Antibiotic sensitivity and horizontal gene transfer in *Escherichia coli*

A genome-wide perspective

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To my family and friends

ABSTRACT

Since their discovery in the early 20th century, antibiotics have truly revolutionized human medicine. They have allowed us to treat diseases that were previously untreatable and have become a staple of modern medicine. However, along with the human use of antibiotics pathogens resistant to antibiotics emerged. Over the last decades, an arms race between bacteria developing resistance and human medicine has been raging. Today, antibiotic resistance is a global problem with even the most potent antibiotics losing their efficiency.

Antibiotic resistance occurs when bacteria develop mechanisms to withstand the antibacterial effects of antibiotics. It is widely known that, once developed, resistance is selected for by the concentrations of antibiotics that are used to treat infections. In addition, it is becoming increasingly evident that even very low levels, often many times lower than those used in a clinical setting, can select for resistance. These low levels of antibiotics are commonly found in the environment where they contribute to the global reservoir of resistance by maintaining a constant level of resistance. Antibiotic resistance can also spread between bacteria through horizontal gene transfer. The major driving force behind this is believed to be bacterial conjugation. Despite this, the underlying mechanisms of conjugation are not fully understood.

In this thesis, antibiotic resistance and horizontal gene transfer is explored from a genome-wide perspective. The results indicate that the presence of resistance genes alone does not give the full picture when it comes to growth at sub-inhibitory levels of antibiotics. We also discuss how conjugation can be inhibited and found both genetic and environmental factors that can impair conjugation. Overall, our findings emphasize the importance of understanding the emergence, selection, and spread of antibiotic resistance at sub-inhibitory concentrations of antibiotics.

Keywords: antibiotics, antibiotic resistance, *Escherichia coli*, heavy metals, bacterial conjugation, pangenome, sub-inhibitory

SAMMANFATTNING

Sedan deras upptäckt i början av 1900-talet har antibiotika revolutionerat den moderna sjukvården. De har gjort det möjligt för oss att behandla sjukdomar som tidigare inte gick att behandla och har därmed blivit en av stöttepelarna inom både human och veterinär sjukvård. I samband med att användningen av antibiotika inom sjukvården ökat har även antalet sjukdomsframkallande bakterier som är resistenta mot antibiotika ökat. Under de senaste årtiondena har en kapprustning mellan bakterier och sjukvården rasat. Idag är antibiotikaresistens ett globalt problem där även de mest effektiva antibiotika börjat tappa sin slagkraft.

Antibiotikaresistens uppstår genom att bakterier utvecklar mekanismer för att oskadliggöra antibiotikans effekt. När resistens har uppstått kan den främjas, eller selekteras för, av användning av antibiotika, speciellt av sådana nivåer som används för att behandla infektioner. Även mycket låga nivåer av antibiotika kan främja förekomsten av antibiotikaresistens. Sådana nivåer är vanligtvis många gånger lägre än de som används inom sjukvård och är således icke-hämmande för bakterier. Dessa låga nivåer förekommer ofta i miljön där de bidrar till den globala reservoaren av resistens genom att bibehålla en konstant nivå av resistens. Dessutom kan antibiotikaresistens spridas mellan bakterier genom horisontell genöverföring. Den huvudsakliga drivkraften bakom detta tros vara bakteriell konjugation. Trots detta är de underliggande mekanismerna för konjugation inte helt fastställda.

I denna avhandling utforskar vi antibiotikaresistens och dess spridning genom bakteriell konjugation ur ett genomomfattande perspektiv. Våra resultat visar på att förekomsten av antibiotikaresistensgener inte alltid ger hela bilden när det gäller tillväxt i närvaro av icke-hämmande nivåer av antibiotika. Vi diskuterar även möjligheten att stoppa konjugation och vilka miljö- och genetiska faktorer som kan hindra konjugation. Över lag betonar våra resultat vikten av att förstå uppkomsten, urvalet och spridningen av antibiotikaresistens vid icke-hämmande nivåer av antibiotika.

LIST OF PAPERS

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

I. **Palm M**., De Chiara M., Rahmqvist I., Jaén-Luchoro D., Khaiwal S., Stenberg S., Boström J., Pueyo Hurtado S., Tous Mohedano M., Owens Uwangue, Smits S., Moore E. R. B., Parts L., Liti G., Warringer J., Farewell A. Mapping the Antibiotic and Heavy Metal Phenotype-Genotype Landscape of *Escherichia coli*.
Unpublished manuscript

II. Benkwitz-Bedford S., **Palm M**., Yasir Demirtas T., Mustonen V., Farewell A., Warringer J., Parts L., Moradigaravand D. Machine Learning Prediction of Resistance to Subinhibitory Antimicrobial Concentrations from *Escherichia coli* Genomes. *mSystems*, 2021, 6, e00456-21

III. Graf F. E., **Palm M**., Warringer J., Farewell A. Inhibiting conjugation as a tool in the fight against antibiotic resistance. *Drug Development Research*, 2019; 80

IV. Alalam H., Graf F. E., **Palm M**., Abadikhah M., Zackrisson M., Boström J., Fransson A., Hadjineophytou C., Persson L., Stenberg S., Mattsson M., Ghiaci P., Sunnerhagen P., Warringer J., Farewell A. A High-Throughput Method for Screening for Genes Controlling Bacterial Conjugation of Antibiotic Resistance. *mSystems*, 2020, 5, e01226-20

V. **Palm M**., Fransson A., Hultén J., Bucáro Stenman K., Allouche A., Chiang O. E., Constandse M. L., van Dijk K. J., Icli S., Klimesova B., Korhonen E., Martinez-Crespo G., Meggers D., Naydenova M., Polychronopoulou M. An., Schuntermann D. B., Unal H., Wasylkowska A., Farewell A. The Effect of Heavy Metals on Conjugation Efficiency of an F-Plasmid in *Escherichia coli*. *Antibiotics*. 2022; 11(8):1123

Co-authored papers that are not included in this thesis

1. Moradigaravand D., **Palm M**., Farewell A., Mustonen V. Warringer J., Parts L. Prediction of antibiotic resistance in *Escherichia coli* from large-scale pan-genome data. *PLoS Computational Biology*, 2018, 14(12).

2. Dimovska Nilsson K., **Palm M**., Hood J., Sheriff J., Farewell A., Fletcher J. S. Chemical Changes On, and Through, The Bacterial Envelope in *Escherichia coli* Mutants Exhibiting Impaired Plasmid Transfer Identified Using Time-of-Flight Secondary Ion Mass Spectrometry. *Analytical Chemistry*, 2019, 91, 17

3. Kvint K., **Palm M**., Farewell A. Teaching about antibiotic resistance to a broad audience: a multidisciplinary approach. *FEMS microbiology letters*, 367, 14, (2020)

4. Murphy R., **Palm M**., Mustonen V., Warringer J., Farewell A., Parts L., Moradigaravand D. Genomic Epidemiology and Evolution of *Escherichia coli* in Wild Animals in Mexico. *mSphere*, 2021, 6, e00738-20

5. Bourgard C., Rodriguez-Hernández D., Rudenko A., Rutgersson C., **Palm M**., Larsson D. G. J., Farewell A., Grøtli M., Sunnerhagen P. Development of Dicationic Bisguanidine-arylfuran Derivatives as Potent Agents Against Gran-negative Bacteria. *Antibiotics*. 2022; 11(8):1115

6. Gamfeldt L., Roger F., Hagan J. G., **Palm M**., Warringer J., Farewell A. Scale and heterogeneity increase transgressive overyielding in biodiversity-ecosystem functioning experiments. *Accepted for publication in Oikos*

7. Graf F. E., Persson L., Tous Mohedano M., **Palm M**., …, Farewell A. Variation in Chromosomal Determinants of antibiotic resistance plasmid conjugation. *Unpublished manuscript*

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ABBREVIATIONS

AAC	Aminoglycoside acetyltransferase
AME	Aminoglycoside modifying enzyme
ANT	Aminoglycoside nucleotidyltransferase
APEC	Avian pathogenic *E. coli*
APH	Aminoglycoside phosphotransferase
DAEC	Diffusely adherent *E. coli*
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *E. coli*
E. coli	*Escherichia coli*
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
ESBL	Extended-spectrum beta-lactamase
HGT	Horizontal gene transfer
Inc group	Incompatibility gtoup
IR	Inverted repeat
IS	Insertion sequence
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
mRNA	Messenger RNA
MSC	Minimum selective concentration
PBP	Penicillin-binding protein
RNA	Ribonucleic acid
TE	Transposable element
THFA	Tetrahydrofolic acid

AIM OF THE THESIS

Antibiotic resistance is a major threat to modern medicine and over time, the severity of this problem has escalated. One of the major driving forces contributing to this is that of horizontal gene transfer, in particular bacterial conjugation.

This thesis compiles a total of five research papers which explore aspects of antibiotic resistance and mechanisms behind bacterial conjugation at a genome-wide scale. The initial part of the thesis introduces antibiotics and their mechanisms of action followed by the various mechanisms of antibiotic resistance employed by bacteria. Next, heavy metal toxicity and resistance, and the co-selection of heavy metal and antibiotic resistance are discussed. Lastly, the various mechanisms of horizontal gene transfer and how they contribute to resistance are reviewed. The papers can be divided into two main groups that explore different aspects of antibiotic resistance.

First, we explore the growth in the presence of sub-inhibitory levels of antibiotics and heavy metals.

a) In **Paper I**, we explore the phenotype-genotype landscape of *Escherichia coli* with regards to growth on sub-inhibitory levels of antibiotics and heavy metals.
b) In **Paper II**, we employ machine learning to predict growth characteristics of *E. coli* growing in the presence of sub-inhibitory concentrations of antibiotics.

Second, we study the spread of antibiotic resistance through conjugation.

c) In **Paper III**, we briefly discuss various ways of inhibiting conjugation in order to stop the spread of antibiotic resistance.
d) In **Paper IV** and **Paper V,** we set out to investigate how genetic and environmental perturbations impact the conjugation of an IncF plasmid.

