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# Antibiotic resistance in the pan-genome of *E. coli*

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**Abstract**

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The pan-genome of a species is made up of all gene families that can be included in any individual isolate of the species. *Escherichia coli* (*E. coli*) has an open pan-genome including at least 128000 gene families, while only about half of the genes found in each individual isolate are common to all isolates. This indicates a great intraspecies genetic diversity that is not often considered when studying antibiotic resistance. This thesis uses a comparatively large collection of isolates to include more intraspecies genetic diversity and assess its impact on resistance.

One angle of this approach was to study the impact of the pan-genome on spontaneous resistance development. For this, we compared the development of resistance to several antibiotics in a 35-strain collection of *E. coli* isolates. We found that frequencies of resistant mutants varied greatly between strains, that this variation was largely independent from the initial resistance level of the isolates, and that an isolate's frequency of mutants for one antibiotic was a poor predictor of the mutant frequencies for other antibiotics. In conclusion, there was a clear impact of genetic diversity on spontaneous antibiotic resistance development.

Using this approach, we observed a previously undescribed pattern of resistance development for tigecycline, a last-line antibiotic, via amplifications of a known efflux pump. In addition, we found a mutated allele of the pump with a reduced level of induction that did not allow for resistance development through amplifications. We showed that a fitness advantage at low antibiotics concentrations and clonal spread were likely contributing to the high occurrence of the mutated pump among *E. coli* isolates. While this efflux pump is common and well-studied, the lack of pre-existing knowledge of the mutated allele highlights the value of including many isolates in studies of antibiotic resistance.

Another angle of this thesis was to determine whether intraspecies genetic diversity also impacts plasmid-borne resistance. For this, we transferred several multiresistance plasmids into a collection of *E. coli* hosts and characterized the plasmid-host combinations. We observed strain- and plasmid-dependent variations in resistance as well as inconsistencies in the clinical resistance categorization of different hosts with the same plasmid.

In conclusion, this work reveals the impact of intraspecies genetic diversity on the development of antibiotic resistance, both through spontaneous mutations and the acquisition of resistance plasmids, highlighting the need to include intraspecies genetic diversity in studies of antibiotic resistance.

*Keywords:* Antibiotic resistance, pan-genome, genetic diversity, plasmids

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*“Nothing in life is to be feared,*
*it is only to be understood. Now*
*is the time to understand more,*
*so that we may fear less.”*

-Marie Curie

*To my daughter*

# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Jagdmann, J., Guliaev, A., Andersson, D.I., Nicoloff, H. (2022). The pan-genome of *Escherichia coli* strongly affects the potential for *de novo* antibiotic resistance evolution. *Manuscript*.

II. Jagdmann, J., Andersson, D.I., Nicoloff, H. (2022). Low levels of tetracyclines select for a mutation that prevents the evolution of high-level resistance to tigecycline. *Provisionally accepted.*

III. Jagdmann, J., Puignau Saubi, C., Andersson, D.I., Nicoloff, H. (2022). Impact of the bacterial genetic background on plasmid-borne antibiotic resistance. *Manuscript*.

# Contents

# Abbreviations

| | |
|---|---|
| ABR | Antibiotic resistance |
| AMR | Antimicrobial resistance |
| AWaRe | Access, Watch, Reserve (WHO antibiotic classification) |
| bp | Base pair |
| DNA | Deoxyribonucleic acid |
| *E. coli* | *Escherichia coli* |
| EHEC | Enterohemorrhagic *E. coli* |
| EIEC | Enteroinvasive *E. coli* |
| EML | Essential Medicines List (WHO) |
| ESBL | Extended spectrum β-lactamase |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| FDA | Food and Drug Administration (USA) |
| FMT | Fecal microbiome transplantation |
| GAS | Group A *Streptococcus* |
| GBS | Group B *Streptococcus* |
| HC | Hemorrhagic colitis |
| HUS | Hemolytic uremic syndrome |
| ICE | Integrating conjugative elements |
| kb | Kilo-base pair |
| MDR | Multi-drug resistant |
| MIC | Minimum inhibitory concentration |
| mRNA | Messenger RNA |
| MSC | Minimum selective concentration |
| PCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| RT-qPCR | Real-time qualitative PCR |
| ST | Sequence type |
| TGC | Tigecycline |
| UPEC | Urinary pathogenic *E. coli* |
| UTI | Urinary tract infection |
| WHO | World Health Organization |
| wt | Wild-type |

# Introduction

*"Antimicrobial resistance poses a catastrophic threat. If we don't act now, any one of us could go into hospital in 20 years for minor surgery and die because of an ordinary infection that can't be treated by antibiotics. And routine operations like hip replacements or organ transplants could be deadly because of the risk of infection."*

Professor Dame Sally Davies,
Chief Medical Officer of England

These words exemplify the risks that humanity faces at the hands of increasing bacterial resistance to antibiotics. Since the introduction of antibiotics in the 1940s[1], new medical advances have surpassed each other, leading to a dramatic increase in quality of life and life expectancy. Many of these advances are dependent on the availability and reliability of antibiotics, to handle the risk of infections that can be caused by, e.g., invasive surgery or immunosuppressant therapy[2]. Several widely-published reports[3,4] with calculations of future death tolls and economic impacts of antibiotic resistance (ABR) attempt to assess what lies ahead for humanity, and despite concerns regarding the methods used for these calculations, such reports can give policy-makers some idea of the severity of the issue. Much of the global effort to prevent increasing ABR is focused on developing new antimicrobial therapies. While this must indisputably be one of the central efforts of the global push to prevent ABR, it is not enough. Other necessary actions include international agreements regulating antibiotic use and stewardship, novel economic initiatives and reimbursement plans for antibiotic development and production, and global efforts to improve health infrastructure[5,6]. It is often forgotten that the biggest killer globally is the lack of access to antibiotics, not resistant infections[7].

The complexity of the ABR issue notwithstanding, an improved understanding of microbes themselves, including microbial genetic diversity, spread of resistance genes, and mechanisms of resistance, can significantly contribute to targeting these global efforts to focus on the most effective measures. The work presented here strives to add to the existing knowledge of these topics.

# *Escherichia coli* and bacterial pan-genomes

## *E. coli*: the pathogen

*Escherichia coli* (*E. coli*), a bacterial species of the Enterobacteriaceae family, are Gram-negative and rod-shaped facultative anaerobes. This species is largely made up of opportunistic pathogens carried in the gastrointestinal tract of humans and other animals[8]. As they are excreted from the host through defecation, *E. coli* can also be found in the environment and in water sources[9,10]. The types of disease caused by these pathogens include urinary tract infections (UTIs), bacteremia, neonatal meningitis, intraabdominal infections and various forms of gastroenteritis. For both UTIs and bacteremia, *E. coli* is a leading cause of infection[11,12]. While the *E. coli* species carries a set of common virulence factors including endotoxin (lipid A) and type III secretion systems, subgroups of the species based on pathogenicity and virulence (pathotypes or virotypes) often carry virulence factors specialized to the specific disease caused[8]. Table 1 shows a list of pathotypes, virulence characteristics, and the disease caused by each type[8,13,14].

Table 1. *E. coli* pathotypes, including characterizing features and symptoms of disease.

| ***E. coli pathotype*** | ***Disease*** | ***Characteristics[a]*** |
|---|---|---|
| **Gastrointestinal *E. coli*** | | |
| Enterotoxigenic, ETEC | Severe, acute infant diarrhea, "traveler's diarrhea" | Heat-stable (HST) and heat-labile toxin (HLT) |
| Enteropathogenic, EPEC | Severe, acute infant diarrhea, "traveler's diarrhea" | LEE[b] pathogenicity island, bundle-forming pili (BFP), EAE[c] |
| Enteroaggregative, EAEC | Severe, acute infant diarrhea, "traveler's diarrhea" | Aggregative adherence fimbriae (AAF) |
| Enterohemorrhagic, EHEC | Bloody diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) | Shiga-like toxins (SLTs or Verotoxin) |
| Enteroinvasive, EIEC | Bloody diarrhea, bacillary dysentery | Invasion, lateral cell-to-cell spread, pINV[d] |
| Adherent-Invasive, AIEC | Associated with Crohn's disease | Adherence to and invasion of epithelial cells, replicate in macrophages |

| | | |
|---|---|---|
| **Extraintestinal pathogenic *E. coli*** | | |
| Uropathogenic, UPEC | Urinary tract infection | P (pyelonephritis-associated) pili, adhesins, invasion, exfoliation |
| Sepsis-associated, SEPEC | Septicemia | K1 polysaccharide capsule |
| Neonatal-meningitis-associated, NMEC | Neonatal meningitis | K1 polysaccharide capsule |

a. Including examples of virulence factors
b. Locus of Enterocyte Effacement
c. Enterocyte Attachment and Effacement
d. plasmid carried by EIEC, *Shigella*, allows invasive phenotype

Another way to classify *E. coli* is by multilocus sequence typing, or MLST. This method identifies which alleles of a few genes found in all strains of a species (housekeeping genes) a specific strain carries and correlates the given allele profile to a sequence type (ST)[15,16]. MLST has allowed for the identification of clinically important groups, e.g., the *E. coli* clone ST131[17,18]. This clone has caused many clinical outbreaks, is extensively antibiotic resistant, and has spread around the world[19–21].

In the light of modern techniques allowing for comparatively easy whole-genome sequencing, the increased complexity of species classification has been evident. For example, *Shigella spp,* a genus of Enterobacteriaceae previously thought to be separate from *Escherichia*, has more recently through whole-genome sequencing and molecular phylogeny been determined to be a subclade of *E. coli*[15,16]. *Shigella* are most closely related to EIEC, but carry Shiga-toxin and can cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), similar to EHEC (see Table 1)[14,22]. EHEC, on the other hand, do not have the invasive and cell-to-cell transmission phenotype of EIEC and *Shigella*[14]. Within the *E. coli* species, whole-genome sequencing has also brought forth examples of limitations to pathotyping, where isolates may not easily fit into a specific pathotype, presumably due to genetic transfer between isolates[13]. Overall, whole-genome sequencing has added the complexity of combining molecular phylogeny with the historical classification based on, e.g., serology and biochemical properties.

## *E. coli*: the model organism

*E. coli* have widely been used as the model organism for bacteria, including specifically as a model organism for bacterial genetics and physiology. The majority of the investigative work on *E. coli,* including the first whole-genome sequencing, has been performed in *E. coli* MG1655, a derivative from K12, which was isolated from a diphtheria patient in 1922. The *E. coli* strain

MG1655 has been cleared of the phage λ and the F plasmid [23,24]. Several major molecular biology techniques have been developed in MG1655, and the strain is often considered to be a standard for *E. coli*. As a consequence of decades of use as a model organism, *E. coli* MG1655 has adapted to the laboratory environment and may no longer be entirely representative of clinical, pathogenic *E. coli*[25]. Despite this, *E. coli* MG1655 is often used as a model organism to identify clinically relevant characteristics, such as the ability to develop antibiotic resistance[26–28]. It is worth mentioning that strains described as MG1655 may vary in exact sequence, as the strain has been used so extensively and over a long period of time[29,30]. It is therefore important to identify exactly which MG1655 is used and what unique mutations it contains.

