can continue even in lower temperatures in environmental reservoirs with notable antibiotic pollution.

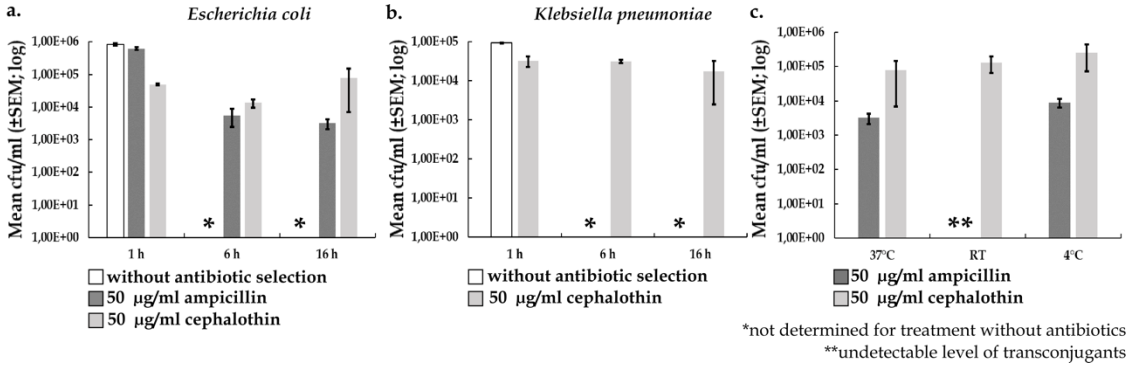

FIGURE 4 The mean cell density (cfu/ml; ±SEM) of *E. coli* (**a.**) and *K. pneumoniae* (**b.**) transconjugants after exposure 50 µm/ml of ampicillin or cephalothin antibiotic selection for 1 h, 6 h and 16 h. **c.** The mean cell density (cfu/ml; ±SEM) of *E. coli* transconjugants after exposure to 50 µg/ml ampicillin or cephalothin in three temperatures: 37°C, room temperature (RT) and 4°C. Three replicates (n=3) were done for each treatment.

The spheroplasts (gram-negative bacteria without cell wall) might explain the long antibiotic tolerance of the studied *Enterobacteriaceae* species. Both *E. coli* (Lederberg 1956) and *K. pneumoniae* (Nakao *et al.* 1981) are known to form spheroplasts by β-lactam induction. Without the cell wall, cells are immune to β-lactams which prevent the crosslinking of peptidoglycan (Walsh 2000). Also, spheroplasts have been isolated in human and animal samples with chronic

infections (Guze and Kalmanson 1964, Gutman *et al.* 1965) and therefore, both spheroplasts and L-forms of gram-positive bacteria, have been suggested to be partly responsible for chronic and persistent infections (Errington *et al.* 2016). In lower temperatures, such as 4°C, the decreased metabolic activity might also enhance the survival, as β-lactams are effective only against actively dividing cells. However, spheroplasts are highly sensitive to changes in osmotic pressure and unable to proliferate through cell division (Mercier *et al.* 2014), therefore, maintaining a stable population is difficult compared to cell wall containing bacteria. The conjugative resistance plasmids can rescue the spheroplast community by providing them antibiotic-resistance gene(s) and hence enabling the reversion back to cell wall containing phenotype and restoring the vegetative growth. It must be noted that microscopy studies are needed to confirm whether spheroplast-mediated β-lactam tolerance indeed explains these results. The difference in the survival under ampicillin and cephalothin selection might be due to the different binding affinities of these β-lactams to various PBPs (Curtis *et al.* 1979). For β-lactams to cause bactericidal effect and bacterial death, the essential PBPs (PBP1a, PBP1b, PBP2 and PBP3) for cell wall synthesis (Spratt 1975, Yousif *et al.* 1985) have to be saturated (Satta *et al.* 1995). Ampicillin can saturate all of these PBPs in lower concentrations than cephalothin, which only saturates PBP3 in low concentration (Curtis *et al.* 1979). Therefore, ampicillin can cause more rapid decrease in viable cell density. As PBSs of different enterobacterial species share homologs and functional similiarities (Curtis *et al.* 1979), the different binding affinities of these two β-lactams could explain the similar rescue results of *E. coli* and *K. pneumoniae*.

Overall, more research is required to understand evolutionary pressures affecting the evolution and dissemination of conjugative multi-resistance plasmids. In this phenomenon, the characteristics of the donor and recipient, their evolution and adaptation, as well as the evolutionary history of the plasmid, play a notable role. In heterogenous communities, the dispersal of conjugative multi-resistance plasmids is enhanced by effective enterobacterial donors (Dionisio *et al.* 2002). These donors can reach similar plasmid transfer frequencies between different *Enterobacteriaceae* species, as well as between the same bacterial species (Dionisio *et al.* 2002). Additionally, in clinal settings, the recipient with low-level resistance mutations might persist longer and, eventually, acquire plasmid-mediated resistance from the surrounding population, even in higher antibiotic concentrations (Komp Lindgren *et al.* 2003). The evolutionary outcome of the host range of a plasmid is dependent on whether the plasmid evolves within a single- or multi-host environment. Due to fitness trade-off, evolution selects for host-specialist plasmids in a single-host environment (Kottara *et al.* 2016), whereas in a multi-host environment, these trade-offs can be circumvented, resulting in the selection of host-generalist plasmids (Kottara *et al.* 2016). Therefore, a diverse bacterial community might select for plasmids with a broad host-range (Kottara *et al.* 2016), and thus, the evolutionary rescue potential of our plasmids may be affected by the co-evolution with their previous hosts. For this reason, our results may be applicable only in the hosts employed in the study. Our plasmids' conjugation efficiency might also vary between different

donors and recipients, which would be an interesting quality to determine in future studies. Furthermore, the ability of plasmids to modify their host range can affect their evolutionary rescue potential. Shufflons, the site-specific recombination system in IncI1 plasmids, can alter host specificity by generating variability in the C-terminus of the adhesin protein PilV (Komano *et al.* 1994, Komano 1999). This protein is a component of the tip of a pilus, which binds to the LPS of the recipient's cell membrane (Komano 1999). Moreover, the integration of transposon into high-frequency transfer (HFT) regions of an IncI1 encoding EBSL-resistant gene (pESBL) has been shown to enhance the plasmid's transfer rate via conjugation by ~20-fold compared to wildtype (Yamaichi *et al.* 2015). The HFT region resides between the conjugal transfer protein TraA and the replication initiator protein gene (Yamaichi *et al.* 2015). According to the BLASTn alignment, all the IncI1s in this study were found to contain the ~270 bp HFT region in the same site as in the IncI1 plasmid studied by Yamaichi *et al.* (2015). HFT region 35 394-35 663 bp of pESBL (Genbank: NC_018659.1) was aligned with all the pEC plasmid sequences with BLASTn. However, the only IncI1 plasmid encoding ESBL-resistance gene was pEC16I. As the HFT region has also been discovered in some IncFII plasmids (Yamaichi *et al.* 2015), it would be interesting to study whether the HFT region could explain the higher evolutionary rescue potential of pEC16 in the higher antibiotic concentration compared to IncFII with an ESBL-resistant gene. The IncFII plasmids studied here did not have the HFT region.

Altogether, this study demonstrates that evolutionary rescue via HGT occurs frequently within a simple study set-up where one donor and one recipient strain are employed. These events potentially take place during antibiotic treatments within human-associated microbiomes, such as gut microbiota. Therefore, future research directions should involve the investigation of evolutionary rescue via HGT in complex, multi-species microbial communities, for example via experimental evolution. Furthermore, HGT affects the dispersal of multi-resistance elements in farms and sewage, environments where varying (but often sub-inhibitory) antibiotic concentrations are commonly present (Andersson and Hughes 2012). Therefore, there is great interest in developing approaches that allow the elimination of plasmids, for example ESBL-encoding ones, within bacterial communities such as gut microflora.

4.2 The conjugative CRISPR-Cas9 system can remove the ESBL-phenotype by cutting the β-lactamase gene (III)

ESBL-carriage, asymptomatic carriage of ESBL-resistant bacteria within gut microflora, is a significant problem as it enables the spread of resistance genes to new bacterial hosts, even under lethal antibiotic concentrations as demonstrated in the previous section. Therefore, it appears beneficial to eradicate bacteria carrying ESBL-encoding plasmids or the plasmids themselves.

CRISPR-Cas9 provides a potential solution to try cure ESBL-carriage by re-sensitizing ESBL-bacteria to β-lactams. In this study, we developed a conjugative CRISPR-Cas9 system and examined its ability to delete ESBL-resistance genes from a bacterial population. We programmed the system to target conjugative ESBL plasmids (pEC13 and pEC15), originating from bacteria introduced in part I. The developed system was named midbiotic and consisted of two plasmids: the conjugative IncP plasmid, RP4, designated as the delivery plasmid, and the mobilised plasmid encoding the *Streptococcus pyogenes* CRISPR-Cas9 system, referred to as pCRISPR plasmid (Jiang *et al.* 2013). The pCRISPR plasmid encoded all the components of CRISPR-Cas9 needed for sequence-specific targeting: tracrRNA, cas9, and the leader and CRISPR locus with crRNA(s) targeting ESBL gene(s). Enabling horizontal transfer via conjugation by the relaxosome of RP4, part of the RP4 oriT site, was cloned into the pCRISPR plasmid (Pansegrau *et al.* 1988, Bates *et al.* 1998). The crRNAs were designed to target a conserved site of either the β-lactamase gene blaTEM or blaCTX-M. The conserved regions were determined by aligning all the known gene variants of both gene classes separately. The sequence that was located next to PAM and had the broadest coverage among the gene variants was selected as crRNA. The selected sequence of blaTEM crRNA covered the majority of the *blaTEM* gene variants used in the alignment, whereas slightly less than half of the *blaCTX-M* genes contained the conserved crRNA target site. This variation can be explained by the *blaCTX-M* gene class' high recombination rate (Novais *et al.* 2006, Cantón *et al.* 2012). However, in practice, the target coverage could be even wider than the alignment suggests as the 5'-end of the crRNA can contain some mismatches in the complementary sequence without affecting the efficacy of Cas9 (Jiang *et al.* 2013). We constructed a mobilisable pCRISPR plasmid with crRNA targeting only *blaTEM*, referred to as pCRISPR-crRNA, and pCRISPR with multiple crRNAs targeting both *blaTEM* and *blaCTX-M,* referred to as pCRISPR-multi-crRNA. pCRISPR-control was otherwise similar to the pCRISPR plasmid but without targeting-crRNA. The delivery plasmid was prevented from self-targeting by deleting the conserved target site from *blaTEM-2* of RP4.

We investigated the effectiveness of midbiotics *in vitro* by conjugating pCRISPR-crRNA into ESBL-producing recipient bacterium harbouring *blaTEM-52b* (in pEC15), which provides resistance to various β-lactams, including ampicillin. After 24 hours, the cell density of transconjugants carrying pCRISPR-crRNA (3×10^4 cfu/ml) was almost four orders of magnitude lower than the cell density of control cells without crRNA (pCRISPR-control; $7{,}7\times10^7$ cfu/ml) under ampicillin selection. To rule out uneven conjugation efficiency in pCRISPR-crRNA and pCRISPR-control that could explain this result, the plasmids were conjugated in a similar manner into an ESBL-free recipient strain. Both plasmids conjugated evenly, producing ~10^7 colony-forming units/milliliter (cfu/ml) of transconjugants over a 24 h observation period. When transformed into recipient bacterium carrying either *blaTEM-52b* or *blaCTX-M-14*, pCRISPR-multi-crRNA caused a ~2.5 orders of magnitude decline in the cell density of transformants (*blaTEM-52b*: $5{,}6\times10^2$ cfu/ml, *blaCTX-M-14*: $8{,}3\times10^2$ cfu/ml) compared to controls (*blaTEM-52b*: $5{,}6\times10^5$ cfu/ml, *blaCTX-M-14*: $3{,}3\times10^5$ cfu/ml). This

indicates that by combining several crRNAs into the same CRISPR locus, a single pCRISPR plasmid can be used to target several different ESBL-producing bacteria. The fold changes in the cell density of midbiotic-treated cells from both conjugation and transformation assays were in line with previous studies where CRISPR-Cas9 has been used to eradicate multi-resistance plasmids from bacterial communities (Citorik *et al.* 2014, Bikard *et al.* 2014).

Despite the midbiotic treatment, some ESBL-producing pCRISPR-transconjugants retained resistance. A closer examination of these escape mutants from both experiments revealed a complete or partial deletion of the CRISPR locus from the pCRISPR plasmid. The spontaneous deletion of spacer sequences was also the main cause of failed CRISPR-Cas9 activity in other studies (Citorik *et al.* 2014). This could be avoided by cloning several CRISPR loci into the pCRISPR plasmid. Additionally, some of the escape mutants transformed with pCRISPR-multi-crRNA had an intact CRISPR locus. Tolerance against midbiotics, in these cases, was probably due to a mutation in other parts of the CRISPR-Cas9 system, for example, in the tracrRNA site or *cas9* gene (Citorik *et al.* 2014), or in the targeted resistance genes.

We further studied the dispersal of midbiotic plasmids in the bacterial population after 72 h. We found that the delivery plasmid began to conjugate independently without the pCRISPR-crRNA plasmid as the density of cells with a pCRISPR plasmid ($3{,}0\times10^6$ cfu/ml) was two-fold lower compared to the density of cells with a delivery plasmid ($1{,}8\times10^8$ cfu/ml). Ninety colonies harbouring either the delivery or pCRISPR plasmid were further examined to study the presence of the other midbiotic plasmid. All the isolates with the delivery plasmid had lost the pCRISPR plasmid, whereas all those isolated with a pCRISPR plasmid contained the delivery plasmid. This independent dispersal of the delivery plasmid could possibly be prevented by combining the two plasmids into one or dividing the TA system between the two midbiotic plasmids: the toxin being encoded by the delivery plasmid and the antitoxin by pCRISPR. The dispersal of only the delivery plasmid without pCRISPR would lead to a cytotoxic response and cell death in the recipient cell (Yang and Walsh 2017).

In a previous study, genes encoding conjugation machinery mediating the CRISPR-Cas9 delivery were integrated into the chromosome of the donor bacterium (Citorik *et al.* 2014). Our approach, which utilises the complete conjugative plasmid, allows the system to transfer to the stable resident flora and, thus, remain in the bacterial community regardless of the survival of the initial donor bacterium. In addition to being a well-studied plasmid (Adamczyk and Jagura-Burdzy 2003), the benefits of utilising RP4 as a delivery plasmid are its wide host range (Schmidhauser and Helinski 1985, Popowska and Krawczyk-Balska 2013) and the low prevalence of IncP plasmids among clinical bacterial isolates (Carattoli 2009). If IncP plasmids were common among the resistant bacteria, the entry exclusion could diminish the system's efficacy in the targeted bacterial communities. Compared to phage-mediated delivery, conjugation is slower, but its advantage is independence from cell-surface receptors, which are required for successful phage entry. Furthermore, bacteria easily develop

resistance to phages by masking or losing receptors, and hence, also prevent the CRISPR-Cas9 system from being introduced into the targeted cell.

The preferable application of midbiotics could be as a preventive eradication of the undesirable and harmful genes from the bacterial population rather than as an antimicrobial treatment. However, there are also risks to be considered when using this kind of a plasmid system. One of the risks is that the delivery plasmid acquires and spreads unwanted genes. However, it must be noted that any targeted bacterial community is very likely to already contain various MGEs with resistance genes (Jernberg *et al.* 2007, Sommer *et al.* 2009, Hu *et al.* 2013, Forslund *et al.* 2013, Pérez-Cobas *et al.* 2013). Nevertheless, if the midbiotic system is to be introduced into the target system *in vivo,* the delivery plasmid's resistance or virulence genes should be deleted, and plasmid-dependent phages could be used to eradicate the midbiotic system from the bacterial community after the eradication of targeted resistance genes (Ojala *et al.* 2013).

In conclusion, the nearly four orders of magnitude decline in the cell density of two different ESBL bacteria indicates that midbiotics are a possible option for developing applications that seek to eradicate or prevent the spread of ESBL plasmids in bacterial communities. By targeting the conserved sequences, a single crRNA can be used to cleave multiple variants of the same gene class. The conserved target sites of crRNAs for *blaTEM* and *blaCTX-M* genes contain the nucleotide codon encoding the conserved amino acids 107 and 264, respectively (Philippon *et al.* 2016). Conserved sites are good targets because they mostly encode the essential parts for protein function, such as substrate binding or catalytic activity (Philippon *et al.* 2016). Bacteria primarily repair double-strand DNA breakage via homologous recombination (Cubbon *et al.* 2018). However, at least *Pseudomonas aeruginosa* and *E. coli* have alternative repair mechanisms called non-homologous end joining (NJEH) (Zhu and Shuman 2005) and alternative end-joining (A-EJ) (Chayot *et al.* 2010), respectively. A-EJ is an error-prone repair system and is associated with insertion and deletion mutations (Chayot *et al.* 2010). Thus, although the bacterium may be able to repair double-strand DNA breakage, the resulting mutation may still lead to the production of defective β-lactamase. Further studies are needed to examine the efficacy and dissemination rate of midbiotics in more complex bacterial systems. For *in vivo* experiments, a midbiotic system could be conjugated into bacterium isolated from the commensal gut flora of the patient and delivered back to the gut as a midbiotic-harbouring probiotic. Thus, the eradication of the introduced bacterium would be unnecessary, and the midbiotic system could disperse in the microflora more efficiently (Lawley and Walker 2013, Pamer 2016). Bacteriophages could be utilized to increase the efficacy of midbiotics by reducing the number of primary ESBL-plasmid hosts. As such, the effect of occasionally emerged spacer lacking midbiotics and the transfer of the ESBL-plasmid to unconventional hosts would be minimized. Nevertheless, phages could be harnessed also alone as prophylactic treatment to eradicate ESBL-resistant bacteria from the gut microflora. In the next section I explore the feasibility of this approach.

4.3 The successful isolation of new phages for *Enterobacteriaceae* associated with ESBL carriage (IV)

In addition to conjugative CRISPR-Cas9 system, premade phage cocktails would be a convenient way to eradicate ESBL bacteria from the gut. However, such cocktails are unlikely to be available for all ESBL carriers due to the unique diversity of the gut's bacterial communities (Turnbaugh *et al.* 2010). Therefore, new phages could be isolated as needed to personalise phage therapy for individual communities. To evaluate the potential of on-demand phage isolation as a way to respond to ESBL carriage in the gut, we conducted an experiment covering the isolation success rate of new phages for two different bacterial genera: *Escherichia* and *Klebsiella*. Both these genera carry multi-resistance elements, and their colonisation in the gut has been associated with bacterial infections in humans (Rychlik and Barrow 2005, Aloush *et al.* 2006, Dubinsky-Pertzov *et al.* 2018). Two preliminary settling tanks in a wastewater treatment plant in Nenäinniemi (Jyväskylä, Finland) were used as the environmental source for new phages. Because these bacteria are abundant in sewage (Rizzo *et al.* 2013), and the co-occurrence of bacterial hosts and their phages is tightly linked (Drulis-Kawa *et al.* 2012), wastewater treatment plants are considered a convenient source of phage isolation.

