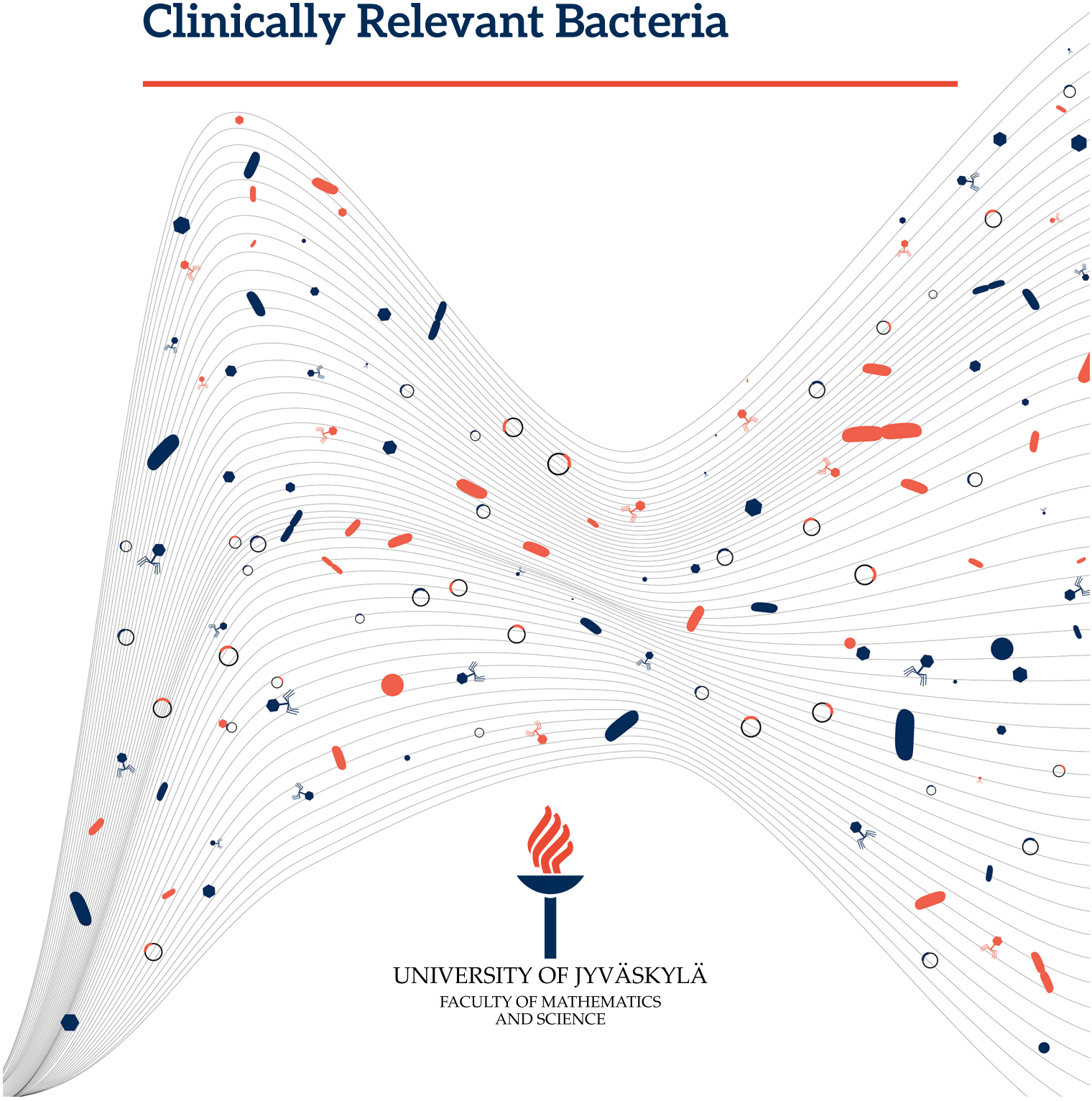
JYU DISSERTATIONS 805

Ilmur Jonsdottir

Evolutionary Trajectories of Conjugative Resistance Plasmids and Their Interplay in the Ecology of Clinically Relevant Bacteria



UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF MATHEMATICS
AND SCIENCE

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Evolutionary Trajectories of Conjugative Resistance Plasmids and Their Interplay in the Ecology of Clinically Relevant Bacteria

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ABSTRACT

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Antimicrobial resistance (AMR) has emerged as a pressing global crisis that threatens to undermine a century of medical advancements and poses significant implications to healthcare worldwide. The complexity of AMR, particularly evident in notorious pathogens like those within the Enterobacteriaceae family, is exacerbated by the horizontal dissemination of antimicrobial resistance encoding genes (ARGs). This propagation within bacterial communities is further accelerated by self-replicating and self-transferrable mobile genetic elements (MGEs), known as conjugative plasmids. This thesis delves into the antimicrobial resistance and plasmid dynamics of Enterobacteriaceae members E. coli and K. pneumoniae. The overarching aim of this thesis is to explore various aspects of microbial dynamics and antibiotic resistance evolution. Firstly, the investigation aims to delve into the dynamics of plasmids and resistance following antibiotic therapy (Study I). Secondly, it examines the evolutionary dynamics of plasmid lineages and their potential for evolutionary rescue (Study II). Furthermore, it aims to explore the environmental determinants influencing the development of phage resistance (Study III). Lastly, it aims to investigate the evolution and persistence of an ESBL plasmid targeted by CRISPR antimicrobial strategies (Study IV). Findings reveal significant AMR and plasmid content diversity among multiresistant E. coli and K. pneumoniae gut isolates. Notably, the research underscores the critical role of ecological factors in shaping the efficacy of interventions like phage therapy and CRISPR antimicrobials. Moreover, it highlights the profound influence of evolutionary history on plasmid dynamics and resistance mechanisms. This thesis expands current knowledge by shedding light on the adaptability of bacteria to their environment and emphasizes the need to consider ecological factors in developing effective strategies against the AMR crisis.

Keywords: Antimicrobial resistance (AMR); bacteriophages; conjugative plasmids; CRISPR; evolutionary rescue; phage resistance; phage therapy.

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TIIVISTELMÄ

Jonsdottir, Ilmur
Konjugatiivisten plasmidien evoluutiokulku ja vuorovaikutukset kliinisesti merkittävien bakteerien ekologiassa
Jyväskylä: Jyväskylän yliopisto, 2024, 96 s.
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Mikrobilääkeresistenssi (AMR) on noussut merkittäväksi globaaliksi ongelmaksi, sillä se hankaloittaa bakteeri-infektioiden hoitamista. AMR on haastava ongelma erityisesti *Enterobacteriaceae*-heimoon kuuluvien antibiooteille vastustuskykyisten patogeenien sekä niiden horisontaalisesti leviävien antibioottiresistenssigeenien (ARG) vuoksi. Näiden geenien leviämistä bakteeriyhteisöissä edesauttavat erityisesti itsenäisesti replikoituvat ja siirtyvät liikkuvat geneettiset elementit (MGE) kuten konjugatiiviset plasmidit. Tämän vuoksi AMR-ongelman ratkaisemiseksi tarvitaan uudenlaisia hoitomuotoja. Faagiterapiassa hyödynnetään bakteereja infektoivia viruksia. Antimikrobiaaliset CRISPR-pohjaiset työkalut taas voisivat olla tulevaisuuden ratkaisu ARG:ien poistamiseksi bakteeriyhteisöistä. Tässä väitöskirjassa tutkittiin *Enterobacteriaceae*-heimoon kuuluvien *Escherichia coli-* ja *Klebsiella pneumoniae* -bakteerien plasmididynamiikkaa ja antibioottiresistenssiä eri näkökulmista. Osatyössä I selvitettiin plasmidien ja antibioottiresistenssin dynamiikkaa antibioottihoidon aikana. Toisessa osatyössä (II) tutkittiin evoluutiohistorian vaikutusta plasmidien evoluutiodynamiikkaan ja niiden potentiaalia pelastaa antibiooteille herkkiä bakteereja konjugatiivisten plasmidien avulla. Lisäksi osatyössä III selvitettiin faagiresistenssin kehittymiseen vaikuttavia ympäristötekijöitä moniresistenteillä bakteereilla. Tutkimuksen viimeisessä osatyössä (IV) tutkittiin antimikrobisen CRISPR-työkalun vaikutusta ESBL-(Extended-Spectrum Beta-lactamase)-plasmidien säilyvyyteen ja evoluutioon. Tutkimustulokset osoittavat, että ekologiset tekijät vaikuttavat kohdennettujen keinojen tehokkuuteen. Lisäksi työn tuloksissa korostuu resistenssiplasmidien kyky sopeutua erilaisiin isäntäbakteeriympäristöihin sekä evoluutiohistorian vaikutus plasmididynamiikkaan. Tämä väitöskirjatyö laajentaa nykyistä tietämystä siitä, miten voimakkaasti sopeutumiskykyiset bakteerit kykenevät adaptoitumaan muuttuviin olosuhteisiin, korostaen erityisesti ekologisten tekijöiden merkitystä kehitettäessä tehokkaita työkaluja antibioottiresistenssiä vastaan.

Avainsanat: Mikrobilääkeresistenssi; bakteriofagit; konjugatiiviset plasmidit; CRISPR; evolutiivinen pelastus; faagiresistenssi; faagiterapia.

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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–IV.

I. **Jonsdottir, I**., Meaden, S., Salminen, P., Kallonen, T., Ravantti, J., Pajander, A., Vanhatalo, S., Jalasvuori, M., Sundberg, L.-R., Westra, E., van Houte, S., Hakanen, A., & Penttinen, R. 2024. " Longitudinal evolutionary dynamics of plasmidome and antibiotic resistance within a gut microbiome subsequent antibiotic therapy: a case study". Manuscript.

II. **Jonsdottir, I**., Given, C., Penttinen, R., & Jalasvuori, M. 2023. Preceding Host History of Conjugative Resistance Plasmids Affects Intra- and Interspecific Transfer Potential from Biofilm. *mSphere* vol: 8 e00107-23. https://doi.org/10.1128/msphere.00107-23

III. **Jonsdottir, I**., Vacker, S., Jalasvuori, M., Sundberg, L.-R., Penttinen, R. 2024. "Unavoidable development of induced phage resistance in clinical multidrug-resistant *E. coli* and *K. pneumoniae* gut isolates". Manuscript.

IV. Given, C., **Jonsdottir, I**., Norvasuo, K., Paananen, P., Ruotsalainen, T., Hiltunen, M., Gunell, A., Hakanen, Jalasvuori, M., Penttinen, R. 2024. "ESBL plasmid compatibility with the surrounding microbial community influences ESBL gene survival under CRISPR-antimicrobial targeting". Manuscript.

TABLE 1 Table of author contribution to the original publications.

Study	**I**	**II**	**III**	**IV**
Original idea	RP, AJH, PS	MJ, RP	IJ, RP, LRS	RP
Study Design	RP, SM, IJ, TK, AP, SV, AJH, PS	IJ, CG	IJ, RP, LRS	RP, CG, MJ
Data Collection	IJ, RP, AP, SV, AJH, PS, MJ	IJ	IJ, RP, SMV	CG, IJ RP, KN, PP, PR, MJ, MG, AH
Data Analysis	IJ, RP, SM, JR, EW, SVH	IJ	IJ, RP, LRS, SMV	CG, IJ, RP
Writing	IJ, RP, SM, TK, AP, SV, AJH, PS, MJ, SM, EW, SVH, LRS, JR	IJ, CG, RP, MJ	IJ, RP, MJ, LRS	CG, RP, MJ, IJ, TP, MG, AH

IJ = Ilmur Jonsdottir, RP = Reetta Penttinen, CG = Cindy Given, MJ = Matti Jalasvuori, LRS = Lotta-Riina Sundberg, TK = Teemu Kallonen, AP = Annaleena Pajander, SV = Sanja Vanhatalo, AH = Antti Hakanen, PS = Paulina Salminen, KN = Krista Norvasuo, PP = Petra Paananen, PR = Pilvi Ruotsalainen, SMV = Sanna Vacker, SM = Sean Meaden, EW = Edze Westra, SVH = Stineke van Houte, JR = Janne Ravantti, MG Marianne Gunnell, TP = Teppo Hiltunen

ABBREVIATIONS

AMR	Antimicrobial resistance
Bp	Base pair
Cas	CRISPR-associated
CFU	Colony Forming Units
Cq	Quantification Cycle
CRICON	CRISPR via Conjugation
CRISPR	Clustered Regularly Interspaced Palindromic Repeats
EtOH	Ethanol
HGT	Horizontal gene transfer
MGE	Mobile genetic elements
NTC	No Template Control
O/N	Overnight
PAM	Protospacer adjacent motif
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
qPCR	quantitative Polymerase Chain Reaction
µL	Microlitre
µM	Micro Molar

Words from a *dóttir*

Dedicated to my mother, Laufey Ýr Sigurðardóttir.
Fortunate enough to be your dóttir,
I know nothing but love and support,
and because of this,
I pursued my desires,
I ventured where I wished,
I achieved what I aspired to,
With no expectations of a return,
I can only thank you,
for all I am and all I do, I owe to you.

Takk elsku mamma mín, þetta er fyrir þig, eins og allt annað.

1 INTRODUCTION

1.1 Microbial dynamics

In the landscape of microbial life, bacteria stand as omnipresent representatives. These unicellular microbial entities serve an essential role within the biosphere and in countless ecological niches, including the human body. Bacteria, ubiquitous ancient life forms that have thrived for billions of years, come in diverse shapes and sizes. Despite from an anthropocentric standpoint often only being thought of as pathogens, these cells fulfill a vital, albeit often invisible, function in the intricate web of life. For instance, it facilitates digestion, synthesizes crucial vitamins, and contributes to digestive system health in virtually all animals (McFall-Ngai *et al.* 2013). The genomes of bacteria are contained within a single chromosome composed of DNA and circular extrachromosomal DNA structures referred to as plasmids. These self-replicating genetic elements exert significant influence on bacterial evolution and can contain genes that confer selective advantages, such as antibiotic resistance. Further, plasmids also serve as essential tools in genetic manipulation research as versatile vectors for introducing, replicating, and expressing specific genes. Research into the symbiotic dance between bacteria and their plasmids allows us to discover their story of adaptation, evolution, and scientific innovation, a testament to how life works on a microscopic scale.

1.1.1 Ecology of the human microbiome

The human microbiome is a complex ecosystem within the human body encompassing a diverse repertoire of bacteria, viruses, and other microbial entities. These entities reside in various locations across the human body and exceed the number of our own cells (Savage 1977, Bianconi et al. 2013, Sender et al. 2016a, b). The estimations of the amount and diversity of genes jump

significantly by broadening the definition of a human to include its bacterial inhabitants, forming a superorganism (Turnbaugh *et al.* 2007). Bacteria flourish in collaborative communities, exemplified by biofilms, structured collaborative communities made of (living and dead) bacterial cells and extracellular matrix. In the human microbiome, biofilms are crucial for adherence to surfaces within the body, playing a significant role in various physiological processes (Costerton *et al.* 1999). These biofilms contribute to the dynamics of the human microbiome by increasing the resilience of the microbial community (Percival et al. 2015, Greene et al. 2016). Moreover, studies have demonstrated a correlation between biofilm formation and increased horizontal gene transfer (HGT) *in vitro* (Burmølle et al. 2006, Madsen et al. 2012). This horizontal exchange of bacterial genetic material fosters genetic diversity and adaptability within microbial populations and frequently engages mobile genetic elements (MGEs).

The discovery of antibiotics a century ago represents a pivotal chapter in the story of the human microbiome as it transformed human health in miraculous yet complex ways (Fleming 1929). With antibiotics came significant positive impacts on life expectancy and quality of life. The complexities of antibiotic use stem from their overuse and misuse and their effect on the natural human flora. Human microbial communities show remarkable diversity among individuals, carrying a multitude of significance towards human health (Turnbaugh et al. 2007, David et al. 2014, Vatanen et al. 2016, Delaroque et al. 2022). Despite recognizing this broad diversity, the underlying factors contributing to the differences and the mechanisms regulating them remain uncertain. A better understanding of these factors is a focal point in microbiome research, and the challenges faced in this exploration provide a fertile ground for continued exploration and discovery in the field.

1.1.2 Gut microbiota

The gut microbiota, an individual-specific and complex commensal and symbiotic microbial community within the gut of each person, is essential to the health of humans (O'Hara and Shanahan 2006, Clemente et al. 2012). The roles that the gut microbiota plays include managing metabolism, preserving the mucosal barrier, and modulating the immune system (Weinstein and Cebra 1991, Xu and Gordon 2003, Menard 2004, Rakoff-Nahoum et al. 2004, Paone and Cani 2020). The most critical time in the development of the gut microbiota occurs during infancy (Palmer et al. 2007, O'Toole and Claesson 2010, Martin et al. 2010, Stewart et al. 2018, Roswall et al. 2021). During early life, there are significant influences on the microbiota from various factors like birthing methods, breastfeeding, and antibiotic exposure (Azad et al. 2016, Vandenplas et al. 2020, Jeong 2022). Following this, the gut microbiota generally maintains stability into adulthood (Rinninella *et al.* 2019). However, just like other human microbial communities, the gut microbiota displays individual variations. The reasons for these differences remain somewhat unclear but likely stem from the unique mix of host genetics, environmental influences, lifestyle decisions, dietary

preferences, and other elements (Ley *et al.* 2005, 2006, Frank *et al.* 2007, Clarke *et al.* 2014, Monda *et al.* 2017, Rinninella *et al.* 2019, Delaroque *et al.* 2022).

Significant research efforts have investigated the impact of antibiotic use on the gut microbiota composition and function. As mentioned earlier, a person's gut microbiota tends to remain relatively stable during adulthood, demonstrating a degree of resilience against external disruptions. Nevertheless, this does not eliminate the fact that this ecosystem is static as it undergoes sporadic changes, sometimes in as little as a day, resulting from factors such as antibiotics (David *et al.* 2014, Lange *et al.* 2016). Despite this, the gut's "stability" encompasses a capacity to revert to its previous homeostasis. This refers to the balanced and stable state maintained within the gastrointestinal tract, which involves the regulation of microbial populations, immune responses, and barrier integrity. However, the duration of this regression of the gut varies significantly among individuals and is a topic of scientific interest (Lozupone *et al.* 2012). The antibiotic-induced disruption is typically characterized by an overall decrease in diversity and even the loss of clinically important taxa (Dethlefsen *et al.* 2008, Heinsen *et al.* 2015). This disruption can lead to the diminished ability of the natural gut community to resist the colonization of invading pathogens, better known as colonization resistance (Stecher *et al.* 2013). The effects of antibiotics on the gut microbiota are particularly evident within the first two years of life due to the lower resilience (Vangay *et al.* 2015). Further, post-antibiotic dysbiosis has been found to promote horizontal gene transfer, contributing to the evolution of drug-resistant pathogens (Stecher *et al.* 2013). This forms the basis of Study I in this thesis, where the microbiome of an individual patient was meticulously examined after undergoing intensive antibiotic therapy.

FIGURE 1 Visualization of the gut microbiota, featuring epithelial cells, bacteria, bacteriophages, and mucin glycoproteins (depicted as Y-shaped structures). Bacteriophages are shown attaching to mucin proteins within the gut environment, highlighting their interaction with the mucosal surface.

In the anatomy of animals, the gut lining is notable for its goblet cells, which specialize in mucus production. The mucosa provides a physical barrier that separates the luminal contents of the gut from the underlying epithelium. This allows it to act as a protective shield against pathogens and harmful substances. Mucin, a component of the mucus layer in the gut, plays a fundamental role in shaping the dynamics and functionality of the gut microbiota (fig. 1) (Thornton and Skeehan 2004, Linden *et al.* 2008, Bakshani *et al.* 2018). Beyond its function as a physical barrier, mucin can serve as a nutrient source for mucin-degrading bacteria (Glover *et al.* 2022). These specialized microbes possess the enzymatic machinery necessary to break down and utilize mucin as an energy source, influencing the composition and metabolic functions of the gut microbiota (Tailford *et al.* 2015, Hansson 2020, Glover *et al.* 2022). Further, mucin helps maintain a balanced host-microbe interaction by fostering tolerance towards commensal bacteria while not disturbing the immune system, still allowing it to detect and respond to potential threats (Dharmani *et al.* 2009, Johansson and Hansson 2016). Due to the high bacterial concentration within the human body, the mucosal environment is an ideal habitat for the viruses that infect bacteria, known as bacteriophages. Some of these phages create a symbiotic niche within the eukaryotic host by interacting with the mucin present on mucosal surfaces (Barr *et al.* 2013a, Almeida *et al.* 2019, Sausset *et al.* 2020, de Freitas Almeida *et al.* 2022). Some phages encode carbohydrate-binding and -degrading proteins that target bacteria and archaea, mediating their binding to mucosal components, such as mucins (Rothschild-Rodriguez *et al.* 2023). Studies have shown that mucin can affect the outcomes of phage predation within the intestine by altering bacterial growth and increasing phage production (Carroll-Portillo *et al.* 2023). Further, the presence of mucin has been linked to changes in phage binding on mucus and its impact on phage-bacterium interactions (Almeida *et al.* 2019). Phages with an affinity towards binding to host mucins can form a protective antibacterial defense mechanism called BAM (bacteriophage adhesion to mucus, fig. 1) (Barr *et al.* 2013b, Chatterjee and Duerkop 2018). In essence, mucin plays a pivotal role as a mediator in nurturing the symbiotic relationship between the gut and its resident microbial community. With its multifaceted functions as a barrier, adhesion mediator, immune modulator, and metabolic regulator, mucin holds profound implications for gut health and disease. This forms the foundation of a segment of the thesis work (Study III), focusing on examining the impact of mucin and antibiotics on the potential emergence of phage resistance in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*.

1.1.3 Enterobacteriaceae

Arguably, no prokaryotic group has exerted more influence on public health and research than the *Enterobacteriaceae* family (Paterson 2006, Pitout 2008). The Gram-negative members of the *Enterobacteriaceae* family, tallying over 50 genera, inhabit the human body as both commensals and symbionts. These bacteria are found primarily in the gastrointestinal (GI) and respiratory tracts, where they play an essential role in numerous physiological functions (Guentzel 1996).

However, studies have reported that the proliferation of *Enterobacteriaceae* contributes to the pathogenesis of a broad range of diseases (Lupp *et al.* 2007, Morgan *et al.* 2012, Gevers *et al.* 2014, Zuo and Ng 2018, Baldelli *et al.* 2021). *Enterobacteriaceae* tend to harbor conjugative plasmids containing antimicrobial resistance (AMR) genes susceptible to evolution, furthering the challenge they pose as opportunistic pathogens (Alonso-del Valle *et al.* 2023). Within the gut microbiota, *Enterobacteriaceae* are commonly found near the epithelial layer, likely due to their high tolerance to oxygen (Zeng *et al.* 2017). Clinical *Enterobacteriaceae* isolates can be categorized for epidemiological purposes by their sequence type (ST). This is determined by the nucleotide sequence of certain multilocus sequence typing (MLST) housekeeping genes (Aanensen and Spratt 2005, Pérez-Losada *et al.* 2013, Maiden *et al.* 2013, Jolley and Maiden 2014).

Escherichia coli, a prominent member of the *Enterobacteriaceae* family, typically exists as a benign resident of the human body. Yet, this versatile bacterium stands out as an opportunistic pathogen capable of transitioning from commensal to pathogenic behavior (Tenaillon *et al.* 2010, Crossman *et al.* 2010, Koli *et al.* 2011, Proença *et al.* 2017). This species of bacteria has become increasingly implicated in a wide array of infections, spanning from intestinal to extraintestinal sites (Russo and Johnson 2003, Köhler and Dobrindt 2011). Beyond its clinical significance, *E. coli* is of great interest in microbiological research for several reasons, including its consideration as a bacterial model system and its amenability to laboratory cultivation. Moreover, the genetic flexibility of *E. coli* and its well-studied molecular biology make it a prime subject for investigating microbial physiology and interactions between hosts and pathogens (Pontrelli *et al.* 2018). *E. coli* is a common pathogen in various infections, comprising various described pathotypes. The most notorious of which are the extraintestinal pathogenic *E. coli* (ExPEC), causing infections outside the intestinal tract such as urinary tract infections (UTI), sepsis, neonatal meningitis, and infections of other extraintestinal organs (Dale and Woodford 2015). Other pathotypes include Enterotoxigenic *E. coli* (ETEC) responsible for diarrhea through the production of endotoxins, Enteropathogenic *E. coli* (EPEC) forming lesions on intestinal cells, and Enterohemorrhagic *E. coli* (EHEC) causing foodborne illnesses such as hemolytic-uremic syndrome (HUS). Here, mobile genetic elements play a role in the evolution and dissemination of virulence factors among the different *E. coli* pathotypes. These elements can carry genes encoding toxins, adherence factors, and other virulence determinants (Donnenberg and Whittam 2001, Gomes *et al.* 2016). *E. coli* is the culprit in 70-90% of UTIs and 10-30% of bloodstream infections (Johnson 1991, Ejrnæs 2010, Doumith *et al.* 2015, Kudinha 2017, Daga *et al.* 2019). It has been widely reported that pathogenic *E. coli* is either majorly or entirely (60-100%) responsible for acute appendicitis (Saxen *et al.* 1996, Chen *et al.* 2012, Jeon *et al.* 2014). Acute appendicitis can manifest either uncomplicated or complicated, with the standard treatment being surgery to remove the appendix. Notable research has focused on less invasive therapeutics (antibiotics) as a treatment for computed tomography (CT)-proven uncomplicated acute appendicitis, bypassing the

inherent risks associated with surgery (Minneci *et al.* 2014, Salminen *et al.* 2015, 2018, Vanhatalo *et al.* 2019, Podda *et al.* 2019, Herrod *et al.* 2022).

Another ubiquitous constituent of the *Enterobacteriaceae* family and of much relevance to human health is *Klebsiella pneumoniae*. Although an inherent inhabitant of the Gastrointestinal tract (GI) tract and the skin, its clinical importance cannot be underestimated, as *K. pneumoniae* is one of the dominant offenders in nosocomial infections, accounting for approximately one-third of these infections (Navon-Venezia *et al.* 2017). This bacterium exhibits a propensity for developing multidrug resistance, posing a considerable challenge from a clinical perspective. Of particular concern is its association with a spectrum of infections, including intestinal, urinary, respiratory, and blood. Immunocompromised individuals are primarily susceptible to *K. pneumoniae* infections (Podschun and Ullmann 1998). This species inclination to develop biofilms *in vivo* can shield the pathogen from host immune responses and antibiotics (Jagnow and Clegg 2003, Vuotto *et al.* 2014, Wang *et al.* 2020). The ability of *K. pneumoniae* to persist in healthcare environments and its increasing antibiotic resistance and spread of resistance urges further understanding of its pathogenic mechanisms.

