TRANSMISSION OF ANTIBIOTIC RESISTANCE GENES ENCODED ON A BROAD HOST RANGE RP4 PLASMID AMONG MEMBERS OF THE HUMAN GUT MICROBIOTA

By

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ABSTRACT

The emergence and global spread of antibiotic resistance among life-threatening pathogens are serious public health threats. Conjugative plasmids are considered the leading cause of spreading antibiotic-resistant genes among pathogens. The human gut microbiota is considered an important reservoir of antibiotic resistance genes. However, little is known about the frequencies and mechanistic drivers of the plasmids-mediated spread of antimicrobial resistance in the human gut. This study aims to determine the frequency and transferability of conjugative RP4 plasmid among enteric bacteria in both *in-vitro* settings and *in-vivo* mouse models transplanted with human gut microbiota.

We performed in-vitro experiments to determine the primary and secondary frequencies of a broad host range plasmid RP4 to multiple naïve host bacteria. We demonstrated that the RP4 plasmid transferred from human gut commensal donor *E. coli* LM715-1 to *Citrobacter rodentium, Salmonella typhimurium, Klebsiella pneumoniae, Pseudomonas putida, Vibrio cholerae*, and three different strains of *E. coli*. However, the plasmid transfer frequency (TF) differed greatly between specific donor-recipient pairings, ranging from 10^{-2} to 10^{-8}. We also observed that recipients of RP4 further transferred that plasmid to commensal *E. coli*. Furthermore, we examined the effect of the antibiotic ampicillin on RP4 plasmid transfer frequency from human gut commensal *E. coli* LM715-1 to *Citrobacter rodentium, Salmonella typhimurium, Klebsiella pneumoniae, Pseudomonas putida, Vibrio cholerae*, and *E. coli*. A serial passage plasmid persistence assay showed that the RP4 plasmid imposed a fitness cost on its host, *E. coli* LM715-1, resulting in the loss of the plasmid over time. However, plasmid-bearing cells persisted at a low proportion of the population for at least ten transfers.

Next, we performed *in vivo* experiments to develop a tractable mouse model transplanted with the adult human gut microbiota to study the RP4 plasmid-mediated spread of ARGs from a human gut commensal donor *E. coli* LM715-1 to resident bacteria of the human gut microbiota. We found that commensal donor strain *E. coli* LM715-1 colonized the mouse gut and persisted throughout the ten-day experiment, while the laboratory donor strain *E. coli* MG1655 was not recovered after 48 hours. Next, we tracked donor and recipient bacteria in a complex microbial community using flow cytometry to sort the transconjugant bacteria. The flow cytometry of the treatment group fecal samples showed an increased spread of detectable cells with the tagged plasmid when compared to those before the gavage. Donor and transconjugant bacteria were recovered from fecal samples and sorted by FACS. A 16S sequencing analysis of sorted cells showed *Lachnospiraceae, Clostridiaceae, Pseudomonadaceae, Rhodanobacteraceae, Erysipelotrichaceae, Oscillospiraceae,* and *Butyricicoccaceae* are the primary target bacterial families of RP4 plasmid acquisition.

In summary, the findings from these studies have paved a path for addressing the spread and persistence of antibiotic-resistant bacteria through horizontal gene transfer in complex environments like the human gut. The transplanted mouse model would likely to serve an important tool to study epidemiologic, evolutionary, and ecological aspects of antibiotic resistance in the human gut.

This dissertation is dedicated to my parents, elders, and all my beloved teachers.

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Chapter 1: Literature Review

Epidemiology of Antimicrobial Resistance

Antimicrobial resistance (AMR) among bacterial pathogens has arisen as a major global public health challenge. The Centers for Disease Control and Prevention (CDC) assessed the national burden of AMR using 2019 surveillance data of 18 leading pathogens in the USA. The CDC reported that antibiotic-resistant (AR) pathogens in the USA caused ~ 3 million infections and 36 thousand deaths in 2019 (CDC., 2019). Additionally, it was reported that diseases caused by these antibiotic-resistant pathogens required more extended hospitalizations and inflicted a substantial economic burden on the US healthcare system. A recent systemic analysis study used 471 million individual records from 204 countries to determine AMR global burden and spread of 23 life-threatening bacterial pathogens. It was estimated that approximately 4.95 million AMR-associated deaths occurred globally, which included 1.27 million deaths directly attributed to drug-resistant bacteria in 2019 (Murray et al., 2022).

In 2014, an independent review on AMR was commissioned by the United Kingdom government to assess the scope and magnitude of AMR worldwide. It was determined in the final report of this review that if actions to address AMR concerns were not undertaken proactively, antibiotic-resistant (AR) bacteria would be expected to become a leading cause of death with approximately 10 million cases occurring annually worldwide by 2050 (J. O'NEILL, 2016). It has been predicted that multi-drug resistant bacteria could cause the next pandemic. O'Neill et al. (2016) also estimated that the AMR crisis could significantly increase the economic burden by up to $100 trillion by 2050 if no actions are taken to address this crisis (J. O'NEILL, 2016).

A research group at the World Bank assessed the economic burden of AMR and published a comprehensive report in 2017 (Jonas et al., 2017). Financial losses by 2050 were determined for both low-impact and high-impact AMR scenarios using a simulation method. They estimated that if AMR is not adequately addressed in a low-impact scenario, it could generate a loss of over 1 trillion dollars after 2030. In high-impact scenarios, they estimated it could create a loss in the world's annual gross domestic product (GDP) of over $3.4 trillion by 2030. Global trade was expected to be seriously affected by unchecked AMR occurrence and spread (Jonas et al., 2017). These findings highlight the fact that the emergence and spread of antibiotic-resistant pathogens have become a major global health challenge.

Antimicrobial Resistance: A Multifaceted Global Health Threat

Antibiotic resistance is an emerging multifaceted problem driven by several complex interlinked clinical, biological, social, and environmental factors. It has been widely studied that over-prescription and unwarranted use of antibiotics in human and animal clinical settings are leading causes of increased antibiotic resistance (Chang et al., 2019; Holmes et al., 2016; Shallcross & Davies, 2014). A study was conducted to assess the proper application of antibiotics during outpatient visits in the United States from 2010 to 2011 (Fleming-Dutra et al., 2016). These investigators estimated that 506 antibiotic prescriptions per 1000 population were made during this period, and 353 of these antibiotics were found to be appropriately prescribed according to the national guidelines provided by medical experts for common health conditions (Fleming-Dutra et al., 2016). Similarly, the CDC reported that 269 million antibiotic prescriptions were

made in the USA in 2015 alone, and about 30 percent were unnecessary based on the national guidelines for antibiotic prescriptions (CDC, 2017).

The CDC has established multiple collaborations with healthcare establishments and professionals to ensure the appropriate use of antibiotics and develop a strong AMR stewardship program nationally. The CDC conducts various surveys, including the National Ambulatory Medical Care Survey (NAMCS) and the National Hospital Ambulatory Medical Care Survey (NHAMCS), to collect data about antibiotic prescriptions by emergency and outpatient departments from the entire country. This data is used to design guidelines and policies to efficiently implement the AMR stewardship program in clinical settings. In 2020, the second national action plan for combating AMR, 2020-2025, was released (Federal Task Force, 2020). It further strengthens ongoing strategic actions and prioritizes reducing the burden of emerging AR bacteria, establishing partnerships following the One Health concept, and conducting research to develop new drugs and treatments (Federal Task Force, 2020).

The environment plays an essential role in the emergence and transmission of antibiotic resistance. Excessive use of antibiotics in agriculture farming, especially livestock, poultry, and aquaculture, has been reported worldwide (Cabello, 2004; Eltayb et al., 2012; Kumar et al., 2019; Shao et al., 2021). In 2011, the Food and Drug Administration (FDA) agency estimated that about 13.6 million kilograms of antibiotics were approved in food-producing animals in the United States (Hollis & Ahmed, 2013). Tetracycline and ionophores are the most used drugs (Administration, 2014). China has

a large livestock farming industry, but the rate of antibiotics usage is higher per unit than in the USA and UK (Shao et al., 2021). Another study estimated that 63,151 tons of antibiotics were used in food-producing animals worldwide in 2010. Authors further projected that usage of antibiotics would increase by 67% by 2030 and would almost double in China, Brazil, India, Russia, and South Africa by this time (van Boeckel et al., 2015). Therefore, a significant amount of antibiotic residues and contaminants from farm settings are released into the environment, which drives the spread of antibiotic resistance, particularly in foodborne pathogens (Holmes et al., 2016; Tian et al., 2021). Furthermore, due to increasing industrialization, many pollutants and chemical agents, especially heavy metals, are released into the environment without proper treatment, which drives antibiotic resistance among pathogens and environmental bacteria (Bengtsson-Palme et al., 2018; Larsson et al., 2018). Many other factors, including socioeconomic, political, climatic changes, urbanization, and explosive human population growth, also play significant roles in the persistence and reemergence of AMR worldwide (Vikesland et al., 2019). Thus, considering the complex nature of antibiotic resistance, there is a dire need to address this global challenge using a multi-disciplinary approach under a one health concept.

Mechanisms of Antibiotic Resistance

Biologically, multiple mechanisms lead to the emergence of antimicrobial resistance among bacteria. First, bacteria can become resistant to antibiotics through intrinsic processes, i.e., altering drug target sites, inactivating, and modifying antibiotics, activating efflux pumps, and blocking uptake of antibiotics (Du et al., 2018; Gottlieb et

al., 1967; Reygaert, 2018). Second, bacteria acquire foreign antibiotic-resistance genes (ARGs) from other bacteria through horizontal gene transfer (HGT). There are three mechanisms of HGT reported in bacteria; transformation (Dubnau, 1999), transduction (Jiang & Paul, 1998; Schicklmaier & Schmieger, 1995), and conjugation (Buchanan-Wollaston et al., 1988; Heinemann & Sprague, 1989). The spread of ARGs through the mechanisms of HGT enables bacteria to evolve rapidly and survive under high selection pressures, including antibiotic treatment (Charpentier et al., 2012; Wiedenbeck & Cohan, 2011). However, the transfer of DNA through conjugation is considered a significant source that produces genetic diversity by the sharing of foreign DNA, including ARGs, among bacteria (de la Cruz & Davies, 2000; Ochman et al., 2000).

Transfer of Antimicrobial Resistance Through Conjugation

It is well known that conjugation is a frequently occurring mechanism among bacteria through which they can share extrachromosomal genetic information located on a circular plasmid. Conjugative plasmids have genetic machinery related to self-replication and mobilization that enables them to invade other bacteria and spread ARGs independently (Billot-Klein et al., 1990; Jarlier et al., 1988). The emergence of antibiotic resistance in bacteria was observed shortly after the discovery of the first antibiotic, penicillin, in 1928. Early evidence identified plasmid-mediated penicillin resistance among *Enterobacteriaceae* in 1965 (Datta & Kontomichalou, 1965). Since then, several studies have concluded that conjugative plasmids encoding antibiotic resistance genes are causing a vast spread of multi-drug antibiotic resistance among

bacteria (Davies & Davies, 2010; de la Cruz & Davies, 2000; Dolejska & Papagiannitsis, 2018; Levy & Bonnie, 2004).

Conjugative plasmids are transferrable, reproducible, and capable of carrying multiple resistance associated genes and likely invade a broad range of microbial communities (Vogwill & Maclean, 2015). Conjugation can be divided into two main stages, mating pair formation among two cells followed by the transfer of plasmid DNA. Mating pair formation is partly due to a type IV secretion system (T4SS), which controls the uptake and release of bacterial cellular molecules like DNA, proteins, etc. (Schröder & Lanka, 2005). It has been identified that the transfer region of the F plasmid contains about 40 genes, which provide overall control of mating pair formation and DNA transfer during conjugation (Frost et al., 1994). The most critical set of genes involved in conjugation is located in the Tra operon. This operon can be divided into the Tra1 region and the Tra2 region. Tra1 region genes regulate DNA replication and transfer, while the Tra2 region encompasses genes controlling the mating pair formation and post-transfer stabilization(Malgorzata et al., 1998). The specific role of many Tra genes remains unknown; however, around 25 different Tra genes have been studied that play an essential role in the successful transfer of DNA through conjugation (Virolle et al., 2020). Transfer of DNA begins from a specific sequence of DNA called the origin of transfer (oriT) that is found in the Tra1 region of the plasmid. Conjugative plasmids also carry relaxases, helicases, and polymerases that can initiate the replication of plasmid DNA independently (Jain & Srivastava, 2013). A complete sequence of the RP4 plasmid (IncPα) of 40kb size showed that it carried 74 distinct closely packed genes, and the

expression of 60 of these genes was studied experimentally (Pansegrau et al., 1994). About 25 different promoters were found in the intergenic regions of this plasmid (Pansegrau et al., 1994). RP4 plasmid carries an oriV for replication and multiple gene operons, especially genes belonging to Tra1 and Tra2 regions, for self-replication, self-transferability, and stability in the host bacterium. (Adamczyk & Jagura-Burdzy, 2003; Malgorzata et al., 1998). These genomic features and characteristics make conjugative plasmids a significant force in spreading antibiotic resistance genes among pathogens and commensal bacteria in human and animal microbial communities.

Conjugative plasmids are mainly classified based on these two different criteria. First, conjugative plasmids can be divided into two groups based on the scale of transferability: narrow versus broad host range plasmids. Narrow host range plasmids are primarily found in closely related bacterial species. However, broad host range plasmids are very promiscuous and capable of invading a wide range of closely and distantly related bacteria. Thus, these plasmids are the leading force in driving ARGs spread among multiple species of bacteria (Jain & Srivastava, 2013; Schlechter et al., 2018; von Wintersdorff et al., 2016). Second, conjugative plasmids are also divided into different incompatibility groups based on their stability during conjugation (Datta & Hedges, 1973). Broad host range plasmids belonging to incompatibility groups (IncA/C, IncL/M, IncN, IncP, IncQ, IncW) have been commonly reported among bacteria (Rozwandowicz et al., 2018). Broad host range plasmids associated with the IncP group, especially the RP4 plasmid, have shown massive potential for spreading ARGs via conjugation based on population studies (Klümper et al., 2015; Lu et al., 2017;

Popowska & Krawczyk-Balska, 2013). The RP4 plasmid (IncP-1α) was first identified among *Enterobacteriaceae* isolated from human clinical samples from Bringham, UK, in 1969. Researchers found a similar transferable RP4 plasmid first in *Pseudomonas aeruginosa* and then *Klebsiella aerogenes* in the same Bringham Hospital setting (Ingram et al., 1973). Since then, the RP4 plasmid has been isolated from multiple bacterial species and has become one of the most studied plasmids (Christopher et al., 1987; Datta & Hedges, 1971; Pansegrau et al., 1994). RP4 plasmids have several genetic features to maintain self-replication, transmissibility, and survival in a wide range of gram-negative and gram-positive bacteria (Adamczyk & Jagura-Burdzy, 2003; Barth & Grinter, 1977). The transfer of RP4 plasmid has been observed in multiple bacterial species, including *Pseudomonas* (Ehlers & Bouwer, 1999), *Acinetobacter* (Geisenberger et al., 1999), *E. coli* and *Salmonella* (Wang et al., 2015). Similarly, multiple studies have shown the transfer of ARGs encoded RP4 plasmid from laboratory strains of *E. coli* and *Pseudomonas putida* to bacteria from soil (Klümper et al., 2015; Musovic et al., 2014), sewage, and activated sludge (Geisenberger et al., 1999; Liu et al., 2019; Soda et al., 2008). These studies show that RP4 plasmids are highly promiscuous and contribute significantly to the spread of antibiotic resistance among bacteria in complex environments.

Microbiome and Antibiotic Resistance Through Horizontal Gene Transfer

The human body hosts over 100 trillion microbial organisms, which is ten times more than the total eukaryotic cells in the body (Savage, 1977). The human gut microbiota is a complex and diverse consortium of $\sim 10^{14}$ microbes from more than 1000

bacterial species (Huttenhower et al., 2012; Lozupone et al., 2012). The gut microbiome is a complex microbial consortium that resides in a symbiotic and mutually beneficial relationship with the host and serves as a critical interface between environmental factors and the host (Costello et al., 2009; Dethlefsen et al., 2007; Eckburg et al., 2005). The gut microbiome has also been shown to have a huge potential for modulating different biological mechanisms of the host, such as immunity (Mezouar et al., 2018), development, growth and dietary patterns (Wu et al., 2011), drug metabolism (Wilson & Nicholson, 2017), and gut-lung (Martin et al., 2018) or gut-brain axis (Dang & Marsland, 2019).

In addition, the gut microbiome is also considered a reservoir of ARGs and a potential milieu for producing AR bacteria that can lead to the emergence of AR pathogens leading to severe disease (Salyers et al., 2004). ARGs encoding plasmids can invade diverse microbial community members and quickly spread antibiotic resistance in the local environment with and without any antibiotic treatment (Davies, 1994; Levy & Bonnie, 2004; Ochman et al., 2000). The human gut, mainly the colon, is considered a conducive environment for ARG transfer between bacteria due to the highly nutrient-enriched, densely populated community of bacteria ($\sim 10^{12}$ bacteria per gram), presence of food particles, and the intestinal surface where adherence and conjugal mating can take place (Shoemaker et al., 2001). Two different studies have shown that plasmids encoding beta-lactamase were transferred in infants' gut microbiota from *Klebsiella pneumoniae* to *Escherichia coli* (Bidet et al., 2005) and *E. coli* to *E. coli* (Karami et al., 2007) following treatment for urinary tract infections. A

longitudinal evolutionary study showed that *E. coli* lineages co-existing in the gut exchanged multi-drug resistant plasmids encoding beta-lactamase, and these resistant transconjugants maintained their plasmid for months in the absence of antibiotic treatment (Gumpert et al., 2017). It suggests that gut resident bacteria carrying ARGs on transferable plasmids can spread to other bacteria with and without antibiotic treatment. With the advances in genome sequencing, multiple studies have been conducted to analyze for the presence of ARGs in the gut microbiota using computational approaches (Ogilvie et al., 2012; Sommer et al., 2009). A study reported the presence of ARGs against 11scherrent antibiotics in a set of 252 fecal samples collected from volunteers from three countries, Denmark, Spain, and the United States (Forslund et al., 2013). These findings show how much ARGs are prevalent among the human population worldwide.

Most of these studies have elucidated the potential of the gut microbiome to harbor and disseminate ARGs among its community members either by directly culturing AR bacterial isolates from fecal samples or by performing PCR on isolates against specific ARGs or by conducting metagenomic analyses on fecal samples. However, we still do not know mechanistically how conjugative plasmids mediate ARG transfer in the complex milieu of the gut microbiota.

Models for Studying Plasmid Mediated Antibiotic Resistance in Gut Microbiota

Different in-vitro, in-situ, and in-vivo experimental approaches have been taken to investigate the plasmid-mediated transfer of antibiotic-resistant genes in gut microbiota.

In vitro Studies

In vitro studies have shown that donor bacteria isolated from humans and animals may be conjugated with recipient bacteria of interest in the lab to determine the ability and frequency of conjugal transfer (Trieu-Cuot et al., 1987; Trobos et al., 2009). The effect of different parameters on conjugation, such as plasmid origin, selection pressure, donor-recipient ratio, and incubation time, can be easily studied in lab-based experiments (Gama et al., 2017). Most conjugation experiments are conducted by mating donor and recipient bacteria in a broth media (Corliss et al., 1981) or on a solid surface like a piece of filter paper (Poole et al., 2017). Thus, in-vitro studies have been critical to investigating the plasmid-mediated transfer of ARGs. However, it is hard to apply these findings in the human gut microbiota due to the many complex microbial and host factors present.

In situ Systems

In situ systems like continuous flow systems, batch fermenters, and CoMiniGut to mimic gut conditions have been used to study the transfer of antibiotic resistance through conjugation. (Gibson & Fuller, 2000; Schjørring & Krogfelt, 2011; Smet et al., 2011). Smet et al. 2010 performed conjugation using a continuous flow culture system where fecal samples from healthy volunteers were mixed with a donor bacterium (Smet et al., 2011). These authors observed a transfer of plasmid-mediated antibiotic resistance gene (blaTEM-52) from an avian-origin *E. coli* strain to a human commensal *E. coli* with and without antibiotics. However, antibiotic selection pressure increased plasmid transfer from the donor to the recipient (Smet et al., 2011). Another

study was conducted using a continuous colonic fermenter to study plasmid transfer among bacteria in infant fecal samples. The authors observed the transfer of a conjugative plasmid from *Enterococcus faecalis* to *Listeria monocytogenes* and commensal bacteria of infant fecal samples (Haug et al., 2011). Similarly, Anjum et al. observed plasmid transfer from *E. coli* carrying a conjugative plasmid to bacteria of human fecal samples grown in a CoMiniGut system developed to mimic the human gut (Anjum et al., 2019; Wiese et al., 2018). These models are good tools for studying antibiotic resistance by simulating the gut conditions; however, due to considerable differences in the growth conditions of gut microbial communities, these in-vitro models are missing the complexity of the human gut and host factors' impact on conjugation.

In vivo Animal Models

It is challenging to study antibiotic resistance in the human gut due to the complex host environment. However, researchers have used different in vivo mouse models to validate in vitro results and investigate the effect of host factors on conjugation-mediated antibiotic resistance spread in the gut.

Germ-free Mouse Model

Germ-free mice are commonly used to study the plasmid transfer among the specific donor and recipient bacteria in the host gut. Different studies have shown the transfer of plasmid-encoded ARGs from *Enterococcus faecalis* to *Escherichia coli* and *Enterococcus faecium* to human *Enterococcus* in the gut of gnotobiotic mice (Doucet-Populaire et al., 1992; Moubareck et al., 2003). This model provides a controlled

environment with a largely intact physiological environment and immune system functions with which to observe conjugation frequency in the gut. However, the germ-free model lacks any microbes and thus becomes very simplistic and does not reflect the complexity of the host (Pollard & Sharon, 1970; R. Ducluzeau, 1984). Indeed, many physiological functions of the host are absent in the absence of the microbiome, and cell development and immune functions are dramatically altered (Cheng et al., 2014; Hooper et al., 2012; Hooper & Gordon, 2001).