## Bacterial pan-genomes

As an increasing number of species and isolates within each species have been whole-genome sequenced, it has become clear that there may be more intra-species diversity than was previously thought to exist. This has challenged the way we look at species classification and genome plasticity. The concept of a "pan-genome" as a way to describe the genetic content of a bacterial species was first introduced by Tettelin *et al.* in 2005[31] after studying genomes of isolates from different serotypes of *Streptococcus agalactiae* (group B *Streptococcus*, or GBS). Tettelin *et al.* defined a pan-genome as the composition of the core genome, or gene families found in all strains of a species, and the dispensable (also called accessory or variable) genome, including gene families that are not present in all strains and can even be unique to a single strain. In the same work, the concepts of an open or closed pan-genome were introduced. Analysis of both the group A (GAS) and group B *Streptococcus* and extrapolation from observed results led to the conclusion that for both species, each additional sequenced genome added new gene families to the total pan-genome. This was described as an open pan-genome. In comparison, a similar analysis of sequenced *Bacillus anthracis* concluded that after the inclusion of four genomes, the predicted number of gene families added to the pan-genome per added genome approached zero. This implies that *B. anthracis* has a closed pan-genome, as portrayed in Figure 1. Another conclusion that can be drawn from this work is that in species with open pan-genomes, the core genome may comprise only a small fraction of the total pan-genome, allowing for vast intra-species diversity.

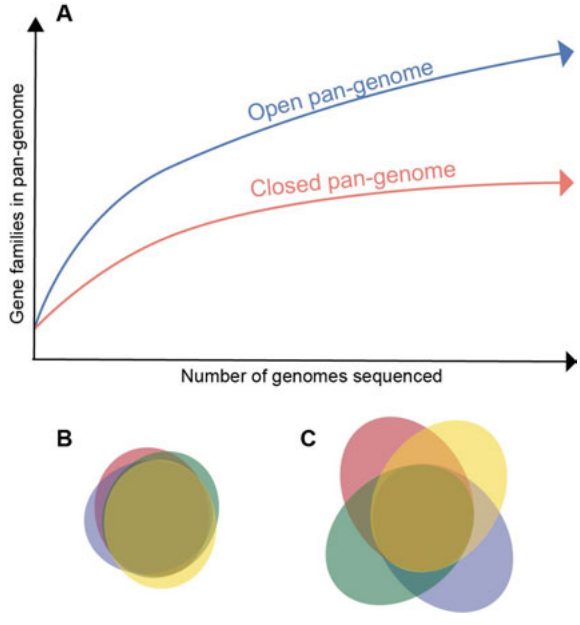


Figure 1. Comparison of open pan-genomes and closed pan-genomes. A). As further genomes are sequenced in species with closed pan-genomes, the increase in total number of gene families approaches zero. For open pan-genomes, the increase in total number of gene families with additional genomes does not approach zero. B). Representative Venn diagram of overlapping genes in four isolates from a closed pan-genome. C). Representative Venn diagram of overlapping genes in four isolates from an open pan-genome.

When attempting to describe the pan-genome of a given species, or subset thereof, there are several factors that impact the outcome of a pan-genome analysis. The sampled genomes used for calculation must be of a large enough quantity and be representative if attempting to draw conclusions regarding a larger group, for example an entire species. The quality of genome sequencing will also play a role, and the quality of the specific gene annotations used and parameters for alignment (i.e., percent identity and sequence length) may change the classification of certain genes, for example from core to variable, impeding comparisons between different pan-genome analyses. The use of a preset threshold to determine if genes are part of the same gene family makes the consistent use of the term "gene families" as opposed to "genes" imperative when speaking of pan-genomes. Several tools for pan-genome analyses have been developed to attempt to streamline and standardize the process and parameters, but various parameters are still used[32].

**The pan-genome of *E. coli***

There are a few descriptions of the pan-genome of the *E. coli* species. As described above, these descriptions can vary depending on number of genomes included and the thresholds used to categorize genes to gene families. Based

on the 186 *E. coli* and *Shigella* sequences available in 2011, Kaas *et al.*[33] determined the pan-genome of *E. coli* to comprise of over 16,000 gene families (described as homolog gene clusters). The "soft" core genome, or the gene families present in at least 95% of all genomes, was determined to comprise of 3,051 gene families, while the "strict" core genome, or gene families present in 100% of all genomes, was determined to be 1,702 gene families. In this study, the variation within gene families was also analyzed, showing that core gene families contained far less variation than accessory gene families. The number of sequenced *E. coli* genomes had increased drastically by 2015, to 2,085 genomes, and a pan-genomic analysis was performed using these genomes[34]. The *E. coli* pan-genome was determined at this point to comprise 90,000 gene families, but uncertainties regarding the one-third of these families that only occur in one single genome and the fact that many of the sequenced genomes were draft assemblies allowing for errors in gene calling led Land *et al.* to a conservative estimate of more than 60,000 gene families in *E. coli*. The core genome was found to be made up of 3,188 gene families. Most recently, an updated pan-genome analysis of *E. coli* published in 2019, including *Shigella* spp, found a pan-genome size of over 128,000 gene families[35]. In this analysis, the core genome was comprised of 2,608 gene families, with each isolate carrying an average of 4,889 genes. As the exact methods and parameters used to determine the numbers described in this study are unclear, they should be considered with caution. There is also a potential issue in assuming that the whole-genome sequenced *E. coli* represent *E. coli* as a species, as there may always be a bias towards human, particularly virulent isolates of *E. coli,* which do not represent the species as a whole. It is clear, however, that the *E. coli* pan-genome, and the diversity included, is vast.

**The evolution of pan-genomes**

The genetic diversity described by a bacterial pan-genome has been attributed to the immense quantity of genetic information simply present in various environments, such as marine water and the human gut, and to genetic transfer (described further below) between different species and strains[36]. There have, however, been questions regarding why these acquired genes would be maintained, as their presence will carry the cost of maintenance and expression, if the product of the gene is not beneficial to the cell. The act of acquiring genes itself can also incur a cost, e.g., if the gene disrupts an essential function in the cell. Also, if the genes acquired are beneficial and therefore maintained, why does the size of individual genomes not increase as the pan-genome does? Why do certain species have closed pan-genomes, while other species have open, vast pan-genomes? Several models have proposed different ways of looking at the issue[37–39]. One leading explanation of this phenomenon is that the pan-genome size is primarily influenced by effective population size and migration. In other words, horizontally transferred genes can be selected for and maintained in cases where there is a large population that resides in

multiple niches. Exposure to various niches and a changing environment with an abundance of genetic content available can give an advantage to the acquired genes, if they are beneficial in certain environments, for example those with fluctuating antibiotic concentrations. Species that reside in a specific niche and do not migrate, such as intracellular pathogens, may, on the other hand, have a limited access to and benefit of horizontally transferred genetic material[37]. Others have instead proposed applying population genetic theory to the gene families themselves rather than species, due to the ability of the same gene to be present across multiple species[39].

**The value of pan-genome analysis**

What can be gained from knowledge of bacterial pan-genomes? Some researchers have suggested that the extensive knowledge of genetic diversity be used to reclassify bacterial species to better fit genetic relationships[40,41]. However, while this reclassification could be considered to be more correct, it may cause unnecessary confusion in applied settings, such as the clinic. Another example of the usefulness of pan-genome analysis is the clarification that the multidrug resistant (MDR) ST131 lineage of *E. coli* is a host-generalist, and findings that support the existence of multiple sub-types of *E. coli* ST131[42]. Recently, using pan-genome analysis of over 1,900 *E. coli* isolates also gave insight into the successes and difficulties of predicting antibiotic resistance through machine learning[43]. Novel β-lactamases have even been discovered by analyzing genomes of many species and metagenomic data, followed by confirmation-of-function in *E. coli*[44]. Such approaches of predicting resistance genes before testing function or using functional metagenomics to screen diverse genetic material for antibiotic resistance are a useful way of taking genetic diversity into account when identifying new resistance genes and mechanisms[45]. However, such studies may remove the impact of the genetic background and natural regulation on the gene, i.e., a gene that may confer resistance in one background and under certain regulation may not confer resistance in another genetic background, under different regulation. Consequently, the original setting of the gene of interest may play an important role. It is, however, clear that in pathogens such as *E. coli*, an increased understanding of the pan-genome and the impact of the genetic background on the phenotypes resulting from expression of known antibiotic resistance genes will play a role in how pathogens are classified and resistance is understood[46].

## Mechanisms of bacterial genetic exchange

An important step in understanding genetic diversity and where it comes from is understanding how genetic diversity arises. Genetic information can be maintained and disseminated in several ways. Primarily, the transfer of genetic

information can be described in two ways: vertical and horizontal transfer (Figure 2).

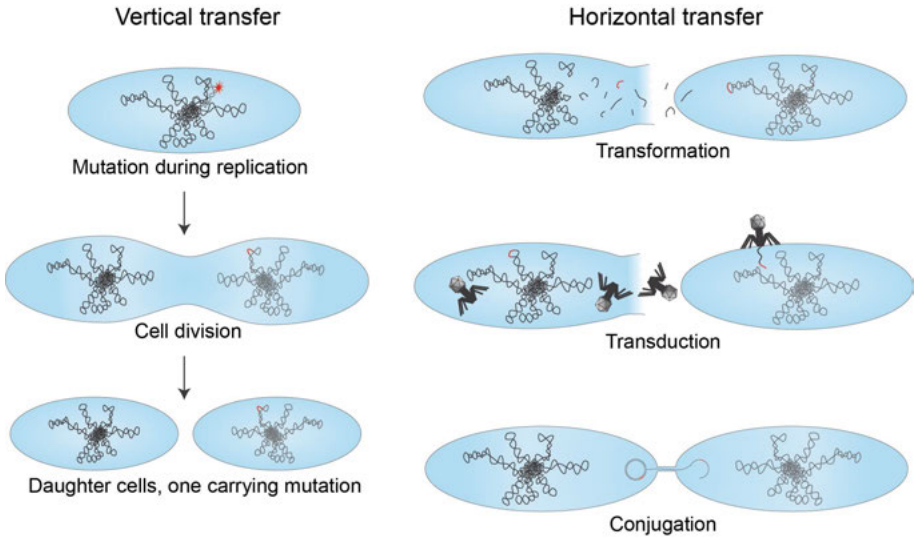


Figure 2. Vertical and horizontal mechanisms of genetic exchange, shown here spreading genes and mutations of interest.

## Mutations can be vertically transferred

Vertical transfer describes the transfer of genetic information to daughter cells after cell division. Most of the genetic material transferred to the daughter cells will be identical to that of the parental cell, but changes to the genetic material during replication will also be transferred. For example, an error in DNA replication that is not corrected, such as a nucleotide substitution (point mutation) in a functional gene, will be inherited by a daughter cell through cell division. If the function of the protein coded by the mutated gene is altered, the daughter cell can be affected by the change in protein function. Point mutations can change an amino acid in the protein, changing or preventing the function of a protein, or insert a premature stop codon that leads to a truncated protein. Point mutations can also have no effect on the coded protein, so-called neutral mutations[47].