Isolation was successful for *Enterobacteriaceae* associated with ESBL carriage. In 35 isolation attempts, the isolation success rate was 90.6 % for *Escherichia coli* (Table 3). Similarly, a new phage was isolated in almost every attempt for *Klebsiella pneumoniae*: the isolation success rate was 83.3 % in 15 isolation attempts (Table 3). The high isolation frequency is unsurprising as phages for these strains have been successfully isolated from sewage in the past (Debarbieux *et al.* 2010, Hung *et al.* 2011, Santos *et al.* 2011, Merabishvili *et al.* 2012). However, between these two species, there were a few strains for which we were unable to isolate phages. To our knowledge, no previous studies have covered the topic of the success rate of isolating new phages to studied *Enterobacteriaceae* species and how many isolation attempts are needed to find one. Instead of a wastewater treatment plant, the human gut could be used as an alternative isolation source, but it might not be a convenient solution because finding suitable donors is laborious, and a metagenomic study suggests that temperate phages dominate the human gut (Reyes *et al.* 2010). For example, the isolation of coliphages from the feces of healthy persons has revealed that the human gut contains primarily temperate coliphages with low titers (Furuse *et al.* 1983), although lytic coliphages with higher titers have been isolated from the samples of ill human patients (Furuse *et al.* 1983). Lytic coliphages have also been shown to be abundant in the horse gut (Clokie *et al.* 2011). The abundance of temperate phages might be explained by evolution in a complex habitat. A human gut provides a habitat with a complex interplay between bacterial hosts, phages, the environment, and the immunologic defence of eukaryotic hosts, which tend to select for lysogenic lifeforms to prevent the extinction of the phage

(Poulsen *et al.* 1995, Brüssow 2009, Clokie *et al.* 2011). The mutualistic lifestyle of prophages benefits the host by providing new metabolic, morphological, or immunogenic traits, as well as resistance to homologous phages (Howard-Varona *et al.* 2017). Nevertheless, as *E. coli* and *K. pneumoniae* are common constituents in ESBL carriage, it appears possible to utilise on-demand isolation as a means to find phages that could help cure carriage.

TABLE 3 The probability of isolating of a new phage (a.) or cross-infecting phage (b.) for *Escherichia coli* or *Klebsiella pneumoniae* pathogens. Also the percentage of cross-infecting phages, which infect three or more alternative bacterial strains, was calculated.

Bacterial pathogen	a. mean hit for isolating a new phage*	b. mean hit% for isolating a cross-infecting phage*	Percentage of cross-infecting phages (3 or more)**	Isolation attempts	Number of strains hit
Escherichia coli	90.6%	78.1%	55.2%	35	15/16
Klebsiella pneumoniae	83.3%	61.7%	0.0%	15	6/6

*as calculated over the bacterial strains of given species
**calculated according to spot test with clear lysis

In phage therapy, phages with a broad host-range are preferred; their pharmacology can be determined more quickly compared to a multi-phage cocktail because the pharmacology must be determined for each phage individually. The probability of isolating a cross-infecting phage against *E. coli* was nearly 80% and somewhat lower (60.1%) for *Klebsiella* pathogens (Table 3). The host range of the obtained phages against other bacterial strains of the same species was determined via spot tests (see Fig. 5). The host range of coliphages was moderate (55,2% of the isolated phages infected three or more bacterial strains), whereas, for the phages infecting *Klebsiella*, it was narrow (Table 3). Two coliphages (EC6P1 and EC6P2) were able to infect over half of the hosts (9 of 16 hosts) (see. Fig. 5). Further research is needed to determine whether they are the same phage. In contrast, none of the *Klebsiella* phages were able to infect three or more bacterial strains and only three of the isolated phages (KP1P2, KP1P4, and KP2P1) were able to clearly infect two of the six bacterial hosts (see. Fig. 5). Most were only able to slightly inhibit the growth of one additional bacterial host. KP1P5 and KP4P1 were exceptions as they were able to weakly inhibit the growth of two other hosts.

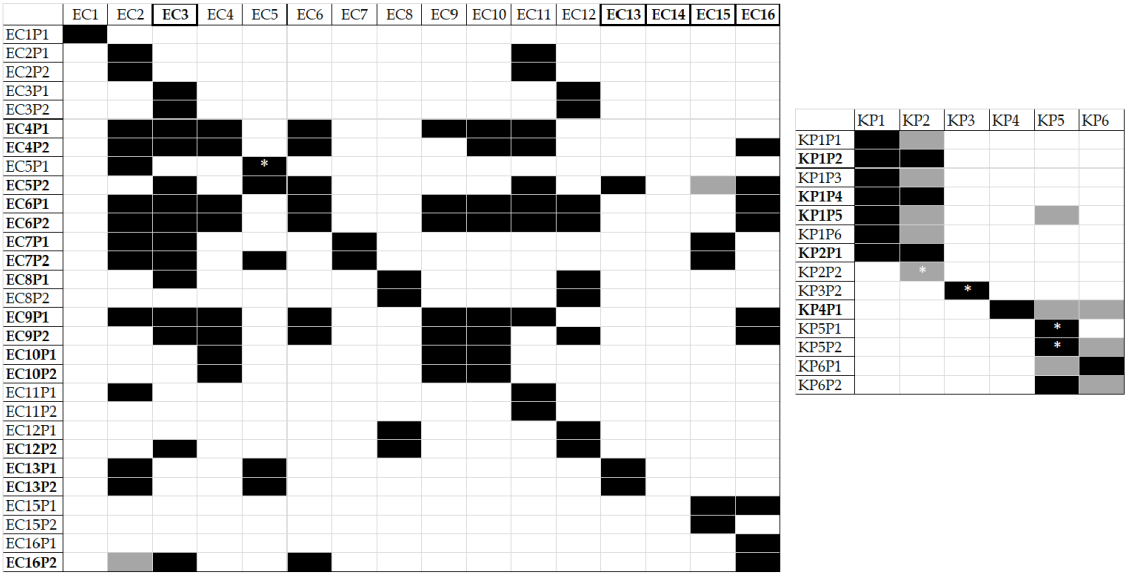

FIGURE 5 The cross-infectivity of isolated phages infecting *Escherichia coli* (EC) and *Klebsiella pneumoniae* (KP). The host strains are indicated in the horizontal axis and the phages in vertical axis. The clear lysis is presented with black colour and the dim lysis with grey background. The white background indicates no lysis. The spot test measurements conducted with phage stocks with less than 10^5 pfu/ml are marked with an asterisk. The bolded name present coliphages which can infect three or more bacterial strains. The bolded *Klebsiella* phages infect one additional host besides their original host used for the isolation.

A phage cocktail can be constructed to attempt the eradication of the *E. coli* strains harbouring the ESBL-plasmid described in study I. The potential phages for constructing this prophylactic phage cocktail are presented in the Table 4. As an example, in addition to EC16P1 and EC16P2, several phages of other *E. coli* hosts (EC4P2, EC5P2, EC6P1, EC6P2, EC9P1, EC9P2, and EC15P1) were able to infect EC16 bacteria and could be used in the phage cocktail against the host of plasmid pEC16. Eliminating these bacteria could be an important step in inhibiting the dispersal of multiresistant plasmids in bacterial communities present, for example, in the gut.

TABLE 4 The potential phages for prophylactic phage cocktail treating *E. coli* strains (EC) carrying conjugative ESBL-resistance plasmids (pEC).

ESBL-resistant *E. coli* strain	Infective phages						
EC3 (pEC3)	EC3P1	EC3P2	EC4P1	EC4P2	EC5P2	EC6P1	EC6P2
	EC7P1	EC7P2	EC8P1	EC9P1	EC9P2	EC12P2	EC16P2
EC13 (pEC13)	EC5P2	EC13P1	EC13P2				
EC14 (pEC14)	no infective phages isolated						
EC15 (pEC15)	EC7P1	EC7P2	EC15P1	EC15P2			
EC16 (pEC16)	EC4P2	EC5P2	EC6P1	EC6P2	EC9P1	EC9P2	EC15P1
	EC16P1	EC16P2					

However, spot testing can overestimate the host range and provide false positive results when lysis occurs due to either the absorption of a high number of phages into the cell or residual endolysins or bacteriocins in the phage lysate (Brüssow 2014). To minimise the absorption of a high number of phages and control the appearance of plaque due to endolysins, we used a diluted phage stock for the spot tests. Hence, clear plaques are likely to be due to phage replication, and without sequencing the genome of both the phage and the bacterial host, we could not rule out the possibility of isolating the same phage multiple times or having several identical host strains. The sequencing of phage genomes is also necessary to exclude temperate phages, which are considered less suitable for phage therapy due to their potential to transmit virulence or resistance genes (Loc-Carrillo and Abedon 2011). Also, the small sample size can skew the statistics of the probability of isolating a new or cross-infecting phage as well as the percentage of cross-infecting phages.

Optimally, phages suitable for therapeutic use remain stable in storage and produce high titers. The enrichment method employed in this study, a modified two-step phage isolation protocol (Van Twest and Kropinski 2009), is biased towards fast propagating phages (Dunbar *et al.* 1997), possibly ruling out phages with a longer latent period with a larger burst size (Mirzaei and Nilsson 2015). Bottlenecks caused by plaque-picking to purify a phage clone also narrows the selection of potential phages. Nevertheless, to evaluate the stability of the isolated phages, titers (plaque-forming units/ml, pfu/ml) of the obtained phage stocks were verified immediately after stock preparation and after one month of storage at +4°C. Phages with both low and high titers were isolated for all bacterial species, and the highest titers of the phage stocks reached the same densities used in phage therapy cocktails, i.e. 10^9-10^{12} pfu/ml (Abedon 2016). An average 0.5 log reduction was seen after a month of storage with the phages of studied bacterial hosts. However, it should be noted that the phage stocks' stability is dependent on the storage buffer and temperature (Skurnik *et al.* 2007), but their optimisation was not within the scope of this thesis.

In conclusion, personalising prophylactic phage therapy by on-demand phage isolation is a potential option for treating the carriage of ESBL-producing *E. coli* and *K. pneumoniae*. However, further improvements are needed so that phages can maintain their stability and high titers in hostile environments, such as the gastrointestinal tract. For example, the tannic acids of rumen have been shown to inhibit phage propagation (Letarov and Kulikov 2009), and the absorption of coliphages into the bacterial host has been observed to be impaired by bile salts and carbohydrates (Gabig *et al.* 2002). The encapsulation and antacids, such as calcium carbonate and bicarbonate, protect phages from the gut's low pH level (Stanford *et al.* 2010, Colom *et al.* 2015, Colom *et al.* 2017). Several studies conducted using variable combinations of different encapsulation materials and methods have shown that they can prevent the decrease of various phages' titers (Stanford *et al.* 2010, Colom *et al.* 2015, Colom *et al.* 2017). Encapsulation also enables a controlled, stable release during the degradation of the capsule material (Malik *et al.* 2017), which further helps the phage titers to

remain longer within the therapeutic range (Singla *et al.* 2015). However, the optimisation of encapsulation for different phages is laborious, and various methods are needed for different phages (Malik *et al.* 2017).

Finally, the two methods described in this thesis, namely ESBL-strain specific phage therapy and conjugative delivery of anti-ESBL CRISPR-system, can also be utilized together. As phage therapy causes selection pressure against specific ESBL bacteria, it might cause the conjugative multi-resistance plasmids to escape to new bacterial hosts. Given that in this case the number of new ESBL hosts may be relatively low, the co-administration of broad-spectrum midbiotic may lead to more complete elimination of the plasmids from the remaining phage-resistant ESBL-positive bacterial community. For example, ESBL carriage in the human gastrointestinal tract could be eradicated by using the combination therapy (see Fig. 6). Naturally, the selected therapeutic phages should be non-infectious to the chosen midbiotic bacterial vector in order for the system to be compatible with the tandem-delivery.

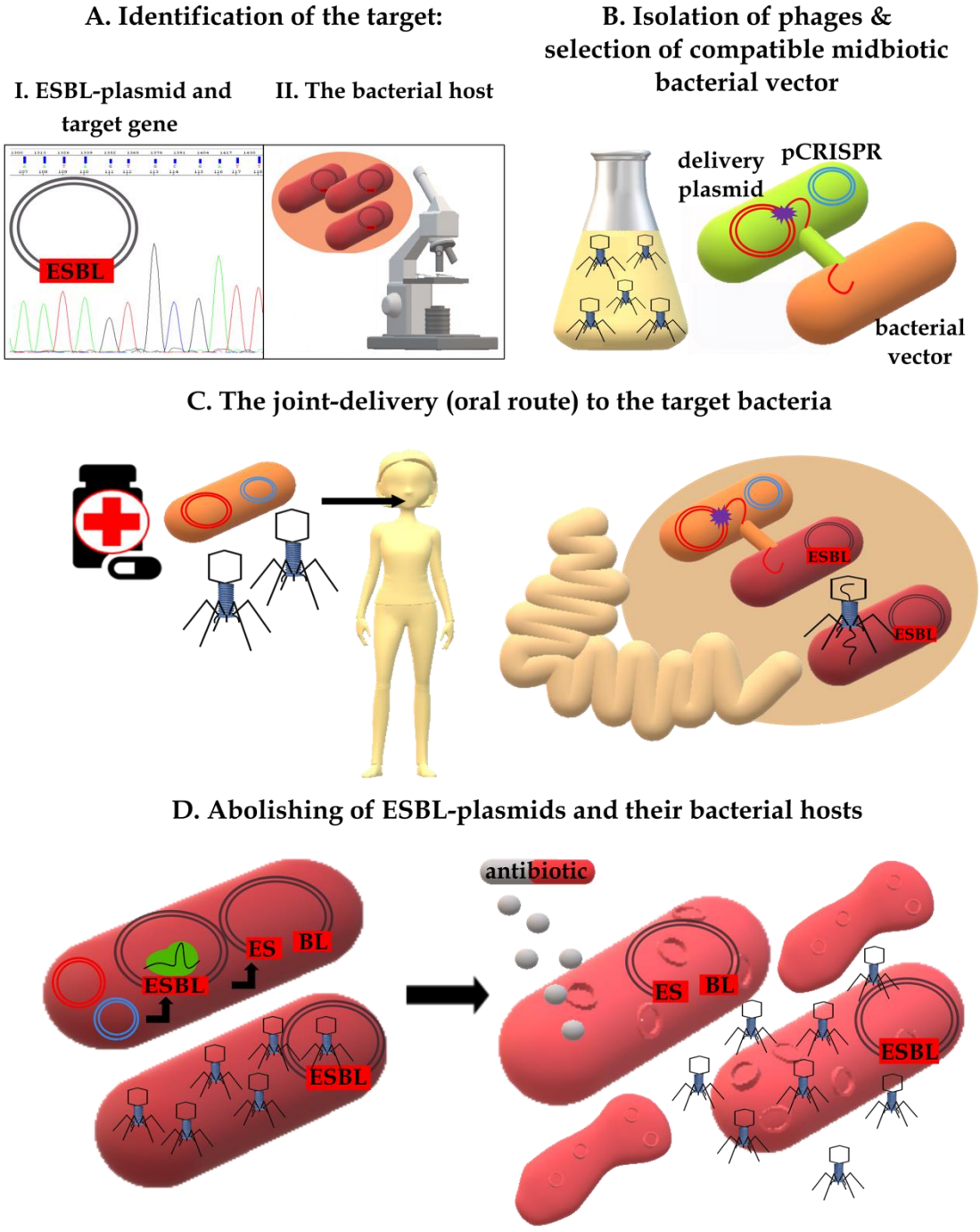

FIGURE 6 An example workflow for the combination therapy to cure ESBL-carriage. **A.** First, the targeted antibiotic resistance gene and the plasmid encoding the gene, as well as the bacterial host carrying the plasmid, are identified. Based on the identification, the suitable crRNA(s) and the bacterial vectors can be selected. **B.** Phages are isolated on-demand for personalised phage therapy and, the midbiotic plasmids (red=delivery plasmid, and blue=pCRISPR) are conjugated for the best-suited bacterial vector. **C.** Encapsulated phages and the midbiotic are delivered together to the gut via oral administration. The phages inject their genome into the targeted bacteria, and the bacterial vector conjugates the therapeutic midbiotic into the antibiotic-resistant cells **D.** The targeted bacteria are largely killed by phages when the release of phage progeny erupts the cell. Additive effect is achieved when the midbiotic treatment re-sensitises the remaining phage-resistant ESBL bacteria to beta-lactams.

5 CONCLUSIONS

The presence of conjugative or mobilisable ESBL plasmids in commensal bacterial flora can compromise the treatment of antibiotic-susceptible pathogens. This is due to the interbacterial transfer of resistance plasmids, an evolutionary rescue mechanism that has not received much attention from researchers but may save pathogens even after they are exposed to lethal β-lactam concentrations (I and II). Because ESBL carriage—an asymptomatic colonisation of ESBL bacteria in the gut—has become more common, the applicability of β-lactams in treating infections is diminishing. Interestingly, however, HGT can also be turned against ESBL bacteria. In this study, an RP4-mobilisable CRISPR-Cas9 plasmid, which efficiently cleaves different ESBL genes, resensitises bacteria to β-lactams (III). In the future, these CRISPR-systems could be incorporated into resistance-free conjugative plasmids that disseminate within commensal bacterial flora and clear potential ESBL genes in the process. However, optimisation is needed to avoid crRNA deletion. One potential method is the incorporation of multiple crRNA arrays into the midbiotic plasmid. Additionally, separation of the 'curing' CRISPR plasmid from the conjugative plasmid poses potential risks as the conjugative plasmid may disseminate on its own. There are also different solutions to this problem, such as the separation of toxin-antitoxin systems between midbiotic plasmids. Altogether, conjugation is an interesting and, currently, mostly unexplored mechanism used to genetically tinker with bacterial communities *in situ*. Nonetheless, this study further demonstrates that isolating bacteriophages against the most common ESBL-pathogens, such as *E. coli* and *K. pneumonia*, is relatively easy (IV). These phages could be harnessed as selective agents to kill commensal ESBL bacteria in the gut and, thus, cure ESBL-carriage. Ultimately, this thesis argues that, in the end, we can use both 'forgotten' (i.e., phage therapy) and state-of-the-art techniques (e.g., CRISPR-Cas9) to help resolve the antibiotic resistance crisis.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Laajakirjoista β-laktamaasia tuottavien enterobakteerien aiheuttamat riskit antibioottihoidon aikana ja näiden enterobakteerien kantajuuden poistaminen uusilla potentiaalisilla menetelmillä

Antibiootit ovat modernin lääketieteen kulmakivi ja ne ovat mahdollistaneet monen lääketieteellisen menetelmän kehittymisen, kuten elinsiirrot ja esimerkiksi aggressiiviset leukemiahoidot. Näiden hoitojen aikana ihmisen oma immuunipuolustus alenee, mikä altistaa potilaan erinäisille infektioille, jotka terveelle ihmisille olisivat vaarattomia. Vuosikymmenten antibioottien valtava ja joskus tarpeeton käyttö sekä terveydenhuollossa että karjataloudessa on johtanut antibioottivastustuskykyisten bakteerien räjähdysmäiseen kasvuun. Erityisen huolestuttavaa on terveiden kantajien määrän kasvu. Kantajilla viitataan oireettomiin ihmisiin, joiden suolistoon on kolonisoitunut laajakirjoista β-laktamaasia tuottava enterobakteeri (mm. *Escherichia coli* ja *Klebsiella pneumoniae*). Tämä mahdollistaa nopeamman antibioottivastustuskyvyn leviämisen sekä suoliston normaalifloorassa, että toisiin ihmisiin tai eläimiin. Lisäksi monien antibiooteille vastustuskykyisten bakteeri-infektioiden hoitovaihtoehdot ovat kaventuneet, ja ilman uusien antibioottien tai vaihtoehtoisten hoitomenetelmien kehittämistä palaamme aikaan ennen antibiootteja. Tällöin bakteeri-infektioihin kuolleisuus oli suurta. Mittavien ihmishenkien menetysten lisäksi tämä kaikki kuormittaisi valtavasti taloutta. Ongelman ratkaisemiseksi ja tilanteen kontrolloimiseksi tarvitaan sekä kansainvälisiä että valtakunnallisia toimia, joilla pystytään estämään antibiooteille vastustuskykyisten bakteerien leviäminen sekä kehittämään uusia hoitomuotoja.