Antibiotic-treated Mouse Model

Antibiotic-treated mice are also used to study the transfer of plasmid-mediated ARGs among bacteria in the gut. A study reported AR plasmid transfer from a clinical isolate of *K*. *pneumoniae* MGH78578 to recipient *E*. *coli* MG1655 in the mouse gut. Mice were treated with 0.5 gram/liter of streptomycin sulfate before inoculation of donor and recipient bacteria (Schjørring et al., 2008). Different antibiotics can be used to eliminate certain bacteria, such as kanamycin and streptomycin against facultative anaerobic bacteria, especially *Enterobacteriaceae*, clindamycin against anaerobes, and ampicillin for broad intestinal microbial communities (D. Hentges et al., 1984; D. J. Hentges et al., 1985). The antibiotic-treated mouse model—considered a disassociated mouse model—also loses colonization resistance by disrupting the gut microbiota, which benefits colonizing foreign bacterial strains in the gut (Freter, 1983). Both germ-free and antibiotic-treated mouse models have the advantage of studying the transfer of ARGs in a simple bacterial ecosystem; however, those do not mimic well the gut of a healthy person (Kennedy et al., 2018). Antibiotic-treated mice

mimic the clinical patients' situation where constant exposure to broad-spectrum antibiotics can lead to the persistence and sharing of ARGs among resident bacteria.

Transplanted Mouse Model

Mice transplanted with bacterial flora of human and other animal species could be used to address different ecology and metabolic aspects of the gut (Hirayama, 1999). Faure et al., 2010 used rats transplanted with human feces to study the plasmid-mediated transfer of β-lactamase (*bla*$_{\text{CTX-M-9}}$) from animal-origin *Salmonella* to foreign recipient *E. coli J5* and resident *Enterobacteriaceae* of human gut microbiota. These authors observed the transfer of β-lactamase encoding plasmid to recipient *E. coli J5* from donor *Salmonella* bacterium. However, no transconjugants were observed in bacteria of the human gut microbiota with or without antibiotic treatment (Faure et al., 2010). Though the transplanted mouse model is an excellent tool to mimic the human gut, it is challenging to transfer and maintain the majority of human gut bacterial communities in rodents. Changes in the gut microbiota are likely to occur during and after inoculation (Kibe et al., 2005).

Human Studies

Trobos et al. 2009 conducted a small study where nine human volunteers were fed with a pig-derived *E. coli* donor strain (carrying a sulfonamide resistant conjugative plasmid) and a human-derived recipient *E. coli* (rifampicin-resistant). These authors observed the transfer of the sulfonamide-encoded plasmid from the donor *E. coli* to the recipient *E. coli* in the human feces (Trobos et al., 2009). They also found that the

transferred plasmid in transconjugant bacteria differed from the plasmid given to the study group of people, suggesting that it came from the person's own gut microbiota (Trobos et al., 2009). This study addresses the possibility of conjugation-mediated spread of ARGs in the human gut; however, due to the low frequency and relying primarily on the ability to culture these bacteria, the authors could not observe plasmid spread among the diverse bacterial population of the gut.

Despite these successes in developing models for the study of conjugative transfer, it is crucial to consider which question is being addressed while choosing the model. These models allow for observing the phenomenon of plasmid transfer among gut bacterial communities. Germ-free mice could be used for bacteria of interest in the gut without having any other bacterial community. Antibiotic-treated mice can be used to study the conjugation-mediated antibiotic resistance in the intestine after disruption in the gut microbiota after antibiotic treatment. Mice with conventional microbiota could be used to study AMR spread; however, the target microbial population in these cases is different from what is present in an intact human gut microbiota. Thus, all these mice transplanted with human fecal microbiota are the best choice of models that most closely mimic the human gut and study the plasmid-mediated spread of antibiotic resistance in a human-derived microbial community.

Previously, most studies used a simple model to study conjugation due to the inability to track these genes on the plasmid. Even though some of the studies used natural gut microbiota mice, the scope of investigating plasmid-mediated ARGs was

limited (Goon et al., 2003; Ronda et al., 2019; Stecher et al., 2012). Most of these studies were conducted using culture, PCR, and restriction enzyme digestion methods. Additionally, the most significant challenge of in-vivo models was reproducibility due to variation in the gut microbiota (Ott et al., 2020). Therefore, there is a dire need to build a tractable mouse model to study the spread and emergence of antibiotic resistance in the gut microbiota. We aim to create a mouse model and conjugation system that can give us insight into primary transfer and secondary transfer from a commensal donor strain. In addition, the persistence of those plasmids transferred to new bacteria.

Rationale

Conjugation, a form of HGT, is considered one of the major mechanisms driving genomic diversity among bacteria due to the frequent sharing of different genes between diverse community members (Ochman et al., 2000). Though conjugation was discovered more than a half-century ago, we still do not understand the transfer rate of ARGs and the key mechanistic factors that drive this transfer of ARGs among bacteria. There is little known about the baseline transfer frequency of ARGs among bacteria in the human and animal gut, which is known as a reservoir of ARGs (Salyers et al., 2004). Challenges that prevent these studies include; 1) inability to track donor and recipient bacteria in a complex microbial community, 2) simultaneous occurrence of other DNA sharing phenomena (transformation, transduction), 3) poor colonization of the mammalian gut by engineered laboratory-modified bacterial strains, and 4) difficulty in identifying and characterizing culturable and non-culturable recipient bacteria from fecal samples. Therefore, in the human gut microbiota, questions related to the transfer

rate of ARGs, mechanistic drivers of conjugation, potential reservoir bacteria of ARGs, plasmid adaptation, and fitness costs in recipient bacteria remain unaddressed. As a result, there is no effective strategy to mitigate the spread of ARGs through HGT in the gut.

We aim to build a robust and tractable human microbiota transplanted mouse model system to investigate the emergence and spread of antibiotic resistance through horizontal gene transfer in the gut microbiota. This study specifically examined the conjugation-mediated spread of broad host range plasmids encoding ARGs among enteric bacteria using both *in-vitro* and a mouse model transplanted with adult human gut microbiota. Findings from this study will be applicable to a broad range of AR bacteria carrying ARGs on mobile elements and establish fundamental knowledge for the study of other mechanisms of HGT in the gut microbiota. The study will provide both qualitative and quantitative assessment of plasmid-mediated transmission of antibiotic resistance in a diverse microbial community of the humanized mouse gut. The model can be used to investigate plasmid fitness costs which will guide finding novel genomic targets to develop new drugs and anti-conjugative strategies to block the spread of AR in the gut microbiota and environment. Quantification and mathematical modeling of plasmid-mediated resistance events; such modeling will help us understand the spread and emergence of AMR among commensal and pathogenic bacteria in humans. This work will also lay the groundwork for screening clinically relevant AR bacteria carrying transferrable ARGs in our developed mouse models. The establishment of this tractable mouse model will help study the effect of various external factors such as antibiotic

overuse, heavy metals exposure, and clinically significant xenobiotics on the transfer of ARGs among the gut microbial community.

Specific Aims

We proposed the following Specific Aims:

Specific Aim 1:

To determine the broad host range conjugative RP4 plasmid transfer to multiple enteric commensal, pathogenic and environmental bacterial strains.

Under this aim, these hypotheses have been addressed.

i. The RP4 would invade multiple recipient strains, including clinical, commensal, and environmental bacterial strains
ii. The RP4 plasmid transfer frequency would be greater among more closely phylogenetically related donor and recipient strains.
iii. The selection of donor strain by ampicillin treatment would increase the transfer frequency among all recipient bacteria.
iv. Broad host range plasmid can adapt to the naïve host commensal gut *E. coli LM 715-1.*

Specific Aim 2:

To assess the persistence of donor bacteria carrying RP4 plasmid in a transplanted mouse model without any selection pressure.

Under this aim, this hypothesis has been addressed.

i. Human derived & mouse gut-adapted commensal *E. coli* LM715-1 donor strain will colonize the gut and persist long enough to facilitate conjugation without any antibiotics treatment.

Specific Aim 3:

To study the plasmid-mediated spread of antimicrobial resistance in a tractable mouse model transplanted with human gut microbiota. Under this aim, these hypotheses have been addressed.

i. An engineered commensal *E. coli* LM715-1 donor strain will spread the RP4 plasmid among gut microbial community members by conjugation.
ii. Transconjugants will be maintained in the gut microbial community without applying selection pressure

Chapter 2 summarizes *in-vitro* experiments to determine primary and secondary frequencies of a broad host range plasmid RP4 to multiple naïve host bacteria *Citrobacter rodentium, Salmonella typhimurium, Klebsiella pneumoniae, Pseudomonas putida*, *Vibrio cholerae,* and three different strains of *E. coli*. The effect of antibiotic ampicillin on RP4 plasmid transfer frequency among bacteria and the persistence of RP4 plasmid in donor strain *E. coli* LM715-1 have been discussed.

Chapter 3 summarizes *in-vivo* experiments to develop a tractable mouse model transplanted with adult human gut microbiota. It includes validating engineered fluorescently labeled commensal donor strain *E. coli* LM715-1 colonized in the transplanted mouse gut. The persistence and spread of broad RP4 plasmid in the gut microbiota using culture, PCR, and flow-cytometry coupled 16S gene sequencing.

Chapter 4 summarizes the main findings of this study and discusses the possible studies to investigate the spread of ARGs driven by different conjugative plasmids in human gut microbiota with and without antibiotic selection pressure.

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Chapter 2: Conjugative plasmid-mediated transfer of antibiotic resistance genes to commensal and multidrug-resistant enteric bacteria *in vitro*

Abstract

Many antibiotic-resistant (AR) bacteria carry antibiotic resistance genes (ARGs) on conjugative plasmids transferable to commensals and pathogens. We seek to understand the plasmid-mediated spread of ARGs among human gut microbiota. Our objective was to determine the ability of multiple enteric bacteria to acquire and retransfer a broad host range plasmid encoding beta-lactamase resistance in *in vitro* conjugation experiments. The donor strain was human-derived commensal *Escherichia coli* LM715-1 carrying a red fluorescent protein gene on the chromosome and a green fluorescent protein (GFP)-labeled broad host range RP4 plasmid with *amp*R, *tet*R, and *kan*R genes. Recipients were strains isolated from humans, including Escherichia (*E. coli* MG1655, *E. coli* Dec5α, *Vibrio cholerae*, *Pseudomonas putida, Pseudomonas aeruginosa, Klebsiella pneumoniae, Citrobacter rodentium,* and *Salmonella* Typhimurium carrying chromosomally encoded rifampicin resistance. We performed *in vitro* matings using different combinations of donor and recipient strains and determined conjugation frequencies using selective media. Transconjugants were confirmed using fluorescence microscopy and PCR to detect the GFP gene.

The RP4 plasmid was transferred from *E. Coli* LM715-1 to *C. rodentium, S.* Typhimurium*, K. pneumoniae, P. putida*, *V. cholerae,* and three *E. coli* strains. However, plasmid transfer frequencies (T.F.s) differed between specific donor-recipient pairings (10^{-2} to 10^{-8}), including among *E. coli* recipients. Similarly, the RP4 plasmid was transferred back to *E. coli* LM715-1 from the recipient strains with T.F.s ranging from 10^{-2} to 10^{-7}. Plasmid T.F.s were not correlated with phylogenetic donor-recipient

relatedness. A serial passage plasmid persistence assay showed loss of the plasmid over time in the absence of antibiotic, indicating that the plasmid imposed a fitness cost on its host; however, plasmid-bearing cells persisted in the population for at least ten transfers. The results reveal that the RP4 plasmid can transfer to multiple clinically relevant bacterial species of the gut microbiota without antibiotic selection pressure; however, transfer frequency varies among donor-recipient pairings.

Introduction

Antimicrobial-resistant pathogens are becoming a leading cause of death worldwide. A recent study estimated that approximately 4.95 million deaths occurred globally due to drug-resistant bacteria in 2019 (Murray et al., 2022). Similarly, the Centers for Disease Control and Prevention (CDC) reported that antibiotic-resistant (AR) pathogens in the USA caused ~ 3 million infections and 36 thousand deaths during 2019 (CDC, 2019). It has been estimated that if antibiotic resistance concerns are not adequately addressed in the near future, AR bacteria are expected to become a leading cause of death, estimated at 10 million each year globally, and could pose a significant economic burden of up to 100 trillion dollars by 2050 (O'Neill, 2016). These findings highlight the fact that the emergence of AR pathogens has become a major global health challenge.

Broad host range (BHR) conjugative plasmids capable of transferring to a wide range of closely and distantly related bacteria are the leading cause of ARG spread among multiple species of bacteria (Aminov, 2011; Jain and Srivastava, 2013; von Wintersdorff et al., 2016; Schlechter et al., 2018), and are divided into different incompatibatibility groups based on their stability during the conjugation process (Datta and Hedges, 1973; Rozwandowicz et al., 2018). BHR plasmids associated with the IncP group, especially the RP4 plasmid (IncP-1α), have shown massive potential for spreading and transferring ARGs to new bacterial hosts (Popowska and Krawczyk-Balska, 2013; Klümper et al., 2015; Lu et al., 2017). The RP4 plasmid (IncP-1α) isolated from human clinical samples is one of the most highly studied plasmids since its

discovery in the 1970s (Datta and Hedges, 1971; Pansegrau et al., 1994). The RP4 plasmid has various genetic features to maintain self-replication, transmissibility, and survival in a wide range of gram-negative and gram-positive bacteria (Barth and Grinter, 1977; Adamczyk and Jagura-Burdzy, 2003). Previous studies have shown the transfer of the RP4 plasmid from laboratory strains of *E. coli* to bacteria isolated from soil (Johannes Sørensen et al., 1999; Musovic et al., 2014; Klümper et al., 2015), sewage, and activated sludge (Geisenberger et al., 1999; Soda et al., 2008; Liu et al., 2019). In a recent study, Heß et al. (2022) reported that in microcosm experiments, RP4 plasmid transferred to multiple bacterial strains from three donor *E. coli* strains (Heß et al., 2022). These past studies on RP4 conjugation were mainly conducted using laboratory or environmental donor *E. coli* strains. However, a major knowledge gap exists in understanding the transfer of a broad host range plasmid such as the RP4 to other bacteria of the human gut microbiota.

Most of the antibiotic-resistant bacteria identified to date are either enteric pathogens or capable of residing in the human gut (CDC., 2019; Murray et al., 2022). Bacterial species belonging to families *Enterobacteriaceae*, *Pseudomonadaceae,* and *Vibrionaceae* are among the leading causes of antibiotic-resistant infections worldwide (Murray et al., 2022). Compared to other bacterial families, members of the *Enterobacteriaceae* serve as hosts for the greatest number of antibiotic-resistance encoding plasmids (Shintani et al., 2015) including both broad and narrow host range plasmids (extensively reviewed by Carattoli, (2009) and Rozwandowicz et al., (2018)). To date, 28 different incompatibility (Inc) groups of plasmids in *Enterobacteriaceae* and

14 in *Pseudomonadaceae* have been reported including the RP4 plasmid (Shintani et al., 2015). However, little is known about the transfer frequencies and persistence of these BHR plasmids among enteric bacteria belonging to the families *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonadaceae*. These clinically relevant AR bacteria live in the human gut, a complex environment where they have great potential to share resistance-gene-bearing plasmids with other commensal and pathogenic bacteria and to convert recipients into potential emerging drug-resistant bacteria. Furthermore, plasmid acquisition imposes a fitness cost on recipient bacteria that determines plasmid persistence in a complex microbial community (San Millan and MacLean, 2017; Li et al., 2020). Thus, it is difficult to develop strategies to mitigate the spread of ARGs without understanding the frequency of spread and the persistence of BHR plasmids among these clinically relevant bacteria. Therefore, we investigated the RP4 plasmid transfer from a commensal human gut *E. coli* bacterium to other closely and distantly related enteric bacteria, which have not been examined previously.

In this study, we determined the potential for primary spread of a broad host range plasmid RP4 from commensal gut *E. coli* to multiple naïve host bacteria of the gut microbiota and, once transferred, the subsequent secondary transfer from those hosts back to plasmid-free commensal *E. coli* (Figure 2.1). We hypothesized that i) the RP4 plasmid would transfer to many species of *Gammaproteobacteria* in the absence of antibiotic treatment, ii) the different pairings of donor and recipient strains would influence plasmid transfer frequency, and iii) the RP4 plasmid can persist in a naïve host without antibiotic selection pressure. To address these hypotheses, we chose a

sample set of clinically relevant bacterial species of the *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonadaceae* families, to act as RP4 donors and recipients (Table 1). In these experimental transfers, we used a commensal Uropathogenic (UPEC) *E. coli* LM715-1 originally isolated from a human infant gut microbiota as the primary donor and recipient strain. RP4 plasmid persistence in this strain was measured using a serial transfer experiment. Overall, our study showed that the i) the RP4 plasmid could transfer to multiple bacterial species effectively, ii) the specific donor-recipient pairing affected the transfer frequency of the plasmid, and iii) the RP4 plasmid imposed a fitness cost on a naïve host but persisted in a small proportion of the population.

Materials and methods

Media, chemicals, and reagents

Luria agar (Acumedia, Lansing, MI), LB-Miller Broth (IBI Scientific, Dubuque, IA), MacConkey agar (Neogen, Lansing, MI), and Bacteriological Agar (Neogen) were used to grow donor, recipient, and transconjugant bacteria. Bacterial culture experiments for *Salmonella enterica* serovar Typhimurium*, Citrobacter rodentium* ATCC 51459*, Klebsiella pneumoniae* IA565*, Vibrio cholerae* O1 biotype El Tor C6706str2*, Pseudomonas aeruginosa, Pseudomonas putida* KT2440, *Escherichia coli* LM715-1, *Escherichia coli* MG1655, and *Escherichia coli* DEC 5a TW00587 were performed aerobically at 37°C either in an incubator (plates) or on a shaker at 150 rpm (liquid cultures). We used antibiotics at the following concentrations, ampicillin (50 µg/ml), kanamycin (50 µg/ml), chloramphenicol (20 µg/ml), tetracycline (15 µg/ml), rifampicin (20 µg/ml), and ceftriaxone (4 µg/ml) throughout the study where required for specific selection. We used antibiotic combinations with the same concentrations to select donor, recipient, and transconjugant bacteria, depending on the donor and recipient bacterial strains used in a specific experiment. The use of specific antibiotics is documented for each experiment in the results section. We prepared phosphate buffered saline (1X PBS) using following recipe (NaCl: 137 mM, KCl: 2.7 mM, Na2HPO4: 4.3 mM, KH2PO4: 1.4 mM). The sources of antibiotics were ampicillin (Millipore Sigma, Burlington, MA), kanamycin (Fischer Scientific, Fairlawn, NJ), chloramphenicol (M.P. Biomedicals, Solon, OH), tetracycline (M.P. Biomedicals, Solon, OH), rifampicin (Alfa Aesar, Tewksbury, MA), and cefotaxime (Alfa Aesar, Tewksbury, MA).

Isolation and characterization of a human-derived donor bacterial strain

Human-derived, mouse-adapted commensal *E. coli* LM715-1 was isolated from a mouse that carried a human infant fecal microbiota transplanted to germ-free mice and passaged vertically to subsequent generations in the Mansfield laboratory colony (Moya and Mansfield, unpublished). This is a Biosafety Level 2 colony managed to prevent any acquisition of bacteria extraneous to the defined human source (Brooks et al., 2017). These mice were transplanted with fecal slurries of children recruited into the Isle of Wight "Third Generation Study" under the U.K. ethics approval numbers 09/H0504/129 (22 December 2019), 14/SC/0133 (22 December 2019), and 14/SC/1191 (15 November 2016).

The Dneasy Blood & Tissue Kit (QIAGEN, Catalogue. No. / ID: 69504, Germantown, MD) was used to extract genomic DNA of an *E. coli* colony isolated from this mouse. Next, we performed multilocus sequence typing (MLST) of the *E. coli* LM715-1 strain using a scheme of seven housekeeping genes; *aspC, clpX, fadD, icdA, lysP*, *mdh*, and *uidA*. This MLST scheme can characterize pathogenic *E. coli* isolates using an existing database of the above seven housekeeping genes. The primers and protocols for MLST available on the website[1] were used to amplify these genes. Sanger sequencing of these amplicons was performed at Michigan State University Research Technology Support Facility (MSU RTSF) Genomics Core. Sequence analysis was done using an online tool available on the database website[2] of Shiga-toxin-producing *E. coli* (STEC). To determine antibiotic resistance-based selection markers on this *E. coli* LM715-1 isolate to conduct conjugation experiments, we performed antibiotic resistance profiling of this *E. coli* isolate against the following antibiotics at

specific concentrations; ampicillin (50 µg/ml), kanamycin (50 µg/ml), chloramphenicol (20 µg/ml), tetracycline (15 µg/ml), Rifampicin (20 µg/ml), and ceftriaxone (4 µg/ml). These specific antibiotic concentrations were selected based on the resistance markers present on the RP4 plasmid, donor, and recipient bacteria and used throughout the study.

Creation of a fluorescently labeled commensal donor strain

To create a trackable donor strain, we obtained a chromosomal insertion toolbox designed by Schlechter et al. (2018) (Schlechter et al., 2018) from the Addgene plasmids repository (Watertown, MA)[3], which consists of a Tn7-based 43scheri-unstable delivery suicide plasmid (pMRE-Tn7-155 plasmid, Addgene Plasmid #118569). We inserted a genomic cassette (mScarlet fluorescent protein gene, KanR, and CamR) into the *E. Coli* LM715-1 bacterial chromosome using a pMRE-Tn7-155 delivery plasmid following the method described by Schlechter et al. (2019) (Schlechter and Remus-Emsermann, 2019). The insertion and activity of the fluorescence gene and antibiotic resistance marker genes were confirmed using fluorescent microscopy, selection on LB agar with chloramphenicol (20 µg/ml) and kanamycin (50 µg/ml), and colony PCR (see below) for the mScarlet fluorescent marker gene.

E. coli MG1655 bearing a broad host range (BHR) RP4 plasmid labeled with a green fluorescent protein marker and antibiotic resistance genes *amp*R, *tet*R, and *kan*R was the kind gift of Dr. Barth Smets' research group at the Technical University of Denmark (Klümper et al., 2015). We mated the fluorescently labeled commensal strain

E. coli LM715-1 (mScarlet, *cam*R, *kan*R) as a recipient strain with donor *E. coli* MG1655 (RP4::GFP, *amp*R, *tet*R, *kan*R) using the filter conjugation method described below. A single colony from the transconjugant selective medium (Luria agar containing chloramphenicol and ampicillin) was picked and streaked on a fresh Luria agar plate containing chloramphenicol and ampicillin, and plates were incubated aerobically at 37°C overnight. After streaking twice for purity from single colonies, a bacterial lawn of *E. coli* LM715-1 carrying the RP4 plasmid was suspended in LB-Miller broth containing 30% glycerol and stored at -80°C. The donor strain was re-streaked from this stock culture for all experiments.

