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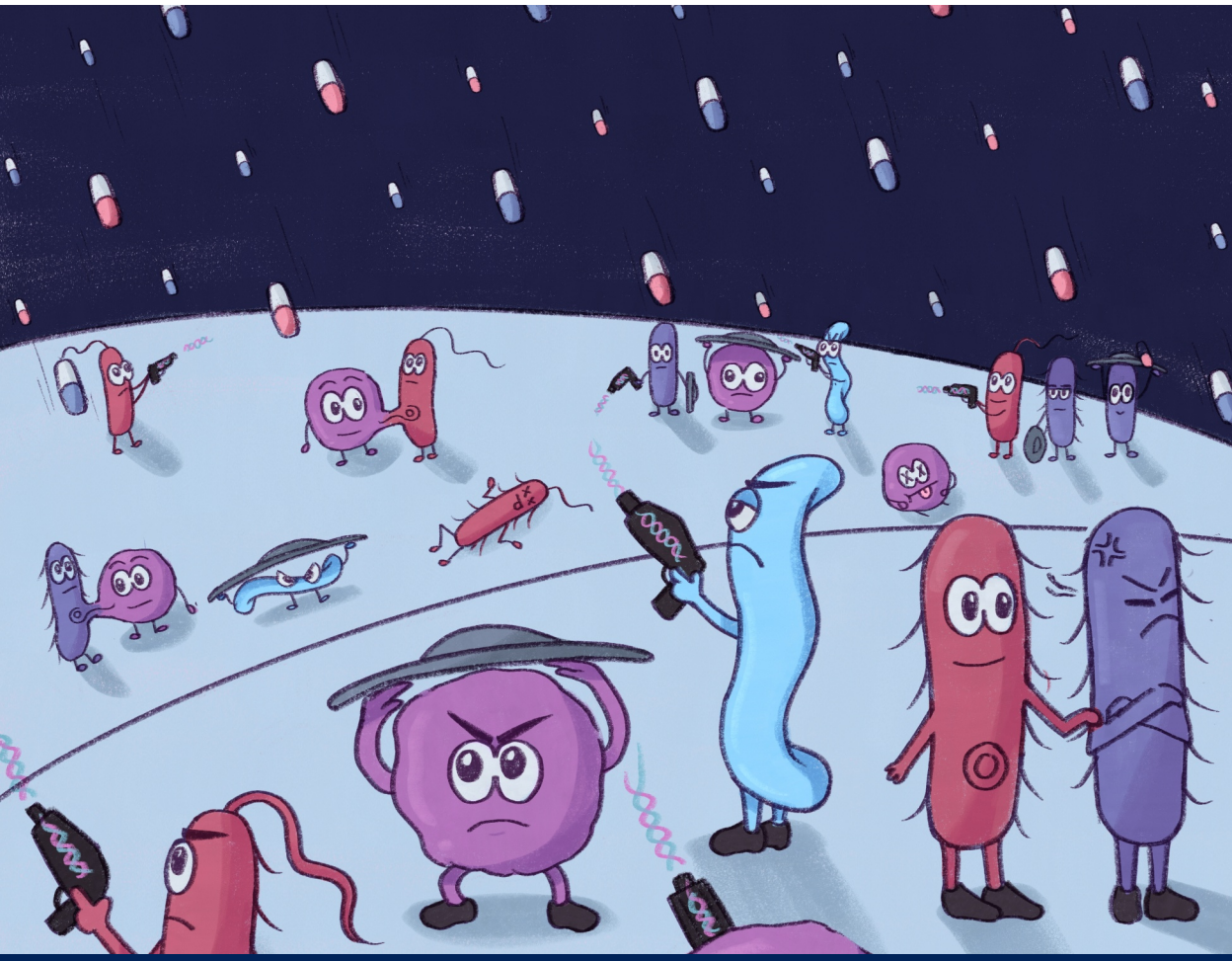
Spread and evolution of antimicrobial resistance in natural bacterial populations and communities

Pilar López-Hernández



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Spread and evolution of antimicrobial resistance in natural bacterial populations and communities

Pilar López-Hernández

Academic dissertation for the Degree of Doctor of Philosophy in Molecular Bioscience at Stockholm University to be publicly defended on Friday 25 April 2025 at 09.30 in Sal K, Undervisningshuset, Almas Allé 8, Uppsala.

Abstract

Antimicrobial resistance (AMR) is the ability of microbes to survive exposure to antimicrobial agents. Even though AMR is not a disease per se, it is claiming more lives (directly and indirectly) than malaria and HIV combined. While a lot of research has been done in this area, most studies do not account for the fact that in nature, bacterial populations are genetically diverse and live in multispecies communities where they interact with each other and the environment. Importantly, the spread and evolution of AMR can be influenced by such interactions. This thesis focuses on two processes that can promote AMR in the environment: horizontal transfer of antibiotic resistance genes (ARGs) in polymicrobial communities and co-selection of resistance-like phenotypes by exposure to other biocides. In **paper I**, bioreactors were established to grow and maintain a stable polymicrobial community over longer timescales, enabling studies of how a promiscuous plasmid (pKJK5) disseminates ARGs among microbial members in the community. The plasmid was assimilated by less abundant community members, while in contrast one of the most abundant members remained plasmid-free. The composition and dynamics of the polymicrobial community changed during and after antibiotic treatment depending on the absence/presence of the plasmid. The presence of the plasmid also seemed to prevent a bloom of a pathogenic member of this microbial community. In **paper II**, we went beyond the taxonomy of plasmid hosts to identify and describe genome-encoded plasmid transfer barriers from metagenomes and single cell genomes. There was a clear barrier for plasmid transfer related to the biochemical Gram classification. Comparing genomes of transconjugants to non-transconjugants, there were multiple enriched genomic differences in genes involved in cell envelopes and post-translational regulations while canonical plasmid transfer barriers such as presence of other incompatible plasmids or bacterial defense systems did not seem to be a major constraint for the spread of pKJK5. In **paper III**, we explored co-selection of zinc and antibiotic resistance through a series of short term experimental evolution incubations with a Gram positive environmental isolate. There was an increased antibiotic tolerance in isolates previously grown in high concentrations of zinc. While no plasmid-encoded resistance genes were found (often causing co-selection), specific chromosomal mutations were distinct to either the zinc-evolved or control isolates. Our results also highlight the need for appropriate control lines that account for domestication, as the control line in our study increased susceptibility to antibiotics tested when compared to the originally isolated parental strain. The studies advances our understanding of evolution and spread of ARGs in natural polymicrobial communities and populations, and can in the long run help us forecast and model such processes in a more mechanistic way. While AMR can spread rapidly across communities, such transfer still encounters barriers that need further investigation. The cell envelope seems to be one important barrier to horizontal transfer of ARGs, but the studies also reveal ecological roles of resistance-plasmids in polymicrobial communities and a role for selective pressures other than antibiotics in fostering AMR.

Keywords: *antimicrobial resistance, antibiotic resistance genes, horizontal gene transfer, plasmid, conjugation, plasmid barriers, zinc.*

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A la Abuela

Abstract

Antimicrobial resistance (AMR) is the ability of microbes to survive exposure to antimicrobial agents. Even though AMR is not a disease *per se*, it is claiming more lives (directly and indirectly) than malaria and HIV combined. While a lot of research has been done in this area, most studies do not account for the fact that in nature, bacterial populations are genetically diverse and live in multispecies communities where they interact with each other and the environment. Importantly, the spread and evolution of AMR can be influenced by such interactions. This thesis focuses on two processes that can promote AMR in the environment: horizontal transfer of antibiotic resistance genes (ARGs) in polymicrobial communities and co-selection of resistance-like phenotypes by exposure to other biocides. In **paper I**, bioreactors were established to grow and maintain a stable polymicrobial community over longer timescales, enabling studies of how a promiscuous plasmid (pKJK5) disseminates ARGs among microbial members in the community. The plasmid was assimilated by less abundant community members, while in contrast one of the most abundant members remained plasmid-free. The composition and dynamics of the polymicrobial community changed during and after antibiotic treatment depending on the absence/presence of the plasmid. The presence of the plasmid also seemed to prevent a bloom of a pathogenic member of this microbial community. In **paper II**, we went beyond the taxonomy of plasmid hosts to identify and describe genome-encoded plasmid transfer barriers from metagenomes and single cell genomes. There was a clear barrier for plasmid transfer related to the biochemical Gram classification. Comparing genomes of transconjugants to non-transconjugants, there were multiple enriched genomic differences in genes involved in cell envelopes and post-translational regulations while canonical plasmid transfer barriers such as presence of other incompatible plasmids or bacterial defense systems did not seem to be a major constraint for the spread of pKJK5. In **paper III**, we explored co-selection of zinc and antibiotic resistance through a series of short term experimental evolution incubations with a Gram-positive environmental isolate. There was an increased antibiotic tolerance in isolates previously grown in high concentrations of zinc. While no plasmid-encoded resistance genes were found (often causing co-selection), specific chromosomal mutations were distinct to either the zinc-evolved or control

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Keywords: antimicrobial resistance, antibiotic resistance genes, horizontal gene transfer, plasmid, conjugation, plasmid barriers, zinc

Popular summary

As a result of widespread and incorrect use of antibiotics (medications that inhibit the growth of bacteria), new antibiotic-resistant bacteria are developing, and the presence of various microorganisms that cannot be controlled with antibiotics is increasing in our environment and society. New studies show that in Europe alone, more than 35,000 people die each year directly due to resistant bacterial infections, and even more concerning is that the corresponding global figure exceeds one million deaths. In addition to the direct overuse of antibiotics, continuous exposure to other harmful substances, such as disinfectants and heavy metals, can promote bacteria that are able to survive and grow under such challenging conditions, which simultaneously promotes antibiotic resistance.

Bacteria are single-celled microorganisms, and their various characteristics, including resistance mechanisms, are derived from their genetic code and so-called genes in the DNA of their genome. Bacteria can not only transfer genes directly between generations when cells divide, but characteristics related to genetic material can also be transferred between different types of bacteria in the form of small circular DNA structures called plasmids. This type of transfer is of great importance for the spread of genes that carry antibiotic resistance in bacteria. Since bacteria normally live close to each other in communities consisting of several different species, their interactions with each other (including the transfer of plasmids containing resistance genes) and the environment they live in (including the presence of antibiotics and other biocides) will have a significant impact on the development of antibiotic resistance.

Until now, most studies on resistant bacteria have been conducted with one or two bacterial species at a time. Most studies have also exclusively focused on the bacteria that cause disease in humans or animals, which has created a biased and incomplete understanding of the biology of antibiotic resistance, one that does not take into account that bacteria (which mostly do not cause disease) interact with each other and with the environment they live in.

In order to manage and counteract current and future crises and risks associated with antibiotic resistance and thereby reduce mortality linked to infectious diseases, we need to create a deeper understanding of how resistant bacteria develop and spread, not only in pure cultures in well-controlled laboratories and not only in well-known disease-causing bacteria but also in our

environment and in the complex communities that are the natural habitat of microorganisms. In this thesis, I use new tools and methods to study the spread and emergence of antibiotic resistance in naturally complex microbial communities.

In **paper I**, I showed that a plasmid carrying antibiotic resistance genes can spread to several different types of bacteria in the human gut microbiota and that dynamic changes in both populations and the community as a whole were altered depending on the presence or absence of the plasmid. The presence of the plasmid also appeared to inhibit the growth of potential disease-causing bacteria in our model system. In **paper II**, in addition to identifying which species of bacteria were recipients of the plasmid, I was able to study whether there were obvious differences in the genetic material of bacteria that carried the plasmid and those that did not. Although we could describe an apparent barrier to plasmid transfer based on the degree of relatedness, there were also many examples where closely related populations either took up the plasmid or lacked it. In these cases, we could identify specific differences in the genetic material related to, among other things, the cell's surface structure, which could explain the varying degree of susceptibility to this horizontal gene transfer. In the final article (**paper III**), I experimentally studied whether a Gram-positive bacterium of the genus Bacillus develops increased antibiotic tolerance after exposure to the heavy metal zinc. The isolates that were exposed to zinc for a longer period of time became, as expected, more resistant to all tested antibiotics, while the control line, which was not exposed to this external environmental stress, instead became significantly more sensitive. Together, these studies contribute to an improved understanding of how antibiotic resistance develops and spreads and provide important knowledge that can help us manage the growing health threat posed by antibiotic-resistant microorganisms.

Populärvetenskaplig sammanfattning

Som följd av utbrett och felaktigt användande av antibiotika (läkemedel som hämmar tillväxten av bakterier) utvecklas nya antibiotikaresistenta bakterier och förekomsten av olika mikroorganismer som inte kan kontrolleras med antibiotika ökar i vår miljö och vårt samhälle. Nya studier visar att det enbart i Europa dör mer än 35 000 personer per år som en direkt följd av resistenta bakteriella infektioner, och än mer oroande är att motsvarande siffra globalt överstiger en miljon dödsfall. Utöver direkt över-användning av antibiotika kan kontinuerlig exponering för andra skadliga ämnen, såsom desinfektionsmedel och tungmetaller, främja bakterier som klarar av att överleva och tillväxa under sådana utmanande förhållanden, vilket samtidigt främjar antibiotikaresistens.