Not all mutations are nucleotide substitutions in protein-coding regions, and they can take many forms. For example, mutations in promoters may alter expression of a gene. Genetic material can also be inserted or deleted. This can lead to a frame-shift in the coding sequence, changing the protein structure, or add or remove a part of or entire genes. The deletion or insertion can also be in-frame, resulting in a protein with the same basic structure but with a few amino acids added at some position, with no disruption to the downstream amino acid sequence[47]. Amplifications of DNA can also occur, where

regions of DNA can be copied multiple times, especially between repeated sequences. These amplifications can be small, comprised of a few nucleotides, or very large, e.g., a few hundred base pairs[48,49]. Such changes in the inherited genetic information can be maintained or lost in a population, depending on, e.g., the effect on the bacterial fitness, or how well bacteria can thrive and produce offspring in a specific environment compared to competitors. The mutations can drive evolution and allow for part of the enormous genetic diversity we see in microorganisms such as *E. coli.*

## Horizontal genetic transfer

Not all genetic changes are caused by mutations and vertical transfer of genetic information. Genetic material can also be transferred between cells, both of the same and different species. This allows for the spread of entire functional genes and traits that can be gained and selected for. This horizontal genetic transfer can occur in three different ways: transformation, transduction, and conjugation.

Transformation is the uptake of DNA from the environment into the cell. Some bacteria are naturally able to accept free DNA, also called "competent", such as *Bacillus subtilis* and *Neisseria gonorrheae*. Transformation in other bacteria, for example *E. coli*, may require treatment to make the cell competent. While it is not always advantageous for a bacterium to be able to pick up foreign DNA, genes of advantage can be picked up and integrated into the recipient cell's DNA through recombination, potentially giving the cell an improved fitness in a specific environment. Transformation is also a very convenient technique in molecular biology, allowing for DNA to be inserted into the desired recipient isolate once it is made competent, e.g., through electroporation[50].

Transduction is the transfer of DNA from one bacterium to another through a bacteriophage, a virus that targets bacteria[51]. There are more bacteriophages, also called phages, on Earth than any living organism, and the diversity of phages is vast[52]. Phages infect bacteria and hijack bacterial replication mechanisms to replicate and package phage particles, before lysing the cell and releasing daughter phage particles. Some phages can nonspecifically package segments of the host bacterial DNA rather than viral DNA into the head of the virus by accident. When such phages carrying DNA from the donor bacterium infect a new cell (called a recipient cell), foreign bacterial DNA rather than viral DNA is injected into the recipient cell. Without viral DNA, no new viruses are produced, but the foreign bacterial DNA can be inserted into the DNA of the new host[51]. While this can happen naturally, transduction has also been manipulated into a useful tool in molecular biology in order to move desired mutations from one isolate into another, e.g., transduction using phage P1 in *E. coli*[53].

Through direct contact and what is sometimes considered "mating", genetic material can also be spread from one bacterium to another in a process called conjugation[54]. Most often, the transferred genetic material is a plasmid, which is a non-chromosomal DNA molecule that can be carried by bacteria, but integrating conjugative elements (ICE) can also transfer by conjugation and usually integrate into the host chromosome[54]. Conjugation is considered to be the most common way for bacteria to acquire resistance genes, making it a central aspect of the spread of antibiotic resistance[55]. As understanding plasmids is a crucial component to understanding antibiotic resistance, they are expanded on in the next section.

# Plasmids

## Plasmid structure and function

Plasmids are usually circular replicons - a self-replicating circular DNA molecule - and can be very small, appearing to only carry genes coding for the machinery necessary for replication, or very large and made up of several thousand base pairs and carrying genes encoding many functions. Many properties of plasmids affect their dynamics, including replication, partitioning, and incompatibility[56].

### Plasmid replication and partition

Plasmids replicate, like the bacterial chromosome, starting from an origin of replication (*ori*-region), and can be allocated to daughter cells after cell division. The *ori*-region plays a significant role in the regulation of the plasmid, in turn affecting plasmid function. Depending on the plasmid in question, replication occurs by theta replication, similar to chromosomal replication, or by rolling circle replication. Regardless, plasmids generally borrow many host proteins to perform replication and encode only a few necessary replication proteins on the plasmid[56].

Plasmid replication largely impacts a number of properties of a plasmid, including the final number of molecules of a particular plasmid that will be found in the host cell, also called the copy number of the plasmid. As too many copies of a plasmid may over-burden the host cell and cause the host to be outcompeted by cells without plasmids, the number of copies of a plasmid in a cell must be regulated. However, plasmids can be high-copy number, with several hundred copies per cell, or low-copy number, with only a few or a single plasmid copy per cell. This regulation is largely determined by the *ori*-region of the plasmid, and can use different mechanisms, including via the Rep protein and corresponding complementary RNA to restrict replication[56]. Another aspect that can be impacted by the *ori*-region of a plasmid is host range. Some plasmids have a very narrow host range, meaning they can only

be replicated and carried by a few hosts, while other plasmids have a broad host range and can be spread between genetically different bacterial species[56].

Consistent distribution to the bacterial daughter cells during cell division is crucial for the plasmid. Proper allocation of plasmids to daughter cells ensures that the plasmid is carried on, so the regulation of partitioning, and thereby the segregation and distribution of replicated plasmids to daughter cells in cell division can be regulated through partition (Par) systems. While such systems are not present on all plasmids, they use a variety of mechanisms to separate replicated plasmids from each other to different locations in the cell, promoting the chances of at least one plasmid copy ending up in each daughter cell after division. In small, high-copy number plasmids, these systems may not be necessary, as the sheer number of plasmid molecules increases the likelihood of at least one copy ending up in each daughter cell[56].

**Plasmid incompatibility**

While many plasmids can be (and are) stably maintained in an isolate, some isolates with overlapping machinery cannot be carried together, called incompatibility. Incompatible plasmids are grouped and labeled, e.g., IncF, IncI, IncN, and many more. For example, presence of two different plasmids with replication regulated by the same mechanism in one bacterial cell would disrupt and prevent proper replication regulation. The regulation system would not distinguish between the plasmids of the same type, and the cell would not have the proper number of each plasmid before division. Proper partition can also be disrupted, as plasmids using the same partition system would not be able to properly spread around the bacterial cell. In both of these cases, while incompatible plasmids may be maintained together by chance for a few generations, plasmid loss (or curing) will occur over time. Incompatibility will therefore affect plasmid dynamics by preventing the co-carriage of certain plasmids in the same cell[56].

**Plasmid transfer and stability**

Plasmids can be spread and transfer themselves to new host cells through conjugation, as described above, requiring for example transfer (*tra*) genes and an origin of transfer (*oriT*). However, some plasmids that are not able to conjugate on their own can be mobilized and transferred through other conjugative plasmids, e.g., by carrying the *oriT* of a conjugative plasmid. The efficiency of plasmid transfer by conjugation is affected by the regulation of, for example, the *tra* genes, as these genes are not constantly expressed in the bacterial cell[54].

Plasmids can additionally carry mechanisms to promote their continued maintenance, e.g., toxin-antitoxin systems[56]. Poor plasmid stability, or loss of plasmid in the host population, can be compensated by evolution and adaption of either the host or the plasmid to allow for increased plasmid stability[57]. The

initial stability of an acquired plasmid will also depend on the host background, as well as the specific plasmid in question[58].

## Plasmid evolution and fitness

Plasmids are common and widespread in the bacterial world, but their existence is not entirely logical. Carriage, that is replication, transfer, gene expression, etc., all should confer a fitness cost to the cell, thereby making it grow more slowly[59]. In addition, any genes carried by a plasmid that could be beneficial in a certain environment could be transferred to and carried on the bacterial chromosome instead, rendering the plasmid useless. While recurring plasmid conjugation[60] or plasmid stability mechanisms such as the toxin-antitoxin systems mentioned above would facilitate the continued carriage of plasmids, the long-term presence of plasmids in bacterial communities remains a sort of paradox[61]. Therefore, one can ask, why and how are plasmids so common? For one, plasmids and their hosts can evolve together, seeing as some host or plasmid mutations can alleviate an associated fitness cost[59,62].

As mentioned previously, tight regulation of several plasmid regulation systems is necessary to minimize the cost of carrying a plasmid. At the same time, it has also been observed that, for example, the loss of conjugation machinery can reduce plasmid cost, but as this prevents the further spread of the plasmid to new bacterial cells, this would not be ideal in the long term[63]. Instead, plasmid or host mutations can limit some of the costs of plasmid carriage without such impacts on plasmid dynamics. In some cases, carriage of a plasmid increases the expression of some chromosomal genes, and correction of this overexpression can mediate fitness costs[64,65]. Interestingly, one study even showed multiple evolutionary trajectories of increased plasmid persistence in different lineages, including plasmid (*repA*/*trfA*) and host (*fur*) mutations, as well as the uptake of a toxin-antitoxin system onto the plasmid[57]. However, despite the interesting progress made in understanding plasmid fitness and evolution, it is important to consider that the genetic diversity of both a host and a plasmid contribute to the fitness of a plasmid in a host, and a plasmid that is costly in one host may even confer a fitness advantage in another[66–68].

## Plasmids and antibiotic resistance

Plasmids have importantly been used as vectors in molecular biology, but understanding the natural function, evolution, and dissemination of plasmids has significant clinical relevance. Plasmids often carry cassettes of resistance genes, and their horizontal spread is a contributing factor to the spread of antibiotic resistance. Also, virulence factors can be selected for in parallel if carried by the same plasmid[69]. Some plasmid groups can be associated with certain bacterial families or with certain resistance genes. For example, IncFII,

IncA/C, IncL/M, and IncI1 plasmids (referring to incompatibility groups) have been found to be prevalent in *Enterobacteriaceae,* with many resistance genes carried[70]. Consequently, understanding plasmids is an important part of studying antibiotic resistance.

## Antibiotics and antibiotic resistance

Antimicrobial compounds targeting bacteria, also called antibiotics, a term coined by Dr. Selman Waksman, have inarguably changed medical practice immensely for the better[71]. Antibiotics have not only allowed us to treat previously lethal infections, but are also vital for medical procedures such as organ transplantation, several surgeries (such as hip replacements), and chemotherapy and other immunocompromising medical treatments. The scope of this improvement may be lost on those who have always lived in an age where common bacterial infections can be treated easily and quickly[72]. After the first antibiotics for widespread use were introduced in the 1940s, the middle of the 20$^{th}$ century followed as a time of great success in antibiotic development, with the discovery of many novel antibiotic classes with different mechanisms of action. This "Golden Era" has unfortunately been followed by a "discovery void", with most recently introduced novel antibiotics belonging to classes that were discovered before 1990[73], with only a few exceptions such as lefamulin[74]. Concomitantly, a pattern of discovery of resistance can be seen for all antibiotics after, or even before, introduction into the clinic[1]. The discovery of teixobactin from natural sources, and thereby a novel antibiotic class, has been a light of hope. However, this drug has yet to reach the market and does not work against some of the target pathogens that are described as having the highest demand for antibiotic research and development by the World Health Organization[75,76]. There are simply very few novel antibiotic classes being successfully developed. Currently, the antibiotics that are being introduced are merely improvements or advancements on existing classes of antibiotics, for example increasing the host range or counteracting some common resistance mechanisms[5]. When resistance is already established in the clinic to a class of antibiotics, one can imagine that the bacteria can more easily evolve to overcome the new challenges, as shown by the emergence of resistance to β-lactamase inhibitor combinations[77].