1.1.4 Bacteriophages

Bacteriophages, colloquially known as phages, are viruses that exclusively target and infect bacteria (Twort 1915, d'Herelle 1917, Ackermann and DuBow 1987). Like all viruses, phages are not "alive," but these microscopic entities assume an essential role in biological functions as predators of bacterial cells. The life cycles of bacteriophages can be categorized into two groups based on their replication mechanism: lytic and temperate (fig. 2) (Ackermann and DuBow 1987). In the lytic cycle, the phage infects its host, overtaking its machinery for replication, culminating in lysis, and liberating new phages. In contrast, the lysogenic cycle does not result in the lysis of the cell. Instead, it entails the integration of the phage genetic material into the bacterial genome, establishing a genetic symbiotic relationship. Phages capable of replicating via the lysogenic cycle are called temperate phages, and they can spontaneously transition their replication strategy to the lytic cycle (Barksdale and Arden 1974).

Phages exhibit a broad range of morphological structures reflecting their evolutionary adaptations to selectively target their specific bacterial hosts. The morphological characteristics of phages primarily revolve around their capsid, additionally on features such as tails, fibers, and spikes (fig. 2) (Ackermann 2007, 2009). All phages share the common feature of containing their genetic material within a proteinaceous structure known as a capsid. Capsid morphology encompasses three predominant categories: icosahedral, helical, and complex (Louten 2016). Icosahedral phages present a symmetrical and polyhedral capsid shape, while helical phages exhibit a spiral structure. In contrast, complex phages manifest more elaborate and often asymmetrical morphologies. A paradigmatic example of phage morphology is seen with the T4-like bacteriophage, typified by an icosahedral capsid and, most notably, a retractable tail (Yap and Rossmann

2014). These phages are within the group of *Myoviruses* and represent the most known and described groups of phages (ICTV 2009, Turner *et al.* 2023). T4-like bacteriophages are known to generally carry the *hoc* gene that encodes for the Highly Antigenic Outer Capsid (Hoc) protein (Sathaliyawala *et al.* 2010). Categorizing phage morphotypes systematically enriches our understanding of their evolutionary connections and diverse functionalities. Taxonomically, phages are classified according to their nucleic acid content (DNA or RNA), morphology (utilized by electron microscopy), and replication strategy (lytic or lysogenic). This systematic classification and nomenclature of phages falls under the responsibility of the International Committee on Taxonomy of Viruses (ICTV).

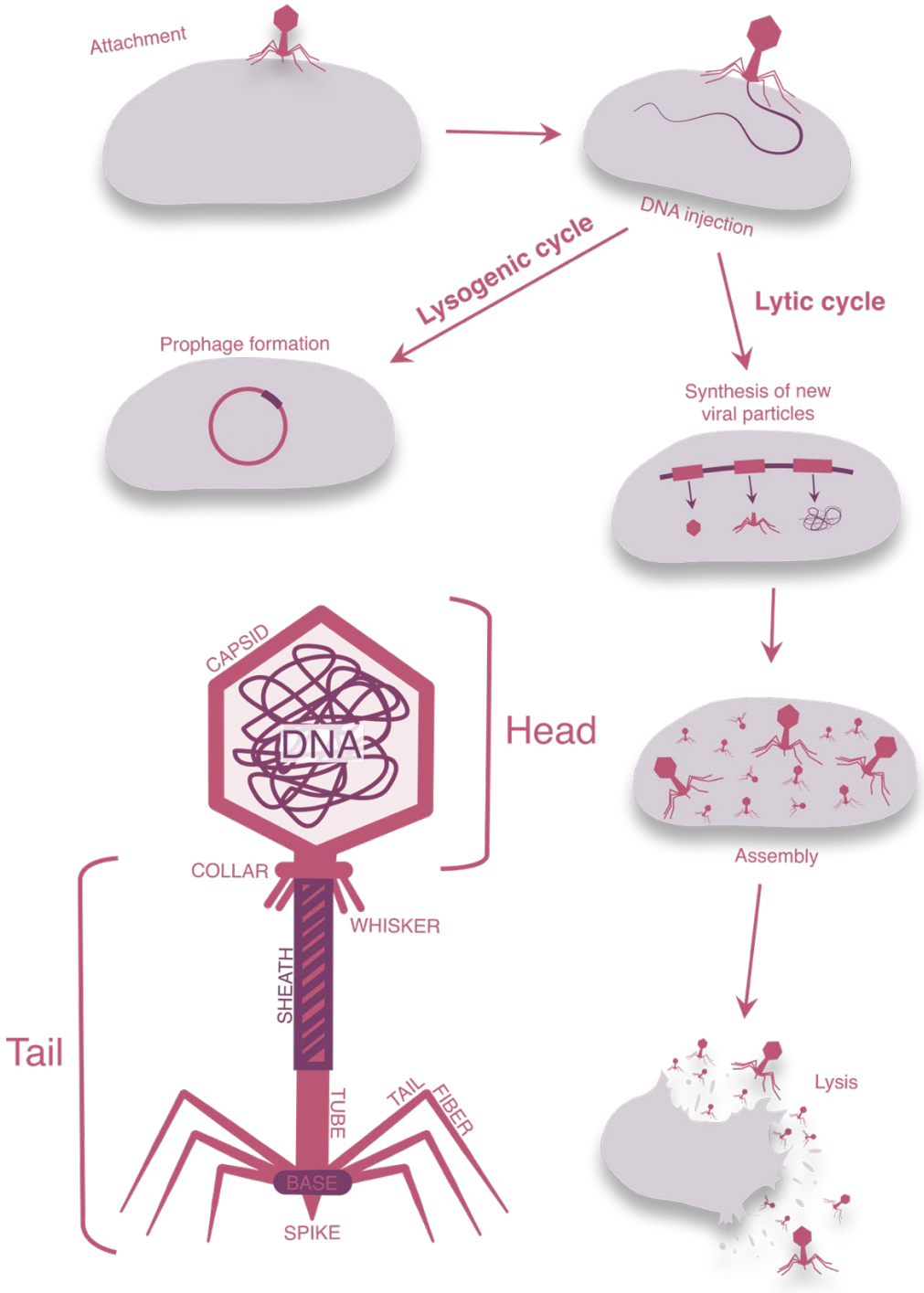

FIGURE 2 Representation of the typical morphology of a head-tail phage, as well as an illustration of the two replication cycles utilized by phages: (1) the lytic cycle and (2) the lysogenic cycle. The lytic cycle involves immediate replication and lysis of the host cell, while the lysogenic cycle integrates the phage genome into the host's DNA, potentially remaining dormant until triggered to enter the lytic cycle.

The specificity of the interaction between phages and their bacterial hosts lies in the unique correspondence between the phage and a specific set of receptors on the bacterial cell surface. This allows the phage to recognize and bind to its target, forming the fundamental basis of phage infectivity. A fascinating subset of bacteriophages is the plasmid-dependent phages, or plasmid-specific phages, which rely on plasmid-encoded features for successful host recognition and infection. These phages evolve to target and leverage plasmids specifically, creating an additional niche for exploitation. However, the interaction between plasmids and phages is not a one-way street, as phages can actively drive bacterial communities to become antibiotic-susceptible by targeting specific structures like the sex pili (Colom *et al.* 2019). Because these viruses depend on plasmids, researchers can utilize this dependency to isolate the viruses and investigate their infectivity patterns. (Ngiam *et al.* 2022). Furthermore, plasmid-dependent phages have been identified as a possible player in constraining horizontal gene transfer, indicating their significant evolutionary role (Quinones-Olvera *et al.* 2023). Research has shown that plasmid-dependent phages can lead to the loss of antibiotic-resistance plasmids in bacterial populations (Jalasvuori *et al.* 2011). Additionally, the close association between conjugative plasmids and male-specific bacteriophages within biofilm communities hints at using phages to regulate biofilm formation (May *et al.* 2011). This highlights the potential of plasmid-dependent phages as tools to combat challenging bacterial pathogens, such as by reducing the carriage of AMR plasmids.

Lately, bacteriophages have become a particular research focus due to their potential for therapeutic application, i.e., phage therapy, where their unique ability to diminish bacterial populations specifically is harnessed as an alternative to antibiotics. Unlike traditional antibiotics that can target a broad spectrum of bacteria, including those that are beneficial, bacteriophages exhibit a remarkable specificity as they recognize and infect particular bacterial strains. This heightened specificity is owed to the discernible molecular interactions between the phage and receptors on the bacterial surface. As scientists delve deeper into bacteriophages, it opens up exciting possibilities for their potential as therapeutic agents.

The infinite arms race between bacteria and bacteriophages has led to the evolution of sophisticated anti-phage defense mechanisms within bacteria. Additionally, the lesser-known "anti"-anti-phage systems are designed to counteract these defenses. Bacterial defenses against phages are a "hot" research topic, with new systems discovered frequently and cumulating into the hundreds (Georjon and Bernheim 2023). Among these, the most prominent are passive defenses, such as surface modifications, or active defenses, such as restriction-modification (RM), CRISPR/Cas, and abortive infection (abi) systems. RM systems work by modifying bacterial DNA and restricting foreign DNA, preventing the replication of the phage (Tock and Dryden 2005). CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats) systems provide adaptive immunity by storing phage DNA sequences or "memorizing" and using them to recognize and destroy similar invaders (Mojica *et al.* 2005, Pourcel *et al.* 2005, Bolotin *et al.* 2005). Abi systems sacrifice the infected bacterial cell to block

phage replication and protect neighboring cells (Lopatina *et al.* 2020). These anti-phage systems showcase the varying bacterial strategies to fend off viral threats. Understanding these defense mechanisms can elucidate the biology of bacteria-phage interactions and hold the potential for innovative applications in biotechnology and therapeutic interventions (Jinek *et al.* 2012).

1.1.5 Horizontal gene transfer

Bacteria are known to exchange genetic material among themselves, and the mechanisms behind this are as various as many. This horizontal gene transfer (HGT) contributes significantly to bacteria's ability to adapt and evolve (Juhas *et al.* 2009). The traditional genetic flow is the vertical form, in which the genetic material is transferred from parental to daughter cell. However, HGT allows for the sharing of genetic material between unrelated individuals, even those of different strains and species (Dutta and Pan 2002, Grohmann *et al.* 2003a). Mobile genetic elements, including plasmids, transposons, and bacteriophages, play a pivotal role in HGT. Conjugative plasmids, for instance, are essential contributors to bacterial conjugation, a process where bacteria transfer genetic information through extended structures called pili. These sex pili facilitate the physical connection between donor and recipient cells, enabling the transfer of genetic material during conjugation. The uptake and incorporation of foreign DNA from the environment is known as transformation. This can be replicated under certain conditions in the laboratory. Additionally, bacteria possess specific cell surface machineries, including some secretion systems and type IV pilus structures, that are known to facilitate DNA uptake (Christie *et al.* 2014). Through their infection life cycle, bacteriophages are introduced into different bacterial cells and can erroneously transfer the bacterial genetic information to another cell. This is called transduction and serves as an additional bacterial HGT mechanism. In addition, bacterial "jumping" genes, or transposons, are HGT elements that utilize transposition to disseminate genetic material between cells. Transposons move with the assistance of enzymes called transposases that recognize and cleave the DNA sequence of the transposon, enabling it to be excised and inserted elsewhere. Bacterial transposons can be composed of both coding and non-coding regions. Bacterial transposons can only contain genes for transposition with no extra functions, or complex ones, that carry additional genes that can present the host bacteria with specific abilities. Transposable elements are categorized into classes based on their transfer method. Retrotransposons move via reverse transcription (RT), while DNA transposons do not rely on them (Feschotte and Pritham 2007). All types of horizontal gene transfers may provide selective advantages to the host bacteria, such as antibiotic resistance genes.

1.2 Conjugative Plasmids

Conjugative plasmids, a subset of plasmids, are key bacterial DNA transfer agents. These specialized plasmids harbor genetic machinery encoding everything necessary to allow for their autonomous transfer from one bacterium to another through conjugation. Their relocation can span large taxonomic distances, highlighting their impact on bacterial diversity, evolution, and dissemination of critical traits, such as antibiotic resistance genes (Norman *et al.* 2009). Conjugative plasmids can extend HGT through mobilization to include nonconjugative mobilizable plasmids (Smillie *et al.* 2010). These exciting elements can be taxing on their hosts, demanding energy and resources allotted to their transfer. The dilemma of explaining the survival of costly plasmids without apparent advantages is known as the "plasmid-paradox" (San Millan and MacLean 2017, Brockhurst and Harrison 2022). This unresolved puzzle is a popular research area due to its enigmatic nature. The extensive research focusing on conjugative plasmids has helped determine their functional dependencies and networks (Firth *et al.* 1996, De La Cruz *et al.* 2010, Guglielmini *et al.* 2013, Johnson and Grossman 2015, Hall *et al.* 2016, Werisch *et al.* 2017, Botelho and Schulenburg 2021, Ares-Arroyo *et al.* 2023). Moreover, continuous research on conjugative plasmids explores their diverse characteristics, including their size, complexity, and their regulatory mechanisms (Bañuelos-Vazquez *et al.* 2017, Sysoeva *et al.* 2020, Virolle *et al.* 2020, Ni *et al.* 2021, Neil *et al.* 2021). Efforts to establish a taxonomic classification system for conjugative plasmids are ongoing within the field (Shintani *et al.* 2015, Fernandez-Lopez *et al.* 2017). The classification is based on replication and transfer systems alongside the taxonomy of the host. Our knowledge of the genetics of pathogenic bacteria relies on a thorough understanding of conjugative plasmids, thus promoting their investigation in a clinical light. This will ideally aid in deciphering the spread of antibiotic resistance and develop strategies to manage it effectively.

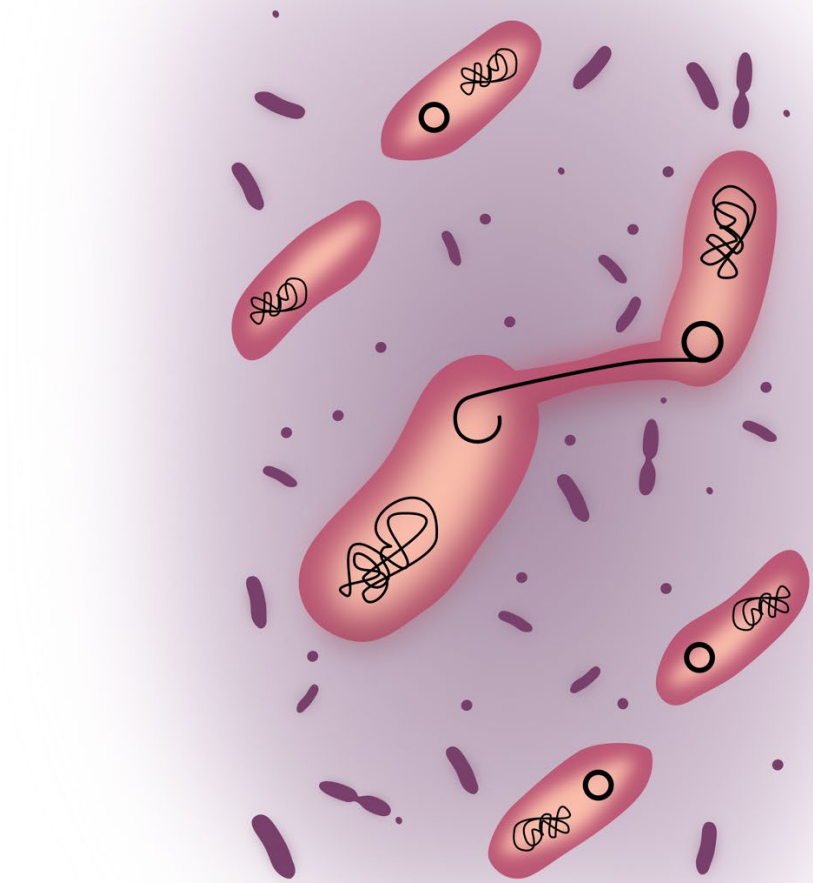

FIGURE 3 Illustration demonstrating bacterial conjugation, where conjugative plasmids are transferred between bacterial cells. Initially, two adjacent bacterial cells, both compatible with the plasmid's host range, connect via sex pili. Subsequently, the plasmid transfers and replicates within the recipient cell, resulting in both the donor and recipient cells carrying the plasmid.

1.2.1 Plasmid characteristics

Conjugative plasmids possess distinctive characteristics that distinguish them within bacterial genetics. The hallmark trait of conjugative plasmids is their ability to self-transfer from one cell to another. The machinery encoded by conjugative plasmids facilitating the transfer is a set of genes, known as the *tra* genes, encoding proteins responsible for the assembly of conjugative pili and the transfer of plasmid DNA during conjugation (Frost *et al.* 1994, Lanka and Wilkins

1995, Firth *et al.* 1996, Zatyka and Thomas 1998, Grohmann *et al.* 2003b). The conjugative pili or sex pili are filamentous structures protruding from the donor cell that mediate contact with the recipient (fig. 3). The *tra* gene core regulates the initiation, formation, and termination of the transfer process. Conjugative plasmid sequences typically contain *ori*T (origin of transfer) sites, serving as starting points for DNA transfer during conjugation.

Categorization of the mobility of plasmids based on their ability to move between bacterial cells is encompassed in their mobility class. This classification entails three main categories: non-mobilizable, mobilizable, and conjugative. Non-mobilizable plasmids lack both the transfer machinery and the *ori*T required for autonomous or assisted transfer. Mobilizable plasmids lack the complete machinery for transfer but contain an origin of transfer (*ori*T), allowing them to be mobilized in conjunction with a helper (conjugative) plasmid. The third class is conjugative plasmids, transfer regions within conjugative plasmids facilitate the formation of a mating pair between donor and recipient bacteria and the subsequent transfer of plasmid DNA, enabling horizontal gene transfer (Norman *et al.* 2009, Smillie *et al.* 2010).

Another system of plasmid classification is based on their ability to stably coexist within the same bacterial cell over generations, known as the Inc (incompatibility) group of plasmids. Plasmids belonging to the same Inc group are unable to coexist through generations within a single cell line due to their similar replication or partitioning systems, resulting in a competitive exclusion phenomenon. This dynamic fosters the diversity of plasmid types in bacterial communities and has significant ramifications for the dissemination of antibiotic-resistance genes carried by plasmids, shaping their prevalence and persistence. The classification system is fundamental to any plasmid research and is denoted by "Inc" followed by a letter or a combination of letters representing different incompatibility groups. For instance, IncF, IncI, IncX, and IncN represent plasmid incompatibility groups of much clinical relevance (Glenn *et al.* 2013, Mutai *et al.* 2019, Chen *et al.* 2024). Within bacterial populations, certain incompatibility groups often carry similar resistance genes, an essential factor when researching them, mainly in the context of genomic epidemiology. The knowledge of Inc types holds the potential for predicting which plasmids might spread or persist in bacterial communities and aids in developing strategies to manage and control their dissemination, especially in clinical settings (Palchaudhuri and Maas 1977, Novick and Hoppensteadt 1978, Bergquist *et al.* 1982, Lanka and Wilkins 1995, Grohmann *et al.* 2003b, Helinski 2022).

The establishment of an intimate physical contact, the extended sex pili, between a plasmid donor and plasmid recipient cell during conjugation is called a mating pair formation (Mpf). Plasmid mating pair formation (Mpf) systems are widely classified based on the gene and protein complexes involved. Additionally, membrane-associated proteins apart of these systems aid in the recognition and engagement of the recipient cell, ensuring the efficient transfer of plasmid DNA (Zatyka *et al.* 1997, Li *et al.* 1998, Schröder and Lanka 2005, Zhong *et al.* 2010). The characteristics of conjugative plasmids provide insights into the mechanisms underlying one of the primary modes of genetic exchange

among bacteria. This directly contributes to our understanding of clinically relevant topics such as AMR as well as microbial evolution and ecology.

1.2.2 Host-plasmid co-evolution

In the construction of bacterial evolution, plasmids are architects, indispensable, steering the course of adaptation and diversity of their hosts. The evolutionary interplay between plasmids and their microbial hosts is versatile and shaped by host specificity, fitness consequences, and the acquisition of essential genes. Plasmids exhibit greater plasticity than the chromosome and evolve through a blend of mutation, horizontal gene transfer, recombination, and selective pressure. Plasmids are known to shape the accessory genome of their hosts and foster the development of plasmid-encoded traits while also bolstering the adaptability of the host chromosome (Rodríguez-Beltrán *et al.* 2021). Like other MGEs, every plasmid has a host range determined by its ability to transfer and replicate efficiently within bacterial cells. This range can potentially span across different strains and species. Due to MGEs integration within their host, the environment of a plasmid extends beyond the confines of its host. With this, environmental determinants, abiotic and/or biotic, affecting the microbial community as a whole also influence host-plasmid interactions. Several studies have highlighted factors contributing to plasmid evolution, including plasticity, host-resource competition, temperature, and biofilm formation (Porse *et al.* 2016, Wein *et al.* 2019, Stalder *et al.* 2020, Lehtinen *et al.* 2021, Metzger *et al.* 2022). Nevertheless, knowledge gaps exist concerning the impact of the environment on plasmid evolution, mostly revolving around bridging plasmid behavior in bacterial consortia between *in vitro* studies and natural environments. These unknowns are understandable given the wide array of environmental factors bacterial populations encounter, particularly within the human body.

Experimental evolution studies unveil the emergence of plasmid stability in non-selective conditions, upholding antibiotic resistance and prompting compensatory adaptations of both plasmid-based and chromosomal, thus sustaining plasmid persistence (Harrison *et al.* 2015, 2016, Loftie-Eaton *et al.* 2017, Zwanzig *et al.* 2019, Wein *et al.* 2019, Hall *et al.* 2021, Bird *et al.* 2022, DelaFuente *et al.* 2022). The trajectory of plasmid evolution profoundly influences bacterial populations, with migration identified as a pivotal determinant of plasmid population dynamics (Harrison *et al.* 2018). Plasmids can facilitate ecological isolation within communities, fostering speciation of host and plasmid via continuous horizontal gene transfer.

The reciprocal evolution of plasmids and their hosts can be a stabilizing factor for antibiotic resistance. *In vitro* experimental evolutionary studies offer insights into how antibiotic treatment influences the co-evolution of plasmids and hosts, contributing to the persistence of antibiotic-resistant plasmids (Stalder *et al.* 2017). Large conjugative plasmids, such as multidrug resistance plasmids, are known to exhibit three strategies to enhance their persistence: mutations in their replication genes, altering the transcriptional regulatory system of their host, and acquiring toxin-antitoxin (TA) systems (Porse *et al.* 2016). Toxin-

antitoxin systems function by deploying a toxic protein (toxin) alongside its corresponding inhibitory counterpart (antitoxin); this creates a delicate balance that, if disrupted, leads to programmed cell death. When encoded on plasmids, it maintains their stability as the cell succumbs to toxicity when the antitoxin is no longer produced (Yang and Walsh 2017). This mechanism is one of many that reinforces the complexity of the interactions between plasmids and their hosts. The evolutionary dance of plasmids with their bacterial hosts bears extensive implications for ecological dynamics and the spread of antibiotic resistance. A better understanding of host-plasmid evolution is vital for tackling challenges associated with antimicrobial resistance and the evolution of bacterial pathogens.

1.3 Antimicrobial resistance

Antimicrobial resistance (AMR) casts a persistent shadow on health care globally, marked by the adaptation of pathogenic bacteria to resist the impact of antimicrobial drugs, rendering these medications ineffective (O'Neill 2016, Murray *et al.* 2022). Antibiotics are categorized into various classes based on their chemical structure, mechanism of action, and spectrum of activity. Some of the most commonly used antibiotic classes include fluoroquinolones, macrolides, tetracyclines, sulfonamides, and beta-lactams such as penicillins, cephalosporins, carbapenems, and monobactams. Each class possesses unique properties that dictate their efficacy, spectrum of activity, and potential side effects. While this bacterial resistance phenomenon happens naturally, it is accelerated by the inappropriate use of antibiotics in both humans and animals, insufficient infection prevention and control measures, and the scarcity of newly discovered antimicrobial agents (Shull 1935, Shlaes *et al.* 2013, Årdal *et al.* 2018, Bhavnani *et al.* 2020, Elisabeth *et al.* 2021, Mancuso *et al.* 2021, Ruzante *et al.* 2022). The implications of AMR are dire, compromising the efficacy of our most common infection treatments and leading to prolonged illnesses, increase in healthcare expenses, and higher mortality rates (ECDC/EMEA 2009, Stoll *et al.* 2010, World Health Organization 2012, 2014, Centres for Disease Control and Prevention 2013, Prestinaci *et al.* 2015). Effectively addressing this issue necessitates a complex and thorough strategy, encompassing solutions to the implications mentioned. To mitigate antimicrobial resistance means safeguarding the effectiveness of seemingly irreplaceable treatments.

1.3.1 Resistance genes and dissemination

Antimicrobial resistance genes (ARGs) represent the genetic components responsible for antimicrobial resistance (Harbottle *et al.* 2006). These genes are prevalent in a wide array of pathogenic and commensal bacteria. Typically, ARGs encode proteins or enzymes that alter, break down, or expel antibiotics from bacterial cells, nullifying the effectiveness of the drug (Christaki *et al.* 2020). The acquisition of ARGs occurs via multiple routes, including horizontal gene

transfer facilitated by plasmids, phages, and transposons. These resistance mechanisms, alongside mutations within pre-existing genes, are selected for under exposure to antibiotics. The extensive diversity and prevalence of ARGs across microbial populations pose a challenge in combatting infectious diseases, especially in the light of multidrug-resistant strains. The spectrum of ARGs culminates in their classification as multidrug-resistant (MDR), extensively drug-resistant (XDR), or pan-drug-resistant (PDR) based on their resistance scope and pattern (Falagas and Karageorgopoulos 2008, Magiorakos *et al.* 2012). Extensive studies have delineated the diversity, mechanisms, and prevalence of ARGs within clinical bacterial populations, grouping them according to their genetics and virulence (Wendlandt *et al.* 2013, Pereira *et al.* 2013, Khan *et al.* 2019, Almeida *et al.* 2020). Antimicrobial resistance genes that work through enzymatic inactivation represent an overwhelming threat to healthcare and include genes encoding beta-lactamases, aminoglycoside-modifying enzymes, tetracycline resistance, and fluoroquinolone resistance. Other resistance mechanisms involving ARGs and even simple point mutations are target modification, efflux pumps, enzymatic modification, and transporter and regulatory mutations (Munita and Arias 2016). Recognizing the different mechanisms and their genetic counterparts directly facilitates antimicrobial resistance surveillance worldwide. A better grasp of the mechanisms, transmission pathways, and regulatory mechanisms governing ARGs is needed to formulate strategies to alleviate their impact and preserve the potency of antimicrobial therapies (Tenover 2006).