ANTIBIOTICS

The discovery of antibiotics has forever changed all aspects of modern medicine, not only because it allowed for the treatment of potentially lethal infections, but also because it made it possible to perform medical procedures such as organ transplants and cancer treatments. The introduction of antibiotics also had a big impact on modern society by indirectly increasing the average lifespan over the course of the 20th century (Lederberg, 2000). Most antibiotics that are in clinical use today were originally isolated from soil microorganisms. Despite this, the role of antibiotics in nature and why microorganisms produce them in the first place is still not fully understood. The most straightforward answer, due to their antimicrobial properties, would be that they act as chemical weapons that microorganisms use to kill or inhibit its neighbors to compete for nutrients. Although, the levels of antibiotics produced in a natural setting are much lower than what is used in a clinical setting and rarely reach inhibitory concentrations for most environmental bacteria (Aminov, 2009). These low levels of antibiotics have led some to speculate that the main function of antibiotics is not to kill other microorganisms, but rather to communicate with them. In fact, low levels of antibiotics have been shown to alter gene expression (Fajardo & Martínez, 2008). Regardless of their natural function, the discovery and introduction of antibiotics are without a doubt one of the most important medical advances of all time.

A BRIEF HISTORY OF ANTIBIOTIC DISCOVERY

1900s-1910s, Salvarsan

The history of antibiotics starts with German physician and scientist Paul Ehrlich and his search for a “magic bullet”, a substance that could kill infectious agents without harming the human host. In the early 1900s, syphilis had a major impact on society. The treatments of syphilis at the time, most commonly using mercury, suffered from poor efficacy and severe side effects making the disease very difficult to cure. In 1907, Paul Ehrlich and Alfred Bertheim synthesized the organoarsenic compound arsphenamine from Atoxyl, a compound known to have some inhibitory activity against

trypanosomes with severe toxic side effects. Two years later, while performing a screen of organic arsenical compounds, Sahachiro Hata discovered that arsphenamine was toxic to the bacterium *Treponema pallidum*, the causative agent of syphilis. The compound was also well tolerated by humans and became in 1910 the first antimicrobial introduced into clinical use, marketed under the name Salvarsan.

1930s-1940s, Prontosil and sulfa drugs

Inspired by Ehrlich's work and success with Salvarsan, the Friedrich Bayer company in Germany set out to find new potential antibacterial compounds. In 1932, the chemists Joseph Klarer and Fritz Mietzsch, synthesized several hundred compounds for bacteriologist Gerhard Domagk to test. The basis for many of these compounds were azo dyes. Among the compounds that were synthesized was KL-695, an azo dye to which a sulfonamide group had been added, which showed a weak antibacterial activity against streptococci in mice. Following the promising results with KL-695, Klarer and Mietzsch performed some further synthetic work to improve the activity of KL-695. This yielded KL-730, later named Prontosil, that had a more consistent and effective antibacterial activity against streptococci in mice (Bentley, 2009). Over the following years, Prontosil started being used in treatment of streptococcal infections in humans. However, the medical community was initially somewhat skeptical about its overall effectiveness. It was not until 1936 that Prontosil was accepted by the broader medical community after Colebrook used it to treat puerperal (postpartum) infections (Colebrook & Kenny, 1936) and several success stories, including the treatment of U.S. President Franklin D. Roosevelt's son (TIME Magazine, 1936), were published. The success of Prontosil prompted several medicinal chemistry labs to investigate and improve Prontosil further. One such group was that of Ernest Fourneau at the Pasteur Institute in Paris. Fourneau's group could show that the azo link of Prontosil could be broken within tissues to yield sulfanilamide and later also showed that sulfanilamide was the sole cause of Prontosil's antibacterial properties (Tréfouël et al., 1935; Wainwright & Kristiansen, 2011). This discovery sparked an increase into the synthesis and investigation of sulfonamides. Between 1935 and 1945 hundreds of new sulfonamide

compounds were produced and introduced to treat infections, in part thanks to the ease with which sulfanilamide could be modified. Thus, the first class of antibiotics was born.

1940s, penicillin

In 1928, prior to the introduction of sulfa drugs, Alexander Fleming made a peculiar find when he returned to his lab at St. Mary's Hospital in London from a holiday. He had noticed that an agar plate with *Staphylococcus aureus* had been contaminated by mold and that the area around the mold had been free of bacteria. He hypothesized that the mold secreted some compound that killed the surrounding bacteria. Seeing the potential of his finding, Fleming went on to isolate the mold and identified it as a member of the *Penicillium* genus (later shown to be *Penicillium rubens*). He obtained an extract from the mold and confirmed his hypothesis by testing the extract on staphylococci and other gram-positive bacteria. He named the active agent of the extract penicillin, after *Penicillium* (Fleming, 1929). However, Fleming and his co-workers were unable to purify penicillin due to its unstable nature. It would not be until 1939 when Ernst Boris Chain and Howard Florey of Oxford University revisited Fleming's findings, as part of an initiative to find other agents with antibacterial properties, that penicillin would finally be purified (Chain et al., 1940). When, large-scale production of penicillin started in 1944, it quickly became one of the most used drugs and was hailed as a "miracle drug".

1940s-1960s, the "golden age" of antibiotic discovery

Shortly after the first clinical use of penicillin, in 1943, a new compound was isolated from *Streptomyces griseus* by Albert Schatz that showed potential as an antibiotic. This compound, named streptomycin, showed antibacterial activity against *Escherichia coli* and the tuberculosis bacterium *Mycobacterium tuberculosis*, for which streptomycin was the first effective treatment. The discovery and introduction of penicillin and streptomycin paved the way for a golden age of antibiotic discovery where many new antibiotic classes were discovered, many of which are still in clinical use today. During these years, the focus shifted from trying to synthesize new compounds to instead find natural products that have antibacterial properties. Some examples

of the antibiotics discovered and introduced during this era are the aminoglycoside kanamycin, the amphenicol chloramphenicol, the glycopeptide vancomycin, tetracycline and many more. While most of the antibiotics discovered during the 1950s and 1960s were natural products and most of which were derived from members of the *Streptomyces* genus, there were also some semi-synthetic and synthetic antibiotics introduced such as the cephalosporin, cefacetrile, and the nitrofuran, nitrofurantoin (Hutchings et al., 2019). The introduction of all these antibiotics forever changed not only human medicine but also that of veterinary medicine. During the 1950s it had been discovered that giving low doses of penicillin or the newly discovered tetracycline to poultry via their feed could increase the growth rate of the birds (Heth & Bird, 1962). This opened a whole new market for the pharmaceutical industry and the use of penicillin used in livestock quickly rose and, in 1964, matched the use in humans (Hewitt, 1967).

Mid-1960s-today, post-golden age and the "discovery void"

In the late 1960s it was generally believed that the time had come to "*close the book on infectious disease*" (Cohen, 2000), because of all the antibiotics that were discovered in the previous decades. However, this positive outlook was not going to last as antibiotic resistance was on the rise. Clinical resistance to penicillin had been detected in staphylococci as early as 1947 (Barber, 1947). The response to the rising levels of resistance from researchers and pharmaceutical companies was to modify the existing antibiotics such that resistant bacteria were susceptible once more. One such antibiotic was methicillin, meant to treat penicillin resistant staphylococci. However, resistance to methicillin was soon detected as well. This quickly became a pattern and soon resistance to most of the antibiotics in clinical use was spreading to the point where all the progress made in the previous decades was threatened. New classes of antibiotics were desperately needed. Unfortunately, starting in the 1980s and lasting until the early 2000s there has been a "discovery void" where no new antibiotic classes were discovered and introduced. The reasons for this are complex but two contributing factors are 1) the pharmaceutical industry was focusing on producing analogues to existing antibiotics to combat rising resistance and 2) the "low-hanging fruit"

of antibiotic classes had already been found. In the early 2000s the discovery void ended when two new classes were introduced: the oxazolidinone linezolid and the cyclic lipopeptide daptomycin (Coates et al., 2011). Resistance to these new classes is still at a relatively low level but it is expected to rise in a similar way to resistance to previous antibiotics. As a result, in 2022, more than a century after the discovery of salvarsan, bacterial infections are still a very current threat to human health.

MECHANISMS OF ACTION

Antibiotics exert their antibacterial properties by blocking or disrupting important parts of the bacterial cell or its metabolism. Many of the structures targeted by antibiotics only exist in bacteria, such as the cell wall or some metabolic enzymes. The targets that do also exist in humans, such as the ribosome, are in most cases sufficiently distinct from each other such that selective toxicity is possible. Generally, antibiotics fall into two major categories based on their mechanism of action: bactericidal or bacteriostatic. Bactericidal antibiotics work by killing the bacteria either through lysis, by interfering with the cell wall or membrane, or by inhibiting DNA synthesis. Bacteriostatic antibiotics work by inhibiting the growth of the bacteria. This is generally achieved by preventing the bacteria from producing new proteins.

Cell wall synthesis inhibitors

The cell wall is an essential part of the bacterial cell that makes up the bacterium's first line of defense against its surrounding environment and gives the cell its structural integrity. It also allows for the intake of nutrients from the outside and the disposal of waste molecules from the inside via various channels and porins integrated into the cell wall. The structural strength of the cell wall comes from a layer of peptidoglycan which is a matrix of polysaccharide strands that are cross-linked by short peptides. The process to synthesize this peptidoglycan layer is complex and is a very common target for antibiotics (Figure 1).