Bakterier är encelliga mikroorganismer och deras olika egenskaper, även resistensmekanismer, utgår från deras genetiska kod och så kallade gener i arvsmassans DNA. Bakterier kan inte bara överföra gener direkt mellan generationer när cellerna delas, utan egenskaper kopplade till genetiskt material kan även överföras mellan olika typer av bakterier i form av små cirkulära DNA strukturer som kallas plasmider. Denna typ av överföring är av stor betydelse för spridningen av gener som medför resistens mot antibiotika i bakterier. Eftersom bakterier normalt lever nära varandra i samhällen där flera olika arter ingår, kommer deras inbördes interaktioner (inklusive överföring av plasmider där resistensgener ingår) och den miljö de lever i (inklusive närvaron av antibiotika och andra biocider) ha en betydande påverkan på utvecklingen av resistens mot antibiotika.

Fram till nu har de flesta studier om resistenta bakterier genomförts med en eller två arter av bakterier åt gången. De flesta studier har dessutom fokuserat uteslutande på de bakterier som orsakar sjukdom hos människor eller djur, vilket skapat en vinklad och ofullständig kunskap om antibiotikaresistensens biologi, som inte tar hänsyn till att bakterier (som till största delen inte orsakar sjukdom) interagerar med varandra och med den miljö de lever i.

För att kunna hantera och motverka nuvarande och framtida kriser och risker associerade med antibiotikaresistens och därigenom minska dödligheten kopplat till infektionssjukdomar, behöver vi skapa en mer långtgående förståelse för hur resistenta bakterier utvecklas och sprids, och då inte bara i renkultur i i välkontrollerade laboratorier och inte bara hos kända sjukdomsorsakande bakterier, utan även i vår miljö och i de komplexa samhällen som är mikroorganismernas naturliga hemvist. I denna avhandling använder jag nya verktyg och metoder för att

studera spridning och uppkomst av antibiotikaresistens i naturligt komplexa mikrobiella samhällen.

I **artikel I** visade jag att en plasmid som bär på gener för antibiotikaresistens kan spridas till flera olika typer av bakterier som ingår i människans tarmflora och att dynamiska förändirngar i såväl påopulationer som samhället som helhet förändrades beroende på plasmidens närvaro eller frånvaro. Närvaron av plasmiden verkade också kunna förhindra tillväxt av potentiella sjukdomsorsakande bakterier i vårt modellsystem. I **artikel II** kunde jag, utöver att identifiera vilka arter av bakterier som var mottagare av plasmiden, studera om det fanns uppenbara skillnader i arvsmassan hos de bakterier som bar på plasmiden och de som inte gjorde det. Även om vi kunde beskriva en synbar barriär för överföring av plasmider som baserades på graden av släktskap, fanns det även många exempel där närbesläktade populationer endera tog upp plasmiden eller saknade densamma. I dessa fall kunde vi identifiera specifika skillnader i arvsmassan kopplat till bland annat cellens ytstruktur som kunde förklara den varierande graden av mottaglighet för denna horisontella genöverföring. I den sista artikels (**artikel III**) studerade jag experimentellt om en grampositiv bakterie av släktet Bacillus utvecklar förhöjd antibiotikatolerans efter att ha exponerats för tungmetallen zink. De isolat som under en längre tid utsatts för zink blev som förväntat mer motståndskraftiga mot alla testade antibiotika, medan kontrollinjen som inte utsatts för denna yttre miljöpåverkan och stress istället blev avsevärt mer känslig. Tillsammans bidrar studierna till en förbättrad kunskap om hur antibiotikaresistens utvecklas och sprids och viktig kunskap som kan hjälpa oss att hantera det växande hälso-hotet från antibiotikaresistenta mikroorganismer.

Resumen de divulgación científica

Como resultado del uso generalizado e incorrecto de antibióticos (medicamentos que inhiben el crecimiento de bacterias), se están desarrollando nuevas bacterias resistentes a los antibióticos, y la presencia de diversos microorganismos que no pueden ser controlados con antibióticos está aumentando en nuestro entorno y en nuestra sociedad. Nuevos estudios muestran que solo en Europa, más de 35,000 personas mueren cada año directamente debido a infecciones bacterianas resistentes, y aún más preocupante es que la cifra correspondiente a nivel mundial supera el millón de muertes. Además del uso excesivo directo de antibióticos, la exposición continua a otras sustancias perjudiciales, como desinfectantes y metales pesados, puede promover bacterias que son capaces de sobrevivir y crecer bajo tales condiciones desafiantes, lo que simultáneamente promueve la resistencia a los antibióticos.

Las bacterias son microorganismos unicelulares, y sus diversas características, incluidos los mecanismos de resistencia, provienen de su código genético y los llamados genes en el ADN de su genoma. Las bacterias no solo pueden transferir genes directamente entre generaciones cuando las células se dividen, sino que las características relacionadas con el material genético también pueden ser transferidas entre diferentes tipos de bacterias en forma de pequeñas estructuras circulares de ADN llamadas plásmidos. Este tipo de transferencia es de gran importancia para la propagación de genes que contienen resistencia a los antibióticos en las bacterias. Dado que las bacterias normalmente viven cerca unas de otras en comunidades que incluyen varias especies diferentes, sus interacciones entre ellas (incluida la transferencia de plásmidos que contienen genes de resistencia) y el entorno en el que viven (incluida la presencia de antibióticos y otros biocidas) tendrán un impacto significativo en el desarrollo de la resistencia a los antibióticos.

Hasta ahora, la mayoría de los estudios sobre bacterias resistentes se han realizado con una o dos especies bacterianas a la vez. La mayoría de los estudios también se han centrado exclusivamente en las bacterias que causan enfermedades en humanos o animales, lo que ha creado una comprensión sesgada e incompleta de la biología de la resistencia a los antibióticos, una que no tiene en cuenta que las bacterias (que en su mayoría no causan enfermedades) interactúan entre sí y con el entorno en el que viven.

Para gestionar y contrarrestar las crisis y riesgos actuales y futuros asociados con la resistencia a los antibióticos y, por lo tanto, reducir la mortalidad vinculada a enfermedades infecciosas, necesitamos crear una comprensión más profunda de cómo las bacterias resistentes se desarrollan y se propagan, no sólo en cultivos puros en laboratorios bien controlados y no solo en bacterias patógenas conocidas, sino también en nuestro entorno y en las comunidades complejas que son el hábitat natural de los microorganismos. En esta tesis, utilizo nuevas herramientas y métodos para estudiar la propagación y el surgimiento de la resistencia a los antibióticos en comunidades microbianas naturalmente complejas.

En el **artículo I**, mostré que un plásmido que transporta genes de resistencia a los antibióticos puede propagarse a varios tipos diferentes de bacterias en la microbiota intestinal humana y que los cambios dinámicos tanto en las poblaciones como en la comunidad en su conjunto se alteraron dependiendo de la presencia o ausencia del plásmido. La presencia del plásmido también pareció inhibir el crecimiento de bacterias potencialmente patógenas en nuestro sistema modelo. En el **artículo II**, además de identificar qué especies de bacterias eran receptoras del plásmido, pude estudiar si existían diferencias evidentes en el material genético de las bacterias que llevaban el plásmido y las que no lo llevaban. Aunque pudimos describir una barrera aparente para la transferencia de plásmidos basada en el grado de parentesco, también hubo muchos ejemplos en los que poblaciones estrechamente relacionadas unas tomaron el plásmido mientras otras no. En estos casos, pudimos identificar diferencias específicas en el material genético relacionadas, entre otras cosas, con la estructura superficial de la célula, lo que podría explicar el grado variable de susceptibilidad a esta transferencia horizontal de genes. En el artículo final (**artículo III**), estudié experimentalmente si una bacteria Gram positiva del género Bacillus desarrolla una mayor tolerancia a los antibióticos después de haber sido expuesta al metal pesado zinc. Los aislamientos que estuvieron expuestos al zinc durante un período de tiempo más largo se volvieron, como era de esperar, más resistentes a todos los antibióticos probados, mientras que la línea de control, que no estuvo expuesta a este estrés ambiental externo, se volvió significativamente más sensible. Juntas, estas investigaciones contribuyen a una comprensión mejorada de cómo se desarrolla y propaga la resistencia a los antibióticos y proporcionan conocimientos importantes que pueden ayudarnos a manejar la creciente amenaza para la salud que representan los microorganismos resistentes a los antibióticos.

List of included papers

This thesis is based on the following work, referred to in the text by roman numerals:

I. **López-Hernández, P.**, Buck M., Bergin C., Udekwu K.* and Bertilsson S.* (2024) Impacts, range and dynamics of a promiscuous conjugative plasmid in an anaerobic poly-microbial bioreactor community. *Under review*

II. **López-Hernández, P.**, Buck M., Kalckar Olesen, A., Raine A., Bergin C., Søren Johannes S., and Bertilsson S. (2025) Identifying Transfer Barriers of a Broad-Host-Range Plasmid in a Wastewater Microbial Community Using Metagenomic and Single-Cell Data. *Under review*

III. **López-Hernández, P.**, Buck M., Pastuszek P., Moet L., Bertilsson S. and Udekwu K. (2025) Concurrent evolution of zinc and antibiotic resistance in *Bacillus altitudinis*. *Manuscript*

* contributed equally to the study

Other publications not included in this thesis:

Westmeijer, G., Escudero, C., Bergin, C., Turner, S., Ståhle, M., Mehrshad, M., Leroy, P., Buck, M., **López-Hernández, P.**, Kallmeyer, J. and Amils, R., Bertilsson, S., Dopson, M. 2024. Continental scientific drilling and microbiology:(extremely) low biomass in bedrock of central Sweden. Biogeosciences, 21(2), pp.591-604.

Respondent contributions

The respondent contributed with laboratory work, data analysis, drafting-writing of the manuscripts and led the scientific discussion for Paper I, Paper II and Paper III. More detailed contributions:

Paper I:
Laboratory work: experimental design, preparation and assembly of the system of bioreactors, run the experiment and sample collection, process of samples for further analysis, nucleic-acid extractions, preparation of libraries and sequencing with both Illumina and Oxford Nanopore.
Data analysis work: data processing of amplicons, leading and planning of analyses
Writing work: drafting and writing of the manuscript

Paper II:
Laboratory work: filter matting experiment, nuclei-acid extraction for metagenomics
Data analysis work: data analysis of single cells, leading and planning of analyses
Writing work: drafting and writing of the manuscript.

Paper III:
Laboratory work: sampling of the urban environment, isolation of strains of interest, experimental design of the short evolution experiment, run evolution experiment line L, MIC assays, disc diffusion assays and nucleic-acid extractions
Data analysis work: whole genome analysis, comparative genomic analysis, data visualization
Writing work: drafting and writing of the manuscript.

Abbreviations

AMR	antimicrobial resistance
ARG	antibiotic resistant gene
ASV	amplicon sequence variant
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic acid
FACS	fluorescence activated cell sorting
GFP	green fluorescence protein
HGT	horizontal gene transfer
MAG	metagenome assembled genome
MDA	multiple displacement amplification
MIC	minimum inhibitory concentration
NGS	next generation sequencing
OM	outer membrane
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RM system	restriction modification system
SAG	single cell amplified genome
SNP	single nucleotide polymorphism
SPLAT	splinted ligation adapter tagging
TMP	trimethoprim
T/AT system	toxin antitoxin system
up_SPLAT	ultra-pooled splinted ligation adapter tagging
RNA	ribonucleic acid

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Introduction

The goal of this introductory section is to provide the reader with context and reasonable reasons to justify the content of this thesis. To achieve such a goal, I will start by recognizing the global health emergency that antibiotic resistant bacteria present. The current antibiotic crisis has multiple facets, some of them are bacterial, some of them are not, but we will be focusing on the former ones for this thesis. Antibiotic resistance will always be part of the microbial world, hence it needs to be studied well enough for us to manage this health threat better from now on. In essence, using antibiotics to our advantage while minimizing the risks (i.g resistance). It is undeniably so that research on antimicrobial resistance is abundantly represented in the scientific literature. This body of literature has increased during the last few decades due to the realization that we humans were facing this serious and global, but largely invisible problem. However, most of the literature has been done with single isolates, model organisms or laboratory strains, neglecting that those lonely domesticated bacterial cells do not adequately represent the real world, but rather selected for practical reasons. With the arrival of culture-independent techniques in the field of microbiology and molecular biology (i.e. Next Generation Sequencing), we have corroborated that microbes live in "multicultural societies" and that the composition of those communities and their interactions (which at same time depends on environmental conditions), determine the evolutionary trajectory of its members, including selection (or not) of antibiotic resistance genes and the emergence of resistant strains. In other words, the microbial ecology matters as it can forecast the evolution and spread of antimicrobial resistance. We need to study the response to antibiotics of species (pathogenic or not but retrieved from natural settings) within a microbial community context to gain a deeper understanding of how this (originally) microbial war works.