The relentless group of enzymes known as beta-lactamases is at the forefront of the global AMR battle. These enzymes, mainly produced by Gram-negative bacteria within the *Enterobacteriaceae* family, exhibit remarkable proficiency in hydrolyzing (breaking) the beta-lactam rings in their namesake antibiotics, including penicillins and cephalosporins (Philippon *et al.* 1989, Turner 2005, Paterson and Bonomo 2005, Livermore 2008, Falagas and Karageorgopoulos 2009, Castanheira *et al.* 2021). Extended-spectrum beta-lactamases (ESBLs), a subtype of beta-lactamases, confer resistance to a wide spectrum of beta-lactam antibiotics. ESBL-producing *Enterobacteriaceae*, notably *Klebsiella pneumoniae* and *Escherichia coli*, are extensively studied due to their prevalence in healthcare settings, although their incidence in community-acquired infections is on the rise (Rodriguez-Bano *et al.* 2009, Khanfar *et al.* 2009, Larramendy *et al.* 2021). The genes responsible for encoding ESBLs are highly receptive to horizontal gene transfer (HGT), facilitating their widespread dissemination in turn, complicating treatment options (Hawkey and Jones 2009, Cantón *et al.* 2012, Hartmann *et al.* 2012, Doi *et al.* 2012, Woerther *et al.* 2013, von Wintersdorff *et al.* 2016).

The dissemination of AMR involves resistant bacteria and ARGs proliferating widely, posing a significant challenge throughout various ecosystems. This process is driven by multiple mechanisms, including horizontal gene transfer, clonal expansion of resistant strains, and the migration of resistant organisms within and between communities. Particularly noteworthy is the promiscuity of plasmid-mediated HGT, *i.e.,* conjugation, often allowing for the exchange of resistance genes among different bacterial species and populations

(Marí-Almirall *et al.* 2021, Kessler *et al.* 2023, Castañeda-Barba *et al.* 2024). The dispersion of ARGs transcends conjugative plasmids and includes transposons, integrons, and bacteriophages (Boerlin and Reid-Smith 2008, Baker *et al.* 2018, Vikesland *et al.* 2019). From an anthropogenetic perspective, AMR extends from clinical settings to the food chain. (Cabello and Godfrey 2016, Arnold *et al.* 2016, Ruiz and Alvarez-Ordóñez 2017, Koutsoumanis *et al.* 2021). Researching the pathways through which AMR pathogens evolve and disseminate is essential for making strides in understanding the drivers of AMR transmission. However, experimental testing of evolutionary rescue (discussed in more detail in section 1.3.2) via HGT within microbial communities poses several challenges. These mainly revolve around the experimental constraints that come with any microbes, as well as their evolutionary rates, community interactions, environmental heterogeneity, quantifying fitness effects, and migration.

1.3.2 Evolutionary rescue

Evolutionary rescue is a key concept in evolutionary biology involving the adaptive change or "rescue" of a population on the brink of extinction due to an environmental challenge. This concept in the context of microbiology can occur in a bacterial population as a response to stressors or threats such as antibiotics (fig. 4). As selective pressures mount from the extensive use of antibiotics, bacteria must adapt to survive. In this adaptive landscape, disseminating ARGs via promiscuous resistance plasmids can mean an evolutionary rescue. The rescue potential of resistance plasmids thus becomes a critical factor in shaping the evolutionary trajectories of bacterial populations. Recent studies have shown that evolutionary rescue via conjugative plasmids can cause susceptible bacteria to become resistant during beta-lactam antimicrobial treatment (Mattila *et al.* 2017, Ruotsalainen *et al.* 2020). With this in mind, conjugative resistance plasmids each carry a transfer potential, with findings in this thesis (Study II) showing that plasmid-host interactions and other evolutionary influences can impact the potential of plasmids to rescue susceptible bacteria from the effects of certain antibiotics. Nevertheless, delving into the study of evolutionary rescue in microbial communities unveils a labyrinth of challenges, given the complex nature of microbial ecology and the dynamics associated with adapting to environmental stressors. With the trajectory from genotype to phenotype in bacteria being unmistakably non-linear, quantifying the fitness effects of adaptive traits within populations proves to be extremely multifaceted (Ruppé *et al.* 2017, Morris *et al.* 2020, Srivastava and Payne 2022). Further, studying evolutionary rescue in bacterial populations would necessitate clearly distinguishing between local adaptation and the migration of pre-adapted individuals. Additionally, clinical investigation of this phenomenon encounters obstacles in the diverse interspecific interactions and navigating the environmental heterogeneity within the human body. However, investigating the interplay between the spontaneous genetic adaptation to selective pressures imposed by antimicrobial agents would clearly allow for a more comprehensive scope of AMR.

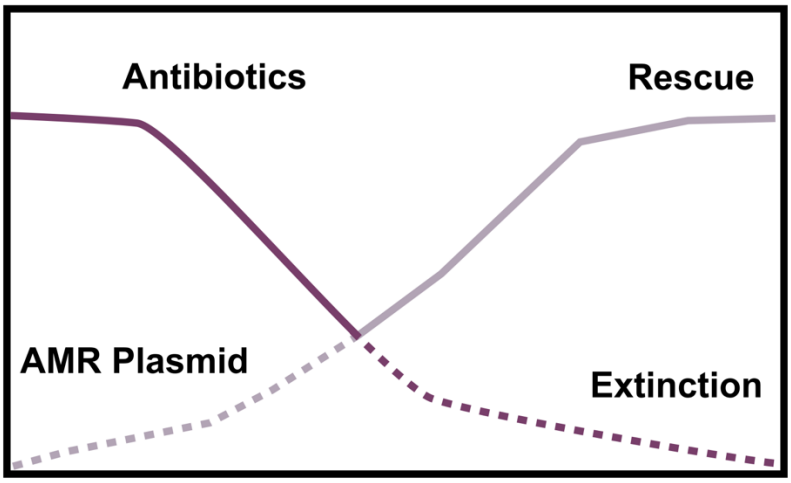

FIGURE 4 Graphic depiction showcasing the concept of evolutionary rescue within microbial communities under antibiotic exposure that harbor conjugative antimicrobial resistance (AMR) plasmids. As antibiotic selective pressure intensifies, specific bacteria harboring advantageous traits, AMR plasmids, may undergo evolutionary adaptations, enabling them to share their beneficial traits and aid the survival and prosperity of the population.

1.4 CRISPR/Cas

Few microbial discoveries have provoked the world as much as the captivating bacterial immunity system CRISPR/Cas (The Nobel Foundation 2020). CRISPR, alongside the CRISPR-associated (Cas) proteins, constitutes an adaptive bacterial immune system defending its host against viruses and foreign DNA such as plasmids. Recently, under the recognition of seemingly the entire scientific community, CRISPR/Cas has been repurposed as a revolutionary genome editing tool (Jinek *et al.* 2012). In its natural pathway, the system consists of two main components: the guide RNA, targeting specific DNA or RNA sequences, and the Cas proteins, acting in various ways, including as molecular scissors (fig. 5) (Jansen *et al.* 2002, Tang *et al.* 2002, Mojica *et al.* 2005, Pourcel *et al.* 2005, Bolotin *et al.* 2005). The CRISPR/Cas systems operate through two phases, encompassing three key steps. In the initial phase, termed adaptation, bacteria respond to viral invasions by incorporating a fragment of the phage genome into its own (CRISPR array). This process or step is known as spacer acquisition. Proteins like Cas1, Cas2, and occasionally Cas4 are instrumental in this step, demonstrated through extensive mutation studies that showed the cessation of spacer acquisition when these proteins were removed (Nuñez *et al.* 2014, 2015, Kieper *et al.* 2018). The subsequent phase, biogenesis or transcription, wherein the CRISPR array is transcribed to produce pre-crRNA, later processed into mature crRNA with the aid of certain Cas proteins. In the interference phase, the mature crRNA combined with Cas proteins form what is known as an effector module, targeting and cleaving foreign nucleic acids in a sequence-specific manner. Of much

importance to this system is the Protospacer Adjacent Motif (PAM), a short DNA sequence immediately following the target sequence (Mojica *et al.* 2009). Cas proteins involved in the cleaving of foreign DNA recognize the PAM. This distinguishes the viral genome, sparing the genetic region of the bacteria iteslf from being targeted. Approximately half of the sequenced bacterial genomes encode CRISPR/Cas immune systems (Ishino *et al.* 2018). In response, many phages have evolved anti-CRISPR (*acr*) genes encoding for Acr proteins that inhibit the CRISPR immune response (Bondy-Denomy *et al.* 2013, Pawluk *et al.* 2017).

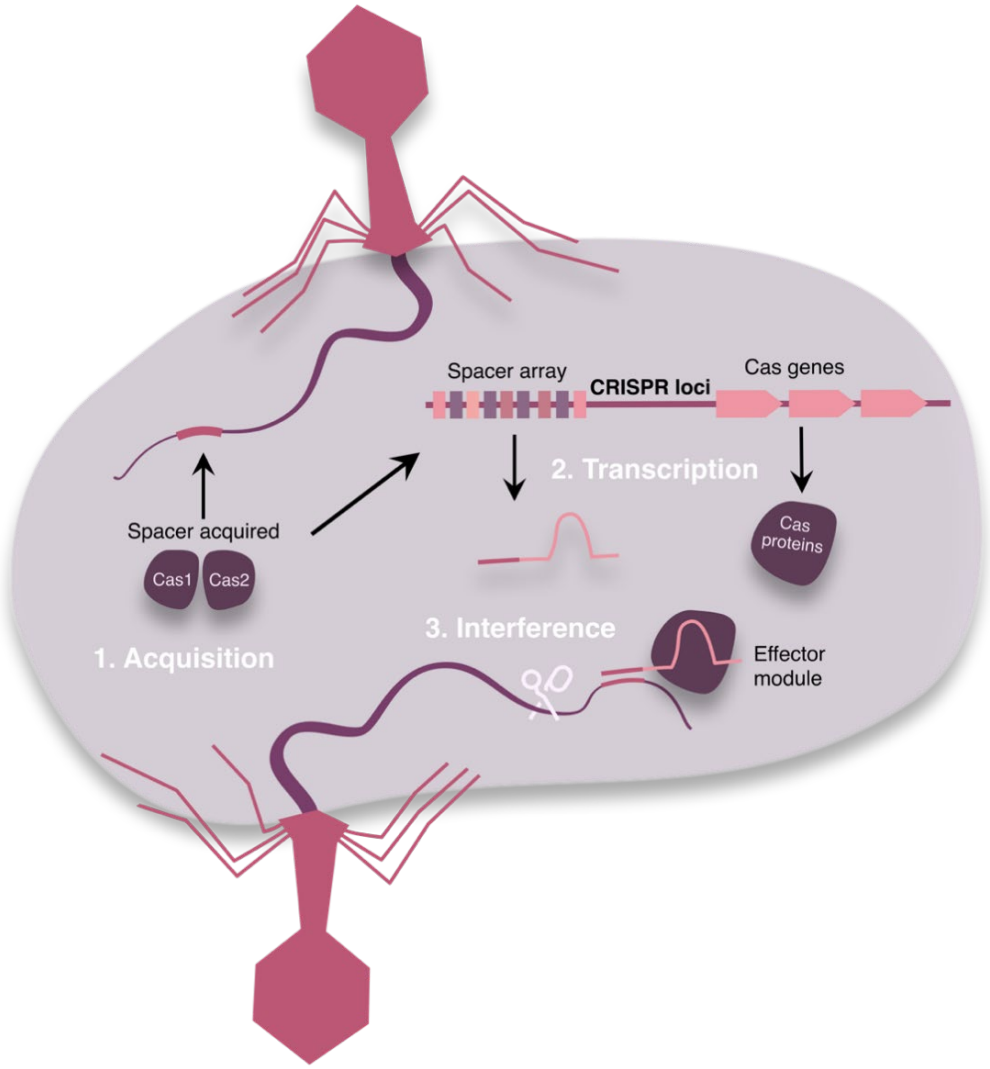

FIGURE 5 Diagram depicts the natural mechanism of CRISPR, which is a defense mechanism found in bacteria against foreign genetic material, such as bacteriophages. The figure illustrates the acquisition of new spacers, facilitated by Cas1 and Cas2 proteins, which are inserted into the spacer array and flanked by repeats. The spacer array alongside the Cas genes make up the CRISPR loci. Transcription and translation processes yield the crRNA from the spacer array and the Cas proteins from the Cas genes. When combined, these components form the effector module, enabling interference with invading foreign genetic material.

The extensive interest and research on CRISPR/Cas systems have prompted a rapid necessity for a structured classification framework. This categorization has proved important in fostering a deeper understanding of the mechanisms and complexities inherent in CRISPR/Cas systems. The molecular machinery and functional attributes of CRISPR/Cas systems exhibit remarkable diversity across their bacterial and archaeal carriers. The classification, primarily anchored in the distinction of effector complexes/modules and associated proteins, delineates two classes based on the effector module involving one large (class 2) or multiple Cas proteins (class 1). Class 1 further branches into Types I, III, and IV, while Class 2 encompasses Types II, V, and VI (Makarova and Koonin 2015, Makarova *et al.* 2020). This classification enhances our understanding of the fundamentals of CRISPR-based bacterial immunity and guides research and the development of innovative applications.

The presence of the Cas9 endonuclease chiefly characterizes Type II CRISPR systems within class 2. The relative structural simplicity of class-1 systems, exemplified by Type II, which rely on a crRNA-effector module with a single subunit, makes them the primary focus for CRISPR programmability. Consequently, they have been extensively studied compared to their seemingly limited representation in nature, constituting only approximately 5% of CRISPR systems (Chylinski *et al.* 2014). Type II CRISPR systems have been exclusively identified in bacteria, with *Streptococcus* species being the most commonly observed, though notably absent in *E. coli* and *K. pneumoniae* (Lier *et al.* 2015, Le Rhun *et al.* 2019, Mosterd and Moineau 2020, Bauer *et al.* 2023). CRISPR/Cas9-based gene editing involves using the Cas9 enzyme, guided by a synthetic RNA molecule, to target and modify specific DNA sequences, this allows for the alteration, insertion, or deletion of genes (Jinek *et al.* 2012).

The Type IV CRISPR/Cas systems represent a subset within the CRISPR landscape that has garnered comparably less attention but is of outmost importance in plasmid biology. These systems are Class 1 CRISPR systems forming multi-subunit effector complexes. They are further subtyped based on their host species, with subtype IV-A3 associated exclusively with *Klebsiella pneumoniae* (Pinilla-Redondo *et al.* 2020, Taylor *et al.* 2021). Recent studies have indicated that what differentiates this type from others is its content of spacers, primarily targeting plasmids (Pinilla-Redondo *et al.* 2020, Newire *et al.* 2020, Moya-Beltrán *et al.* 2021). Moreover, Type IV systems are encoded mainly on plasmids and are thought to be involved in the competition between plasmids. Notably, Type IV systems are known to lack modules involved in spacer acquisition. Instead, they are believed to utilize nearby adaptation modules (Cas1-Cas2) from other systems, such as Type IE in the bacterial genome (Kamruzzaman and Iredell 2020, Benz *et al.* 2023). Because of the largely unknown factors involved in their spacer acquisition, the biological functions of type IV CRISPR/Cas systems are not entirely understood.

1.5 Infection Control

1.5.1 CRISPR tools

In the quest to avert a post-antibiotic era, scientists have harnessed a natural bacterial defense by manipulating CRISPR/Cas systems to target pathogenic bacteria and antimicrobial resistance genes selectively (Bikard *et al.* 2014, Beisel *et al.* 2014, Bikard and Barrangou 2017, Palacios Araya *et al.* 2021). CRISPR-based antimicrobial tools have emerged as a promising strategy to combat AMR and target ARGs. The potential of CRISPR antimicrobials offers an exciting preview of future strategies to combat the proliferation of antimicrobial resistance. They are gaining increased attention amid the surge of resistance, compounded by the stagnation in discovering new antibiotics. While numerous designs of CRISPR antimicrobials have been developed, their clinical potential needs to be addressed, relying on further investigations into the diverse factors influencing their success (Mayorga-Ramos *et al.* 2023). This sequence-specific antimicrobial approach leverages the high specificity and programmability inherent in CRISPR/Cas systems to reduce the abundance of resistant cells and ARGs (Bikard and Barrangou 2017, Ruotsalainen *et al.* 2019, Gholizadeh *et al.* 2020, Palacios Araya *et al.* 2021, Duan *et al.* 2021). The survival of the targeted bacterial cells is contingent upon the location of the antimicrobial resistance gene. Targeting a plasmid-encoded ARG results in plasmid loss, while targeting a chromosomal-located ARG eliminates the entire cell (Gomaa *et al.* 2014, Cui and Bikard 2016, Dong *et al.* 2019). Studies have shown the efficacy of CRISPR antimicrobials against a spectrum of antibiotic-resistant pathogens, including *Klebsiella pneumoniae* and *Escherichia coli* (Kiga *et al.* 2020). However, several areas must be addressed to achieve the full potential of CRISPR-based antimicrobial tools, such as the efficient delivery of DNA vectors and overcoming cells repairing double-stranded breaks induced by CRISPR-based antimicrobials (Uribe *et al.* 2021). In the case of delivery, mobile genetic elements such as conjugative plasmids seem to be ideal candidates. However, the effectiveness of plasmid-encoded CRISPR/Cas antimicrobials can be influenced by competitive factors. The experimental control in this setup involved a targetless CRISPR plasmid. Developing a robust delivery system remains a critical challenge in the practical application of CRISPR-based antimicrobials. Therefore, there is a necessity for studies focused on the limitations and formulation of effective delivery systems (Fagen *et al.* 2017, Pursey *et al.* 2018). During this doctoral research (Study IV), the efficiency and ecological implications of a conjugatively delivered CRISPR/Cas9-based antimicrobial tool (CRICON) were tested. This approach was applied to specifically target and eliminate the ESBL gene *bla*CTX-M-15 from four clinical *E. coli* isolates within a synthetic multi-species community. The CRICON system involved a two-plasmid configuration comprising a mobilizable CRISPR plasmid and an accompanying broad host range conjugative plasmid (RP4) responsible for facilitating delivery. The programmability and specificity of CRISPR-based antimicrobials open avenues

for their repurposing as novel antibiotics. Amidst this, ethical considerations and potential unintended consequences highlight treading with caution for any responsible approach to the expansion of applications of CRISPR/Cas technology (Ruotsalainen *et al.* 2019).

1.5.2 Phage therapy

In the ever-intensifying battle against AMR, efforts must extend further than halting its dissemination and eradicating AMR bacteria and ARGs. Exploring alternatives to antibiotics is needed. Here, bacteriophages, viruses that selectively infect and kill bacteria, have emerged as potential agents for combatting bacterial infections (Housby and Mann 2009, Loc-Carrillo and Abedon 2011, Chan *et al.* 2013, Gordillo Altamirano and Barr 2019). Phage therapy involves using these viruses to precisely target pathogenic bacteria, offering a promising alternative or complementary approach to antimicrobial treatments. Ongoing research is primarily concentrated on the potential of phage therapy as an alternative or adjunctive to conventional antibiotic treatments, with a particular emphasis on its applicability against multidrug-resistant bacterial infections (Viertel *et al.* 2014, Lin *et al.* 2017, Broncano-Lavado *et al.* 2021). Recent studies actively engaged in enhancing the reliability and efficacy of phage therapy in clinical contexts (Letkiewicz *et al.* 2009, Kutter *et al.* 2010, Międzybrodzki *et al.* 2012, McCallin *et al.* 2013, 2018, Petrovic Fabijan *et al.* 2020, Leitner *et al.* 2021). The influence of the immune response on phage therapy has been studied and revealed that certain immunosuppressed patient subgroups may not be eligible for this treatment (Reindel and Fiore 2017, Krut and Bekeredjian-Ding 2018, Jariah and Hakim 2019, Manohar *et al.* 2019). This potentially limits its efficacy in addressing the need for new antibacterial therapies targeting nosocomial infections. Further, understanding phage-host interactions is essential for advancing sustainable phage therapy. These interactions influence the emergence of phage resistance, which practically determines the effectiveness and longevity of phage therapy.

Phage therapy has gained recognition and support from influential entities like the European Union and the US administrations (Naureen *et al.* 2020, Verbeken and Pirnay 2022). Moreover, attention has been directed towards regulatory considerations and adopting legal frameworks for phage therapy, highlighting the potential of phage therapy as a promising alternative to conventional antibiotics. However, a diverse set of challenges must be addressed before phage therapy's widespread clinical implementation, especially the long-term stability and efficacy of phage combinations (Liu *et al.* 2021).

2 AIMS

This thesis explores the interconnections between antibiotic therapy, bacterial evolution, and the dynamics of resistance mechanisms, thereby informing strategies for combating antimicrobial resistance and improving patient outcomes. Here, the dynamics and significance of conjugative resistance plasmids in antibiotic resistance and bacterial evolution are explored through four distinct studies. Each study contributes to understanding the complex interactions among bacteria, plasmids, phages, the environment, and antimicrobials. Through these investigations, the thesis provides comprehensive insights into the intricate relationships between antibiotic resistance, plasmid dynamics, and bacterial evolution. The individual aims of each study are outlined below:

i. To understand the evolutionary pathways of the plasmid population and antibiotic resistance profile within a single patient that underwent antibiotic therapy, through integrating gut metagenomic analysis and the study of gut *Escherichia coli* isolates.
ii. To investigate the potential of conjugative resistance plasmids in rescuing antibiotic-susceptible bacteria, aiming to understand the extent to which these plasmids contribute to bacterial survival during antibiotic therapy.
iii. To ascertain the effects of mucin and antibiotics on the evolution of phage resistance in ESBL-producing *E. coli* and *K. pneumoniae*, providing insights into the dynamic interplay between environmental factors and the employment of resistance mechanisms, potentially influencing phage therapy.
iv. To assess the efficacy of the CRISPR-antimicrobial (CRICON) delivered through conjugation in eradicating ESBL genes from four clinical *E. coli* isolates within a synthetic multispecies community. While also investigating the dynamics and mechanisms governing the evolution and survival of conjugative resistance plasmids.

3 OVERVIEW OF THE METHODS

An overview of the methods used in this thesis is presented Table 2. A detailed description of the materials and methods used can be found in the original articles, indicated by Roman numerals.

TABLE 2. Methods used in the original articles included in the thesis.

Method	**Original Article(s)**
Bacterial strains and culturing	I, II, III, IV
Plasmid conjugation in co-culture	I, II, IV
Evolutionary experiments	II, III, IV
Phage lab work	II, III
Growth rate analysis	II, III, IV
DNA extraction	I, II, III, IV
qPCR	IV
DNA sequencing	I, II, III, IV
Bioinformatic analysis	I, II, III, IV
Statistical analysis	II, III, IV

4 RESULTS

4.1 Unraveling the molecular landscape in clinical drug-resistant *E. coli* and *K. pneumoniae* gut isolates (I, III)

In the present era, we inhabit a world where the effectiveness of our most powerful defenses against bacterial infections, antibiotics, is gradually eroding due to their misapplication and excessive use (Llor and Bjerrum 2014). This global health crisis presents itself as the accelerated development of antimicrobial resistance (AMR) in bacterial pathogens (Prestinaci *et al.* 2015, World Health Organization 2020). A strategic clinical determination of the serious offenders of AMR and their genetic context stands at the forefront of this ongoing battle, aiming not only to grasp the extent of AMR comprehensively but also to discern effective strategies for its mitigation. In Finland, significant efforts have gone into the descriptive analysis of clinical drug-resistant gut isolates. One such has been spearheaded by Turku University Hospital (TYKS) with participation in their multiple clinical studies, including MAPPAC (Microbiology APPendicitis Acuta) and APPAC (APPendicitis Acuta) (Salminen *et al.* 2018, Vanhatalo *et al.* 2019). In the first and third studies of this thesis, genetic characterization of the whole genome sequences of sixteen clinical *Escherichia coli* and four *Klebsiella pneumoniae* gut isolates was conducted (fig. 6). Of the 16 *E. coli* isolates, 12 came from the same MAPPAC patient. The remaining four *E. coli* isolates and the four K. *pneumoniae* isolates came from an ongoing ESBL study at Turku University Hospital. The analysis included examining the sequence types (MLST), the AMR profiling, the CRISPR systems, and the plasmid carriage.