Donor and recipient strains

This study examined the transfer of the broad host range RP4 plasmid in clinically relevant human-derived enteric commensal and pathogen strains. We chose a diverse collection of recipient strains, including *Salmonella enterica* serovar Typhimurium*, Citrobacter rodentium* ATCC 51459*, Klebsiella pneumoniae* IA565*, Vibrio cholerae* O1 biotype El Tor C6706str2*, Pseudomonas aeruginosa, Pseudomonas putida* KT2440, commensal human gut *Escherichia coli* LM715-1, Diarrheagenic *Escherichia coli* DEC 5a TW00587, and K-12-derived *Escherichia coli* MG1655 (Table 1). Three different strains of *E. coli* were included to determine differences in plasmid transfer frequencies within a single bacterial species; Diarrheagenic *E. coli* Dec 5a belongs to the sequence type *ST-73*, commensal *E. coli* LM715-1 belongs to *ST-259,* and *K-12-derived E. coli* MG1655. To identify antibiotic resistance markers for performing conjugation assay, we conducted antibiotic resistance profiling of these strains by

growing them in Luria broth with a panel of antibiotics with the concentrations specified above. We found that none of the bacteria were resistant to Rifampicin. Therefore, we selected for rifampicin-resistant spontaneous mutants as a means of positively identifying RP4 recipients. We isolated rifampicin-resistant spontaneous mutants of all recipient bacteria by streaking on Luria agar plates containing Rifampicin (20 µg/ml) and incubating overnight at 37°C aerobically. Single mutant colonies were transferred to another Luria Agar plate containing Rifampicin (20 µg/ml) and incubated again overnight at 37°C aerobically. After the third repetition of streaking of single mutant colonies on the Luria agar plate with Rifampicin (20 µg/ml), these rifampicin-resistant colonies were stored in LB-Miller broth containing 30% glycerol at -80°C. Before every conjugation experiment, we freshly grew donor and recipient bacteria from the freezer stock to confirm antibiotic resistance markers. We also assessed the antibiotic resistance for cross selections of a donor, recipient, and transconjugant bacteria using LB agar plates with the combination of different antibiotics. We used Rifampicin only when no other antibiotic resistance marker was present on donor and recipient bacteria (Table 1).

In vitro conjugation experiments to confirm plasmid transfer

The plasmid transfer frequencies for all pairs of donor and recipient bacteria were assessed using a filter-based conjugation method (Trieu-Cuot and Courvalin, 1985). Briefly, both donor and recipient bacteria were grown overnight in Luria broth with respective antibiotics as indicated in the experimental design at 37°C and 150 rpm. 750µL of each donor and recipient culture were mixed, and a pellet was obtained after washing twice with phosphate buffered saline (1X PBS) at 10,000 × g for 5 minutes

(Eppendorf™ centrifuge 5415D, F-45-24-11 rotor, Hamburg, Germany). After resuspending the pellet in 100μL of PBS, 20μL of suspension was spread on each of four to five separate filters on a Luria agar plate with or without ampicillin (50 μg/ml). Cellulose filter papers (Whatman catalog# 1001-125, Maidstone, U.K.) were cut with a sterile scissor into small pieces of 2 cm X 2 cm). Filters were placed on Luria agar plates with or without ampicillin (50 μg/ml). After incubating plates at 37°C overnight, each filter was placed in a 1.5 mL microcentrifuge tube, 1000 μL of PBS was added, and the tube was vortexed for 60 seconds before serially diluting the suspension. We spread 100 μL of these dilutions on each of three separate Luria agar plates with antibiotics selecting separately for the donor, recipient, and transconjugants. Plates were incubated aerobically overnight at 37°C; colonies were counted to calculate plasmid transfer frequency using the formula $f = \frac{T}{R+T}$ (plasmid transfer frequency = transconjugants / (transconjugants + recipients)).

Detection of fluorescence in donor and transconjugant bacteria

Bacterial cells from donor and transconjugant selective media plates were suspended in PBS and centrifuged at 10,000 × g for 1 minute at room temperature in an Eppendorf™ centrifuge 5415D with F-45-24-11 rotor (Eppendorf™, Hamburg, Germany). The pellet was resuspended in 100 μL of PBS. We used the agarose pad method to obtain images of fluorescent bacteria (donor and transconjugants) (Skinner et al., 2013). Briefly, a 1% agarose solution was poured on a plain surface bordered with microscope slides to achieve agar pads of the even thickness. After the agarose solidified, coverslip-sized pads were cut using a sterilized scalpel and placed on another

microscope slide. 2-5 µL of bacteria suspended in PBS was spread over the agar pad and covered with a coverslip. A Nikon Eclipse N*i*-U upright microscope (Nikon, Tokyo, Japan) was used with bright field, GFP, and RFP filters, to record and analyze the fluorescent bacteria at magnifications of 20X and 40X. For rapid screening of fluorescent bacterial colonies, we took a small number of bacteria directly from the individual colonies grown on the plate using a sterile toothpick and mixed them with 10 µL of PBS or deionized water on a microscope slide. After placing a coverslip and air-drying the slide for ten minutes, we observed fluorescent mScarlet and GFP expression in the donors, recipients, and transconjugants under the microscope.

Confirmation of the plasmid in transconjugant bacteria using colony PCR

Colony PCR was performed to confirm the presence of RP4 plasmid carriage by the transconjugant bacteria using primers for the GFP marker located on the plasmid (Table 2). Colonies from transconjugant selective media plates were sampled using a sterilized toothpick and mixed with a 25 µL reaction mixture in a PCR tube. Each reaction mixture contained 2.5 µL 10X Buffer ($MgCl_2$ free), 2.5 µL $MgCl_2$ (50 mM), 2.0 µL dNTPs (2.5 mM), 0.25 µL Taq DNA polymerase (New England BioLabs, Woburn, MA), and 1.0 µL both forward and reverse primers (25 pM/µL); final volume was adjusted with sterile distilled water up to 25 µL. DNA amplification was done in a thermocycler (Eppendorf™, Model # AG 22331, Hamburg, Germany) using an initial denaturation step at 95°C for 10 min followed by 30 cycles of amplification (denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min), and ending with a final extension at 72°C for 5 min. The PCR product was visualized by

agarose (1.5%) gel electrophoresis to confirm the predicted 181 base pair band for GFP present in the transconjugant colonies.

Bacteriocin assay

A cross-streaking method was used to determine the bacteria-killing effect (Williston et al., 1947). We streaked the Luria agar plate with *P. aeruginosa* using a sterilized cotton swab from fresh overnight culture and incubated the streaked plate for 24 hours. The next day, we cross-streaked the plate with indicator strain *E. coli* LM715-1 using a sterilized cotton swab from fresh overnight culture and incubated it for 24 hours. The cross-streaked plate was photographed for bacteria-killing activity on the following day.

Plasmid persistence assay

In order to test the ability of the RP4 conjugative plasmid to persist long-term in its bacterial host, we streaked donor *E. coli* LM715-1 (mScarlet *cam*R, *kan*R, and RP4::GFP *amp*R *tet*R *kan*R) from the frozen stock onto Luria agar plates containing ampicillin (50 µg/ml) and chloramphenicol (20 µg/ml) and incubated the plates aerobically overnight at 37°C. A single colony was harvested and inoculated on the following day into 3mL of Luria broth containing ampicillin (50 µg/ml) and chloramphenicol (20 µg/ml) and incubated aerobically at 37°C for 18 hours in a shaker at 150 rpm. Next, we inoculated 30 µL of this overnight culture into each of 8 tubes containing 3 mL Luria broth; four tubes had no antibiotic, and the other four tubes contained ampicillin (50 µg/ml). These tubes were incubated aerobically overnight at

37°C in a shaker at 150 rpm. On each of the following ten days, the overnight culture from each tube (30 µL) was transferred into 3 mL fresh Luria broth (1:100 dilution), and ampicillin was added to the respective tubes at 50 µg/mL. The optical density (600 nm) of all cultures was measured every 24 hours. We diluted 100 µL of overnight culture from each tube serially in ten-fold steps and poured 100 µL of these dilutions on each of three separate Luria agar plates with specific antibiotics on days 1, 2, 3, 5, 7, and 10. Luria agar plates containing chloramphenicol (20 µg/ml) were used to select for total bacteria with and without plasmid, and Luria agar plates containing ampicillin (50 µg/ml) and chloramphenicol (20 µg/ml) were used to select for bacteria bearing plasmid. These plates were incubated aerobically overnight at 37°C; colonies were then counted to calculate the proportion of plasmid-bearing cells using the formula $p = \frac{D^+}{D^+ + D^-}$ (proportion of plasmid-bearing cells = plasmid-bearing cells (donors) / total bacteria with and without plasmid).

Statistical Analysis:

We used an F-test to determine the equality of variances among replicates in the primary and secondary transfers of RP4 plasmid from donor to recipient pairings. Based on the results from the F-test, a *t*-test was chosen to calculate statistical significance among treatment groups. An unpaired two-sample *t*-test (independent samples *t*-test) was used to compare statistical differences in mean values of two groups having equal variance. If an unequal variance was observed between groups, Welch's t-test was used to calculate p-values and significance levels (Welch, 1938). In all statistical analyses, we used $p \leq 0.05$ as the cutoff level to determine significance. We also

performed statistical analyses after log transformation of the data. However, there were no differences in identifying significant comparisons between groups when compared to using the raw data that is, no additional significant comparisons were detected, although in most cases, these log transformation analyses produced p values that were lower than 0.05. Thus, all the data and analyses presented here are based on the original data.

Results

Characterization of donor and recipient strains

To study the conjugation-mediated transfer of ARGs, we developed a set of marked donor and recipient strains to perform traceable *in-vitro* conjugation experiments to document transfer and its frequency. We used a fluorescently labeled commensal donor strain human gut *E. coli* LM715-1 carrying the mScarlet fluorescent protein gene and chloramphenicol and kanamycin antibiotic markers on the bacterial chromosome. *E. coli* LM715-1 was characterized Uropathogenic (UPEC) *E. coli* strain based on the MLST scheme based on seven housekeeping genes; see method for more details. A broad host range plasmid of RP4 origin carrying green fluorescent protein and ampicillin, kanamycin, and tetracycline antibiotic resistance markers was transferred to the fluorescently labeled donor strain *E. coli* LM715-1. The differential antibiotic and fluorescence markers on the donor bacterial chromosome and plasmid allowed us to select and trace donors, recipients, and transconjugant bacteria using selective media and fluorescence microscopy. Next, we collected a diverse group of strains to test as potential recipients of the RP4 plasmid, including *S.* Typhimurium*, C. rodentium, K. pneumoniae, V. cholerae, P. aeruginosa, P. putida,* and three different *E. coli* strains, DEC 5a, LM715-1, and MG1655 belong to ST-73, ST-259, and K-12 groups (Table 1). All recipient bacteria also carried unique selection markers not present on the donor bacterial chromosome and RP4 plasmid to determine the selection of genetic markers for the donor, recipient, and transconjugant cells (Table 1).

The labeled RP4 plasmid transferred to multiple drug-resistant bacterial strains

To test if this broad host range RP4 plasmid carrying multiple antibiotic-resistant genes can transfer to a diverse set of clinical, commensal, environmental, and opportunistic bacterial strains, we performed *in vitro* conjugation experiments mating the mouse-adapted commensal human gut *E. coli* LM715-1 plasmid donor with multiple recipient strains. We found that the *E. coli* LM715-1 commensal donor strain successfully transferred the RP4 plasmid to multiple clinically relevant recipient strains (Figure 2.2), supporting the conclusion that the plasmid can effectively transfer to various bacteria belonging to the *Gammaproteobacteria*. However, when we performed a conjugation experiment with *P. Aeruginosa,* we found that this recipient killed the donor bacteria during incubation on filter paper for conjugation, suggesting that the *P. aeruginosa* strain carries a bacteriocin or other toxins. Further testing by cross-streaking the two strains on an agar surface supported this conclusion (Figure 2.2 B).

Next, we tested the hypothesis that the RP4 plasmid transfer frequency would be greater among phylogenetically closely related donor and recipient strains. Phylogenetic relatedness based on 16S rRNA has been studied in *Enterobacteriaceae* (*E. coli*, *C. rodentium, S.* Typhimurium*, K. pneumoniae*) (Brenner and Farmer, 2015; Zhang et al., 2015), *Vibrionaceae* (*V. cholerae*) (Farmer and Janda, 2015), and *Pseudomonadaceae* (*P. aeruginosa, P. putida*) (Garrity et al., 2015). We found that the transfer frequency of the plasmid was different for each donor and recipient combination regardless of phylogenetic relatedness (Figure 2.2). We performed a statistical analysis using an unpaired independent samples *t*-test to compare the mean values of transfer frequencies from one donor *E. coli* LM715-1 strain to all recipients. The mean self-

transfer frequency of donor *E. coli* LM715-1 to recipient *E. coli* LM715-1 was used as a reference value. It was observed that, except for *V. cholerae,* plasmid transfer frequencies were higher among more distantly related bacteria (*K. pneumoniae, P. putida*) than closely related bacteria (*E. coli* Dec 5a*, E. coli* MG1655, *C. rodentium, S.* Typhimurium*)*. These findings indicate that the transfer of the RP4 plasmid is not dependent on the phylogenetic relatedness of the donor strain to the recipient strain; thus, we rejected our hypothesis.

Next, we assessed the effect of the antibiotic ampicillin on the transfer frequency of the RP4 plasmid carrying the beta-lactamase gene from the commensal donor strain *E. coli* LM715-1 to different recipient bacterial strains. Based on the antibiotic resistance profiling of recipient bacteria, we divided them into two groups: susceptible to ampicillin (50 µg/ml) and resistant to ampicillin (50 µg/ml). We hypothesized that first, the presence of ampicillin would increase the transfer frequency among recipient bacteria susceptible to ampicillin, and second, ampicillin would not increase plasmid transfer frequency among those resistant to ampicillin. We used ampicillin (50 µg/ml) for selection pressure in these conjugation experiments. Overall, we found that ampicillin selection pressure increased plasmid transfer among all recipient strains except *K. pneumoniae* (Figure 2.3A). For the comparison of conjugation in the presence and absence of ampicillin, we performed an unpaired independent samples t-test between no antibiotic and antibiotic treatment groups. The RP4 plasmid transfer frequencies were significantly increased in ampicillin susceptible recipient strains *E. coli* MG1655 and *C. rodentium* exposed to ampicillin.

Similarly, a significant increase in the plasmid transfer frequency was observed in ampicillin-resistant *V. cholerae*. However, the presence of ampicillin significantly decreased plasmid transfer to *K. pneumoniae.* We also calculated the donor-recipient ratio for both groups treated with and without ampicillin during conjugation. We determined that antibiotic treatment increased the donor-recipient ratio among all bacteria except *V. cholerae* (Figure 2.3B). This outcome suggests that this dose of ampicillin was selecting for carriage of the plasmid in donor and transconjugant cells—however, the influence of the donor-recipient ratio on plasmid transfer frequency was not examined further.

All recipient bacterial strains can mediate secondary transfer of the BHR plasmid to the human commensal E. coli LM715-1 recipient strain.

After observing transfer of the BHR RP4 plasmid from one commensal *E. coli* donor strain to multiple bacterial strains, we tested whether these recipients of the plasmid could transfer it to a commensal *E. coli* strain by acting in turn as plasmid donors. Transconjugant bacteria from the previous experiment were used as donor strains including *S.* Typhimurium*, C. rodentium, K. pneumoniae, V. cholerae, P. putida,* and three different *E. coli* strains. The *E. coli* LM715-1 mScarlet labeled chloramphenicol resistant strain was used as a recipient strain in this series of experiments. We performed a statistical analysis using an unpaired independent samples *t*-test to compare the mean values of transfer frequencies individually from each donor strain to the recipient *E. coli* LM715-1 strain. The mean self-transfer frequency of donor *E. coli* LM715-1 to recipient *E. coli* LM715-1 camR was used as a

control to provide a reference value. We found that all transconjugant strains transferred the RP4 plasmid to the naïve *E. Coli* LM715-1 *cam*R recipient strain at frequencies that ranged from 10^{-2} to 10^{-7} (Figure 2.4A). These results show that this RP4 plasmid carrying antibiotic resistance genes can efficiently move among multiple bacterial strains in this *in vitro* model, and, once transferred, the original recipients can then serve as donors of the plasmid for further transfers. After determining the frequencies for these secondary transfers, we also compared plasmid transfer frequencies for each bacterial strain in the primary transfer, in which those strains acted as the recipient, and in the secondary transfer, in which the recipients of primary transfer acted as the donor (Figure 2.4B). As donors, *E. coli* DEC 5a, *S.* Typhimurium*, P. putida*, and *V. cholerae* showed higher plasmid transfer frequencies than they did as a recipient strain receiving the conjugative RP4 plasmid in the primary transfer. *E. coli* MG1655 showed higher plasmid acquisition in the primary transfer than did the *E. coli* LM715-1 when receiving RP4 from *E. coli* MG1655 in the secondary transfer. However, in terms of frequencies, *C. rodentium,* and *K. pneumoniae* showed similar abilities to act as a recipient when acquiring RP4 and as a donor when transferring the RP4 plasmid (Figure 2.4B). These findings show that one must consider how transfer frequencies characteristic of each specific bacterial strain acting in the role of donor or recipient during conjugation affect the efficiency of the spread of the RP4 plasmid.

We were also interested in studying the spread of the RP4 plasmid from *P. putida* and *V. cholerae* to different coliform bacteria other than the donor *E. coli* LM715-1. In these trials, we observed that both donors *P. putida* and *V. cholerae* successfully

transferred the plasmid to two coliform bacteria, *C. rodentium* and *K. pneumoniae* in frequencies ranging from 10^{-2} to 10^{-7} (Figure 2.5). These findings further demonstrate that donor bacteria carrying the RP4 plasmid can effectively spread antibiotic resistance genes to diverse groups of bacteria from the gut microbiota.

Fitness cost and persistence of broad host range plasmid during adaptation and evolution in a naïve host bacterium

The RP4 plasmid is a large broad host range plasmid (~60 Kbp, 2-6 copies of plasmid per cell) and carries self-replication and transmission machinery (Figurski and Helinski, 1979; Pansegrau et al., 1994). We tested whether this plasmid would be maintained or lost by a naïve host donor bacterium such as the human gut commensal *E. coli* LM715-1 due to a fitness cost to the host cell. We hypothesized that the RP4 plasmid would persist in a naïve host without antibiotic selection pressure. We used an experimental evolution approach to passage *E. coli* LM715-1 with mScarlet, *cam*R, *kan*R and RP4::GFP *amp*R *tet*R *kan*R markers in fresh growth media daily in the presence and absence of the antibiotic ampicillin (50 µg/ml) for 10 days to test the hypothesis. We calculated the proportion of plasmid-bearing cells (bacteria with plasmid divided by total bacteria in the culture) every 24 hours during this host-plasmid evolution and adaptation experiment. The proportion of plasmid-bearing cells declined rapidly to 20 percent on day two post-inoculation in cultures with and without antibiotic selection and then decreased slowly throughout the remainder of the 10-day experiment (Figure 3.6 A, B). This result suggests plasmid carriage imposed a fitness cost in this environment, which led to the quick loss of plasmid from the donor strain at the start of

the experiment. However, the plasmid-bearing *E. coli* LM715-1 cell population remained persistently present at a low proportion (3-4%) for up to 10 days. Despite the fact that the proportion of donor bacteria carrying plasmid was lower in the population on days 2-10 of the experiment, a significant number of colony-forming units ($1x10^8$ cfu) of donor *E. coli* LM715-1 bacteria persisted on day 10 (Figure 3.6 B), enough to give rise to further plasmid transfer.

Discussion

Horizontal gene transfer (HGT), which allows bacterial pathogens to acquire resistance genes from other bacteria, has contributed significantly to the spread of antimicrobial resistance among life-threatening pathogens (Aminov, 2011; Huddleston, 2014; Lerminiaux and Cameron, 2019). Our study has shown that the RP4 plasmid effectively transferred to clinically important and emerging drug-resistant strains of *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonadaceae* when transferred by a human-derived commensal *E. coli* donor and tested in the absence of antibiotic selection pressure. Next, we demonstrated that further secondary transmission of the RP4 plasmid to a new recipient occurred and that the primary and secondary transfer frequencies of the RP4 plasmid varied across multiple donor-recipient pairings; this finding is consistent with previous studies (Soda et al., 2008; Benz et al., 2021; Heß et al., 2022). Thus, the broad host range RP4 plasmid is able to replicate and initiate the conjugation process, making it capable of transferring to numerous bacteria and spreading drug resistance, including to bacteria of the human gut microbiota (Adamczyk and Jagura-Burdzy, 2003; Heß et al., 2022).

We found that the selection pressure imposed by the presence of the antibiotic ampicillin increased RP4 plasmid transfer in most donor and recipient combinations. However, in the case of *K. pneumoniae,* the conjugation frequency decreased under antibiotic selection with ampicillin. Other studies have reported that both high and subinhibitory concentrations of beta-lactam antibiotics did not increase the plasmid transfer frequency in *K. pneumoniae* and *E. coli* (Lopatkin et al., 2016; Headd and

Bradford, 2018). The effect of antibiotic selection pressure on the conjugation rate has also been shown to vary based on donor-recipient pairing, plasmid type, and ecological factors (Andersson and Hughes, 2014; Lopatkin et al., 2016; Cairns et al., 2018). We observed that the ratio of donor to recipient bacteria increased in the antibiotic-treated group compared to the no antibiotic group, suggesting that plasmid selection and maintenance in donor bacteria may increase conjugative efficiency. However, the plasmid transfer frequency under antibiotic treatment could be influenced by other factors such as death of recipient bacteria caused by the drug, growth rate of transconjugant bacteria, and secondary transfer of plasmid (Lopatkin et al., 2016). All of these factors must be considered when trying to understand transfer frequencies in more complex communities such as the human gut microbiota.