For the reasons above, with this thesis, I contribute to the field by challenging natural bacterial populations (including diverse communities) to selective pressures such as antibiotics and heavy metals while exploring culturing and analytical ways to study the spread and evolution of antibiotic resistance genes.

CHAPTER 1: The antimicrobial resistance pandemic

Antimicrobial resistance (AMR) is the ability of microbes to resist antimicrobials. Since the 1980´s, AMR has developed into a major threat to global public health and is now recognized as one of the top health topics priorities according to the World Health Organization (WHO). During the last decade, a few reports have been done to study the present state, history and trends of antimicrobial resistance around the globe.
Nowadays, we are experiencing an antibiotic crisis where bacterial infections that were previously easily treated with a first choice of antibiotics are now much harder to cure due to resistant bacterial strains that can cause long term health issues and even death. It has been estimated that in 2021 there were 4.95 million deaths associated with antibiotic resistant bacteria, of which more than a million were directly caused by (or attributable to) resistant bacterial infections (*Naghavi et al.*, 2024). If this trend continues, we are facing bigger numbers in all age groups by 2050 (Figure1) with an estimated 10 million deaths globally associated to AMR (*Review on Antimicrobial Resistance,* 2016; *Antimicrobial Resistance Collaborators, Murray et al*., 2022), with 39 millions between now and 2050.

In addition, AMR causes a great economic burden for our society and the collapse in hospitals causes other health issues to worsen *(Fonou et al*., 2017; *Zhu et al*., 2024). After the mentioned alarming reports, efforts to reduce the global use of antibiotics (including clinics, veterinarian and livestock settings) and reach-out to the general public have started to pay off (*Chukwu et al*., 2024). Furthermore, ongoing fundamental research on antimicrobial resistance is essential to develop strategies aimed at reducing the emergence of resistant strains and identifying additional targets for future therapeutic interventions.

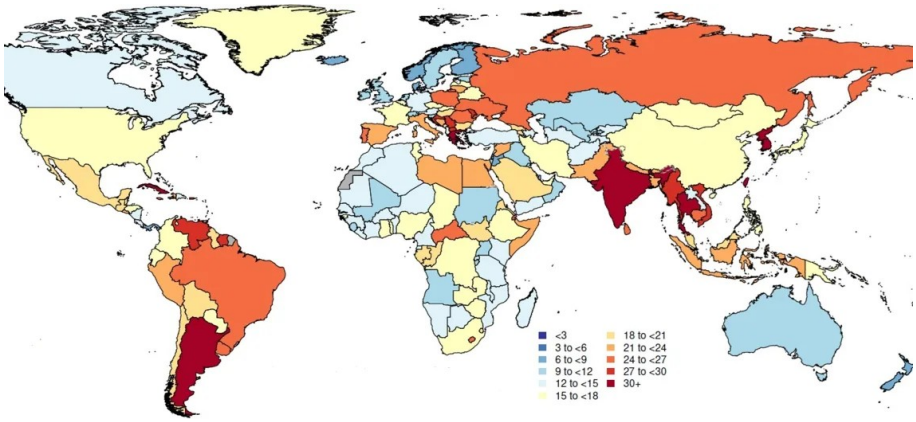

Figure 1. *Estimated death rate attributable to AMR, all ages in 2050, according to an extensive report based on 1990-2021 data (Adapted from Naghavi et al, 2024. CC BY 4.0).*

Antibiotics (and other biocides)

Antimicrobials (from the greek: *anti* (against), *mickos* (small), *bios* (life)) are small molecules that inhibit the growth or reproduction of microorganisms (bacteria, viruses, fungi and microscopic parasites). Antibiotics are a type of antimicrobials that specifically target bacterial cell components slowing down their growth (bacteriostatic antibiotics) or killing them (bactericidal antibiotics). There are hundreds of different types of antibiotics but generally they are classified according to their chemical nature and structure resulting in 6-8 broader groups of compounds. The ability to produce these secondary metabolites that we call antibiotics, are traits that bacteria evolved a long time ago and occur naturally in the environment. Most of them are produced by Actinomycetes, a group of Gram-positive bacteria (terrestrial or aquatic with high GC content) whose most known members are Streptomyces spp. This group produce, for example, lincosamides, chloramphenicol, carbapenems, macrolides, fosfomycin, aminoglycosides and tetracyclines. There are other antibiotic-producing bacteria and also fungi can secrete antibiotics such as the famous penicillins or cephalosporins and fusidic acid. Nowadays there are even semi-synthetic antibiotics (originally produced by microorganisms but later modified in laboratories (e.g. penicillins v and g) and entirely synthetic ones such as cephalosporins and sulphonamides.

Humans have been using drugs with antimicrobial properties since ancient civilizations when it was observed that applying mold on open wounds reduced skin infections (presumably caused by bacteria). Around 1910, Paul

Ehrlich developed arsphenamine (known as Salvarsan), which was presented as a "magic bullet" after helping people recover from infections cause by *Treponema pallidum* (syphilis). This became the standard treatment until the 1940s when the current antibiotic chemotherapy came into practice with penicillin (discovered by Alexander Fleming in 1928). Since then, several new antibiotics have been discovered and developed, presenting different mechanisms of action against bacteria and we can classify them according to their cellular targets (*Allison and Lambert*, 2024) (i) cell wall synthesis, (ii) protein synthesis, (iii) nucleic acid synthesis, (iv) metabolic processes and (v) cell membrane. Often, only a few years after starting to use a new antibiotic, resistance will emerge (*Stennett et al.*, 2022).

Just as they evolved antibiotics, bacteria also evolved mechanisms of resistance against antibiotics to cope with such stressors. Bacterial resistance mechanisms to antimicrobials can also be organized into classes depending on the mechanism of action (*Darby et al.*, 2022) into (i) reduced permeability, bacteria can alter their cell envelope structure preventing the drug from entering the cell (ii) active transport of antibiotics by efflux pumps, bacteria can expel antibiotics and heavy metals out of the cell using transmembrane transporters (iii) target alteration, modification and protection by altering the antibiotic specific target in the bacterial cell which usually requires high affinity (iv) inactivation and modification of the drug, where the antibiotic gets either degraded or modified with chemical groups by bacterial enzymes and (v) target bypass, bacteria can produce an alternative product to the antibiotics target that would have less affinity for the drug as well as producing more of the target.

Apart from antibiotics, there are other biocides that can contribute to the emergence and maintenance of antibiotic resistant bacteria. For instance, certain compounds that occur in high concentrations in the environment can have for bacteria similar toxicity effects to antibiotics (i.e. disinfectants like chlorine, hydrogen peroxide, alcohols or heavy metals) (*Maillard*, 2018). It has been shown that high concentrations of heavy metals (e.g. mercury, copper or zinc) can be detrimental to bacteria, while some bacterial cells may nevertheless survive (*Pal et al*., 2022). Such heavy metal survivors would often be well equipped to overcome the presence of antibiotics due to general resistance mechanisms such as efflux pumps as well as more specific resistance genes frequently found in mobile genetic elements like plasmids (small pieces of extra chromosomal cirular DNA). Plasmids are also known to frequently carry genes encoding for resistance mechanisms of action to both toxicants being co-selection for antibiotic and heavy metals broadly recognized and accepted in the scientific community (*Nguyen et al.*, 2019). Accordingly, different biocides than antibiotics themselves (e.g. heavy metal

pollution) can increase antibiotic resistance within natural microbial populations.

Origin, evolution and spread of resistance to antibiotics

But how do resistant bacteria evolve and emerge?

For millions of years bacteria have been developing the production of secondary metabolites that can have a variety of functions, being one of them able to restrict the growth of other microorganisms (i.e. antibiotics) in close proximity (e.g competitors of other species). It would be expected that those antibiotic-producing bacteria would be "immune" to their own products (else they simply couldn't exist) either by lacking the antibiotic target or by having some sort of molecular mechanism that avoids the drug or its mode of action encoded in genes that we call now resistance genes (*Peterson and Kaur*, 2018). Bacteria can multiply fast (hours or days) doubling quickly their population size. During each cell division, DNA mutations in the bacterial genome can occur and depending on the environmental conditions those mutations can be advantageous to the bacterial cell (*Imhof and Schlöttere*, 2001; *Giraud et al.*, 2001; *Denamur and Matic*, 2006; *Gordo et al.*, 2012; *Perfeito et al.*, 2017). When environmental conditions are challenging for bacteria (like the presence of antibiotics in their surroundings) not all of them will overcome those conditions while others, the most fit (sometimes due to the advantages of mutations) will be able to continue reproducing, this is called natural selection . The challenging conditions are referred to as selective pressures, as such conditions apply pressure to the cell population and ultimately will select for those who are able to manage the "new" environment. As a consequence, the susceptible ones would have a higher risk of extinction while the fit ones will make now at least the majority of the population. In the case of antibiotics as selective pressure, the bacterial variant selected after the presence of antibiotics is what we call a resistant strain (Figure 2).

In nature, antibiotic-producing organisms are secreting these compounds at sub-lethal concentrations (*Aminov*, 2009). In a natural way, resistant strains are commonly present in bacterial populations as they are part of the genetic diversity (mutations and genetic drift). As long as this diversity is maintained, resistant strains do not present the same severity of a problem as when antibiotics are being used at anthropogenic concentrations and frequency. Hence, the more often we use (and abuse) antibiotics the more resistant bacteria will be present as a result of positive selection.

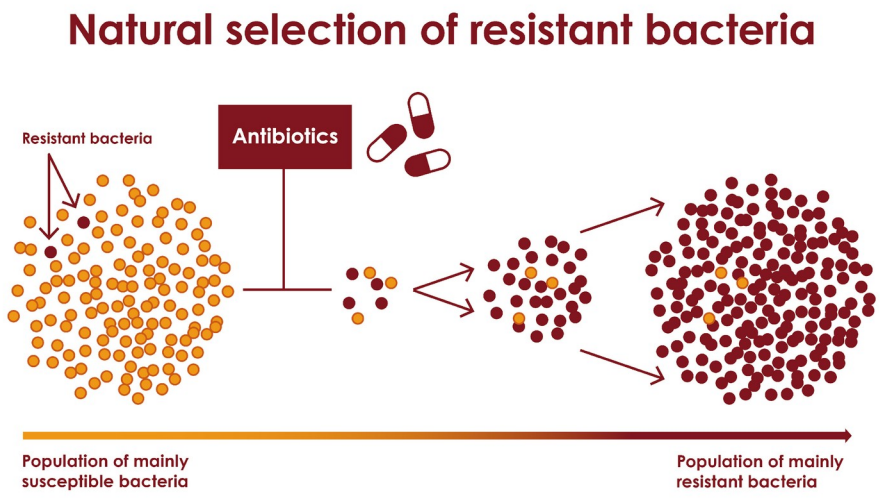

Figure 2. *Very nice imagine showing how antibiotic resistant strains naturally occur as a minority but with the use of antibiotics they can evolve by selection towards a majority. (Source: Uppsala Antibiotic Center).*

But how do resistance genes spread?

Until now, we've said that some bacteria will have beneficial genetic differences that will be selected for under antibiotic conditions, giving rise to emergence of resistant populations, but how can this trait spread to other bacteria?. The first line of inheritance is from parental cell to offspring, when bacteria divide they do so by binary fission, resulting in two identical cells that include any mutations seen in the parental cell along with additional *de novo* mutations that occur during the imminent cell division. Hence, if the parental cell present resistance genes, these will also be carried by the offspring. But bacteria also have another types of "inheritance" that is key for the fast dissemination of antibiotic resistant genes (ARGs); horizontal gene transfer. Horizontal gene transfer is the transfer of genetic material between non-genealogical organisms (*Goldenfeld and Woese*, 2007) and as this is a central topic in the thesis, an entire introductory chapter will be dedicated to describe our current understanding of horizontal transfer of ARGs by means of conjugation (Chapter 2).

Contributing factors to the current antimicrobial resistance pandemic

The antibiotic resistance crisis is not the outcome of a single source but rather of various interconnected factors. However, there are two clear main drivers of AMR: overuse and misuse of antibiotics (*Reghukumar*, 2023).

Since the discovery of penicillin in the 1920's, we have been massively using antibiotics not only to resolve ongoing bacterial infections but also to prevent them, including in animal husbandry, where they are even used as growth promoters at a sub-lethal concentrations (*Nhung et al.*, 2016; *Hassan et al.*, 2018) and agriculture *(Zalewska et al*., 2021, *Wu et al*, 2022). Animal husbandry is where the biggest overuse of antibiotics occurs. According to reports (*Boeckel et al.*, 2017; *Tiseo et al*., 2020), around 70% of antibiotics are used by animals and a good part of those antibiotics are medically important to humans. It is also of interest to notice that the use of antibiotics varies significantly between countries with a gap over 300mg/PCU (PCU stands for population correction unit and it takes into account the animal population and the size of each animal to calculate the antibiotic treatment dose) between Thailand (the highest) and Norway (the lowest) (*Mulchandani et al.*, 2023), and that in Europe, particularly in the Nordics, the use of antibiotics has been reduced significantly in animals since the 1980's having nowadays one of lowest resistance rates of the world (Figure 1 and Figure 3), leaving evidence that limiting antibiotics use in animal farming significantly decreases resistances while continuing keeping food from animal sources safe to eat.