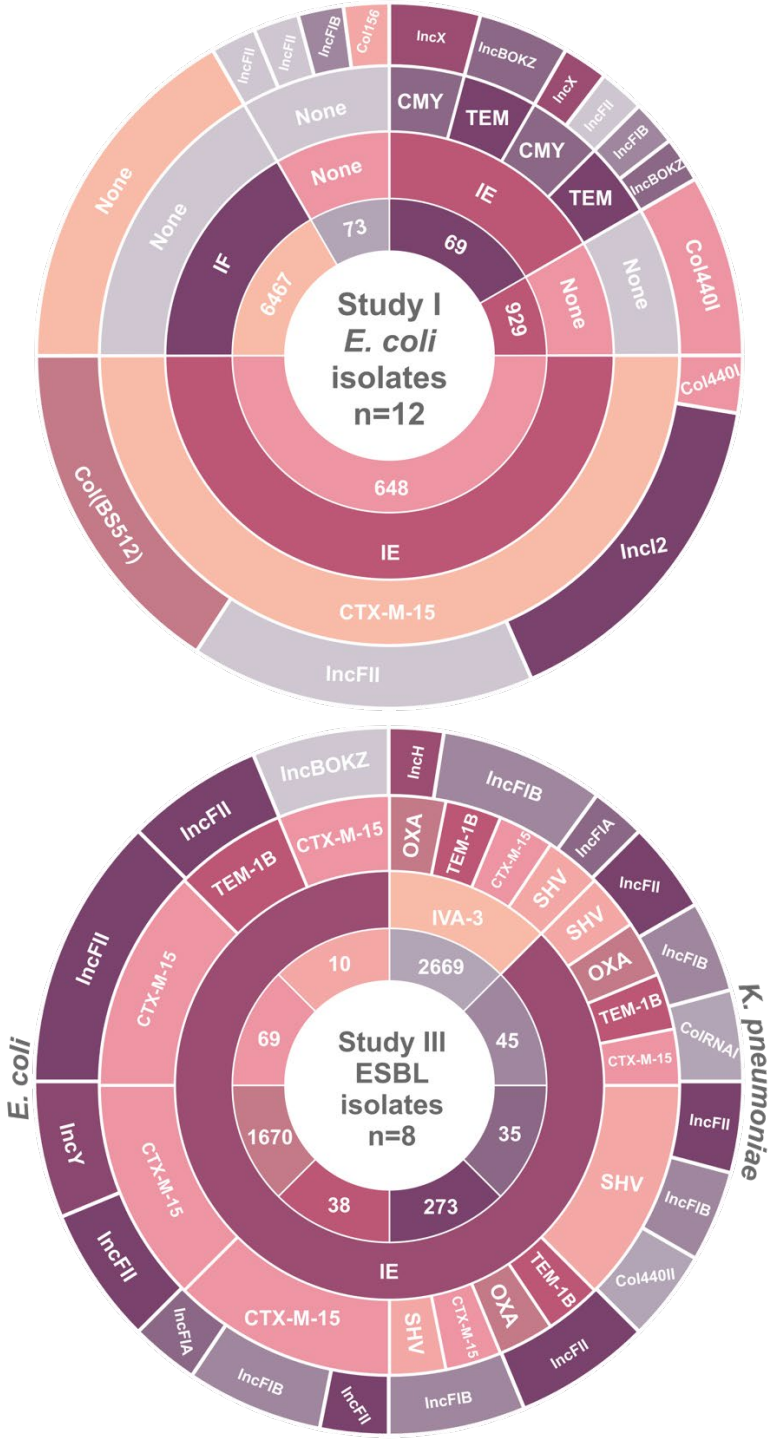

FIGURE 6 Genetic characterization of the clinical *E. coli* (12) gut isolates analyzed in Study I (upper figure) and the clinical ESBL-producing *E. coli* (4 strains, left side) and *K. pneumoniae* (4 strains, right side) isolates analyzed in Study III (lower figure). The inner most ring represents the sequence type (ST), the second ring represent the CRISPR systems, the third ring represent the beta-lactamase producing genes (*bla*), the outermost ring represent the plasmid markers found.

4.1.1 Extensive genetic lineage and resistance profile of *E. coli* and *K. pneumoniae* gut isolates from Finland

MLST is a widely acknowledged genotyping technique that has served as the benchmark for characterizing many bacterial strains and pathogens. MLST encompasses comparing multiple loci, usually highly conserved housekeeping genes, to detect sequence differences. This facilitates monitoring the global dissemination of drug-resistant variants, thereby advancing clinical diagnostics, pathogenicity, and epidemiological surveillance (Maiden *et al.* 1998, Urwin and Maiden 2003, Maiden 2006). The twelve longitudinal *E. coli* isolates from the single MAPPAC patient (Study I) were identified as belonging to four sequence types (see full details in Table 3). These included ST69, ST73, ST929, and ST648. The most common sequence type in these *E. coli* isolates was ST648, which was found to be the same strain isolated multiple times from this patient. The remaining four *E. coli* isolates from the ESBL study (Study III) were identified as ST10, ST38, ST69, and ST1670 (see full details in Table 4). Interestingly, ST69 displayed the most extensive presence, isolated as two different strains from both studies. This lineage (ST69) has been widely reported as resistance-carrying and a determinant of community-acquired infections (Riley 2014, Goswami *et al.* 2018, Fox *et al.* 2020, Mattioni Marchetti *et al.* 2020). Three *E. coli* sequence types, ST6467, ST929, and ST73, did not carry ARGs. Notably, ST73 is recognized as a prevalent antibiotic-susceptible *E. coli* lineage (Riley 2014, Goswami *et al.* 2018). Examining the four *Klebsiella pneumoniae* isolates from the ESBL study revealed that each isolate belonged to a unique sequence type. The identified sequence types for *K. pneumoniae* were ST273, ST35, ST45, and ST2669. All of these lineages, with the notable exclusion of ST2669, are well-recognized as multi-resistant pathogens (MARCADE *et al.* 2013, Ito *et al.* 2015, Chou *et al.* 2016, Liu *et al.* 2018, Literacka *et al.* 2020, Cienfuegos-Gallet *et al.* 2022, Shi *et al.* 2022a).

This investigation explored the prevalence of ESBL-producing isolates, recognizing the critical importance of distinguishing between strains carrying resistance and elucidating whether this carriage is plasmid or chromosomal-based. ESBL production is commonly facilitated by conjugative plasmids and is closely linked to certain plasmids, such as IncF (Carattoli 2011, Pungpian *et al.* 2022). Seven were identified as beta-lactamase-producing isolates of the nine *E. coli* strains collectively analyzed (through Studies I and III). The genes responsible for encoding the beta-lactamases included *bla*CTX-M-15 (carriage rate of 56%), *bla*TEM-1B (carriage rate of 22%), and a variant of *bla*CMY (carriage rate of 11%). The significant prevalence of *bla*CTX-M-15 gene carriage aligns with the ongoing increasing trend of beta-lactamase encoding *E. coli*, even referred to as the CTX-M beta-lactamase pandemic (Cantón and Coque 2006). Additionally, in the first study, the plasmid carriage of the *bla* genes identified AMR plasmids carrying the *bla*TEM-1b and the *bla*CMY variant genes. Beyond identifying beta-lactamase encoding genes, the analysis revealed other notable antimicrobial resistance genes (ARGs), specifically *sul*2, *qnr*S1, and *mdf*A.

Klebsiella pneumoniae is well recognized for its propensity to harbor ARGs, particularly in the context of hospital-acquired infections (Khanfar *et al.* 2009,

Navon-Venezia *et al.* 2017, Wang *et al.* 2020). This poses obvious and significant challenges in effectively managing antimicrobial resistance in healthcare settings. The examination of four *K. pneumoniae* isolates (Study III) revealed each as extensively multi-resistant strains (Table 4). Six distinct beta-lactamase encoding genes were found in all four *K. pneumoniae* isolates, some of the strains carrying up to four *bla* genes. These included *bla*SHV variants, along with *bla*CTX-M-15, *bla*OXA-1, and *bla*TEM-1b, commonly described to be carried within the *K. pneumoniae* species (Babini and Livermore 2000, Park *et al.* 2015, Dziri *et al.* 2018, Shi *et al.* 2022b, Meng *et al.* 2023). However, this sequence type is not strictly beta-lactam susceptible as ESBL gene *bla*SHV has been described in this lineage. Twenty-five additional resistance genes were discovered within the four genomes of *K. pneumoniae*. Particularly noteworthy were those linked to imparting resistance against sulfonamides (*sul*1, *sul*2), tetracyclines (*tet*A), and quinolones (*qnr*S1).

4.1.2 Pervasive plasmid occurrence characterized by consistent incompatibility groups in *E. coli* and *K. pneumoniae*

In light of the well-established role of conjugative plasmids in disseminating antimicrobial resistance, exploring plasmid carriage in clinical bacterial strains is crucial in addressing this threat. However, this bears significant practical complexity, as these genetic elements are intricate, displaying substantial variability in both their advantages and associated costs. In certain instances, the fitness aspects of these plasmids may seem nearly imperceptible, posing a challenge in evaluating their impact when explicit selection markers are absent. This is described by the infamous enigma within the field of plasmid biology coined the "plasmid-paradox" (Carroll and Wong 2018, Brockhurst and Harrison 2022). Clinical plasmid biology research lies with the categorization based on traits such as compatibility for cohabitation, associated ARGs, and mobility. Within the strains of *E. coli* and *K. pneumoniae* analyzed in Study I and III, 34 plasmid Inc types were identified, with only a single *E. coli* strain lacking any markers. These plasmids belonged to 12 plasmid groups, with IncF being the most prevalent, notably represented within three subtypes: IncFII, IncFIA, and IncFIB.

The IncF plasmid group is the most extensively studied and recognized and is exclusively found within members of the *Enterobacteriaceae* family (Carattoli 2009, Mathers *et al.* 2015). Typically characterized as a low copy number plasmid, IncF plasmids are classified under mobility class MOBF. Mobility classes characterize plasmid mobility depending on the conjugation machinery used for transfer. Notably, IncF plasmids exhibit a unique characteristic by deviating from traditional "incompatibility", allowing them to coexist within the same bacterial cell over generations. This ability is facilitated by the presence of multiple replicons encoded by these plasmids, which serve as the foundation for their subtyping (Rozwandowicz *et al.* 2018). A total of 22 IncF markers were identified (Study I, III), constituting 65% of all the identified plasmids. Among the subtypes, IncFIA was the least frequently observed, comprising only two strains.

Interestingly, it was found in both an *E. coli* and a *K. pneumoniae* strain. On the other hand, ten plasmids in each of the IncFII and IncFIB groups were identified, once again showing overlap across both bacterial species. An intriguing discovery was an IncFIB phage-plasmid in one of the *E. coli* strains (ST38; Study III). These genetic elements are unique in that they can transfer horizontally between cells as viruses and vertically within cellular lineages as plasmids (Pfeifer and Rocha 2024). Notably, the analyzed strains harboring IncF plasmids tended to carry multiple IncF plasmids, a phenomenon not surprising given the nature of IncF plasmids to harbor numerous replicons. These findings are likely attributable to the presence of plasmids harboring multiple replicons rather than the existence of multiple individual plasmids. The analysis did not extensively explore the plasmid carriage of the ARGs found due to the limitations of short-read sequencing technologies such as Illumina and DNBSEQ, which do not always provide complete plasmid sequences, thereby complicating the assembly of entire plasmids. Nonetheless, the presence of *bla*TEM-1B in ST69 was noted as being situated on an IncFIB plasmid. This plasmid also harbored ARGs for tetracycline (*tet*A), streptomycin (*aad*A1), fluoroquinolones (*qnr*S1), and macrolides (*mph*A). According to literature, among the most frequently identified antimicrobial resistance genes associated with IncF plasmids are ESBL and Plasmid-Mediated Quinolone Resistance (PMQR) genes (Matsumura *et al.* 2013, Markovska *et al.* 2014, Brolund and Sandegren 2016, Agyekum *et al.* 2016). However, it is vital to acknowledge the potential bias in the mass reporting of plasmid associated ESBL genes, likely influenced by the attention and research efforts directed toward combatting ESBL resistance.

Two IncB/O/K/Z contigs were identified in two different *E. coli* strains, specifically in ST69 (strain 6.2/12.1 from Study I) and ST10 (strain 18B1 from Study III). This plasmid group exhibits a narrow host range, having been previously reported in both *E. coli* and *K. pneumoniae*, albeit more frequently in *E. coli*. The distinctive feature of this group is the challenge of distinguishing between IncB/O, IncK, and IncZ due to their shared membership in the I-plasmid complex, whose members share significant morphological and serological similarities in their pili (Rozwandowicz 2020). These plasmids are notably recognized for their tendency to carry *bla*CMY genes and sulfonamide resistance genes *sul*1 and *sul*2 (Tschäpe and Tietze 1983, Dierikx *et al.* 2010a, 2013, Mnif *et al.* 2012, Hordijk *et al.* 2013, Guo *et al.* 2014). Directly pertinent to this, the ST69 *E. coli* strains in Study I harbored an IncB/O/K/Z plasmid carrying both a *bla*CMY variant gene and a *sul*2 gene. Another constituent of the I-plasmid complex is the IncI plasmid group, distinguishable by its expression of a small counter-transcript RNA, RNAI. Among its subvariants, IncI2 is recognized for its ability to migrate between species. In Study I, an IncI plasmid was identified in an *E. coli* strain (ST648). While this plasmid group has been associated with carrying ARGs, further long-read sequencing could resolve plasmid carriage. A single *K. pneumoniae* strain (ST2669) was found to harbor an IncH plasmid. This group of plasmids is known for its broad host range, and some members can display incompatibility with IncF plasmids. The *K. pneumoniae* strain (ST2669) carrying the IncH plasmid also carried IncF plasmids.

Within the infrequently identified Inc types, we discovered plasmid markers belonging to the IncX (ST69; *E. coli* Study I) and IncY (ST1670; *E. coli* Study III). IncX is a relatively obscure group of plasmids documented in *E. coli* and *Salmonella* (Jones and Stanley 1992, Pál *et al.* 2017, Espinal *et al.* 2018). Interestingly, the IncY group, although rare, represents a collection of prophages that replicate autonomously as plasmids (Meyer *et al.* 1986). IncY is recognized for its association with other plasmid groups, particularly IncF, IncI, and IncH (Novais *et al.* 2006, Kang *et al.* 2008, Dierikx *et al.* 2010b, Rodrigues *et al.* 2013, Jones-Dias *et al.* 2013, Dotto *et al.* 2014, Ben Sallem *et al.* 2014, Vogt *et al.* 2014). This group has been confirmed to carry the *bla*SHV gene but not substantiated within this thesis (Billard-Pomares *et al.* 2014). Additionally, a few Colicinogenic (Col) plasmids were identified in both species. Col plasmids belong to a family of bacteriocins and are known to be produced by certain *E. coli*. Our results revealed the identification of Col-like plasmids Col440II (ST35) and ColRNAI (ST45) in *K. pneumoniae*, comparable to previously described results (Cao *et al.* 2002, Zioga *et al.* 2009). This group is named after the protein colicin, which can eliminate surrounding bacteria and serve as a defense mechanism for the host bacterium. Certain Col plasmids have been observed to be mobilizable and carry multiple ARGs, providing a multi-resistant phenotype (San Millan *et al.* 2009, Hammerl *et al.* 2010, Pallecchi *et al.* 2010, Herrera-Leon *et al.* 2011).

4.1.3 Convergent CRISPR Systems in *E. coli* and *K. pneumoniae*

On the battlefield of microbial ecosystems, bacteria engage in intricate survival strategies, deploying variable mechanisms to defend themselves against foreign threats. CRISPR-Cas systems, widespread throughout bacteria, are one such defense that targets foreign DNA, including conjugative plasmids and phages. In Study I and III, the CRISPR analysis revealed the identification of intersecting CRISPR systems across the *E. coli* and *K. pneumoniae* isolates. The most prevalent system in both species was the class 1 system Type IE, found in 6 of the 9 unique strains, while the adjacent Type IF was identified in a single *E. coli* strain (Table 3). Notably, two *E. coli* strains showed no identifiable CRISPR systems. Further, the investigation uncovered a relatively unique *K. pneumoniae* plasmid CRISPR system belonging to Type IVA-3. While the same type of CRISPR system (Type IE) was identified in both species, the systems themselves often exhibit distinct interspecies characteristics. Although not explored in this study, further investigation into these differences, particularly regarding the repeat and PAM sequences, would be intriguing.

For context, the most extensively described CRISPR system within *Enterobacteriaceae* is the Type IE system. Currently, no class 2 systems have been reported in *E. coli* or *K. pneumoniae* (Medina-Aparicio *et al.* 2018). The unique Type IV systems constitute the least understood group of CRISPR systems. However, there have been recent expansions in the knowledge, including the subgroup Type IVA-3 primarily identified in the *K. pneumoniae* species (Pinilla-Redondo *et al.* 2020). A few recent studies have made substantial progress in comprehending Type IV systems. Notably, research has indicated that the spacers identified in

the CRISPR arrays within these systems predominantly target plasmids (Pinilla-Redondo *et al.* 2020, 2022). However, this observation may be biased due to the limits to phage genomics. The bias stems mainly from the challenges encountered when sequencing and analyzing phage genomes. This involves insufficient data representation due to the difficulties in isolating phage nucleic acids and the presence of temperate phages. Furthermore, even when phage genomes are successfully isolated and sequenced, limitations arise in capturing complete phage genomes, hindered by their unexplored genetic diversity. Nevertheless, these findings suggest the potential for "plasmid warfare", whereas plasmids fight for compatibility within a host. Experimental testing in recent years has sought to elucidate the functions of Type IV CRISPR systems, given their lack of the adaptation module necessary for spacer uptake. Reports indicate that Type IV systems rely on other CRISPR systems, such as Type IE, present in the cell to acquire new spacers (Kamruzzaman and Iredell 2020). Interestingly, the strain (ST2669) harboring the Type IV system in Study III did not carry other CRISPR systems, suggesting that this system is incapable of acquiring new spacers in its current host (ST2669). Additionally, certain Type IVA systems have been exclusively identified on IncFIB and IncHI1B replicons, both found within the ST2669 genome (Newire *et al.* 2020). However, the analysis in Study III did not go into the plasmid location of the identified CRISPR system . Further evaluation is necessary to determine whether Type IV CRISPR systems possess the functionality to carry out CRISPR interference.

TABLE 3 Genetic characterization of the clinical *E. coli* (12) gut isolates analyzed in Study I (MAPPAC).

Study	Strain	Species	ST	Plasmid(s)	*bla* genes	ARG	Mutations	CRISPR
I	0.1	*E. coli*	6467	None	None	None	*gyr*A, *gyr*B, *par*C, *pmr*A, *fol*P, *rpo*B, 16S-*rrs*H	Type IF
I	0.2	*E. coli*	648	Col(BS512), IncFII, IncI2(Delta)	*bla*CTX-M-15	*sul*2	*gyr*A, *gyr*B, *par*C, *pmr*A, *pmr*B, *fol*P, *rpo*B, *amp*C-promoter, 16S-*rrs*H	Type IE
I	1.1	*E. coli*	648	Col(BS512), IncFII, IncI2(Delta)	*bla*CTX-M-15	*sul*2	*gyr*A, *gyr*B, *par*C, *pmr*A, *pmr*B, *fol*P, *rpo*B, *amp*C-promoter, 16S-*rrs*H	Type IE
I	1.2	*E. coli*	6467	None	None	None	*gyr*A, *gyr*B, *par*C, *pmr*A, *fol*P, *rpo*B	Type IF
I	3.1	*E. coli*	648	Col(BS512), IncFII,IncI2(Delta)	*bla*CTX-M-15	*sul*2	*gyr*A, *gyr*B, *par*C, *pmr*A, *pmr*B, *fol*P, *rpo*B, *amp*C-promoter	Type IE
I	3.2	*E. coli*	648	Col(BS512), IncFII, IncI2(Delta)	*bla*CTX-M-15	*sul*2	*gyr*A, *gyr*B, *par*C, *pmr*A, *pmr*B, *fol*P, *rpo*B, *amp*C-promoter	Type IE
I	7.1	*E. coli*	648	Col(BS512), IncFII, IncI2(Delta), Col440I	*bla*CTX-M-15	*sul*2	*gyr*A, *gyr*B, *par*C, *pmr*A, *pmr*B, *fol*P, *rpo*B, *amp*C-promoter, 16S-*rrs*H	Type IE
I	7.2	*E. coli*	648	Col(BS512),IncFII,IncI2(Delta)	*bla*CTX-M-15	*sul*2	*gyr*A, *gyr*B, *par*C, *pmr*A, *pmr*B, *fol*P, *rpo*B, *amp*C-promoter, 16S-*rrs*H	Type IE
I	6.1	*E. coli*	929	Col440I	None	*sit*ABCD	*gyr*A, *gyr*B, *par*C, *pmr*A, *pmr*B, *fol*P, *rpo*B, *amp*C-promoter	None
I	6.2	*E. coli*	69	IncB/O/K/Z,IncFIB,IncFII,IncX4	*bla*TEM-1B*, *bla*CMY-variant*	*qnr*S1, *mphA*, *sul*2, *dfr*A1, *tetA*, *aad*A1, *sit*ABCD, *aph6-Id*, *aad*A1, *aph3''-Ib*, *qac*E	*gyr*A, *gyr*B, *par*C, *par*E, *pmr*A, *pmr*B, *fol*P, *rpo*B, *amp*C-promoter, 16S-*rrs*B, 16S-*rrsC*	Type IE
I	12.1	*E. coli*	69	IncB/O/K/Z,IncX4	*bla*TEM-1B*, *bla*CMY-variant*	*sul*2, *sit*ABCD, *aph6-Id*, *aph3''-Ib*	*gyr*A, *gyr*B, *par*C, *par*E, *pmr*A, *pmr*B, *fol*P, *rpo*B, *amp*C-promoter	Type IE
I	12.2	*E. coli*	73	Col156,IncFIB,IncFII,IncFII(29)	None	*sit*ABCD	*gyr*A, *gyr*B, *par*C, *pmr*A, *pmr*B, *fol*P, *rpo*B, *amp*C-promoter, 16S-*rrs*B, 16S-*rrsC*	None

*plasmid-encoded

TABLE 4 Genetic characterization of the clinical ESBL-producing *E. coli* (4) and *K. pneumoniae* (4) isolates analyzed in Study III (ESBL).

Study	Strain	Species	ST	Plasmid(s)	*bla* genes	ARGs	AMR Mutations	CRISPR
III	18B1	*E. coli*	10	IncB/O/K/Z, IncFII(pCoo)	*bla*CTX-M-15, *bla*TEM-1B	*mdfA*	None	Type IE
III	26B1	*E. coli*	69	IncFII	*bla*CTX-M-15	*mdfA*, *qnr*S1, *sit*ABCD	None	Type IE
III	40B1	*E. coli*	1670	IncFII(pCoo), IncY	*bla*CTX-M-15	*mdfA*, *sit*ABCD, *par*E	*par*E	Type IE
III	53B1	*E. coli*	38	IncFIA,IncFIB(AP001918), IncFIB(H89-PhagePlasmid), IncFII(29)	*bla*CTX-M-15	aph6-Id, mdfA, sitABCD, gyrA	*gyr*A	Type IE
III	28B1	*K. pneumoniae*	273	IncFIB(K), IncFII(K)	*bla*SHV-67/*bla*SHV-11, *bla*CTX-M-15, *bla*OXA-1, *bla*TEM-1B	*aph6-Id, aph3''*-Ib, *aac6'*-Ib-cr, *aac3*-IIa, *sul*2, *dfr*A14, *tetA*, *cat*B3, *fosA*, *oqx*B, *qnr*B1, *oqx*A,	*acr*R, *omp*K36, *omp*K37	Tynpe IE
III	48B1	*K. pneumoniae*	35	Col440II, IncFIB(K), IncFII(K)	*bla*SHV-NN	aadA1, *dfr*A1, *sul*1, *oqx*B, *oqx*A, *tetA*, *cat*A1, *fosA*, *acr*R, *qac*E, *omp*K36, *omp*K37	*acr*R, *omp*K36, *omp*K37	Type IE
III	55B1	*K. pneumoniae*	45	ColRNAI, IncFIB(K), IncFII(K)	*bla*CTX-M-15, *bla*TEM-1B, *bla*OXA-1, *bla*SHV-NN	*aph6-Id, aph3''*-Ib, *aac6'*-Ib-cr, *aac3*-IIa, *dfr*A14, *sul*2, *oqx*B, *oqx*A, *tetA*, *cat*B3, *fosA*, *acr*R, *omp*K36, *omp*K37	*acr*R, *omp*K36, *omp*K37	Type IE
III	93B1	*K. pneumoniae*	2669	IncFIA(pBK30683),IncFIB(K), IncFIB(K)(pCAV1099-114), IncFIB(pNDM-Mar), IncHI1B(pNDM-MAR)	*bla*SHV-65, *bla*CTX-M-15, *bla*TEM-1B, *bla*OXA-1	*aph6-Id, aph3''*-Ib, *aac6'*-Ib-cr, *aad*A16, *sul*2, *sul*1, *dfr*A27, *dfr*A14, *mdfA*, *gyr*A, *sit*ABCD, *cat*B3, *fosA*, *oqx*B, *oqx*A, *qnr*B1, ARR-3	*acr*R, *omp*K36, *omp*K37, *ram*R, *gyr*B, *rps*L	Type IV-A*

4.2 The success of targeted solutions against AMR depends on a greater ecological understanding of the microbial tools available

In the effort to combat AMR, there has been an ongoing pursuit of targeted solutions, including interventions based on viruses and CRISPR technology. The efficient development of these approaches relies on more knowledge surrounding the ecological and evolutionary aspects of AMR. The complexity of the molecular mechanisms behind the evolution of resistances highlights the importance of delving deeper. This instigated the impetus behind Studies II and III presented in this thesis with the dual investigation into ecological and evolutionary factors shaping the efficiency of CRISPR antimicrobials and the development of phage resistance.

4.2.1 The development of resistance to phages is multifaceted across environmental and genetic contexts (III and II)

Phage therapy is the application of bacteriophages to combat infections, including clinical ones, caused by bacteria, such as elusive antimicrobial-resistant pathogens (Housby and Mann 2009, Kortright *et al.* 2019, Zalewska-Piątek 2023). The significance of phage therapy is rooted in its ability to present a targeted and innovative strategy for treating bacterial infections. This avenue offers a promising alternative or supplementary option to conventional antibiotic treatments. Phage resistance development in bacteria, particularly in pathogens like *E. coli* and *K. pneumoniae*, poses a major obstacle to the sustainable implementation of phage therapy. Nonetheless, the development of phage resistance is highly intricate, given the complexity of the co-evolutionary dynamics between phages and bacteria. Strategies to address phage resistance involve isolating and characterizing novel phages targeting pathogenic strains and understanding the development of resistance (Li *et al.* 2021, Jo *et al.* 2023). Additionally, research has delved into the potential of combining phages with antibiotics to surmount multidrug-resistant strains (Qurat-ul-Ain *et al.* 2021).