We also found significant differences in a specific bacterial strains' primary and secondary plasmid transfer frequency depending on whether it was acting as a donor or as a recipient during the conjugation process. It is known that HGT of a conjugative plasmid involves multiple cellular processes (Virolle et al., 2020), and that a success of a transfer is determined by genetic and physical characteristics of the plasmid, donor, and recipient strains (Thomas and Nielsen, 2005). For example, each bacterial species has different immunity or defense mechanisms against foreign genetic material. Restriction and modification systems (RMS) (Purdy et al., 2002), anti-phage defense system (Doron et al., 2018), and the CRISPR-Cas system (Price et al., 2019) are the most well-known bacterial arsenals to prevent the acquisition of foreign DNA, and can all alter the transfer frequency of plasmids among bacterial species. Similarly, studies

have shown that many bacteria secrete bacteriocins to compete against different species ((Hibbing et al., 2010; Ghequire and Öztürk, 2018). We observed such killing in a conjugation experiment in which a *P. aeruginosa* strain used a recipient killed the commensal donor *E. coli* LM715-1 strain, so that no cells were left to donate the plasmid. It is important to note that conjugation efficiency can also vary if the doubling times are different for each donor and recipient bacteria, leading to different donor-recipient ratios during the conjugation experiment (Händel et al., 2015). Similarly, other physicochemical factors such as growth phase, growth media, temperature, and mating surface (liquid or solid) can affect conjugation efficiency (David and Bradley, 1980; Lopatkin et al., 2016; Headd and Bradford, 2018). In our experiments, we used equal numbers of cfus for each donor and recipient strain tested, but further work would be required to determine effects of dose on the response of these donor-recipient plasmid transfers.

In a serial passage experiment, we found that the fraction of RP4 plasmid-bearing cells rapidly decreased to 20 percent of the population after 48 hours, but afterward, the rate of decline was slow, and the donor bacteria remained in a low proportion throughout the ten-day experiment. Most plasmid adaptation studies have been conducted for more extended periods of 75 days (Lenski and Bouma, 1987; Bouma and Lenski, 1988). However, another study showed a much more rapid adaptation of a pKP33 plasmid encoding CTX-M-15 extended spectrum beta-lactamases (ESBL) and carbapenemases in *E. coli* with in a 10-day time-course experiment (Porse et al., 2016). Based on our results, we find that it is entirely possible that presence of the RP4 plasmid in a host gut microbiota for even a short period may

be sufficient time to enable further spread of this broad host range plasmid to other bacterial species. Critical studies in other environments support this conclusion. For instance, multiple studies have shown that laboratory donor *E. coli* strains carrying conjugative plasmids can spread the plasmid in complex systems like gut microbiota in even a few hours (Ronda et al., 2019), and in soil and sewage microbiota (Musovic et al., 2014; Klümper et al., 2015; Li et al., 2020). Moreover, it has been shown that multiple conjugative plasmids, including RP4, can persist within host bacteria even in the absence of antibiotics (Lopatkin et al., 2017).

One must also consider that conjugative plasmids can adapt to host bacteria by several mechanisms, including ameliorating fitness costs through compensatory mutations either on the chromosome or plasmid (Porse et al., 2016; San Millan, 2018), carriage if beneficial resistance genes are present (Bahl et al., 2009; Andersson and Hughes, 2011), co-evolution under antibiotic selection (Bottery et al., 2018), presence of multiple copies of plasmid (Rodriguez-Beltran et al., 2018) or promotion of a high frequency of plasmid transfer (Stewart and Levin, 1977). Conjugative plasmids, especially broad host range plasmids, can initiate conjugation and transfer to multiple bacteria without antibiotic selection pressures as we have shown here. Through continuous conjugation among a network of multiple bacterial cells, such plasmids have another mechanism to persist in host bacterial populations or in complex communities even with low level of conjugation frequency (Salyers et al., 1997; Bahl et al., 2007; Lopatkin et al., 2017). Also, we found that the presence of the antibiotic ampicillin did not increase the proportion of plasmid-bearing cells compared to the no antibiotic group

in the conjugation experiments. It has been shown that positive selection of plasmid does not always maintain the plasmid in the host but can also cause plasmid instability and loss (Bottery et al., 2018; Nicoloff et al., 2019); however, the primary mechanism behind plasmid instability remains unknown. We used ampicillin, which falls into the beta-lactam class of antibiotics, as our selective drug in this study. It is possible that *E. coli* LM715-1 carrying this plasmid produced a beta-lactamase that degraded the ampicillin leading to no effect of the antibiotic on overall bacterial growth during the evolution experiment (Moya et al., 2009; Zeng and Lin, 2013).

Our study implies that broad host range plasmids like RP4 carrying ARGs are likely to spread antibiotic resistance quickly to multiple species in complex communities even when under no antibiotic selection pressure. We expect that RP4 plasmids encoding beta-lactamase can maintain themselves in a complex community for more extended periods because they mediate sufficient conjugation frequency for transfer to a diverse range of bacteria to occur (Geisenberger et al., 1999; Heß et al., 2022). The plasmid transfer frequencies generated in this study could be used to build a mathematical model to predict the spread of the conjugative RP4 plasmid across multiple species in a complex environment like the gut microbiota, where it is difficult to determine actual plasmid transfer frequencies. Such a model could be further extended by adding other clinically relevant plasmids from the IncF, IncI, IncN, and IncQ subgroups (Rozwandowicz et al., 2018). In addition to transfer frequencies, the incompatibility type of broad host range plasmids, donor-recipient pairings, and

environmental factors related to the conjugation process are also important and should be considered in such a model (Bahl et al., 2009; Carattoli, 2009).

Footnotes.

1. http://shigatox.net/ecmlst/cgi-bin/scheme
2. http://shigatox.net/ecmlst/cgi-bin/dbquery
3. https://www.addgene.org

Tables and figures

Tables:

Description of Donor and Recipient Strains Included in This Study			
Bacterial Strain	**Antibiotic resistance phenotype**	**Isolate Type**	**Source (attached references)**
Escherichia coli LM715-1	CamR, KanR	Human *E. coli* Strain (UPEC) – ST 259	Linda S. Mansfield lab (This study)
Escherichia coli MG1655	RifR	K-12 *E. coli* Laboratory Strain	ATCC/ Lixin Zhang lab
Escherichia coli DEC 5a TW00587	AmpR, RifR	Human Diarrheagenic *E. coli* Strain (DEC) – ST 73	STEC/Shannon Manning lab (Reid et al., 1999)
Citrobacter rodentium ATCC 51459	RifR	Pathogen Strain	ATCC/Linda S. Mansfield lab
Pseudomonas putida KT2440	AmpR, CtxR, RifR	Environmental Strain	ATCC/Lixin Zhang lab
Pseudomonas aeruginosa	AmpR, CtxR, CamR	Human Pathogen Strain Isolated from CF Patient	Robert Quin Lab (personal communication)
Klebsiella pneumoniae IA565	AmpR, RifR	Human Pathogen Strain	Christopher Waters lab (Jagnow and Clegg, 2003; Sambanthamoorthy et al., 2011)
Salmonella enterica serovar Typhimurium	RifR	Clinical Strain Isolated from Chicken	Srinand Sreevatsan lab (Joshi et al., 2009)
Vibrio cholerae O1 biotype El Tor C6706str2	AmpR, StrepR	Human Pathogen Strain	Christopher Waters lab (Helene Thelin and Taylor, 1996)
CamR, chloramphenicol; KanR, kanamycin; AmpR, ampicillin; CtxR, ceftriaxone; RifR, rifampicin; StrepR, streptomycin; UPEC, Uropathogenic *E. coli; DEC,* Diarrheagenic *E. coli; ST, sequence type; CF, cystic fibrosis.*			

Table 2. 1 Bacterial strains with their antibiotic resistance profiles and isolation sources.

Primers used for donor and transconjugants confirmation				
Primer	**Product Size (in bp)**	**Primer Sequence (5′-3′)**	**Target gene**	**Gene Bank accession no. References**
*gfp*F	182	*ggtgaaggtgaaggtgatgc*	*gfp*	U73901.1
*gfp*R		*cttctggcatggcagacttg*		
*mScarlet*F	371	*cgcgtgatgaactttgaaga*	*mScarlet-I*	KY021424.1
*mScarlet*R		*tcgctgcgttcatactgttc*		

Table 2. 2 These primers were used for the detection of fluorescent markers in the donor and recipient strains.

Figures:

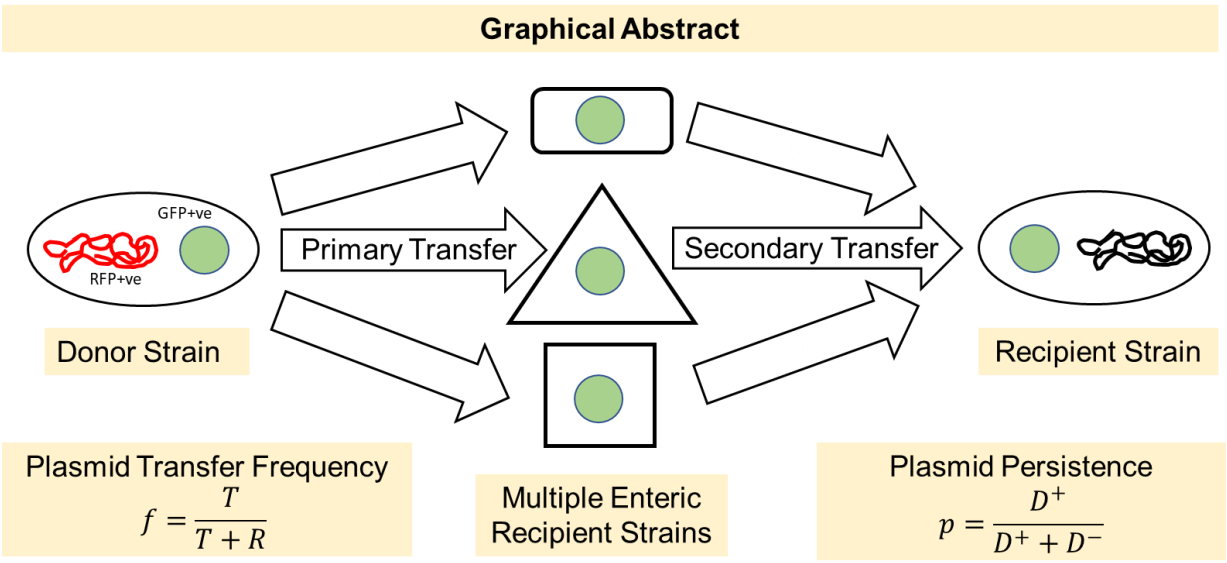
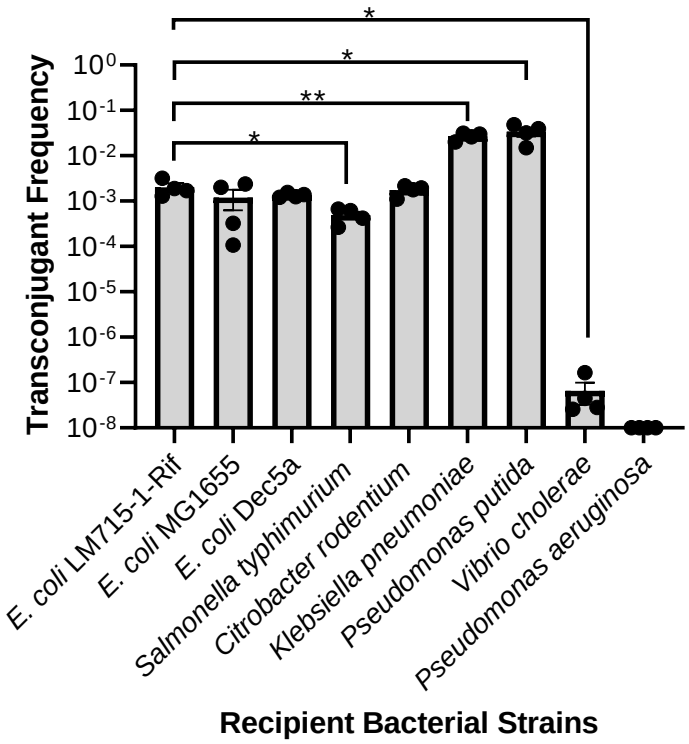


Figure 2. 1 Graphical abstract showing the experimental design used in this study.

We studied the transfer frequencies of a broad host range RP4 plasmid among multiple clinically relevant bacteria. In the primary transfer, the RP4 plasmid was transferred from *E. coli* LM715-1 to different enteric commensal and pathogen bacteria, and in the secondary transfer, we observed the transfer of RP4 plasmid to *E. coli* LM715-1 from all plasmid recipients of primary transfer. Plasmid persistence was also studied in *E. coli* LM715-1 using a serial passage approach.

(A)



(B)

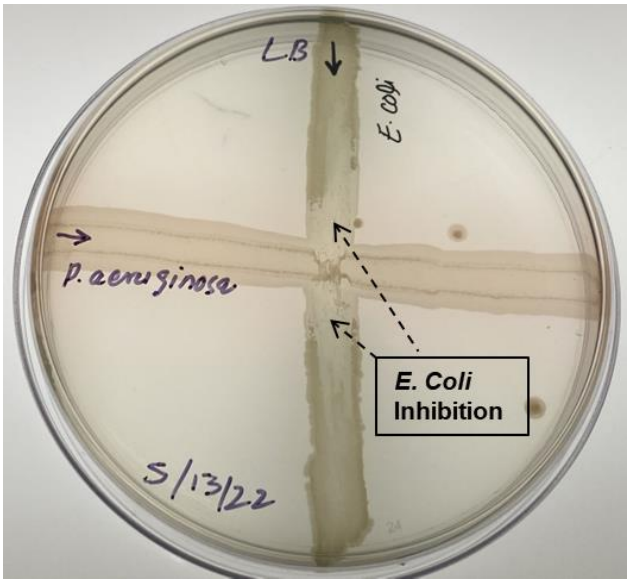
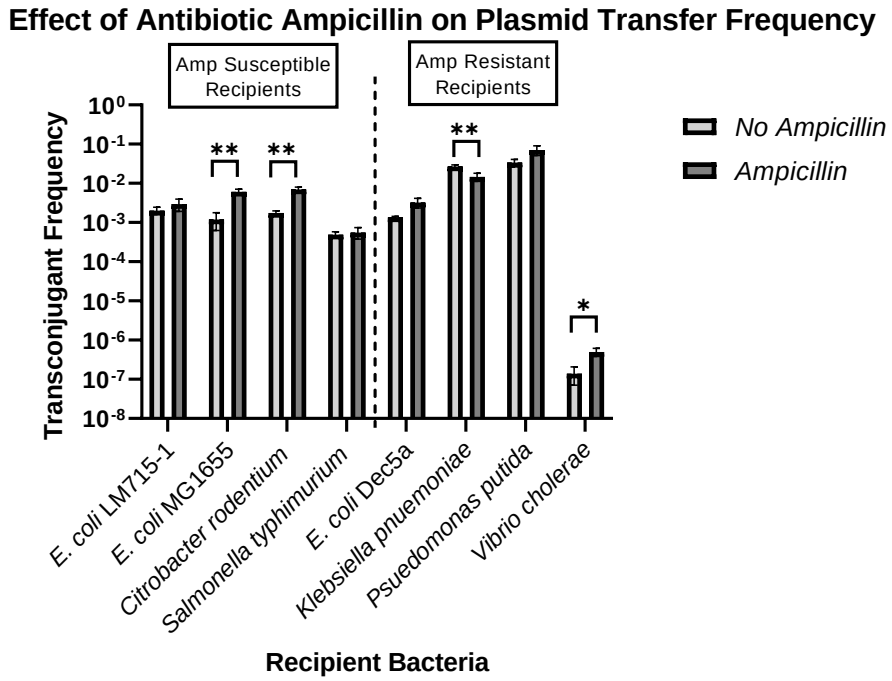


Figure 2. 2 Transfer of RP4 plasmid from commensal strain *E. coli* LM715-1 to multiple recipient bacteria and inhibition by *P. aeruginosa*.

Figure 2.2 (Cont'd)

Figure 2.2A shows the plasmid transfer frequencies for all recipients calculated using the formula (plasmid transfer frequency = transconjugants / (transconjugants + recipients)). Bars show mean values ± standard error of the mean (SEM) based on four independent replicates (n=4) performed for each conjugation experiment. Plasmid transfer frequencies from *E. coli* LM715-1:RP4 to all other isolates were compared to the plasmid transfer frequency from *E. Coli* 715-1:RP4 to *E. coli* LM715-1 RifR. We used Welch's *t*-test instead of an unpaired independent samples *t*-test if there was unequal variance between compared groups to calculate *p* values. Only significant *p* values are annotated in the graph: ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. Figure 2.2B shows the growth inhibitory activity of *P. aeruginosa* against commensal strain *E. coli* LM715-1; zones of growth and inhibition are indicated by complete and dotted arrows, respectively. The growth of *E. coli* significantly diminished near the cross-section compared to opposite ends of the streak.

(A)



(B)

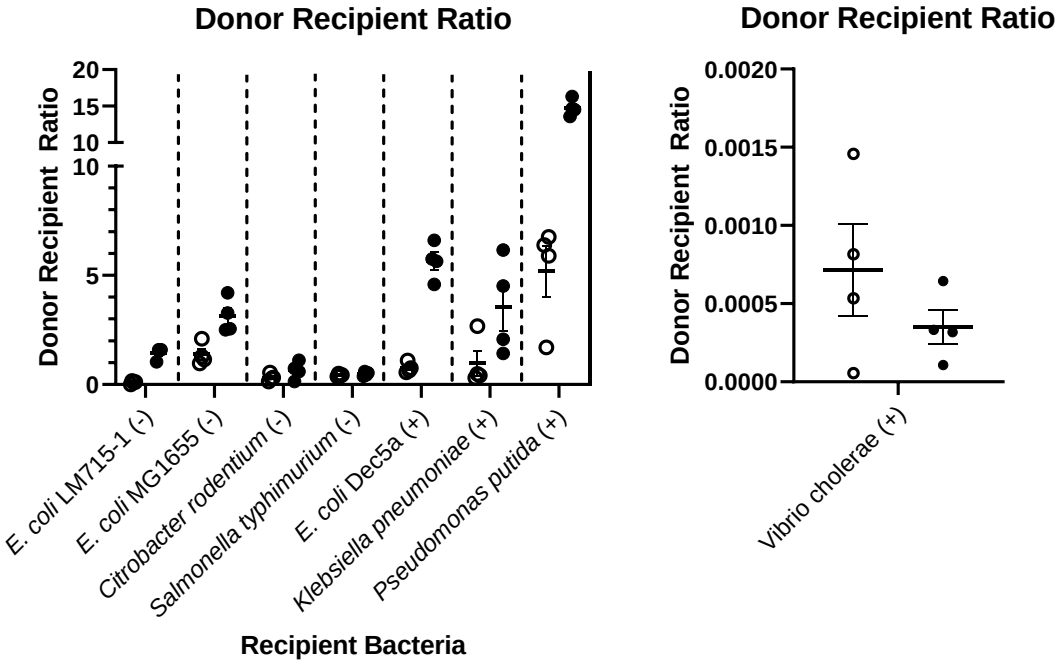
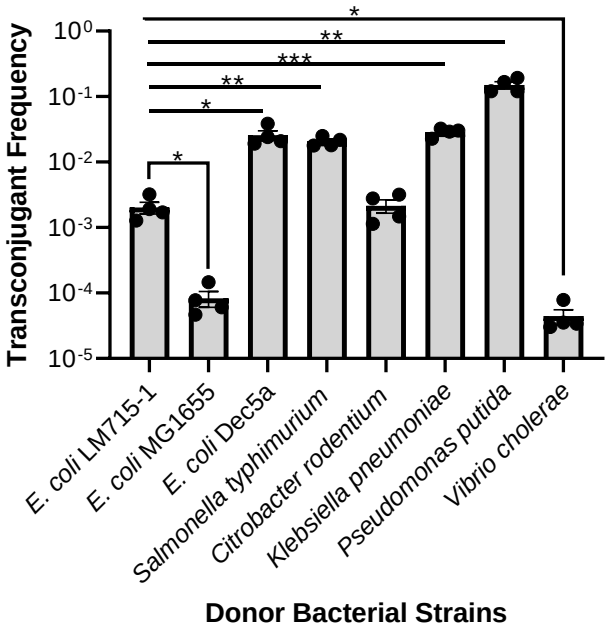


Figure 2. 3 Effect of ampicillin on plasmid transfer frequency.

Figure 2.3 (Cont'd)

Figure 2.3A shows the plasmid transfer frequencies for all recipients as calculated using the formula (plasmid transfer frequency = transconjugants / (transconjugants + recipients)). Bars show mean values ± standard error of the mean (SEM) based on four independent replicates (n=4). For each recipient, frequency of transfer from *E. coli* LM715-1:RP4 in the absence of ampicillin was compared to the transfer frequency in the presence of ampicillin. An unpaired independent samples *t*-test was performed to calculate *p* values; only significant *p* values are annotated in the graph: ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. Figure B shows the donor to recipient ratio with and without ampicillin. The ratio was calculated using the total number of donor and recipient bacteria after 24 hours of incubation on filter paper for conjugation on Luria agar plates with and without ampicillin. Circles show four independent replicates (n=4) performed for each ampicillin treated (filled circles) and untreated (empty circles) group. Bars show mean values ± standard error of the mean (SEM) based on replicates in each group. A negative symbol in parenthesis shows susceptibility to ampicillin, and a positive sign shows resistance to ampicillin. The transconjugant frequency shown in Figure A was computed using the same number of recipient bacteria used here to calculate the donor-to-recipient ratio.