This brings forth the following questions: what are the characteristics of a good antibiotic, and why are they so difficult to find? Also, what are the mechanisms of action of currently known antibiotic classes? How does resistance develop, and what are the mechanisms of this resistance? Understanding the answers to these questions is an essential step to managing the global problem that is antibiotic resistance.

# Antibiotics

## Antibiotic discovery and development

Historically, many antibiotics have been discovered in nature, e.g., the discovery of penicillin by the observation of Sir Alexander Fleming of the inhibition of bacteria by a *Penicillium* mold[78]. However, this method of screening natural products for antimicrobial properties has led to something of a dead-end, and it is now difficult to sort through the so-called "low hanging fruit" that have already been discovered to find novel classes. That being said, a recent discovery of a novel antibiotic class was found in nature, albeit with enough difficulty to require a creative approach[75]. In addition, attempts to synthesize novel antibiotics have not been as successful as hoped, and there are few fully synthetic antimicrobials, e.g., fluoroquinolones, in use today.[73] As a result, and in combination with increasing antibiotic resistance, we are left with a decreasing number of functional antibiotics, and very few new antibiotics are being added to the mix.

Several traits are required or desirable for an effective antibiotic. Aside from the obvious answer of antimicrobial function, an antibiotic must also specifically target bacteria; in other words, the antibiotic must not be too toxic to the human, animal, or other organism that is being treated at the concentrations required to give antimicrobial function, and side effects should be minimal. The pharmacokinetics and bioavailability of the antibiotic also need to allow the compound to reach the site of infection in the body, at a high enough concentration to work. Furthermore, especially from the perspectives of drug developers and many clinicians alike, an antibiotic should be functional against many different kinds of bacteria, or have a broad spectrum of activity, as antibiotic treatment is often necessary before the cause of infection can be identified and some antibiotics are, for example, not able to cross the outer membrane of Gram-negative bacteria. This perspective is changing, however, as the understanding of the effects of wide-ranging disruptions on a patient's microbiome, as caused by broad-spectrum antibiotics, is increasing, and the merit of narrow-range antibiotics is starting to be appreciated. Moreover, properties such as easy administration are desirable[72]. It is worth noting that several antibiotics that have been considered undesirable due to issues such as toxicity are returning to use or still in use due to the increase in resistant infections, e.g., colistin and tigecycline[79,80].

Aside from the above-mentioned desirable antibiotic properties, certain pathogens present a more urgent need for new antibiotics. There are a few different ways to categorize these pathogens, including the WHO global priority pathogens list of antibiotic resistant bacteria[76] and the ESKAPE pathogen grouping[81]. Many organizations that fund or otherwise promote the development of new antibiotics have targeted initiatives for candidates that are active against these sorts of pathogens, e.g., CARB-X.

**Grouping antibiotics**

Antibiotics can be grouped in several ways. To start, antibiotics can either be bactericidal or bacteriostatic. Bactericidal antibiotics function by killing the bacteria, while bacteriostatic compounds inhibit bacterial growth. As the bacterial target also varies, antibiotics can also be grouped depending on target. Antibiotics that target the same pathway or molecule in the cell can still function differently, and thereby belong to different classes, as seen in Figure 3. For example, β-lactams and glycopeptides both target peptidoglycan synthesis, more specifically transpeptidation, but in different ways. β-lactams target the enzymes performing the transpeptidation reaction, while glycopeptides target the substrate of the reaction, namely the D-Ala-D-Ala part of UDP-muramyl-pentapeptide (a building block of peptidoglycan, Figure 3). As the cell wall (and peptidoglycan therein) is specific to bacteria and not present in eukaryotic cells, it makes an excellent antibiotic target, and the most commonly used antibiotics share this target[72]. Antibiotics that function in this way include, e.g., the previously mentioned and clinically-important β-lactams, as well as glycopeptides and fosfomycin[72]. The cell membrane can also be a target, e.g., for colistin[1]. There are, of course, a range of other targets and antibiotic classes, for example protein synthesis (targeted by aminoglycosides and tetracyclines), DNA and RNA synthesis (targeted by fluoroquinolones and rifampicin, respectively), and essential biosynthesis pathways, such as tetrahydrofolate biosynthesis (targeted by trimethoprim and sulfonamides) (Figure 3)[72]. There are also antibiotics in use, for example nitrofurantoin, where the mechanism of action of is, despite the long time since discovery, less precisely understood. It is however clear that the reduced form of nitrofurantoin is toxic and disrupts DNA, RNA, and protein synthesis, indicating that nitrofurantoin is a prodrug and the activated, reduced form in the cell has the antibacterial function[82].

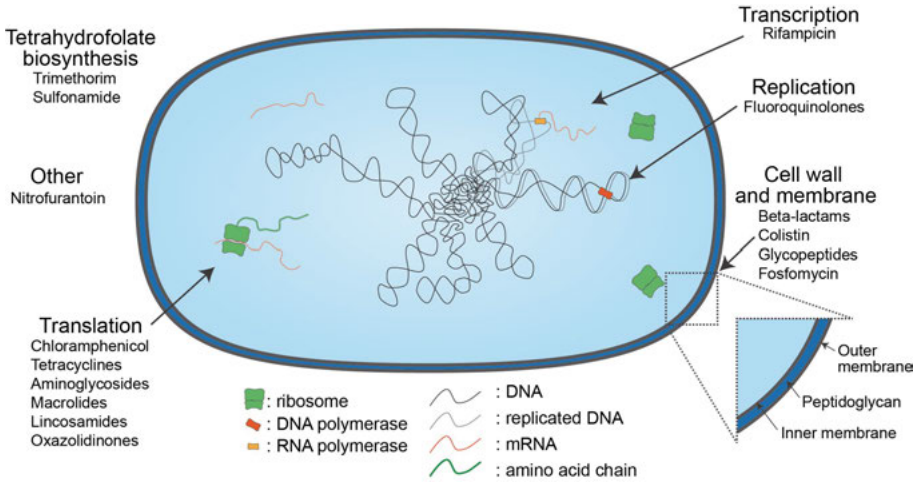


Figure 3. Antibiotic targets in the Gram-negative bacterial cell and examples of antibiotics and antibiotic classes with those targets.

Another way of grouping antibiotic is by their optimal use. For example, certain antibiotics are traditionally considered to be first-choice, or to be the first options for use. Others can be considered last-line (or last-resort), to be saved for the most severe cases. As these classifications have not always been uniform globally and can be challenging to harmonize, an expert committee was commissioned in 2017 for that purpose, in association with the 2017 update of the WHO Essential Medicines List (EML). This committee adapted a new classification system for antibiotics, AWaRe (Access, Watch, and Reserve). Access antibiotics are to be the first- and second-line choices in the clinic, especially the antibiotics considered to be "core-access" (e.g., ampicillin, chloramphenicol, doxycycline, nitrofurantoin, sulfamethoxazole and trimethoprim). Watch antibiotics have a higher risk of resistance or toxicity issues, and should be used cautiously or for certain indications (e.g., carbapenems, glycopeptides, and fluoroquinolones). Reserve antibiotics should, as the name entails, be reserved and only used as a last-line option (e.g., fosfomycin, colistin, tigecycline). These antibiotics should be available but only used in very serious cases, such as infections where few other options for treatment remain, as to not lose their efficacy[83].

## Mechanisms of antibiotic resistance

As mentioned above, the first antibiotics, sulfonamides, were introduced in the 1930s and in the late 1940s penicillin came into common use, and resistance to these first antibiotics was discovered shortly after their discovery. Resistance to antibiotics is increasing the occurrence of infections that are challenging, if not impossible, to treat. This increase is in part caused by the horizontal spread of resistance genes between isolates, as previously

described, most often through conjugation of plasmids (studied in **Paper III**). Many of the horizontally transferred resistance genes originate in the environment, often from a natural producer of the antibiotic. Isolates can also become resistant, even during treatment, through spontaneous mutations (studied in **Paper I**)[1]. In bacteria with quite distinct and reserved niches, such as *Mycobacterium tuberculosis*, resistance is only caused by these spontaneous mutations[84].

There are several ways a bacterium can become resistant to an antibiotic, but the mechanisms can be generalized into the three following categories: i) decreasing the intracellular antibiotic concentration by increased efflux/reducing influx, ii) inactivation or lack of activation of the antibiotic, or iii) alteration of the target by modification or protection[55]. These mechanisms are shown in Figure 4 and are described more in detail below.

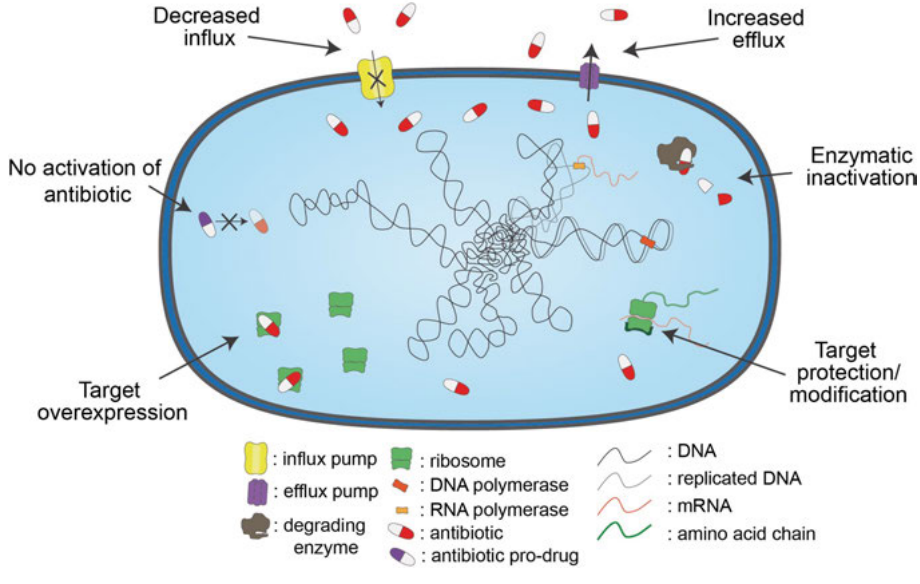


Figure 4. Generalized mechanisms of antibiotic resistance in the bacterial cell. In the example given, the target of the antibiotic is the ribosome.

For one, the intracellular antibiotic concentration can be reduced through an increase in antibiotic efflux or a decrease in influx. An increase in efflux of the antibiotic from the cell can occur through for example the introduction of an antibiotic specific efflux pump, such as *tet*(A) for tetracyclines (as described in **Paper II**) or the overexpression of innate efflux pumps, such as AcrAB-TolC which can reduce susceptibility to several antibiotics including (but not limited to) tetracyclines, ß-lactams, and nitrofurantoin[85–87].