Study III of this thesis examined the potential impact of environmental factors on the development of phage resistance in clinical gut isolates of *E. coli* and *K. pneumoniae*. The motivation for Study III was to investigate the development of resistance against potential phage therapy delegates infective towards ESBL-producing *E. coli* and *K. pneumoniae*. This study incorporated environmental variables, mucin and antibiotics, to investigate their influence on the emergence of phage resistance. Multiple phages were assessed against individual representatives of *E. coli* (26B1 – ST69) and *K. pneumoniae* (93B1 – ST2669). Resistance emerged against all examined phages, with no notable impact by the presence of mucin and/or antibiotics. Research has shown that *E. coli* mutants resistant to a particular phage strain also tend to resist other phages with similar adsorption mechanisms or the same receptors (McGee *et al.* 2023).

Mutations responsible for this phage-resistant trait often impact the structure and synthesis of lipopolysaccharides. A recent case study into personalized phage therapy against multi-resistant *K. pneumoniae* reported that the development of phage resistance was associated with reduced virulence. There, the resistance mechanism was associated with a gene deletion altering the lipopolysaccharide structure (Li *et al.* 2023). It has already been established that phage adherence to mucin can offer preventative protection against pathogenic bacteria (Almeida *et al.* 2019). Further, the use of mucin in the experimental testing in Study III was observed to influence bacterial growth and biofilm formation in some of the bacterial strains. This prompts the need for additional experimental testing of the effect of mucin on phage resistance, particularly focused on biofilm dynamics. This holds clinical significance as bacterial populations within the human body commonly exist in biofilm.

Study II aimed to investigate the concept of evolutionary rescue via HGT within biofilms, a setting more akin to the human body, by introducing various host-specific lineages of the broad-host-range plasmid RP4 to examine the influence of intra- and interspecific host histories on both rescue and transfer potential. Plasmid-host pairs were coevolved to evaluate their impact on plasmid rescue potential. Unexpected findings emerged from this, revealing induced host-specific plasmid lineages associated with phage resistance. Long-term exposure (300 generations) to *K. pneumoniae* prompted the emergence of evolved RP4 plasmid lineages, marked by the loss of essential genes within the mating pair formation system, Tra2. Notably, phage PRD1 relies on the mating pair formation system of the RP4 plasmid for infection (Lessl *et al.* 1993, Haase *et al.* 1995, Grahn *et al.* 1997, 1999). This underscores the active involvement of plasmids in the development of phage resistance. Moreover, it raises questions about using plasmid-dependent phages as delegates for phage therapy, considering the additional potential for manipulation associated with their dependency on plasmids.

4.2.2 The efficacy of CRISPR antimicrobials against plasmid encoded ARGs relies on the evolutionary and ecological factors (IV)

In Study IV, the effectiveness of the conjugatively delivered CRISPR antimicrobial (CRICON system) in targeting and removing the ESBL gene *bla*CTX-M-15 was tested. This was evaluated using four different *E. coli* isolates (carrying the *bla* gene) introduced into the synthetic multispecies community (*E. coli*, *Klebsiella sp., K. pneumoniae,* and *C. freundii*). The experiment ran with a migration treatment that included the migration of plasmid-free *E. coli* strain and a non-migration treatment that did not include this. Due to the insignificance between these treatments, the results are exclusive to the migration treatment. Each of the four clinical *E. coli* isolates harbored the *bla*CTX-M-15 gene, representing both plasmid (E4 and E8) and chromosomal (E6 and E11) genetic contexts. These isolates underwent a 10-day microcosm experiment within the synthetic multispecies community, with limited selection pressure for the ESBL gene. A single antibiotic pulse (cephalothin) was administered on fifth day of the

experiment, and the CRICON system (consisting of a spacer-containing and control without spacer) was added with each daily refreshment. Quantitative PCR analysis revealed that three of the four setups saw the ESBL gene eliminated without the assistance of the CRISPR antimicrobial (E4, E6, E11) (fig. 7). A single strain, *Klebsiella* sp, dominated all communities. A relatively explicit limitation of the CRICON system lies in the reliance on two plasmids instead of a single plasmid capable of both delivery and CRISPR interference. In one *E. coli* strain (E8 - ST69), the abundance of the ESBL gene decreased successfully with the help of the CRICON system (fig. 7). Interestingly, this ESBL gene was plasmid-encoded and thus perhaps capable of "escaping" the CRICON system. Further analysis demonstrated that although the CRISPR antimicrobial effectively diminished the gene, the gene was also efficiently transferred to a more competitive host, *Klebsiella* sp., within the community. This was confirmed by analyzing the ESBL-harboring clones isolated from the experimental endpoint using 16S RNA sequencing. The competitiveness of *Klebsiella* sp. was assessed based on the efficiency of pE8 to transfer to this host and the growth rate of the bacterial strain. This plasmid transfer into the dominating strain, *Klebsiella sp.*, would allow the community to keep the antibiotic resistance (ESBL gene) while the original plasmid host, *E. coli*, practically disappeared (very low abundance).

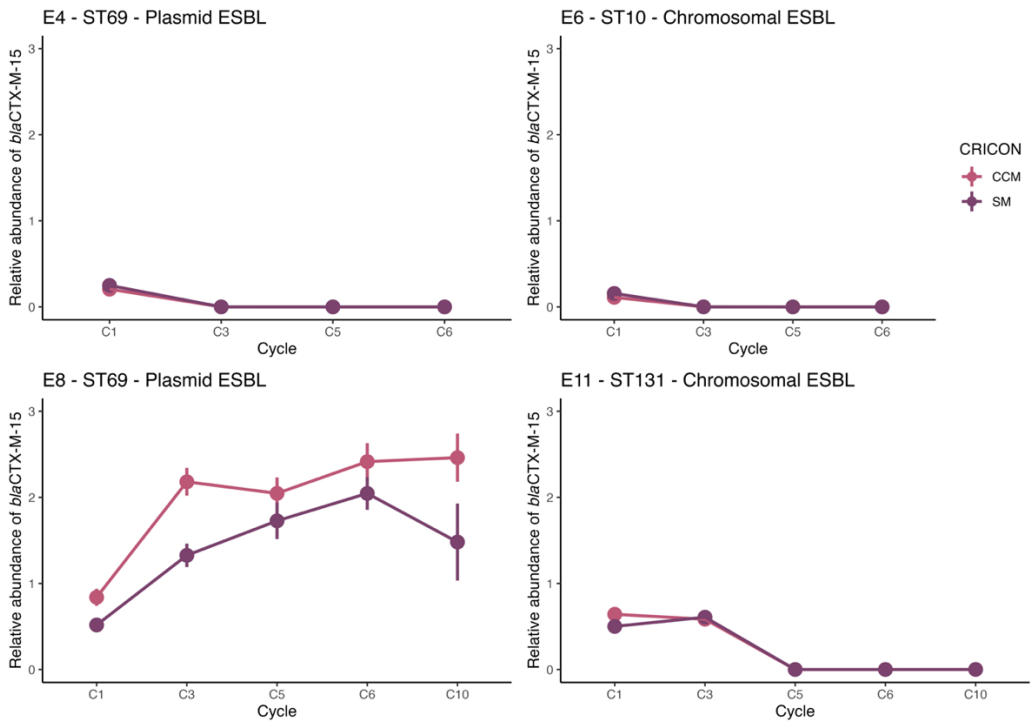

FIGURE 7 Abundance of blaCTX-M-15 within the synthetic multispecies communities, distinguished by the residing ESBL-producing *E. coli* isolates (E4, E6, E8, E11), during the 10-day evolution microcosms experiments. CCM (pink) = CRICON control treatment without target, and SM (purple) = CRICON spacer treatment with spacer targeting ESBL gene. Both treatments included the migration of plasmid-free host cells. The communities containing *E. coli isolates* E4 and E6 did not have measurement at the endpoint (C10) since the ESBL gene had clearly disappeared.

4.3 Evolutionary history impacts plasmid dynamics and antimicrobial resistance

In the tapestry of microbial ecological systems woven inside the human body, the evolution of opportunistic bacteria holds important implications for the health of its host, particularly in terms of antimicrobial resistance. Among the drivers of bacterial adaptation are mobile genetic elements (MGEs), notably conjugative plasmids, often facilitating the horizontal gene transfer (HGT) of ARGs among bacterial populations. The historical interactions between bacteria and their environment, including exposure to antibiotics, shape the genetic makeup of both the bacteria and their plasmids. This evolutionary and ecological context plays a crucial role in determining how plasmids acquire, carry, and transfer antimicrobial resistance genes. Understanding the evolutionary history of bacterial populations provides valuable insights into the mechanisms driving

plasmid dynamics and the emergence of antimicrobial resistance, aiding efforts to devise effective strategies.

4.3.1 Evolutionary trajectory of conjugative resistance plasmids in a multispecies community targeted by CRISPR (IV)

Study IV scrutinized the plasmid evolution of an ESBL (*bla*CTX-M-15) encoding plasmid pE8 (from *E. coli* strain E8 – ST69) after exposure to a targeted CRISPR antimicrobial. Here, the objective was to determine if the selective pressure exerted by CRISPR applications drives the evolution of AMR plasmids, as previously pointed out (Uribe *et al.* 2021). The potential impact of this experimental environment on the transfer rate of the plasmid was tested. The results revealed that the ESBL plasmid underwent evolution, leading to a heightened transfer rate when exposed to the multispecies community (fig. 8). Reports have shown that plasmid stability can evolve through mechanisms such as deleting costly regions from the plasmid backbone, thereby broadening the host-range of the plasmid (Porse *et al.* 2016). Further, the number of plasmids a cell harbors also impacts plasmid transfer rates, with strains carrying two or more plasmids exhibiting faster transfer rates as well as the environment significantly affecting plasmid transfer rates (Darphorn *et al.* 2022). Bacteria from natural populations tend to transfer plasmids predominantly to their kin, indicating a level of selectivity in plasmid transfer (Dimitriu *et al.* 2019). In this study, the genetic changes in the plasmids were not determined. This is a limitation, as sequencing the evolved clones could help address the plasmid evolution further. Interestingly, the evolved plasmid lineage did not exhibit an altered transfer rate when specifically targeted by CRISPR. This is evident by the comparable transfer rates observed in the plasmid exposed to the targeted CRICON and the one exposed to the CRICON control that lacked a specific target. This suggests that the CRISPR intervention had no discernible effect on the increased transfer rate observed in the evolved plasmid lineage. The evolutionary and ecological impacts of CRISPR antimicrobials on microbial communities remain largely unknown.

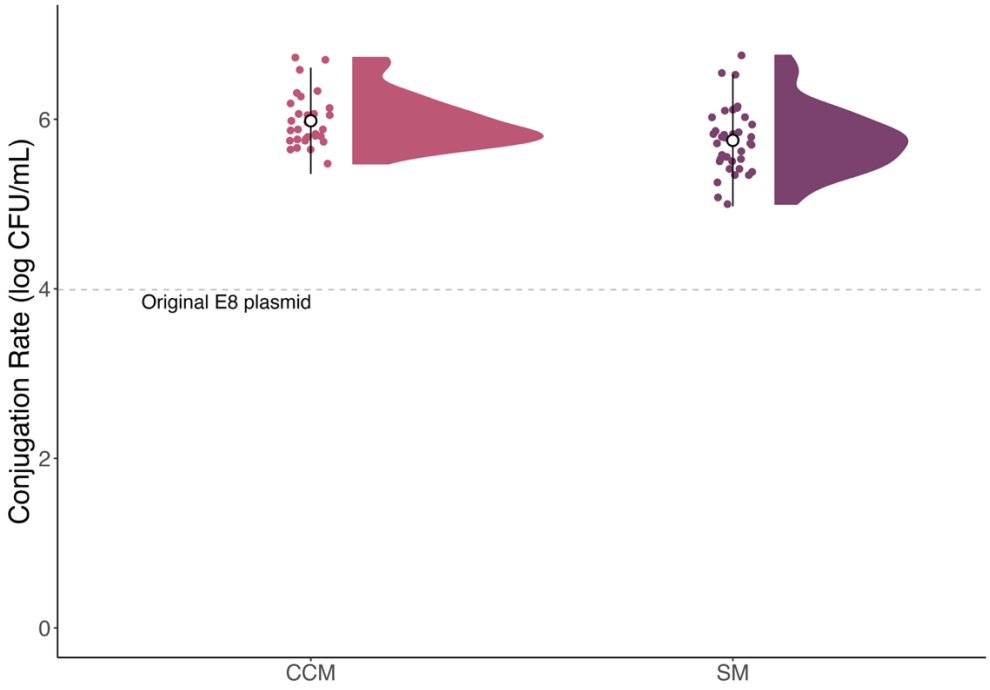

FIGURE 8 Conjugation rate of evolved pE8 compared to the original unevolved pE8. Evolved variations from the *Klebsiella* sp. clones were taken from the endpoint of the microcosm experiment. The evolved pE8 plasmids are distinguished by treatment of CRICON control (CCM; pink) and CRICON spacer (SM; purple). The mean conjugation rate of the original E8 ESBL plasmid is represented in the dashed line. All the plasmids were conjugated from *E. coli* HB101 into *E. coli* HMS174 to be able to compare using the same genetic background.

While it appeared that the CRICON antimicrobial did not impact the transfer rate of the evolved pE8, an evaluation of the efficiency of the CRICON system against the evolved pE8 was conducted. In this test, the CRICON system was delivered to strains harboring the evolved pE8 plasmids, distinguished through the previous exposure to CRISPR targeting (CCM and SM). The results revealed a difference in the overall pattern between the non-CRISPR targeted evolved pE8-CCM plasmid (CRICON control; CCM) and the CRISPR targeted evolved pE8-SM plasmid (CRICON spacer; SM) (fig. 9). It appears evident that the pE8 plasmid, when exposed to CRICON targeting, evolved to exhibit reduced susceptibility to the CRICON system. Limited experimental results have been published regarding the impact and sustainability of CRISPR antimicrobial approaches. Nonetheless, the overarching objective within the field is evident through recent perspectives, and this is to scrutinize the limitations of these systems (Bikard and Barrangou 2017, Fagen *et al.* 2017, Pursey *et al.* 2018, Mayorga-Ramos *et al.* 2023). This emphasizes the need for further research on the adaptive responses of AMR plasmids to ensure the sustained effectiveness of such innovative interventions.

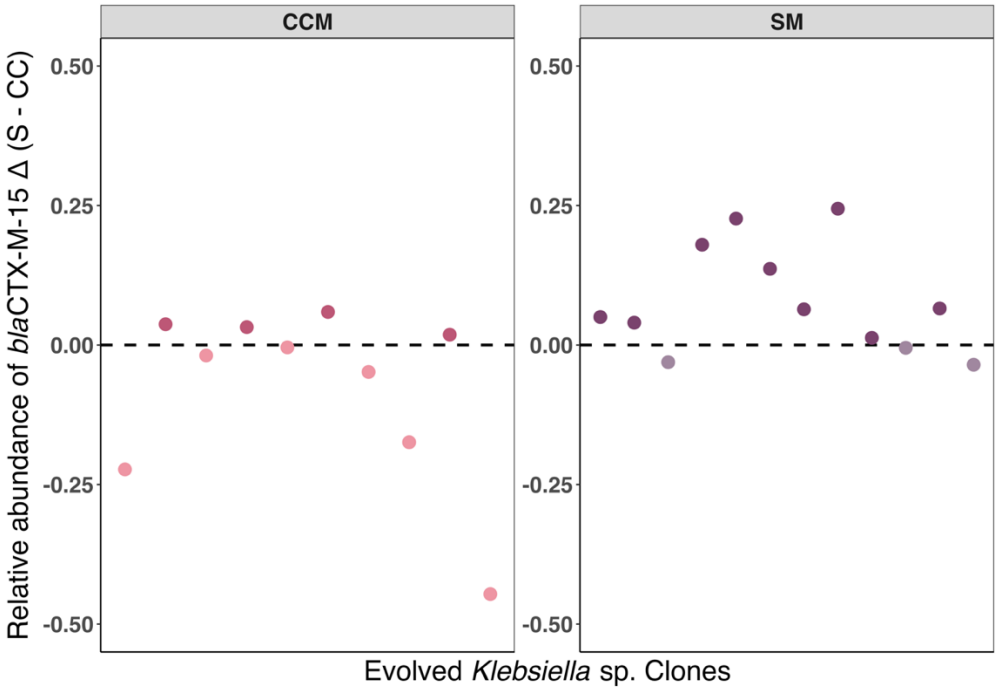

FIGURE 9 Efficiency of CRICON system against the evolved pE8 *Klebsiella* sp. transconjugant clones. ΔblaCTX-M-15 gene abundance (CRICON Spacer – CRICON Control) after delivery of the CRICON system to the *Klebsiella* sp. transconjugant clones. The plots are separated by their CRICON treatment during the microcosm experiment, CRICON control (CCM; pink – left panel) and CRICON spacer (SM; purple – right panel).

4.3.2 Host-plasmid coevolution affects the rescue potential of conjugative resistance plasmids (II)

The phenomenon of evolutionary rescue via HGT remains relatively obscure. Given the documented higher frequency of interactions among neighbors in biofilms compared to planktonic communities, it can be anticipated that an increased likelihood of plasmid transfer and evolutionary rescue happens within biofilms (Molin and Tolker-Nielsen 2003, Madsen *et al.* 2012, Stalder and Top 2016, Stalder *et al.* 2020). Study II examined the potential of the RP4 plasmid within a biofilm to rescue susceptible planktonic cells. The tested RP4 plasmid exhibited four distinct lineages, coevoled with *E. coli*, *K. pneumoniae*, and interspecifically between these species. This coevolution may contribute to a potential stabilization effect, possibly resulting in the development of a host generalist or a host specialist in terms of plasmid rescue potential (Stalder *et al.* 2017). This is evident through experimental findings suggesting that compensatory mutations following coevolutionary dynamics can reduce the costs of plasmid maintenance, favoring the persistence of resistance genes on plasmids (Harrison *et al.* 2015, 2016, Loftie-Eaton *et al.* 2017, Zwanzig *et al.* 2019, Hall *et al.* 2021, Bird *et al.* 2022). Moreover, individuals in a microbial community carrying conjugative resistance plasmids can safeguard susceptible cells in their vicinity through HGT, even after exposure to antibiotics (Mattila *et al.* 2017,

Ruotsalainen *et al.* 2020). The findings from Study II revealed a notable impact of the coevolution history, specifically with *K. pneumoniae* (RP4K and RP4EK), compared to the absence of influence from the *E. coli* plasmid lineage (RP4E). This distinction was evident in the overall diminished rescue potential of *K. pneumoniae* evolved plasmids (RP4K and EP4EK) in the *E. coli* – *E. coli* (EE) setup (fig. 10; EE). Notably, the exclusively *K. pneumoniae* evolved plasmid (RP4K) exhibited host specificity when transferring between *K. pneumoniae* strains, indicating a shift toward host specialists (fig. 10; KK). Our results are consistent with existing research, reinforcing *K. pneumoniae* as a poor interspecific plasmid donor, especially with *E. coli* as the recipient (fig. 10; KE) (Jordt *et al.* 2020). Earlier studies proposed that the absence of essential genes in the Tra2 mating pair formation system of the RP4 plasmid would ultimately impede plasmid transfer. However, the results from Study II indicate a sustained albeit reduced transfer rate even in the absence of these crucial Tra2 genes. The loss of these genes can be attributed to the coevolution with *K. pneumoniae* (RP4K and RP4EK). Importantly, this genetic loss seen in the *K. pneumoniae* RP4 plasmid lineages (RP4K and RP4EK) likely contributed to the overall lower potential for evolutionary rescue than the *E. coli* lineage (RP4E). A recent study discovered that the loss of conjugal transfer genes enhanced plasmid persistence in planktonic communities, while in biofilm communities, these genes were retained (Metzger *et al.* 2022). Deletions in the conjugal transfer region are frequent when the plasmid substantially burdens the host. This seems to be independent of specific species but depends on the host-plasmid interactions (Porse *et al.* 2016).

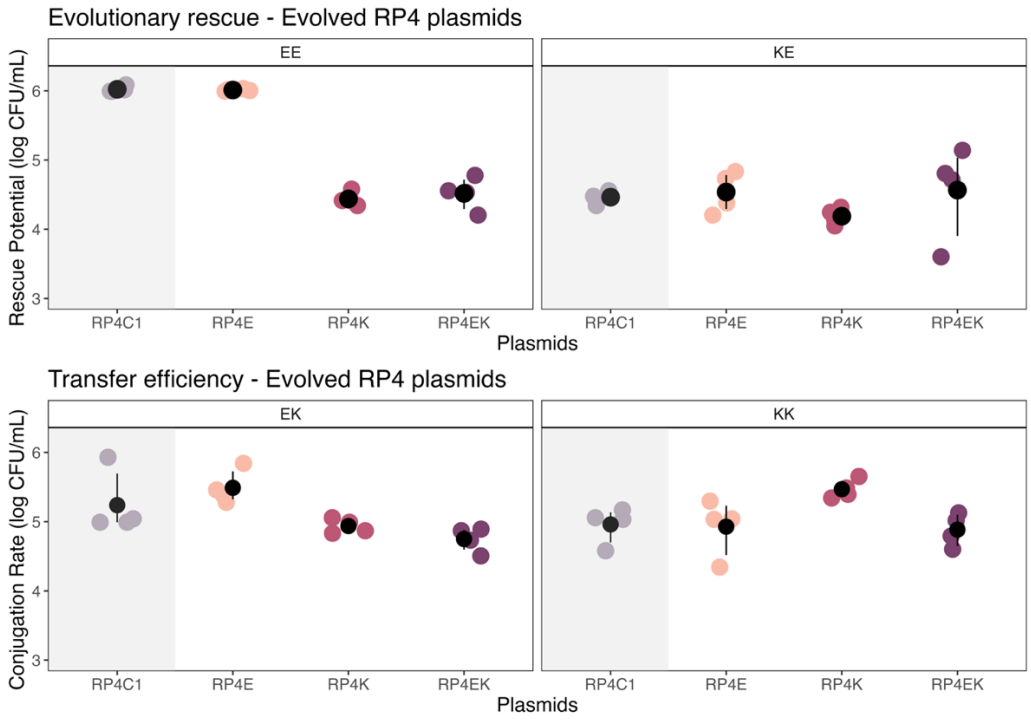

FIGURE 10 Evolutionary rescue and transfer potential of evolved RP4 plasmids (RP4E; green, RP4K; pink, RP4EK; purple) and the unevolved RP4 (RP4C1; blue) from biofilm to susceptible planktonic bacteria. In the upper row: *E. coli* to *E. coli* (EE) and *K. pneumoniae* to *E. coli* (KE) rescue setups, and lower row: *E. coli* to *K. pneumoniae* (EK), and *K. pneumoniae* to *K. pneumoniae* (KK) conjugation setups.

4.3.3 Longitudinal plasmid population dynamics following antibiotic therapy (I)

Understanding temporal patterns and evolution within human microbial communities is particularly important in the context of understanding the role of AMR plasmids. In Study I of this thesis, an in-depth examination of the plasmid population within the gut of a single patient was conducted using metagenomic analysis. This investigation spanned a temporal landscape, encompassing the entire antibiotic treatment and subsequent follow-up time points. Studies have shown that antibiotic exposure induces changes in the gut microbiota composition, which can persist for years (Gough *et al.* 2014). The disturbance caused by antibiotics can result in a decrease in microbiota diversity and an increase in antibiotic resistance. As mentioned, previous studies have reported on experimental plasmid persistence and stability throughout various selective pressures, such as antibiotics and evolution (Ridenhour *et al.* 2017, Carroll and Wong 2018, Top *et al.* 2020, Wang and You 2020, Bottery 2022). Obtaining a comprehensive "moving picture" of the plasmid population within the microbiota of an individual before, during, and after antibiotic therapy has not been done before and could provide insights into the dynamic changes in response to treatment. In Study I, a patient was treated with antibiotic therapy

(ertapenem followed by levofloxacin and metronidazole) for uncomplicated acute appendicitis. This treatment option, instead of surgery, is being considered for uncomplicated forms of appendicitis as it would offer a less invasive option and saving on reasources and costs. By following the relative abundance of the plasmid contigs, we found that the antibiotic treatment exerted a profound impact on the plasmid dynamics, particularly evident by day 7, coinciding with the treatment of the second antibiotic regimen (levofloxacin and metronidazole) (fig. 11). At this juncture, the plasmid diversity experienced a significant reduction, with only six plasmid contigs identified. Here, the plasmid population changes seen on day 7 reflected those of the hosts, with the microbial composition showing an *E. coli* dominance. Day 3 also exhibited discernible changes, albeit less drastic. However, as subsequent follow-up time points were analyzed, it became evident that the plasmid diversity had, at least partially, recovered. However, plasmid population similarity, in turn, did not recover correspondingly. Dynamic changes persisted even between the two follow-up timepoints (6 and 12 months), underscoring the resilience of the plasmid population within the gut microbial community. However, it needs to be addressed that additional antibiotic treatment was administered to the patient at month 10. Future exploration into this approach could offer a more nuanced understanding of how AMR plasmids evolve and adapt over time, enhancing our ability to develop targeted interventions and strategies for mitigating the spread of antibiotic resistance in clinical settings.