(A)



(B)

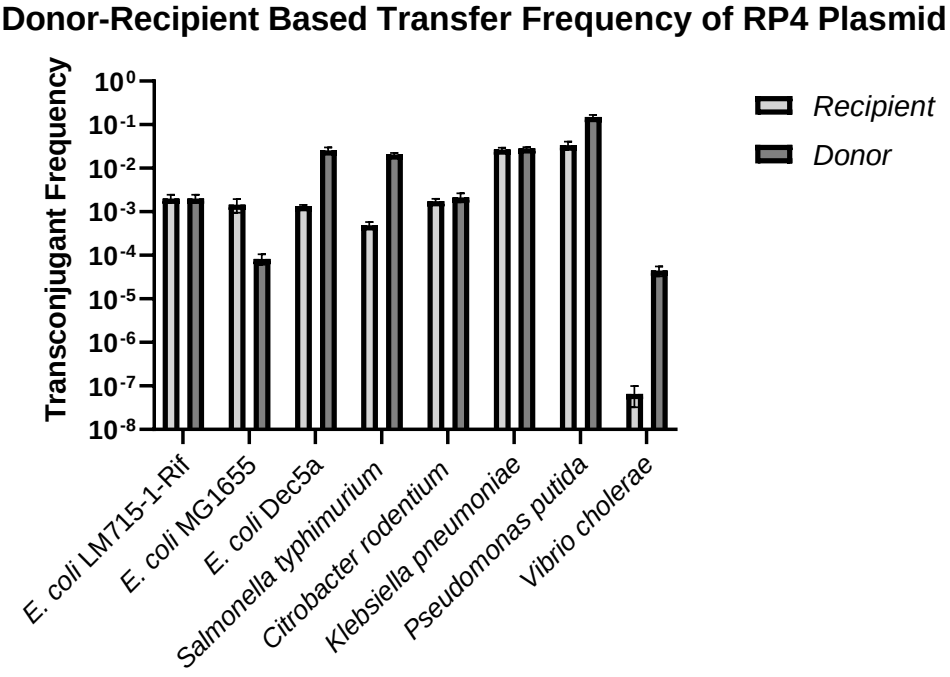


Figure 2. 4 Transfer frequencies of RP4 plasmid to *E. coli* LM715-1 from different donor strains.

Figure 2.4 (Cont'd)

Figure 2.4A shows the secondary transfer frequencies of the BHR RP4 plasmid from different recipient bacterial strains shown in Figure 2.3 back to the commensal recipient *E. coli* LM715-1 strain. The plasmid transfer frequencies for all recipients were calculated using the formula (plasmid transfer frequency = transconjugants / (transconjugants + recipients)). Bars show mean values ± standard error of the mean (SEM) based on four independent replicates (n=4) performed for each conjugation experiment. For each donor, frequency of transfer to *E. coli* LM715-1 was compared to transfer frequency from *E. coli* LM715-1:RP4 to *E. coli* LM715-1 RifR. We used Welch's *t*-test instead of an unpaired independent samples *t*-test if there were unequal variances between compared groups; only significant *p* values are annotated in the graph: ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. Figure 2.4B shows the transfer frequencies for each strain in the primary transfer (when acting as recipient) and the secondary transfer (when acting as donor) of the RP4 plasmid.

(A)

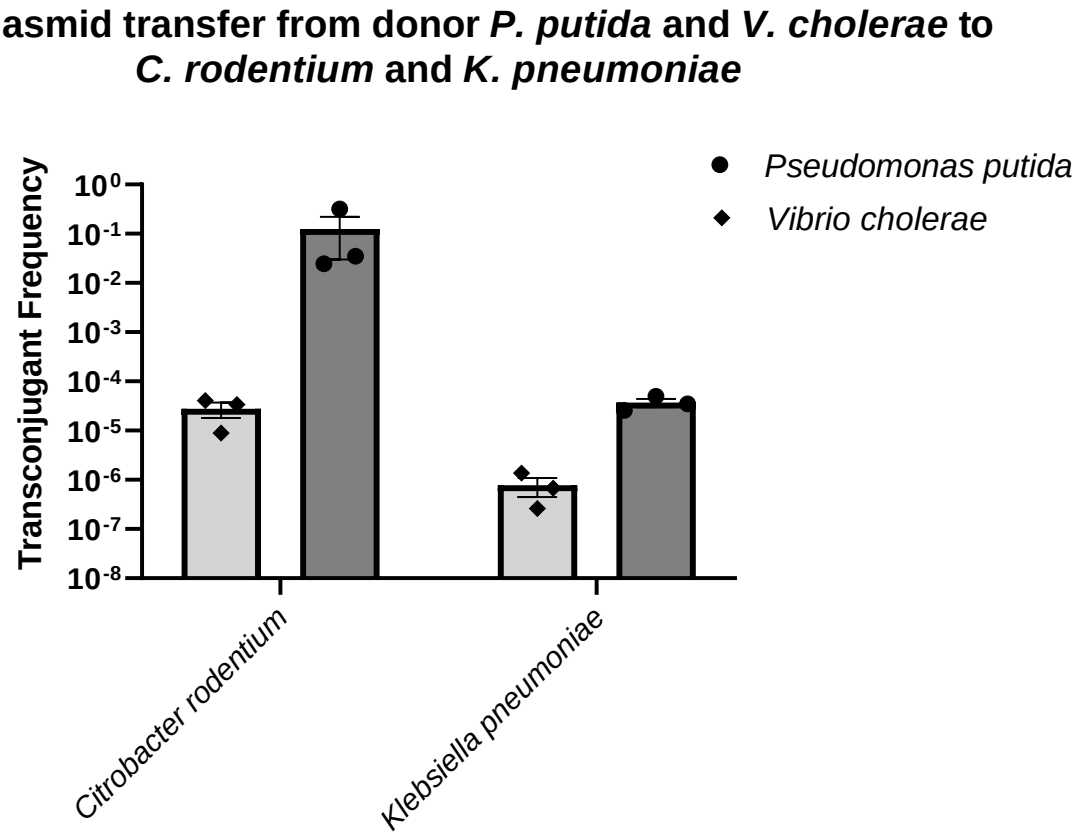
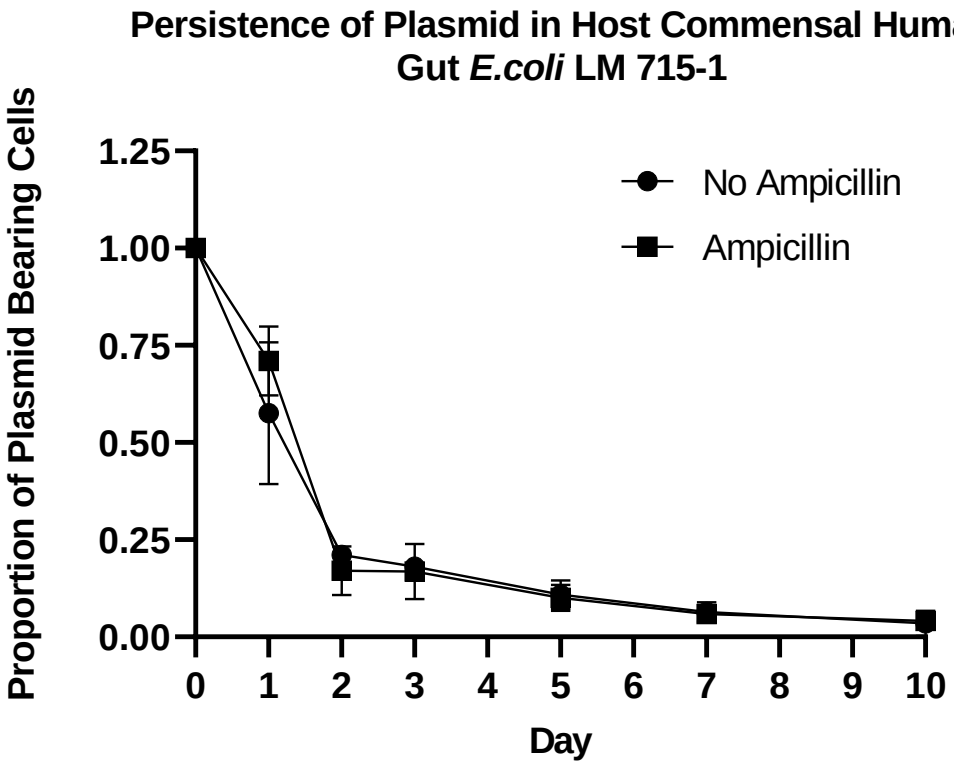


Figure 2. 5 Transfer frequencies of the BHR RP4 plasmid from donors *Pseudomonas putida* and *Vibrio cholerae* to recipient coliform bacteria *Citrobacter rodentium* and *Klebsiella pneumoniae*.

The plasmid transfer frequencies for all recipients were calculated using the formula (plasmid transfer frequency = transconjugants / (transconjugants + recipients)). Bars show mean values ± standard error of the mean (SEM) based on three independent replicates (n=3) performed for each conjugation experiment.

(A)



(B)

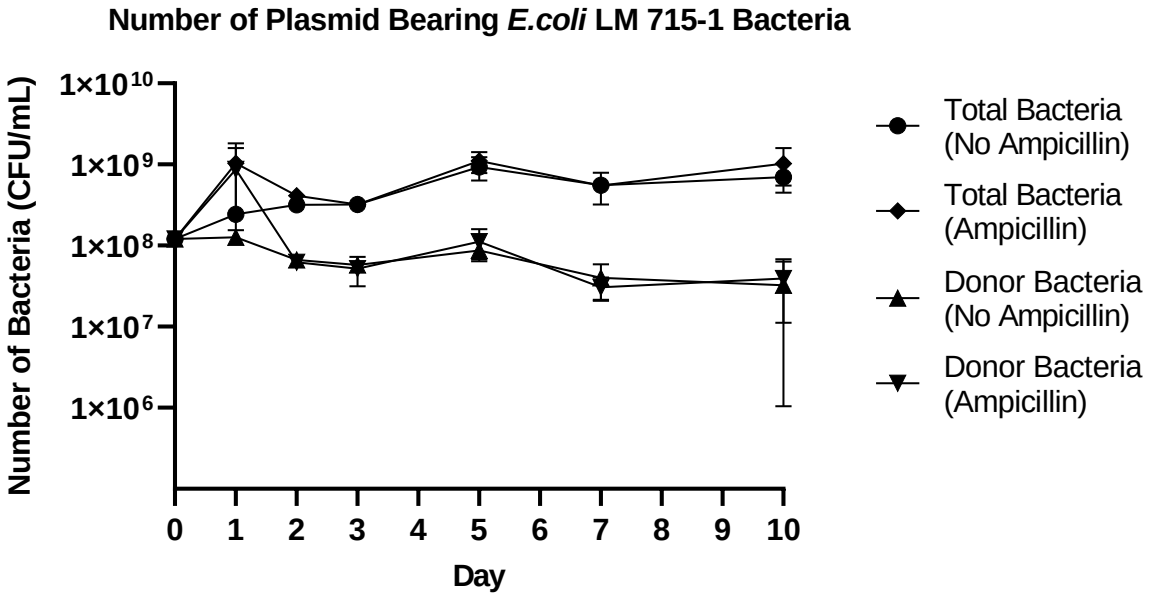


Figure 2. 6 Proportion and number of donor bacteria carrying the RP4 plasmid during serial passage in medium with and without ampicillin.

Figure 2.6 (Cont'd)

Figure 2.6A) Proportions of bacterial cells carrying RP4 plasmid (donor bacteria) and total bacteria (with and without plasmid) were calculated in both groups (ampicillin treated and untreated) on the following days (0,1,2,3,5,7,10). Bars show mean values ± standard error of the mean (SEM) based on three independent replicates (n=3) of each experimental group. Figure 2.6 B) The actual number of bacteria carrying plasmid and of total bacteria with and without plasmid is shown for each treatment group. Bars show mean values ± standard error of the mean (SEM) based on three independent replicates (n=3) of each group of the experiment.

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Chapter 3: *In vivo* plasmid-mediated spread of antibiotic resistance determinants in mice transplanted with human gut microbiota

Abstract

Antibiotic resistance (AR) in bacterial pathogens is a serious threat to public health. Conjugation, a plasmid-driven horizontal gene transfer (HGT) mechanism, can transfer genetic material from one cell to another, including antibiotic resistance genes (ARGs). This study investigates the spread of ARG-bearing plasmids in a complex, diverse population of human gut bacteria using C57BL/6 mice carrying human-derived fecal microbiota. We hypothesized that commensal donor bacteria *E. coli* mediates the transfer of ARGs to many different phylogenetic groups of bacteria in the gut microbiota through conjugation without antibiotic selection pressure.

To address this hypothesis, we created labeled donor *E. coli* bacterial strains carrying red fluorescent protein on the chromosome and green fluorescent protein (GFP) on a broad host range RP4 plasmid. Donor bacteria, including laboratory strain *E. coli* MG1655 and a human-derived commensal *E. coli* LM715-1, were fed orally to mice carrying a human *Bacteroides-Lachnospiraceae* dominant human gut microbiota with undetectable levels of *E. coli.* Fecal samples were collected every 24 hours, stored at -80°C, and colonization of donor strains was assessed by culturing fecal samples on selective media. Fluorescence-activated cell sorting (FACS) coupled with 16S rRNA sequencing was performed to trace and determine ARG-bearing plasmid spread among the gut microbiota community members.

We found that commensal donor strain *E. coli* LM715-1 colonized the mouse gut throughout the ten-day experiment, while the laboratory donor strain *E. coli* MG1655

was not recovered after 48 hours. Flow cytometry analysis of fecal samples from the mice given the donor strain showed an increase in detectable transconjugant bacterial cells expressing GFP located on RP4 plasmid compared to the control fecal samples collected before the gavage. Donor and transconjugant bacteria were recovered from fecal samples and sorted by FACS on days 1 and 3 of post gavage. 16S sequencing analysis of FACS sorted cells showed *Lachnospiraceae, Clostridiaceae, Pseudomonadaceae, Rhodanobacteraceae, Erysipelotrichaceae, Oscillospiraceae,* and *Butyricicoccaceae* were the primary recipient bacterial families of RP4 plasmid in this experiment.

This study's findings show that a tractable human fecal microbiota transplanted mouse model will be an innovative way to investigate the transferability of AR plasmids and leading drivers of HGT in a complex human gut microbial community.

Introduction

The emergence and global spread of antimicrobial resistance against life-saving drugs among bacterial pathogens have become a major challenge in clinical settings. It has been estimated that approximately 4.95 million AMR-associated deaths occurred globally in 2019, including 1.27 million deaths directly attributed to drug-resistant bacteria per clinical record (Murray et al., 2022). Based on a 2019 report by the Centers for Disease Control and Prevention (CDC), more than 2.8 million infections and 35,000 deaths occur in the United States every year caused by antibiotic-resistant (AR) bacteria (CDC., 2019). If antibiotic resistance concerns are not adequately addressed soon, AR bacteria could be a leading cause of death, around 10 million globally, and could significantly increase the economic burden by up to 10 trillion dollars by 2050 (J. O'NEILL, 2016). AR bacteria are causing more fatal infections with lengthier hospitalizations and higher mortality in clinical settings than non-resistant bacteria (CDC, 2013; CDC., 2019) and thus have become a critical public health concern. Most of the AR bacteria reported in the USA and worldwide are enteric pathogens or are capable of living in the human gut (CDC., 2019; Murray et al., 2022).

The spread of transferable antibiotic-resistant genes (ARGs) among enteric pathogens, opportunistic pathogens, and commensal gut bacteria is a serious concern (Wallace et al., 2020). Every year more and more bacteria are emerging as multi-drug resistant bacteria (CDC., 2019). Bacteria often develop antibiotic resistance by creating spontaneous genomic mutations followed by selection pressure due to antibiotic use (Davies & Davies, 2010). It becomes further challenging when these ARGs are located

on mobile elements that can transfer them to other bacteria. Multiple studies have shown that horizontal gene transfer (HGT), which allows bacterial pathogens to acquire resistance from other bacteria, has significantly contributed to the spread of antibiotic resistance (Alekshun & Levy, 2007; Barlow, 2009; Mazel & Davies, 1999). There are three mechanisms of HGT reported in bacteria; transformation (Dubnau, 1999), transduction (S. C. Jiang & Paul, 1998; Schicklmaier & Schmieger, 1995), and conjugation (Buchanan-Wollaston et al., 1988; Heinemann & Sprague, 1989). The human gut is densely populated by diverse bacteria and provides ample opportunity for HGT. The gut microbiota is a known reservoir of antibiotic resistance genes (ARGs) (Anthony et al., 2021; Baron et al., 2018), and its members are potential donors and recipients of ARGs transfer. Considering the presence of opportunistic bacteria and transient pathogens, conjugation in the human gut arguably would have the most direct impact on the spread of ARGs to previously susceptible pathogens. Numerous laboratory studies and genomic data support the occurrence of conjugation in different microbiotas, but this phenomenon has been primarily studied in environmental niches such as soil, animal manures, and wastewater treatment plants (Heuer et al., 2002; Klümper et al., 2015).

However, little is known about the baseline transfer frequencies of ARGs, the potential reservoir of bacterial hosts of transferable ARGs, and drivers of conjugation among bacteria in the human and animal gut, a known hotspot of ARGs (Baron et al., 2018). The inability to track conjugation events in the gut, difficulty in identifying and

characterizing non-culturable plasmid-recipient bacteria and impracticality of controlled experiments in humans has hampered research progress.

In these experiments, we begin to fill this critical knowledge gap by building a robust and tractable human microbiota transplanted mouse model system to address the spread of antibiotic resistance in the gut microbiota. We hypothesized that a broad host range RP4 plasmid carried by a commensal donor bacterium *E. coli* LM715-1 would be transferred to resident bacteria of human gut bacteria. To address this hypothesis, we used in-house bred C57B/6 mice transplanted with adult human gut microbiota and shown to be carrying a stable microbial community over generations. We created a fluorescently labeled commensal 88scherichia88c *E. coli* LM715-1 (UPEC) originally isolated from a human infant gut microbiota. Next, we integrated high-throughput cell sorting, 16S rRNA amplicon sequencing, and culture to track fluorescently labeled donor and transconjugant bacteria from fecal samples of C57B/6 mice transplanted with adult human gut microbiota (Figure 3.1). We found that the labeled commensal donor strain *E. coli* LM715-1 colonized the mouse gut throughout the ten-day *in-vivo* experiment without any antibiotic treatment. The broad host range RP4 plasmid carried by the donor *E. coli* strain invaded diverse bacterial families of human gut microbiota members. Overall, this study's findings suggest that using a human gut transplanted mouse model integrated with high-throughput sorting and sequencing techniques will be an innovative way to investigate the spread of transferable ARGs through HGT in the gut microbiota.

Materials and methods

Animal ethics statement:

All mouse experiments were conducted according to guidelines provided by the American Veterinary Medical Association (AVMA) and the National Institute of Health (NIH). The animal use protocol (06/18-080-00) was approved by the Michigan State University Committee on Use and Care of Animals (IACUC).

Media, chemicals, and reagents:

MacConkey agar (Neogen), Bacteriological Agar (Neogen), Luria agar (Accumedia), LB-Miller Broth (IBI Scientific), and Mueller Hinton II agar (Becton Dickinson) were used to grow donor, recipient, and transconjugant bacteria and screen fecal pellets for the presence of antibiotic-resistant bacteria. All bacterial culture experiments were done aerobically or on microaerophilic conditions using a gas mixture of 80% N2, 10% CO2, and 10% H2 at 37°C either in an incubator or on a shaker at 150 rpm. We used antibiotics with the following concentrations, ampicillin (50 µg/ml), chloramphenicol (20 µg/ml), kanamycin (50 µg/ml), tetracycline (15 µg/ml), rifampicin (20 µg/ml) throughout the study. We used MacConkey agar or LB agar containing chloramphenicol (20 µg/ml) and ampicillin (50 µg/ml) to select for the donor strain. LB agar containing rifampicin (20 µg/ml) was used to select for the recipient strain, and LB agar containing rifampicin (20 µg/ml) and ampicillin (50 µg/ml) was used to select for transconjugants. Phosphate buffered saline (PBS-1X) was used for washing and diluting bacterial culture.

Labeling of commensal donor strain:

We isolated human-derived, mouse gut-adapted commensal *E. coli* LM715-1 from an infant fecal microbiota transplanted C57B/6 mouse from the Mansfield laboratory colony at Michigan State University. These mice were transplanted with fecal slurries of children recruited into the "Third Generation Study" under ethics approval numbers 09/H0504/129 (22 December 2019), 14/SC/0133 (22nd December 2019), and 14/SC/1191 (15 November 2016) at Michigan State University. To create a traceable donor strain, we obtained a chromosomal insertion toolbox designed by Schlechter et al. (2018) (Schlechter et al., 2018) from the Addgene plasmids repository (https://www.addgene.org), which consists of a Tn7-based 90scheri-unstable delivery suicide plasmid (pMRE-Tn7-155 plasmid, Addgene Plasmid #118569, Watertown, MA)**.** We inserted a genomic cassette (mScarlet fluorescent protein, KanR, and CamR) into the bacterial chromosome using a pMRE-Tn7-155 delivery plasmid following the method described by Schlechter et al. (2019) (Schlechter & Remus-Emsermann, 2019). The insertion of fluorescence and antibiotic resistance marker genes was confirmed using fluorescent microscopy, selection on LB agar with chloramphenicol (20 µg/ml) and kanamycin (50 µg/ml), and colony PCR (see below) for mScarlet fluorescent marker.

E. coli MG1655 bearing a broad host range (BHR) RP4 plasmid labeled with a green fluorescent protein marker and antibiotic resistance genes *amp*R*, tet*R*,* and *kan*R was the gift of Dr. Barth Smets' research group at the Technical University of Denmark (Klümper et al., 2015). We mated the fluorescently labeled commensal strain *E. coli* 715-1 (mScarlet, *cam*R, *kan*R) as a recipient strain with donor *E. coli* MG1655

(RP4::GFP, *amp*R, *tet*R, *kan*R) using the filter conjugation method described below. A single colony from the transconjugant selective medium (LB agar with chloramphenicol and ampicillin) was picked and streaked on a fresh LB agar plate with chloramphenicol and ampicillin. After streaking twice, the bacterial lawn of *E. coli* LM715-1 carrying RP4 plasmid was suspended in LB-Miller broth containing 30% glycerol and stored at -80°C. The donor strain was re-streaked from this stock culture for all experiments. The presence of plasmid in commensal recipient strain *E. coli* LM715-1 was confirmed by fluorescence microscopy and colony PCR.