With regards to human consumption of antibiotics in the clinical settings, an alarmingly high percentage of prescriptions are actually unnecessary (*Lopez-Vazquez et al*., 2012; *Kitano et al*., 2021). In addition, patients often do not follow the guidelines for antibiotic treatments, i.e. not finishing the treatments because they often start feeling well before the end of the treatment (giving the possibility to re-infections due to a small number of bacteria remaining). In addition, due to lack of understanding patients also take antibiotics without prescription when they actually have a viral infection like the seasonal flu (for which antibiotics do not have any effect as they specifically target bacterial cell components) exposing their microbiota to antibiotics unnecessarily. In fact, low- and middle-income countries still have antibiotics as an over the counter medicine being more accessible for sick people than a medical practitioner (*Cabral et al*., 2024) also increasing the wrongly use of antibiotics.

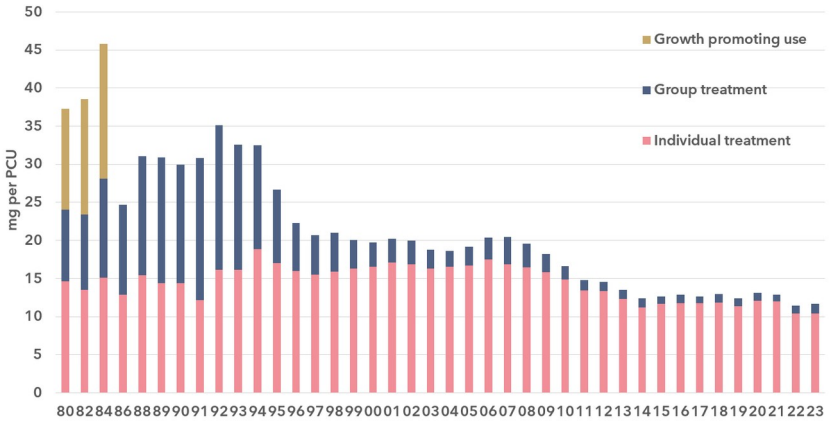

Figure 3. *Yearly sales of veterinary medicines with antibiotics expressed as mg per population correction unit (PCU) in Sweden. Sales of antibiotics for use in animals decreased 70% since the 80's. (Source: Statens Veterinärmedicinska Anstalt, Swedish veterinary agency (SVA)).*

Improper disposal of antibiotics can lead to contamination of the environment with active compounds that can promote the development of resistance phenotypes. Furthermore, non-metabolized antibiotics excreted by humans and animals can enter and persist in wastewater systems. This potentially contaminated water is then used in agricultural practices, resulting in the consumption of food products, including meat and plants, that may contain traces of antibiotics and potentially resistant strains as well (*Landers et al*., 2012; *Arsene et al.*, 2022; *Ugoeze et al.*, 2024).

Another contributing factor to the current antibiotic crisis has been the lack of developing new antibiotics since the 1970s while resistance against all previously used antibiotics was being detected (*Brüssow,* 2024). This situation presents a challenge for physicians faced with patients who have multi-drug resistant infections, as these pathogens can continue to persist despite the administration of the current last-resort antibiotics.

And last but not least, bacterial genetics. Bacteria have a great genetic plasticity (including lateral acquisition of genetic material) and a fast generational time, that allow them to rapidly adapt and thrive in new and changing environments.

CHAPTER 2: Horizontal transfer of antibiotic resistant genes

Horizontal gene transfer (HGT) is the transfer of genetic material between phylogenetically unrelated organisms and can play a significant role in the adaptation and evolution of species (*Keeling and Palmer*, 2008; *Soucy et al.*, 2015; *Rodriguez-Beltrán et al.*, 2021; *Arnold et al.*, 2022). HGT was first reported by the British bacteriologist Frederick Griffith in 1928 (*Griffith*, 1928). Evidence of transfer of resistance genes through HGT was initially observed between different species of bacteria (*Ochiai et al.*, 1959). Since then, the evolution and spread of AMR via conjugation has been repeatedly observed in complex microbial communities in the environment and is often responsible for generating multi-drug resistance strains and clinical setting outbreaks (*Devanga Ragupathi et al.*, 2019; *Che et al.*, 2021; *Alonso-del Valle et al.*, 2023). There is no doubt that a major driver of the current high emergence of resistant strains is the ability of bacteria to transfer of ARGs both within and between species.

There are different types of HGT (Figure 4) including (a) conjugation where DNA is pass during cell-cell contact in the form of a plasmid, (b) transformation, where there is an uptake of free DNA directly from the environment, (c) transduction, where DNA is passed by bacteriophages while infecting one bacterial cell after the other and (d) vesiduction, which is a newly discovered mechanism mediated by outer membrane vesicles (OMVs) generated during bacterial cell growth (*Liu et al.*, 2020). Although all four processes can impact the spread of AMR (and other virulence factors), the content of this chapter will focus on conjugation since antibiotic resistance genes are most often transmitted through plasmids acquired via conjugation that can be broadly disseminated within natural poly-microbial communities in the environment (*Aminod*, 2009; *Von et al.*, 2016, *Holmes et al.*, 2016).

Conjugative plasmids (structure, properties and host range)

Antibiotic resistance genes are enriched in mobile genetic elements called plasmids (*Che et al.*, 2021). Plasmids are circular pieces of extra-chromosomal double-stranded DNA (dsDNA) that vary greatly in size (5-500 kb) and are present in all types of bacteria where they can play an important role in bacterial evolution and adaptation.

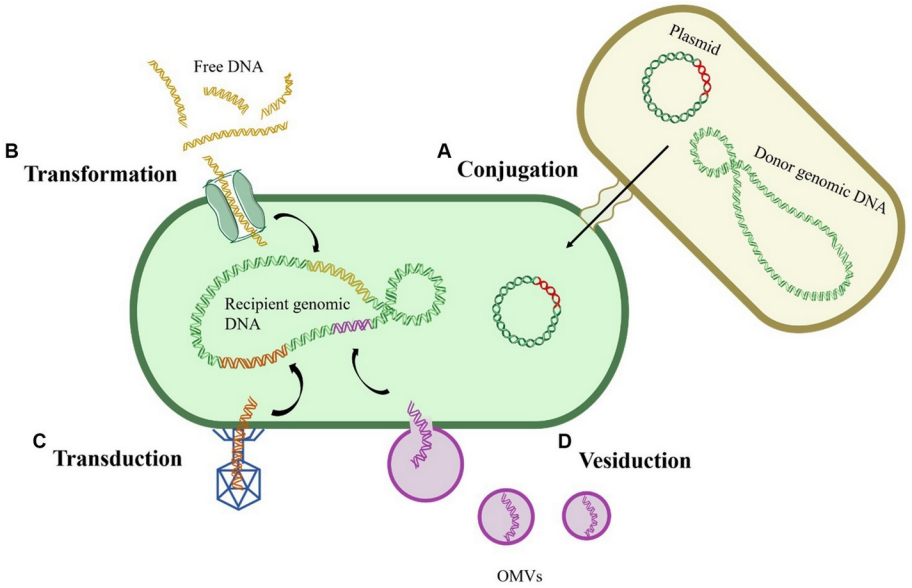

Figure 4. *The four different HGT mechanisms identified until now. (Source: Liu et al, 2024. CC BY 4.0).*

Typically, plasmids do not contain so-called “housekeeping” genes (essential for the growth of the cell) but rather carry accessory or adaptive genes (e.g. antibiotics resistance genes) with potential benefits to their hosts under certain environmental conditions (e.g. the presence of antibiotics). There are different types of plasmids, some are self-transmissible and others are mobilizable plasmids. The difference between the two is that self-transmissible plasmids can transfer themselves from cell to cell while the mobilizable plasmids need assistance of a self-transmissible plasmid for this to happen (*Smillie et al.*, 2010).

Conjugative plasmids are autonomous genetic elements that carry all the necessary information for their replication and transfer (*Carranza et al.*, 2021; *Fernández-López et al., 2017*). Their genetic structure can be split into two big modules, one containing the backbone elements of the plasmid (for plasmid-selfish things such as transfer and replication) and another one containing adaptive elements (variable between plasmids) that typically provide their host with genome-encoded traits that confer fitness advantages (e.g. antibiotic resistance genes) in return for the cost of carrying the plasmid (*Fernández-López et al.,* 2017). Within the backbone elements, we find the region where transfer begins on the plasmid DNA, known as the origin of transfer (*oriT*). This is a short (up to 500 bp) sequence of central importance that contain several elements. Upstream of the *oriT* region, there is a"leading region," which is the first part of the plasmid to enter the recipient cell. This

region contains genes that produce proteins essential for plasmid acceptance in the recipient cell upon the entry of single-stranded DNA (ssDNA). Additionally, genes necessary for the plasmid's synthesis and maintenance are encoded in the *rep* region, enabling the plasmid to replicate independently of the bacterial chromosome as well as maintaining plasmid copy number within the host cell and thereby ensure plasmid persistence. Moreover, a significant portion of the genetic material that makes a conjugative plasmid is dedicated to the transfer (*tra*) genes. The *tra* region is divided into two key components (*Henkin and Peters,* 2020):

1. The *Dtr* component encode for proteins that will break and open one of the strands of the circular piece of DNA (plasmid) so that the transfer of a single stranded piece of DNA can reach the recipient. This includes the relaxase (enzyme that recognizes the origin of transfer sequence on the plasmid and subsequently cleave one of the strands of the plasmid DNA to initiate transfer. This enzyme is part of the relaxosome (proteins that bind to the *oriT* sequence and facilitate the unwinding of double stranded DNA).

2. The *Mpf (mating pair formation)* component, which is responsible for the formation of the sex pilus and the establishment of the cell-to-cell contact that is essential for successful plasmid transfer.

Conjugative plasmids can have a narrow selection of hosts and are only shared between closely related bacterial clades. Other plasmids are very promiscuous and can be found in a wide variety of bacterial taxa (*Acman et al.,* 2020). These limits are sometimes imposed due of compatibility issues, when use of the molecular machinery of the host is needed for multiple plasmids to be stably maintained. Plasmid incompatibility groups, known as "Inc" groups (*Couturier et al*., 1988; *Fernandez-Lopez et al*., 2017), refer to groups of plasmids that share similar replication and partitioning systems. When two plasmids are under the same replicon (replication origin), they would typically require the same (and compete for) regulatory proteins and enzymes within the host and their minimum copy number needed for maintenance might not be reached since the cell doesn't distinguish between the two, leading to a high risk of extinction of at least one of the plasmids. In addition, plasmids count with partitioning and segregating systems that control during cell division that both cells mother and daughter have one copy of the plasmid. When sharing replicons, it is common to not have distinct segregation mechanisms that can ensure both plasmids segregating equally. Incompatibility can also be a consequence of regulatory proteins since some plasmids can encode for their own regulation. If two plasmids under the same replicon are in the same cell, the replication of one could be inhibited by the regulatory system of the other one.

Early classification of plasmids, before sequencing technologies were developed, was based on the observation that certain plasmids could not coexist in the same host cell, leading to the concept of incompatibility where two plasmids of the same Inc group cannot co-exist within the same host. Further studies have shown that plasmids from the same Inc group share similar backbone structures, although their diversity can vary within each group.

Conjugation

Beyond the plasmid itself, conjugation is a complex process that involves multiple steps within both the donor (plasmid-positive) and recipient (plasmid-negative) cells. The process is initiated by cell-to-cell contact, which is mediated by a protein-like structure known as the pilus. The pilus is part of the Type IV secretion system (T4SS) in bacteria and forms a cellular bridge through which genetic material can be transferred between donor and recipient cells (*Costa et al.*, 2021). The initiation of bacterial conjugation is driven by the activation of *tra* genes on the plasmid carried by the donor. This activation can be triggered by various factors such as bacterial cell density (via quorum sensing), environmental stressors (e.g. antibiotics), or physical contact with a potential recipient cell (*Frost and Koraimann, 2010; Sheppard et al., 2020*). Once activated, the *tra* genes encode proteins that facilitate the formation of the pilus and other necessary components for the conjugative process. In the donor cell, the plasmid is prepared for transfer by the relaxosome. During this process, the T4SS also plays a critical role in stabilizing the mating pair by retracting the pilus and bringing the two cells into close proximity, ensuring the successful transfer of the plasmid (*Schröder and Lanka, 2005*). Once the donor cell has transferred the single strand of plasmid DNA, the recipient cell must “accept” and synthesize the complementary strand for the plasmid to become functional within the recipient.