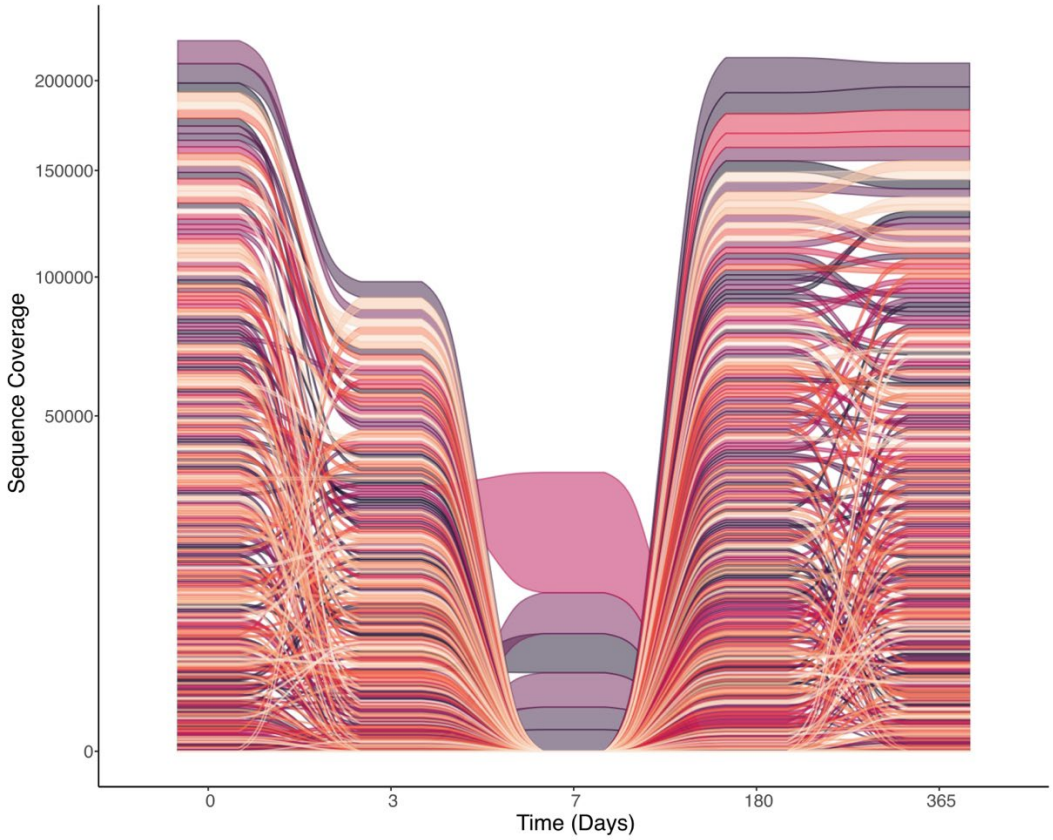

FIGURE 11 Temporal plasmid population dynamics within a single patient that underwent antibiotic treatment. Relative sequence coverage of plasmid contigs throughout the sampling timepoints, day 0; prior to treatment, day 3; third day of treatment and day of antibiotic cycling, day 7; last day of treatment, day 180; 6-month follow-up, day 365; 1-year follow-up.

In the analysis of Study I, the antimicrobial resistance profile of the patient was investigated, revealing intriguing patterns. The ESBL gene *bla*CTX-M-15 exhibited an exponential increase during the antibiotic treatment, peaking by day 7. Notably, this gene was not plasmid-encoded and was found in one of the *E. coli* strains (ST648) isolated from the patient involved in Study I. Additionally, the plasmid-encoded sulfonamide resistance gene *sul*2 mirrored the exact trend of the ESBL gene, reaching its peak at day 7. Both these genes (*sul2* and *bla*CTX-M-15) represented the predominant ARGs shaping the AMR profile during the treatment period. Interestingly, ARGs *erm*G, *mef*A, and *msr*D, encoded by the same plasmid contig, initially declined during treatment but reasserted dominance within the overall AMR landscape during the follow-up time points. A similar trend was observed with the beta-lactamase resistance gene *cfx*A6, present on both plasmid and chromosome. However, it displayed a slightly different pattern than the aforementioned plasmid-encoded ARGs. Once again, it is pertinent to note the antibiotic cycling regimen administered to the patient. The more pronounced effect observed on day 7 stemmed from administering levofloxacin, a fluoroquinolone, and metronidazole treatments. However, the impact of the initial antibiotic ertapenem, a beta-lactam, appears to have been

less pronounced. Different classes of antibiotics can lead to varying alterations in the composition and function of the gut (Knecht *et al.* 2014). Beta-lactams and fluoroquinolones have been reported to disrupt the gut microbiota, reducing microbial diversity (Gu *et al.* 2020). Further, each can potentially contribute to the development of resistance, with beta-lactam usage being found to increase the prevalence of beta-lactam resistance genes, and fluoroquinolone treatments increased the rate of gut carriage of fluoroquinolone-resistance (de Lastours and Fantin 2015, Loo *et al.* 2020, Tchesnokova *et al.* 2022). Studies have shown that the mode of action of antibiotics plays a crucial role in modulating the gut microbiota composition and increasing the gut resistome (MacPherson *et al.* 2018). However, the impact of antibiotics on the gut microbiota depends on the class of antibiotics and factors such as dosage and duration of treatment (Holman and Chénier 2014). This emphasizes the complex interplay of antibiotic usage and the emergence and dissemination of resistance elements within microbial communities.

5 CONCLUSIONS

Throughout this work, I embarked on an eclectic exploration of antimicrobial resistance and the role of conjugative plasmids in its propagation. This journey began with examining the reality of AMR and evaluating its implications within a single patient. Subsequently, I explored the interplay of ecological and environmental factors shaping the two primary modes of targeted solutions against AMR, phage therapy and CRISPR antimicrobials. Finally, with the primary objective of this thesis, I ventured into the evolutionary trajectory of AMR plasmids when faced with environmental changes and selective pressures.

The findings shed light on various facets of antibiotic therapy, bacterial and plasmid evolution, and resistance mechanisms, revealing their interconnected dynamics. Integrating gut metagenomic analysis and bacterial isolates elucidated the evolutionary pathways of plasmid populations and antibiotic resistance profiles in treated patients. The transient alteration observed in microbial composition, AMR profile, and plasmid population during treatment underscores the temporary impact of antibiotic therapy on the gut microbiome (Study I). Furthermore, the investigation into the potential of conjugative resistance plasmids in rescuing antibiotic-susceptible bacteria has revealed the influence of evolutionary history on host-specific plasmid lineages and their role in bacterial survival during therapy (Study II). Additionally, examining mucin and antibiotic effects on phage resistance has demonstrated the complex interplay between environmental factors and resistance mechanisms (Study III). Lastly, assessing CRISPR-antimicrobial efficacy in targeting ESBL genes within a multispecies community uncovered unexpected survival strategies of ESBL-plasmids and the development of evading the CRISPR-based antimicrobials.

The findings in this thesis underscore the complex and dynamic nature of the genetic landscape within *Enterobacteriaceae*, exhibiting both broad inter and intraspecific variability while also sharing intersecting components. This emphasizes the necessity for adjoining research within this family. Furthermore, individual *Enterobacteriaceae* members may contribute more significantly to antimicrobial resistance. Still, the presence of AMR-containing conjugative plasmids mandates the evaluation of the entire community as collectively AMR-

containing. There is an imperative need for ecological research when assessing the efficacy and sustainability of targeted solutions like CRISPR antimicrobials and phage therapy. While these approaches may seem straightforward in theory, their success hinges on an articulate understanding of ecological and evolutionary influences. Lastly, exploring plasmids supports the general theory of them being highly adaptable and dynamic entities, responding extensively to environmental changes. This context provides a foundation for comprehending the evolution and persistence of AMR plasmids, which is vital for informing strategies in the ongoing battle.

RESUME (SUMMARY FOR A GENERAL AUDIENCE)

Bacteria, tiny, invisible (to us humans) organisms, are unicellular microorganisms that often live in communities and come in diverse shapes and cell structures. Notably, bacteria are prokaryotes, meaning they are a different kind of cell than those that makeup humans and other eukaryotic (multicellular) beings. Bacteria are everywhere around us, even inside our own bodies. The bacteria residing in our bodies, known as the human microbiota, play essential roles in maintaining our health. While many bacteria in our bodies are beneficial, there are also harmful bacteria that can cause infections and diseases. The difference between good and bad bacteria lies in their interactions with our bodies. Good bacteria, often called probiotics, typically coexist with us peacefully, providing various benefits such as aiding digestion and supporting our immune system. In contrast, harmful bacteria, known as pathogens, can disrupt this balance and cause illness by producing toxins, invading tissues, or triggering immune responses. Antibiotics are medications used to treat bacterial infections. They work by targeting specific components of bacterial cells, such as their cell walls, proteins, or DNA, to either kill the bacteria or inhibit their growth. By disrupting essential bacterial processes, antibiotics help our immune system fight off the infection more effectively. However, it's important to note that antibiotics are only effective against bacterial infections and are not effective against viral infections like the common cold or flu. Overuse or misuse of antibiotics can lead to antibiotic resistance, where bacteria develop the ability to survive and grow despite the presence of antibiotics, making infections more complicated to treat. As bacteria evolve and become increasingly resistant to antibiotics, a phenomenon known as antimicrobial resistance (AMR) has become a growing threat to public health. Both antibiotic resistance and AMR make infections harder to treat, leading to longer illness, increased mortality rates, and higher healthcare costs. Therefore, finding innovative solutions to tackle both phenomena is becoming more urgent than ever.

Like other biological entities, bacteria carry their own genetic material, DNA, both in their chromosome and in smaller molecules known as plasmids. These plasmids can contain genes that provide bacteria with various abilities, including antibiotic resistance. Among these plasmids are conjugative plasmids, which have a remarkable ability to transfer themselves from one bacterial cell to another, spreading traits such as antibiotic resistance throughout bacterial populations. An important aspect of conjugative plasmids is their host range. Host range refers to the range of bacterial species or strains to which a particular conjugative plasmid can transfer genetic material. A broad host range means that the plasmid can transfer itself and its genes to many different bacterial species, while a narrow host range indicates that it can only interact with a limited number of bacterial species. Another facet of bacterial life is bacteriophages, or simply phages, which are viruses that infect only bacteria, hence their name. These viruses work by injecting their genetic material into the bacterial cell, hijacking them to produce more of themselves until the cells are completely full

and burst open. This allows for releasing new phages with a single mission: to find and infect more bacteria. Interestingly, researchers are exploring the potential of phages as an alternative to antibiotics in combating bacterial infections, especially those resistant to antibiotics. Phages, with their ability to target specific bacteria and their potential to be customized to combat antibiotic-resistant strains, offer hope in the ongoing battle against drug-resistant infections. Similar to plasmids, phages have a host range that can be broad or narrow, depending on the amount of different bacteria they can infect.

In this thesis, divided into four studies, we explore various aspects of antimicrobial resistance. In the first study, we delve into the effects of antibiotic treatment on the gut microbiome of a single patient that received antibiotics for appendicitis instead of the typical surgical appendectomy. Rectal swabs and stool samples were collected before and throughout the 7-day treatment, and at the 6 month and one year follow-up. By analyzing the microbiome and isolated bacterial strains, we observed significant changes following the administration of antibiotics. The antibiotics reduced the diversity of the bacteria and plasmid population and altered their resistance pattern. Fortunately partial recovery of these aspects was observed during the extended follow up period, although it did not return to pre-treatment levels. In the second study, we conducted experiments to test whether bacteria carrying conjugative resistance plasmids could rescue bacteria that harbour no antibiotic resistance. The bacterial population was exposed to lethal antibiotics and was close to extinction. In the next step bacteria carrying resistance plasmids, which could transfer resistance to and rescue previously sensitive bacterial strain were introduced to the bacterial medium. For further exploration of this concept, we evolved the plasmids with different bacteria (of different species) before the rescue in an attempt to see if the previous exposure affected their rescue potential. Our findings revealed that the coevolution of bacteria and plasmids is highly dynamic and can vary significantly between bacterial species. In other words, some bacteria led to changes in the plasmids by being exposed to each other for prolonged periods of time, while others did not. In the third study, we explored resistance to phages instead of focusing on resistance to antibiotics. To recap, phages are viruses that can potentially be used as alternatives to antibiotics. In this experiment, we wanted to see if certain factors, like antibiotics and mucin (component in the gut lining), might affect the development of resistance to phages. Certain phages attach to mucin, and the hypothesis was that the mucin might influence phage resistance. However, the results suggested that neither mucin nor antibiotics seemed to affect the development of phage resistance. Instead, resistance to phages was highly specific to particular combinations of bacteria and phages. In the final study, we tested the effectiveness of a CRISPR antimicrobial tool to remove genes that cause antimicrobial resistance. CRISPR is a fascinating defense mechanism bacteria use to protect themselves against phage infections. Scientists have manipulated this mechanism to edit genes and genomes. Essentially, CRISPR acts like a precise pair of scissors that memorize DNA sequences and can target and cut the specific DNA sequences they memorize. In our study, we gave the CRISPR system a code that matched a

resistance gene to see if it could successfully remove the gene. Interestingly, we discovered that bacteria often got rid of the host, carrying the AMR gene, independently without the help of CRISPR. However, in one instance, CRISPR successfully removed the AMR gene, which was located on a plasmid. Yet, as bacteria are incredibly adaptive, we did observe that the gene was finding ways to evade the CRISPR mechanism in cases where CRISPR succeeded.

The research in this thesis shows that bacteria have a diverse and ever-changing genetic makeup, which helps them learn to thrive in any situation. Understanding that bacteria are diverse and come with a diverse arsenal to help them survive is crucial in studying how bacteria develop antibiotic resistance. Even though some types of bacteria may contribute more to antibiotic resistance than others, it is essential to consider the whole bacterial community, as antibiotic resistance can spread between different types of bacteria via conjugative plasmids. To effectively combat antibiotic resistance, we must study how these bacteria interact with each other and their environment. This includes exploring new treatments like CRISPR antimicrobials and phage therapy, but ultimately, the success of this depends on understanding how bacteria evolve and adapt. All of this surmounts to knowledge essential for developing strategies to fight antimicrobial resistance and protect public health.

RESUME (ICELANDIC)

Bakteríur eru agnarsmáar lífverur, ósýnilegar mannlegu auga. Þær eru örverur af gerð einfrumunga, búa gjarnan í samfélögum og geta verið margskonar að lögun og byggingu. Geta má þess að bakteríur eru dreifkjörnungar og ólíkar þeim frumum sem byggja upp mannverur og fleiri heilkjörnunga. Bakteríur eru alls staðar í umhverfinu og meira að segja inni í mannslíkamanum. Þær bakteríur sem þar búa hafa mikilvægu hlutverki að gegna í því sambandi að viðhalda heilbrigði manna og kallast örverumengi mannsins. Þótt margar bakteríur í líkamanum láti gott af sér leiða þá er þar líka að finna bakteríur sem geta valdið ígerðum og sjúkdómum. Munurinn á góðum og slæmum bakteríum liggur í því hvernig samspil þeirra við líkamann er. Það er dæmigert fyrir góða bakteríu, sem oft er kölluð bætibaktería, að hún lifir með manninum í sátt og samlyndi og leggur sitt af mörkum til að auðvelda meltingu og styrkja ónæmiskerfi hans. Á hinn bóginn getur skaðleg baktería, svokallaður sýkill, riðlað slíku jafnvægi og valdið veikindum með því að framleiða eiturefni, ráðast inn í vefi eða koma af stað viðbrögðum frá ónæmiskerfinu. Sýklalyf eru notuð til að vinna á bakteríusýkingum. Það gera þau með því að ráðast á tiltekna þætti bakteríu-frumanna, svo sem frumuveggi, prótín þeirra eða DNA til að drepa bakteríurnar eða stöðva vöxt þeirra. Með því að rjúfa mikilvæga þróunarferla baktería leggja sýklalyf ónæmiskerfi mannsins lið við að vinna á sýkingum á skilvirkari hátt. En það er mikilvægt að gefa því gaum að sýklalyf gagnast einungis gegn bakteríusýkingum en eru haldlaus gegn veirusýkingum eins og kvefi og flensu. Ofnotkun eða misnotkun á sýklalyfjum getur leitt til ónæmis fyrir þeim, en það gerist þegar bakteríur þróa með sér þann eiginleika að geta lifað af og vaxið þrátt fyrir návist lyfjanna, og þar með verður flóknara að meðhöndla sýkingar. Eftir því sem bakteríur hafa þróast og myndað sífellt aukið þol gegn sýklalyfjum hefur ónæmi fyrir örverueyðandi lyfjum, oft kallað AMR (antimicrobial resistance), orðið síaukin ógn við lýðheilsu. Bæði sýklalyfjaónæmi og AMR valda því að erfiðara er að lækna sýkingar, veikindi vara lengur, dánartíðni eykst sem og kostnaður heilbrigðisþjónustunnar. Það er því brýnna en nokkru sinni fyrr að finna skapandi lausnir til að ráðast gegn báðum þessum fyrirbrigðum.

Líkt og gildir um aðrar líffræðilegar einingar þá er erðaefni baktería, DNA, að finna í litningum þeirra og enn minni mólekúlum sem kallast plasmíð. Þessi plasmíð geta innihaldið gen sem sjá bakteríum fyrir ýmsum eiginleikum, þeirra á meðal ónæmi fyrir sýklalyfjum. Sum þessara plasmíða eru svokölluð samtengjandi plasmíð sem búa yfir þeim undraverða hæfileika að geta flutt sig sjálf frá einni bakteríufrumu til annarrar og á þann hátt dreift eiginleikum á borð við sýklalyfjaónæmi um gjörvallt bakteríuþýði. Mikilvægt atriði varðandi samtengjandi plasmíð er hýsilsvið þeirra. Með hýsilsviði samtengjandi plasmíðs er átt við þá flokka eða ættleggi af bakteríum sem plasmíðið getur flutt erfðaefni til. Breitt hýsilsvið þýðir að plasmíðið getur flutt sig og sín gen í margar ólíkar bakteríutegundir, þröngt hýsilsvið þýðir hinsvegar að þær eru fáar. Annar flötur á lífi baktería snýr að svokölluðum bakteríuveirum sem eru veirur sem sýkja einungis bakteríur og draga nafn sitt af því. Þessar veirur vinna þannig að þær

dæla erfðaefni sínu inn í bakteríufrumu og valda niðurbroti frumunnar með því að þvinga hana til að framleiða sífellt meira af efninu uns fruman springur. Þá hefur losnað um nýjar bakteríuveirur sem stefna að því einu að finna og sýkja fleiri bakteríur. Það áhugaverða við þetta er að vísindamenn vinna nú að rannsóknum á því hve miklir möguleikar felist í að nota bakteríuveirur sem valkost við sýklalyf í baráttunni við bakteríusýkingar, einkum þegar um ónæmi bakteríunnar fyrir lyfjunum er að ræða. Bakteríuveirur, með þeim hæfileika sínum að ráðast gegn tilteknum bakteríum og þeim eiginleika að þær megi sérhæfa til að herja á sýklalyfjaónæma bakteríustofna, vekja von í hinni linnulausu baráttu við sýkingar sem eru ónæmar fyrir lyfjum. Líkt og gildir um plasmíð þá er hýsilsvið bakteríuveira ýmist breitt eða þröngt og fer það eftir því hve margar ólíkar bakteríutegundir þær geta smitað.

Í þessari ritgerð sem samanstendur af fjórum rannsóknarverkefnum könnum við ýmsa fleti á þoli gegn örverueyðandi lyfjum. Í fyrstu rannsókninni eru brotin til mergjar áhrifin af sýklalyfjameðferð á örverulífbelti í görn sjúklings sem fékk sýklalyf við botnlangabólgu í stað hefðbundins botnlangaskurðar. Strok frá endaþarmi og hægðasýni voru tekin meðan á sjö daga sýklalyfjameðferð stóð og við 6 mánaða og eins árs eftirfylgni. Með því að rannsaka bæði örverulífbelti sjúklingsins og einstaka bakteríustofna mátti sjá greinilegar breytingar sem sýklalyfin höfðu í för með sér. Þau drógu úr fjölbreytileika bæði bakteríu- og plasmíðstofnanna og breyttu þoli þeirra. Sem betur fer mátti sjá, við eftirfylgnina, að ástand þessara þátta var að hluta til komið í fyrra horf, þótt ekki næðist sama ástand og var fyrir meðferð. Í annarri rannsókninni voru gerðar prófanir á því hvort bakteríur með samtengjandi lyfjaónæm plasmíð geti bjargað bakteríum sem búa yfir engu sýklalyfjaþoli. Beitt var sýklalyfi gegn óþolnum bakteríum uns þær voru deyjandi. Þá var bakteríum með lyfjaónæm samtengjandi plasmíð bætt út í ætið þeim til bjargar. Til að kanna hugmyndina frekar þá voru plasmíðin þróuð með öðrum bakteríum (af ólíkum tegundum) áður en til björgunarinnar kom til að sjá hvort slíkt hefði áhrif á björgunarmátt plasmíðanna. Niðurstaðan var sú að samþróun baktería og plasmíða sé mjög lífleg en miklu geti munað milli baktería af ólíkum tegundum. Sumar bakteríurnar breyttu plasmíðunum ef návígi þeirra varði lengi, en aðrar gerðu það ekki. Í þriðju rannsókninni var kannað þol gegn bakteríuveirum í stað þess að beina sjónum að þoli gegn sýklalyfjum. Eins og komið hefur fram þá eru bakteríuveirur veirur sem hugsanlega má nota í stað sýklalyfja. Í þessari tilraun lék okkur grunur á að vita hvort tilteknir þættir eins og sýklalyf eða músín (prótín innan á garnavegg) kynnu að hafa áhrif á þróun þols gegn bakteríuveirum. Það er þekkt að sumar bakteríuveirur loða við músín svo ætla mætti að efnið hefði áhrif á ónæmi fyrir bakteríuveirum. Hinsvegar sýndu niðurstöðurnar að hvorki músín né sýklalyf virtust hafa áhrif á þróun slíks ónæmis. Í síðustu rannsókninni könnuðum við hve skilvirk CRISPR-tækni er til að fjarlægja gen sem valda ónæmi fyrir örverueyðandi lyfjum. CRISPR táknar afar áhugaverða varnarhætti sem bakteríur nota til að verjast sýkingum af völdum bakteríuveiru. Vísindamenn hafa hagrætt þessu gangvirki í því skyni að meðhöndla gen og genamengi. Í raun þá vinnur CRISPR eins og um nákvæm skæri væri að ræða sem muna DNA-raðir, geta fundið þær og klippt burt. Í rannsókninni létum við CRISPR-tólið fá kóða

sem passaði við ónæmt gen til að sjá hvort það réði við að fjarlægja genið. Það var áhugavert að komast að því að bakteríuþýðið gat oft losað sig við hýsilinn sem bar AMR genið upp á eigin spýtur og án aðstoðar CRISPR. Að vísu fjarlægði CRISPR genið farsællega í einu tifelli. Bakteríur hafa ótrúlega aðlögunarhæfni og við tókum eftir að genið fann leiðir til að komast undan CRISPR-tólinu í þeim tilfellum þar sem CRISPR skilaði árangri.

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ORIGINAL PAPERS

I

LONGITUDINAL EVOLUTIONARY DYNAMICS OF PLASMIDOME AND ANTIBIOTIC RESISTANCE WITHIN A GUT MICROBIOME SUBSEQUENT ANTIBIOTIC THERAPY: A CASE STUDY

by

Ilmur Jonsdottir, Sean Meaden, Paulina Salminen, Teemu Kallonen, Janne Ravantti, Annaleena Pajander, Sanja Vanhatalo, Matti Jalasvuori, Lotta-Riina Sundberg, Edze Westra, Stineke van Houte, Antti J. Hakanen & Reetta Penttinen 2024

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II

PRECEDING HOST HISTORY OF CONJUGATIVE RESISTANCE PLASMIDS AFFECTS INTRA- AND INTERSPECIFIC TRANSFER POTENTIAL FROM BIOFILM

by

Ilmur Jonsdottir, Cindy J. Given, Reetta Penttinen & Matti Jalasvuori 2023

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RESEARCH ARTICLE

Preceding Host History of Conjugative Resistance Plasmids Affects Intra- and Interspecific Transfer Potential from Biofilm

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ABSTRACT Conjugative plasmids can confer antimicrobial resistance (AMR) to their host bacterium. The plasmids disperse even between distantly related host species, rescuing the host from otherwise detrimental effects of antibiotics. Little is known about the role of these plasmids in the spread of AMR during antibiotic treatment. One unstudied question is whether the past evolutionary history of a plasmid in a particular species creates host specificity in its rescue potential or if interspecific coevolution can improve interspecific rescues. To study this, we coevolved the plasmid RP4 under three different host settings; solely *Escherichia coli* or *Klebsiella pneumoniae*, or alternating between both of them. The ability of evolved plasmids in bacterial biofilm to rescue susceptible planktonic host bacteria of either the same or different species during beta-lactam treatment was tested. The interspecific coevolution seemed to decrease rescue potential for the RP4 plasmid, while the *K. pneumoniae* evolved plasmid became more host specific. Large deletion in the region encoding the mating pair formation (Tra2) apparatus was detected in the plasmids evolved with *K. pneumoniae*. This adaptation resulted in the exapted evolution of resistance against a plasmid-dependent bacteriophage PRD1. Further, previous studies have suggested that mutations in this region completely abolish the plasmid's ability to conjugate; however, our study shows it is not essential for conjugation but rather affects the host-specific conjugation efficiency. Overall, the results suggest that previous evolutionary history can result in the separation of host-specific plasmid lineages that may be further amplified by unselected exaptations such as phage resistance.

IMPORTANCE Antimicrobial resistance (AMR) is a major global public health threat which can rapidly spread in microbial communities via conjugative plasmids. Here, we advance with evolutionary rescue via conjugation in a more natural setting, namely, biofilm, and incorporate a broad-host range plasmid RP4 to test whether intra- and interspecific host histories affect its transfer potential. *Escherichia coli* and *Klebsiella pneumoniae* hosts were seen to elicit different evolutionary influences on the RP4 plasmid, leading to clear differences in the rescue potential and underlining the significant role of the plasmid-host interactions in the spread of AMR. We also contradicted previous reports that established certain conjugal transfer genes of RP4 as essential. This work enhances the understanding of how plasmid host range evolve in different host settings and further, the potential effects it may have on the horizontal spread of AMR in complex environments such as biofilms.

KEYWORDS antibiotic resistance, biofilms, evolutionary rescue, experimental evolution, horizontal gene transfer (HGT), host-plasmid interactions, plasmids

Plasmids are self-replicating extrachromosomal genetic elements of bacteria. Conjugative plasmids are able to encode a bridge between their current host and suitable neighboring bacteria, allowing for horizontal gene transfer (HGT) via conjugation (1, 2). These plasmids are part of the antimicrobial resistance (AMR) global health

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problem as they can harbor and confer AMR genes (3–11). Plasmids and bacteria are intrinsically linked through their shared environment (12–15). Through conjugation certain plasmids can spread intra- and interspecifically depending on their host-range (16, 17). However, plasmids carry a fitness-cost that is often associated with their maintenance in the host cell. Compensatory mutations within the host chromosome and the plasmid can alleviate the plasmid fitness cost to help promote their persistence in the community (18–27). Nevertheless, the long-term survival of plasmids in communities remains puzzling due to their costs to the host (sometimes referred as "the plasmid-paradox") (28).