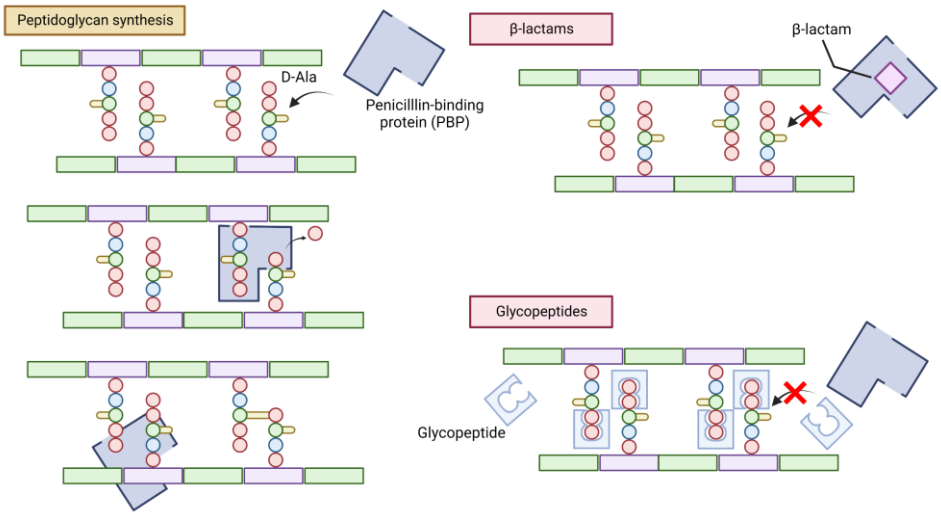


Figure 1. Cell wall synthesis inhibitors. The last steps of peptidoglycan synthesis involve the cross-linking of peptidoglycan strands. This is catalyzed through the action of penicillin-binding proteins (PBPs). PBPs bind to the D-Ala-D-Ala moiety found at the end of the peptidoglycan to cross-link two peptidoglycan strands together. β-lactam antibiotics inhibit cell wall synthesis by binding to and inhibiting PBPs, which leads to a structurally weakened cell wall that is unable to withstand the increasing pressure as the cell grows, leading to cell death. Similarly, glycopeptide antibiotics also prevent the function of PBPs. However, rather than binding directly to the enzyme itself, it binds to and blocks access to the D-Ala-D-Ala moiety. Figure created with BioRender.com.

β-lactams

The β-lactams are a class of bactericidal antibiotics and are the most widely used group of antibiotics. They are characterized by the presence of a β-lactam ring in their chemical structure. This rather unique structure allows them to covalently bind to and inhibit a group of proteins called penicillin-binding proteins (PBPs) in the cell wall. PBPs are involved in the later stages of peptidoglycan synthesis, in which the polysaccharide strands are cross-linked. PBPs bind to a D-Ala-D-Ala moiety at the end of the peptidoglycan and perform the cross-linking reaction with another peptidoglycan strand (Figure 1). The structure of the β-lactam antibiotics is similar to that of the D-Ala-D-Ala moiety, allowing them to bind irreversibly to the active site of the PBPs (Tipper & Strominger, 1965; Spratt & Cromie, 1988). By inhibiting these

proteins, the peptidoglycan layer and the cell wall as a whole is structurally weakened. This weakened cell wall is unable to withstand the increasing pressure that is generated as the cell grows bigger and the cell bursts, like that of a balloon that is inflated too far.

The β-lactam class is typically divided into subgroups. These subgroups are penicillins, cephalosporins, monobactams and carbapenems. Cephalosporins are commonly used as chemical scaffolds which led to a continuous development of new and improved versions. There are currently five generations of cephalosporins and the most recent ones, such as ceftaroline, are used in the treatment of several important pathogens (T. E. Long & Williams, 2014). Monobactams are, in contrast to the other β-lactams, exclusively used against Gram-negative bacteria as they have a low affinity for the PBPs of Gram-positive bacteria (Brewer & Hellinger, 1991). Carbapenems have the broadest spectrum of activity among the β-lactams. They are typically reserved for the use of multidrug resistant (MDR) bacteria as they are generally less susceptible to β-lactamases than other β-lactams and are thus often used as "last resort antibiotics" (Livermore, 1998).

Glycopeptides

Glycopeptides are bactericidal antibiotics that kill bacteria by, like β-lactams, inhibiting peptidoglycan synthesis, albeit in a slightly different way. Rather than binding to PBPs to inhibit their function, glycopeptides bind to the D-Ala-D-Ala moiety itself to block the PBPs from accessing the peptidoglycan precursor (Figure 1). This ultimately prevents the PBPs from cross-linking the peptidoglycan and the cell wall is weakened. Glycopeptides are, in general, only active against Gram-positive bacteria. This is because of the size of the glycopeptide molecule which prevents it from passing the outer membrane of Gram-negative bacteria. There are, however, some findings that indicate that this can be circumvented by modifying the glycopeptide (Yarlagadda et al., 2016). Many glycopeptide antibiotics, such as vancomycin, teicoplanin and telavancin, are used to treat infections that are resistant to β-lactams (Zeng et al., 2016).

Protein synthesis inhibitors

Bacterial 70S ribosomes are large macromolecular complexes consisting of a small 30S and a large 50S subunit, that translate the information stored in mRNA into a polypeptide chain that is folded into a functioning protein. During translation, the 30S subunit will bind to an mRNA after which the 50S subunit and other accessory components assemble to initiate translation. The 70S ribosome has three sites for binding of tRNA: the A, P and E sites. The A site is where an aminoacyl-tRNA, a tRNA carrying a specific amino acid, binds to the ribosome. Before binding, a decoding center located on the 30S subunit ensures that the interaction between the anticodon of the incoming tRNA and the codon of the mRNA matches to ensure that the correct amino acid is added to the polypeptide. Once bound to the ribosome, the forming polypeptide chain is transferred to the aminoacyl-tRNA in the A site by the formation of a peptide bond between the polypeptide chain and the amino acid. This reaction is catalyzed by a peptidyl transferase center on the 50S subunit and as it takes place, the ribosome shifts along the mRNA and the tRNA in the A site, now a peptidyl-tRNA, moves to the P site. At the same time, the tRNA that occupied the P site passes to the E site where it exits the ribosome, allowing a new aminoacyl-tRNA to bind to the A site. This process repeats until the entire mRNA has been translated after which the 70S ribosome is split into its subunits that are recycled for the next round of translation. Protein synthesis, the ribosome in particular, is the target of a wide variety of antibiotics, most of which are bacteriostatic (Figure 2).

Aminoglycosides

Aminoglycosides are a group of antibiotics that bind to sites on the ribosome. Most aminoglycosides, such as kanamycin and gentamicin, have a bactericidal effect. These antibiotics kill the cell by binding to and inhibiting the decoding center of the 30S ribosomal subunit (Figure 2). This will not stop the translation process but rather induce misreading of the mRNA, causing the ribosome to produce non-functional and sometimes toxic proteins (Krause et al., 2016). These proteins are subsequently released into the cell where they will cause damage to various cellular components, such as the cell membrane, leading to cell death (Davis et al., 1986). Some aminoglycosides, such as spectinomycin,

have a bacteriostatic activity. These antibiotics bind to the A site of the ribosome and prevent translocation of the tRNA from the A site to the P site (Burns & Cundliffe, 1973). This will cause the ribosome to stall, and protein synthesis will be interrupted leading to bacteriostasis.

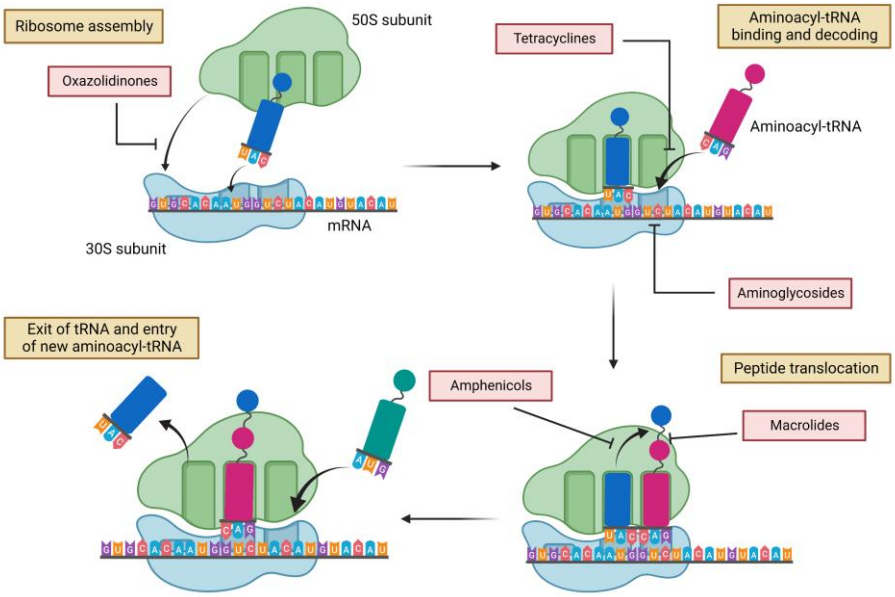


Figure 2. Protein synthesis inhibitors. The ribosome is the target of a large number of antibiotics and inhibiting any of these steps leads to either cell death or growth inhibition. Oxazolidinones bind to the 30S subunit and prevent ribosome assembly. Tetracyclines binds to the A site of the 30S subunit and prevent the binding of incoming tRNA molecules. Aminoglycosides inhibit the decoding function of the 30S subunit, leading to the production of non-functional proteins. Amphenicols interfere with the peptidyl transferase activity of the 50S subunit, preventing the formation of a peptide bond between the polypeptide and the incoming tRNA. Similarly, some macrolides also inhibit peptidyl transferase activity while others bind to and block the tunnel through which polypeptide chain exits the ribosome, leading to the premature dissociation of the peptidyl-tRNA from the ribosome. Figure created with BioRender.com.

Amphenicols

Amphenicols is a class of broad-spectrum antibiotics that have a bacteriostatic activity against Gram-positive and Gram-negative bacteria. They stop bacterial growth by inhibiting the elongation of the polypeptide chain during translation by binding to and inhibiting the peptidyl transferase activity of the 50S

ribosomal subunit (Figure 2). This prevents the ribosome from forming a peptide bond between the incoming amino acid and the nascent polypeptide chain, leading to a disruption of protein synthesis and growth ceases. Amphenicols, such as chloramphenicol, are rarely used systemically in humans due to severe side effects but are still used to treat topical and eye infections (Lam et al., 2002).