In vitro conjugation experiments to confirm plasmid transfer:

The plasmid transfer frequency for donor and recipient bacteria was assessed using a filter-based conjugation method (Trieu-Cuot' And & Courvalin, 1985). Briefly, both donor and recipient bacteria were grown in Luria broth with respective antibiotics at 37°C and 150 rpm overnight. 750µL of each donor and recipient culture were mixed, and a pellet was obtained after washing twice with phosphate-buffered saline (PBS) at 10,000 × g for 5 minutes (Eppendorf centrifuge 5415D, F-45-24-11 Rotor). After resuspending the pellet in 100µL of PBS, 20µL was spread on each of four to five separate filters on a Luria agar plate. We used cellulose filter papers manufactured by Whatman (Catalogue# Whatman 1001-125) and cut them into small pieces of 2cmX2cm). After incubating plates at 37°C overnight, each filter was placed in a 1.5 mL microcentrifuge tube, 1000µL of PBS was added, and the tube was vortexed for 60 seconds before diluting the suspension serially. We spread 100 µL of these dilutions on each of three separate Luria agar plates with specific antibiotics selected for the donor,

recipient, and transconjugants. Plates were incubated aerobically overnight at 37°C; colonies were counted to calculate plasmid transfer frequency using the formula $f = \frac{T}{R+T}$ (plasmid transfer frequency = transconjugants / (transconjugants + recipients)).

Inoculation of mice with donor strain:

We used C57BL/6 wild-type mice transplanted with human adult fecal microbiota for all experiments (Brooks et al., 2017). These mice were created, bred, and reared by Mansfield laboratory at Michigan State University. Based on the 16S analysis, these mice carry a human *Bacteroides-Lachnospiraceae* dominant human gut microbiota with undetectable levels of *E. coli.* We also confirmed the absence of *E. coli* by plating fecal samples on MacConkey agar collected from five mice of two generations. Therefore, we chose this transplanted mouse model to avoid background noise for our donor bacterial strain.

The labeled commensal donor strain *E. coli* LM715-1 (mScarlet, KanR, and CamR) carrying RP4 conjugative plasmid was inoculated into Luria broth containing ampicillin and chloramphenicol and incubated overnight in a shaker at 37°C and 150 rpm. The next day, the overnight culture was washed twice and resuspended in phosphate-buffered saline (PBS) to remove antibiotics. The optical density (OD_{600}) was adjusted to 1.0 (approximately 10^9 cells per mL) before inoculating the mice with 100uL bacterial culture, equivalent to 10^8 donor cells. All experimental mice were fed a single time with 100 µL of the volume of the respective group treatment (donor strain and PBS)

at the start of the experiment using a pipet tip and dripping the suspension into the mouth carefully.

All experiments were conducted with singly housed mice to minimize the cage effect and prevent the transfer of transconjugants between group-housed mice, thereby overestimating the transfer of ARGs. Every experiment had five mice in the treatment group (donor strain) and two to three mice in each control group (PBS). Fecal pellets were collected periodically before and after inoculation, suspended in LB with 30% glycerol, and stored at -80°C throughout the experiment. All mice were humanely euthanized at the end of the experiments by CO_2 asphyxiation; fecal pellets and different parts of the GI tract were collected at necropsy and suspended in LB with 30% glycerol and stored at -80°C**.** These stored samples were later processed to measure colonization of donor strain by culture and perform flow cytometry to detect the donor and any other bacteria that acquired the plasmid for DNA extraction and 16S sequencing to characterize the gut microbiota.

Assessing colonization of donor strain in fecal and GI samples:

The stored fecal and GI samples were thawed on ice. 100 µL of each sample was then serially diluted in ten-fold steps in PBS. Next, we used MacConkey agar plates with ampicillin and chloramphenicol to select for the donor strain carrying the RP4 plasmid and MacConkey agar plates with chloramphenicol only to select for the donor strain with or without plasmid. Plates were incubated aerobically overnight at 37°C. Individual colonies from incubated plates were counted, and the colonization of donor

strain in fecal and GI part samples of every mouse in the treatment and control groups was calculated.

Screening of human adult fecal microbiota transplanted mice for the presence of antibiotic resistance:

Fecal pellets were collected from three to four group-housed mice in different cages twice from two different generations of human fecal microbiota transplanted mice. We used two growth media: MacConkey agar to select only for coliform bacteria and Mueller Hinton agar to select for a broader bacterial community. Both growth media were supplemented with individual antibiotics (kanamycin (50 µg/ml), chloramphenicol (20 µg/ml), rifampicin (20 µg/ml), ceftriaxone (4 µg/ml), and ampicillin (50 µg/ml)). All plates spread with fecal bacterial suspension were incubated at 37°C for 24 to 48 hours.

Detection of fluorescence in donor and transconjugant bacteria:

Bacterial cells from donor and transconjugants selective media plates were suspended in PBS and centrifuged at 10,000 × g for 1 minute at room temperature in an Eppendorf centrifuge 5415D with F-45-24-11 Rotor. The pellet was resuspended in 100 µL of PBS. We used the agar pad method to obtain images of fluorescent bacteria (donor and transconjugants) (Skinner et al., 2013). 1% agarose solution was poured on a plain surface bordered with microscopic slides to get the same thickness agar pads. After solidifying the agar, coverslip size pads were cut using a sterilized scalpel and placed on another microscope slide. 2-5 µL of bacteria suspended in PBS was spread over the agar pad and covered with a coverslip. Nikon eclipse N*i* microscope with bright

field, GFP, and RFP filters was used to record and analyze the fluorescent bacteria at magnifications of 20X and 40X. For rapid screening of fluorescent bacterial colonies, we took a small number of bacteria directly from the individual colonies grown on the plate and mixed with 10 µL of PBS or deionized water on a microscopic slide. After covering it with a microscopic coverslip and air-drying it for five-ten minutes, we observed fluorescent mScarlet and GFP expression in the donor, recipient, and transconjugants under the microscope.

Validation of antibiotic resistance marker and fluorescent in donor and transconjugants:

To confirm the presence of antibiotic resistance markers in donor and transconjugant cells, we used two strategies: one was to directly plate bacterial suspension of fecal pellets on media containing specific selective antibiotic(s), and the second was to use colony PCR using specific primers against antibiotic resistance genes present on plasmid and donor ‘s chromosome.

We performed a colony PCR to confirm the presence of RP4 plasmid in transconjugant bacteria using primers for the GFP marker located on the plasmid (Table 1). A small amount of bacterial culture was picked using sterilized toothpicks and mixed with a 25 µL reaction mixture in PCR tubes. Each tube reaction mixture contained 2.5 µL 10X Buffer ($MgCl_2$ free), 2.5 µL $MgCl_2$ (50 mM), 2.0 µL dNTPs (2.5 mM), 0.25 µL Taq DNA polymerase (New England BioLabs), 1.0 µL both forward and reverse primers (25 pM/µL) and final volume was adjusted with sterile distilled water up to 25 µL. DNA

amplification was done in an Eppendorf thermocycler using an initial denaturation step at 95°C for 10 min followed by 30 cycles of amplification (denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min), and ending with a final extension at 72°C for 5 min. The PCR product was visualized by agarose (1.5%) gel electrophoresis to confirm the predicted 181 base pair band for GFP present in the transconjugant colonies.

Whole-genome sequencing of bacteria:

Bacteria were grown on Luria agar with chloramphenicol and ampicillin overnight at 37°C. Bacterial cells from the streaked plate were suspended in 1000 µL of PBS and pelleted by centrifuging for 10 minutes at 5000 × g. According to the manufacturer's instructions, we used the QIAGEN Dneasy Blood and Tissue Kit to extract DNA from the pelleted bacterial cells. Sequencing was performed at the Michigan State University Research Technology Support Facility (MSU RTSF) using Illumina TruSeq Nano DNA Library Prep Kit. We sequenced the commensal donor strain *E. coli* LM715-1 through Oxford Nanopore technology by preparing a sample library using the 1D Ligation Sequencing Kit.

Fluorescence-activated cell sorting of donor and transconjugant bacteria:

Fecal pellets stored in LB containing 30% glycerol were thawed and processed for isolating bacterial cells using the method described by Ronda et al. 2019 with the following modifications. Fecal pellets were mechanically homogenized with a pestle. 750 µL of PBS was added and vortexed for 15 seconds before centrifuging at 1,000 rpm for 30 seconds. 500-750 µL of supernatant was carefully removed and placed in a fresh

microcentrifuge tube. Next, three more iterations of washing the bacterial suspension were done by adding and replacing 750 µL of PBS with centrifugation at 1,000 rpm for 30 seconds. The saved supernatant from these four iterations was spun down at 6600 rpm for 5 minutes, and the pellet was retrieved. To remove more debris, we further did two more iterations at 1,000 rpm for 30 seconds. The final supernatant was centrifuged at 66000 rpm for 5 minutes. The pellet was retrieved and resuspended into 500 µL of PBS. All samples were filtered through a 40 µM cell strainer before diluting into PBS, filtered with 2 µM, for flow cytometry or FACS sorting of bacteria performed at the Flow Cytometry Core Facility at Michigan State University.

The BD Influx cell sorter was used to acquire, analyze and sort donors (mScarlet+/GFP+), transconjugants (GFP+ only), and resident fecal bacteria (mScarlet-/GFP-). We used the 488 laser (bandpass filter 530/40) and 552 laser (bandpass filter 585/29) to detect GFP and mScarlet fluorescing bacteria, respectively. The background was assessed by analyzing fecal samples before gavaging mice with donor strain and fecal samples collected from control mice over time. The gating strategy to sort bacteria was based on the positive control bacteria grown in the laboratory expressing different fluorescent proteins alone and in combination, which was effectively used in another study (Klümper et al., 2015). FACS Express 7 Plus software was used to analyze the FACS data and create plots for the manuscript.

DNA extraction from FACS sorted transconjugant bacterial cells:

Dneasy Blood & Tissue Kit (QIAGEN, Catalogue. No. / ID: 69504) was used to extract genomic DNA from FACS sorted cells. Bacterial cells were pelleted by centrifuging at 7500 rpm for 20 minutes, and the supernatant was carefully discarded. Cells were resuspended in 180 µl enzymatic lysis buffer and incubated for 60 minutes at 37°C. The enzymatic lysis buffer containing 20 mM Tris-HCl, 2 mM EDTA, and 1.2% Triton was made using this protocol (Julie Haendiges et al., 2020). Next, we followed the Instructions provided by the manufacturer for extracting DNA from bacterial cells in Dneasy Blood & Tissue Handbook (07/2020). The extracted DNA was immediately stored at -80°C.

16S rRNA gene sequencing and analyses:

We used a two-step PCR approach for amplifying the 16S V4 region of bacterial DNA extracted from FACS sorted transconjugant cells using the following dual-indexed primers pair (515-Forward: GTGCCAGCMGCCGCGGTA, and 806-Reverse: GGACTACHVGGGTWTCTAAT) (Kozich et al., 2013). First PCR was performed at the laboratory using the above specified V4 region target primers with underlined tags on the 5' ends.

CS1-TS-F: 5’ – <u>ACACTGACGACATGGTTCTACA</u>GTGCCAGCMGCCGCGGTA – 3’

CS2-TS-R: 5’ – <u>TACGGTAGCAGAGACTTGGTCT</u> GGACTACHVGGGTWTCTAAT – 3’

We used around 10 nanogram template DNA from each sample to 25 µL reaction mixture in PCR tubes. Each tube reaction mixture contained 12.5 µL of DreamTax PCR Master Mix (ThermoFisher, Catalog # K1071), 1.0 µL of both forward and reverse primers (10µM), and the final volume was adjusted with sterile distilled water up to 25 µL. DNA amplification was done in an Eppendorf thermocycler using an initial denaturation step at 95°C for 2 min followed by 30 cycles of amplification (denaturation at 95°C for 40 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 60 seconds), and ending with a final extension at 72°C for 7 min. The PCR product was visualized by agarose (1.5%) gel electrophoresis to confirm the amplification of the V4 region. The concentration of PCR product was measured using Qubit 4 fluorometer (Invitrogen), and samples were normalized in the range from 15 ng/uL to 25 ng/uL. These total of 20 primary PCR products prepared and normalized in our laboratory were submitted to the Michigan State University Research Technology Support Facility (MSU RTSF) Genomics Core, where these samples were amplified using primers with the Fluidigm common oligos CS1/CS2 fused to their 5' ends. The Genomics Core performed secondary PCR using dual indexed, Illumina compatible primers, which target the Fluidigm CS1/CS2 oligomers at the ends of our primary PCR products. The PCR reaction recipe and cycling conditions were the same as described above in primary PCR. More details can be found on the MSU RTSF website: https://rtsf.natsci.msu.edu/genomics/sample-requirements/illumina-sequencing-sample-requirements/#Other. Amplicons were batch normalized using a SequalPrep Normalization plate (ThermoFisher Scientific/Applied Biosystems part # A1051001) and recovered product was pooled. The pool was concentrated using an Amicon Ultra-0.5

Centrifugal Filter Unit (Millipore Sigma part # UFC5050). The pool was QC'd and quantified using a combination of Qubit dsDNA HS, Agilent 4200 TapeStation HS DNA1000 and Invitrogen Collibri Library Quantification qPCR assays.

This pool was loaded onto one Illumina MiSeq v2 nano flow cell and sequencing was carried out in a 2x250bp paired end format using a MiSeq v2 500 cycle reagent cartridge. Custom sequencing and index primers complementary to the Fluidigm CS1 and CS2 oligomers were added to appropriate wells of the reagent cartridge. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54, and the output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.20.0. A FastQC report of the run output was created to determine the quality scores of the sequenced data.

We analyzed the 16S rRNA gene sequencing data using the QIIME2 16S pipeline (Bolyen et al., 2019) on the web-based platform, Nephlene, developed and managed by scientists at the National Institute of Health (NIH), USA (Weber et al., 2018). The Nephlene platform uses standardized data-analysis steps recommended by QIIME2 authors to process submitted samples. The Silva 16S ribosomal gene database (version 4) was used to align 16S rRNA gene amplicons (Quast et al., 2013). Next, sequences with 97% identity were clustered together into single operational taxonomic units (I). An Excel sheet with all assigned OTUs was downloaded, and the data was analyzed further to calculate relative abundance, diversity indexes, principal component analyses, and statistical measures.

Results

Fluorescently labeled commensal E. coli strain colonized the gut longer than the labeled laboratory strain of E. coli

We isolated a human-derived and gut-adapted commensal *E. coli* LM715-1 strain from infant microbiota transplanted mice and inserted a cassette containing mScarlet, chloramphenicol resistance, and Kanamycin resistance (*mScarlet, CamR, KanR*) genes into the chromosome. Inserted markers were validated through polymerase chain reaction (PCR) and fluorescent microscopy.

Next, to assess the persistence of markers on the chromosome of fluorescently labeled commensal human *E.* coli LM715-1, we inoculated four mice transplanted with healthy adult human gut microbiota with this commensal modified strain and collected fecal pellets daily for 14 days; two mice mouse were given PBS and served as a negative control. Then fecal pellets were screened for labeled commensal *E. coli* bacteria using MacConkey agar plates containing chloramphenicol and kanamycin, antibiotic resistance markers present on the chromosome. It was found that the commensal strain successfully colonized the gut of four mice without applying antibiotic selection pressure, along with maintaining all three markers inserted on the chromosome (*mScarlet, CamR, KanR*) (Figure 3.2A). The fluorescent marker of mScarlet was confirmed by fluorescent microscopy. We also tested the colonization of the fluorescently labeled laboratory strain, *E. coli* MG1655, in the mouse gut in an identical experiment. We found that the labeled laboratory strain of *E. coli* MG1655 did not colonize longer than 72 hours (Figure 3.2B). However, the *E. coli* MG1655

bacterium also maintained fluorescent and antibiotic markers during transit in the gut. These findings suggest that using a commensal gut-adapted *E. coli* will be more helpful in studying horizontal gene transfer for an extended period, especially the plasmid-mediated spread of ARGs in the gut microbiota.

Commensal donor E. coli LM715-1 carrying RP4 plasmid can persist in the gut without antibiotic treatment

After assessing the stability of traceable markers on the labeled commensal *E. coli* in the mouse gut, we transferred the RP4 plasmid to this commensal strain. The RP4 plasmid is also tagged with a green fluorescent protein marker and antibiotic resistance genes *amp*R*, tet*R*,* and *kan*R. We hypothesized that gut-adapted commensal *E. coli* LM715-1 carrying RP4 would colonize the gut of human fecal microbiota transplanted mice without applying any antibiotic selection pressure. Commensal donor bacteria were given to five mice, and daily fecal samples were collected for ten days; a sixth mouse served as a negative control. Mice were screened for the presence of labeled commensal bacteria with and without plasmid using selective growth media containing antibiotics. It was found that the commensal donor strain *E. coli* IM715-1 strain carrying RP4 colonized around 80% of mice (4/5) on day 7 and 40% (2/5) on day 10 without any antibiotic selection pressure (Figure 3.3A). However, all 5 mice carried the labeled *E. Coli* strain without RP4 plasmid till the end of the ten-day experiment (Figure 3.3B). Similarly, we also found that *E. coli* strain RP4 persisted in the mouse gut transplanted with infant A microbiota for around 60% of mice (3/5) on days 7 and 10 without any antibiotic selection pressure. It suggests a fitness cost associated with the

RP4 plasmid, which ultimately caused the loss of the plasmid in two different mouse models.

At the end of the ten-day experiment, we also determined the spatial distribution of labeled *E. coli* strain in different parts of the gastrointestinal (GI) tract (Figure 3.4). The labeled *E. coli* strain largely colonized distal parts of the GI tract, cecum, and colon, ranging from 10^3 to 10^4 bacterial cells per gram of intestinal content. The same level of *E. coli* was detected in fecal samples collected before necropsy on day 10 (Figure 3.4). Donor *E. coli* cells carrying the RP4 plasmid were isolated in two out of five mice in both cecum and colon, ranging from 10^2 to 10^3 bacterial cells per gram of intestinal content (Figure 3.4). These findings showed that the commensal *E. coli* strain mainly colonizes the cecum and colon, and fecal samples are a good proxy for assessing colonization of the donor *E. coli* strain.

Sorting of transconjugant bacteria that received the labeled RP4 plasmid from commensal donor E. coli LM715-1

The presence of unique fluorescent markers on donor chromosome (mScarlet) and transferable RP4 plasmid (GFP) enabled us to distinguish transconjugant bacteria carrying only the GFP marker located on the plasmid from both plasmid-bearing donor *E. coli* (bearing both mScarlet and GFP markers) and from donor *E. coli* that had lost the RP4 plasmid (mScarlet only) in the fecal samples by flow cytometry. We determined the gating strategy using control bacterial culture samples with and without fluorescent markers by following the methods described in these studies (Klümper et al., 2015, 2016). Figure 2.5 illustrates the gates for identifying and sorting bacteria with no

fluorescence, red only, green only, and red and green. After determining the gating strategy using pure bacterial cultures, fecal samples from mice before gavage of fluorescently labeled donor strain. Most fecal bacterial communities appeared in the red-negative green-negative gate for no fluorescence. However, some autofluorescence was detected in the indicated gates for red only, green only, and red and green while sorting fecal samples before gavage. Considering autofluorescent cells in the pre-gavage samples, we used a two-stage sorting strategy to accurately detect true transconjugant bacteria expressing GFP-only in post gavage samples.

In the first sorting (sort-I), fecal samples after gavage were sorted with all four gates shown in column III and row b of figure 3.5. Then cells only expressing GFP were sorted a second time to get purely GFP-positive bacteria, excluding all other bacteria. In this way, these two consecutive sorts (I, II) reduced the chances of false-positive cells and the effect of autofluorescence (Figure 3.6). We selected two time points, 24 and 72 hours post gavage of donor bacteria, to detect the transconjugants bacteria in the fecal samples. Following the two-stage sorting strategy, we carried out fluorescence-activated cell sorting (FACS) on fecal samples collected on day 1 and day 3 post gavage of C57B/6 mice with transplanted adult human gut microbiota with donor strain *E. coli* LM715-1. Overall, we collected FACS-sorted transconjugant bacteria from all five mice, ~ 1,000,000 on day 1 and 769,000 on day 3 (totals from all 5 mice). About 200,000 transconjugant bacteria expressing plasmid-encoded GFP were FACS-sorted and collected from each of five mice on day 1. Similarly, on day 3, three fecal samples yielded around 200,000 FACS-sorted cells, and two mice yielded 101,000 and 69,000

cells. These findings suggest that transconjugant bacteria expressing plasmid-encoded GFP could be sorted from complex microbial communities of the gut microbiota.

The RP4 plasmid bearing ARGs was transferred from commensal donor E. coli LM715-1 to a diverse bacterial population of human gut microbiota members.

We hypothesized that after introducing labeled plasmid-bearing commensal donor bacterium *E. coli* LM715-1, the RP4 plasmid encoding ARGs would spread among resident bacteria of the gut microbiota. After successful FACS sorting of transconjugant bacteria, we performed 16S rRNA amplicon sequencing to characterize the bacteria that acquired the plasmid in the mouse gut. Green-fluorescent FACS-sorted cells from each of five mice from days 1 and 3 were separately sequenced, resulting in 23,123 to 29,890 sequences per sample. These 16S sequences were analyzed using the Qiime2 pipeline to identify operational taxonomic units (OTUs) in the pool of potential transconjugant bacteria. The 16S sequencing analysis showed that RP4 plasmid effectively invaded diverse microbial communities of gut bacteria. About 115 unique OTUs on day 1 and 137 OUTs on day 3 were identified in the FACS-sorted potential transconjugants pool. Members of the following bacterial families, *Lachnospiraceae, Clostridiaceae, Pseudomonadaceae, Rhodanobacteraceae, Erysipelotrichaceae, Oscillospiraceae,* and *Butyricicoccaceae,* were the leading recipients of RP4 plasmid (Figure 3.7). We found that the RP4 plasmid invaded 30 different genera of the *Lachnospiraceae* bacterial family in the mouse gut, resulting in the most target bacterial family for RP4 plasmid-mediated transfer of ARGs. Most importantly, OTUs representing recipients of RP4 plasmid found on day 1 were also

mainly present on day 3, suggesting that these recipient bacteria harbored plasmid even after 72 hours (Figure 3.7, 3.8). Principal component analyses showed that sorted cells from day 1 and day 3 were more similar and grouped together while preprocessing samples and after cleaning samples appeared together on PCA axes (Figure 3.8). Overall, these findings indicate that a broad host range RP4 plasmid encoding beta-lactamase and other ARGs invaded a diverse population of resident bacteria of the human gut microbiota.