Biofilms are one microbial formation where plasmids are maintained (29, 30). The individuals of these connected ecosystems interact more frequently with their neighbors, allowing for better mating pair formation and ultimately higher chance of plasmid transfer (31–34). Recently, biofilms were shown to improve the persistence of AMR plasmids (25, 35). Worryingly, plasmid-carrying resistant bacteria can save susceptible cells in their vicinity via HGT even after exposure to antibiotics (36, 37). However, this so-called evolutionary rescue via HGT has not been studied for biofilm associated bacteria despite the ubiquity of biofilms in nature.

The overall survival and success of all plasmids are influenced by "built-in" evolutionary trade-offs in a multihost environment (38, 39). Namely, natural selection within a single host strain allows plasmids to coevolve with their hosts and compensate any detrimental plasmid fitness effects (23, 26, 40). However, a long-term adaptation to a specific host can increase the fitness cost of the plasmid in less similar hosts as these changes are only continuously checked against that particular within-host environment (41). The adaptive changes in one host may cause conflicts (on a molecular level) in others, similar to speciation in sexually reproducing organisms (38, 42).

Alternatively, plasmids that regularly change host species are likely to maintain lower fitness effects in all their regularly "evaluated" hosts, as well as more likely to be devoid of specific adaptations that help in one host but cause conflicts in others (38, 43). Therefore, initially homogenous plasmid population could diverge to "host-generalists" and "host-specialists." To what extent this occurs, is still unclear. Without strong selection for any particular host species, the existence of such plasmid groups in a community may be negligible. However, in specific situations the preceding host history may become relevant. For example, sudden change in environmental conditions (such as administration of antibiotics) can favor different subpopulations of plasmids that may have adapted to their current host species, to multiple species, or to a specific alternative species. Further, the plasmid donor species may play a vital role as conjugation intra- and interspecifically may affect the transfer rate of the plasmid to sensitive hosts.

We aimed to better understand the potential of plasmids on rescuing susceptible bacteria from the effects of lethal antibiotics. To determine the factors that affect the rescue potential, we utilized plasmids with different characteristics and different evolutionary histories (Fig. 1). We hypothesized that a plasmid that was coevolved intraspecifically with its host (*E. coli* or *K. pneumoniae*) would exhibit host specificity in its rescue and a plasmid with a history of interspecific coevolution (between *E. coli* and *K. pneumoniae*) would broaden its rescue prospects. We observed a clear difference in the plasmid adaptation with a stronger evolutionary response linked to *K. pneumoniae*, which resulted in a major deletion of RP4's mating pair formation gene core (Tra2). Without the transfer genes the rescue potential of the plasmid decreased but did not dissipate entirely and conferred plasmid-dependent phage (PRD1) resistance.

RESULTS

Rescue potential differs between plasmids of different characteristics. We sought out to investigate if different genotypic characteristics of plasmids effected their rescue potential by testing the rescue potential of six plasmids harboring separate features in the same rescue setup (*E. coli* to *E. coli*; EE). The density of rescued cells varied across 4 orders of magnitude (Fig. 2). Plasmid pEC15 was unable to rescue any sensitive hosts

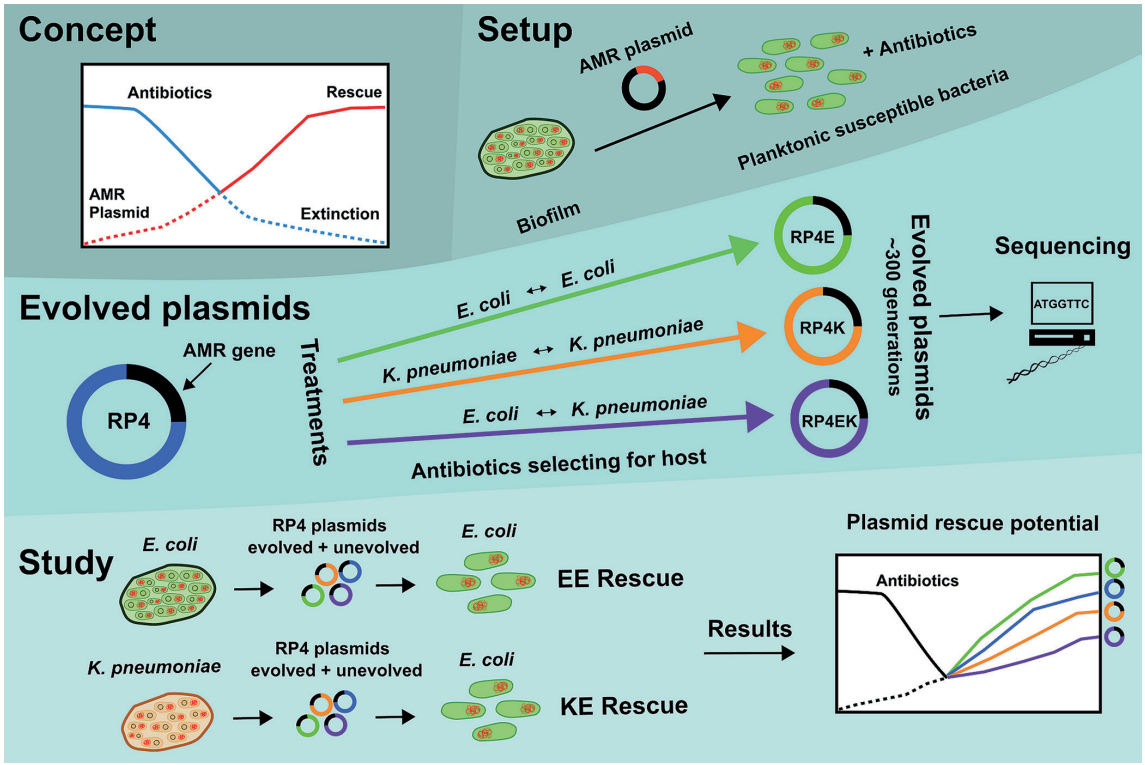

FIG 1 Schematic design of this study. The key concepts in this experimental design were host history and evolutionary rescue via HGT. The rescue occurred from biofilm, rescuing planktonic antibiotic-susceptible bacteria after 1-h antibiotic exposure. The plasmids used in the rescue were RP4 and variants of RP4 after 300 generations of coevolution with *E. coli* (RP4E), *K. pneumoniae* (RP4K) or alternating between them both (RP4EK). Two rescue setups were performed; intraspecific: *E. coli* rescuing *E. coli* (EE) and interspecific: *K. pneumoniae* rescuing *E. coli* (KE), with the results measuring the rescue potential of each plasmid.

and was therefore omitted from the subsequent analysis. Each plasmid in the evolutionary rescue experiment showed statistically significant differences in their rescue potential (ANOVA; Tukey-HSD, $P < 0.001$). The plasmid pEC14 carried the lowest rescue potential aside from pEC15, with two of the replicates for pEC14 omitted as they did not rescue any planktonic cells. RP4 plasmid showed the highest rescue potential.

Past evolutionary history with *K. pneumoniae* significantly affects the rescue potential of RP4 plasmid. In the *E. coli* to *E. coli* (EE) evolutionary rescue setup, the *E. coli* plasmid (RP4E) had the same rescue potential as the unevolved plasmid (RP4C1) (Fig. 3A). However, the rescue potential significantly decreased with the *K. pneumoniae* evolved plasmid (RP4K) and the interspecific evolved plasmid (RP4EK), with both evolved plasmids having similar mean rescue potentials. This suggests that evolutionary history involving *K. pneumoniae* decreased RP4 rescue potential between biofilm-associated and planktonic *E. coli* (Kruskal-Wallis; Dunn, $P < 0.01$). Further, *K. pneumoniae* as a plasmid donor had significantly less potential in rescuing planktonic *E. coli* regardless of the past host-history. This is seen clearly in the *K. pneumoniae* to *E. coli* (KE) evolutionary rescue setup, in which all plasmids (RP4C1, RP4E, RP4K, RP4EK) give similar mean rescue potential with no statistical difference (Kruskal-Wallis; Dunn, $P = 0.174$) (Fig. 3B).

Intraspecific coevolution with *K. pneumoniae* caused significant host specificity of RP4 plasmid. We studied the effects of past host-history on the conjugation rates from biofilm to planktonic *K. pneumoniae* in a similar setup as above except the antibiotic concentration was not lethal for the recipient. The *E. coli* evolved plasmid (RP4E)

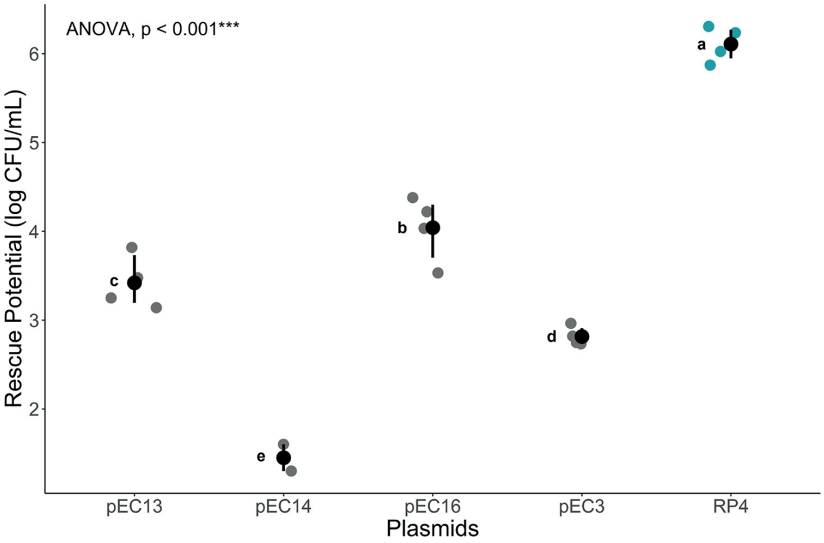

FIG 2 Evolutionary rescue potential of pEC(3,13,14,and 16) (gray) and RP4 plasmid (blue) in *E. coli* from biofilm to susceptible planktonic bacteria. Rescue potential was measured as the conjugation rate (CFU/mL) of each plasmid (N = 4). Two replicates for pEC14 did not produce any transconjugants and were omitted from this figure. The mean and bootstrap confidence interval of each plasmid are represented by point ranges. A one-way ANOVA with Tukey-HSD post hoc was performed between all plasmid-carrying strains. The P-value is shown, and the Tukey's HSD letters (a-e) next to each point range indicate whether there is a statistical difference.

had a higher mean conjugation rate from *E. coli* to *K. pneumoniae* (EK) in comparison to plasmids that evolved entirely or partly with *K. pneumoniae* (RP4K, RP4EK) (Kruskal-Wallis; Dunn, $P = 0.015$) (Fig. 4A). In the interspecific conjugation setup from *E. coli* to *K. pneumoniae* (EK), the interspecifically evolved plasmid (RP4EK) had the lowest conjugation rate. In the intraspecific K. pneumoniae to *K. pneumoniae* (KK) conjugation setup, the K. pneumoniae evolved plasmid (RP4K) had the highest conjugation rate (Fig. 4B). This indicates that evolution solely in *K. pneumoniae* improved the within-species horizontal transfer of the plasmid. Interestingly, however, the lowest conjugation rate was seen with the interspecifically evolved plasmid (RP4EK) (Kruskal-Wallis; Dunn, $P = 0.033$) (Fig. 4B). In the KK setup, the RP4C1 and RP4E plasmids had decreased conjugation rate compared to the EK setup, supporting host specificity of the RP4K plasmid. In both of these setups (KK and EK), the *K. pneumoniae* evolved plasmid (RP4K) confers a higher mean conjugation rate compared to the interspecifically evolved plasmid (RP4EK). However, this was not seen in the evolutionary rescue setups, where RP4K and RP4EK plasmids showed similar results.

Significant evolution in *K. pneumoniae* coevolved plasmids generates phenotypic effects. We sequenced the evolved RP4 plasmids to determine the genetic changes that may confer the phenotypic properties that were observed in plasmids with different host history. We found a major 2,232 bp deletion (with zero coverage in plasmid location 18,827-21,058) located in the Tra2 conjugal transfer region in RP4K and RP4EK plasmids, that were coevolved within setups involving *K. pneumoniae* (Fig. 5). This deletion affected four genes of the Tra2 complex, *trbB* (960 bp), *trbC* (438 bp), *trbD* (312 bp), and *trbE* (2,559 bp). Almost complete deletion (3-960 bp) was seen for gene *trbB*, complete deletions of *trbC* and *trbD*, and *trbE*, the largest gene of the complex, had a partial deletion of the first 514 bp. Through previous studies, the Tra2 region of RP4 is well established in its role of PRD1 phage propagation (44–49). To test whether the deletion in this region in the RP4K and RP4EK plasmids affects the infectivity of PRD1, we performed spot tests. Bacteria carrying the evolved RP4 plasmids (RP4E, RP4K, and RP4EK) or the unevolved RP4 (RP4C1) as a control were all tested

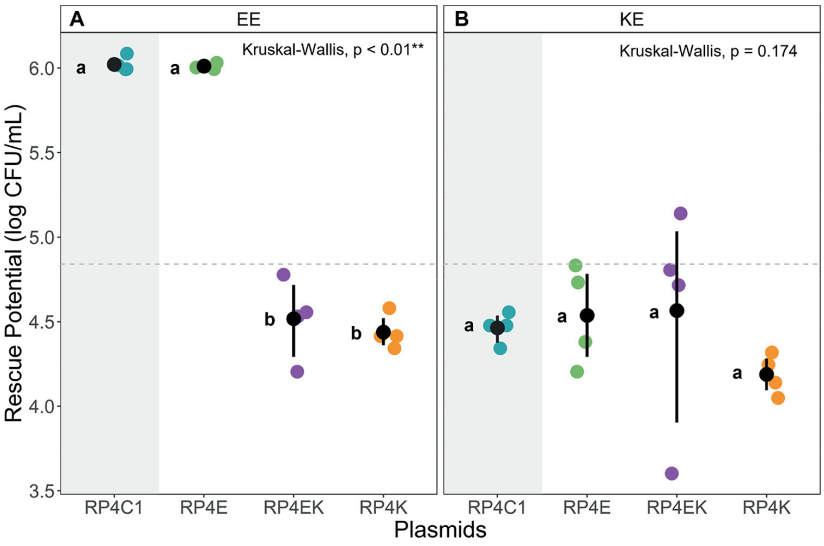

FIG 3 Evolutionary rescue potential of evolved RP4 plasmids (RP4E; green, RP4EK; purple, RP4K; orange) and the unevolved RP4 (RP4C1; blue) from biofilm to susceptible planktonic bacteria in: (A) *E. coli* to *E. coli* (EE), and (B) *K. pneumoniae* to *E. coli* (KE) rescue setups. Rescue potential was measured as the conjugation rate (CFU/mL) of each plasmid (N = 4) in the rescue setup. The mean and bootstrap confidence interval of each plasmid can be seen represented by point ranges. A Kruskal-Wallis with Dunn test for post hoc was performed between all plasmid-carrying strains. The P-value is shown, and the letters (a-e) next to each point range indicate whether there was a statistical difference between the plasmids found in the Dunn test. The mean of the entire data can be seen represented in the dashed line.

for the susceptibility to PRD1 phage. We observed that while PRD1 was highly infective against bacteria carrying RP4C1 and RP4E plasmids, the bacteria harboring RP4K and RP4EK, that had the partial deletion of the Tra2 transfer region, were found to be immune to this plasmid-dependent phage (Table S2).

DISCUSSION

The usage of antibiotics along with its resistance is on the rise (50). This is because through consumption of antibiotics, resistance is selected for if it is present in the community. Through numerous studies on HGT, in particular via conjugative plasmids, and how it plays in the spread of AMR, its role is well established (3, 51–55). In our study, we investigated a phenomenon known as evolutionary rescue via HGT, which in this case would rely on AMR plasmids being present in the community and spreading during antibiotic treatment to save susceptible bacteria from extinction. This process is associated with certain antibiotics such as beta-lactams and resistance genes that encode degrading enzymes like beta-lactamases or even extended-spectrum beta-lactamases (ESBLs). This is due to the mechanism of the antibiotics which continues to allow for conjugation while the cell is still viable, as well as the resistance mechanism in the case of resistant individuals being present in the community as they lower the antibiotic concentration in their proximity through degradation (36, 37). The evolutionary rescue and conjugation setups performed in this study involved coevolved plasmids and biofilms, which are common bacterial habitats found in the human body. However, we acknowledge that the *in vitro* conditions of this work (LB media, laboratory strains and plasmids) possess limitations on the clinical relevancy of our findings.

In the initial biofilm evolutionary rescue experiment, we investigated the effects of different plasmid characteristics on the rescue potential of the plasmids. There was a significant distinction in the higher rescue potential of the RP4 plasmid compared to the other pEC plasmids. Certain pEC plasmids, pEC15 and pEC14, had little to no rescue

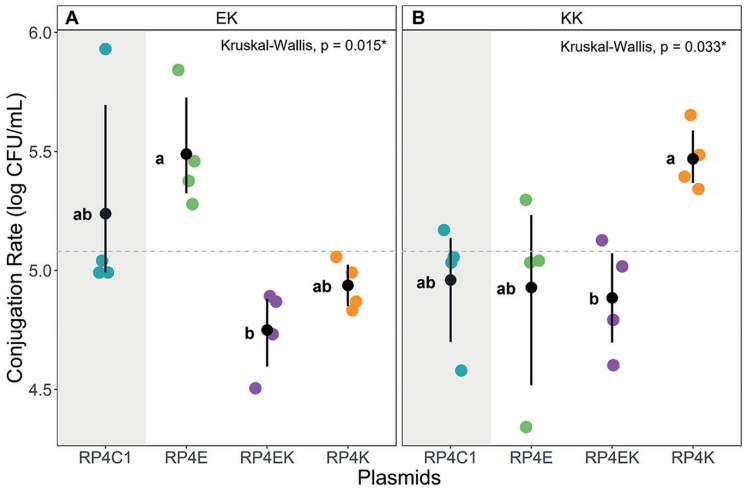

FIG 4 Conjugation rate of evolved RP4 plasmids (RP4E; green, RP4EK; purple, RP4K; orange) and the unevolved RP4 (RP4C1; blue) from biofilm to susceptible planktonic bacteria in: (A). *E. coli* to *K. pneumoniae* (EK), and (B). *K. pneumoniae* to *K. pneumoniae* (KK) setups. Transfer potential was measured as the conjugation rate (CFU/mL) of each plasmid (N = 4) in the setup. The mean and bootstrap confidence interval of each plasmid can be seen represented by point ranges. A Kruskal-Wallis with Dunn test for post hoc was performed between all plasmid-carrying strains. The P-value is shown, and the letters (a-e) next to each point range indicate whether there is a statistical difference between the plasmids found in the Dunn test. The mean of the entire data can be seen represented in the dashed line.

potential, which is consistent with the previous studies using these plasmids (36, 56). Little correlation could be drawn between the rescue potential and certain plasmid characteristics such as Inc type, mobility class, and mating pair formation systems. All of these plasmid characteristics were seen to be shared between RP4 and at least one of the pEC plasmids. In natural environments, plasmids are likely to come into contact with each other and interact within cells. The future studies on evolutionary rescue could benefit from involving setups with multiple plasmids which would allow within-host plasmid interactions and might bring differing results than what was presented here. Further, given the different AMR genes encoding various beta-lactamases for each plasmid in this experiment, it is pertinent to investigate whether the rescue potential is affected by the enzyme efficiency.

The different evolutionary histories we created in the RP4 plasmid were tested in the biofilm to planktonic rescue and conjugation setups. It was anticipated that the intraspecifically evolved plasmids (RP4E and RP4K) would have host specificity inferring increased rescue potential or conjugation rate in the intraspecific setup involving their host. Additionally, we were interested in examining the rescue potential or conjugation rate of the interspecifically evolved plasmid (RP4EK) in the interspecific setups. Previous research showed that intraspecific evolutionary history led to host-specialist while interspecific host-plasmid coevolution could lead to host-generalist (57). The interspecific KE rescue setup clearly showed poor plasmid transfer from *K. pneumoniae* to *E. coli*. This is consistent with previous results in planktonic setups (23). Throughout our biofilm setups, the *E. coli* evolved plasmid (RP4E) gave consistent results with the unevolved plasmid (RP4C1). Therefore, it appears reasonable to expect that there was little to no evolutionary influence from the *E. coli* host during the coevolution. Overall, the *K. pneumoniae* evolved plasmid (RP4K) and the interspecifically evolved plasmid (RP4EK) grouped together in their relatively low rescue potential and conjugation rate. This grouping seems to indicate a strong evolutionary influence of *K. pneumoniae* during the coevolution. However, this does not demonstrate RP4EK as a host-generalist

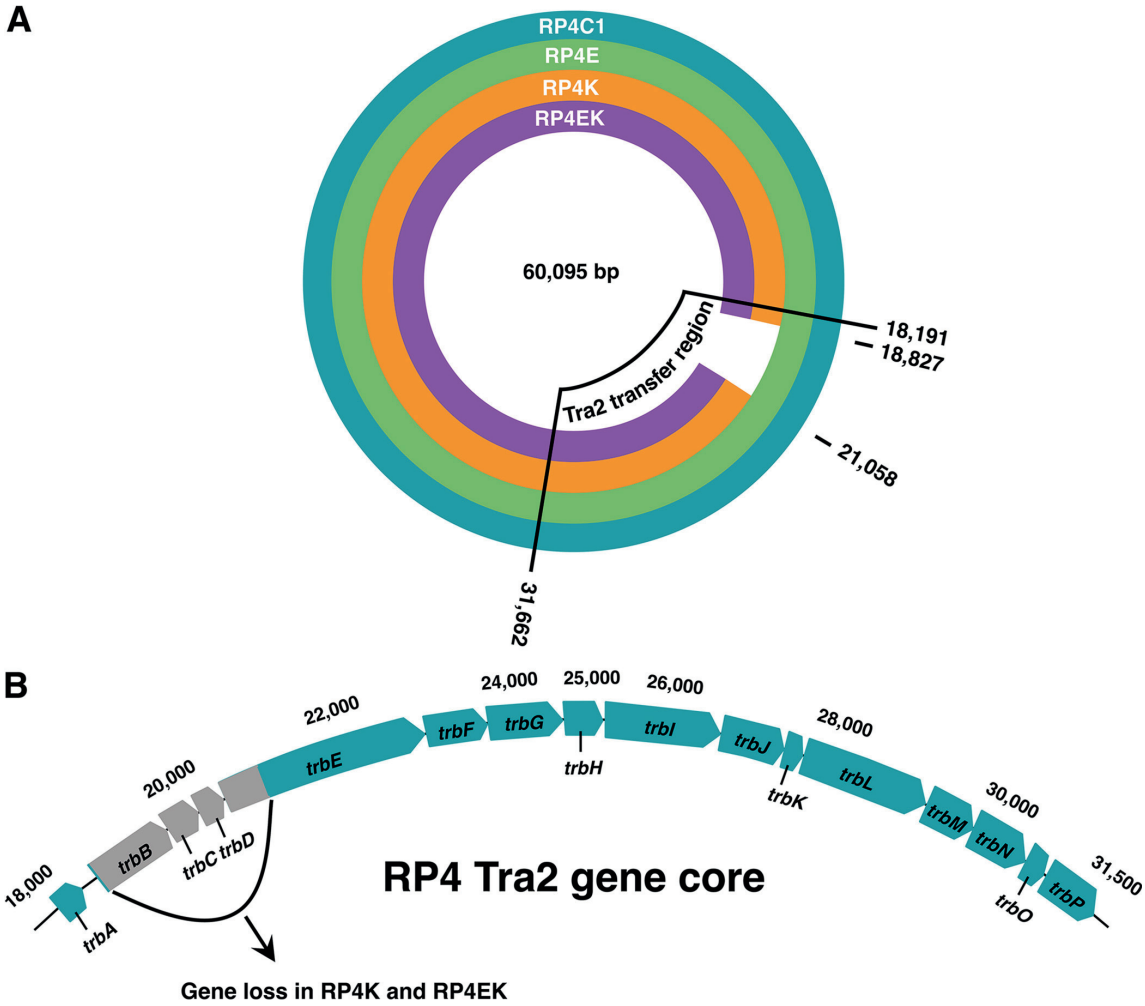

FIG 5 Comparison of the evolved RP4 plasmid sequences. (A) Sequences of RP4 evolved intraspecifically with *E. coli* (RP4E; green) or *K. pneumoniae* (RP4K; orange), or interspecifically with them both (RP4EK; purple) were compared with unevolved plasmid (RP4C1; blue). The Tra2 transfer region responsible for the mating-pair formation is highlighted. (B) The genetic organization of Tra2 region with the deletion detected in evolved plasmids RP4K and RP4EK shown in gray.

with increased rescue potential toward a broader spectrum of hosts. Based on earlier studies involving host switching lineages which might promote adaptation to a new unfavorable host, RP4EK should be tested further on its potential to rescue with an unfamiliar host (43).

Analyzing the growth curves, growth rate, and maximum yield of each evolved plasmid with their coevolved host clearly shows the similarities of RP4K and RP4EK and the distinction of RP4E (Fig. S1, Fig. S2, and Fig. S3). Following this trajectory, the change in rescue potential of the RP4K and RP4EK plasmids was observed in comparison to the unevolved plasmid RP4C1 in the EE setup. Additionally, when examining the significant differences in the rescue potential of RP4EK and the *E. coli* evolved plasmid RP4E, in the EE rescue, provided that RP4EK was coevolved equally with *E. coli* and *K. pneumoniae*. The results seen in the EE rescue setup, indicating clear evolutionary variance distinguishing RP4E and RP4C1 to RP4K and RP4EK was supported by the conventional planktonic conjugation assay (Table S1).