Macrolides

Macrolides is a class of bacteriostatic antibiotics mainly used against Gram-positive bacteria. Macrolides exert their bacteriostatic mechanisms through two general mechanisms depending on their chemical structures. First, some macrolides such as spiramycin stop bacterial growth by preventing polypeptide chain elongation by inhibiting the peptidyl transferase activity of the 50S subunit, similar to amphenicols (Poulsen et al., 2000). Second, macrolides such as erythromycin bind to and block the entrance to the tunnel through which the nascent polypeptide chain exits the ribosome (Figure 2). This blockage will cause the peptidyl-tRNA to prematurely dissociate from the ribosome shortly after translation initiation, leading to the formation of short and non-functional peptide chains (Menninger & Otto, 1982).

Oxazolidinones

Oxazolidinones is a recently discovered class of bacteriostatic antibiotics that have a rather unique mechanism of action. Oxazolidinones, such as linezolid, inhibit protein synthesis by preventing the assembly of the translation initiation complex (Swaney et al., 1998). The translation initiation complex consists of the 70S ribosome, a tRNA charged with N-formylmethionine and the mRNA to be translated. Linezolid binds to the 50S subunit near the interface with the 30S subunit, causing a distortion of the site. This distortion has been suggested to prevent the translation initiation complex from properly forming, leading to the inhibition of translation initiation (Figure 2) (A. H. Lin et al., 1997).

Tetracyclines

Tetracyclines are a class of antibiotics that can be divided into two subgroups, typical and atypical. Typical tetracyclines, such as tetracycline and tigecycline,

are bacteriostatic antibiotics that target the binding of the charged tRNA to the translation complex. They bind to the A site of the 30S ribosomal subunit and prevent the binding of incoming charged tRNAs (Figure 2) (Wurmbach & Nierhaus, 1983). This leads to a stop in the translation process as the ribosome will be unable to receive and incorporate new amino acids. Atypical tetracyclines, such as anhydrotetracycline, are derivatives of tetracyclines that exhibit a bactericidal activity. Rather than targeting the ribosome and protein synthesis, they have been suggested to target the cytoplasmic membrane (Oliva et al., 1992). However, atypical tetracyclines are not used clinically due to their side effects.

Folate synthesis inhibitors

Tetrahydrofolic acid (THFA) is a cofactor that is involved in many different biosynthetic reactions in the cell. It is especially important in nucleic acid biosynthesis where it is an essential precursor for the biosynthesis of thymine, one of the bases of DNA. While THFA is essential in both humans and bacteria, they acquire it in different ways. Humans, and other mammals, have active folate transporters in the membrane that transport folate into the cell while in bacteria it needs to be synthesized through folate biosynthetic pathways (Henderson & Huennekens, 1986). The process to synthesize THFA involves many different enzymes. The two final steps of the process are the conversion of *p*-aminobenzoic acid (PABA) and dihydropteroate diphosphate into dihydrofolic acid, and the reduction of dihydrofolic acid into THFA. These steps are catalyzed by dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) respectively (Figure 3). These two steps are also the target of antibiotics. DHPS is inhibited by sulfonamides and DHFR is inhibited by trimethoprim (Burman, 1986). The inhibition of either of these steps leads to the depletion of THFA which in turn will lead to thymine deprivation and bacteriostasis. Trimethoprim and sulfamethoxazole, a sulfonamide, are commonly combined to achieve a synergistic effect to further shut down the synthesis of THFA. When used on their own, trimethoprim and sulfamethoxazole have a bacteriostatic effect on bacteria. When combined,

typically referred to as co-trimoxazole, they exert a bactericidal effect (Garrod & Waterworth, 1968).

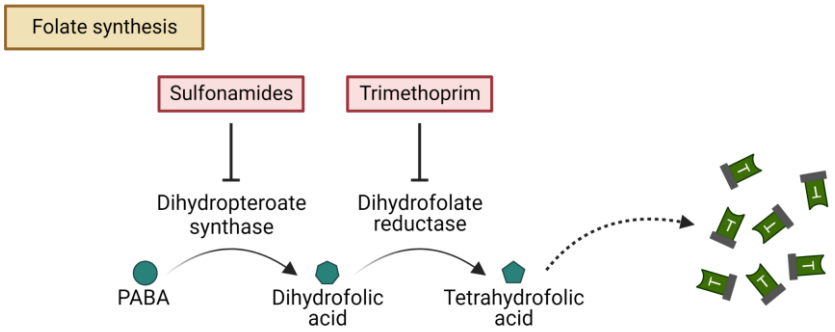


Figure 3. Folate synthesis inhibitors. Tetrahydrofolic acid (THFA) is an important precursor for the synthesis of thymine. The last steps in the synthesis of THFA involves the conversion of PABA to dihydrofolic acid, catalyzed by dihydropteroate synthase (DHPS), and the reduction of dihydrofolic acid into THFA, catalyzed by dihydrofolate reductase (DHFR). These enzymes are the targets of sulfonamide antibiotics and trimethoprim respectively. Inhibiting either of these steps lead to the depletion of thymine and stalled bacterial growth. Figure created with BioRender.com.

RNA polymerase inhibitors

RNA polymerase is an essential component of the cell as it is responsible for the transcription of DNA to mRNA, the first step to produce proteins. The bacterial RNA polymerase core enzyme consists of five subunits: α (two copies), β, β´ and ω. These subunits all serve different functions. The α subunits act as a scaffold for assembly by forming a dimer and interacting with the β and β' subunits. The β and β' subunits make up the bulk of the RNA polymerase enzyme (Figure 4). Once assembled, the β and β' subunits form the cleft though which DNA enters and can reach the active site where the elongation reaction takes place. They also make up a secondary channel that allows for the entry of nucleotides and an exit channel where the nascent mRNA leaves the RNA polymerase (Sutherland & Murakami, 2018). The role of the ω subunit is still not fully understood but it is speculated that it plays a structural and functional role (Mathew & Chatterji, 2006). RNA polymerase is the target of the rifamycin class of antibiotics. Rifamycins are bactericidal antibiotics that bind to the β subunit in the main channel of the enzyme, near

the active site (Campbell et al., 2001). By binding here, it sterically blocks the elongation of the mRNA and the RNA polymerase, and thus transcription, stalls. The most used rifamycin is rifampicin which is commonly used to treat infections caused by mycobacteria such as *M. tuberculosis*.

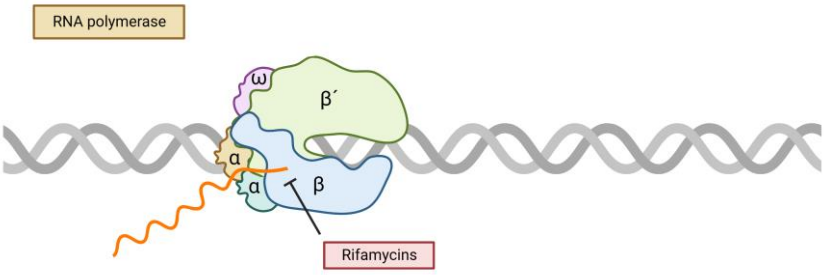


Figure 4. RNA polymerase inhibitors. The core bacterial RNA polymerase is made up of five subunits: α (two copies), β, β´ and ω. Rifamycin antibiotics, such as rifampicin, bind close to the active site of the β subunit where it blocks mRNA elongation and transcription stops. Figure created with BioRender.com.

DNA synthesis inhibitors

As cells grow, they often need to separate the two strands of its DNA for various processes, such as DNA replication and transcription, to allow enzymes access to the DNA bases. Opening the DNA is, however, not a straightforward process and various problems can be encountered. For example, during DNA replication, the two DNA strands are separated by helicases, and the DNA is replicated by DNA polymerase. However, this separation of the DNA strands will eventually lead to the formation of positive supercoiling in front of the replication fork. This supercoiling can cause the replication complex to stall as it is unable to move forward along the DNA. For DNA replication to proceed this supercoiling needs to be resolved. In bacteria, this is resolved by the topoisomerases, topoisomerase IV and DNA gyrase (Figure 5). They do this by introducing a double-strand break in the DNA after which the enzyme unwinds the supercoiled DNA. Once the supercoiling has been resolved, the topoisomerase repairs the double-strand break through ligation. Both topoisomerase IV and DNA gyrase are targets of antibiotics. The major class of antibiotics that targets this process is the

quinolones. Quinolones are bactericidal antibiotics that will inhibit topoisomerase IV and DNA gyrase by binding to the site responsible for the re-ligation of the DNA. By doing so the topoisomerases will introduce a double-strand break, unwind the DNA but will be unable to repair the double-strand break, leading to cell death (Aldred et al., 2014). Quinolones, such as nalidixic acid, were initially used to urinary tract infections. The second generation of quinolones are called fluoroquinolones, due to the addition of a fluorine to their chemical structure. Fluoroquinolones, such as ciprofloxacin, ofloxacin and levofloxacin, make up the majority of quinolones in clinical use and are used to treat a wide variety of infections (King et al., 2000).