The establishment of mating pair formation (MPF, note “MPF” refers to the process and “Mpf” refers to a set of genes (component) in the plasmid) is a crucial step in conjugation. A stable MPF is necessary to ensure efficient DNA transfer. Research has shown that unstable mating pairs result in decreased conjugation rates (*Low et al.*, 2022), highlighting the importance of MPF stabilization in the success of DNA transfer via conjugation.

In summary, bacterial conjugation is a highly regulated process involving precise coordination of multiple factors. Successful conjugation depends on activation of *tra* genes in the donor, the formation of a stable mating pair between donor and recipient, and the recipient's ability to accept and replicate

the plasmid DNA. This process plays a vital role in the horizontal transfer of genetic material, including traits such as antibiotic resistance, between bacterial populations.

Barriers to horizontal transfer of antibiotic resistant genes

Similar to the apparent evolutionary arms race between antibiotics and antibiotic resistance genes, bacteria face an ongoing arms race in the context of HGT and HGT barriers. Bacteria continuously evolve molecular mechanisms to avoid being parasite by selfish genetic elements (*Mayo-Muñoz et al*., 2024), which can also be used against other foreign DNA such plasmids. For instance, when bacteria detect single-stranded DNA (ssDNA) like plasmids in the cytoplasm, they activate an SOS response that degrades the foreign DNA, impeding conjugation. To circumvent this defense, conjugative plasmids can encode single-stranded binding (SSB) proteins in their leading region, that bind to and protect the plasmid ssDNA from degradation by the SOS response of the recipient. Plasmids can also deploy anti-restriction modification (anti-RM) proteins to avoid recognition by bacterial RM systems (*Tock and Dryden*, 2005).

In the context of antimicrobial resistance spread via HGT, it is crucial to examine the potential defense mechanisms that bacteria may utilize to protect themselves from non-self genetic elements such as plasmids. Bacterial immune defense systems are widespread across the bacterial domain and while some bacteria rely on innate mechanisms, such as Restriction-Modification (RM) systems or Toxin-Antitoxin (TA) systems, others have acquired and deployed adaptive immunity, including Clustered Regularly Interspaced Short Palindromic Repeats and associated enzymes (CRISPR-Cas).

RM systems have been among the most extensively studied bacterial defense mechanisms since their discovery in the 1960s (*Boyer*, 1964). Restriction enzymes protect bacterial cells from mobile genetic elements, such as phages and plasmids, essentially by recognizing and differentiating their own "signature marks" (methyl groups) from that of invading genetic material. This process involves two enzymes: a methylase, which modifies specific sequences along the bacterial chromosome by adding methyl groups to protect the bacterial DNA, and an endonuclease, which can then cut foreign DNA devoid of such modifications. Over 3,000 restriction endonucleases have been characterized, with variation across different bacterial species.

The CRISPR-Cas system, now widely known as a gene-editing tool, originally evolved as an adaptive immune system in bacteria (*Makarova et al.*,

2006). CRISPR sequences consist of spacers, interspersed with short palindromic DNA sequences derived from phages that previously infected the cell. The system actively monitors incoming DNA (in the form of plasmids or phages) and integrates short segments of foreign DNA into its sequence. When a matching sequence is encountered, the CRISPR-associated (Cas) enzyme is activated to cleave the foreign DNA. This system enables bacteria to acquire immunity to specific genetic elements, making CRISPR-Cas an important barrier to horizontal gene transfer, including conjugation (*Wheatley and MacLean*, 2021).

While these defense systems have been studied extensively, there may be additional barriers to plasmid transfer that remain less well understood. Before the plasmid enters the host and immune defense systems are activated, the T4SS and MPF must succeed in physically facilitating plasmid transfer. The structural composition, protein availability, and conformation of proteins and fatty acids in the cell envelope, could play crucial roles in this process as they interact directly during cell-cell contact. Some studies have for example identified specific fatty acids as conjugation inhibitors (COINS) (*Getino et al.*, 2015).

As plasmids spread via conjugation, it is essential to consider factors that either facilitate or restrict this process. While plasmid incompatibility is an important consideration, it is equally important to evaluate the broader context of conjugation. For example, while Inc groups relate to plasmid incompatibility, they do not necessarily impact the MPF. These nuances will be more extensively discussed at the conclusion of the thesis, where the distinctions between barriers to the plasmid itself and barriers to conjugation could be explored in more detail using results observed during the research presented in this thesis.

CHAPTER 3: Antibiotic resistance in natural bacterial communities

Antibiotics and antibiotic resistance are naturally present in the environment as part of the global microbiome. In that environmental settings, bacteria typically live in spaces where multiple species coexist and interact via cooperation, competition and symbiosis. Environmental conditions and other selective pressures can also impact those interactions. When we use antibiotics, pathogens are typically the targets while they represent a minority of the total microbiome, with their ecology and evolution critically dependent on their relationships with the rest of the microbiome. Hence, trying to understand the evolution and spread of AMR with an exclusive focus on the pathogenic microbial minority will not provide us with the necessary and comprehensive knowledge about the nature of the problem. A more inclusive microbial approach, one that incorporates non-pathogenic but functionally important members of microbial ecosystems along with environmental factors, will undoubtedly enhance our understanding of the dynamics and trajectory of AMR.

AMR in the environment

Even though antibiotics are well known for having inhibitory effects on bacteria when we use them in the clinics at high concentrations to de-activate pathogenic activity, the situation is quite different in nature where such substances are often present at sub-inhibitory concentrations that in addition to having antimicrobial effect can also modulate different functions of the microbial community. For instance, they are involved in quorum sensing, biofilm formation and virulence factor production (*Sengupta et al*, 2013).

Microbes have co-evolved for billions of years while being engaged in an evolutionary arms race, where competition through adaptation and selection for antibiotic-producers and antibiotic-resistant populations takes place (*Baquero et al., 2021*). This evolutionary history has led to a situation where there is a large diversity of resistance mechanisms distributed among a high number of clades across the tree of life. Having knowledge of which mechanisms are enriched in certain environments and taxonomic groups of bacteria could expand our understanding of AMR and its ecological role. It should however be noted that it is difficult to accurately determine what type of environment harbor the most AMR, due to the fact that it is challenging to identifying ARGs and current biases generated by bioinformatic analysis of environmental data.

Nevertheless, *Inda-Diaz et al* (2023) shows that soil and freshwater have a large diversity of ARGs which is comparable to for example skin or activated sludge. ARGs have been found in all environments (*Agudo and Reche*, 2024), including the most remote and pristine parts of our planet, such as the Antarctic (*Hernandez et al*., 2019; *Hwengwere et al.*, 2022; *Bargagli and Rota*, 2024), or deep sea sediments (*Zhang et al*., 2024). These traits seem to have evolved million of years before the first use of antibiotics by humans (*Perry et al*., 2016). To explain the cosmopolitan presence of ARGs in the global microbiome, both transfer between clades and transfer between habitats need to be considered. However, the natural distribution of ARGs before humans started using antibiotics (and polluting with biocides the environment) is still not clear, making it difficult to know how much of the currently observed ARG distribution has been shaped by anthropogenic impacts. Independent of this question, it has been shown that human activity contributes to ARG transfer between habitats (Figure 5) (*Salazar et al.*, 2022; *Da Costa* et al., 2013).

As discussed in previous chapters, ARGs can be widely disseminated within microbiomes by means of HGT, with plasmids as main vectors for transfer of ARGs between clades. Plasmids have been found in a wide variety of bacterial taxa retrieved from the environment (*Rodríguez-Beltrán et al*, 2021; *Coluzzi et al.,2022*) including resistant conjugative plasmids (*Che et al., 2021*). Even though the study of the AMR ecology is advancing fast, it is still in its infancy. Hence, the dynamics and ecological roles of plasmids encoding for resistance within natural microbial ecosystems remain poorly understood.

An example of a global concept recognizing that different environments are interconnected is “One Health”. In the One Health framework it is acknowledged that the health of humans, animals and the state of the environment are tightly connected and that we can make major advances by integrating and collaboration across the different fields of research to promote the health of one global ecosystem. The One Health concepts also covers antimicrobial resistance and it validates the importance of understanding the complexity of microbial interactions through which ARGs persist in natural reservoirs (*Read et al*, 2024; *Javvadi and Mohan*, 2024).

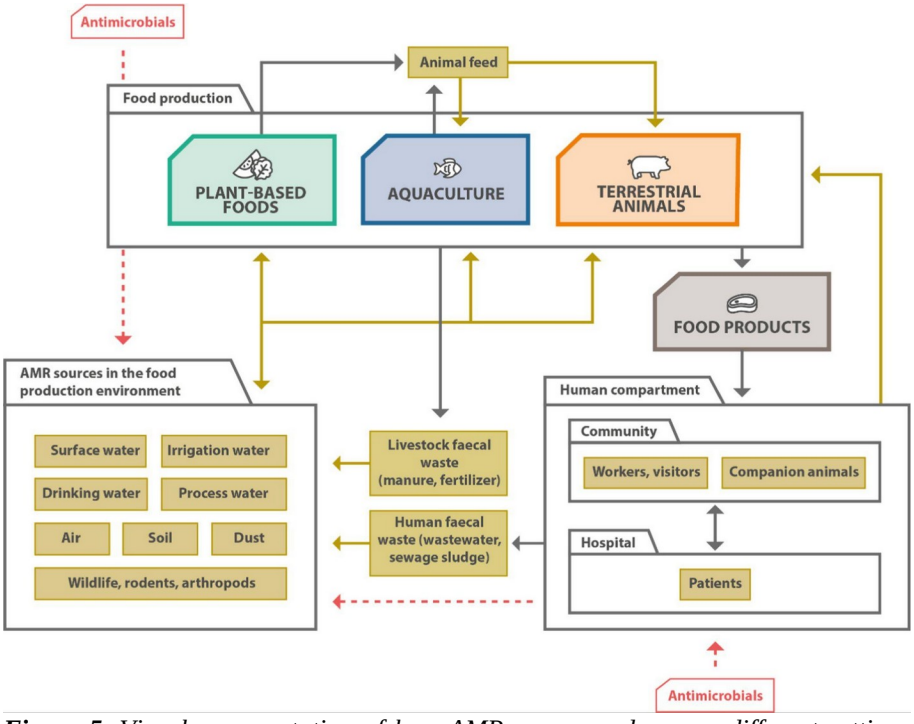

Figure 5. *Visual representation of how AMR can spread among different settings that are interconnected. Transmission routes are represented as dark gold arrows, black arrows depict the food production chain and interaction between human groups, red arrows depict the usage of antimicrobial agents (Source: Koutsoumanis et al, 2021. CC BY-ND 4.0).*

Antibiotic resistance research within a microbial ecology context

Microbial ecology is the study of the diversity, distribution, and interactions of microorganisms in ecosystems, and their role in recycling matter and energy. (Encyclopedia of ecology).

We could say that, the beginning of microbial ecology started in the late 1600's when Leeuwenhoek built his first microscope and observed diverse microbial life in drops of water (*Lane*, 2015), writing in his journal: *Examining this water...I found floating therein divers earthy particles, and some green streaks, spirally wound serpent-wise...and I judge that some of these little creatures were above a thousand times smaller than the smallest ones I have ever yet seen, upon the rind of cheese, in wheaten flour, mould, and the like*.

Until the recent development of sequencing technologies (i.e. next generation sequencing) and other molecular techniques (e.g. polymerase chain reaction), the field of microbial ecology was predominantly relying on more traditional microbiological methods dependent on culturing and visual and biochemical phenotyping. These methods limited our view of microbial diversity to the culturable minority (*Harwani*, 2013). Similarly, the study of AMR was limited to well established model systems and pathogenic isolates in single or highly simplified co-cultures. Since then, the field of microbial ecology has developed to integrate and use a wide variety of molecular, genomic, microscopy and mass spectrosscopy tools as well as bioinformatic methods to study complete microbial communities (not only the culturable minority) in a wide range of natural habitats (aquatic, terrestrial, host, extreme and even ancient) (Figure 7). These new methods allow us to study the complexity of natural microbial systems that typically offer a multitude of host genomes for ARGs.

The study of such complex systems still presents a variety of limitations though. For instance, in experimental microbiology, recreating natural ecosystems that have developed over billions of years in a laboratory laboratory settings is not an easy task (*Cao et al*, 2021). First and foremost there is microbial "dark matter" (*Rinke et al*, 2013), meaning that there are many microbes whose biology is hidden because we cannot grow them in isolation under laboratory conditions, most likely because we don't mimic their natural environment or niche well enough *(Renwick et al*, 2021). In natural ecosystems microbes often are metabolically co-dependent of each other. For instance, some microbes might need the waste byproducts of others to obtain energy (cross-feeding) or are able to utilize a compound that has been previously modified by another microbe (nutrient cycling) (*Fritts et al.*, 2021; *Culp and Goodman*, 2023). Hence cultivating only a fraction of a total microbiome could present difficulties for the growth of some.

There have been some attempts to mimic natural microbiomes, including the human gut (*Molly et al.*, 1993), but while some of these studies have been rather successful (bringing the culturing of multiple species together long term a reality), they are very specific and context-dependent and also require a large budget and highly specialized trained staff. Hence we could benefit from using simpler model systems such as continuous cultures, that provide realistic conditions while being reproducible and tractable model systems for the wider research community.