Antibioottivastustuskyvyn tuottavat geenit sijaitsevat useimmiten konjugatiivisissa plasmideissa, jotka ovat DNA-renkaita ja erillisiä bakteerigenomista. Lisäksi ne pystyvät itse ohjaamaan siirtymistään bakteerista toiseen niin kutsutun pariutumis-kanavan läpi. Tätä kutsutaan konjugaatioksi. Yksi konjugatiivinen plasmidi voi tuottaa vastustuskyvyn useita eri antibiootteja vastaan, eli on niin sanotusti moniresistentti. Juuri moniresistenttien konjugatiivisten plasmidien leviäminen jopa eri bakteerilajien välillä, on mahdollistanut nopean antibioottivastustuskyvyn tuottavien geenien leviämisen ympäri maailman. Tätä siirtymistä bakteerien välillä kutsutaan horisontaaliseksi geenien siirroksi. Tämän vuoksi on tärkeää ymmärtää mitkä ekologiset ja evolutiiviset tekijät vaikuttavat näiden plasmidien leviämiseen koko ekosysteemin läpi, maaperästä lihakarjaan ja aina ihmiseen asti. Uusia menetelmiä tarvitaan pikaisesti konjugatiivisten moniresistenttiplasmidien leviämisen kontrolloimiseksi, moniresistentti bakteerien poistamiseksi normaalifloorasta ja kyseisten bakteeri-infektioiden hoitamiseksi.

Tämän väitöskirjatyön ensimmäisessä osatyössä tutkin sairaalabakteereista eristettyjen moniresistenttien plasmidien siirtymistä bakteereihin letaalin β-laktaami—antibioottikäsittelyn aikana. Osa moniresistenttiplasmideista pystyi pelastamaan antibiooteille alttiit bakteerit siirtämällä niihin moniresistenttiplas-

midin, jolloin alttiista bakteereista tuli antibiooteille vastustuskykyisiä. Erilaisten moniresistenttiplasmidien siirtymistehokkuuksien välillä oli kuitenkin eroja, mutta osa pystyi pelastamaan alttiit bakteerit jopa kaikkein korkeimmissakin antibioottipitoisuuksissa. Lisäksi toisessa osatyössäni selvisi, että konjugatiivisten moniresistenttiplasmidien pelastustehokkuuteen vaikuttaa käytetty β-laktaami antibiootti sekä kasvulämpötila. Tällainen plasmidien siirtyminen bakteerien välillä voi tuhota antibioottihoidon vaikutuksen ja johtaa uusien moniresistenttien bakteerikantojen muodostumiseen ja pitkittyneisiin antibioottihoitoihin. Konjugatiivisten plasmidien leviämisen ja uusien moniresistenttien kantojen muodostumisen voisi estää käyttämällä hyödyksi bakteerien omaa immuunipuolustuskeinoa nimeltään geenisakset (CRISPR-Cas9). Kolmannessa osatyössäni muokkasin geenisakset tuhoamaan monia eri β-laktaami-antibiooteille vastustuskyvyn antavia geenejä. Menetelmällä geenisakset voitiin siirtää konjugoimalla ne moniresistentin bakteerin sisään, missä ne katkaisivat antibioottivastustuskyvyn antavan geenin, jolloin bakteereista tuli taas alttiita β-laktaami-antibiooteille. Näin bakteerit pystyttiin taas tappamaan β-laktaameilla, mutta samalla estettiin moniresistenttiplasmidin leviäminen, koska useimmiten plasmidin katkaisu johtaa plasmidin tuhoutumiseen bakteerin sisällä.

Moniresistenttien bakteeri-infektioiden hoitoon tai poistamiseksi suoliston normaalifloorasta voitaisiin käyttää myös faagiterapiaa, hoitomuotoa, jota on käytetty jo 1900-luvun alussa ennen antibioottien löytymistä. Faagiterapiassa käytetään bakteeriviruksia, eli faageja, jotka infektoivat ainoastaan bakteereja. Faagit ovat bakteerien luonnollisia vihollisia ja aivan kuten kausittainen influessa-virus, se voi muuntua loputtomasti. Näin ollen, vaikka bakteerit tulisivat vastustuskykyisiksi faagille, faagi voi muuntautua ja uudestaan infektoida kyseistä bakteeria. Lisäksi yksittäinen faagi voi infektoida useimmiten vain yhtä bakteerilajia, joten toisin kuin antibiootit, se ei tuhoa muuta suolistomikrobistoa vaan ainoastaan halutun kohdebakteerin. Faagit ovat runsaslukuisin kaikista biologisista organismeista ja onkin arveltu, että jokaista bakteerisolua kohden on kymmenen sitä infektoivaa faagia. Teoriassa tämä tekee faageista ehtymättömän hoitomuodon bakteereja vastaan. Viimeisessä osakokeessani tutkinkin, miten helposti erilaisille moniresistenteille bakteerilajeille löytyy faageja jätevesinäytteistä ennaltaehkäisevää faagiterapiaa varten. Erityisesti laajakirjoista β-laktamaasia tuottavat *E. coli* ja *K. pneumoniae* enterobakteerit on yhdistettty suolistoflooraan kolonisoitumiseen ja siitä aiheutuviin infektioihin. Näiden bakteerien poistaminen normaalifloorasta ehkäisee myös antibioottivastustuskyvyn leviämisen mikrobifloorassa. Faageja eristettiin jätevedestä, koska niiden tiedetään sisältävän paljon faageja. Moniresistenteille *E. coli* ja *K. pneumoniae* bakteereille löytyi lähes jokaisella eristyskerralla uusi faagi. Kuitenkin niitä tilanteita varten milloin uusia faageja ei saada eristettyä, olisi hyvä luoda kansainvälinen faagi-kirjasto, josta voisi tilata kyseistä bakteeria infektoivan faagin. Faagiterapian kehittäminen hoitomenetelmäksi on kuitenkin vielä alkutekijöissään, joten uusien hoitomuotojen nopeaan kehittämiseen tarvitaan merkittäviä taloudellisia sijoituksia ja poliittista tahtoa.

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ORIGINAL PAPERS

I

CONJUGATIVE ESBL-PLASMIDS DIFFER IN THEIR POTENTIAL TO RESCUE SUSCEPTIBLE BACTERIA VIA HORIZONTAL GENE TRANSFER

by

Sari Mattila, Pilvi Ruotsalainen, Ville Ojala, Timo Tuononen, Teppo Hiltunen & Matti Jalasvuori 2017

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Conjugative ESBL-plasmids differ in their potential to rescue susceptible bacteria via horizontal gene transfer in lethal antibiotic concentrations

Running title: Evolutionary rescue of bacteria via conjugation

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Description

Emergence (and proliferation) of resistant pathogens under strong antibiotic selection is an evolutionary process where bacteria overcome the otherwise growth inhibiting or lethal concentration of antimicrobial substances. In this study, we set to investigate a largely unexplored mechanism, namely evolutionary rescue (i.e. adaptive evolutionary change that restores positive growth to declining population and prevents extinction) via horizontal gene transfer, by which new resistant bacteria may emerge both in and out of clinical environments.

At sufficiently high concentrations, the likelihood of pre-existing single-step resistant mutants to exist in the population is very low, hence therapies aim to maintain the drug-levels above this so-called "mutation selection window" (MSW)[1]. However, when we take a look at the multi-resistant bacteria in hospital settings, they often carry mobile genetic elements such as conjugative plasmids[2]. These elements contain genes that encode molecular machineries for mediating the transfer of the mobile element from one bacterium to another allowing the spread of antibiotic resistance. Often, it is the mobile elements that carry resistance genes and thus provide pathogens with their phenotype. Clearly, the evolution of resistance is not just a matter of mutations but also, and even more so, it is about the lateral movement of selfish genetic replicators among (even distantly) related bacterial cells[3]. This notion served as an incentive for our study: even if it is reasonable to maintain antibiotic-level above the MSW in order to avoid the emergence of resistant mutants, the presence of other - even harmless - bacteria that carry mobile resistance conferring elements may nevertheless compromise the outcome of antibiotic treatments. In other words, it is possible that resistance element gets transferred horizontally to the susceptible bacteria during antibiotic therapy, thus generating novel resistant pathogens.

Studies have shown that beta-lactamase producing bacteria allow other susceptible (non producer) individuals to co-exist with them as "cheaters"[4]. That is, non-resistant bacteria take the benefit of the "altruistic" nature of the resistance mechanism as beta-lactamases reduce the concentration of the antibiotic for everyone in their immediate vicinity. Indeed, it was shown that a conjugative resistance plasmid can get transferred to the "cheaters" even when the "altruistic" plasmid-harboring bacteria were added only afterwards to the high-antibiotic environment[5]. Due to the transfer, cheaters became genuinely resistant entities. Extrapolating this result to clinical context would hint that the effectiveness of antibiotic treatment might depend on the bacterial community to which the patient is exposed during treatment.

We selected sixteen ESBL *E. coli* -strains isolated from patients from the University Hospital of Turku, Finland, and transferred the resistance plasmids from these strains to a second bacterium (*E. coli* K-12 HMS174) then to third strain (*E. coli* K-12 JM109(pSU18)) and finally back to HMS174. We isolated total plasmid-DNA from HMS174 strains after the third transfer and selected five strains with differing DNA-profiles for detailed analysis (Supplementary Figure 1). All the plasmids were sequenced, resulting in total of ten plasmid sequences originating from five ESBL *E. coli* strains. The plasmids were named pEC3I, pEC3II, pEC13I, pEC14I, pEC14II, pEC14III, pEC15I, pEC15II, pEC16I and pEC16II, where ECx is the name of the original host strain[6] and the Roman numeral is the number of the plasmid. Three of the strains (EC3, EC15, EC16) carried two mobilizable plasmids whereas EC14 had three plasmids and EC13 had only one. Hereafter, pEC14, for example, describes all the different plasmids originally derived from the strain EC14.

General features of the isolated plasmids are listed in Table 1. All the sequences are available on GenBank (accession numbers KU932021-KU932034). The incompatibility types of the isolated plasmids (including IncI, IncF, IncX) resemble those of ESBL-plasmids in bacteria causing nosocomial infections[7,8]. The beta-lactamase genes similarly provided a good coverage of the most common types (TEM, CTX-M, SHV[9]). One of the plasmids, pEC14I, contains a Class 1 integron

residing next to several resistance genes, suggesting that this plasmid carries a DNA-integrating resistance island. Indeed, mobile genetic islands such as these are common among multi-drug resistant bacteria. Also, sequence assembly of four of the plasmids revealed variable sequence within the original DNA sample, resulting in two versions of the plasmid sequences (named pEC3II_1 and pEC3II_2, pEC14II_1 and pEC14II_2, pEC15I_1 and pEC15I_2 and pEC16I_1 and pEC16I_2). In all cases, this sequence variability was observed in IncI plasmid shufflon area[10]. Overall, the selected plasmids provide a decent coverage of common features of mobile resistance elements.

In order to evaluate the evolutionary rescue potential of each of the plasmid combinations, an ampicillin-susceptible strain HMS174 was used as the recipient for the resistance plasmids. Around 2*10^6 HMS174 cells were transferred to a medium containing differing concentrations of ampicillin (0, 15, 75 and 150 mg/l). Few minutes later, 5 µl of overnight grown plasmid-harboring JM109-cells (their respective average cell densities are listed in Supplementary table 1) was added to the medium. Notably, bacteria carrying plasmid pEC3 reached about four times higher density than the rest of the donor cells and were thus diluted accordingly before the rescue experiments. The co-culture was let to grow for 24 hours in 37 °C. The number of beta-lactam resistant HMS174 cells in these cultures was measured with colony forming assay (Figure 1a).

The rate of horizontal transfer of beta-lactam resistance providing elements differed substantially between the strains. In the absence of antibiotics, pEC13, pEC15 and pEC16 were the most efficient in getting transferred to the recipient strain. This conjugation frequency reflected their rescue potential in 15 mg/ml ampicillin concentrations. However, when the antibiotic level increased, there were substantial differences between plasmids. In particular, pEC15 lost most of its rescue potential in higher concentrations since the number of resulting transconjugants was almost four orders of magnitude lower than that of pEC13 and pEC16. On the other hand, pEC3 was less efficient in transferring its plasmids to the recipient in the absence of antibiotics but it relatively

well maintained the rescue potential as the concentration increased. It is also notable that pEC14, which consists of three different large plasmids along with the plasmid-integrated resistance cassette, was very poor at disseminating the resistance to susceptible bacteria even in the absence of antibiotics. In practice, pEC14's potential for evolutionarily rescuing other bacteria in its vicinity was abolished as the concentration of antibiotic increased.

We measured the number of cheaters (i.e. bacteria that are not resistant but which survive due to the presence of "altruistic" beta-lactamase producers) in an attempt to explain the differences in rescue potentials (Figure 1b). Indeed, in the sole case of pEC15, the lower levels of cheaters could explain its rapidly diminishing rescue potential as the antibiotic concentration rises. Interestingly, however, the potential to support cheaters did not differ much for other plasmid combinations, thus the prevalence of cheaters is not directly related to their capability to rescue susceptible bacteria. Also, we tested whether only some of the plasmids (in those strains that harbor multiple plasmids) are transferred during the rescue event by amplifying each plasmid sequence with specific primers from the rescued clones. In most cases it appears that even in adverse conditions for the recipient strain, all plasmids get through the conjugation channel. However, pEC3II plasmid was detected only in half of the tested transconjugants, indicating that sometimes rescue via horizontal gene transfer can lead to the loss of plasmids that are not coding for the necessary resistance. Furthermore, we attempted to evaluate the sensitivity of rescue frequency on resource availability. As suspected, lower concentration of resources (5% L-broth) significantly reduces the rescue potential in 150 mg/l ampicillin concentration, but the rescue pattern remained similar (i.e. the best rescuing strains were the same in both nutrient levels) (Figure 1c).

In practical and evolutionary terms, the obtained results could help infer the resistance dynamics during antibiotic treatments as well as in farming environments and sewage. In particular, ESBL-carriage (i.e. people diagnosed with ESBL-positive bacteria, but with no acute infections) is becoming more common among healthy individuals[11]. Often carriage itself is not dangerous, but it

may compromise the outcome of future antibiotic therapies. Previous studies have highlighted the importance of maintaining antibiotic concentration above MSW[12]. However, given that ESBL-genes often reside in mobilizable elements, the efficiency of treatment may be more dependent on preventing the pathogen's access to global gene pool rather than preventing novel mutations *per se.* Measures that block horizontal gene transfer[13] could help prevent evolutionary rescue during treatment and thus improve the success-rates of treating ESBL-positive patients. Especially in the case of ESBL-carriage, the mobilizable resistance element(s) can be identified beforehand and thus the treatment, if meaningful, could be modified accordingly. Using our results as an example, in case of pEC14, the evolutionary rescue can be prevented simply by increasing the effective concentration of antibiotics. On the contrary, such approach would be ineffective for most of the other plasmid combinations, thus calling for alternative ways to prevent conjugation. Speculatively, these could utilize plasmid-dependent phages, pilus-binding phage-derived proteins or other components that disrupt plasmid transfer and/or maintenance[14,15,16,17]. Overall, extending the use of existing antibiotics requires us to acknowledge that resistance among bacteria is often a feature of the whole microbial community where lateral genetic transfer can play a notable role.

Experimental procedures

Conjugation of ESBL plasmids, plasmid sequencing and analysis

Escherichia coli strains 10UU11258, 57253, 55027, 56895 and 57361 were obtained from Medix Laboratories and Turku University Hospital and were named EC3, EC13, EC14, EC15 and EC16, respectively (see 6). Plasmids were isolated HMS174 with QIAGEN Large-construct Kit according to manufacturer's instructions. Their sequences were determined with PacBio next-generation

sequencing technology (DNA sequencing and Genomics laboratory, University of Helsinki, Finland). Sequence analysis was performed with NCBI's Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP). Highly similar plasmid sequences were searched using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Plasmid Inc groups were determined using PlasmidFinder[18] (https://cge.cbs.dtu.dk//services/PlasmidFinder/) and antimicrobial resistance genes using ResFinder[19] (https://cge.cbs.dtu.dk/services/ResFinder/). Mating pair formation (MPF) types and Mobility (MOB) groups of plasmids were determined using Geneious version 9.0.5 and BLASTx to compare the amino acid and nucleotide sequences of mobility region proteins and relaxase, respectively, with reference sequences described before[20].

Evolutionary rescue experiments

Five donor strains (see above) and recipient strain HMS174 were grown to carrying capacity 46 h at +37 °C, 200 rpm in the presence of appropriate antibiotics. To initiate the experiments, equal volumes of recipient and donor strain (5 µl) were added in 5 ml of L-broth (containing varying concentrations of ampicillin, when appropriate) in this order. After 24 h incubation (+37 °C, 200 rpm) number of different cell types were determined by plating various dilutions on appropriate antibiotic-containing plates. Three replications of each experiment were performed in the presence of lethal ampicillin concentrations (15, 75 or 150 mg/l). Natural transfer rates of plasmids were conducted in similar experimental conditions lacking the antibiotic. Additionally, all used ampicillin concentrations were shown to be lethal in experimental conditions without the presence of resistance plasmid. In other words, after using the same amounts of HMS174 and JM109(pSU18) cultures as in original evolutionary rescue experiments, no colonies formed on L-plates.