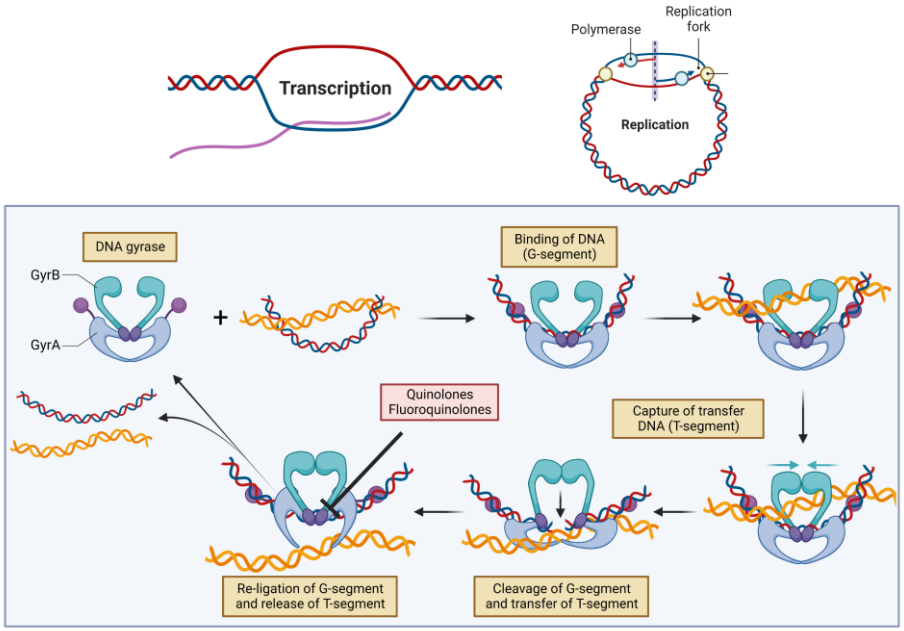


Figure 5. DNA synthesis inhibitors. DNA replication and transcription lead to the formation of supercoiling in the bacterial DNA. This supercoiling can cause problems in DNA processing and thus must be resolved. The supercoiling is alleviated by topoisomerase IV and DNA gyrase. The general mechanism of DNA gyrase is shown. DNA gyrase binds to double-stranded DNA (denoted the G-segment). It then captures another molecule of double-stranded DNA (denoted the T-segment). A double-strand break is introduced into the G-segment after which the T-segment is passed through the now open G-segment. DNA gyrase then repairs the double-strand break and releases the two DNA molecules. Quinolone and fluoroquinolone antibiotics both bind to topoisomerases and inhibit their ability to re-ligate the G-segment. This leads to the introduction of double-stranded breaks in the chromosome, leading to cell death. Figure created with BioRender.com.

Membrane disruption

The bacterial cell membrane, like the cell wall, plays an important role in protecting the cell from outside threats. It is also crucial for maintaining a proton motive force through the exchange of protons across the membrane. The proton motive force is important for various important bacterial processes such as ATP synthesis, nutrient import, and cell division (Strahl & Hamoen, 2010).

Polymyxins

One class of antibiotics that acts on the cell membrane is the polymyxins. There are only two polymyxins in clinical use, polymyxin B and colistin, but they are rarely used because of their severe side effects. Polymyxins kill Gram-negative bacteria by interacting with lipopolysaccharides (LPS) in the outer membrane. This interaction will cause the displacement of Ca^{2+} and Mg^{2+} ions that help stabilize the membrane (Figure 6). This will cause the outer membrane to be weakened and lead to the uptake of molecules from the environment and leakage of periplasmic proteins. The permeabilization of the outer membrane will also allow the polymyxin to penetrate the periplasm and attack the cytoplasmic membrane, causing lysis of the cell (Trimble et al., 2016).

Lipopeptides

Lipopeptides is a class of bactericidal antibiotics that is one of the most recently discovered classes of antibiotics, together with oxazolidinones. The most used member of this class is daptomycin. Daptomycin exerts its bactericidal activity by disrupting the membrane potential of the cytoplasmic membrane, leading to a loss of the proton motive force (Figure 6). The loss of the proton motive force causes a disruption of DNA, RNA, and protein synthesis in the cell which in turn leads to cell death. It achieves this by inserting into the cytoplasmic membrane in a Ca^{2+}- and phosphatidylglycerol-dependent manner (Jung et al., 2004). In the membrane, it oligomerizes and aggregates to form pore-like structures. These structures disrupt the integrity of the membrane and cause a release of intracellular ions leading to a loss of membrane potential (Silverman et al., 2003). The antibacterial activity of

lipopeptides is limited Gram-positive bacteria as it is unable to pass through the outer membrane of Gram-negative bacteria.

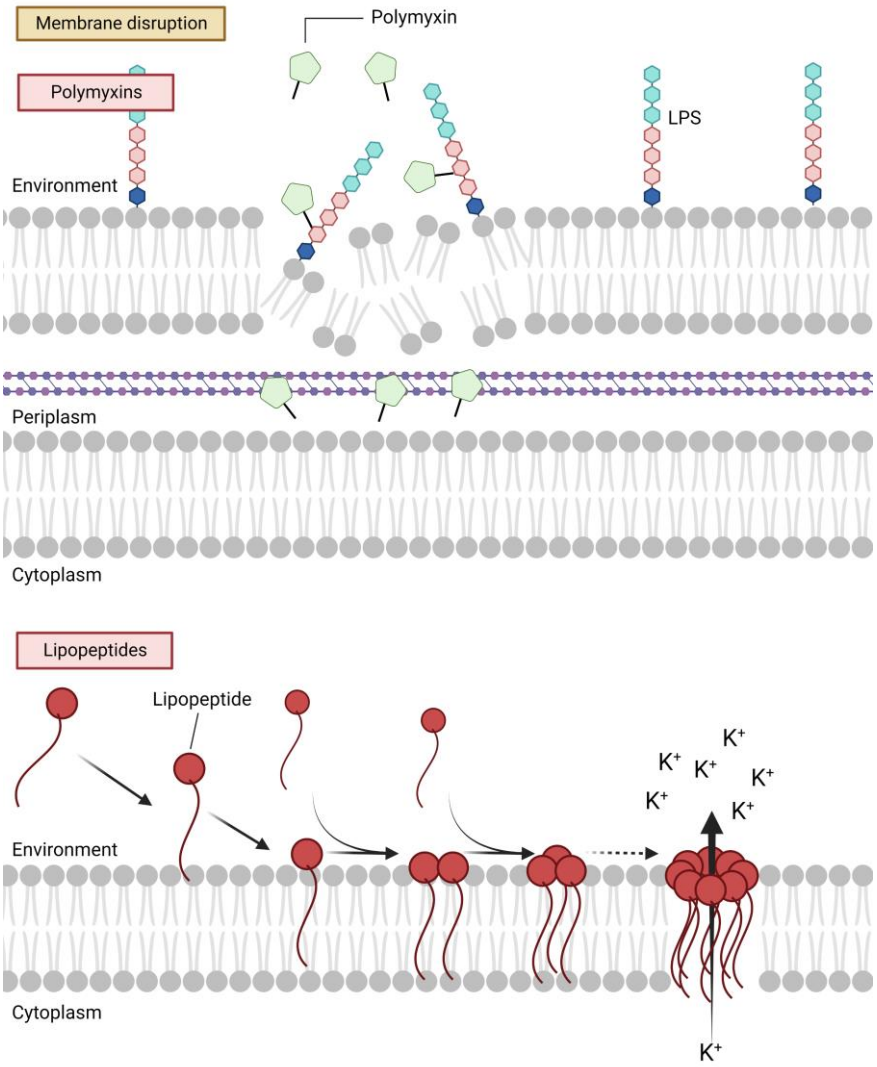


Figure 6. Antibiotics targeting the bacterial membrane. Polymyxins interact with the lipopolysaccharides (LPS) in the Gram-negative outer membrane, causing the displacement of Ca^{2+} and Mg^{2+} which leads to the destabilization of the membrane. Lipopeptides insert into the cytoplasmic membrane of Gram-positive bacteria where they oligomerize and aggregate to form pore-like structures. These structures will disrupt membrane integrity and cause the release of intracellular ions leading to a loss of membrane potential. Figure created with BioRender.com.

ANTIBIOTIC RESISTANCE

Antibiotic resistance is defined as a bacterium's ability to withstand the antibacterial activities of antibiotics. Antibiotic resistance has existed for as long as there have been antibiotics as a way for antibiotic-producing microorganisms to protect themselves or as a defense against antibiotics released by other microorganisms. Some studies suggest that antibiotic resistance, and presumably antibiotics, are ancient with genes encoding resistance to clinically relevant antibiotics being found in 30,000-year-old permafrost (D'Costa et al., 2011; Waglechner et al., 2021). While antibiotic resistance has been around for thousands of years, it is only during the last century that it has started to have a negative impact on human health, where resistance to antibiotics have made infections that were previously easily treatable untreatable. This increase of antibiotic resistant infections has primarily been caused by the over- and misuse of antibiotics, and the lack of new classes of antibiotics being developed (Ventola, 2015). This has led to resistance emerging among clinically important pathogens, such as *Escherichia coli* and the ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. In 2018, the World Health Organization published a priority list where resistant *E. coli* and ESKAPE pathogens are among the top priorities (Tacconelli et al., 2018). Today, antibiotic resistant infections are an all too familiar occurrence in hospitals worldwide and it has been estimated that approximately 1.27 million deaths could be directly attributed to antibiotic resistant infections in 2019 (Murray et al., 2022). Of these deaths, more than 250,000 were attributable to resistant *E. coli* infections alone. In 2017, England's chief medical officer Sally Davies warned that the increasing levels of resistance could lead to "*an end of modern medicine*" and that if no action is taken, we are facing "*a dreadful post-antibiotic apocalypse*" (The Guardian, 2017).

MECHANISMS OF RESISTANCE

Mechanisms of resistance differ depending on the bacterial species and the antibiotic, and are commonly divided into three groups: intrinsic, acquired, and phenotypic resistance. Intrinsic resistance is resistance that occurs naturally among some bacterial species. This occurs without any genetic or physiological changes to the cell. Most commonly this is because the bacteria lack the structure or process that is targeted by the antibiotic, or because the antibiotic is unable to reach its target (Nikaido, 1994). Acquired resistance is when a bacterium obtains the ability to survive the effects of antibiotics that they are not naturally resistant to. This can occur primarily through chromosomal mutations or the acquisition of resistance genes through horizontal gene transfer (HGT, see later section). Lastly, phenotypic resistance refers to resistance, or tolerance, that arises because of transient changes to the cell's physiology such as altered gene expression or decreased metabolism (Corona & Martinez, 2013).