In summary, the diverse nature of microbiomes, the importance of AMR within microbial interactions and the promiscuity of ARGs means that excluding the microbial ecology dimension of AMR in our studies limits our understanding of this research field.

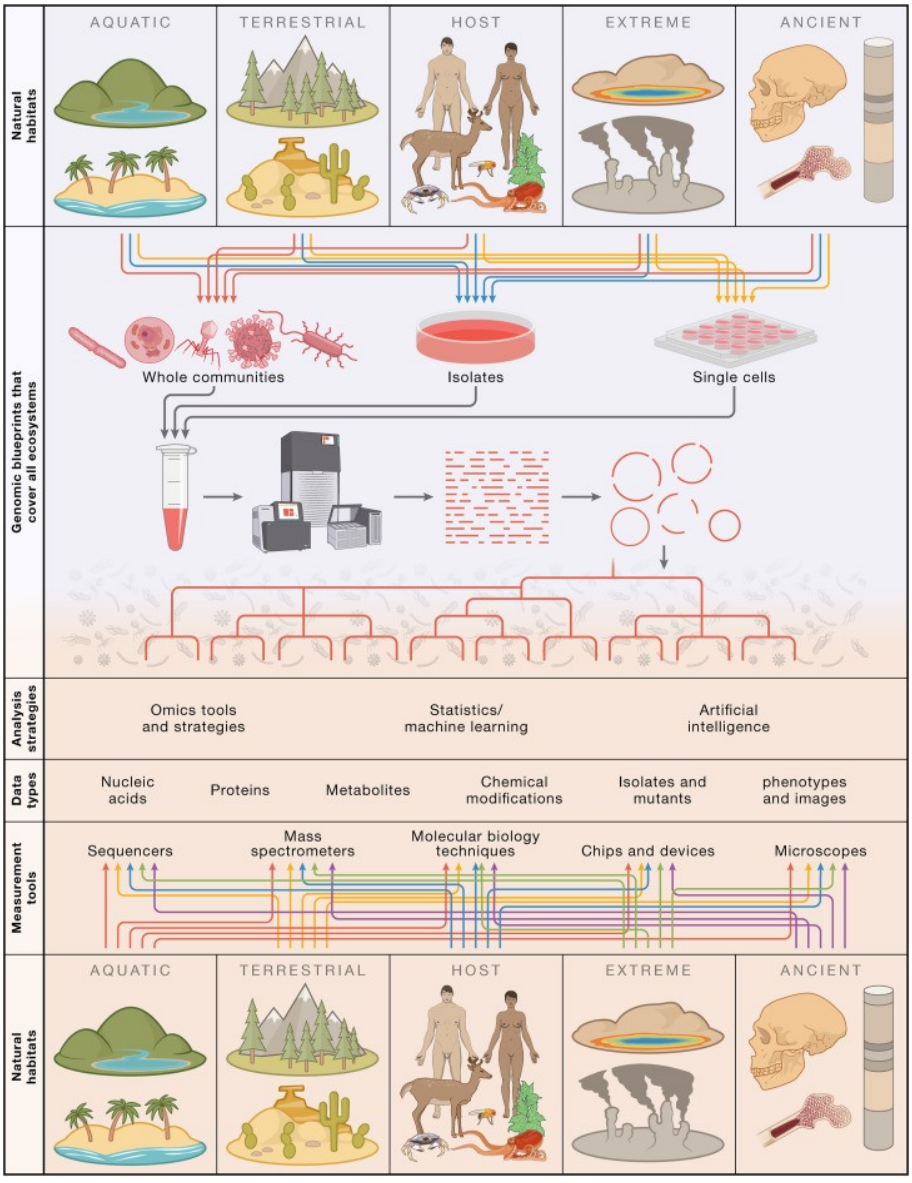

Figure 7*. Modern microbial ecology techniques, a combination of computational and molecular tools to investigate the microbial ecology of complex microbial systems. (Source: Eren and Banfield, 2024. CC-BY).*

CHAPTER 4: Methods for detection and study of AMR

This chapter contains a variety of methods available nowadays to study genotypes and phenotypes associated to AMR. I split this chapter in two: genomic and non-genomic methods.

Non-genomic methods

Prior to the genomic era we mainly studied antibiotic resistance by observing activity and phenotype of bacterial isolates cultured in the laboratory. These methods are broadly termed antimicrobial susceptibility testing (AST) and they detect resistance phenotypes with the goal of identifying so called minimum inhibitory concentration (MIC) of the drug, at which the microbe is not able to reproduce. Some of these methods are:

- Disc diffusion assays, where you place filter discs containing specific concentrations of antibiotics of choice on freshly inoculated (with bacteria subject to study) and measure the zone of inhibition that the drug makes on the growing bacteria. The larger the zone of inhibition, the bigger the antimicrobial effect (*Balouiri et al., 2016*).

- E-test, where you also plate a mat of your bacteria but instead of discs you place a filter strip containing a concentration-gradient of the antibiotic where you can then directly read out the concentration of antibiotic (indicated in the filter strip) where the bacteria under scrutiny is not reproducing (*Citron et al., 1991*).

- Broth microdilution assays (can also be done on agar), using liquid media with serial concentrations of antibiotics inoculated with bacteria. Incubations are then typically carried out overnight and optical density (OD_{600}) is measured as a proxy for growth. The lowest concentration of antibiotic showing no increase in the culture's OD is the MIC (*Weseler et al., 2005*).

- Time-kill curves are also often used to determine the ability of microbes to survive the exposure of specific drugs. In this method, multiple measurements over time are collected to generate a growth curve for each microbe-drug concentration combination, which will help identify not only the inhibitory net effect of a drug, but also the response time. Time-Kill assays can also help identify the antibiotic's mode of action on the microbe as bacteriostatic (slows

the growth but not killing of the bacteria) or bactericidal (kills) (*Van Os et al., 2021*).

- Flow cytometry, where we can count cell density which would change if the cell growth is affected by for example presence of antibiotics, or by observing other virulence factors associated with a higher tolerance to drugs like biofilms (*Saint-Ruf et al., 2016*).

The data obtained from measurement of inhibition zones and optical densities would then be used to estimate the dose needed to apply antibiotics for an ongoing infection in the medical setting. So it was useful, but of course, very limited. We could not know what genes were involved or how resistance spread. Or could not know anything about any microbe we can actually not grow in laboratory conditions.

Genomic approaches

The broad development and application of nucleic acid sequencing technology and related genomic approaches enabled new opportunities in AMR research, with the possibility to identify genes responsible for resistant phenotype, elucidate molecular mechanisms involved and assess how genes responsible for resistance spread and evolve. Some genomic methods are:

- Knock-out collections are a classic tool where genomes are edited in microbial clones, deleting individual genes and observing the phenotype that was produced for each mutant and thus linking genes to a function (*Liu et al., 2010*).

- Polymerase chain reaction (PCR) assays to score presence/absence of targeted ARGs. PCR is a method used to amplify a specific DNA sequence where a pair of sequences complementary to the target DNA region are used to prime the chain reaction, cycling through denaturation, annealing, and extension steps with the help of DNA polymerase. The method can be highly sensitive, hence used for detection of specific genes, and the amplified fragments can be sequenced for genomic analyses (*Adams, 2020*).

- Plasmid isolation. As ARGs are often found in plasmids, plasmidomes (the set of plasmids within an organisms or community) of bacterial communities and isolates are a good predictor of the resistance present in our cultures (*Smalla et al., 2015*). There are commercial kits available that can retrieve the

plasmid DNA while degrading the chromosomal DNA due to their size differences and supercoiled form of the plasmid. Sequencing of the extracted plasmids can be done to identify possible resistances that they might carry.

- Whole Genome Sequencing (WGS), the most comprehensive analysis to link phenotype to genotype. Since the whole set of genes that comprise a genome is sequenced, genetic variants and mutations can be identify. It is used for identification of resistant genes and detection of novel mutations that might confer resistance. For instance, it is used in the clinical setting to identify pathogenic strains and detect resistant outbreaks through tracking and monitoring. In research it is the method of choice to study evolution of resistance in single isolates using comparative genomic analysis (*Loman and Pallan, 2015*). Due to the capacity of linking a resistant phenotype to a particular genotype variant, this method can help expanding lists of resistance genes for databases. Sometimes (and probably to a higher degree than we might think) genes which have been assigned other functions in the cell are also responsible for resistant phenotypes (examples). Validation of those candidate genes to confer resistance can be done through knock-out collections after identification through WGS.

Databases of verified resistance genes remain limited to culturable microbes. But, what about all the species we cannot culture?, and how can we tell what is the distribution in natural populations of those genes and plasmids?. As of today, there is a before and after in the genomic methods for microbial ecology. Up to the early 2000's, genomic methods applied to study the ecology of microbial ecosystems were heavily culture-dependent, meaning we could only study deeply those microbes that we were able to isolate in laboratory settings. However, since massive parallel or Next Generation Sequencing (e.g. Illumina platform) technologies arrive we have been able to significantly expand our knowledge of the microbial ecology by sequencing environmental samples without the need of cultures getting a better representation of the microbial community composition as well as the functional potential of such ecosystems (*De Mandal et al*, 2015). Genomic approaches with Next Generation Sequencing (NGS):

- Metagenomics: a culture-independent technique (if you want to) that not only provides taxonomy but also functional identity through the sequencing of the total DNA in the (environmental) sample. In this method, we sequence whatever is in your sample and process the data (the sequences) either as raw reads that are directly annotated

against databases (quantification of functions and taxonomy) or genome resolved metagenomics (where the reads are assembled and then clustered into metagenomes assembled genomes). Limitations: binning (clustering the assembly by microbial sps) is…complicated to say the least. With this technique we can sequence samples without culturing so we get a much better representation of the reality while sequencing samples from outbreaks, hospitals or patients (*Yadav and Kapley, 2021*). We can get an idea of resistance genes, microbial composition including pathogens, but it is still difficult to link the taxonomy identity of MAGs to plasmids when working with metagenomic data. But work is on the way! We, for the first time just gave it a go to a novel way of using this method to study plasmid barriers associating genomic content or variability to lateral transfer of genetic material in polymicrobial communities. Mini-metagenomes, metagenomes of a sub-sorted cell population.

A special version of metagenomics is the Hi-C method. Hi-C is a genomic technique that can map proximity of genomic regions. DNA is cross-linked with formaldehyde before DNA-extraction and the non-crosslinked DNA is digested, followed by a ligation of the cross-linked DNA-fragments. After reversal of cross-linking the left-over ligated DNA fragments are sequenced, and matched against a reference genomic dataset to obtain a "map" of DNA proximity (*Castaneda-Barba et al., 2023*).

- Dual fluorescence reporter systems are based on fluorescence proteins as gene reporters in both the chromosome and plasmid. As the gene protein in the plasmid is constitutively repress from the chromosome of the donor they will express only the fluorescent protein encoded in their chromosomal DNA (e.g red), while the fluorescence protein encoded in the plasmid (e.g. green) will only be allow to properly express within new recipients cells (transconjugants) where there is no restriction control. Cells can then get sorted depending on their fluorescence through fluorescence activated cell sorting (FACS) and collect only those expressing the plasmid fluorescence protein as they are assumed to contain the plasmid and are not a donor (*Pinilla-Redondo et al. 2018*).

- In combination with FACS or other methods to isolate or that enrich resistant bacteria (through ARGs or plasmids with fluorescence reporter genes) in the samples, 16S rRNA genes amplicons can be useful (Figure 10). 16S rRNA gene amplicons: is a type of gene amplification that targets the conserved (but variably between taxa) region of the gene for the 16 ribosomal subunit in bacteria. This can also be done in environmental samples for which the culturing step

can be skipped. It also has its bias and limitations like over and under-estimation of taxa abundance as different bacterial species can have different copies of the 16S ribosomal RNA gene (or even during DNA extraction as well as different cell walls will require different treatments for DNA extraction). 16S rRNA gene amplicon sequencing can also be use in combination with another single cell isolation technique, the epic-PCR method (Emulsion, Paired Isolation and Concatenation PCR), based on a fusion-PCR allowing us to link a functional sequence of interest to a 16S rRNA gene fragment and use the mass sequencing of the resulting amplicons for taxonomic assignment of the functional sequence-carrying bacteria (*Roman et al.*, 2021). In the case of plasmid tracking within a diverse microbial community the taxonomy given by the 16S rRNA gene can be linked to plasmid hosts via also targeting specific genes within the plasmid.