In order to determine which plasmids actually transferred during experiments, the plasmid contents of at least five transconjugants from each experiment were analyzed with colony PCR using plasmid-specific primers (Supplementary table 2). The PCR-products were analyzed with

agarose gel electrophoresis In case we were unable to assure the presence of a certain plasmid via colony PCR, the total plasmid content was isolated with Agencourt CosMCPrep (Beckman Coulter) kit according to manufacturers' instructions before PCR.

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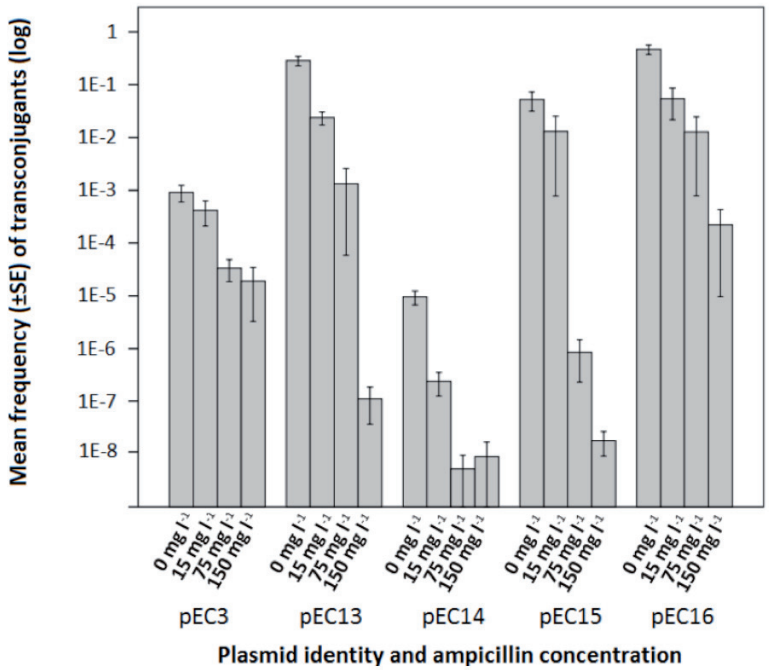
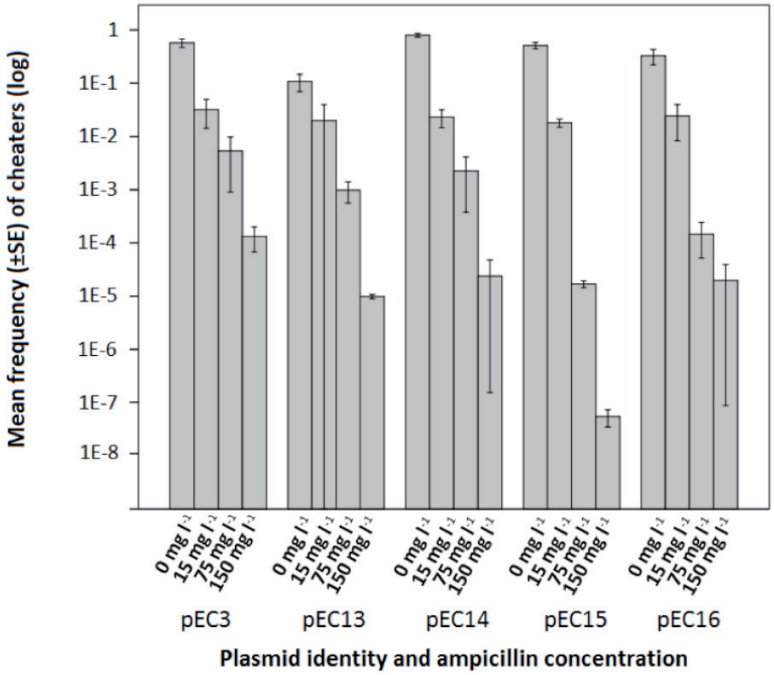
Figure legends

Figure 1: **(A)** Number of transconjugants (HMS174) after 24 h in different ampicillin concentrations at 37 °C (n=3). **(B)** Number of cheaters (HMS174) after 24 h in different ampicillin concentrations at 37 °C (n=3). In the absence of antibiotics (0 mg ampicillin), the value presents the standard frequency of the recipient bacterial strain. **(C)** Number of transconjugants (HMS174) after 24 h in 5 % L-broth at 37 °C with and without antibiotic selection (150 mg l^{-1}, n=5).

A


Mean frequency (±SE) of transconjugants (log)
1
1E-1
1E-2
1E-3
1E-4
1E-5
1E-6
1E-7
1E-8
0 mg l-1
15 mg l-1
75 mg l-1
150 mg l-1
pEC3
pEC13
pEC14
pEC15
pEC16
Plasmid identity and ampicillin concentration

B


Mean frequency (±SE) of cheaters (log)
1
1E-1
1E-2
1E-3
1E-4
1E-5
1E-6
1E-7
1E-8
0 mg l-1
15 mg l-1
75 mg l-1
150 mg l-1
pEC3
pEC13
pEC14
pEC15
pEC16
Plasmid identity and ampicillin concentration

C

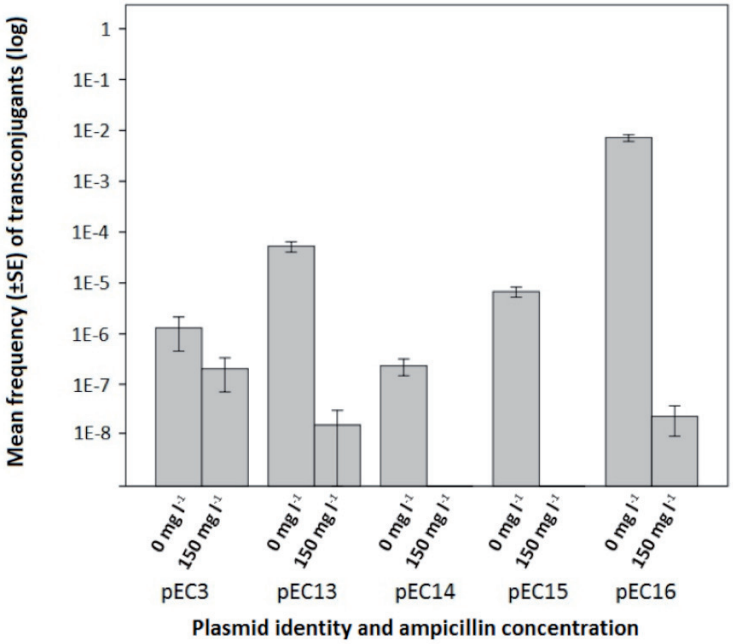

Mean frequency (±SE) of transconjugants (log)
1
1E-1
1E-2
1E-3
1E-4
1E-5
1E-6
1E-7
1E-8
0 mg l-1
150 mg l-1
pEC3
pEC13
pEC14
pEC15
pEC16
Plasmid identity and ampicillin concentration

Table 1: Plasmid features. Alterations to plasmid size due to shufflon area are indicated in parenthesis.

Plasmid	Plasmid size bp	Inc type	MPF type	MOB class	β-Lactamase identified	Other resistance genes
pEC3I	91 885	*IncB/O/K/Z*	MPFI	MOBP	*blaTEM-1C*	*strA, strB, sul2*
pEC3II	59 192 (59 192)	*IncI2*	MPFT	MOBP	-	-
pEC13	71 656	*IncFII*	MPFF	MOBF	*blaCTX-M-14*	-
pEC14I	143 590	*IncFII, IncQ1, IncP, IncFIB(AP001918)*	MPFF	MOBF	*blaTEM-1B*	*strA, strB, aadA1, mph(B) sul1, sul2, tet(A), dfrA1*
pEC14II	87 848 (87 666)	*IncI1*	MPFI	MOBP	-	-
pEC14III	80 057	*IncFII*	MPFF	MOBF	-	-
pEC15I	87 811 (87 767)	*IncI1*	MPFI	MOBP	-	-
pEC15II	38 611	*IncX1*	MPFT	MOBQ	*blaTEM-52B*	-
pEC16I	94 325 (95 380)	*IncI1*	MPFF	MOBP	*blaSHV-12*	-
pEC16II*	7 939	*ColRNAI*	-	MOBP	-	-

* non-conjugative mobilizable plasmid

Supplementary material

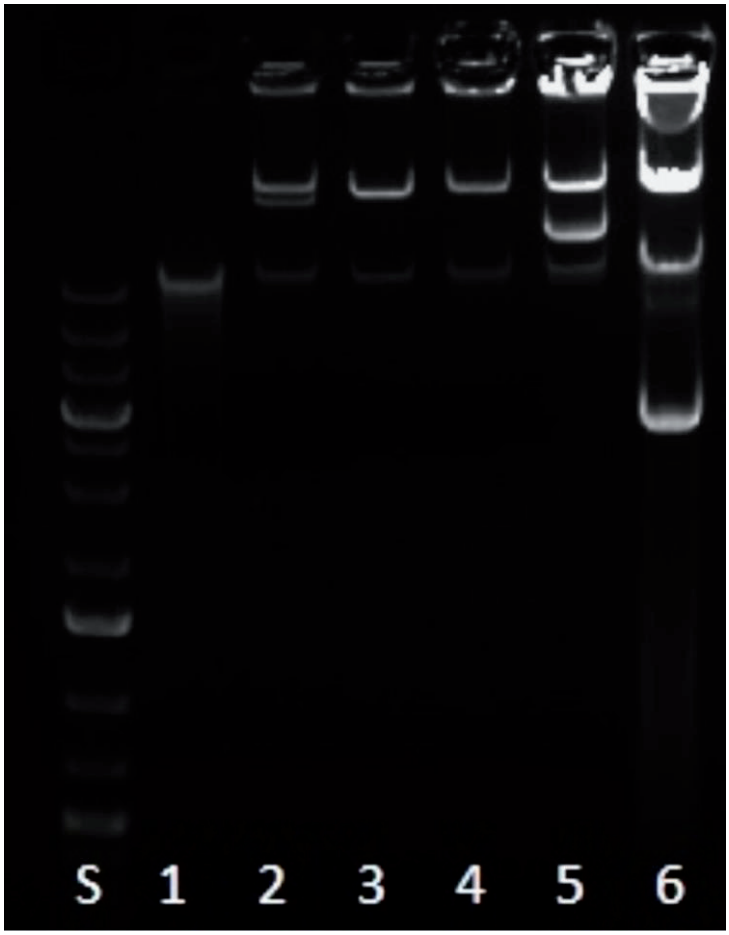


Figure S1. AGE (0.8 %) profiles of selected plasmids. S= GeneRuler 1kb Plus DNA Ladder, 1=HMS174 genome 2=pEC3, 3=pEC13, 4=pEC14, 5=pEC15, 6=pEC16.

Table S1: Average cell densities at the beginning of the evolutionary rescue experiments.

Cell strain JM109	cfu/ml	cells/ experiment
pEC3	$8.1*10^8$	$1.01*10^6$**
pEC13	$2.4*10^8$	$1.20*10^6$
pEC14	$2.5*10^8$	$1.25*10^6$
pEC15	$2.1*10^8$	$1.05*10^6$
pEC16	$2.5*10^8$	$1.25*10^6$

** 1:4 dilution of saturated culture was used

Table S2: Primers used in the study

Target sequence	Forward primer	Reverse primer
pEC3 I	CGGACATATGGACTGGAACAG	GACAGGTGTTTCCCAGCGCAG
pEC3 II	CTGTCGGCATGTCTGTCTCCC	TTCACCAGATCAACTCCCAGC
pEC14 I	GTCCGCAACGGCGATGCGCCG	CTTTTGACGACACCAAGGCCAG
pEC14 II	GCAAAACGATAGTTTCCCCTG	TTTCTTGTCACCTTCCACATC
pEC14 III	AGATGCTCTGTCTGTAATAC	ACCCTTATCCGGAGAGAG
pEC15 I	GACTCTCATTTCCGACGCTC	GAAGAACTGCTGAATGATAC
pEC15 II	CTGCCGAAAATCTGAAAGCTG	CCTATCAGTTTATATTTTCTG
pEC16 I	TCCGTGGGTATGTAATAACTG	GAAATCAACTATACATCTTAG
pEC16 II	GAAGAAGTTCGGGAACTCATC	CCTGGTGCTCCACCCACAGG

II

EVOLUTIONARY RESCUE BY ESBL PLASMID-CONJUGATION IN LETHAL PENAM AND CEPHEM CONCENTRATIONS

by

Pilvi Ruotsalainen, Reetta Penttinen & Matti Jalasvuori 2019

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III

MIDBIOTICS: CONJUGATIVE PLASMIDS FOR GENETIC ENGINEERING OF NATURAL GUT FLORA

by

Pilvi Ruotsalainen, Reetta Penttinen, Sari Mattila & Matti Jalasvuori 2019

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BRIEF REPORT

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Midbiotics: conjugative plasmids for genetic engineering of natural gut flora

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ABSTRACT

The possibility to modify gut bacterial flora has become an important goal, and various approaches are used to achieve desirable communities. However, the genetic engineering of existing microbes in the gut, which are already compatible with the rest of the community and host immune system, has not received much attention. Here, we discuss and experimentally evaluate the possibility to use modified and mobilizable CRISPR-Cas9-endocing plasmid as a tool to induce changes in bacterial communities. This plasmid system (briefly midbiotic) is delivered from bacterial vector into target bacteria via conjugation. Compared to, for example, bacteriophage-based applications, the benefits of conjugative plasmids include their independence of any particular receptor(s) on host bacteria and their relative immunity to bacterial defense mechanisms (such as restriction-modification systems) due to the synthesis of the complementary strand with host-specific epigenetic modifications. We show that conjugative plasmid in association with a mobilizable antibiotic resistance gene targeting CRISPR-plasmid efficiently causes ESBL-positive transconjugants to lose their resistance, and multiple gene types can be targeted simultaneously by introducing several CRISPR RNA encoding segments into the transferred plasmids. In the rare cases where the midbiotic plasmids failed to resensitize bacteria to antibiotics, the CRISPR spacer-(s) and their adjacent repeats or larger regions were found to be lost. Results also revealed potential caveats in the design of conjugative engineering systems as well as workarounds to minimize these risks.

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Genetic engineering; antibiotic resistance; ESBL carriage; conjugative plasmid; CRISPR editing; enterobacteria

Introduction

The possibility to engineer gut microbiome has become a notable avenue of research. Restoration of microbial balance in the gut can provide a cure to a multitude of complex diseases. Nonetheless, stable installation of foreign beneficial microbes in the gut is problematic. Studies have shown that dietary supplement bacteria (probiotics) disappear from the community soon after their ingestion ceases.[1,2] This has led many teams to compile bacterial cocktails that would establish a more stable population within the gut.[3] Also, the near-complete replacement of gut flora has been used to revert dysbiosis. This so-called bacterial transplantation is an effective approach to cure especially recurrent diarrhea caused by *Clostridium difficile*,[4-6] but could also be used to improve various other conditions.[7] The composition of gut flora is also sensitive to diet, and, for example, increase of fiber can result in notable shifts in the community composition.[8] In some circumstances, however, the possibility to modify the genomes of existing bacteria in the gut could provide an alternative to remodel the system.

So far, the genetic engineering of bacterial communities *in situ* has mainly focused on bacteriophage-based applications.[9,10] Conjugative plasmids offer an alternative route with differing engineering qualities. They are circular antagonistic genetic elements that can mediate their own transfer from one bacterium to another. In addition, these self-transmissible plasmids can co-transfer non-conjugative plasmids with appropriate *oriT* site.[11] The relaxosome of the conjugative plasmid recognizes the similar *oriT* site in non-conjugative plasmid and mobilizes it through conjugation.[12] The exact conjugation mechanisms vary between plasmids, but they all form a channel between the cells through which the plasmid is usually transported as

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a single-stranded DNA molecule to the recipient bacterium. Plasmids can be readily modified with various molecular biology methods, thus providing a relatively simple platform for carrying out *in situ* genetic engineering of bacterial cells. Given that the diversity of gut microbiota varies even between genetically identical twins,[13] the attempts to colonize maladapted (engineered) bacteria within an already established community can be a challenging if not an impossible task. In this respect, the introduction of an engineered mobile element into the existing community instead of relying on the establishment of an entire bacterium provides a potential workaround for deploying desired functionalities within the system. Given the established concepts of probiotics (health-promoting bacteria) and prebiotics (nutrients that promote the growth of beneficial bacteria), "midbiotics" (plasmid-probiotics in a sense) provide yet an alternative form of biotic substances that can be used to acquire beneficial changes in the gut flora. Naturally, such plasmids have only limited use, albeit, in certain instances, they may be even a preferable choice over probiotics such as when only particular genes need to be removed from the community.

Plasmids are divided into incompatibility groups (Inc) based on their potential to stably coexist in a bacterial cell. In other words, two plasmids that share the same Inc-group cannot be maintained in a single cell indefinitely. Conjugative plasmids also often encode entry-exclusion mechanisms that prevent related plasmids from entering the cell. Due to these natural features, the plasmids used for engineering should be uncommon in the targeted flora. Naturally, determining the existence of certain plasmid types routinely from a heterogenous community is a laborious task. Yet, certain antibiotic resistance–conferring plasmids of Enterobacteriaceae, for instance, are relatively rare in patients. Indeed, in a metastudy, Carattoli reviewed the prevalence of different resistance plasmid families in Extended Spectrum Beta-Lactamase (ESBL) strains.[14] Among the rarest were IncP-type plasmids. Despite this, conjugative IncP-plasmids are well-studied, they have a robust conjugation machinery and a broad host range. As such, they provide an example of potential backbones that could be utilized for engineering purposes.

In bacteriophage-based applications, the genetic material within the phage is replaced, and as it infects a cell after the attachment to a specific receptor on the host cell surface, it delivers the genomic cargo into the bacterial host.[9,10] Phage-based tools have acquired notable attention and are currently under development toward drugs. The advantage of phages is that they have a narrow host range, and thus, they target specifically only the desired fraction of the bacterial community. Yet, bacteria rapidly become resistant to phages, and phages cannot be easily used to exert activity against even all variants of certain species. In this regard, conjugative plasmids provide qualities that could be useful for alternative and more generally applicable engineering purposes. As noted above, conjugative plasmids are usually delivered as a single DNA strand to the recipient cell. The complementing strand is synthesized in the recipient bacterium and thus it contains all the host-specific modifications in the nucleic acids.[15] This way the host does not recognize the incoming plasmid as foreign genetic material, which, in turn, allows the plasmid to establish itself into a natural community without prior knowledge of the features of bacteria therein. Additionally, unlike phages, conjugative plasmids are not dependent on specific receptors on host cells as plasmids require only cell-cell contact. And once plasmid gets into natural bacteria, it can further disseminate itself into the next host. The transfer rate from one bacterium to another is, of course, slower and less-precise than phage-mediated delivery of DNA. This sets certain boundaries for the utilization of plasmids. Nevertheless, conjugative plasmids can provide a broad host range for introducing genetic material into the gut flora.