Discussion

In this study, we used a mouse model (Brooks et al., 2017) that mimics the human gut microbiota combined with a traceable commensal donor *E. coli* strain to study the conjugative plasmid-mediated spread of ARGs among resident bacteria of the gut. This transplanted mouse model has been shown to carry similar gut microbiota over the generations (Brooks et al., 2017), allowing for experimental reproducibility and offering the opportunity to ask multiple questions related to HGT using the same target microbial population.

Studying the spread of antibiotic resistance in healthy individuals is as important as in patients. One study has shown that ARGs in healthy people remained persistent and resilient to short-term changes in gut microbiota (Kang et al., 2021). Therefore, we used a commensal human gut-derived donor strain *E. coli* LM715-1 to investigate plasmid spread in a mouse model colonized with a human-derived gut microbiota without applying antibiotic selection pressure or inducing any unhealthy conditions in the host.

In past studies, computational genomic methods have been used to identify and confirm ARG transmission in pathogens and resident bacteria of gut microbiota (Forsberg et al., 2012; Gumpert et al., 2017; X. Jiang et al., 2017). Some studies have also reported plasmid-mediated transfer of ARGs in animal models; conventional mice (Bakkeren et al., 2019; Ronda et al., 2019), chicken (Hadziabdic et al., 2018), zebrafish (Fu et al., 2017) and pigs (Johnson et al., 2015). These studies either used germ-free

models or animals pretreated with antibiotics to observe plasmid transfer, without mimicking the complexity of human gut microbiota. To our knowledge, this is the first study using a tractable transplanted mouse model that mimics a human gut microbiota and provides a practical means of investigating the spread of ARGs via conjugation and other mechanisms of HGT.

Using a non-laboratory-adapted labeled commensal donor strain able to persist in the gut environment would be an effective way to investigate the spread of ARGs in the gut microbiota for an extended period of time. Our work showed that commensal donor bacteria *E. coli* LM715-1 persisted in the gut throughout the ten-day experiments without administration of antibiotic, while the laboratory donor strain didn't colonize for more than 3 days. Ronda et al. (2019) also found that a laboratory donor strain was undetectable after 48 hours (Ronda et al., 2019). Nevertheless, there was an apparent fitness cost to maintenance of the labeled RP4 plasmid in the donor strain which resulted in a decrease of the potential donor population. The possible influence of plasmid maintenance on donor fitness is a phenomenon that is likely to affect the degree of plasmid spread in natural settings.

Finally, we observed that labeled commensal donor strain *E. coli* LM715-1 spread a broad host range RP4 plasmid to diverse bacterial communities of the human gut microbiota. 16S sequencing analysis of FACS-sorted cells showed *Lachnospiraceae, Clostridiaceae, Pseudomonadaceae, Rhodanobacteraceae, Erysipelotrichaceae, Oscillospiraceae,* and *Butyricicoccaceae* are the primary potential

recipient bacterial families of RP4 plasmid. Interestingly, Ronda et al. (2019) studied the editing of mouse gut microbiota using different lab-based conjugative plasmids, including RP4 backbone plasmid, carrying genetic inserts. They also identified the transfer of plasmid-mediated genetic insets in multiple genera of *Lachnospiraceae, Clostridiaceae,* and *Pseudomonadaceae* in conventional mouse gut microbiota (Ronda et al., 2019). Previous studies have shown the transfer of RP4 plasmid from laboratory strains of *E. coli* to diverse populations of bacteria in soil (Klümper et al., 2015; Musovic et al., 2014), sewage, and activated sludge (Geisenberger et al., 1999; Liu et al., 2019; Soda et al., 2008), and conventional mouse gut (Ronda et al., 2019). Here we have shown first time that the RP4 plasmid potentially invaded diverse resident bacteria derived from the human gut from a commensal donor bacteria *E. coli* LM715-1. Longitudinal analyses also showed that most OTUs identified in the potential transconjugant pools appeared similar on days 1 and 3, suggesting that the RP4 plasmid was maintained in gut microbiota.

Overall, our study findings demonstrate the use of a tractable mouse model transplanted with the human gut microbiota to study plasmid-mediated transfer ARGs longitudinally. The use of such models could address the impracticality of studying the conjugation among human gut microbiotas. Similar studies are required to determine the spread of broad host range plasmids from other commensal enteric bacterial species.

However, there are some limitations that must be addressed in future experiments. Our study does not address secondary plasmid transfer; that is, whether these bacteria were further transferring plasmid to each other in the gut bacteria or not. Such a study would require the ability to differentiate between primary and secondary recipients of the plasmid. Using a non-self-conjugative plasmid that relies on the host for conjugation or a plasmid with transposons that can insert fluorescent markers on the recipient bacteria could help track primary and secondary transfers in the gut microbiota (Ronda et al., 2019). For this study, we relied solely on high-throughput flow cytometry coupled with 16S rRNA amplicon sequencing to characterize potential transconjugants in the gut microbiota. This method has been previously used and validated in multiple environmental studies using the similar RP4 plasmid (Klümper et al., 2015, 2016; Musovic et al., 2014). Because we studied the plasmid transfer in a natural healthy gut environment of transplanted mice, we were unable to isolate transconjugants by directly culturing from fecal samples, possibly due to the low frequency of the plasmid transfer in the natural gut and the inability of the recipient bacteria to grow in the laboratory conditions. To increase the study's robustness, we used two consecutive sortings of transconjugant bacteria expressing GFP, and this repetitive exposure of bacteria to a laser beam likely killed the cells. This problem can be resolved by creating a strong fluorescent-tagged plasmid to avoid autofluorescence in fecal samples and collect a distinct bright transconjugant pool just after the first sort through flow cytometry. There are many brightest fluorescent proteins (sGFP, mScarlet, mCardinal) that can be used to create labeled donor strains and plasmid (Schlechter et al., 2018).

In future studies, epicPCR can be used to confirm the transconjugant bacteria carrying RP4 plasmid directly before and after FACS sorting of fecal samples. epicPCR couples the antibiotic resistance gene located on plasmid and phylogenetic gene (16S rRNA gene) in individual bacterium level (Spencer et al., 2016). This technique would add a second layer of confirmation of plasmid transfer from a diverse community of the mouse gut and reduce or eliminate the confounding effect of green autofluorescence on identification of transconjugants. Finally, we only used RP4 plasmid in this study, so more broad host range plasmids belonging to other incompatibility groups (IncA/C, IncL/M, IncN, IncP, IncQ, IncW) needs to be tested in this mouse model to understand transferable ARGs in depth and make study findings more generalized and clinical applicable.

Tables and figures

Table:

Primer	Product Size (in bp)	Primer Sequence (5′-3′)	Target gene	Gene Bank accession no. References
*gfp*F	182	*ggtgaaggtgaaggtgatgc*	*gfp*	U73901.1
*gfp*R		*cttctggcatggcagacttg*		
*mScarlet*F	371	*cgcgtgatgaactttgaaga*	*mScarlet-I*	KY021424.1
*mScarlet*R		*tcgctgcgttcatactgttc*		

Table 3. 1 These primers were used for the detection of fluorescent markers in the donor and recipient strains.

Figures:

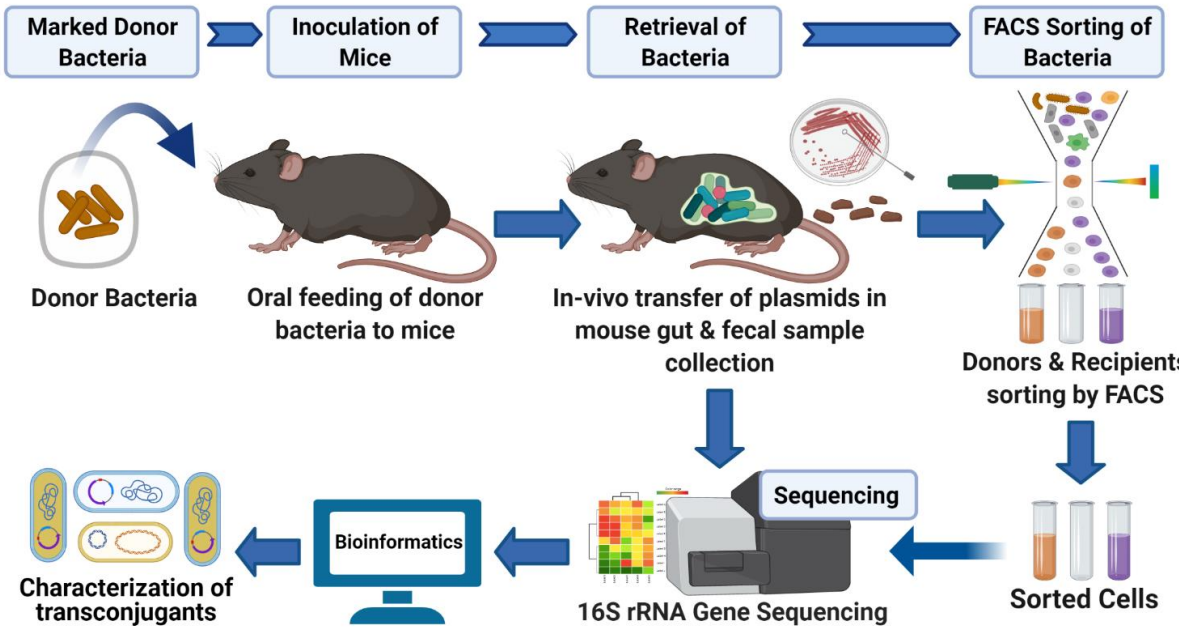
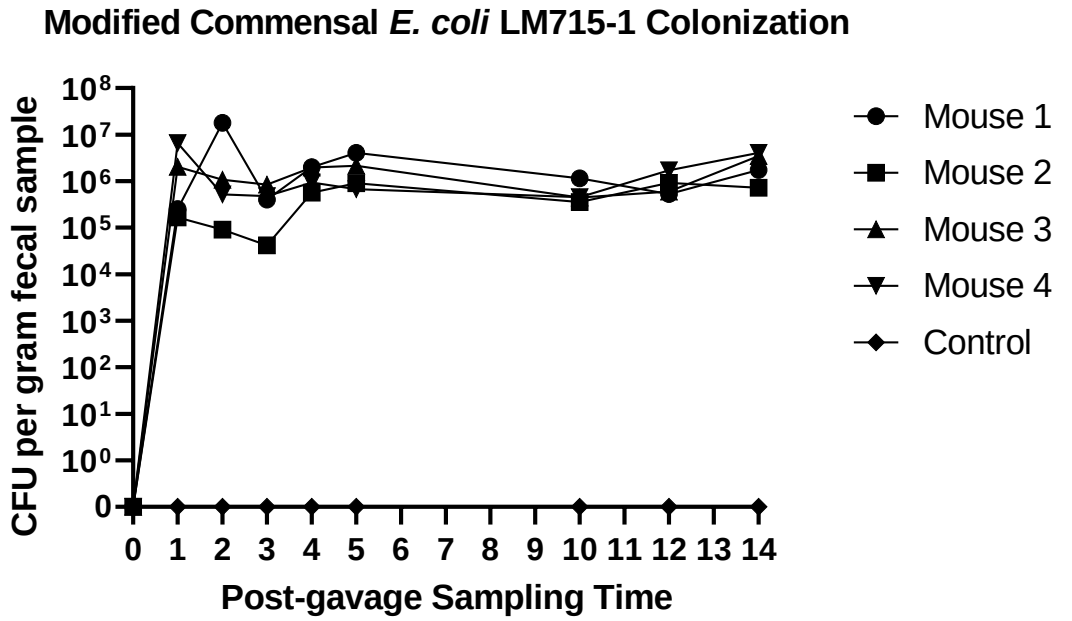


Figure 3. 1 This graphical abstract shows the experimental design and methods used in this study.

Mice transplanted with human adult fecal microbiota were fed fluorescently labeled donor *E. coli* bacteria carrying GFP-encoded RP4 plasmid. Colonization of donor bacteria was assessed by culturing bacteria directly from mouse fecal samples. FACS was performed on fecal samples to detect and sorted transconjugant bacteria expressing only GFP encoded on the RP4 plasmid. 16S rRNA amplicon sequencing was done on sorted bacteria to characterize the potential recipient of broad host range RP4 plasmid in the gut microbiota.

(A)



(B)

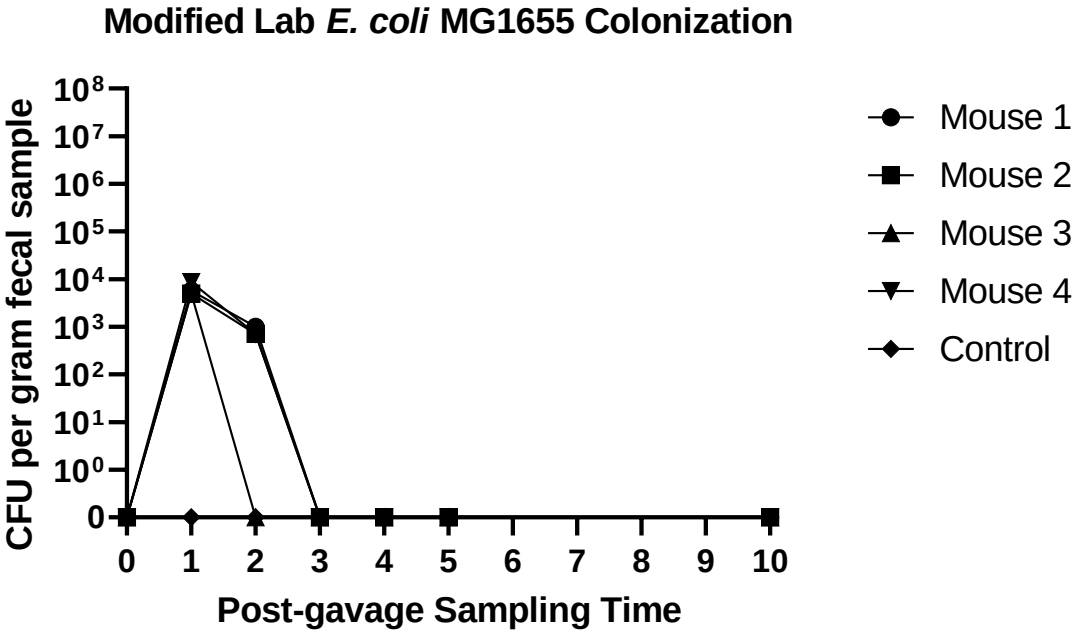
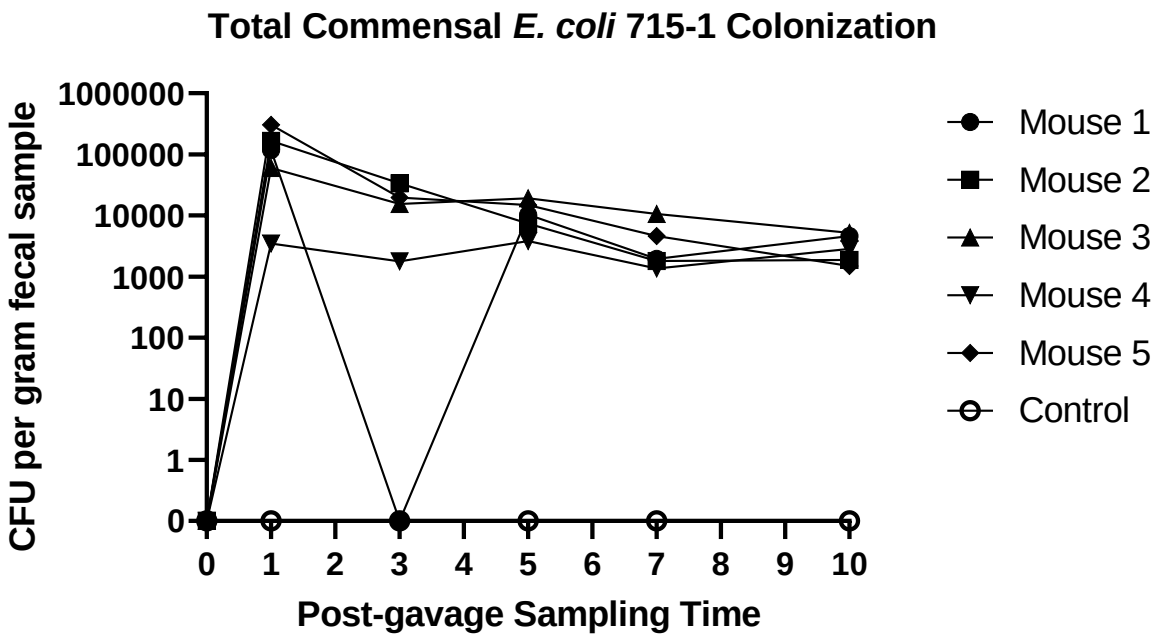


Figure 3. 2 Colonization of fluorescently labeled commensal and laboratory strains in the mouse gut.

Figure 3.2 (Cont'd)

Figure 3.2A shows the total colony forming units (CFUs) of the human-derived *E. coli* LM715-1 bacterium identified throughout the 14-day experiment as determined by serial dilution plating on MacConkey agar containing chloramphenicol. Figure B shows the colonization of labeled laboratory strain of *E. coli* MG1655 in the mouse gut as determined by serial dilution plating MacConkey agar containing ampicillin. Each symbol represents the data points of the induvial mouse of the experiment (n=4). 100 CFUs per gram fecal material was the lower limit detection.

(A)



(B)

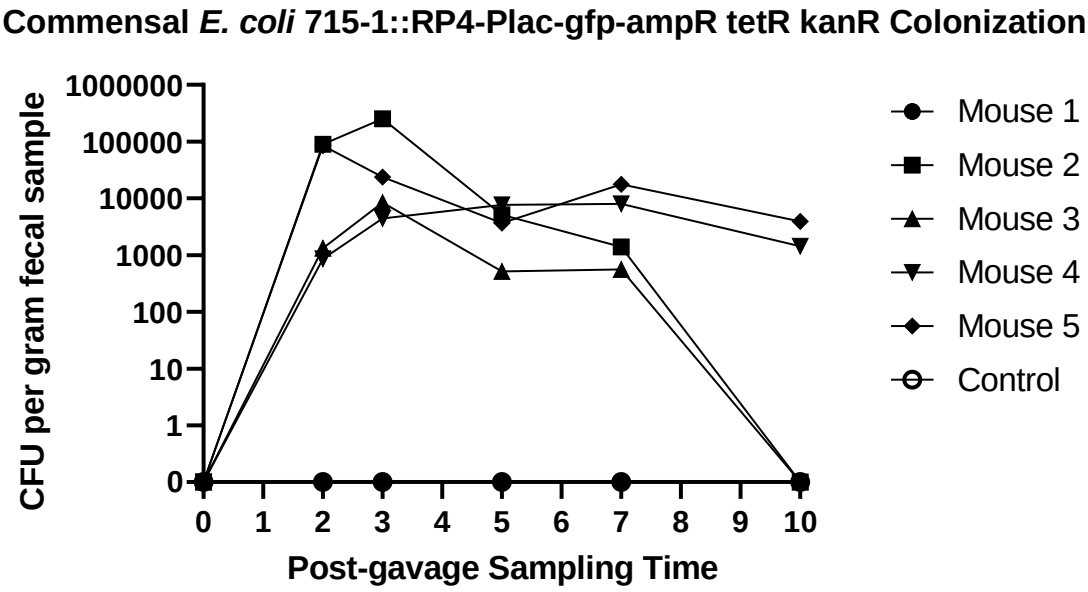


Figure 3. 3 A modified commensal donor *E. coli* strain colonized the mouse gut without any antibiotic selection pressure.

Figure 3.3 (Cont'd)

Figure 3.3 A) The total number of bacteria with and without RP4 plasmid is shown for each mouse as determined by serial dilution plating on MacConkey agar containing chloramphenicol. Figure 3.3 B) Number of bacterial cells carrying RP4 plasmid (donor bacteria) on different days of the ten-day experiment as determined by serial dilution plating on MacConkey agar containing chloramphenicol and ampicillin. Each symbol represents the data points of the induvial mouse of the experiment (n=5). Mouse 1 remained negative for donor bacteria after 24 hours. Two mice (n=2) were in the control group given Luria broth only. 100 CFUs per gram fecal material was the lower limit detection.

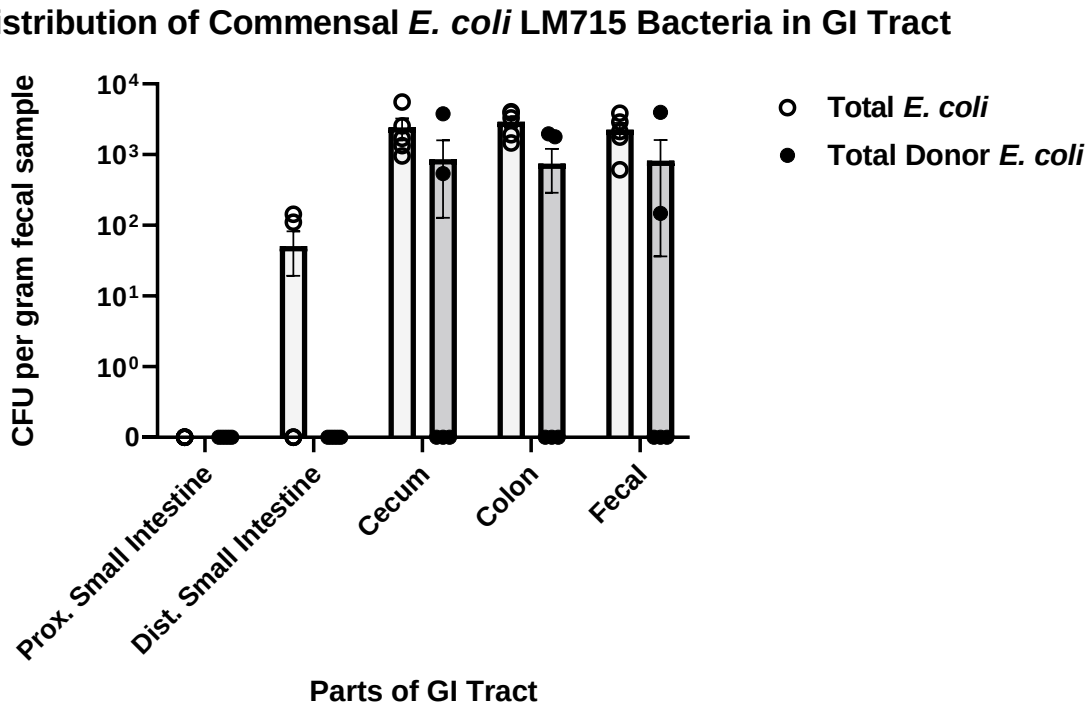


Figure 3. 4 Distribution of modified commensal donor *E. coli* strain in the mouse gut.