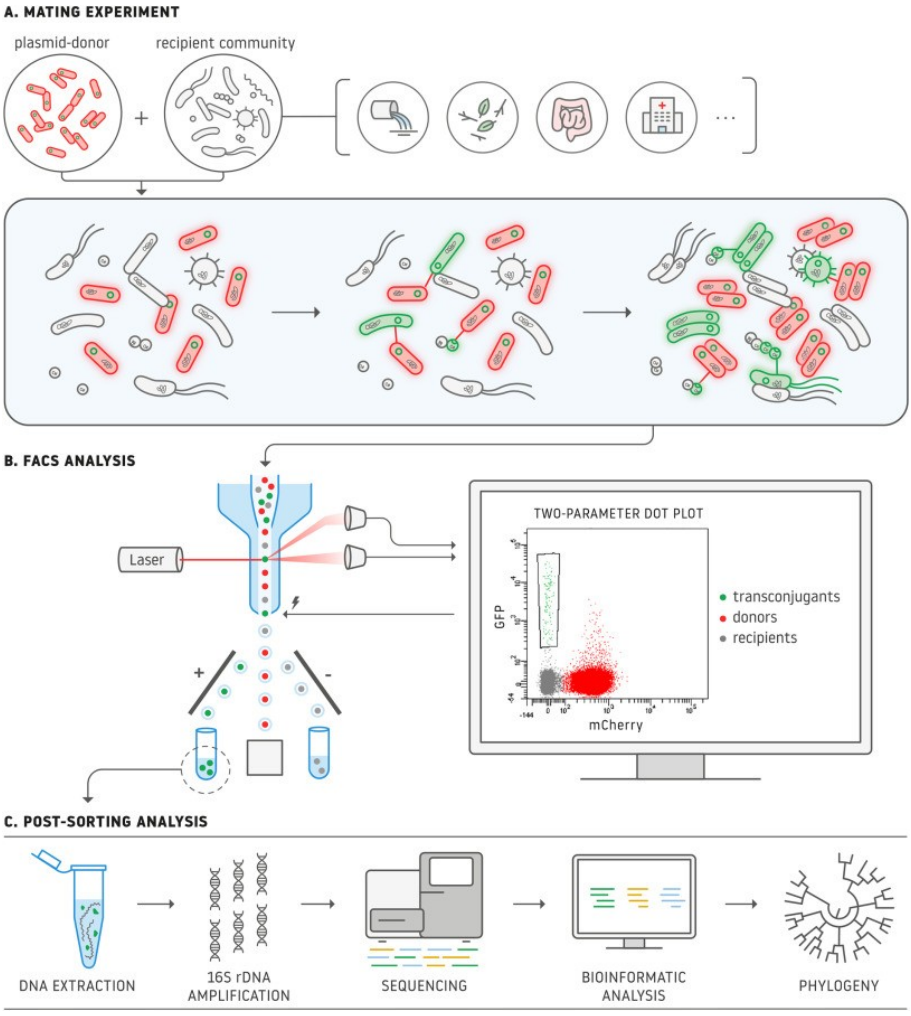

Figure 10. *Current methodology to track ARGs acquired through HGT in complex microbial communities (Source: Pinilla-Redondo et al., 2018. Permission for re-used under the licence CC-BY-NC-ND was granted).*

Present investigation

Aims

The overarching goal of this thesis was to expand our knowledge on how antibiotic resistance evolves and spreads in natural bacterial communities and populations. The following more specific aims are addressed in the different papers/chapters:

- Explore the dissemination limits of a promiscuous resistance-plasmid among bacterial members of clinically and environmentally relevant natural polymicrobial communities (**Paper I, Paper II**)

- Study how resistance-plasmids can influence microbial composition and dynamics of polymicrobial communities during and after antibiotic treatments (**Paper I**)

- Gain knowledge of barriers to plasmid transfer within polymicrobial communities (**Paper II**)

- Develop more affordable methods (bioreactors) for long term experiments with polymicrobial communities to enable further studies of plasmid barriers in polymicrobial communities (**Paper I, Paper II**)

- Investigate how exposure to other biocides, like heavy metals, can impact the tolerance range to different classes of antibiotics of relevant environmental bacteria (**Paper III**)

Results and Discussion

Paper I

Impacts, range and dynamics of a promiscuous conjugative plasmid in an anaerobic poly-microbial bioreactor community

(Aims 1, 2 and 4, p. 18)

We wanted to explore the dissemination limits of the very promiscuous resistance-plasmid pKJK5 among bacterial members of a clinically relevant polymicrobial community from human gut microbiota. An associated aim was to assess how a plasmid that confer antibiotic resistance impacts bacterial community its function and interactions.
To this end we designed a continuous culture system (Figure 8, Figure 9 and Illustration 1) where we could mix a human gut-derivative bacterial community (potential recipients) with a plasmid donor containing ARGs while mimicking the gut conditions. We then observed changes in community composition during the different periods of the experimental incubations and plasmid recipients were taxonomically identified.

Results

pKJK5 was found to invade most bacterial taxa (including Gram-positives) in the polymicrobial gut community

The taxonomic profile observed from this experimental study of conjugation in bioreactor conditions and the identity of the transconjugants obtained from sequence data imply an even broader mobility of pKJK5 than previously assumed. Most of the major taxa in our mixed community were able to take up the plasmid, and unexpectedly this also included the genus Enterococcus represented by the pathogenic species *Enterococcus faecalis*, even though it carried a chromosome resistance gene against the antibiotic used.

Enterococcus faecium, originally a major component of this community, remained plasmid-free

The absence of conjugative transfer to this abundant Gram-positive population in the model community clearly demonstrates that the plasmid transfer is not frequency dependent in the sense of being equal across the community. Our more in depth analysis of its genome revealed an absence of the *esp* gene (enterococcal surface protein involved in biofilm formation)

which has been reported in previous studies to affect conjugation rates positively when present (*Lund et al*., 2006).

Community dynamics under antibiotic selection is influenced by pKJK5

The impact of a plasmid-encoding resistance to an antibiotic would critically depend on the promiscuity and host range of the plasmid in the given community setting. We observed that this broad host range plasmid can have a big impact on community dynamics and the composition of polymicrobial communities. While the relative abundance of the pathogen *Enterococcus faecalis* increased in the reactor that contained no donor or plasmid, they remained rather rare in the three bioreactors where pKJK5 was present, with a 10% difference in relative abundance between control reactor and treated reactors.

The plasmid pKJK5 seems to not only be widely distributing ARGs among a wide variety of taxa but also playing an ecologically significant role in this particular community, both by contributing to the community stability under antibiotic selective pressure and preventing blooms of pathobionts such as *E. faecalis* that carry chromosomal ARGs.

In this thesis (paper I) a system of “homemade” bioreactors is present (Figure 8-9 and Illustration 1) where key variables of culture (i.e. temperature, pH, nutrient and cell density) can be maintained for longer periods of time to study the dynamics of polymicrobial communities during for example antibiotic treatments, keeping both reproducibility and accessibility to the researchers in the field.

Figure 8. *System of bioreactors to study long term cultures of polymicrobial communities including anaerobic conditions.*

Figure 9*. Detail of the individual bioreactors.*

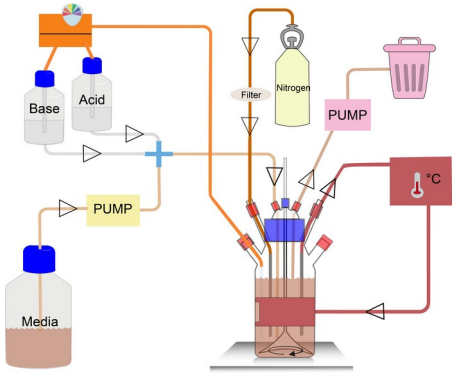

Illustration 1. *Experimental operation of the continuous culture using the Electrolab FerMac 200 system*

Paper II

Identifying transfer barriers of a broad host range plasmid in a wastewater microbial community using metagenomic and single cell data

(Aims 1, 3 and 4, p. 18)

To take one step further in studying lateral transfer of resistance genes (encoded in pKJK5) in mixed (more complex) bacterial communities, an experiment was conducted to identify genome-encoded plasmid barriers in members of a wastewater microbial community using a combination of metagenomics and single cell genomics.

Results

Phylogeny drives dissemination of pKJK5 in our wastewater microbial community

Results from the conjugation assay revealed a transfer-barrier for pKJK5 at the Gram classification boundary and with ample potential for spread of the plasmid and associated AMR to pathogenic Gram-negative species. Gram-positives are contained within a thicker and more rigid peptidoglycan cell wall than Gran-negatives that could be preventing of conjugation during the mating pair formation step, while both cell envelops of donor and recipient are physically in contact.
However, among the Gram-negatives we found plasmid-positive as well as plasmid-negative populations within the same taxonomic clades. We therefore wanted to identify genomic differences between the respective groups to help explain the contrasting responses.

Plasmid barriers beyond phylogeny: defense systems, genetic content variation and the plasmidome

We first wanted to check the expected or most obvious known barriers to plasmid dissemination, such as plasmid incompatibilities and bacterial immune defense systems. We found a variety of defense systems within this complex microbial community, but none of them were exclusive to non-transconjugants, making them less likely suspects of being the reason we didn't detect the plasmid within those plasmid-negative genomes.. Similar results were found when looking into the plasmidome of transconjugants versus non-transconjugants. No other Inc-P1 plasmids were found exclusively in the non-transconjugants.
A few genetic differences were found between the plasmid-positive and plasmid-negative Gammaproteobacteria and Bacteroidota populations. A

variant of the *FabG* gene (involved in the biosynthesis of fatty acids) was found as a genetic trait common to non-transconjugants. Fatty acids are important are important parts of the cell membranes in bacteria and can vary within groups as well as with environmental conditions. Previous studies have observed that unsaturated fatty acids and some synthethic ones can inhibit conjugation (*Fernández-López et al.*, 2005; *Getino et al.*, 2015; *Palencia-Gándara et al*., 2021) and this could be the case in our study.
For the plasmid-positive Gammaproteobacteria we found an incomplete version of the histidine operon that differs to the plasmid-negative Gammaproteobacteria (where we detected a complete version). No previous studies have shown a direct link between histidine biosynthesis and conjugation but we could speculate that as histidine is essential to proteins with methal chelation functions, it could posible modulate the binding sites of the outer membrane affecting conjugation during the mating pair formation step.

SNP profile of *Aeromonas media* pangenome

Since most of our single cell genomes belonged to the pathogenic species *Aeromonas media*, we analyzed this species in detail, including both Aeromonas from transconjugant population and single cell genomes, and compared them to Aeromonas from the controls in order to identify possible plasmid barriers within this constrained taxonomic group. An enrichment in SNPs was observed when comparing the MAGs of *Aeromons media* plasmid-positives versus plasmid-negatives for genes involved in post-translational regulation, cell envelopes and membrane transport. These results point to new research directions for exploring the barriers to HGT of ARGs where the previous focus has predominantly been on genomic editing systems that inactivate the plasmid once it is already inside of the cell. Even though post-translational regulation like methylation can be related to those genomic processes, it is also part of the way bacteria regulate the structure and composition of their outer membrane in Gram-negatives like Aeromonas.

To conclude, we identified a phylogenetically constrained dissemination of pKJK5 in the wastewater bacterial community and comparative genome analyses highlighted membrane structure and composition as having important roles for plasmid transfer. We struggled with some technical issues related to scalable single cell genome sequencing, and solved this by combining hundreds to thousands of transconjugant cells into one single library pool, making the methodology more feasible. With this complementary methodological approach, we could look into the genomes of individual cells, their composition and genetic content while getting supporting data from combined metagenomes and mini-metagenomes. This enabled us to identify potential genomic transfer barriers.

Paper III

Concurrent evolution of zinc and antibiotic resistance in Bacillus altitudinis

(Aim 5, p. 18)

We wanted to investigate how exposure to other biocides, such heavy metals, can impact bacterial tolerance to different classes of antibiotics. His end we isolated a Gram-positive *Bacillus altitudinis* from a metro station and did short term experimental evolution incubations where increasing concentrations of zinc were added to the media. The different cell lines (controls and zinc-exposed) were then assessed for susceptibility to different antibiotics and their genomes were analyzed.

Results

Isolates evolved under elevated zinc levels are more tolerant to all antibiotics tested than parallel control cell lines.

Measurements of the zone of inhibition from the disc diffusion assay showed smaller diameter for all antibiotics in isolates that had previously been evolved under high zinc concentrations, while the control line isolate had a larger inhibition zone than zinc-exposed cell lines, but was also more sensitive than the parental strain. This translates into a higher tolerance to antibiotics of *B. altitudinis* when pre-adapted to high concentrations of zinc and an increased susceptibility of the control line due to domestication under laboratory conditions where ambient selective pressures are absent or weak.