The advent of CRISPR-Cas9 editing has provoked numerous studies where specific target sequences within various host organisms are modified,[16] even enabling strain-specific elimination of bacteria from heterologous communities.[10] Introduction of CRISPR-Cas9 editing components into conjugative plasmids provides a potential mean to remove unwanted genes such as those conferring antibiotic resistance from diverse bacterial systems. ESBL carriage refers to non-symptomatic colonization of the gut by bacteria

which are resistant to a wide range of different beta-lactam antibiotics.[17,18] This is of major concern, as beta-lactams are the most commonly used class of antimicrobials, owing to their broad spectrum and minimal side effects. They are abundantly administered to treat and prevent bacterial infections during various medical procedures. Over the past few decades, ESBL carriage has become increasingly more common among long-term hospitalized patients as well as in the community.[19] ESBL carriage serves as a reservoir of resistance genes and significantly increases the risk of clinical infections.[20-22] As such, we here set to evaluate the possibility to use conjugatively transferred plasmids to induce the loss of ESBL genes (located either in plasmids or in the chromosome) from a bacterial community.

Results and discussion

We constructed a midbiotic system consisting of a conjugative IncP plasmid RP4[23] and a mobilizable pCas9 plasmid containing *Streptococcus pyogenes*–derived CRISPR/Cas9[24] that targets conserved sites in two different beta-lactamase genes via plasmid-encoded CRISPR RNA (crRNA). Part of RP4 origin-of-transfer (*oriT*) site was cloned into pCas9 plasmid in order to make it horizontally transferrable by the RP4-encoded relaxosome complex. Further, 543 bp region, including the target site of the CRISPR/Cas9 system, was deleted from the beta-lactamase gene *blaTEM-2* of RP4 to prevent the system from self-targeting. From now on, the RP4$^{blaTEM-2\Delta172-714}$ plasmid is referred to as delivery plasmid and the modified pCas9 as pCRISPR plasmid, crRNA/multi-crRNA referring to spacer(s) targeting the beta-lactamase gene(s).

A donor bacterium (*Escherichia coli* HMS174) harboring midbiotic plasmids (delivery and pCRISPR-crRNA plasmids) was cocultured together with recipient *E. coli* strain (HB101) carrying a conjugative ESBL-plasmid pEC15 that encodes *blaTEM-52b* target gene.[25] The transfer of these plasmids to ESBL-positive bacteria and the subsequent coexpression of endonuclease Cas9 and crRNA should induce the loss of resistance by guiding the Cas9 complex to ESBL gene and create a double-stranded nick within the target site (Figure 1a). Nicking linearizes the plasmid and prevents its replication. Indeed, after 24 h, only approximately 1:10 000 transconjugants retained the resistance in comparison to a control treatment lacking the crRNA (Figure 1b). To rule out the possibility that this might result from the unequal conjugation rates between pCRISPR-crRNA and pCRISPR-control plasmid, both were conjugated independently to a recipient HB101 lacking the target plasmid (Figure 2a). Altogether, this suggests that in principle the dispersal of such midbiotics in the bacterial flora would relatively efficiently resensitize the ESBL-harbouring recipients to beta-lactams. Yet, while this approach appears promising in accelerating ESBL loss, there are still potential obstacles to be taken into account when specific genes are targeted with Cas9. These obstacles would be relevant to most *in situ* applications that seek to delete specific functions from the community (and sometimes in applications that attempt to introduce them); hence, we decided to take a closer look at the caveats and the realistic prospects of midbiotic engineering.

In many cases, there can be multiple variants of the genes that encode undesired phenotypes. For example, there is no single guiding crRNA sequence that would direct Cas9 to all possible ESBL variants. However, all classes of beta-lactamase genes share sequences that are usually conserved within the class (Figure 3). Targeting these sites would provide a broad activity against the class regardless of specific knowledge of the variant in any particular case. When various crRNAs are combined into the same plasmid similarly to spacer arrays of natural CRISPR systems, several targets could be abolished with a single pCRISPR plasmid. We tested this by adding two crRNA coding sites separated by a repeat into the pCRISPR plasmid. This pCRISPR-multi-crRNA plasmid was then transferred into two bacterial strains each harboring a different type of an ESBL gene (*blaTEM-52b* and *blaCTX-M-14*). The plasmid exhibited the activity against both ESBL types, leading to a nearly 500-fold decrease in cell density in treated bacteria compared to control, suggesting that combination of crRNA sites could indeed be utilized to achieve broad activity (Figure 1c).

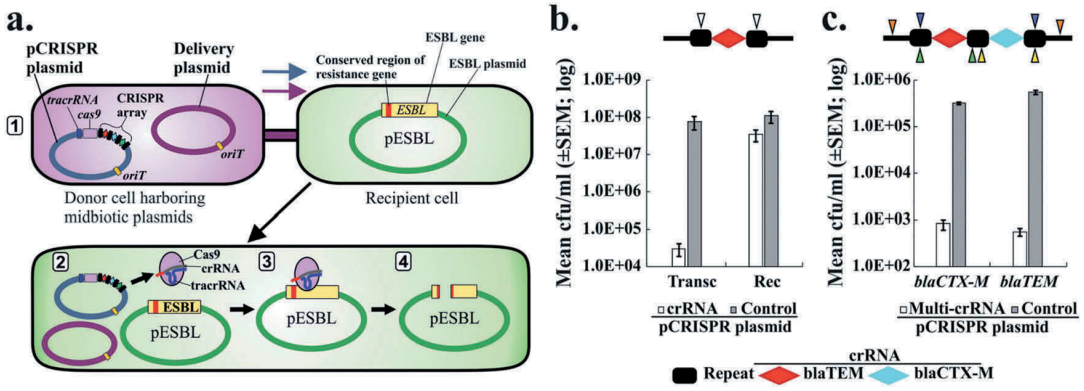

Figure 1. Midbiotic plasmids against ESBL-positive bacteria. (a). 1) Donor cell delivers midbiotic plasmid system (pCRISPR plasmid and delivery plasmid both of which share the same origin of transfer site, oriT) via conjugation into recipient target cell that harbors ESBL plasmid (pESBL). 2) After a successful delivery of the plasmids, the new host cell starts producing the components required for CRISPR/Cas9-activity (endonuclease Cas9, crRNA and tracrRNA, encoded by pCRISPR). 3) Cas9 cleaves the ESBL gene based on crRNA that is programmed to target a conserved region within the gene. 4) This results in degradation of ESBL plasmid. (b). Among the transconjugants (Transc) receiving the pCRISPR-crRNA, a difference of nearly four orders of magnitude in ESBL-positive bacteria was observed. Rec denotes the total number of recipient bacteria. Out of the survivors, the deletion of the spacer in the CRISPR locus of pCRISPR-crRNA plasmid (white arrowheads) explained the loss of activity. The mean cell density (cfu/ml) is calculated from a total of six replicates from two different experiments ($n = 6$). The black bars indicate the standard error of mean (SEM). (c). Transformation of pCRISPR-multi-crRNA into target bacteria caused the cell density of HB101(pEC13) (blaCTX-M) to decline by two orders of magnitude and HB101(pEC15) (blaTEM) by three orders of magnitude. The deletion of either one (green and yellow arrowheads) or both of the spacers (blue arrowheads) resulted in the survival of transformants. Larger deletion in CRISPR locus was most likely the reason for the unsuccessful amplification of some escape mutants, as the primer binding sites were located in the deletion (orange arrowheads). Some survivors contained the intact spacers, suggesting that Cas9 gene or the target sequence might carry mutations. The mean cell density is calculated from three replicates ($n = 3$). The black bars indicate the standard error of mean (SEM).

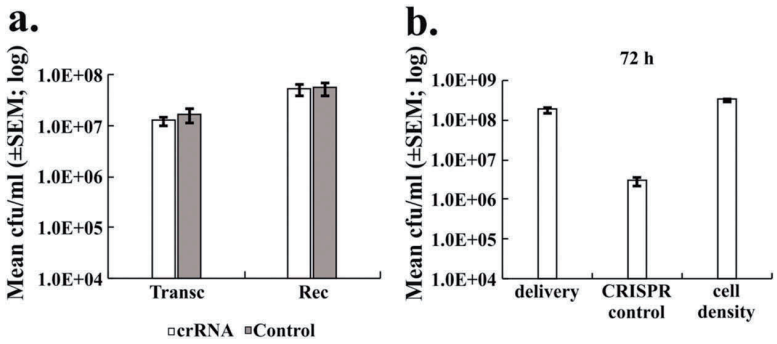

Figure 2. Conjugation of midbiotic system. (a). The conjugation rates of pCRISPR-crRNA and pCRISPR-control plasmid are equal, determined by measuring the mobilization frequencies after 24 h conjugation. Therefore, the presence of spacers does not itself hinder the mobilization rate of the pCRISPR plasmid. The mean cell density was calculated from three replicates ($n = 3$). The black bars indicate the standard error of mean (SEM). (b). After 72 h, the delivery plasmid was observed to conjugate independently without the mobilizable pCRISPR-control plasmid, as the density of cells containing pCRISPR-control plasmid was two orders of magnitude lower than cells with delivery plasmid. Also, when 90 colonies from delivery plasmid selection plate were streaked on plate selecting for pCRISPR-control plasmid, none of them was observed to contain the pCRISPR-control plasmid. On the contrary, all the 90 colonies with pCRISPR-control plasmid also contained the delivery plasmid. The mean cell density was calculated from three replicates ($n = 3$). The black bars indicate the standard error of mean (SEM).

We further studied the individual bacteria that appeared to have avoided the anti-ESBL effect despite having been introduced with the midbiotic system. In other words, some bacteria which had received the pCRISPR-crRNA/multicrRNA plasmid still retained the resistance to beta-lactams (Figure 1b-c). Sequencing of CRISPR spacer locus of these plasmids (8 escape colonies/replicate/experiment) revealed that the observed tolerance to the midbiotic treatment after 24

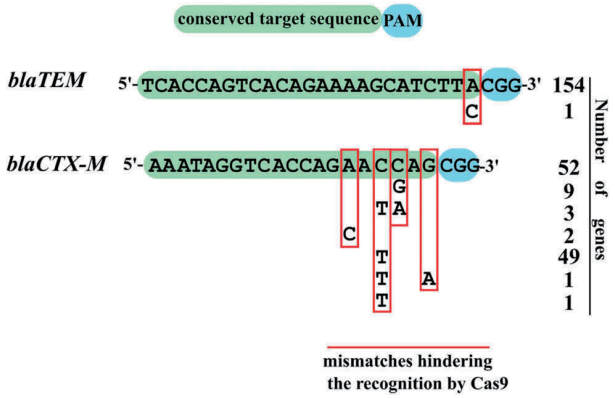

Figure 3. Designing of guide RNA for conserved sites in beta-lactamase genes. A potential obstacle in gene deletion by midbiotic application is the diversity of the genes that need to be targeted. By combining multiple spacers into a single plasmid and selecting conserved sites within target genes, it is possible to increase the coverage. As the beta-lactamase genes belonging to the same class share conserved sites in nucleotide level, these sites can be used to design spacers for CRISPR/Cas9 system in order to target several resistance gene variants with a single spacer. Majority of the genes in class blaTEM (154) contain the conserved target sequence (green bar). The target sequence selected for the class of blaCTX-M genes (green bar) is not as highly conserved as in the blaTEM class, only 52 genes contain the exact sequence. Only one gene in blaTEM class has a point mutation (red rectangle) in the first nucleotide next to PAM (blue), whereas genes of blaCTX-M class have more variation in these nucleotides. These mismatches in the first seven nucleotides next to PAM might hinder the recognition of the target by Cas9[24] and thus the efficiency of the spacer.

h was mainly due to loss of the beta-lactamase-targeting spacer(s) and their adjacent repeat (see the graphic illustration of spacer deletions in Figure 1b-c). In some cases, however, we did not succeed to amplify the crRNA coding region at all, suggesting that a larger deletion might have occurred within the region. On the other hand, sometimes the crRNA site was unaltered, indicating potential changes elsewhere, such as mutations in tracrRNA, Cas9 or PAM sequence.[10] Nevertheless, the emergence of mutants may be difficult to prevent, but in principle several copies of the crRNA regions, for example, could be included in the plasmid, hence allowing it to retain its activity even if one of the sites is lost.

Another potential concern derives from the separation of the midbiotic into two or more plasmids. It is possible that the delivery plasmid mobilizing the pCRISPR plasmid goes 'rogue' and spreads alone in the community, thus attenuating the desired effect. We investigated this possibility by cultivating midbiotic bacteria (harboring pCRISPR control plasmid) together with ESBL-positive strain for 72 h during which the culture was refreshed once a day. All of the studied clones (90 colonies) with the mobilizable pCRISPR plasmid also contained the delivery plasmid. In contrast to this, all bacteria harboring the delivery plasmid had lost the pCRISPR plasmid (Figure 2b). This indicates that the mobilizable pCRISPR plasmid is not always delivered together with the conjugative plasmid, thus requiring countermeasures to minimize the probability of such events. There are at least two possibilities to achieve this: either the pCRISPR plasmid and delivery plasmid could be combined into a single plasmid or the toxin–antitoxin system could be separated so that the pCRISPR plasmid carries the gene for antitoxin and the delivery plasmid encodes the toxin. In the latter case, the dispersal of the delivery plasmid alone would lead to cytotoxic response and death of the recipient cell.

Conjugative plasmids are agents in natural microbial communities, albeit not an inherent part of any particular strain or species. In the recent bloom in microbiota research, they have so far been a seldom utilized tool for inducing genetic changes in existing bacterial communities. Plasmids could be used both to introduce desired genes or remove existing ones. Whether they have applications beyond laboratories is yet to be demonstrated, and the possible spread of malevolent traits via horizontal gene transfer may be a deterrent against using plasmids for engineering purposes. Indeed, the obvious risk in introducing a conjugative plasmid into a bacterial community is that the element may pick up an unwanted gene and disperse it further into other hosts. Before introduction into clinical applications, the resistance genes of delivery plasmid should be deleted to prevent dispersal of new resistance genes. However, it must be noted that the communities aimed to be engineered will nevertheless harbor various types of mobile genetic elements, and, thus, if there is notable selection within the population for acquiring a particular gene, it is likely to disperse anyway. In any event, if the plasmid used for midbiotic-like engineering must be removed

from the community, the plasmid-dependent bacteriophages could provide a way to induce direct selection against the plasmid. However, while *in vitro* experiments suggest that this would result in plasmid loss,[26,27] it is yet to be determined whether this occurs also *in vivo*.

Overall, the fraction of the community that can be engineered with conjugative plasmids is equal to the fraction of the flora that receives them. Studies suggest that plasmid dynamics and persistence in a community is a complicated matter where trophic levels and various characteristics of plasmids, their hosts and the environment play an indispensable role.[28,29] Without extensive selection for the midbiotic plasmid, it is unlikely to spread to even all possible hosts. Therefore, as in the case of ESBL carriage, the midbiotic system could be considered as a booster which accelerates ESBL curing rather than an outright treatment. Sometimes, however, even a small fraction of engineered bacteria may be enough, such as in the case of making the midbiotics encode externally secreted bacteriocins against unwanted bacterial species. Yet, the overall improved understanding of the survival conditions of plasmids can help us find ways both to get rid of conjugative plasmids and, if necessary, to facilitate their dispersal. Nevertheless, while caution is necessary, the ability to introduce or remove genes within natural bacterial communities is a real possibility that could be considered as a potential tool for genetic engineering of existing bacterial systems or, for example, modification of gut microbe transplants prior to their implementation.

Materials and methods

Plasmids, bacterial strains and culture conditions

In this study, the so-called midbiotic system consists of the conjugative RP4$^{blaTEM-2\Delta172-714}$ plasmid (delivery plasmid) and mobilizable pCas9 plasmid (pCRISPR plasmid, a gift from Luciano Marraffini, Addgene plasmid # 42876) encoding the *S. pyogenes* CRISPR/Cas9 system[24] with crRNA(s) targeting conservative sites of different beta-lactamase resistance genes in ESBL plasmids (Table 1). pCas9 was made mobilizable by cloning RP4 *oriT* site[12,30] (50980–51793 bps, amplified with primers RP4oriT-F and RP4oriT-R, Supplementary Table 1) into pCas9 digested with SalI (ThermoScientific; Waltham, Massachusetts, United States) into region spanning 7377–7486 bps. The phosphorylated ESBL-gene-targeting crRNA oligonucleotides (2 µM each) were first annealed together in 50 µl reaction with 1x of T4 ligase buffer (New England Biolabs; Ipswich, Massachusetts, United States) and 0.05 M NaCl by heating first at 95°C for 5 min and then cooling it down gradually (1°C/35 sec) to 20°C. Then, crRNA insert was ligated into BsaI (ThermoScientific) digested pCas9 plasmid by T4 ligase in T4 ligase buffer (New England Biolabs; Ipswich, Massachusetts, United States). In order to prepare the pCRISPR-multi-crRNA plasmid, the multi-crRNA insert was multiplied by PCR from a synthetic plasmid (GenScript; Nanjing, China) with primers spacer-multi-crRNA-F and spacer-multi-crRNA-R (Supplementary Table 1). PCR product was purified according to instructions of Qiagen PCR purification kit before being ligated (similarly as above) into the plasmid. The pCRISPR-control plasmid was otherwise similar but lacked the crRNA (Table 1). If not mentioned otherwise, all the PCRs were done according to instructions of Phusion Hot Start II High-Fidelity PCR mastermix (ThermoScientific), except for an extended initial denaturation (from 5 min to 7 min 30 s), using C1000 Thermal Cycler (Bio-Rad Laboratories Inc.; Hercules, California, United States). Both ESBL plasmids, pEC13 and pEC15, in recipient strains, originate from nosocomial isolates,[25] and the conserved sites of their respective beta-lactamase genes (Table 1) were selected as targets for the CRISPR/Cas9 system of pCRISPR plasmids.

All the bacterial cultures were grown at +37°C in Luria Bertani Lennox-broth (LB)[31] and, as necessary, plated on LB-agar (1%) plates. When appropriate, the following antibiotic concentrations were used: rifampicin (50 µg/ml), streptomycin (25 µg/ml), kanamycin (25 µg/ml), chloramphenicol (25 µg/ml) and ampicillin (150 µg/ml). Liquid cultures were shaken at 220 rpm.

Partial deletion of blaTEM-2 in RP4

The part of *blaTEM-2* gene (172–714 bp) containing the crRNA target site was deleted from RP4 to prevent the midbiotic system from self-targeting the

Table 1. Bacterial strains and plasmids used in the experiments and the spacer sequences of pCRISPR plasmid. Only the resistance genes relevant to the experiments are mentioned here.