Decreasing the uptake of an antibiotic can be mediated by, for example, mutations in porins, as can be seen for ß-lactams and nitrofurantoin[88]. Reducing the intracellular concentration of an antibiotic through these routes would, logically, reduce the effect of an antibiotic on the bacterial cell.

Also logically, an antibiotic must be in an active form to have an antibacterial effect. Some antibiotics, such as nitrofurantoin, enter the bacterial cell in an inactive form and must be activated in the cell (also known as a prodrug). Consequently, mutations or deletions in *nfsA* and *nfsB*, encoding nitroreductases that activate nitrofurantoin into an active antibiotic, lead to nitrofurantoin resistance[89]. Conversely, antibiotics can be deactivated in the bacterial cell by enzymes either modifying or degrading the molecules into harmless forms. Some clear examples of this type of resistance include ß-lactamases of many types (such as OXA, TEM, CTX, and variants of each), aminoglycoside modifying enzymes (such as AAC and APH variants) and Tet(X) variants, which degrade tetracyclines. Such degrading enzymes are often horizontally transferred and carried on plasmids[90–93]. In the past decade, plasmid-borne enzymes that degrade or modify last-line antibiotics such as tigecycline have been identified (e.g., Tet(X4))[94].

Antibiotic resistance can also be caused by modification or protection of the target as well as overexpression or "bypassing" of the target. For example, the gene *tet*(M) encodes a ribosomal protection protein protecting from tetracyclines, and mutations in *rpsJ* (encoding ribosomal protein S10) can confer tigecycline resistance[90,95]. Trimethoprim resistance, on the other hand, can be mediated by DHFR (dihydrofolate reductase, the chromosomal target of trimethoprim) variants that are not targeted by the antibiotic, often carried on plasmids[96]. Common first step mutations conferring resistance to ciprofloxacin are also target alterations, such as *gyrA/gyrB* and *parC* mutations[97].

The examples given here are not comprehensive, and there are a wide variety of resistance genes and mechanisms, even for the same antibiotic. For example, resistance to tetracyclines can be mediated by a wide variety of mechanism, as described above[90]. Additionally, several types of resistance mechanisms might be necessary, and necessary to combine, to be able to reach clinical resistance levels in an isolate. For ciprofloxacin, for example, one single resistance mutation is not enough to confer clinically relevant resistance. Rather, bacteria develop resistance in multiple steps, often with both an accumulation of mutations in target genes and plasmid-mediated target protection genes[98]. Such cases remind us of the complexity of resistance evolution.

## Evolution and selection of resistance

As mentioned above, resistance to antibiotics can arise from mutations or horizontal gene transfer, or even a combination of both. The benefit of these resistance genes and mutations is easy to anticipate when considering lethally selecting antibiotic concentrations, in other words concentrations of

antibiotics where the bacteria without resistance mechanisms will not be able to grow. Many resistance genes and mutations do, however, carry a fitness cost (described above) in absence of antibiotics, and may be selected against in absence of antibiotic pressure[99]. Conversely, this cost can be reduced or reversed by compensatory mutations either in the mutated gene or in a different gene entirely[100]. Therefore, evolution dynamics and the selection of antibiotic resistance are also central concepts to understanding antibiotic resistance. Evolution is itself a broad, complex topic that cannot be thoroughly covered here, but it is nonetheless important to introduce and discuss some aspects.

So, how can resistance evolve? Reaching high resistance levels may be dependent on a genetic context made up of many, low-level resistance mutations and genes[101]. Transposable resistance genes are also not static but evolve themselves, as is clear from an analysis of, e.g., β-lactamases from the TEM family. The parental genes TEM-1 and TEM-2 are not functional against the 3rd generation cephalosporins that were produced to circumvent resistance to early β-lactams. However, derivatives of these parental genes with extended spectrum β-lactamase (ESBL) activity have since been discovered[91,92]. In another example, mutations in the tetracycline efflux pump TetA(A) can confer resistance to tigecycline[102]. On the other hand, tandem amplifications of resistance genes are more dynamic mutations causing an increase in gene dosage and often a corresponding increase in resistance, a phenomenon observed in **Paper II.** These amplifications can also allow for an intermediate step before point mutations, improving the function of a resistance gene, as more copies of the resistance gene would be present in which a mutation could occur[49]. These sorts of amplifications, with a corresponding increase in resistance, have been observed in clinical strains and on clinical resistance plasmids for genes such as *aphA1* and β-lactamases[103,104].

**Environment impacts resistance development**

The fitness of a bacterium is context-dependent, and the environment of the bacterium will not always be constant, or dichotomous, e.g., lethal antibiotic selection or no antibiotic present at all. Many clinical antibiotics are found in varying concentrations in several environments, and even blood serum levels in a patient will vary over the course of an antibiotic treatment[72,105–107]. Therefore, antibiotics may be present in concentrations below the MIC (minimum inhibitory concentration) of susceptible bacteria that still affect the fitness of these and give strains carrying resistance genes or mutations a fitness advantage (see Figure 5). The minimal antibiotic concentration at which a resistant strain will out-compete a sensitive strain is also known as the minimum selective concentration (MSC), and can be significantly lower than the MIC of the susceptible strain, or the concentration where the susceptible strain would no longer be able to grow[108]. Also, at different antibiotic concentrations,

evolutionary trajectories can vary for the same isolate and antibiotic, emphasizing the effect that changes in antibiotic concentrations can have on evolution of resistance[109].

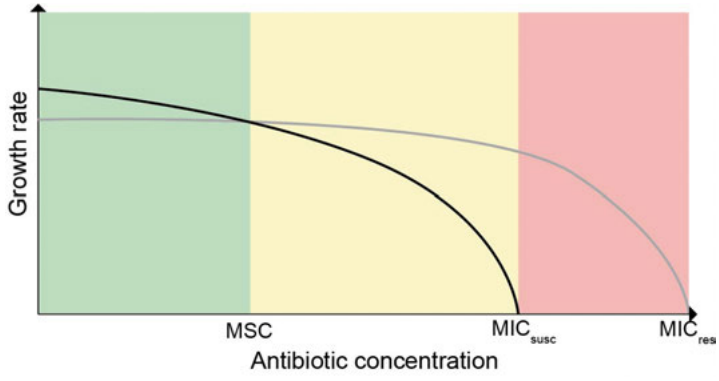


Figure 5. A figurative depiction of the change in fitness, as observed by growth rate, with increasing antibiotic concentrations. The growth rate of a resistant isolate (gray line) is initially lower than the growth rate of the susceptible isolate (black line, green range), but with increasing antibiotic concentrations, there will be a concentration where the growth rate of the wild-type isolate is lower than the growth rate of the mutant (MSC, followed by yellow range). After the MIC of the susceptible ($MIC_{susc}$) has been reached, only the resistant isolate will grow (red range) up to the MIC of the resistant isolate ($MIC_{res}$).

An additional level of complexity is the relatively rapid fluctuation of antibiotic concentrations that bacteria can be exposed to. For example, an infecting bacterial strain can, through the course of antibiotic treatment, be exposed to no antibiotic (pre-treatment), high antibiotic concentrations (during treatment), and waning antibiotic concentrations (during and post-treatment) in a matter of hours, and the specific concentrations would of course also vary depending on the antibiotic, site of infection, and patient in question[110]. Therefore, plasticity in resistance gene expression can be advantageous to bacteria, for example through gene amplifications mentioned above. The advantage of gene amplifications in this situation is largely their transient nature, where quick adaptation to an environmental change is balanced by the ability to return to the original susceptible state without any permanent alteration of DNA. This speedy adaptation is mediated by the relatively high rates of gene amplifications compared to point mutations, which can also be lost at similarly high rates in absence of selection[49].

The ecological surroundings of a bacterial cell also have a direct effect on horizontal transfer of resistance genes: any horizontal genetic transfer requires ecological opportunity. For example, plasmid transfer between cells requires direct contact and that the plasmid can be carried by the host, both regarding host range and incompatibility[56]. This can be exacerbated in biofilms, for

example, as genetic transfer is mediated and the cells can be protected from antibiotic exposure and other external factors[111]. With all these influences combined, fully understanding the evolution dynamics leading to antibiotic is clearly a challenge.

**Epistasis and resistance**

The effects of mutations in bacteria are dependent on the genetic background of the mutation. Two mutations that each cause a 5% decrease in fitness may have a very different effect when combined, for example a 20% decrease in fitness compared to the parental strain. Conversely, the two mutations together could cause only a 7% decrease in fitness. These examples portray a phenomenon called epistasis, in this case epistasis of fitness, which can be very important when studying antibiotic resistance and resistance selection[112]. Combined mutations giving resistance to different antibiotics can show negative fitness epistasis (a higher cost of fitness for the combined resistances than the additive fitness cost for both mutations alone), positive epistasis (a lower cost of fitness for the combined resistances than the additive fitness cost for both mutations alone), or even sign epistasis (a lower fitness cost than one of the mutations alone, but not the other). Trindade *et al.* [113] discovered that several resistance combinations in *E. coli*, e.g., streptomycin (*rpsL* K43R) and nalidixic acid (*gyrA* D87G) resistance, resulted in sign epistasis and, additionally for this example, no fitness disadvantage relative to the wild-type. Considering the potential diversity of *E. coli* strains, even within core genes such as *rpsL* and *gyrA* in this example, it is likely that one mutation will have varying effects in different strains, not only on phenotypic resistance but also fitness and selection thereby.

# Measures to counter antibiotic resistance

Dealing with a problem as complex as antibiotic resistance requires efforts on many fronts simultaneously. The following section is in no way comprehensive, but introduces the diversity of approaches (Figure 6).

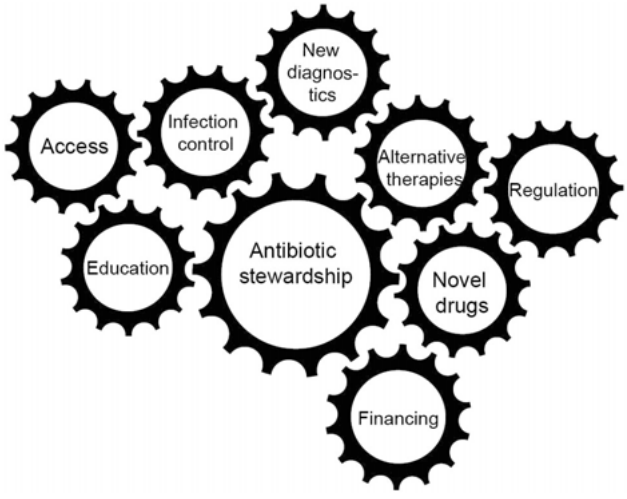


Figure 6. Efforts to counter antibiotic resistance. Simplified schematic representing the interplay of various efforts to reduce or manage antibiotic resistance. Note, more efforts than those in the figure exist.

## Developing updated antibiotics, combination drugs

As mentioned previously, the global antibiotic development pipeline, while including some hopeful and novel candidates, is insufficient for dealing with the antibiotic resistance problem. Most antibiotic candidates in the clinical pipeline, that is recently approved (but not marketed) or currently in phase 1-3 clinical trials, are not from novel classes (see Figure 7)[114,115]. Additionally, a large number of candidates in early clinical development do not make it through to market approval due to a variety of issues including toxicity, resistance development, or ineffectiveness. While there are several promising antimicrobial candidates through various stages of pre-clinical and clinical testing, the clear drop in candidates in phase 1 to phase 3, shown in Figure 8, highlights the need to bring more candidates forward[114].