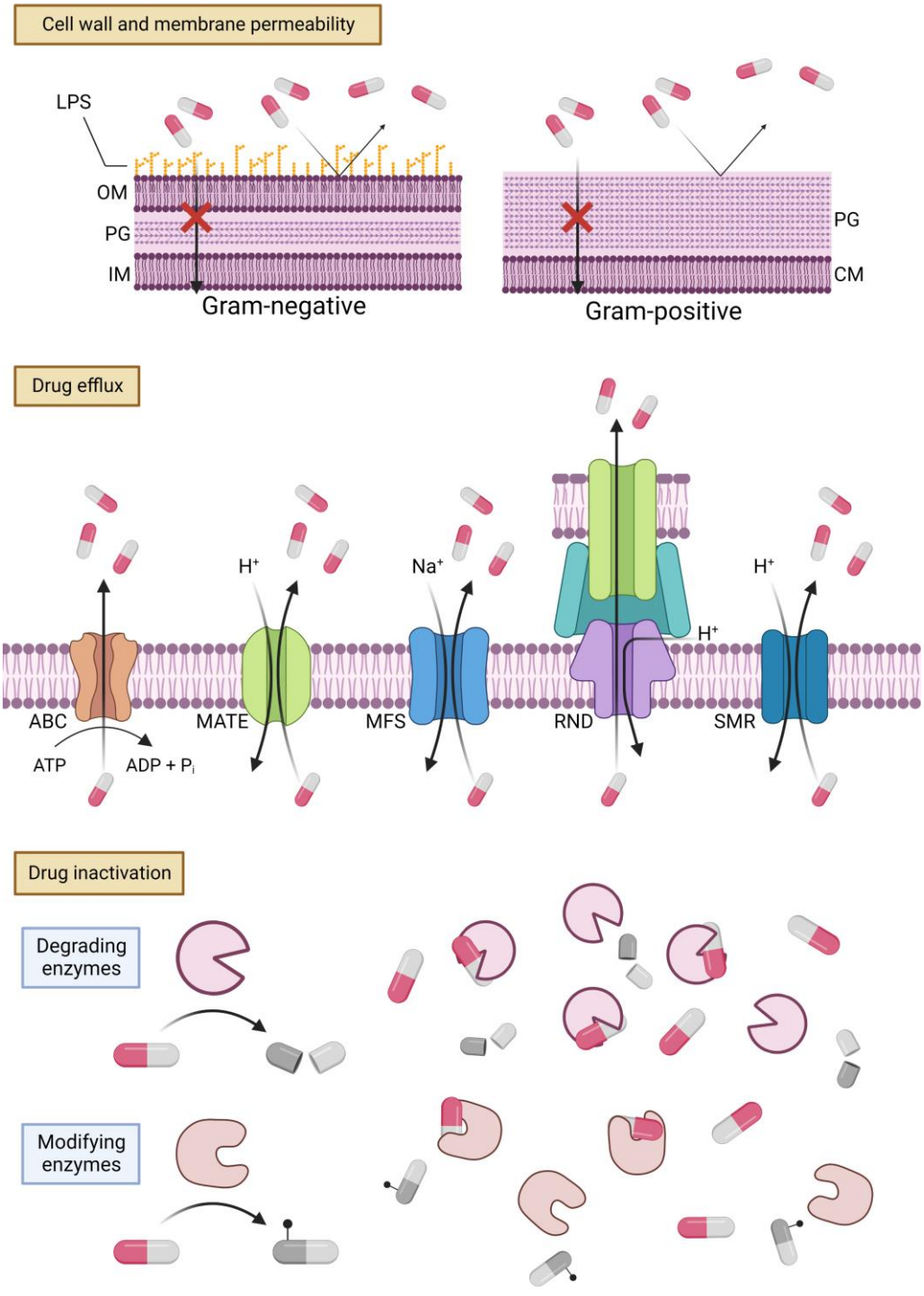
Cell wall and membrane impermeability

The first obstacle that all antibiotics encounter is how to get past the bacterial cell wall and cytoplasmic membrane. Therefore, the permeability of these barriers plays a crucial role in the cell's ability to survive against antibiotics. In addition, the chemical properties of the antibiotic will affect its ability to penetrate the membrane. Hydrophobic antibiotics can penetrate in a lipid-mediated manner while hydrophilic antibiotics pass through channels in the membrane to enter the cell. The composition of the cell wall differs between different bacterial species. The cell wall of Gram-positive bacteria consists of the cytoplasmic membrane and a thick layer of peptidoglycan while that of Gram-negative bacteria consists of the cytoplasmic membrane, a thin layer of peptidoglycan and an outer membrane (Figure 7). This cell wall structure of both Gram-positive and negative bacteria can confer intrinsic resistance to different antibiotics, but Gram-negative bacteria are generally more resistant to various antibiotics because of their outer membrane. The outer membrane is different from that of the cytoplasmic membrane in that it contains lipopolysaccharides (LPS) which are complex molecules that consists of a lipid

and a polysaccharide part. The presence of these molecules makes the outer membrane much less permeable to hydrophobic molecules, compared to the cytoplasmic membrane (Vaara et al., 1990). In contrast, the lack of an outer membrane in Gram-positive bacteria means that limiting drug intake is not as prevalent as the thick peptidoglycan layer does not affect the permeability of the cell wall. While the cell wall shields the bacterium from the environment, it can also prevent nutrients and other important molecules from entering the cell. To allow the uptake of these molecules, bacteria produce transmembrane channel proteins called porins throughout their membranes. These channels can also allow hydrophilic antibiotics through and reducing the number of porins in the outer membrane has been shown to lead to increased resistance to the carbapenems imipenem and meropenem in *E. coli* and *E. cloacae* by reducing the uptake of the antibiotic (Cornaglia et al., 1996).

Drug efflux

A major contributor to the problem of multidrug resistance is the action of efflux pumps. Efflux pumps are transmembrane protein channels that serve as a way for the cell to get rid of toxic substances and metabolites by transporting them from the cytoplasm to the environment (Figure 7). Over time, these systems have evolved to allow them to also transport antibiotics and they can confer resistance to antibiotics by decreasing the concentration of a given antibiotic in the cytoplasm by transporting it out of the cell. This will prevent the antibiotic from reaching the necessary concentration it needs to exert its antibacterial effect. In bacteria, there are five clinically relevant families of efflux pumps: the ATP-binding cassette (ABC), multidrug and toxic compound extrusion (MATE), major facilitator superfamily (MFS), resistance-nodulation-division (RND), and small multidrug resistance (SMR) families. Among these, the RND family is exclusive to Gram-negative bacteria while the ABC, MATE, MFS and SMR families exist in both Gram-negatives and positives. These families differ in structure, energy source and substrate specificity. For example, efflux pumps of the RND family make up a tripartite complex that spans across the inner membrane, periplasm, and outer membrane while the other families are only distributed in the inner membrane. The ABC family utilizes ATP hydrolysis to transport molecules from across


Cell wall and membrane permeability
LPS
OM
PG
IM
Gram-negative
PG
CM
Gram-positive
Drug efflux
ABC
ATP
ADP + Pi
MATE
H+
MFS
Na+
RND
H+
SMR
H+
Drug inactivation
Degrading enzymes
Modifying enzymes

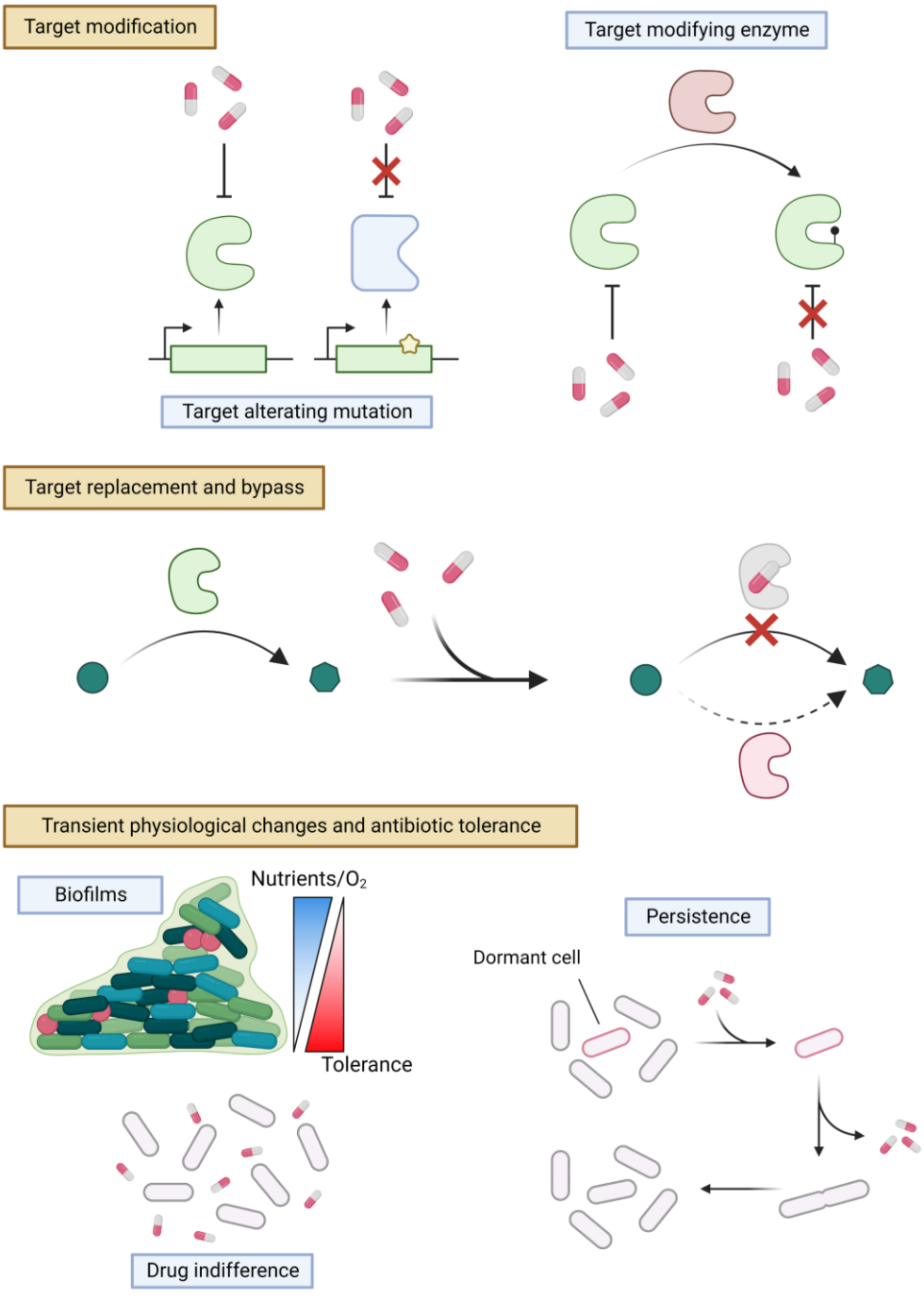


Figure 7. Mechanisms of antibiotic resistance. An overview of the mechanisms deployed by bacteria to achieve antibiotic resistance. See text for more details. Figure created with BioRender.com.

the membrane and the remainder rely on the proton motive force as an energy source. All five of the efflux pump families contribute to antibiotic resistance but members of the RND and MFS families, such as AcrAB-TolC and NorA respectively, are the most clinically relevant (Piddock, 2006).