	Strain features	Plasmid	Relevant characteristics		Resistance genes
DONOR HMS174	*E. coli* K-12, chromosomal rifampicin-resistance	RP4$^{blaTEM-2\Delta172-714}$	IncP plasmid		*aph(3′)-Ib, tet,* ***blaTEM-2Δ172–714***
		pCRISPR-crRNA	*oriT* site of RP4 (50 980-51 793 bp)	A spacer targeting conservative site of *blaTEM* genes	*cat*
		pCRISPR-multi-crRNA[c]		3 spacers targeting conservative sites of *blaTEM*, *blaCTX-M, blaSHV* genes, respectively	
		pCRISPR-control		Without crRNA	
RECIPIENT HB101	*E. coli* K-12, chromosomal streptomycin resistance	pEC13	Target of pCRISPR-multi-crRNA		*blaCTX-M-14*
		pEC15	Target of pCRISPR-crRNA/multi-crRNA		*blaTEM-52b*
RECIPIENT BL21 Gold	*E. coli* B, chromosomal tetracyclin resistance	pCRISPR-crRNA	A spacer targeting conservative site of *blaTEM* genes		*cat*
Sequence of the crRNA (5 →3′)					
crRNA[a]		AAACTCACCAGTCACAGAAAAGCATCTTAG			
multi-crRNA[b]		AAACTCACCAGTCACAGAAAAGCATCTTAGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACAAATAGGTCACCAGAACCAGGTTTTA GAGCTATGCTGTTTTGAATGGTCCCAAAACAACTGAATGAGGCGCTTCCCG			

[a] Sequence of crRNA of pCRISPR-crRNA
[b] Sequence of crRNAs of pCRISPR-multi-crRNA
[c] The plasmid was isolated from the DH5α strain.

delivery plasmid. The deletion was first created in the RP4 *blaTEM-2* gene cloned in pET24 plasmid by polymerase chain reaction (PCR) using 0.2 µM of primers deletion-F and deletion-R (Supplementary Table 1) with elongation time (5 min 15 s) adjusted so that the plasmid without the unwanted sequence was amplified with extension rate 0.5 kb/min.[32] As the deletion was confirmed with agarose gel electrophoresis, pET24-blaTEM -2Δ172–714 PCR-product was recombined back to circular plasmid by Red/ET recombination in recombineering-proficient *E. coli* strain GB08-red ^{RifR} (Gene Bridges; Heidelberg, Germany) according to the manufacturer's instructions, with the exception of using 10 ng of DNA for transformation. Briefly, the truncated *blaTEM-2Δ172–714* gene was amplified by PCR with primers delblaTEM2-F and delblaTEM2-R (Supplementary Table 1). Template of the PCR was removed by DpnI treatment (ThermoScientific), and *blaTEM*-2Δ172–714 PCR product was purified from the gel according to instructions of Qiagen's Gel purification kit (Hilden, Germany). RP4 plasmid containing the deletion was obtained by recombining *blaTEM*-2Δ172–714 PCR product into RP4 in GB08-red^{RifR} strain. This RP4$^{blaTEM-2\Delta172-714}$ was then conjugated from GB08-red^{RifR} to BL21 Gold(pCRISPR-crRNA) with a donor to recipient ratio of 2:1 in 3 ml and then cultivated at +37°C, 220 rpm, for 16 h. Transconjugant bacteria were selected on LB agar plates with chloramphenicol-kanamycin selection. The colonies were picked and transferred into LB medium with same antibiotic selection as above and cultivated overnight without shaking. The colonies containing the deletion (RP4$^{blaTEM-2\Delta172-714}$) were identified by negative selection by plating on LB agar plates with and without ampicillin selection. This RP4$^{blaTEM-2\Delta172-714}$ plasmid was conjugated to HMS174 by incubating donor and recipient in ratio 1:1 in 5 ml cultivation for 2 h at +37°C, 220 rpm. Transconjugants were selected by plating on LB agar plates with rifampicin-kanamycin selection.

Midbiotic conjugation

The efficiency of the midbiotic plasmids in inducing ESBL loss from the transconjugants was investigated with the following setup. Before the experiments, donors HMS174(RP4$^{blaTEM-2\Delta172-714}$)(pCRISPR-crRNA) and control HMS174(RP4$^{blaTEM-2\Delta172-714}$) (pCRISPR-control) and the recipient strain HB101 (pEC15) were cultivated overnight with antibiotic selection. To mix the equal number of cells for conjugation experiments, the cell density (colony forming units; cfu/ml) of all the cultures were determined either by plating or by measuring the optical density at OD_{595} (Multiskan FC, ThermoScientific; Waltham, Massachusetts, United States). The experiments were performed in two sets with slightly different concentrations of bacteria (see below), and each conjugation setup was replicated six times in total. The recipient strain (~3.0×10^5 cfu/ml in the first experiment and ~7.0×10^5 cfu/ml in the second) was mixed with the donor (~1.0×10^5 cfu/ml in the first and ~2.0×10^6 cfu/ml in the second) and cultivated 24 h in 5 ml LB without antibiotics. After the experiment, plating was used to measure the cell density of transconjugants (streptomycin-ampicillin-chloramphenicol), recipients (streptomycin-ampicillin), donors (rifampicin-kanamycin-chloramphenicol in the first experiment and rifampicin-chloramphenicol in the second) and of the community (no antibiotics). Donor and recipient strains were distinguished by differing resistance for rifampicin and streptomycin, respectively. The presence of RP4$^{blaTEM-2\Delta172-714}$ was controlled by kanamycin and the pCRISPR by chloramphenicol selection. In order to observe the potential for different midbiotic plasmids to disperse separately, RP4$^{blaTEM-2\Delta172-714}$ and pCRISPR-control plasmid were cultivated for 72 h during which the culture was renewed daily by transferring 50 µl of culture into fresh 5 ml LB medium. After 72 h, the density of bacteria carrying either RP4$^{blaTEM-2\Delta172-714}$ or pCRISPR-control plasmid and the total cell density was determined by plating with appropriate antibiotics. From the total of 90 colonies (30 colonies/replicate), we determined whether RP4$^{blaTEM-2\Delta172-714}$ or pCRISPR-control plasmid containing colonies also accommodated the other midbiotic plasmid.

The conjugation efficiencies of the pCRISPR-crRNA and pCRISPR-control plasmid were determined to be equal by conjugating the plasmids into HB101 without target ESBL plasmid. The donors were mixed with the recipient in ratio ~1–1.65:100 in 5 ml LB and cultivated overnight in the absence of antibiotics. The cell density (cfu/ml) of transconjugants with pCRISPR plasmids (streptomycin-chloramphenicol), recipients (streptomycin) and

donors (rifampicin-chloramphenicol) as well as the total cell density (no antibiotics) were determined by plating.

pCRISPR plasmid with multiple ESBL targets

To test the activity of multi-crRNA, electroporation was used to transform the pCRISRP-multi-crRNA plasmid to ESBL-plasmid harboring strains. Electroporation was performed according to the protocol in manual of recombineering-proficient *E. coli* strain GB08-red (Gene Bridges; Heidelberg, Germany). The optimal density was measured with UV-mini-1240 UV-VIS Spectrophotometer (Shimadzu; Kyoto, Japan) using 1.5 ml semimicro cuvettes (Brand; Germany). A 6.5×10^5 cfu/ml of HB101(pEC13) and 4.0×10^6 cfu/ml of HB101 (pEC15) strain were used for each plasmid transformation. Every plasmid transformation (pCRISPR-multi-crRNA and pCRISPR-control) was conducted in triplicates by using 20 ng of plasmid DNA. One negative control, transformed with 1 µl of water, per bacterial strain was done. The DNA concentration of plasmids was measured according to the protocol of Qubit™ dsDNA HS Assay Kit (Invitrogen; Carlsbad, California, United States) by using Qubit® 2.0 Fluorometer (Invitrogen; Carlsbad, California, United States). Transformants were plated on LB agar plates without antibiotics and with the combination of chloramphenicol and ampicillin. Negative control was plated with and without chloramphenicol selection. The activity of different crRNA sites was determined by counting the colonies on each plate.

Target site selection

The conserved regions of the *blaTEM* and *blaCTX-M* beta-lactamase genes for crRNA targets were determined by aligning sequence samples of these classes (obtained from the ResFinder 3.0 database)[33] separately with MUSCLE algorithm with default settings by Geneious 8.1.9 (Biomatters Ltd; Auckland, New Zealand). The most conserved sites with the appropriate PAM sequence were selected for the crRNA spacer sequences.

Escape mutants

The survived escape mutant colonies from the conjugation and transformation were re-isolated by plating them on chloramphenicol-ampicillin. Eight colonies/replicate, except two colonies per control replicate (altogether 32 colonies/experiment), were grown in LB media with chloramphenicol-ampicillin at +37°C without shaking. CRISPR locus of pCRISPR-crRNA, pCRISPR-multi-crRNA and pCRISPR-control plasmid were amplified with PCR using one bacterial colony as a template with primers spacerseqF and spacerseqR (Supplementary Table 1). PCR product was purified from primers and nucleotides with 0.4 U of Exonuclease I (20 U/µl, ThermoScientific; Waltham, Massachusetts, United States) and 0.4 U of FastAP Thermosensitive Alkaline phosphatase (1U/µl, ThermoScientific; Waltham, Massachusetts, United States). These reactions were incubated at +37°C for 20 min and then at +80°C for 15 min in order to inactivate the enzymes. Sequencing-PCR of ExoSAP-treated DNA was performed with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems; Foster City, California, United States) according to the manufacturer's protocol. The sequencing reactions were purified using the protocol of BigDye Terminator v3.1 Cycle Sequencing kit except centrifugation was performed with 1109 × g and 100 × g and, before adding formamide, samples were dried at +37°C for 10 min. Sequencing was carried out with 3130*xl* Genetic Analyzer (Applied Biosystems/HITACHI; Foster City, California, United States). The basecalling was performed with Sequencing Analysis Software v6.0 (Applied Biosystems; Foster City, California, United States), and the sequences were analyzed for deletions or mutations in CRISPR locus by mapping them against the original sequence by using Geneious 8.1.9 (Biomatters Ltd; Auckland, New Zealand).

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No potential conflicts of interest were disclosed.

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***Supplementary Table 1**: Primers and their sequences used in the experiments.*

PRIMER NAME	**PRIMER SEQUENCE**
RP4oriT-F	TATATAGTCGACCGACAGGCTCATGCC
RP4oriT-R	ATATATGTCGACAGCCTTGCCCCCCT
spacer-blaTEM-F	[Phos]AAACTCACCAGTCACAGAAAAGCATCTTAG
spacer-blaTEM-R	[Phos]AAAACTAAGATGCTTTTCTGTGACTGGTGA
spacerseq-F	TGCCACTCTTATCCATCAATCCA
spacerseq-R	TCACACTACTCTTCTTTTGCCTATTATAACAT
deletion-F	AAGATCCTTGAGAGTGGATCTCGCGGTATCATTGCA
deletion-R	ATGATACCGCGAGATCCACTCTCAAGGATCTTACCGCTGTT
delblaTEM2-F	ATGAGTATTCAACATTTCCGT
delblaTEM2-R	TTACCAATGCTTAATCAGTGA
spacer-multi-crRNA-F	TATATAGGTCTCGAAACTCACCA
spacer-multi-crRNA-R	ATATATGGTCTCGAAAACG

IV

ON-DEMAND ISOLATION OF BACTERIOPHAGES AGAINST DRUG-RESISTANT BACTERIA FOR PERSONALIZED PHAGE THERAPY

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On-Demand Isolation of Bacteriophages Against Drug-Resistant Bacteria for Personalized Phage Therapy

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Bacteriophages are bacterial viruses, capable of killing even multi-drug resistant bacterial cells. For this reason, therapeutic use of phages is considered as a possible alternative to conventional antibiotics. However, phages are very host specific in comparison to wide-spectrum antibiotics and thus preparation of phage-cocktails beforehand against pathogens can be difficult. In this study, we evaluate whether it may be possible to isolate phages on-demand from environmental reservoir. We attempted to enrich infectious bacteriophages from sewage against nosocomial drug-resistant bacterial strains of different medically important species in order to evaluate the probability of discovering novel therapeutic phages. Stability and host-range were determined for the acquired phages. Our results suggest that on-demand isolation of phages is possible against *Pseudomonas aeruginosa, Salmonella* and extended spectrum beta-lactamase *Escherichia coli* and *Klebsiella pneumoniae*. The probability of finding suitable phages was less than 40% against vancomycin resistant *Enterococcus* and *Acinetobacter baumannii* strains. Furthermore, isolation of new phages against methicillin resistant *Staphylococcus aureus* strains was found to be very difficult.

Keywords: antibiotic resistance, ESBL, MRSA, phage therapy, phage cocktails, bacteriophages

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INTRODUCTION

Antibiotic resistance is an emerging global health crisis, resulting from the continuous use (and misuse) of antibiotics in healthcare, farming industry, and elsewhere (Cantas et al., 2013; World Health Organization [WHO], 2014). Phage therapy refers to the utilization of bacteriophages (or just phages, viruses infecting bacteria) to treat bacterial diseases (Abedon et al., 2011). Given the increasing number of drug-resistant bacterial infections, especially within hospital settings, the exploration of alternatives to conventional antibiotics has become an important research objective (Finch, 2011; Sommer and Dantas, 2011). Bacteriophages are very abundant (Hendrix et al., 1999) and every bacterium is likely to have their own specific viruses that could be utilized as antibacterial agents (Clokie et al., 2011; Flores et al., 2011; Örmälä and Jalasvuori, 2013). Historically, phages were used therapeutically already in the early 20th century (Sulakvelidze et al., 2001). Yet, the discovery of broadly effective antibiotics led to the demise of the development of phage therapy in western countries and only as the antibiotics are starting to fail there has been a serious attempt to restore the old tool. However, the second coming of phage therapy faces challenges regarding

to the strict regulatory guidelines and the development of effective therapeutic practices (Gill and Hyman, 2010; Lu and Koeris, 2011; Keen, 2012). Yet, phage therapy can provide an evolutionarily sustainable alternative to conventional antibiotics, should we be able to adjust our regulations and procedures to meet the special requirements of phage based medicine (Keen, 2012; Örmälä and Jalasvuori, 2013).

It is important to note that phages infect bacterial hosts very selectively. Often, the narrow host-range is considered as an advantage over traditional antibiotics since phage treatment can focus accurately on the pathogen without harming commensal bacterial flora (Loc-Carrillo and Abedon, 2011). On the other hand, bacteria develop resistance also to phages rapidly, and thus the achieved antibacterial effect may be transient (Hyman and Abedon, 2010; Labrie et al., 2010). When multiple different phages are used simultaneously in a phage cocktail, development of resistance is less likely (Skurnik et al., 2007; Chan et al., 2013). However, it is challenging to obtain a set of phages that is effective against all variants of a given pathogen (Pirnay et al., 2011; Chan et al., 2013). There can be a tradeoff between the host range and the therapeutic efficacy of a cocktail for a specific species of bacteria: when the number of phages in a cocktail increases in an effort to increase the host range of the cocktail, the number of phages against a specific strain of bacteria may decrease. Therefore, the host specificity of phages, while in theory beneficial, poses a practical problem when combined with the rapidly emerging resistant phenotypes.

In principle, it is possible to acquire bacteriophages on-demand to treat, for example, infections that are resistant to all known antibiotics and off-the-shelf (standardized) phage-therapy products (Keen, 2012; Örmälä and Jalasvuori, 2013). Tailoring a therapeutic cocktail personally for each patient would allow the cocktails to comprise phages that are effective against the bacterial strains responsible of the infection (Pirnay et al., 2011; Chan et al., 2013). Therefore and in comparison to premade cocktails, a personalized phage therapy does not carry a surplus of ineffective phages. Indeed, there are older studies suggesting that tailored phage treatments are several times more effective compared to standardized cocktails (Zhukov-Verezhnikov et al., 1978), and thus effective phage-therapy practices to treat constantly changing bacterial pathogens may depend on the adjustment of the treatment to the causative agent (Keen, 2012).

Generating a personal set of phages requires that the pathogen is isolated and, then, effective bacteriophages obtained against it. One possible way for identifying suitable viruses is to have a variety of bacteriophages isolated and prepared beforehand and then the causative pathogen screened through the phage-library (Chan et al., 2013). Alternatively, phages may be isolated as needed from environmental reservoirs. In some cases, the latter option may be inevitable due to the lack of infectious phages in the premade libraries against all possible bacterial variants. Ultimately, environment serves as the only source of practically endless phage variety and thus exploitation of the environmental resources forms the basis for personalized phage medicine.

While phages are known to be abundant, it is obvious that all environments cannot contain infective phages against all different bacterial hosts (see e.g., Flores et al., 2011; Atanasova et al., 2012). To the best of our knowledge, the probability of finding therapeutically useful phages against different resistant pathogens on-demand has not been studied *per se* despite the fact that it is likely to be the limiting factor in attempts to update premade cocktails or to generate on-demand personalized therapies (Chan et al., 2013). As an example, hospital acquired wound infections have been suggested to be especially suitable target for phage therapy as the causative agents are generally resistant to various antibiotics (Loc-Carrillo et al., 2012). Yet, there might be multiple different bacterial species present in these infections, including, e.g., *Staphylococcus aureus*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Acinetobacter baumannii* (Agnihotri et al., 2004). Therefore, a successful phage-based treatment can be dependent on the practicality of being able to simultaneously and rapidly isolate new durable phages against very different pathogens.

In this study, we provide an evaluation of the on-demand isolation of phages against the most common hospital borne resistant pathogens: methicillin resistant *S. aureus* (MRSA), extended spectrum beta-lactamase (ESBL) *E. coli* and *K. pneumoniae*, multi-drug resistant (MDR) *P. aeruginosa*, vancomycin resistant *Enterococcus* (VRE), *A. baumannii* and different *Salmonella* species. All aforementioned species are also listed in CDC's report on the top 18 drug-resistant threats to the United States in 2013 (CDC, 2013). These bacteria commonly cause infections of skin, lung and urinary tract, as well as foodborne infections among others and affect people all around the world disregarding their background (CDC/FDA/NIH, 2011).

Sewage is known to be an optimal resource of phages (Lobocka et al., 2014), thus a wastewater treatment plant in Jyväskylä, Finland (Nenäinniemi) was used as the environmental reservoir for phage hunt. The stability of the acquired viruses and their cross-infectivity on other potential host strains were determined.

We demonstrate vast differences in probabilities of finding novel phages against different hosts by using enrichment method for isolation. There appears to be severe constraints in isolating phages on-demand against pathogens like MRSA. On the other hand, it seems feasible to obtain phages against ESBL positive *E. coli* and *K. pneumoniae* as well as *P. aeruginosa*.