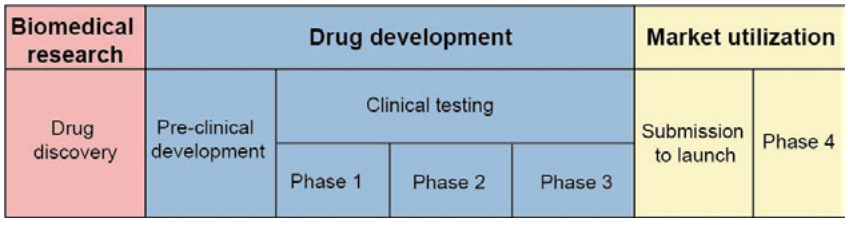


Figure 7. The stages of drug development. Adapted from Cama *et al.* 2021[116].

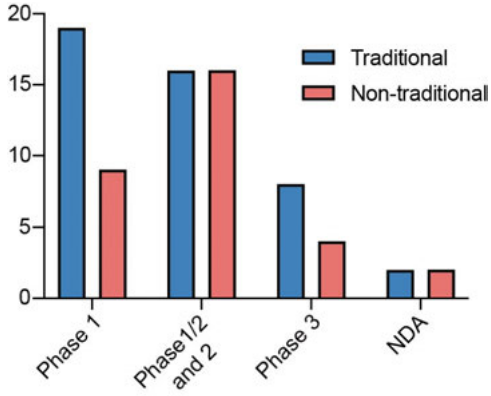


Figure 8. Number of candidates at different stages of clinical development, split into traditional antibiotics and non-traditional alternative therapies (described further below. NDA: Submitted new drug application. Figure adapted from Butler *et al.* 2022[114].

One issue with updating and improving an existing antibiotic class is exemplified in **Paper II**, where resistance to two relatively novel tetracycline antibiotics (tigecycline and the recently approved omadacycline) is facilitated by amplifications of the common tetracycline efflux pump Tet(A). This type of resistance development is also seen for other modern antibiotics, as shown by current research[77,103]. Some interesting, novel combinations of existing antibiotics have been approved in the last 5 years, e.g., Vabomere (meropenem-verobactam) and cefiderocol (siderophore-cephalosporin)[114]. However, for cefiderocol, current research shows this level of novelty may not be sufficient and resistance via known resistance genes can be observed[117].

## Altered financing models

The current financing model for new drugs based on market sales is not compatible with the nature of antibiotics. Not only should new antibiotics ideally be safe, cheap, and easy to administer, but we also want several new, novel antibiotics to reserve for the most severe cases of resistant infections. This means that market returns on a new antibiotic will be low, despite the high medical value of the drug itself. This issue became strikingly clear with the bankruptcy of the biopharmaceutical company Achaogen and its antibiotic plazomicin, approved in 2018[118]. While plazomicin, an aminoglycoside, is not of a novel class, it was almost immediately classified as a "reserve" antibiotic according to WHO's AWaRe classification and included on the WHO essential medicines list (EML)[83,119,120]. However, Achaogen filed for bankruptcy in 2019, with the high cost of drug development and low return on antibiotic marketing highlighted as main factors to blame[118,121]. Achaogen was not alone, as antibiotic developer Melinta suffered a similar fate shortly after[122]. Several approaches to circumvent this issue have been suggested to be used together

or separately, including so-called push or pull incentives, and "delinking" antibiotic reimbursement from sales.

**Push incentives**

Push-funding policies are designed to fund drug development costs early in the process, often in the form of grants, tax incentives, and public/private partnerships[116]. These incentives can be set up to aid at different stages of the drug development process, e.g., the non-profit partnership CARB-X which funds candidates in the preclinical phase up to phase 1 (early clinical) testing (see Figure x), while the AMR Action Fund aims to fund projects across all phases of clinical development[123]. One central challenge for these efforts is to identify and fund promising candidates. As mentioned previously, many candidates in clinical development do not make it to market for a variety of reasons, which are not always related to financing. Unfortunately, these incentives may not be sufficient either, as plazomicin was funded in part by CARB-X before the company producing the approved antibiotic, Achaogen, went bankrupt[118].

**Pull incentives and delinking**

Pull-funding incentives essentially provide rewards or benefits for companies that succeed in developing antibiotics through market approval or another set clinical development stage. These incentives can, for example, look like market entry rewards or extended exclusivity vouchers[124]. Pull incentives can utilize delinkage, or the concept of breaking the dependency on sales volumes for reimbursement of research and development costs, with the knowledge that we do not want modern antibiotics to be used more than necessary and therefore do not want to promote high sales[123]. For example, market entry rewards could include demands on the developer limiting drug costs, promoting fair availability, etc., and be fully delinked from sales or used to "top up" more conventional sales based reimbursement[124].

Recently, some countries have started piloting projects with so-called "subscription" models for antibiotics[125]. With this set-up, a government is essentially paying an antibiotic developer for access to the antibiotic rather than for the units actually used, compensating for the cost of development and ensuring availability while avoiding over- or misuse and providing the developer with predictable revenue. Different models of this system have been piloted in Sweden and the UK, and other countries have similar plans in progress, such as the PASTEUR Act in the US[123].

## Alternative therapies

As struggles with the discovery and development of antibiotic drugs have continued, some efforts have turned to alternative strategies (see Figure 8 for non-traditional alternatives in the clinical pipeline). These strategies include, but

are not limited to, phage therapy, antivirulence therapies, microbiome-modifying therapies, and agents targeting bacterial conjugation.

### Phage therapy

As phages can lyze, or kill, bacteria, they have been used to treat bacterial infections for nearly a century in agriculture, and in some parts of the world on humans. Over the last few decades, interest in phage therapy as a treatment option has surged in light of increasing antibiotic resistance[126–128]. In fact, some cases of difficult-to-treat infections being managed by phage therapy have made headlines in recent years[129,130]. Phage therapy can use different approaches, including using fixed or individualized cocktails or even using genetically engineered phages to treat an infection. However, there are many challenges to using phage therapy, including manufacturing and regulatory difficulties, phage specificity (requiring accurate and timely diagnostics), and the emergence of phage resistance[127].

### Targeting virulence

Another approach has been targeting bacterial virulence, in other words targeting the ability of bacteria to cause disease. This can be achieved by, e.g., neutralizing bacterial toxins[131], disrupting secretion systems[132] or bacterial communication[133], or preventing adhesion and/or biofilm production[134]. While there are, for example, FDA-approved monoclonal antibodies targeting bacterial toxins[131], such anti-virulence therapies are difficult to get approved, as the design of clinical trials and the approval process of new treatments is not optimized to determine their efficacy. In addition, there is the same need for accurate diagnostics as for phage therapy, and a risk of resistance remains[127].

### Microbiome modulation

As our understanding of the importance of the microbiome increases, so do our attempts to use our microbiomes to aid in the treatment and prevention of bacterial infections[127]. For example, fecal microbiome transplantation (FMT) can be used to reestablish a healthy microbiome in patients suffering from recurrent *Clostridiodes difficile* infections[135]. The use of FMT to decolonize individuals carrying, for example, carbapenem-resistant *Enterobacteriaceae* has also been considered, but once again, clinical superiority trials have not been successful[136]. In addition, there have been safety concerns with FMT, including the spread of antibiotic resistant bacteria[137], and its regulation and international availability varies[137,138].

### Bacterial conjugation

Inhibiting bacterial conjugation has also been considered as a tool to prevent the spread of antibiotic resistance, potentially in combination with traditional antibiotic treatment. Such an approach could, in theory, inhibit the donor cell, the plasmid itself, or target the recipient cell of conjugation[139]. Any of these

approaches could reduce the horizontal transfer of resistance genes and be helpful in cases where, for example, a patient is colonized by an isolate known to carry a high risk, multiresistance plasmid, but a broader approach may be difficult to achieve.

## Stewardship

Antimicrobial stewardship, including antibiotic stewardship, describes the work to track and improve the way antimicrobials are prescribed and used, and is key to preserving any new antibiotics that come to market[140,141]. Despite the difficulty in making such calculations, various studies have found that 50% or more of our antimicrobial use could be unnecessary or inappropriate[142]. If we do not continue to improve how we use antibiotics, we will only run into the same difficulties in the future.

One goal of effective antibiotic stewardship is to balance the need of the individual, namely rapid and efficient treatment, with the need of the collective, which is to preserve functioning antibiotics for the future[143]. Achieving this requires a multifaceted approach, with appropriate, adapted interventions for each relevant setting. Efforts need to be made in hospital, community, and veterinary settings, for example, and can all fall under the umbrella term "antimicrobial stewardship". In addition, many interventions focus on a specific hospital setting or specific prescribers and need to be appropriate for that setting, such as support to a physician in determining appropriate treatment, educating prescribers, and various means of restricting the prescription of certain antimicrobials[144]. In contrast, overreaching policies can be implemented to promote large scale changes, such as banning antibiotics as growth promotors in agriculture[145] or the sale of antimicrobials without a prescription[146]. In short, antimicrobial stewardship is complex but necessary, and must be included in future efforts to control antibiotic resistance.

# Current investigations and future perspectives

## Paper I: The pan-genome of *Escherichia coli* strongly affects the potential for *de novo* antibiotic resistance evolution

Jennifer Jagdmann, Andrei Guliaev, Dan I. Andersson, and Hervé Nicoloff
*Manuscript*

While our appreciation of the intra-species genetic diversity of the important pathogen *E. coli* has grown with the increase in whole-genome sequencing and the expansion of pan-genomic analysis, this diversity is often not considered when studying antibiotic resistance. A recent pan-genome analysis showed that a collection of more than 4,000 *E. coli* and *Shigella spp* strains had a pan-genome size of over 128,000 gene families, with a core genome of only 2,608 genes (53% of the genes found in an isolate on average)[35]. However, many studies of antibiotic resistance development, especially in *E. coli*, only include a single laboratory isolate such as MG1655 or a small set of isolates[89,147]. In addition, other studies of antibiotic resistance tend to look at already resistant isolates and attempt to identify the cause of resistance[148]. These approaches do not sufficiently acknowledge the impact of the vast genetic diversity within *E. coli*.

To assess the impact that the *E. coli* pan-genome might have on spontaneous resistance development, we put together a comparatively large (35-isolate) collection of whole-genome sequenced *E. coli* isolates from different sources, attempting to include diverse isolates, and screened for spontaneous resistance development to several antibiotics (Figure 9). A pan-genome analysis of the collection described a pan-genome size of 17,134 gene families and a core genome (present in >95% of all isolates) size of 3,119 gene families. As the number of isolates needed to be manageable for screening and analysis, this diversity was, while not entirely optimal, deemed sufficient for this project.


Figure 9. Phylogenetic tree based on the core genome of our 35-isolate collection of *E. coli*.