Although drug efflux usually leads to a lower level of resistance compared to other resistance mechanisms, the resistance conferred by efflux pumps is generally broader since some efflux pumps are able to extrude a wide variety of antibiotics leading to multidrug resistance (Piddock, 2006). Resistance conferred by drug efflux can occur at an intrinsic, acquired, or phenotypic level. At an intrinsic level, efflux pumps can be expressed constitutively, such as the MexAB-OprM and AcrAB-TolC systems in *Pseudomonas aeruginosa* and *E. coli* respectively that confers resistance to various antibiotics (Nikaido & Zgurskaya, 2001; Poole et al., 1993). Acquired resistance can occur through HGT of genes encoding efflux pumps that are commonly encoded on plasmids. Notably, these efflux pumps typically have a narrower range than those encoded on the chromosome. Examples of this are the Tet, Qac and OqxAB gene families of efflux pumps, encoding resistance to tetracyclines, antiseptics and quinolones respectively, which are found in both Gram-negative and Gram-positive bacteria (Hernando-Amado et al., 2016). Resistance can also be acquired by chromosomal mutations in regulatory elements that lead to an increased expression of naturally occurring efflux pumps, such as mutations in *mvaT* in *P. aeruginosa* which leads to an increase in the expression of the MexEF-OprN efflux system (Westfall et al., 2006). Finally, the expression of some efflux systems is induced by the presence of antibiotics, leading to phenotypic resistance. An example of this is the TtgABC efflux pump in *Pseudomonas putida*, the expression of which is induced by chloramphenicol and tetracycline through the regulator TtgR (Terán et al., 2003).

Drug inactivation

A common way of achieving resistance to an antibiotic is through the production of drug-inactivating enzymes. This inactivation can occur either through the direct degradation of the antibiotic or by chemically modifying the antibiotic, rendering it unable to bind to its target (Figure 7). One of the most common types of resistance conferred by drug degradation is β-lactam resistance. This is mediated by enzymes called β-lactamases that can bind to and destroy the β-lactam ring of β-lactam antibiotics, rendering them inactive. β-lactamases have been studied extensively due to their prevalence in antibiotic resistant infections. The first enzyme able to degrade penicillin, then referred to as a penicillinase, was isolated already in 1940, even before the clinical use of penicillin had started (Abraham & Chain, 1940). Since then, the clinical prevalence and the number of β-lactamases has skyrocketed. Today, β-lactamases are a major clinical problem with the emergence of so-called extended-spectrum β-lactamases (ESBL). ESBLs are β-lactamases that have evolved to be able to degrade even the latest generation of β-lactam antibiotics, including fifth generation cephalosporins and carbapenems. β-lactamases are divided into four major classes based on their sequence similarity: class A, B, C, and D (Ambler, 1980). These are also broadly divided into two larger groups based on their active site, and thus mechanism of action: Class A, C and D are serine β-lactamases (SBL), with a serine residue in their active site, while members of class B are metallo-β-lactamases (MBL), with a zinc ion in their active site. Although all these classes of β-lactamases are widely distributed among bacterial species, there are a few enzyme families that are widely disseminated among important bacterial pathogens, including *E. coli* and the ESKAPE pathogens. Some of the clinically most important β-lactamase families of each class are TEM, SHV, CTX-M and KPC (class A), NDM (class B), CMY (class C) and OXA (class D) (Tooke et al., 2019). A major driving force for the success of these enzymes is their dissemination on conjugative plasmids that spread through horizontal gene transfer (see later section). Due to the prevalence of β-lactamases in clinical infections, resources have been put into finding and developing β-lactamase inhibitors. β-lactamase inhibitors, such as clavulanic acid, are commonly used in combination with β-lactam

antibiotics and work by reversibly or irreversibly binding close to the active site of β-lactamases to inhibit their hydrolytic function (Bush, 1988).

Drug inactivation can also occur through chemical modification (Figure 7); most commonly by acetylation, phosphorylation, or adenylation of the antibiotic. These reactions are catalyzed by acetyl-, phospho- and nucleotidyltransferases respectively. Chemical modification is a common mechanism of resistance for many antibiotics such as chloramphenicol and rifampin, conferred by the chloramphenicol acetyltransferase (CAT) and rifampin phosphotransferase (RPH) enzymes respectively (Schwarz et al., 2004; Spanogiannopoulos et al., 2014). Further, the aminoglycoside class of antibiotics are particularly susceptible to chemical modification due to their chemical structure. Aminoglycoside modifying enzymes (AMEs) are split into three major groups, based on their function: aminoglycoside acetyltransferase (AAC), aminoglycoside phosphotransferase (APH) and aminoglycoside nucleotidyltransferase (ANT). The modifications catalyzed by these enzymes prevent the aminoglycoside from effectively binding to the ribosome (Llano-Sotelo et al., 2002). These three functional groups can be divided into several subgroups based on which site on the aminoglycoside they modify, and the subgroups specify the resistance phenotype. These groups all include enzymes capable of modifying clinically relevant aminoglycoside antibiotics and some of them are commonly found on plasmids and other mobile genetic elements such as integrons and transposons (Ramirez & Tolmasky, 2010). While most antibiotic modifying enzymes are substrate specific, a variant of the aminoglycoside acetyltransferase AAC(6´)-Ib has been shown to also reduce the activity of ciprofloxacin, designated AAC(6´)-Ib-cr (Robicsek et al., 2006). In addition, aminoglycoside modifying enzymes can be bifunctional. One such enzyme is AAC(6´)-APH(2´´) which exhibit both AAC and APH activity against virtually all aminoglycosides (Ferretti et al., 1986). These bifunctional enzymes are of particular clinical importance as they can confer resistance to a wide range of aminoglycoside antibiotics.

Target modification

The chemical structure of an antibiotic is important for its ability to bind to and inhibit its target. In a similar manner, the structure of the target structure is just as important. This means that mutational changes or modifications that alter the structure of an antibiotic target, without impeding its function, can change its susceptibility to the antibiotic (Figure 7). Examples of resistance conferred in this way is that of rifamycin and quinolone resistance where point mutations can confer partial or full resistance (Wehrli, 1983). Rifamycin resistance occurs through mutations in *rpoB*, the gene encoding the β subunit of RNA polymerase, resulting in amino acid alterations that prevent rifamycin antibiotics from binding (Campbell et al., 2001). Quinolone resistance typically occurs through point mutations in the genes encoding the subunits of topoisomerase IV and DNA gyrase, *parC* and *parE*, and *gyrA* and *gyrB* respectively. Of these, point mutations in *parC* and *gyrA* are the most prevalent mutations conferring resistance. These mutations most commonly occur in a region of the two genes close to the active site, called the quinolone resistance-determining region (QRDR) (Yoshida et al., 1990). These mutations will change the structure of the enzyme in a way that prevents the binding of quinolone antibiotics, such as ciprofloxacin. In addition to these point mutations, quinolone resistance can be conferred by the *qnr* family of genes, typically found on plasmids (Vetting et al., 2011). These genes encode proteins that can bind to topoisomerase IV and DNA gyrase to protect it from the inhibitory action of quinolone antibiotics. It has been suggested that these proteins interact with topoisomerase-quinolone complex and promote the release of the quinolone, allowing the topoisomerase to continue its function (Vetting et al., 2011).

The target structure can also be chemically modified to change its susceptibility to the antibiotic. One such target structure is the ribosome in which the modification of the ribosomal RNAs (rRNA) that make up its subunits can be modified. This type of resistance has been found to be clinically relevant as it confers resistance to many antibiotics in clinical use. The most common type of modification is methylation catalyzed by methyltransferases. Two important methyltransferases are the erythromycin

ribosome methylase (*erm*) family and chloramphenicol-florfenicol resistance (*cfr*) methyltransferase. The *erm* family of methylases methylate the 16S rRNA and alter the binding site of antibiotics such as macrolides (Leclercq, 2002). Similarly, the *cfr* methyltransferase methylates 23S rRNA which will prevent the binding of for example amphenicols and oxazolidinones such as linezolid (K. S. Long et al., 2006). As with most successful resistance genes, the genes encoding these methyltransferases can be found on plasmids that have allowed them to spread between species (Zhang et al., 2014). Methylation of the target has also been shown to be a relevant mechanism of resistance for aminoglycosides. Recently, methyltransferases capable of conferring aminoglycoside resistance by modifying the ribosome, encoding the *armA* and *rmt* genes, were found in isolates of Enterobacteriaceae across the world (Fritsche et al., 2008).

Target replacement and bypass

In addition to altering the native target of an antibiotic, bacteria can also, through HGT, acquire alternative versions of the protein that can perform the same function but is less sensitive to the antibiotic. This will allow the cell to bypass various enzymatic steps in processes that are blocked by antibiotics (Figure 7). One of the best examples of this type of resistance is trimethoprim resistance. As previously described, trimethoprim inhibits dihydrofolate reductase (DHFR) in the last step of tetrahydrofolic acid synthesis to stop bacterial growth. Resistance to trimethoprim commonly occurs through the acquisition of an alternative version of DHFR, or an enzyme that can perform the same reaction, but has a lower affinity to trimethoprim. This will mean that trimethoprim will not be able to fully inhibit the production of THFA, which will allow the cell to grow even in the presence of trimethoprim. Resistance to trimethoprim can also occur through mutations that lead to an increase in the production of DHFR where the higher levels of DHFR compensate for the inhibition by trimethoprim (Flensburg & Sköld, 1987). Other examples are that of β-lactam resistance in methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistance in vancomycin-resistant Enterococci (VRE) conferred by the transferable *mecA* gene and *vanA* gene cluster respectively (Munita & Arias, 2016). The *mecA* gene encodes a PBP that has a lower

affinity to β-lactams (Ubukata et al., 1989), making it resistant to the action of the β-lactams, while the *van* gene cluster encodes a biochemical machinery that modifies the D-Ala-D-Ala moiety in the peptidoglycan layer, to which vancomycin binds, into D-Ala-D-lactate (Miller et al., 2014). In both cases, these changes result in the antibiotic being unable to bind, leading to resistance.