MATERIALS AND METHODS

Bacteria Strains and Culturing Conditions

Bacterial strains used in this study were mostly purchased from Medix Laboratories or acquired from Turku University Hospital (Supplementary Table S1). One *Klebsiella* strain and four *Enterococccus* strains were obtained from commercial culture collections. Aside from six bacterial strains, all had caused (antibiotic resistant) human infections and thus they represent pathogens that could have been treated with phages. Overall, we obtained 12 MRSA strains, 16 *E. coli* ESBL strains, 6 *K. pneumonia* (ESBL) strains, 17 *P. aeruginosa* MDR strains, 9 *A. baumannii* strains, 10 *E. faecium* (VRE) strains, 4 *Enterococcus faecalis* (VRE) strains, and 9 different *Salmonella* strains. Detailed

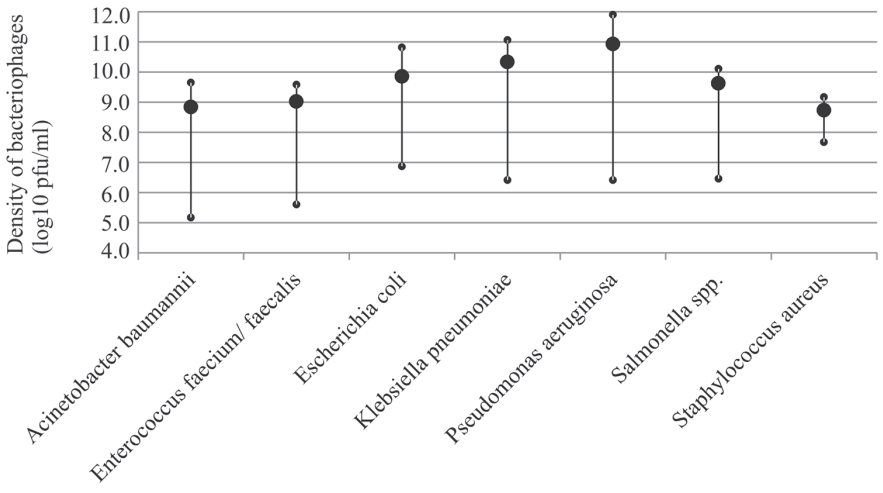

FIGURE 1 | Average density of infectious bacteriophage particles in the prepared stocks of each host species (large circle). Small circles indicate the maximum and minimum values observed.

TABLE 1 | Summary of the decrease in phage titers as observed after 1-month storage at +4°C.

Host bacterium	Average decrease in titer (log10)
Acinetobacter baumannii	0.973
Enterococcus faecium/ faecalis	0.222
Escherichia coli	0.496
Klebsiella pneumoniae	0.594
Pseudomonas aeruginosa	0.437
Salmonella sp.	0.529
Staphylococcus aureus	0.491

characterization of the bacterial strains was beyond the scope of this paper.

All bacteria were cultured in Lysogeny Broth (LB) -medium (Sambrook et al., 1989) at +37°C shaken 230 rpm (*Enterococcus* strains were cultivated without shaking).

Isolation Protocol

The following isolation protocol with slight modification in individual experiments was used throughout the study. Either unprocessed sewage samples or supernatants of turbid samples (centrifuged 3000–6000 *g* in Megafuge 1.0R, Heraeus, or in Eppendorf centrifuge 5702 R, 10–15 min at +4°C) were used in the enrichment steps. In cases where previous isolation attempts had failed to yield phages, the supernatant was also filtrated through a 0.45 μm filter to remove all remaining bacterial cells. The first enrichment step was conducted using 20–30 ml of sewage water filled up to 30–40 ml with LB-broth, depending on the volume of collected sewage samples. The target bacterial strain was added (50–200 μl o/n culture grown in LB-broth, 300 μl in case of *E. faecium* and *E. faecalis*) to enrich (potential) phages in the sample. These enrichments were cultivated overnight at +37°C, shaken 230 rpm. Bacteria from this enrichment culture were removed by centrifugation (3000–6000 *g* in Megafuge 1.0R, Heraeus, or in Eppendorf centrifuge 5702 R, 15–20 min, +4°C) and filtration (0.2 or 0.45 μm filter). The amount of potential phages in a 2.5 ml sample of the bacteria-free enrichment were further amplified by adding 2.5 ml of LB-broth and 50–100 μl of the target host bacterium and were grown overnight as above. The sample from this second enrichment step was centrifuged at 13 000 *g* for 15 min at room temperature and at least 10 μl the supernatant was plated on a LB-agar containing petri dish along with 100–300 μl of the host strain and 3 ml of melted 0.7% soft-agar. The plates were incubated overnight at +37°C. If plaques were observed on the bacterial lawn, a separate plaque was picked and transferred into 500 μl of LB-broth. A sample from this plaque-stock was further plated on the same host strain. Plaque-purification was performed three times for all discovered phages in order to isolate a single homogenous phage from the potentially heterogeneous phage mix that may have been present in the initial enrichment.

Due to poor isolation success for *S. aureus*, different modifications of the above-described method were used for enriching phages. The volume of the first enrichment step as well as the number of enrichment steps was increased (120 ml sewage sample + 70 ml L broth + 1 ml host overnight cultures in the first step). Rotation speed during shaken cultivation steps was varied between 100, 120, 180, or 360 rpm. In addition, samples from different sources were used for phage enrichment (River in Ljubljana, Slovenia, a water-lock sample from the Helsinki university hospital and soil samples from a livestock farm). These samples were not included in analysis of isolation success from sewage.

Preparation of Phage Stock

Semi-confluent plates (i.e., plates of which about half of the area is covered by phage induced plaques and the rest is bacterial lawn) were prepared by plating 100 μl of host strain (300 μl

TABLE 2 | Probability for discovering a bacteriophage from a sewage sample against different pathogens.

Bacterial pathogen	Mean hit %*	Isolation attempts	Number of strains hit
Acinetobacter baumannii	38.9	34	5/9
Enterococcus faecium/faecalis	33.9	27	5/14
Escherichia coli	90.6	35	15/16
Klebsiella pneumoniae	83.3	15	6/6
Pseudomonas aeruginosa	79.4	44	15/17
Salmonella sp.	88.9	11	8/9
Staphylococcus aureus	6.1	117	1/12

**As calculated over the bacterial strains of the given species.*

of *Enterococcus* strains) and 3 ml of melted soft-agar with appropriate dilution of the phage stock. Plates were incubated overnight at +37°C. The soft-agar layers of semi-confluent plates were combined with 2.5–5 ml of LB-broth/plate. The combination was incubated for 4 h at +37°C, 230 rpm, and centrifuged at 6000 *g* for 15 min at +4°C (Megafuge 1.0R, Heraeus). If we were unable to get semi-confluent plates, we used as a combination "over-infected" plates supplied with 100–700 μl of the overnight-cultivated host strain. The supernatant was filtered (0.2 μm filter) and stored at +4°C.

Cross Infection Tests

All phages were used to cross-infect all different bacterial strains of its original host species (excluding *P. aeruginosa* phages as only half of them were used) for preliminary evaluation of their host range. Cross-infection tests were done by spotting 8 μl of phage stock dilution (1:10 or 1:100) on 100 μl bacterial overnight culture in soft-agar (0.7%). Plates were incubated at +37°C overnight. Formation of less opaque spots on the bacterial lawn was scored as a successful infection.

Phage Stock Stability

The titer of each phage stock was determined by standard double agar overlay method by plating a dilution series (10^{-2}–10^{-8}) immediately after preparation of the stock. Titer of the stock was determined again after 1-month storage (+4°C) to estimate the stability of the stock in LB-medium.

RESULTS

We evaluated the feasibility for generating a personalized phage-product on-demand against different bacterial pathogens. We chose bacterial species from seven different genuses that are responsible for the majority of hospital acquired bacterial infections, namely *Escherichia*, *Salmonella*, *Klebsiella*, *Pseudomonas*, *Staphylococcus*, *Enterococcus,* and *Acinetobacer*. Total of 283 phage isolation attempts were conducted for 83 different host strains. Overall 108 bacteriophages were discovered. All of these viruses were characterized for their plaque morphology and stability (described individually for each virus in Supplementary Table S2).

Phages were isolated via three consecutive plaque-picking steps to avoid mixed-culture stocks. Due to different plaque morphologies and titers, the preparation of phage stocks was adjusted for each phage. However, no actual optimization of phage production was carried out. The density of viable phage particles was measured immediately after the preparation of the stock (**Figure 1**). In order to determine their viability for acute use, the number of viable particles was re-measured 1 month later (see summary in **Table 1**). On average, the titers of the stocks decreased around 0.5 log10 during the 1-month storage in L-broth in 4°C. However, for some phages of *Enterococcus*, the titers could no longer be resolved. Phage-specific titers and plaque morphologies are listed in Supplementary Table S2.

The probability for finding an infectious bacteriophage from sewage for different host bacterium varied substantially (**Table 2**). Namely, phages for only a single *S. aureus* strain, SA10, were discovered in total of 117 enrichment attempts (the phages specific to the one *S. aureus* strain were obtained at the same time and they produced visually identical plaques, thus we selected only one of these phages for subsequent analyses). Conversely, almost every isolation attempt yielded a bacteriophage for *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *Salmonella* strains. Phage isolation for *Acinetobacter* and *Enterococcus* had success rates between 30 and 40%. Given the medical importance of MRSA, we decided to investigate whether alternative source materials would be more suitable for discovering phages. We obtained water samples from a water lock situated in a room used to treat MRSA-patients in Helsinki University Hospital. Two phages for a single strain (SA10) were found from these samples. A single MRSA-specific bacteriophage was isolated from a set of soil samples acquired from a livestock farm. Also, a water sample from river Ljubljana, Slovenia, produced a single bacteriophage for strain SA10. Yet, we failed to find a single phage for any of the ten other MRSA-strains used in the isolation attempts.

As presented in **Figure 2**, we studied the host-range of the obtained phages in order to determine their cross-infectivity and thus the potential to combine previously isolated phages into phage-cocktails. Aside from a couple of exceptions, almost all phages isolated for any given *P. aeruginosa* strain could also infect majority of the other strains. However, we neither found any phages for strain PA15 nor did any of the other phages infect this strain. In addition, only 4 out of 20 tested *Pseudomonas* phages infected strain PA6. Detailed characterization of these particular strains was beyond the scope of this paper.

Along with *Pseudomonas* phages, some of the *Salmonella* phages had a wide host range. *E. coli* phages tended to infect more than one strain, except EC1P1, EC11P2, EC15P2, and EC16P1. For other bacterial species, isolated phages generally had less

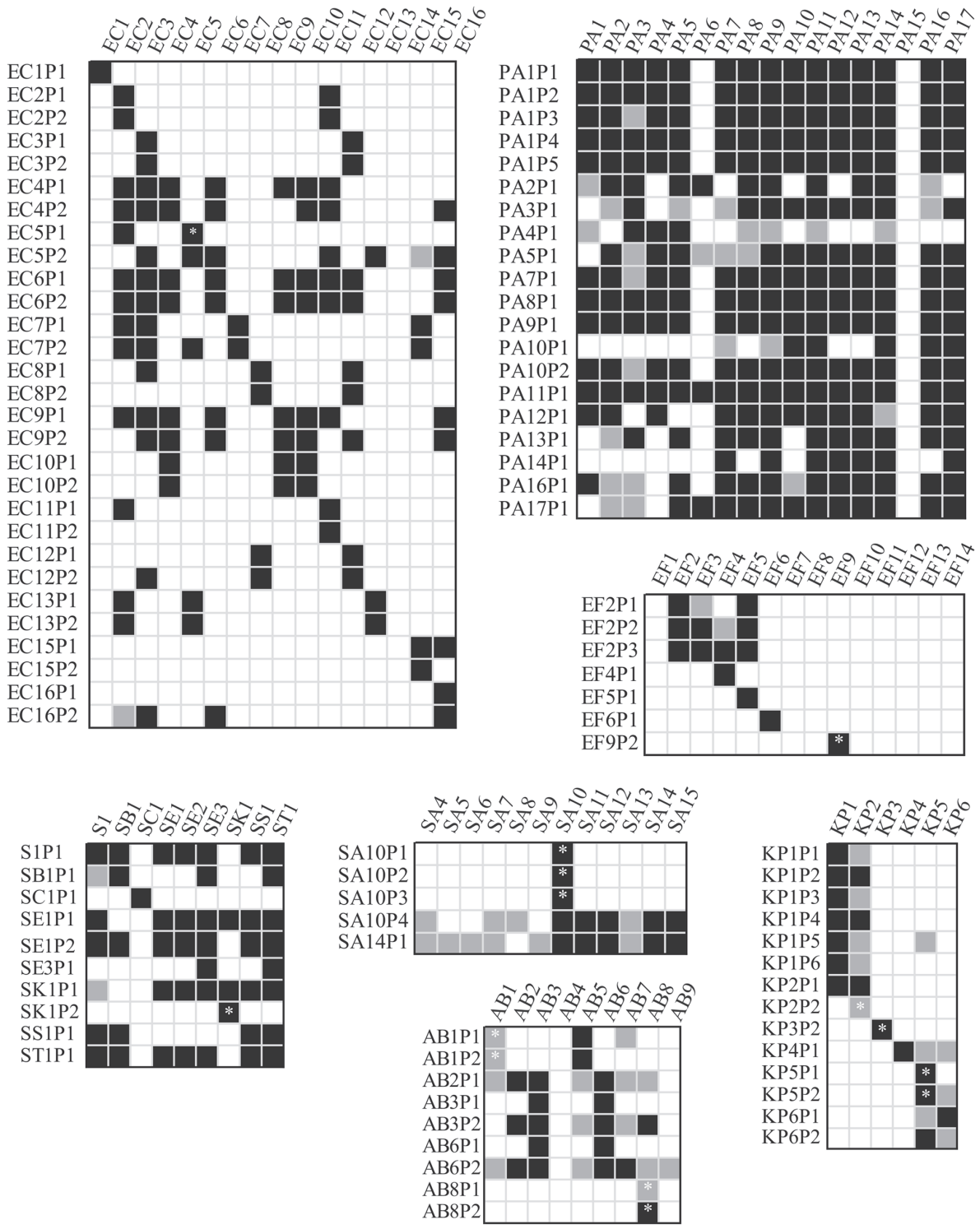

FIGURE 2 | Cross-infectivity of the isolated bacteriophages. Measurements where less than 10^5 pfu/ml were used are indicated with an asterisk. Only half of the isolated *Pseudomonas* phages were used in the experiments. White background indicates no lysis area, black marks clearly detected lysis area and light gray indicates very dim lysis area in the spot test. AB, *Acinetobacter baumannii*; EC, *Escherichia coli*; EF, *Enterococcus faecium* or *faecalis*; KB, *Klebsiella pneumoniae*; PA, *Pseudomonas aeruginosa*; S, *Salmonella* sp., and SA, *Staphylococcus aureus*.

alternative hosts, if any, indicating that a rapid preparation of a personalized phage-cocktail is likely to require multiple separate but simultaneous phage enrichments using a single bacterial strain as a host. Especially, *Klebsiella* and *Enterococcus* phages are very host specific. Sometimes phage stocks produced only a dim inhibition area on alternative hosts (presented as light gray coloring in **Figure 2**). This suggests that something, but not necessarily the phage in the prepared stocks was restricting the growth of the bacterium. Furthermore, phages isolated for any particular host often had similar infection patterns. This suggests that additional isolation attempts using the same isolation source for enrichment may not be the best choice for improving the host-range of the cocktail.

DISCUSSION

Due to the enormous variety of bacteriophages in environmental reservoirs, on-demand isolation of novel phage-antibacterials is a potential way to generate a personalized medicine for treating bacterial infections that are resistant to conventional drugs. In this study, we evaluated the feasibility of isolating phages for such therapeutic cocktails.

The efforts required to find phages differs substantially between bacterial species. Phages can be readily discovered for *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *Salmonella* species. Although virus production was neither optimized nor standardized in this study, phages of these hosts also readily generated high-density virus stocks (**Figure 1**). In contrast, we found it very challenging to isolate phages against *Staphylococcus* strains despite of several attempts that were conducted at different times of year and from multiple sources (sewage, river, hospital water lock, and livestock farm soil samples). It was also more laborious to isolate phages for *E. faecium* and *faecalis* and *A. baumannii,* although it must be noted that we had only handful of these strains and we performed only few isolation attempts for them. Nevertheless, based on the results, the on-demand discovery of phages appears to be feasible for some but not all bacteria. This highlights the importance of premade wide-host range cocktails or the existence of other antimicrobial solutions against species such as *S. aureus* (such as the one developed by Kelly et al., 2011). Also, teixobactin, the first new potential antibiotic to be discovered in 30 years is very effective against bacteria lacking the outer membrane (such as *S. aureus* and *Enterococcus*; Ling et al., 2015). Yet, gram-negative pathogens with the impermeable outer membrane (e.g., *E. coli*, *Salmonella*, *K. pneumonia,* and *P. aeruginosa*) are inherently resistant to antibiotics like teixobactin, but contrastingly appear to be suitable targets for obtaining a cocktail from environmental reservoir (sewage) as needed. Also, better preservability and wider host-range of these phages supports the on-demand isolation approach. While conventional tools for antibiotic development may still remain relevant, in the face of worsening world-wide antibiotic resistance crisis we should be actively exploring these promising alternatives in order to retain the upper hand against all pathogens.

Generalization of the obtained results must be done while acknowledging the potential sources of error. First, while we collected our sewage samples at different times (over the timespan of almost 2 years), only a single wastewater management plant was used. Although the biological material in these plants changes constantly, the phage populations may still be substantially different in different plants, thus possibly skewing the chances for finding phages against certain species. Moreover, the host ranges of some phages appear identical, suggesting that the hosts themselves may be genetically very close to one another. Second, albeit we performed several hundred isolation attempts, just a few isolations were performed for any particular strain and thus the achieved probabilities should be treated as a case study rather than an exhaustive evaluation. Third, we did not perform an in-detail characterization for the isolated phages. Such characterization, at least to some extent, will be necessary during actual therapy practices (Skurnik et al., 2007; Merabishvili et al., 2009; Keen, 2012), as bacteriophages are known to carry undesirable genes coding for toxins and antibiotic resistances (Loc-Carrillo and Abedon, 2011). However, separating lytic phages from temperate phages (possibly when accompanied with genome sequencing and analysis) should be enough and feasible for the rapid assessment of safety (Chan et al., 2013). Also, phage stocks have to be purified from (host-bacterium generated) endotoxins before therapeutic use (Keen, 2012). These steps were not performed or their effects on phages evaluated in this study.

CONCLUSION

The success of on-demand isolation of phages appears to be critically dependent on the bacterial host. Promisingly, against pathogens for which conventional antibiotics are becoming the least useful, such as ESBL *E. coli* and *K. pneumoniae*, personalized phage therapy could be considered as a potential alternative.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01271

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Table S1. Bacterial strains used in this study.