Different genotypes selected after "adaptation" to high concentrations of zinc sustained a high tolerance to antibiotics, while adaptation to laboratory conditions seems to increase susceptibility in the control

Often, plasmids are found to be the causative agent of co-selection for antibiotics and heavy metals (e.g. zinc). However, in our study we didn't find any plasmid in the data obtained from shot-sequencing of *B. altitudinis* but instead we detected an "early" selection of a different genotype with high frequency of those mutated alleles.
The comparative genome analysis of the zinc-evolved isolates using the WT as the reference genome revealed SNPs in only one gene directly linked to antibiotic resistance (*ponA*, penicillin binding protein) but remarkably it was found in both independent/parallel lines of cultures S4 and L4, both exposed to the highest concentration of zinc. Other SNPs were found in genes directly related to resistance but only in the control (*bmr3*, *emrB*). Since the control showed a decrease tolerance for all antibiotics tested, differences in

these genes selected for under the respective laboratory conditions might influence susceptibility. This genotype-phenotype link represent promising gene candidates for studying reversible resistant phenotypes.
The rest of the genomic differences when compared to the WT were found in genes that were not, as of yet, directly related to antibiotic resistance. Even if 4 additional mutations were found in the L4 isolate where zinc was introduced more abruptly at each passage, L4 tolerance was similar to S4, indicating that a higher number of mutational changes in the genome doesn't necessarily translate into higher tolerance.

Observation of phenotype variation in zinc-evolved isolates

While an increased tolerance to antibiotics was observed for zinc-evolved isolates, a trade-off in fitness was also seen in terms of growth rate, carrying capacity and lag phase for those Bacillus growing in high concentrations of zinc when compared to both the parental strain and the control. This significant fitness reduction (including smaller colony size of zinc-evolved isolate compared to WT and control) does not seem to keep *B. altitudinis* from resisting higher concentrations of antibiotics, just like persisters do. Bacterial persisters avoid the toxic effects of antibiotics by entering a state of metabolic arrest and after removing the stressor (in this case the zinc) they continue their normal lifestyle (*Urbaniec et al*., 2022).

I found this study easy to discuss but hard to come to a conclusion. I do think that in our case we initially had a genetically diverse population of *B. altitudinis* in one single colony, and after throwing them into liquid rich media in the laboratory, a particular sub-population of cells were selected for in each cultured cell line. Hence the genomic differences seen between evolved lines and WT were seen with 80-100% allele variation frequencies even in early stages of the evolution experiment. To me, this doesn't indicate *de novo* mutations induced by environmental conditions but rather selection of a sub-population of *B. altitudinis* at the beginning of the experiment. However the mutations that "arise" later on the experiment in each respective experimental line, have higher chances of being *de novo* mutations cause by the environmental changes.

Concluding remarks and outlook

The more you search, the more questions you will find, but at least now you'll know better where to look.

This thesis is address broader questions driven by exploratory curiosity rather than "strict" hypotheses, leaving behind many open questions but at the same time pointing us in promising directions to have a deeper understanding of barriers for AMR dispersal and how AMR is maintained in microbial communities. This knowledge can later be applied to design new antibiotic treatment strategies and bacterial treatment targets. Meanwhile, I have also developed and tested methodological strategies that can be of use in future studies about the spread and evolution of AMR in complex environmental bacterial communities and populations.

Plasmid transfer via conjugation, a Gram barrier for pKJK5?

This is now the right place for me to finally discuss the seemingly contradictory findings reported in paper I and paper II. While we used the same donor and plasmid for these two papers, results in terms of plasmid barriers differed. While pKJK5 was detected in our data of even the less abundant members of the human fecal derived-sample including Gram-positives, pKJK5 was not found in any of the Gram-positives of the wastewater sample (which also reflect fecal microbiomes to some extent) but seem to spread to most Gram-negative clades/taxa. Such a contrast might be explained by differences in growing conditions between the two studies. The growth medium used were "rich" for both studies, aiming to mimic and reproduce the natural environment where the microbes originate and should therefore not give any advantages or disadvantages for conjugation of pKJK5 in either of the studies. Additional environmental parameters such as temperature, was slightly higher in paper I where the bioreactors were constantly at 37^{o}C while the wastewater community was kept at 30^{o}C, and there was also a small pH different from 6.4-6.8 in the bioreactors compared to 7 for the wastewater synthetic media. Notably, no previous studies have linked temperature or pH to differences in conjugation rates. A variable that was drastically different between the two studies is oxygen availability. Paper II was done in normal atmospheric conditions but paper I was done in the absence of oxygen. This could be important since there is some support in the literature that oxygen deficiency could have a positive impact on conjugation rates of another very promiscuous plasmid (pRP4) in *Escherichia coli* as donor (*Jong et al*., 2020).
Another big difference would be the spatial structure where the respective microbial communities were reproducing. The solid surface of the filters

used in paper II differed from the liquid medium used in paper I, and this could surely make a big difference since conjugation can be facilitated by biofilms that would form supposedly in solid medium rather than in liquid, constantly stirred as on paper I (which could physically disrupt biofilms and impede biofilm formation). However, we see more different bacterial taxa as transconjugants in our experiments in liquid media, this could be due to the disruption of the positive assortment leading to a more even sharing of the plasmid among the different members of this polymicrobial community. That being said, the tempo in which the experiments were performed, i.e. 20 hours versus 42 days (of which 15 were under antibiotic selection) could also have an impact in the expansion of transconjugant taxa. In addition, longer incubation times could facilitate the formation of biofilms in our bioreactors, possibly functioning as "mating surfaces" for conjugation (even though liquid media and stirring would theoretically decrease the chances of having biofilms as mentioned a few lines above). In fact, we did observe biofilm formation around the stirrer of the bioreactors. We never tested such biofilm-like structure but we speculate that it is most likely due to the genus Bacillus (*B. Subtilis*, *B. thurigiensis*) as discussed in paper I ("While we did not specifically test whether these biofilms contained *B. subtilis* such structures would readily facilitate conjugation due to increased microbial densities and proximity as shown by *Oliviera et al., (2015*). We could also consider cell density and the possibilities of donor-recipient encounters that depend on the abundance of the potential new plasmid host. Some of the non-transconjugant members of the wastewater community in paper II were pretty abundant but they still did not receive the plasmid. This applies to all Gram-positives in paper II and *E. faecium*, another Gram-positive and abundant member in paper I, and suggest that they will likely have encountered the donor, while there must be other barriers for the transfer of pKJK5. Hence, I conclude that abundance does not seem to be an important factor in plasmid transfer when other barriers do exist.

In summary, while the Gram "status" of the potential new plasmid hosts could definitely be key for conjugation (as observed in paper II), environmental conditions, such as spatio-temporal structure, oxygen availability, physical medium and the composition of the microbial communities that pKJK5 encounter, may influence the spread of ARGs though conjugation (contrasting results between paper I and paper II). Future research could test these factors one by one to observe which ones can alter conjugation rates or the composition of the transconjugant population, while keeping the total microbial community, donor and plasmid constant.

Mate pair formation, an overlooked event in the spread of ARGs via conjugative plasmids

Bacterial cell envelops can have different structures and composition and these can influence their interactions with their external environment and other community members. During conjugation, donor and recipient bacterial cell envelopes are physically in contact as the pilus is inserted into the recipient and retracts bringing both cells closer. This event of cell to cell contact and the secreting machinery involved (pilus) is called mating pair formation. Without a stable mating pair formation, the plasmid transfer runs a high risk of not being successful as both cells are likely to be disconnected. Two *fab* genes (*fabG* and *fabL*, related to fatty acid production and availability in the bacterial cell) were found and highlighted in paper II and paper III respectively. Since fatty acids are important components of bacterial membranes and cell wall lipids, the *fab* genes could be an interesting candidate family of genes to investigate the role of membrane function and composition in the spread and emergence of AMR since fatty acid composition of the bacterial membranes could be impacting conjugation by destabilizing mating pair formation (paper II) through changes in the cell membranes that consequently affect interactions with the bacterial cell environment allowing them to survive during challenging conditions (paper III) like the presence of antibiotics where a well known mechanisms of resistance is modulation of the membrane permeability.

We could also consider the *esp* (for enterococcal surface protein, ESP) genes and speculate that they may also be involved in the "quality" of the mating pair formation since they are responsible for biofilm formation. While *esp* is related to biofilm formation and has already a link to conjugation, it hasn't been directly linked to mating pair formation. Maybe studies to directly link the *esp* with mating pair formation could be an interesting path for future research.

Why does *E. faecium* remain plasmid-free while closely related *E. faecalis* conjugates succesfully?

E. faecium, a Gram-positive member of our bioreactor community, did not seem to have acquired pKJK5 during the 20 days of treatment and subsequent recovery period. When we analyzed the *E. faecium* MAG we found out that the *esp* gene was absent and probably responsible for the lack of conjugation as the *esp* gene has been previously linked to higher conjugation rates when present. This align with our results where *E. faecium* lacke the *esp* gene and lack the plasmid, and *E. faecalis* which is both *esp* and plasmid-positive.

In contrast, the enterococcal surface protein was not essential for cell adhesion and intestinal colonization of *E. faecium* in a study with mice (*Heikens et al*, 2009). This contrast about the impacts os the esps gene in

different studies could by explained by the different roles of the protein ESP, depending on the organisms of study and the environment provided to bacteria (i.e. bacterial cultures versus the intestines of mice).

Future research could investigate genes responsible for biofilm formation or other types of bacterial aggregation as possible candidates for important roles in conjugation (particularly the mating pair formation) impacting the transfer of ARGs between phylogenetically distant bacteria.

Important key findings in Gram-negative genomes

During our analysis of Gammaproteobacteria and Bacteroidia in paper II, we did not find any evidence of bacterial defense systems that were specifc to MAGs of non-transconjugants as a barrier to pKJK5. Similarly, no plasmids were found that could cause incompatibility issues with pKJK5.

Most genes revealed by this analysis, to potentially have an involvement with plasmid barriers, could be related in some way to membrane structure and composition aspects. This is mirrored in the SNP analysis of the Aeromonas populations in our study, which revealed an enrichment in genes involved in post-translational regulation (e.g. of peptides building blocks of the cell envelops), and cell envelope biogenesis and outer membrane.

Method development for the study of AMR in poly-microbial communities

To study the dissemination of AMR in polymicrobial communities and their dynamics, time series sampling over longer periods of time is key. In paper I, we incubated a mix of species using a “homemade” continuous cultures that allowed us to control different environmental variables permitting to apply treatment options while sampling frequently.
In paper II, where we tried to link plasmids to genomes assembled from metagenomic data, we discussed the difficulties and uncertainties in bioinformatically linking plasmids to metagenomes assembled genomes, highlighting the need to develop methods and tools to do so. Single cell genomics is a perfect method to get rid of this specific issue, however, in our study we mostly isolated single cells of the species *Aeromonas media*, as it was the most abundant member of the community. To get a good representation of different members of high diverse communities, a large number of single cells need to be sequenced but the economic costs of covering the production of that amount of data makes it not truly available yet. That is the reason for our collaboration with the National Genomics Infrastructure and Science for Life Laboratory (NGI and SciLifeLab) in developing an ultra pool library preparation (i.e. up-SPLAT) that would allow to sequence a bulk of single cell bacterial genomes at a more feasible

price. Our pilot up-SPLAT sequencing results, brings hope into the community and the NGI is following up a publication about this method.
A combination of continuous culture systems like our bioreactors that keep the diversity of polymicrobial communities for long periods of time, single cell and metagenomics could give us a more mechanistic understanding of the evolution of AMR within diverse microbial communities.

Stressors other than antibiotics can support the emergence of resistant-like phenotypes to different types of antibiotics

Co-evolution of resistance to multiple toxic compounds (e.g. heavy metals and antibiotics) can be an important event in fostering temporal selection of resistance-like phenotypes through a persister strategy (or similar) in the environment since pollution of heavy metals and other biocides is not uncommon (*Pal et al., 2015*). Environmental agencies should do AMR screening in highly polluted areas that might present hotspots for resistant bacteria.

Our methods, settings, experimental design and growing conditions can have a big impact in our results

For instance, like mentioned before, transfer for pKJK5 is different depending on the study. In paper III, the fast domestication of the control line really grabbed our attention since it is the isolate that presents the most mutations in genes directly related to resistances and phenotypically became clearly more susceptible to antibiotics than both WT and zinc-evolved isolates. The lack of controls (or consideration thereof) could blurry our data interpretation, leading to false conclusions.

I think that something is clear, mating pair formation during conjugation could be key in the dissemination of ARGs in poly-microbial communities. The interaction between donor and recipient through their cell envelope likely depends on differences or compatibility of the chemical composition of membranes and cell wall present to establish and maintain good cell-cell contact long enough for the plasmid to completely be transferred. The role of bacterial cell walls and membranes during conjugation remains under-explored and future research needs to be done (maybe in the field of proteomics, lipidomics) to confirm (or reject) these speculations. The use of functional genomics added to dual fluorescence systems for the study of HGT of ARGs in complex microbial communities seems to be the right path to continue developing the methods as they can not only reveal the identity of new resistant bacterial hosts, but also answer the question of “why”.

Acknowledgments

I am writing this section at the last minute and I just hope it comes out nicely and that I don't forget anyone!

First to my first and former supervisor **Klas**, thanks for giving me the chance to pursue a PhD and believe I could make it. Thanks for the freedom of choice, I m not sure that paid off but whatever, thanks for the scientific and non-scientific conversations and I hope you are not scared of me anymore. Actually, I've always been scared of you. Even though you moved to another continent we managed to not lose all contact and finish our studies together. I am proud of both of us.
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