Empty circles show the total number of bacterial cells with and without RP4 plasmid as determined by serial dilution plating on MacConkey agar containing chloramphenicol, while filed circles show mice donor bacteria carrying RP4 plasmid as determined by serial dilution plating on MacConkey agar containing chloramphenicol and ampicillin at the end of the ten-day experiment. Each symbol represents the data points of an individual mouse in the experiment (n=5). 100 CFUs per gram fecal material was the lower limit detection.

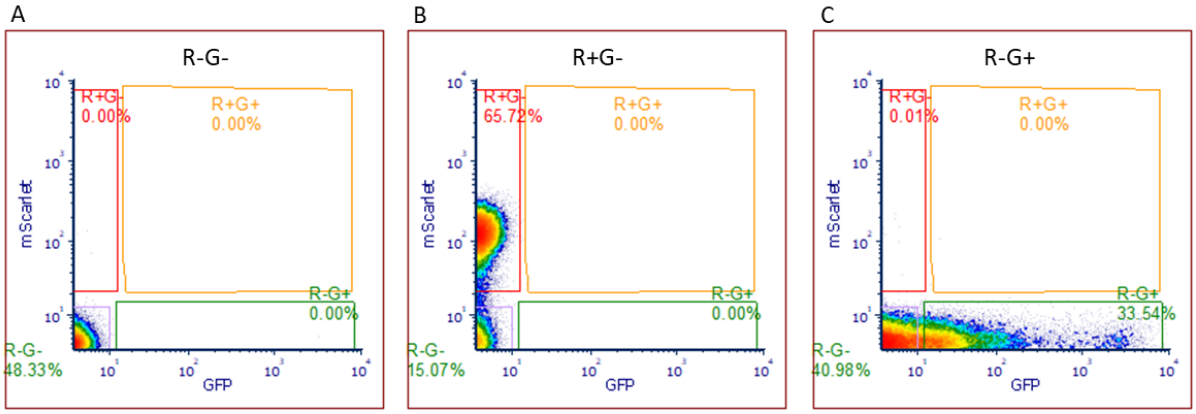


Figure 3. 5 Flow cytometry sorting of bacterial cells with and without fluorescent proteins.

Gates were drawn based on the following pure cultures of bacteria. A) *E. coli* LM715-1 bacteria without any fluorescent protein (R-G-), B) *E. coli* LM715-1 bacteria with a red fluorescent protein (R+G-), and C) *E. coli* LM715-1 bacteria with a green fluorescent protein (R-G+).

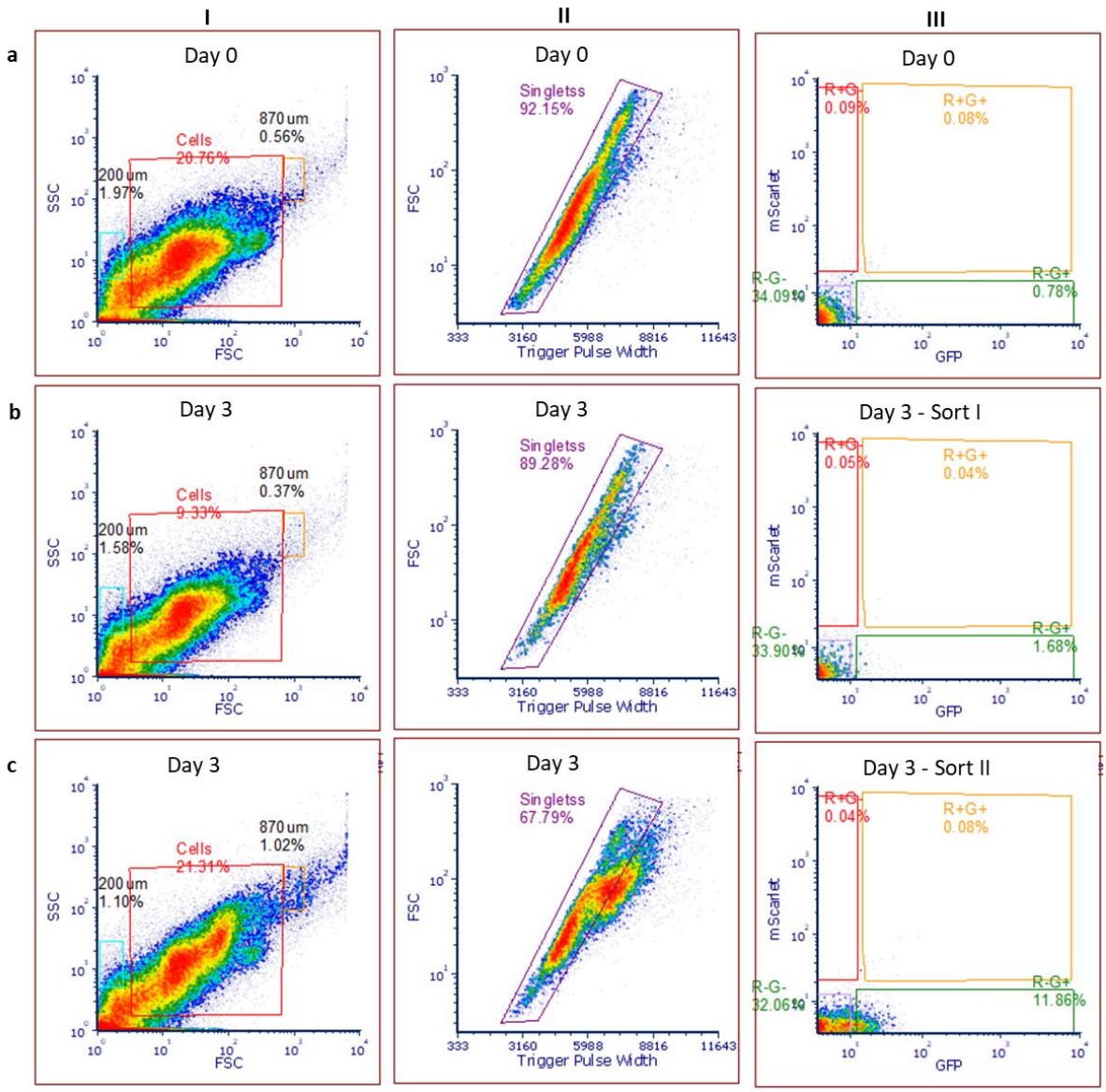


Figure 3. 6 FACS sorting of transconjugant bacteria from fecal samples of mice fed with modified commensal donor *E. coli* strain carrying RP4 plasmid.

The sorting was performed using three successive gates shown in three columns (I, II, III). Gate I sorts for bacterial size based on forward scatter (FSC) and side scatter (SSC); Gate II sorts for singlets; and Gate III sorts the cells expressing green only, red only, green and red, and no fluorescence. **A**) first row shows the sorting of the fecal samples collected on day 0 before the gavage of the mice with donor stain. Autofluorescence was detected in a drawn gate for GFP only while sorting fecal samples before gavage. Thus, two consecutive sorts were performed to minimize autofluorescence. **B**) The second row shows the first sorting (sort-I) of cells from fecal samples collected on day 3 (72 hours after gavage). **C**) The third row shows the second sorting of previously sorted GFP-positive cells in the sort-I to select only those green

Figure 3.6 (Cont’d)

cells that display only green and no red fluorescence. These two consecutive sorts (I, II) reduced the chances of false-positive cells and the effect of autofluorescence.

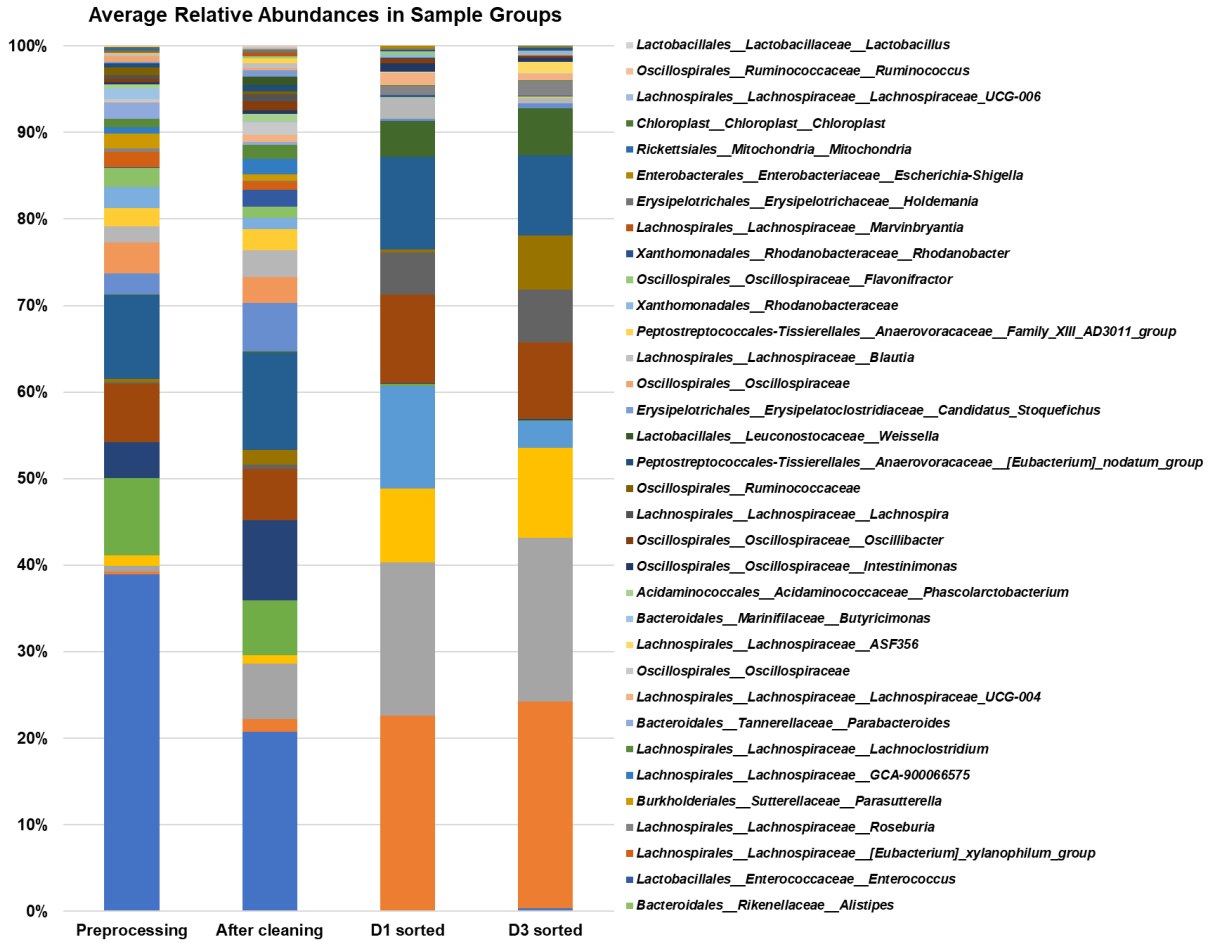


Figure 3. 7 Characterization and relative abundance of gut microbial community and FACS-sorted transconjugant bacteria.

The left y-axis represents the average relative abundance of OTUs identified in the fecal samples of five mice (n=5). Different time points for 16S sequencing analyses are shown on X-axis. Preprocessing shows the reference gut microbial community before gavaging mice with donor bacterium *E. coli* LM715-1 carrying RP4 plasmid. After cleaning bar presents the microbial community before FACS sorting after preparing fecal samples. D1 sorted and D3 sorted show the relative abundance of transconjugant bacteria identified on day 1 and day 3 of post gavage of mice with donor *E. coli* LM715-1. Legends present the most abundant OTUs identified in the gut microbiota before and after sorting the fecal samples.

(A) (B)

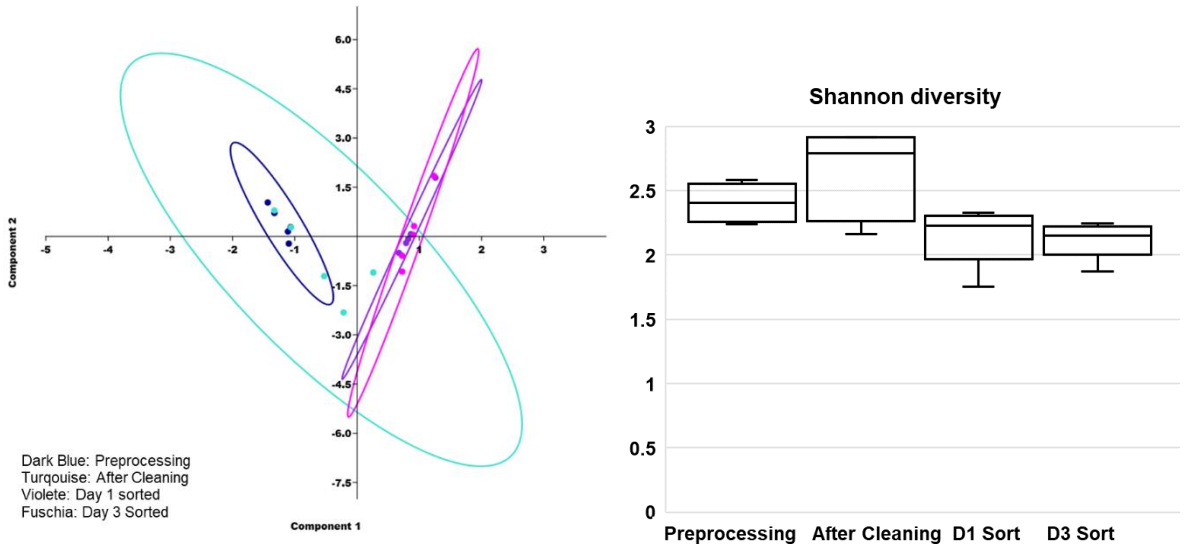


Figure 3. 8 Principal component analysis (PCA) and Shannon diversity of different groups samples.

Figure A describes PCA1 and PCA2 of 16S rRNA gene sequencing data of gut microbiota at different time points (preprocessing, after cleaning, D1 sorted and D3 sorted). PCA axis 1 accounts for 69.3% and PCA axis 2 captures 9.1% inters ample variation among different samples. Figure B present Shannon diversity of gut microbial communities in fecal samples collected at different stages.

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Chapter 4: Summaries and Future Directions

Conjugation-mediated spread of antibiotic resistance among bacteria has become a serious public health challenge worldwide. Most of the known multi-drug resistant bacteria are enteric pathogens. Little is known about the baseline transfer frequency of ARGs among bacteria in the human and animal gut, which is known as a reservoir of ARGs. The long-term goal of this study was to build a tractable mouse model to study the spread of ARGs in the human gut microbiota. This study aimed to determine the transferability of conjugative RP4 plasmid among enteric bacteria in both *in-vitro* settings and *in-vivo* mouse models. The findings from these studies paved a path for addressing the spread and persistence of antibiotic-resistant bacteria through horizontal gene transfer mechanisms in mice transplanted with human gut microbiotas. Numerous questions can be addressed further to advance the knowledge of the spread of plasmid-mediated ARGs among enteric pathogens and commensal in the human gut microbiota.

In Chapter 2, in-vitro experiments were performed to determine primary and secondary frequencies of a broad host range plasmid RP4 to multiple naïve host bacteria. Results showed that the RP4 plasmid transferred from human gut commensal donor *E. coli* LM715-1 to *Citrobacter rodentium, Salmonella typhimurium, Klebsiella pneumoniae, Pseudomonas putida, Vibrio cholerae*, and three different strains of *E. coli*. However, the plasmid transfer frequency (TF) differed between specific donor-recipient pairings, ranging from 10^{-2} to 10^{-8}. It was observed that recipient bacteria of RP4 plasmid further transferred to commensal *E. coli*. Furthermore, the effect of antibiotic ampicillin on beta-lactamase encoded RP4 plasmid transfer frequency from human gut commensal *E. coli* LM715-1 to *Citrobacter rodentium, Salmonella typhimurium,*

Klebsiella pneumoniae, Pseudomonas putida, Vibrio cholerae, and *E. coli*. By performing a serial passage plasmid persistence assay, we showed that the RP4 plasmid imposed a fitness cost on its host, *E. coli* LM715-1, resulting in the loss of the plasmid over time, but plasmid-bearing cells persisted in a low proportion of the population for at least ten transfers. These findings gave us an insight into the transferability of a broad host range RP4 plasmid among multiple enteric bacteria. But still, numerous research questions can be addressed in future studies.

Proposed Experiments:

We only determined the plasmid transfer frequencies among gram-negative bacteria belonging to families *Enterobacteriaceae*, *Pseudomonadaceae,* and *Vibrionaceae*. It will be important to conduct similar experiments to calculate frequencies for RP4 plasmid among gram-positive bacteria, including *Enterococcus faecium, Enterococcus faecalis, Staphylococcus aureus, Streptococcus pneumoniae.* These bacteria are widely known for carrying multi-drug antibiotic resistance and causing huge numbers of life-threatening nosocomial infections worldwide (CDC., 2019; Murray et al., 2022). Furthermore, *Lactobacillus reuteri* can be included in the experiment as a commensal gram-positive bacterium in the human gut. *L. reuteri* is commonly used in many probiotic products, and it would mimic transient donor and recipient of ARGs-encoded plasmid in the gut. Also, AR plasmid has been reported in *L. reuteri* probiotic strain (Egervärn et al., 2010; Rosander et al., 2008). The findings from these experiments will enhance the knowledge about plasmid-mediate ARGs spread among both gram-positive and gram-negative bacteria, and these frequencies could be used to build mathematical models for predicting the spread of ARGs in the human gut.

Next, we determined the persistence of RP4 plasmid in *E. coli* LM715-1 bacteria through serial passaging for ten days. The retainability of the plasmid among other transconjugant bacteria (*Citrobacter rodentium, Salmonella typhimurium, Klebsiella pneumoniae, Pseudomonas putida, Vibrio cholerae*) should be studied. This study will help understand how a broad host range plasmid maintains in a diverse group of naïve host enteric bacteria.

In Chapter 3, we presented *in vivo* experiments conducted to develop a tractable mouse model transplanted with adult human gut microbiota for studying the RP4 plasmid-mediated spread of ARGs from a human gut commensal donor *E. coli* LM715-1 to resident bacteria of the human gut microbiota. We found that commensal donor strain *E. coli* LM715-1 colonized the mouse gut throughout the ten-day experiment, while the laboratory donor strain *E. coli* MG1655 was not recovered after 48 hours. In this way, we showed that poor colonization of the mammalian gut by engineered laboratory-modified bacterial strains could be overcome using a gut-adapted commensal strain. Next, we addressed the inability to track donor and recipient bacteria in a complex microbial community using flow cytometry to sort the transconjugant bacteria. The flow cytometry of the treatment group samples showed an increased spread of plasmid when compared to fecal samples before the gavage. Donor and transconjugant bacteria were recovered from fecal samples and sorted by FACS. 16S sequencing analysis of sorted cells showed *Lachnospiraceae, Clostridiaceae, Pseudomonadaceae, Rhodanobacteraceae, Erysipelotrichaceae, Oscillospiraceae,* and *Butyricicoccaceae* are the primary target bacterial families of RP4 plasmid Invasion.

Proposed Experiments:

The findings from this study established foundations for designing future experiments to address more complex questions. We showed our *in vitro* studies that RP4 was transferred to members of *Enterobacteriaceae* at different transfer frequencies. However, we only used one commensal donor strain *E. coli* LM715-1 carrying RP4 plasmid to determine the spread of plasmid in the gut microbiota. It will be essential to conduct *in vivo* experiments, including other donor strains belonging to *Enterobacteriaceae* like *Salmonella typhimurium* and *Klebsiella pneumoniae*. Findings from these studies will advance the understanding of RP4 plasmid-mediated ARGs from different donor strains to resident members of the human gut microbiota.

Similarly, besides the RP4 plasmid, there is a need to understand the spread of transferable ARGs from broad host range plasmids belonging to different incompatibility groups IncA/C, IncL/M, IncN, IncP, IncQ, IncW (Jain & Srivastava, 2013) in the gut microbiota. Little is known about the presence and maintenance of these conjugative plasmids in the gut microbiota. Future studies on different broad host range plasmids will help identify the host bacteria of these plasmids and the extent of the spread of ARGs driven by these plasmids in the gut microbiota.

Studies could be designed to measure the transferability of RP4 plasmid carrying ARGs from commensal donor bacteria *E. coli* LM715-1 to resident bacteria of the different gut microbiota. In our lab, we have three of these transplanted microbiotas (Infant A, Infant B, and Adult C) taxonomically distinct and stable over generations. It allows us to ask different questions, such as the impact of microbial diversity, selection pressures, and host factors on the transfer of ARGs through conjugation in the gut

microbiota. Findings from these studies will determine the effect of different mouse gut microbiotas on the transfer of plasmid-mediated ARGs in the gut by using single donor bacteria carrying conjugative plasmid.

In this study, we performed 16S amplicon sequencing on transconjugant bacteria sorted by FACS. These findings can be further confirmed using epicPCR (Spencer et al., 2016) to characterize donor and transconjugant bacteria without sorting through FACS. The epicPCR couples the phylogenetic gene of 16S rRNA and ARG located on the plasmid in an individual bacterium. The epicPCR has been previously used to link the abundance of functional genes with the taxonomy of the microbial community in environmental samples (Spencer et al., 2016). This technique can be directly applied to fecal samples and will reduce or eliminate the effect of the fluorescent expression on identifying transconjugants from a diverse community of the mouse gut. Similarly, Hi-C is another culture-independent method to characterize bacteria carrying AR plasmid (Stalder et al., 2019). Method Hi-C is a proximity-ligation of genes present in one bacteria and links plasmid-based ARGs with chromosomal genes. In this way, bacteria with AR plasmids can be identified using metagenomics of DNA after the Hi-C ligation process. Both of these methods can be an alternative to confirm the findings of flowcytometry-based sorting or can be applied jointly with FACS to enhance the robustness of the study.

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