Bacterial strains	Short name	Source
Acinetobacter baumannii 57163	AB1	Turku University Hospital
Acinetobacter baumannii 59999	AB2	Turku University Hospital
Acinetobacter baumannii 62060	AB3	Turku University Hospital
Acinetobacter baumannii 20692	AB4	Turku University Hospital
Acinetobacter baumannii 57163	AB5	Turku University Hospital
Acinetobacter baumannii 18243	AB6	Turku University Hospital
Acinetobacter baumannii 26006	AB7	Turku University Hospital
Acinetobacter baumannii 18510	AB8	Turku University Hospital
Acinetobacter species 18560	AB9	Turku University Hospital
Enterococcus faecium 61285	EF1	Turku University Hospital
Enterococcus faecalis 58897	EF2	Turku University Hospital
Enterococcus faecalis	EF3	ATCC 19433
Enterococcus faecalis	EF4	ATCC 29212
Enterococcus faecalis	EF5	ATCC 33186
Enterococcus faecium	EF6	ATCC 9790
Enterococcus faecium 59776	EF7	Turku University Hospital
Enterococcus faecium 61244	EF8	Turku University Hospital
Enterococcus faecium 61027	EF9	Turku University Hospital
Enterococcus faecium 60803	EF10	Turku University Hospital
Enterococcus faecium 60734	EF11	Turku University Hospital
Enterococcus faecium 60703	EF12	Turku University Hospital
Enterococcus faecium 60457	EF13	Turku University Hospital
Enterococcus faecium 60145	EF14	Turku University Hospital
Escherichia coli 10AE5909	EC1	Medix
Escherichia coli 12UM05186	EC2	Medix
Escherichia coli 10UU11258	EC3	Medix
Escherichia coli 11UT10019	EC4	Medix
Escherichia coli 11AN03027	EC5	Medix
Escherichia coli 11UT12639	EC6	Medix
Escherichia coli 11UO03492	EC7	Medix
Escherichia coli 11UU07697	EC8	Medix
Escherichia coli 11UM05271	EC9	Medix
Escherichia coli 57262	EC10	Turku University Hospital
Escherichia coli 57294	EC11	Turku University Hospital
Escherichia coli 57189	EC12	Turku University Hospital
Escherichia coli 57253	EC13	Turku University Hospital
Escherichia coli 55027	EC14	Turku University Hospital
Escherichia coli 56895	EC15	Turku University Hospital
Escherichia coli 57361	EC16	Turku University Hospital
Klebsiella pneumoniae	KP1	DSM681
Klebsiella pneumoniae 10UO03898	KP2	Medix
Klebsiella pneumoniae 61705	KP3	Turku University Hospital
Klebsiella pneumoniae 61784	KP4	Turku University Hospital
Klebsiella pneumoniae 61837	KP5	Turku University Hospital
Klebsiella pneumoniae 61794	KP6	Turku University Hospital
Pseudomonas aeruginosa 61841	PA1	Turku University Hospital
Pseudomonas aeruginosa 61823	PA2	Turku University Hospital
Pseudomonas aeruginosa 61790	PA3	Turku University Hospital
Pseudomonas aeruginosa 61432	PA4	Turku University Hospital
Pseudomonas aeruginosa 11AN03663	PA5	Medix
Pseudomonas aeruginosa 26153	PA6	Turku University Hospital
Pseudomonas aeruginosa 62314	PA7	Turku University Hospital
Pseudomonas aeruginosa 62263	PA8	Turku University Hospital
Pseudomonas aeruginosa 62224	PA9	Turku University Hospital
Pseudomonas aeruginosa 62206	PA10	Turku University Hospital
Pseudomonas aeruginosa 62180	PA11	Turku University Hospital
Pseudomonas aeruginosa 62181	PA12	Turku University Hospital
Pseudomonas aeruginosa 62172	PA13	Turku University Hospital
Pseudomonas aeruginosa 62109	PA14	Turku University Hospital
Pseudomonas aeruginosa 62090	PA15	Turku University Hospital
Pseudomonas aeruginosa 62069	PA16	Turku University Hospital
Pseudomonas aeruginosa 61932	PA17	Turku University Hospital
Salmonella, Group B, ESBL strain 12FB01687	SB1	Turku University Hospital
Salmonella, Group C, 13FB10784	SC1	Turku University Hospital
Salmonella enteritis (36.)	SE1	Turku University Hospital
Salmonella enteritidis 13FB9205	SE2	Turku University Hospital
Salmonella enteritidis FB11214	SE3	Turku University Hospital
Salmonella Kreber (37.)	SK1	Turku University Hospital
Salmonella stanley FB3820	SS1	Turku University Hospital
Salmonella typhimurium FB7595	ST1	Turku University Hospital
Salmonella, quality control strain 18048	S1	Turku University Hospital
Staphylococcus aureus 11AN02972	SA4	Medix
Staphylococcus aureus 10MR01905	SA5	Medix
Staphylococcus aureus 11AE06590	SA6	Medix
Staphylococcus aureus 11AE07165	SA7	Medix
Staphylococcus aureus 11AN03312	SA8	Medix
Staphylococcus aureus 10AN02929	SA9	Medix
Staphylococcus aureus 10AE05905	SA10	Medix
Staphylococcus aureus 10AE06192	SA11	Medix
Staphylococcus aureus 60820	SA12	Turku University Hospital
Staphylococcus aureus 61765	SA13	Turku University Hospital
Staphylococcus aureus 60881	SA14	Turku University Hospital
Staphylococcus aureus 60689	SA15	Turku University Hospital

Table S2. Isolated phages.

Phage	Host	Plaque morphology	Titer	Titer after 1 month
AB1P1	*Acinetobacter baumannii* 57163	small, bright and some dim	4.2×10^{6}	3.0×10^{3}
AB1P2	*Acinetobacter baumannii* 57163	small, bright and some dim	2.0×10^{6}	7.2×10^{3}
AB2P1	*Acinetobacter baumannii* 59999	small, dim, rough edges, roundish	6.9×10^{7}	3.9×10^{7}
AB3P1	*Acinetobacter baumannii* 62060	medium-size, bright, big halo	4.5×10^{9}	9.1×10^{8}
AB3P2	*Acinetobacter baumannii* 62060	small, ,bright, round	1.8×10^{8}	3.5×10^{7}
AB6P1	*Acinetobacter baumannii* 18243	small and medium-size, dim, big halo	3.5×10^{8}	5.7×10^{8}
AB6P2	*Acinetobacter baumannii* 18243	small, dim, rough edges, roundish	1.1×10^{9}	1.5×10^{8}
AB8P1	*Acinetobacter baumanni* 18510	extremely small, slightly dim	1.5×10^{5}	2.6×10^{5}
AB8P2	*Acinetobacter baumannii* 18510	small, dim, rough edges, roundish	3.4×10^{5}	7.2×10^{4}
EC1P1	*Escherichia coli* 10AE5909	small, bright center surrounded by misty ring	6.0×10^{7}	5.6×10^{7}
EC2P1	*Escherichia coli* 12UM05186	big, bright	1.2×10^{10}	6.7×10^{9}
EC2P2	*Escherichia coli* 12UM05186	big, bright	7.6×10^{8}	1.3×10^{9}
EC3P1	*Escherichia coli* 10UU11258	medium-size, bright	1.7×10^{8}	8.4×10^{6}
EC3P2	*Escherichia coli* 10UU11258	medium-size, bright	4.6×10^{8}	1.3×10^{7}
EC4P1	*Escherichia coli* 11UT10019	small, bright	2.1×10^{7}	8.9×10^{6}
EC4P2	*Escherichia coli* 11UT10019	small, bright	1.6×10^{8}	2.6×10^{8}
EC5P1	*Escherichia coli* 11AN03027	small, bright	7.4×10^{6}	8.8×10^{5}
EC5P2	*Escherichia coli* 11AN03027	extremely small, bright	3.5×10^{4}	N/A
EC6P1	*Escherichia coli* 11UT12639	small, bright	1.6×10^{9}	2.9×10^{9}
EC6P2	*Escherichia coli* 11UT12639	small, bright	1.9×10^{9}	3.1×10^{9}
EC7P1	*Escherichia coli* 11UO03492	small, slightly dim	3.9×10^{9}	2.8×10^{9}
EC7P2	*Escherichia coli* 11UO03492	small, slightly dim	2.1×10^{10}	3.6×10^{10}
EC8P1	*Escherichia coli* 11UU07697	extremely small, slightly dim	2.4×10^{7}	7.3×10^{7}
EC8P2	*Escherichia coli* 11UU07697	extremely small, slightly dim	1.2×10^{7}	4.0×10^{7}
EC9P1	*Escherichia coli* 11UM05271	extremely small, bright	1.3×10^{9}	1.5×10^{9}
EC9P2	*Escherichia coli* 11UM05271	extremely small, bright	1.5×10^{10}	7.3×10^{9}
EC10P1	*Escherichia coli* 57262	small, bright	6.6×10^{10}	6.7×10^{10}
EC10P2	*Escherichia coli* 57262	small, bright	6.3×10^{10}	5.4×10^{10}
EC11P1	*Escherichia coli* 57294	big, bacterial growth in the middle	2.5×10^{9}	2.4×10^{8}
EC11P2	*Escherichia coli* 57294	small, some bright and some dim	1.6×10^{9}	4.0×10^{8}
EC12P1	*Escherichia coli* 57189	small, dim	1.2×10^{8}	8.5×10^{7}
EC12P2	*Escherichia coli* 57189	small, slightly dim	7.0×10^{7}	2.6×10^{7}
EC13P1	*Escherichia coli* 57253	small, dim	2.0×10^{8}	1.1×10^{8}
EC13P2	*Escherichia coli* 57253	small, dim	4.2×10^{8}	8.0×10^{7}
EC15P1	*Escherichia coli* 56895	small, bright, halo	9.0×10^{9}	9.6×10^{4}
EC15P2	*Escherichia coli* 56895	medium-size, dim, vague halo	2.3×10^{9}	4.0×10^{8}
EC16P1	*Escherichia coli* 57361	big and medium-size, bright, vague ring	3.7×10^{9}	6.3×10^{9}
EC16P2	*Escherichia coli* 57361	small/medium-size, bright	6.0×10^{8}	6.0×10^{8}
EF2P1	*Enterococcus faecalis* 58897	small, bright	3.8×10^{9}	1.7×10^{9}
EF2P2	*Enterococcus faecalis* 58897	small, bright	2.3×10^{9}	1.3×10^{9}
EF2P3	*Enterococcus faecalis* 58897	medium-size, brigth	8.8×10^{8}	7.3×10^{8}
EF4P1	*Enterococcus faecalis* ATCC 29212	big, bright, difficult to determine titer	3.5×10^{9}	N/A
EF5P1	*Enterococcus faecalis* ATCC 33186	big, bright, difficult to determine titer	7.0×10^{7}	N/A
EF6P1	*Enterococcus faecium* ATCC 9790	medium-size, bright, difficult to determine titer	8.0×10^{5}	N/A
EF9P2	*Enterococcus faecium* 61027	medium-size, round, dim, difficult to determine titer	4.0×10^{5}	N/A
KP1P1	*Klebsiella pneumoniae* DSM681	big, bacterial growth in the middle of plaque	6.8×10^{10}	1.7×10^{10}
KP1P2	*Klebsiella pneumoniae* DSM681	small, bright center surrounded by misty ring, some big	4.9×10^{8}	2.3×10^{7}
KP1P3	*Klebsiella pneumoniae* DSM681	medium-size, slightly turbid center	3.0×10^{10}	3.2×10^{9}
KP1P4	*Klebsiella pneumoniae* DSM681	medium-size, slightly turbid center	4.7×10^{10}	1.8×10^{10}
KP1P5	*Klebsiella pneumoniae* DSM681	big, turbid, bacterial growth in the middle of plaque	1.2×10^{11}	1.1×10^{10}
KP1P6	*Klebsiella pneumoniae* DSM681	variable size, some bright, some had bacterial growth in the middle	3.0×10^{10}	6.1×10^{9}
KP2P1	*Klebsiella pneumoniae* 10UO03898	medium-size, bright	1.5×10^{8}	2.5×10^{8}
KP2P2	*Klebsiella pneumoniae* 10UO03898	small, bright	2.6×10^{6}	2.1×10^{5}
KP3P2	*Klebsiella pneumoniae* 61705	medium-size, dim	1.0×10^{7}	1.5×10^{7}
KP4P1	*Klebsiella pneumoniae* 61784	small, bright, halo	1.4×10^{9}	4.4×10^{8}
KP5P1	*Klebsiella pneumoniae* 61837	small, bright	1.7×10^{9}	7.0×10^{8}
KP5P2	*Klebsiella pneumoniae* 61837	small, bright	5.0×10^{8}	2.9×10^{8}
KP6P1	*Klebsiella pneumoniae* 61794	small, slightly dim	4.0×10^{9}	1.8×10^{9}
KP6P2	*Klebsiella pneumoniae* 61794	small, dim	4.1×10^{8}	8.0×10^{7}
PA1P1	*Pseudomonas aeruginosa* 61841	small, bright, vague thin ring around	2.0×10^{11}	1.0×10^{11}
PA1P2	*Pseudomonas aeruginosa* 61841	small, bright, vague thin ring around	9.3×10^{10}	6.5×10^{10}
PA1P3	*Pseudomonas aeruginosa* 61841	small, bright, vague thin ring around	8.1×10^{10}	3.6×10^{10}
PA1P4	*Pseudomonas aeruginosa* 61841	small, bright, vague thin ring around	1.1×10^{11}	6.8×10^{10}
PA1P5	*Pseudomonas aeruginosa* 61841	small, bright, vague thin ring around	1.6×10^{11}	1.1×10^{11}
PA2P1	*Pseudomonas aeruginosa* 61823	small, dim	9.5×10^{8}	1.8×10^{11}
PA2P2	*Pseudomonas aeruginosa* 61823	small, round, turbid center surrounded by bright ring	5.0×10^{6}	5.0×10^{7}
PA3P1	*Pseudomonas aeruginosa* 61790	small/medium-size, dim	3.3×10^{10}	2.9×10^{8}
PA3P2	*Pseudomonas aeruginosa* 61790	small/medium-size, very dim, disfigured plaques	3.4×10^{9}	1.9×10^{9}
PA4P1	*Pseudomonas aeruginosa* 61432	small, dim	5.8×10^{6}	1.3×10^{7}
PA4P2	*Pseudomonas aeruginosa* 61432	round, bright	3.7×10^{9}	2.2×10^{9}
PA5P1	*Pseudomonas aeruginosa* 11AN03663	medium-size, bright	6.0×10^{9}	7.0×10^{9}
PA5P2	*Pseudomonas aeruginosa* 11AN03663	medium-size, bright, round, some small and dim	6.7×10^{8}	1.7×10^{6}
PA7P1	*Pseudomonas aeruginosa* 62314	small, dim, rough edges	2.1×10^{10}	3.9×10^{10}
PA7P2	*Pseudomonas aeruginosa* 62314	medium-size, slightly dim	1.5×10^{11}	5.5×10^{10}
PA8P1	*Pseudomonas aeruginosa* 62263	small, halo	1.4×10^{11}	1.3×10^{11}
PA8P2	*Pseudomonas aeruginosa* 62263	small, bright	6.1×10^{8}	2.9×10^{6}
PA9P1	*Pseudomonas aeruginosa* 62224	small, bright, halo	1.4×10^{11}	1.9×10^{11}
PA9P2	*Pseudomonas aeruginosa* 62224	big, bright	1.2×10^{9}	3.4×10^{7}
PA10P1	*Pseudomonas aeruginosa* 62206	big, dim	7.0×10^{9}	6.0×10^{9}
PA10P2	*Pseudomonas aeruginosa* 62206	small, bright, halo	1.0×10^{11}	1.1×10^{11}
PA10P3	*Pseudomonas aeruginosa* 62206	small, dim, vague halo	6.7×10^{10}	6.8×10^{9}
PA11P1	*Pseudomonas aeruginosa* 62180	small, dim, rough edges	2.0×10^{11}	1.4×10^{11}
PA11P2	*Pseudomonas aeruginosa* 62180	small, round, dim	8.0×10^{11}	5.4×10^{11}
PA12P1	*Pseudomonas aeruginosa* 62181	medium-size, slightly dim, rough edges	1.7×10^{8}	2.5×10^{8}
PA12P2	*Pseudomonas aeruginosa* 62181	small/medium-size, slightly dim, round	1.4×10^{11}	1.2×10^{11}
PA13P1	*Pseudomonas aeruginosa* 62172	medium-size, bright	1.2×10^{11}	1.1×10^{11}
PA13P2	*Pseudomonas aeruginosa* 62172	small, dim, some had halo	2.7×10^{11}	4.2×10^{11}
PA14P1	*Pseudomonas aeruginosa* 62109	extremely small, slightly dim	2.6×10^{6}	2.1×10^{4}
PA14P2	*Pseudomonas aeruginosa* 62109	big, slightly dim, round	4.1×10^{9}	4.2×10^{9}
PA16P1	*Pseudomonas aeruginosa* 62069	extremely small, dim, disfigured plaques, difficult to determine titer	1.4×10^{8}	6.7×10^{7}
PA16P2	*Pseudomonas aeruginosa* 62069	small, round (slightly rough edges), dim	9.5×10^{7}	6.6×10^{7}
PA17P1	*Pseudomonas aeruginosa* 61932	small/medium-size, slightly dim,	3.0×10^{9}	3.9×10^{9}
PA17P2	*Pseudomonas aeruginosa* 61932	small/medium-size, slightly dim, round, halo	2.4×10^{9}	1.9×10^{9}
SA10P1	*Staphylococcus aureus* 10AE05905	small, slightly dim	7.4×10^{8}	2.3×10^{9}
SA10P2	*Staphylococcus aureus* 10AE05905	small, slightly dim	1.5×10^{9}	7.0×10^{9}
SA10P3	*Staphylococcus aureus* 10AE05905	small, slightly dim, roundish	4.0×10^{8}	1.0×10^{8}
SA10P4	*Staphylococcus aureus* 10AE05905	small, dim, round	5.0×10^{7}	3.3×10^{7}
SA14P1	*Staphylococcus aureus* 60881	small, slightly dim	4.7×10^{7}	1.0×10^{6}
S1P1	*Salmonella* , quality control strain 18048	small, dim, round	1.2×10^{10}	2.1×10^{10}
SB1P1	*Salmonella* , Group B, ESBL-strain 12FB01687	small and medium-size, dim, roundish	3.5×10^{8}	1.8×10^{8}
SC1P1	*Salmonella* , Group C, 13FB10784	extremely small, bright, round	1.3×10^{10}	3.0×10^{8}
SE1P1	*Salmonella enteritis* (36.)	small, dim	1.1×10^{8}	9.2×10^{7}
SE1P2	*Salmonella enteritis* (36.)	in high density: small, dim, round / in lower density: big, dim halo, bright center, round	1.3×10^{7}	1.0×10^{8}
SE3P1	*Salmonella enteritidis* FB11214	big, turbid center, bright halo, round	7.8×10^{7}	5.0×10^{7}
SK1P1	*Salmonella Kreber* (37.)	extremely small, bright	1.9×10^{7}	9.8×10^{6}
SK1P2	*Salmonella Kreber* (37.)	extremely small, slightly dim, round	2.9×10^{6}	1.6×10^{6}
SS1P1	*Salmonella stanley* FB3820	small, extremely dim, roundish	N/A	N/A
ST1P1	*Salmonella typhimurium* FB7595	big, turbid center, bright halo, round	1.2×10^{10}	2.4×10^{10}