A reasonable explanation for the close results of RP4K and RP4EK is the identical deletion found in both of their sequences, localized in the Tra2 core complex, responsible for mating pair formation in the RP4 plasmid. This deletion was not seen with the RP4E plasmid, indicating less adaptation involving *E. coli* host lineage. This is contrary to previous research where *E. coli* host lineage caused major deletions in a plasmid, including the conjugative machinery, while evolutionary history with *K. pneumoniae* brought no major genetic changes (58). Three genes were practically completely deleted, *trbB*, *trbC*, and *trbD* and a fourth gene *trbE* had a partial deletion. Previous studies have reported that these genes are both essential for the conjugal transfer as well as phage propagation for the plasmid-dependent phage PRD1 (44–49, 59). This study clearly shows continued conjugal transfer in all setups although decreased for RP4K and RP4EK plasmids presumably due to the lack of mating pair formation genes that were previously noted to be essential (45, 59). This may suggest that biofilms better preserve the transfer of the RP4 plasmid even in the absence of seemingly essential conjugal transfer genes as the previous studies tested conjugation in liquid planktonic conjugation setups (45, 59). In this study, the host-plasmid coevolution was performed in liquid planktonic cultures. Although there was a constant antibiotic selection for the plasmid, the host-plasmid coevolution could have provided alternative results had they been performed in biofilm, as it may support the preservation of the conjugal transfer genes that were lost in RP4K and RP4EK. This is also supported by a recent study that found that plasmid persistence in planktonic communities was improved by loss of conjugal transfer genes, compared to biofilm communities in which the genes were retained (60). It seems that deletions of the conjugal transfer region are common when the plasmid confers a high cost to the host. This does not seem to be linked to a specific species, but rather how favorable the host-plasmid pairings are (58). In our study, *K. pneumoniae* could have selective pressure for alleviating the cost of the plasmid and confer fitness advantages through the deletion of the costly genes.

Interestingly, one difference from the grouping pattern of RP4K and RP4EK was observed. In the KK conjugation setup, the RP4K plasmid clearly inferred a higher rescue potential compared to the other plasmids, suggesting increased host specificity. This host specificity seemingly due to the intraspecific coevolution is what we had anticipated. However, this strays away from the genotypic-to-phenotypic patterns for the RP4K and RP4EK plasmids, as they have identical sequences and highly similar results in the other setups. The RP4K plasmid in the KK conjugation setup provides a better conjugation rate that cannot be explained by the plasmid sequence. This could be due to an unknown interaction between the evolved plasmid and host, perhaps epigenetic modifications. As we mentioned above, the deleted genes in RP4K and RP4EK had previously been described as essential in conjugal transfer and for plasmid-dependent-phage PRD1 propagation. Through a simple spot test assay, it was clear that strains carrying the evolved plasmids RP4K and RP4EK inferred immunity to the PRD1 phage. This supports the previous research on the Tra2 core and its essential role in the phage propagation for PRD1 (44). Along with the conceivable fitness advantages in the coevolved planktonic community, the loss of the conjugal transfer genes of RP4K and RP4EK could serve as an exaptation that provide significant advantage in the presence of a phage.

Our findings demonstrate that evolutionary rescue via conjugative plasmids is possible in a biofilm to planktonic setup, even with the lack of conjugal transfer genes. We show here that even relatively short periods of history in specific host can have a significant effect on plasmid's rescue potential and conjugation rate. The hosts used in this study, *E. coli* and *K. pneumoniae*, clearly exhibit different evolutionary influence on the RP4 plasmid, although the hosts are relatively similar. As such, it is possible that plasmid populations are continuously balancing between the benefits and costs of intra- and interspecific adaptations. These adaptations may determine the plasmid's survival in highly adverse conditions (for their hosts) such as in the sudden presence of lethal antibiotic doses and lytic bacteriophages.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial plasmid hosts used in this study were strains of two species of *Enterobacteriaceae*, *Escherichia coli* and *Klebsiella pneumoniae*. The plasmids used in this study were RP4, a broad-host-range conjugative plasmid that has a high conjugation rate (61–64), and five ESBL-plasmids isolated from clinical *E. coli* strains (pEC plasmids; [36]) (Table 1). Lysogeny broth (LB) (65), supplemented with 1% agar and/or antibiotics as indicated, were used for bacterial cultivation. Bacterial cultures were grown at 37°C with 200 rpm agitation unless otherwise specified and on agar plates incubated at 37°C.

Host-plasmid coevolution experiments. The purpose of the experiments was to evolve the RP4 plasmids to intra- and interspecific host systems involving *E. coli* and/or *K. pneumoniae*. The naming of each treatment was the RP4 plasmid indicated by the first letter of the genus name of the host strains used (RP4E, RP4K, RP4EK). The host-plasmid coevolution treatments were initiated in 5 mL LB broth with host bacterial strains containing the RP4 plasmid and carried out for 30 cycles. The bacterial hosts were JM109(pSU19) harboring plasmid pSU19 encoding chloramphenicol resistance (camR) and DSM681 with chromosomal mutations encoding rifampicin resistance (rifR) (66, 67). For each cycle, the culture transfers were done at 1:1000 dilution with appropriate antibiotic selection to select for host and plasmid. For treatment RP4E, the medium was supplemented with 25 μg/mL chloramphenicol and 25 μg/mL kanamycin, and for treatment RP4K with 150 μg/mL rifampicin and 25 μg/mL kanamycin. For treatment RP4EK, each host was resistant to a separate antibiotic, this allowed host antibiotics to be swapped sequentially to encourage plasmid transfer between the two host species, allowing one host strain to maintain the plasmid at once. The antibiotic selection for treatment RP4EK involved the following five cycle supplementation that was repeated six times for a total of 30 cycles: (I) 25 μg/mL kanamycin, (II) 15 μg/mL rifampicin and 2.5 μg/mL kanamycin, (III) 150 μg/mL rifampicin and 25 μg/mL kanamycin, (IV) 2.5 μg/mL chloramphenicol and 2.5 μg/mL kanamycin, (V) 25 μg/mL chloramphenicol and 25 μg/mL kanamycin. This experiment was run with four biological replicates per population. Growth analysis was done to compare the starting point (cycle 1) to the endpoint (cycle 30) of each population, as well as with the plasmid-free host. Overnight cultures were initiated in LB broth (with 25 μg/mL kanamycin for RP4 plasmid carrying strains) before 1:100 dilution was performed into fresh LB medium and mixed thoroughly. The growth of the bacterial strains was determined with a Bioscreen C MBR machine (Bioscreen, Oy Growth Curves Ab Ltd.) for 24 h as described previously (56). The growth curves, growth rate (r), and maximum yield (K) were calculated from the data using RStudio (R version 4.2.1), with R source code based on a previously described MATLAB code (68).

Biofilm experiments: rescue potential and conjugation efficiency. The evolutionary rescue potential of the plasmids was studied by the capacity of plasmid-carrying biofilm to rescue the planktonic antibiotic-susceptible bacteria. The setup involved the evolved RP4 plasmids (RP4E, RP4K, and RP4EK) and unevolved RP4C1, from cycle 1, tested under two rescue setups through a combination of two donor strains, *E. coli* HMS174^{rifR} and *K. pneumoniae* DSM681^{rifR}, and the susceptible recipient strain *E. coli* JM109 (pSU19)^{camR}. The setups were given abbreviations indicating the donor and recipient strains, respectively (EE and KE; E for *E. coli* and K for *K. pneumoniae*). Additionally, five previously characterized ESBL-conferring plasmids (pEC3, pEC13-16) were tested in the EE rescue setup. The conjugation efficiency of the evolved RP4 plasmids from biofilm to planktonic cells was tested in setups EK and KK, with the same donor strains as the rescue setups and the nonsusceptible *K. pneumoniae* DSM681^{strepR} as the recipient strain.

The biofilm experiments were started by inoculating 25 μL of the overnight donor cultures, grown in LB supplemented with 150 μg/mL ampicillin, into fresh 5 mL LB with 150 μg/mL ampicillin and 180 μL were aliquoted into wells of a 96-well plate (Nunc MicroWell, Thermo Scientific) in 4 replicates/culture. Plasmid-free strains (*E. coli* HMS174^{rifR}, *K. pneumoniae* DSM681^{rifR}) were used as a control. The plate was closed with a 96-pin lid (Nunc Immuno TSP Lid, Thermo Scientific), sealed with parafilm, to allow biofilm to grow onto the pins for 5 days at 37°C without shaking. After the 5-day incubation, the lid with the biofilm-covered pins was washed two times with 1× PBS before being introduced to a new 96-well plate containing 180 μL planktonic recipient strain. The recipient strain had been grown overnight, before being transferred at 1:1000 dilution into fresh media supplemented with 150 μg/mL ampicillin for 1 h. The length of antibiotic exposure before rescue and type of antibiotics used were based on previous research (36, 37). The plate was sealed with parafilm and grown overnight without shaking. Dilutions of the product of each well were plated on LB agar plates with appropriate antibiotics selecting for only the recipient and plasmid to determine the density of the formed transconjugants as CFU (CFU). Rescue setup EE selected for transconjugants with 25 μg/mL chloramphenicol and 150 μg/mL ampicillin, while setup KE selected with 25 μg/mL chloramphenicol and 25 μg/mL kanamycin. Conjugation setups EK and KK selected for transconjugants with 25 μg/mL streptomycin and 25 μg/mL kanamycin.

Conjugation assay. Conventional planktonic conjugation assay with the evolved and unevolved RP4 plasmids were performed alongside the biofilm experiments with the same donors and recipients. This was done to measure the mean conjugation frequency (CFU/mL) per donor cell and compare with the biofilm experiments as they were unable to determine the donor cell density. The conjugation was done by adding 5 μL of the plasmid-carrying donor overnight culture and 500 μL of the recipient overnight culture in 5 mL LB for 2 h at 37°C, 200 rpm. The product of the conjugation was then plated on LB agar with appropriate antibiotics to determine the cell density (CFU/mL) of the formed transconjugants. Transconjugants for conjugation assays EE and KE were selected for with 25 μg/mL chloramphenicol and 25 μg/mL kanamycin, and for conjugation assays EK and KK, transconjugants were selected with 25 μg/mL streptomycin and 25 μg/mL kanamycin. The donor strains were plated on LB agar

TABLE 1 The bacterial strains and plasmids used in this study

Strain	Plasmid(s)	Plasmid size (bp)	Inc type	MPF[d] type	MOB[d] class	β-lactamase identified	Other resistance genes
E. coli JM109 (pSU19)^{CamR}	pSU19[a]	2340	-	-	-	-	*cat*
E. coli HMS174 (plasmid-free)^{RifR}	-	-	-	-	-	-	-
E. coli HMS174 (pEC3)^{RifR,AmpR}	pEC3pl1	91,885	IncB/ O/ K/ Z	MPFI	MOBP	*blaTEM-1C*	*strA, strB, sul2*
	pEC3pl2	59,192 (59,192)[c]	IncI2	MPFT	MOBP	-	-
E. coli HMS174 (pEC13)^{RifR,AmpR}	pEC13	71,656	IncFII	MPFF	MOBF	*blaCTX-M-14*	-
E. coli HMS174 (pEC14)^{RifR,AmpR}	pEC14pl1	143,590	IncFII, IncQ1, IncP,	MPFF	MOBF	*blaTEM-1B*	*strA,strB, aadA1, mph(B),*
	pEC14pl2	87,848 (87,666)[c]	IncFIB IncI1	MPFI	MOBP	-	*sul1, sul2, tet(A), dfrA1* -
E. coli HMS174 (pEC15)^{RifR,AmpR}	pEC15pl1	87,811	IncI1	MPFI	MOBP	-	-
	pEC15pl2	(87,767)[c] 38,611	IncX1	MPFT	MOBQ	*blaTEM-52B*	-
E. coli HMS174 (pEC16)^{RifR,AmpR}	pEC16pl1	94,325	IncI1	MPFF	MOBP	*blaSHV-12*	-
	pEC16pl2[b]	(95,380)[c] 7,939	ColRNAI	-	MOBP	-	-
E. coli JM109(pSU19) (RP4)$^{CamR,\ AmpR,\ KanR,\ TetR}$	pSU19[a] RP4	60,095	IncP-1α	MPFT	MOBP	*blaTEM−2*	*cat* *tet(A), aph(3′)-Ib*
E. coli HMS174(RP4)$^{RifR,\ AmpR,\ KanR,\ TetR}$	RP4	60,095	IncP-1α	MPFT	MOBP	*blaTEM-2*	*tet(A), aph(3′)-Ib*
K. pneumoniae DSM681 (plasmid-free)$^{RifR,\ AmpR}$	-	-	-	-	-	*blaSHV-28*	-
K. pneumoniae DSM681 (plasmid-free)$^{StrepR,\ AmpR}$	-	-	-	-	-	*blaSHV-28*	-
K. pneumoniae DSM681(RP4)$^{RifR,\ AmpR,\ KanR,\ TetR}$	RP4	60,095	IncP-1α	MPFT	MOBP	*blaTEM-2, blaSHV-28*	*tet(A), aph(3′)-Ib*
K. pneumoniae DSM681(RP4)$^{StrepR,\ AmpR,\ KanR,\ TetR}$	RP4	60,095	IncP-1α	MPFT	MOBP	*blaTEM-2, blaSHV-28*	*tet(A), aph(3′)-Ib*

[a]Nonconjugative plasmid.
[b]Nonconjugative mobilizable plasmid.
[c]Alterations to plasmid size due to the shufflon area are indicated in parentheses.
[d]MPF = mating pair formation; MOB = mobility.

supplemented with 150 μg/mL rifampicin to determine their cell number (CFU). The mean conjugation frequency was given as the transconjugant cell density divided by donor cell number.

Plaque assay. To determine the infectivity of the PRD1 phage, which is dependent on the RP4 mating pair formation complex, spot test plaque assays were performed (69). The plaque assays were initiated by combining 3 mL of melted LB soft-agar (0.7%) with 100 μL of overnight grown plasmid-carrying host and then poured onto LB agar plates. PRD1 viral lysate (6.9×10^{10} PFU/mL; plaque forming units) was then spotted (10 μL) onto the plates. The plates were grown overnight at 37°C.

Plasmid sequencing and bioinformatic analyses. To explore the possible mutations in the evolved RP4 plasmids, the total DNA from clonal populations of the *E. coli* HMS174^{rifR} strain carrying RP4E, RP4K, RP4EK, and RP4C1 (as reference) was isolated using Wizard genomic DNA purification kit (Promega) according to the manufacturer's instructions. The DNA concentration was determined with a Qubit 3.0 fluorometer using the dsDNA HS kit (Invitrogen, ThermoFisher Scientific). The sequencing library preparation was done with NEB Next Ultra DNA Library Prep kit and 2×150 bp paired-end (PE150) DNA sequencing was performed on Illumina NovaSeq 6000 platform with S4 flowcell for the strains carrying the evolved plasmids. The illumina reads were trimmed with trimmomatic (0.39) (70). Trimmomatic was run as paired end mode to trim for illumina adapters (ILLUMINACLIP with the following settings: 2 for seed mismatches; 30 for palindrome clip threshold; 10 for simple clip threshold; and 2 as minimum adapter length in palindrome mode in keepBothReads setting) and for quality (SLIDINGWINDOW with 3 for window size and 21 for average quality threshold). Reads with length under 100 bp after trimming were discarded from the analysis.

RP4C1 served as an unevolved control and was used for short-insert library preparation and sequenced with DNBSEQ platform (PE150). The reads were quality- and adapter-trimmed with SOAPnuke (71) by the sequencing service; reads containing more than 1% of N, more than 40% of the bases in a read have quality value under 20, or reads with length under 150 bp were removed. The corrected reads were mapped to reference RP4 sequence (BN000925.1) to detect possible genetic changes using the mutation prediction pipeline breseq (0.37.0) with consensus mode to detect mutations that exist in the clonal samples at 100% frequency (72). The mutations detected in RP4C1 were filtered out from the evolved plasmid mutations manually. The overall coverages were 435 (RP4C1), 1127 (RP4E), 471 (RP4K) and 522 (RP4EK). The sequencing coverage of the deletion site was 477 for RP4K and 547 for RP4EK. The Geneious Prime software version 2022.2.2 (Geneious) was used to further visualize specific mutations in the mapped plasmid sequences.

Statistical analysis. All statistical analysis was carried out in RStudio (R version 4.2.1) (see code in supplemental material, Supplemental Text File S1). The dependent variable (CFU/mL) was transformed on a log scale. The possible statistical significance of each plasmid in each rescue and conjugation setup was determined through either a one-way ANOVA with Tukey-HSD as *post hoc* comparisons or a Kruskal-Wallis with Dunn test as *post hoc* comparisons. The statistical significance between plasmids found with the Tukey and Dunn tests was indicated through a compact letter display on the figures.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, PDF file, 0.05 MB.

FIG S1, TIF file, 3.3 MB.

FIG S2, TIF file, 2.9 MB.

FIG S3, TIF file, 3.5 MB.

TABLE S1, PDF file, 0.01 MB.

TABLE S2, PDF file, 0.01 MB.

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Conceptualization, I.J., R.P., and M.J.; methodology, I.J., C.G., and R.P.; formal analysis, I.J. and R.P.; data curation, I.J. and R.P.; writing—original draft preparation, I.J.; writing—review and editing, M.J., R.P., and C.G.; visualization, I.J.; supervision, M.J. and R.P.; funding acquisition, R.P. and M.J. All authors have read and agreed to the published version of the manuscript.

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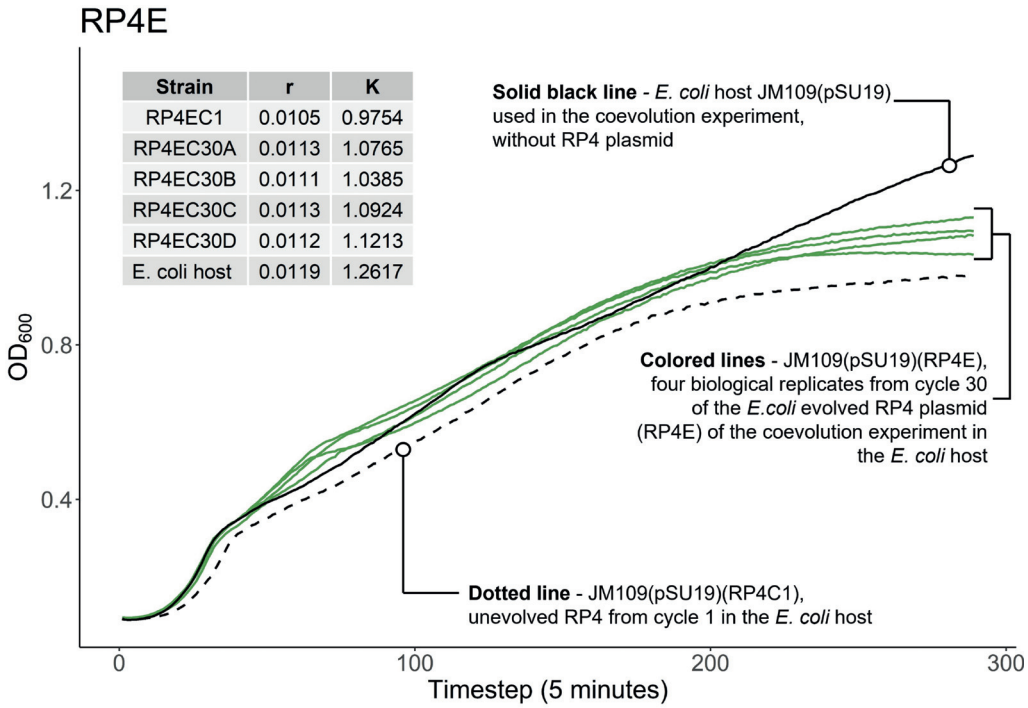
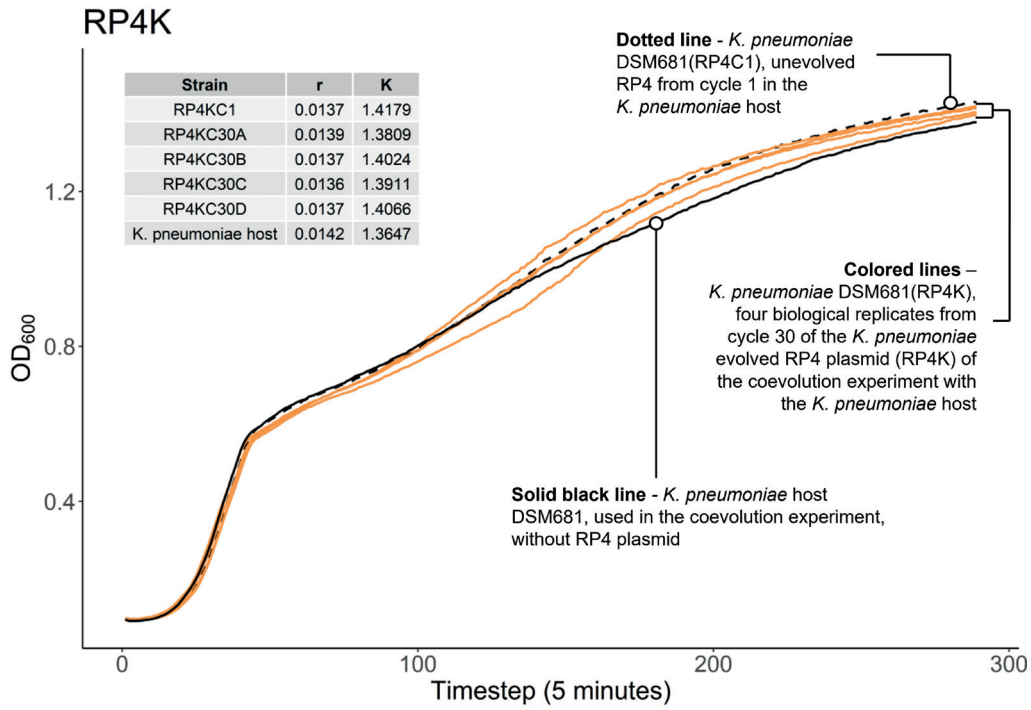
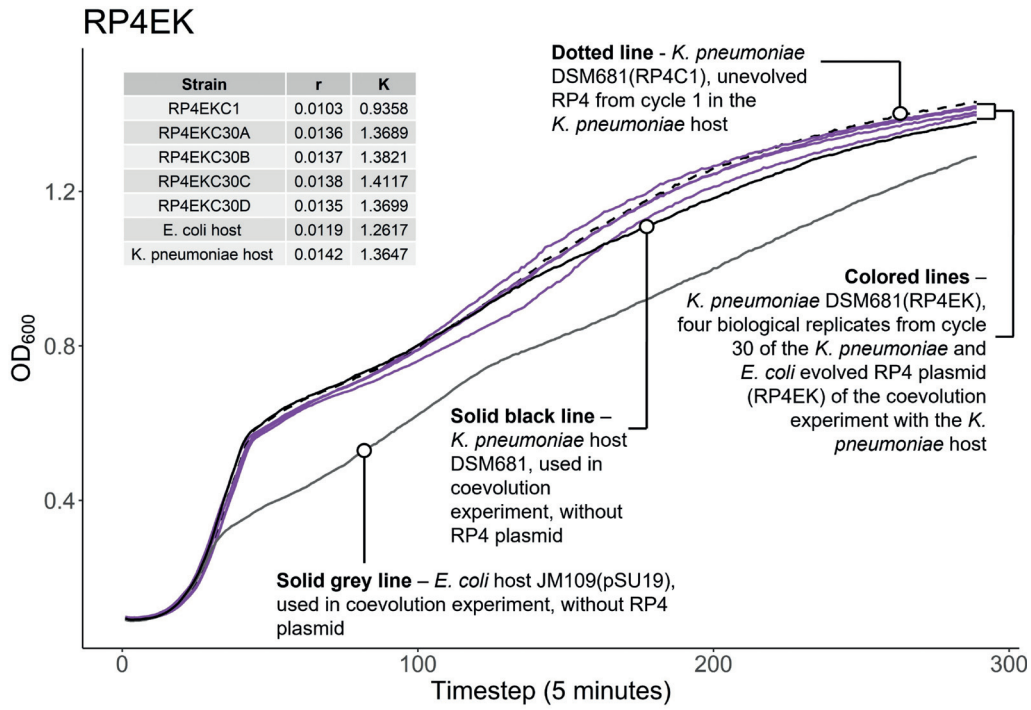
SUPPLEMENTAL MATERIAL



SFigure 1. Evolution of RP4E plasmids coevolved with *E. coli* host JM109(pSU19). Growth curves were measured as optical density (OD600) in 5 min-intervals of JM109(pSU19) harboring evolved (RP4EC30A-D) or unevolved RP4 (RP4EC1) and plasmid-free bacteria to determine the growth rate (r) and maximum yield (K).



SFigure 2. Evolution of RP4K plasmids evolved with *K. pneumoniae* host DSM681. Growth curves were measured as optical density (OD_{600}) in 5 min-intervals of DSM681 harboring evolved (RP4KC30A-D) or unevolved RP4 (RP4KC1) and plasmid-free bacteria to determine the growth rate (r) and maximum yield (K).



SFigure 3. Evolution of RP4EK plasmids evolved with *E. coli* host JM109(pSU19) and *K. pneumoniae* host DSM681. Growth curves were measured as optical density (OD600) in 5 min-intervals of DSM681 harboring evolved (RP4EKC30A-D) or unevolved RP4 (RP4EKC1) and plasmid-free bacteria to determine the growth rate (r) and maximum yield (K).

STable 1. Mean conjugation frequency per donor cell from the conjugation assay replicating the setup of the biofilm experiments (N = 4)

Conjugation System	Plasmids			
	RP4C1	RP4E	RP4K	RP4EK
EE	6.533333e-03	1.153333e-02	5.054054e-06	5.714286e-06
EK	3.181481e-05	2.770000e-05	8.933333e-06	6.085714e-06
KE	7.017544e-06	3.361345e-06	1.250000e-05	6.993007e-06
KK	4.351724e-06	4.506494e-06	4.853333e-06	2.879747e-06

STable 2. Drop plaque assay results involving phage PRD1 and the evolved RP4 plasmid as well as unevolved RP4C1 in the bacterial host *E. coli* HMS174rifR.

Plasmid	PRD1 plaques (viral lysate drop: 6.9×10^8 pfu/10 µL)
RP4C1	Plaque formed
RP4E	Plaque formed
RP4K	No plaque
RP4EK	No plaque

III

UNAVOIDABLE DEVELOPMENT OF INDUCED PHAGE RESISTANCE IN CLINICAL MULTIDRUG-RESISTANT *E. COLI* AND *K. PNEUMONIAE* GUT ISOLATES

by

Ilmur Jonsdottir, Sanna Vacker, Matti Jalasvuori, Lotta-Riina Sundberg & Reetta Penttinen 2024

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IV

ESBL PLASMID COMPATIBILITY WITH THE SURROUNDING MICROBIAL COMMUNITY INFLUENCES ESBL GENE SURVIVAL UNDER CRISPR-ANTIMICROBIAL TARGETING

by

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