We performed a simplified fluctuation assay to determine mutant frequencies for four different antibiotics of interest, and at concentrations corresponding to i) the clinical resistance breakpoint according to EUCAST, ii) the intermediate resistance breakpoint, if one exists, and iii) 0.25x the intermediate resistance breakpoint, or if there is none, 0.25x the resistance breakpoint. The chosen antibiotics were either newer, last-line antibiotics (tigecycline and meropenem) or older antibiotics of renewed clinical relevance where resistance is not fully understood (fosfomycin and nitrofurantoin). In addition, mutation rates were determined by a full fluctuation assay for rifampicin to confirm that no hypermutators were included in our isolate collection.

Mutant frequencies varied greatly (ranges in mutant frequencies spanning 4-to-6 $\log_{10}$) for fosfomycin (at resistance breakpoint) as well as the lowest concentrations for nitrofurantoin and tigecycline. This range was much greater than that observed for rifampicin (about 1 $\log_{10}$). Few mutants were selected at the resistance breakpoint for nitrofurantoin, the intermediate and resistance breakpoint for tigecycline, or at any concentration of meropenem. Exceptions to this were in isolates carrying resistance genes for antibiotics in the same class (*tet*(A) for tigecycline, **see Paper II**, and $bla_{\text{KPC-2}}$ for meropenem). We observed that the mutant frequency for one antibiotic was a poor predictor of mutant frequencies for others. In addition, while parental MICs did positively correlate with mutant frequencies for some antibiotics and concentrations, these correlations were relatively weak ($R^2<0.5$).

Sequencing of select mutants from isolates with high or low mutant frequencies to a few of the antibiotics tested revealed some expected mutants, e.g., *nfsA* and *nfsB* mutations for nitrofurantoin resistance and lps-associated mutations for tigecycline resistance. However, for nitrofurantoin, the exact mutation in *nfsA* or *nfsB* could vary, as did their effect on the MIC of the mutants. In addition, some isolates carried mutations not previously associated with nitrofurantoin resistance, such as large deletions in a plasmid. For mutants selected on tigecycline, we observed an interesting pattern of apparently strain specific targets for resistance mutations, where different mutations were observed in the same target in independent mutants. Remarkably, most

mutational targets appear to be core genes, but further work is needed to confirm this.

While this study is merely scratching the surface of the genetic diversity present in *E. coli*, it is clear that there is greater variation in the ability to develop resistance to antibiotics than has previously been appreciated. The lack of strong correlation between mutant frequencies observed for other antibiotics or between mutant frequencies and parental MICs, as well as the diversity of mutations and mutational targets, highlights this finding.

## Paper I: Future perspectives

While this work is progressive in regard to including multiple, diverse isolates when studying spontaneous resistance development, we acknowledge that the collection is still small when compared to the vast, open pan-genome of *E. coli*. If possible, similar studies with even larger collections, potentially focusing on single antibiotics, would provide additional insight. With the current study design, however, there are some clear steps forward.

i. Sequencing a larger number of mutants would help to support (or discount) some of our preliminary findings, including the apparent strain-specificity of the mutational targets for tigecycline. Additional sequenced mutants from both high and low mutant frequency isolates for all antibiotics could also clarify if there are any patterns in resistance development for high mutant frequency isolates that are missing in low mutant frequency isolates.
ii. A deeper analysis of some of the conserved genes across our isolates would be useful, e.g., comparing the similarity of *nfsA* and *nfsB* across isolates in an attempt to understand the varied effects of different mutations in these targets.
iii. If some mutations are consistently found in high mutant frequency isolates, it would be of interest to reconstruct the same mutations in low mutant frequency isolates. Then, we could test if a similar increase in resistance would be observed or if the genetic background of those isolates interferes and prevents the selection of such mutants.

# Paper II: Low levels of tetracyclines select for a mutation that prevents the evolution of high-level resistance to tigecycline

Jennifer Jagdmann, Dan I. Andersson, and Hervé Nicoloff
*Provisionally accepted*

Clinical resistance to tigecycline (TGC), a last-line tetracycline derivative, has been observed but not fully explained[148,149]. In the process of screening a large collection of *E. coli* isolates for spontaneous resistance development to several different antibiotics in **Paper I**, a novel pattern of TGC resistance was discovered. Tandem amplifications of *tet*(A), encoding the common tetracycline class A efflux pump TetA(A) and its corresponding repressor protein TetR(A) (Figure 10), resulted in MICs above the EUCAST clinical breakpoint for TGC (0.5 mg/L)[150]. While overexpression of this, for TGC, relatively ineffective efflux pump has previously been shown to increase resistance to TGC, the spontaneous occurrence of this resistance mechanism had not been observed[151]. Additionally, unlike many other known TGC resistance mutations, *tet*(A) amplification alone led to clinical resistance in many isolates[147]. Tandem amplifications also lead to an increase in resistance to TGC in *Klebsiella pneumonieae* and to omadacycline (a more recently approved tetracycline derivative) resistance in *E. coli*.

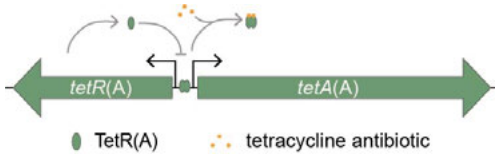


Figure 10. Regulation of *tet*(A).

In this work, we also observed a functional variant of *tetR*(A) with a 24-bp deletion. Resistance development via tandem amplifications of *tet*(A) was not observed in isolates carrying this *tet*(A) variant (designated *tet*(A)$^{\Delta tetR}$). As seen by RT-qPCR and β-galactosidase assays, induction of *tetA*(A) and TetA(A), respectively, was reduced in isolates with the deletion in *tetR*(A). The decrease in induction varied depending on the tetracycline antibiotic used but was especially pronounced for tigecycline, with only about 15% of wild-type mRNA expression and at most 27% of wild-type β-galactosidase induction observed with *tet*(A)$^{\Delta tetR}$. However, no decrease in leaky *tetA*(A) expression in the absence of antibiotic was observed. We concluded that the mutated repressor protein TetR(A) has a reduced affinity for the tetracycline antibiotics

(see Figure 10), to a varying degree depending on the specific antibiotic in question, thereby reducing the expression of TetA(A).

Isolates carrying either allele of *tet*(A) unamplified would both be classified as sensitive following AST and would not be discernable. Therefore, we designed a multiplexed PCR screen to accurately identify carriage of *tet*(A) and the allele thereof to provide insight into the ability of an isolate to develop tigecycline resistance.

The *tet*(A)$^{\Delta tetR}$ allele was common: between 10.5 and 36.8 % of *tet*(A)-carrying *E. coli* isolates had the *tet*(A)$^{\Delta tetR}$ allele depending on the *E. coli* collection analyzed. Interestingly, phylogenetic analysis showed a linkage of *tet*(A)$^{\Delta tetR}$ with the successful clone ST131. Moreover, plasmid typing revealed that a different plasmid type was connected with *tet*(A)$^{\Delta tetR}$ than *tet*(A)$^{wt}$ in ST131 isolates. These factors were not exclusive, however, and other factors that could contribute to the high frequency of this allele were therefore also studied.

In addition to the association with ST131 and specific plasmids, we studied if *tet*(A)$^{\Delta tetR}$ would be advantageous under certain growth conditions. Through head-to-head competition of isogenic strains, we observed that at high tetracycline antibiotic concentrations near wild-type MICs, isolates carrying *tet*(A)$^{wt}$ with its higher expression had a fitness advantage over isolates carrying *tet*(A)$^{\Delta tetR}$. However, at lower antibiotic concentrations, isolates carrying *tet*(A)$^{\Delta tetR}$ had a fitness advantage (almost 8%) over isolates carrying *tet*(A)$^{wt}$. Furthermore, *tet*(A)$^{\Delta tetR}$ was not outcompeted by an isogenic strain without any *tet*(A) at any tested antibiotic concentration. The extent of this effect varied depending on the tetracycline antibiotic used, but the general pattern was consistent.

As the region surrounding the deletion in *tetR*(A) consisted of many short repeated or similar sequences that could facilitate spontaneous deletion, we also attempted to observe the deletion in *tetR*(A) occurring and enriching under laboratory settings. For this, we used amplicon sequencing of *tetR*(A) following experimental evolution under selective concentrations of a tetracycline antibiotic, minocycline, or without antibiotic. The deletion occurred spontaneously and was enriched to a greater extent in lineages exposed to sub-MIC concentrations of minocycline than among lineages evolved without antibiotic selection pressure.

Altogether, this work both introduces a novel and apparently common mechanism of resistance development to TGC and a previously undescribed but frequent allele of *tet*(A). Being able to predict a negative outcome following TGC treatment could aid clinicians when making prescribing decisions. That being said, as *tet*(A)$^{\Delta tetR}$ does not allow for TGC resistance development in the same way due to the altered induction dynamics, it is important to understand the function of this allele and its dissemination. Many potential factors appear to play into the spread of the *tet*(A)$^{\Delta tetR}$ allele, including association

with a successful clone and positive selection under certain growth conditions. However, the dynamics are evidently complex and the precise contribution of the different factors is difficult to discern.

## Paper II: Future perspectives

i. Animal experiments and studies of clinical outcomes with TGC treatment of infections that were caused by isolates carrying *tet*(A)$^{wt}$ would clarify the clinical importance of this mechanism of resistance. Depending on the design, such experiments could also determine if different dosage regimes for infections caused by *tet*(A)-carrying strains could prevent resistance development.

ii. A detailed analysis of the structure of TetR(A)$^{24bp\Delta}$ would aid in understanding the effect of this deletion. Crystallography with the various tetracycline antibiotics associated to the mutated protein as well as more detailed analysis of the affinities of these antibiotics would further describe this allele of TetR(A).

# Paper III: Impact of the bacterial genetic background on plasmid-borne antibiotic resistance

Jennifer Jagdmann, Cristina Saubi Puignau, Dan I. Andersson, and Hervé Nicoloff
*Manuscript*

Plasmids play an integral role in antibiotic resistance. As many resistance genes are carried on plasmids, which can both impact their expression and allow for rapid dissemination to other isolates, understanding plasmids and the effect that plasmid and host diversity may have on antibiotic resistance is crucial[68,152,153]. We therefore continued our approach of including intra-species diversity to the best of our ability by selecting a subset of 16 *E. coli* isolates from our original collection (**Paper I**) as hosts for seven multiresistance plasmids. These plasmids carried a variety of resistance genes (25 different genes and variants in total) and, depending on the plasmid, were successfully transferred them into between eight and 16 hosts. After a successful transfer, isolates were screened for loss of innate plasmids, exponential growth rate was compared to the parental host isolate, and MICs of the parental host and host carrying the plasmid of interest were measured for a selected set of relevant antibiotics.

A few hosts were not able to receive many plasmids, and we observed a possible trend of difficulty transferring plasmids into hosts already carrying many innate plasmids. Three of the 16 hosts carried five or six innate plasmids, and two of these hosts could only receive one plasmid. However, the third host, carrying six innate plasmids, could receive all seven plasmids used. However, in this host, acquisition of new plasmids was accompanied by a discernable fitness cost for six of the seven plasmids.

We observed an apparent impact of the host genetic background on MICs, as well as differences in resistance levels between plasmids carrying the same resistance gene (e.g., *tet*(A) and $bla_{\text{TEM-1B}}$). There were also cases where carriage of the same plasmid in different hosts could result in a wide range of MICs, sometimes reaching above the clinical breakpoint for some hosts and not for others. This highlights the clinical relevance of understanding the interplay with a host's genetic background. Other interesting findings include the trend of different plasmids in one isolate (DA44557) often leading to MICs lower than those observed in other hosts.

While this study is only scratching the surface of the potential genetic diversity that could affect plasmid-borne resistance, it is clear that there is a complex interplay between hosts and plasmids which impacts antibiotic resistance. The effects of plasmid carriage in one isolate are not entirely indicative of the full spectrum of effects in a species. The causes of this variation are

not yet, and may never be, fully understood. Nevertheless, acknowledging such variation is crucial, especially considering the potential clinical relevance.

## Paper III: Future perspectives

Attempting to elucidate some of the specific factors impacting the varied resistance levels reached following plasmid acquisition would be a clear first step, including the following approaches.

i. One important control is to analyze if the plasmids of interest have different copy numbers in different hosts, and, if so, to try to determine why such differences might be observed.
ii. For genes such as *tet*(A) and $bla_{\text{TEM1-B}}$, expression analysis of the specific gene would also be of value as it could indicate strain-specific differences.
iii. In some cases, additional bioinformatic analysis would be beneficial; for example, such analysis could help identify why one isolate had consistently higher than average aminoglycoside MICs following plasmid transfer.
iv. Determining plasmid fitness costs in a more precise manner would also be useful.

# Concluding remarks

From a microbiologist's standpoint, the world today does not look like the world at the end of the 19th century. In about 150 years, we have moved from only beginning to accept germ theory to developing tools for genetic adaptation and potential gene therapy based on bacterial defense mechanisms[154–156]. We have innovative treatments and therapies based on the ability to decrease or deplete one's immune response and still survive[157–159]. The advances have been great, and we have reaped the rewards. We live longer, healthier, fast-paced lives. In the process, we have also forgotten some difficulties of the past.

As a society, we face many great challenges: climate change, rising geopolitical instability, and, of course, increasing antibiotic resistance are only a few. All of these challenges require thorough, complex, and multi-faceted approaches to mitigate the effects in a fair and quick way. Antibiotic resistance is an excellent example, demanding new drugs and treatments, which in turn requires changes in financing models and improved, fair distribution. We also need ethical stewardship and a renewed, universal effort to prevent infections by providing access to clean food and water, as well as adequate health care. There is no quick fix or silver bullet. There will only be hard work.

When faced with such a challenge, with wide-reaching impacts and requiring extensive interventions, one's instinct can be to withdraw or even deny. Facing the need to change your way of life, or view of the world, can be terrifying. However, the solution is in many ways that which gave us the great progress of the past centuries: we must understand more. To respond to difficulty as best we can, we must try to understand as much as possible about the problems themselves. This work is my attempt to contribute to the larger effort by providing a miniscule crumb of knowledge about bacterial genetic diversity and its complex effect on the development of antibiotic resistance. It is not complete or comprehensive, nor is it much in the grand scheme of things, but I am proud, and I hope this contribution won't be my last.

*"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."*

-Marie Curie

# Popular science summary

While many people might recognize *E. coli* from news reports of disease outbreaks, this bacterial species is very diverse and can be part of a healthy gut flora. This variety comes, in part, from the genetic diversity of the species. Each individual *E. coli* strain contains about 4000-5000 genes, but just under half of those genes can differ when comparing one *E. coli* strain to another. In fact, there are at least 128000 genes, plus variations of these, that could be included in an *E. coli* strain. Some strains can carry genes directly causing resistance, for example by coding for an enzyme that breaks down the antibiotic. However, since carrying certain genes (or variants thereof) can affect how other genes are expressed, a gene being present or absent is not all that matters. Despite this, most studies of antibiotic resistance only use on or a few strains, and miss a lot of this genetic diversity. In this thesis, we used a larger-than-usual number of *E. coli* strains to study antibiotic resistance from two main angles: resistance arising from spontaneous mutations and resistance genes that can be transferred between cells.

Antibiotic resistance can be caused by mutations, or changes in the genetic material of a cell. These mutations can stop an antibiotic from working properly, for example by changing how a building block of the cell is structured so the antibiotic can no longer disrupt it. We looked at how often mutations causing resistance to four different antibiotics happened in a set of 35 *E. coli* strains. In this, we found that the frequencies of these mutations were very different between strains and discovered some new mutations that have not been seen before for the antibiotics in question.

As a part of this first project, we found a new path to resistance where some strains carrying a pump that removes a specific type of antibiotic from the cell could make too many copies of the gene coding for this pump. While one copy of the gene is not enough for the cell to efficiently remove the updated antibiotic tigecycline, cells with extra copies of the gene were resistant to tigecycline. We also found a mutated version in some strains where extra copies of the gene did not make the cell resistant, since less of the pump was produced for each gene copy. Since this mutated version has not been described before, we also looked in to different factors that might explain why it is common. The fact that this common mutant of this pump had not been noticed before confirms how important it is to use many different strains when studying antibiotic resistance.

Antibiotic resistance genes can be carried on plasmids (circular pieces of DNA that can be different sizes and usually carry a set of genes) that can move between bacterial cells. To look into this angle of antibiotic resistance, we moved plasmids carrying several resistance genes into a smaller set of *E. coli* hosts from the collection in our first project and studied how the hosts were affected. We found that the increase in antibiotic resistance was not consistent in all hosts for almost all plasmids and discovered some particularly interesting cases where, for example, some strains with a plasmid reached levels considered to be resistant while other strains with the same plasmid did not.

In general, we showed that the genetic diversity of *E. coli* can impact antibiotic resistance in this species. While other bacterial species have different levels of genetic diversity, we can conclude that studying antibiotic resistance in more than one strain of a species should be standard, and drawing conclusions on how antibiotic resistance develops in a species without looking at a larger set of bacterial strains should be avoided.

# Populärvetenskaplig sammanfattning

Många kanske känner igen *E. coli* från nyhetsrapporter om sjukdomsutbrott men färre inser sannolikt hur varierad arten är. Faktum är att många *E. coli*-stammar kan ingå i en hälsosam magflora. Denna variation har till stor del sitt ursprung i artens genetiska mångfald. Medan varje individuell stam innehåller ca 4 000–5 000 gener, så kan strax under hälften av dessa gener skilja sig mellan olika *E. coli* stammar. I själva verket finns det minst 128 000 gener som kan ingå i en individuell *E. coli* stam, samt varianter av alla dessa gener. Bakterierna kan därför bära på gener som direkt leder till resistens, till exempel en som kodar för ett enzym som bryter ner antibiotikan. Dessutom kan vissa gener (eller varianter av gener) påverka hur andra gener uttryckts. Det är således viktigt att ta hänsyn till den genetiska mångfalden när man studerar antibiotikaresistens. Vi har därför använt oss av en jämförelsevis stor samling av *E. coli* stammar för att studera två typer av resistensbildning, det vill säga genom mutationer och genom resistensgener som kan överföras mellan celler.

Antibiotikaresistens kan orsakas av mutationer, eller förändring i cellens genetiska material. Dessa mutationer kan förhindra effekten av en antibiotika, till exempel genom att ändra strukturen av proteinet i cellen som antibiotikan ska binda till så den inte kan få fäste. Därför undersökte vi hur ofta spontana mutationer sker mot fyra olika typer av antibiotika i en kollektion av 35 *E. coli* stammar. Vi upptäckte att mutantfrekvensen varierade stort mellan stammar och antibiotikatyper. Dessutom hittade vi nya mutationer som inte tidigare har beskrivits som resistensmutationer för den antibiotikan.

I samband med det första projektet upptäcktes det ett nytt resistensmönster. Vi såg att vissa stammar som bar på en pump som rensar bort en typ av antibiotika från cellen kunde få extra kopior av genen som kodar för denna pump. Med dessa extra kopior kan den annars ineffektiva pumpen även rensa bort tigecyklin, en uppdaterad antibiotika, till den graden att bakterien blir resistent. Under tiden har vi även upptäckt en muterad version av pumpen där extra kopior av genen inte leder till resistens, då färre pumpar producerades för varje genkopia. Då den muterade varianten inte har beskrivits tidigare undersökte vi även olika faktorer som kan förklara varför den var frekvent. Att en ofta förekommande muterad variant inte hade upptäckts tidigare bekräftar vikten med att använda många olika stammar i liknande studier.

Antibiotikaresistensgener kan bäras på plasmider (cirkulära DNA-fragment i olika storlekar som ofta bär på flera gener) som kan överföras till andra

bakterieceller. För att undersöka denna typ av resistensbildning flyttade vi plasmider med ett flertal resistensgener till en mindre samling av *E. coli* värdstammar från vår ursprungskollektion och tog reda på hur dessa stammar påverkades. Vi såg att ökningen i mängden antibiotika som cellerna kunde tåla skilde sig åt mellan olika stammar. Dessutom upptäckte vi intressanta fall där vissa värdstammar med en plasmid nådde resistensnivåer medan andra värdstammar med samma plasmid inte gjorde det.

Överlag såg vi att den genetiska mångfalden i *E. coli* påverkar resistensutvecklingen inom arten. Medan nivån av genetisk mångfald varierar mellan bakteriearter, bör man undvika att dra slutsatser om resistensutveckling baserat på ett fåtal stammar och sträva efter att istället använda större och varierade kollektioner.

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and I hope we can sometime do an on-site outreach event together again! I'm proud of what we've done with the podcast, and I think you've done an amazing job with your work! I've said it before and will again, I have no idea how you get things done.

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Outside of the lab, there is a huge number of friends who have supported me and been there for me over these last years. I'm sorry if I don't get everyone here, but know that I appreciate each and every one of you. I have to start with **Frida** though, you have been an amazing friend, I'm so happy you moved in with me 11 years ago. You helped me learn how to live here and be an adult. Thank you doesn't feel like enough. And **Magnus N,** I'm also so happy you decided to join our program! You're one of my favorite people to talk to. I can't even guess how many times I've come to you guys to complain and whine, thank you for being there. **Magnus L,** you've been a great friend for a long time, and I'm so grateful to know I can get a sympathetic/sarcastic comment from you pretty much any time I need one. I'm so glad you met **Sara G**! **Anna**, I'm so happy we stay in touch, even if it isn't as often. I hope we get to travel together again in 2023! Thank you, my oldest friend. **Skrea** and **Laura,** I've enjoyed so many game nights with you, and lots of fun stories. **Adam** and **Luciano**, I look forward to more holidays/brunches/drinks/games! **Valle, Jennifer, Jonas, Hanna, Fredrik,** and **Lotta,** I hope to see more of you now and play more Secret Hitler (even though I'm a terrible liar). **Johan, Madeleine, Annica, Jon**, **Malin, Jonas, Fredrik, Rebecka, Gadd,** and many

more, I hope to play beer pong at Kräftis again soon (even if it has to be kid-friendly now)!

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