Transient physiological changes and antibiotic tolerance

Antibiotic tolerance differs from antibiotic resistance in that antibiotic tolerance is typically transient and is not inherited by daughter cells while antibiotic resistance requires the acquisition of new genetic information. Antibiotic tolerance is usually achieved through changes to the cell's physiology, either before or in response to encountering antibiotics. These changes are reversible, and the cell becomes susceptible to the antibiotics again after returning to a more normal physiological state.

Biofilms

Biofilms are multicellular structures that bacteria form when growing on surfaces, held together by a matrix of polysaccharides and proteins. This matrix can impede the diffusion of antibiotics throughout the biofilm and thus protect the cells within it (Kumon et al., 1994). In addition, bacteria that have acquired ways to degrade the antibiotic can also protect other bacteria in the biofilm by degrading the diffusing antibiotic. While growing in the biofilm, cells are exposed to a gradient of nutrients and oxygen depending on their depth in the biofilm. Cells growing close to the top of the biofilm has access to more nutrients and oxygen while the cells at the bottom, close to the surface on which the biofilm is growing, has access to very little (Figure 7). This lack of nutrients and oxygen can cause the cells at the bottom to enter a non-dividing state, making them more tolerant to antibiotics such as β-lactams (Corona & Martinez, 2013).

Drug indifference

Many antibiotics, like the β-lactams, only exert an effect on actively growing cells (Lee et al., 1944). This means that the cell's growth rate and stage are important for the function of the antibiotic and that changes to these can lead

to tolerance to certain antibiotics (Figure 7). One example of this is that of stationary-phase cells that are more tolerant to certain antibiotics than exponentially growing cells (Levin & Rozen, 2006). It has also been shown that the concentration of penicillin needed to kill off an infection increases the longer the infection lasts (Eagle, 1952). The reason for this is that as the infection progresses, the growth rate of the bacteria will decrease and eventually stop, likely due to the depletion of nutrients, leading to an increase in tolerance to the antibiotic. This kind of dormancy or growth retardation that leads to antibiotic tolerance is commonly referred to as "drug indifference" (McDermott, 1958).

Persistence

During an antibiotic treatment, most of the cells of a bacterial population susceptible to the antibiotic are killed. However, many bacterial species produce subpopulations that are not killed by antibiotic treatment. These cells are called persister cells, as they can persist through the antibiotic treatment without being genetically resistant. These persister cells are cells that have entered a dormant, non-dividing state (Van den Bergh et al., 2017). In this state, various processes of the cell's metabolism are shut down. This protects the cells from the action of many antibiotics that would target these processes. Once the antibiotic treatment is over, the persister cells can leave their dormant state and grow to form a new healthy, albeit antibiotic susceptible, bacterial population again (Figure 7). It is unclear whether, and if so how, this dormant state differs from other non-dividing states like the ones described above. Persister cells can also be found in biofilms and have been suggested to be one of the reasons why biofilms are so difficult to treat (Spoering & Lewis, 2001).

RESISTANCE AND MOBILE GENETIC ELEMENTS

As has been alluded to in earlier sections, antibiotic resistance and mobile genetic elements (MGEs) go hand in hand. This association has been largely driven by the clinical use of antibiotics, as MGEs have existed long before the introduction of antibiotics. There are three major types of MGEs: integrons, transposable elements, and plasmids. These are all genetic elements that are

able to move genes throughout the bacterial genome. In addition, MGEs can be exchanged horizontally between bacteria through various mechanisms, as will be discussed in a later section.

Integrons

Integrons are highly dynamic recombination based genetic systems that contribute to bacterial genetic diversity. They do this by integrating and expressing genes located on small mobile elements called gene cassettes (Figure 8). Gene cassettes are circular non-replicative elements that typically consist of one or two promoterless open reading frames, a ribosome binding site, and a recombination site important for integron integration called *attC*. The integron itself carries the machinery necessary for cassette integration: a gene encoding the integrase protein that is responsible for catalyzing the recombination reaction, a recombination site called *attI* and a promoter (P_C). During integration, the integrase protein recognizes the *attI* site on the integron and the *attC* site in the gene cassette. It then integrates the gene cassette into the integron through site-specific recombination (Domingues et al., 2012). Once integrated, the gene(s) in the gene cassette are expressed from the P_C promoter.

It has been estimated that more than 15% of genome-sequenced bacteria have at least one integron in their genome (Cambray et al., 2010) and they are commonly found in environmental isolates isolated in soils and water (Gillings, 2014). Integrons are also of clinical relevance due to their strong association with genes conferring antibiotic resistance. One integron can carry several gene cassettes, in some cases even in the hundreds (Rowe-Magnus et al., 2003), which in turn can contain one or two genes. This creates a huge potential for accumulating resistance genes and many clinically relevant integrons confer multidrug resistance. The integrons carrying antibiotic resistance genes, such as In53, are usually mobile, i.e., associated with transposons and/or plasmids, allowing them to be disseminated to other bacteria and bacterial species through HGT (Naas et al., 2001).

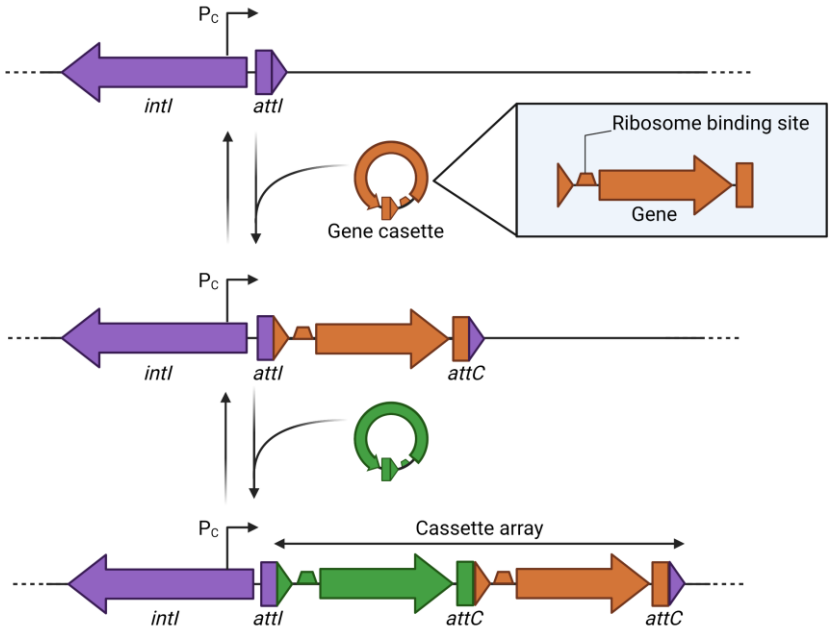


Figure 8. Integrons. Integrons consist of a gene encoding an integrase protein, a recombination site called *attI* and a promoter (P_C). They are able to capture and express gene cassettes. Gene cassettes typically consist of one or two promoterless genes, a ribosome binding site and a recombination site called *attC*. During integration, the integrase protein recognizes the *attI* and *attC* sites and integrates the gene cassette into the integron through site-specific recombination. Once integrated, the gene(s) of the gene cassette are expressed from the P_C promoter. Figure created with BioRender.com.

Transposable elements

Transposable elements (TE), or "jumping genes", are genetic elements able to move, or "transpose", between various genetic locations. This transposition is mediated by an enzyme called transposase, typically encoded on the TE. The transposase recognizes specific inverted repeat (IR) sequences that are on the flanking ends of the TE. Once bound to the IR sequences, the transposase catalyzes the transposition of the TE. This can be done in a non-replicative or replicative manner. Non-replicative transposition uses a 'cut-and-paste' mechanism, where the TE is excised and inserted in another location. Replicative transposition occurs using a 'copy-and-paste' mechanism where the TE is replicated before being transposed, leaving a copy of it in its original

site (Figure 9) (Hallet & Sherratt, 1997). The insertion of TEs into new genetic locations does not require sequence homology and can thus occur randomly. This random insertion can be destructive for the cell as the TE can insert into and disrupt important genes. Due to this, transposition of TEs is a very regulated process that only occurs rarely (Nagy & Chandler, 2004).

The first and simplest type of TEs is called insertion sequence (IS) elements. These typically only consist of a gene encoding a transposase protein and flanking IR sequences. The second and larger type of TEs is transposons. The two main types of transposons are composite and non-composite, or unit, transposons (Figure 9). Composite transposons are composed of two IS elements that are flanking various accessory genes. When these IS elements are transposed, the accessory genes will also be moved together with them (Partridge et al., 2018). These accessory genes can encode a wide variety of functions, including antibiotic and heavy metal resistance. A composite transposon can be created *de novo* by the random insertion of two IS elements in close proximity of each other. In contrast, unit transposons consist of a gene encoding transposase and accessory genes flanked by IR sequences.

Transposons are clinically important due to their wide association with genes encoding antibiotic resistance. Both composite and unit transposons can carry integrons as part of their accessory genes, making them efficient vectors of multidrug resistance. In addition, the transposition of IS elements and transposons are not limited to the chromosome. They can also be transposed onto plasmids where they can be further mobilized through HGT, as is the case for Tn*21* which is located on the conjugative plasmid NR1 (R100) (Womble & Rownd, 1988). Some of the most clinically important transposons include the Tn*3*, Tn5 and Tn*10* transposons, carrying genes encoding β-lactam, aminoglycoside, and tetracycline resistance respectively, found in Gram-negative bacteria (Partridge et al., 2018).

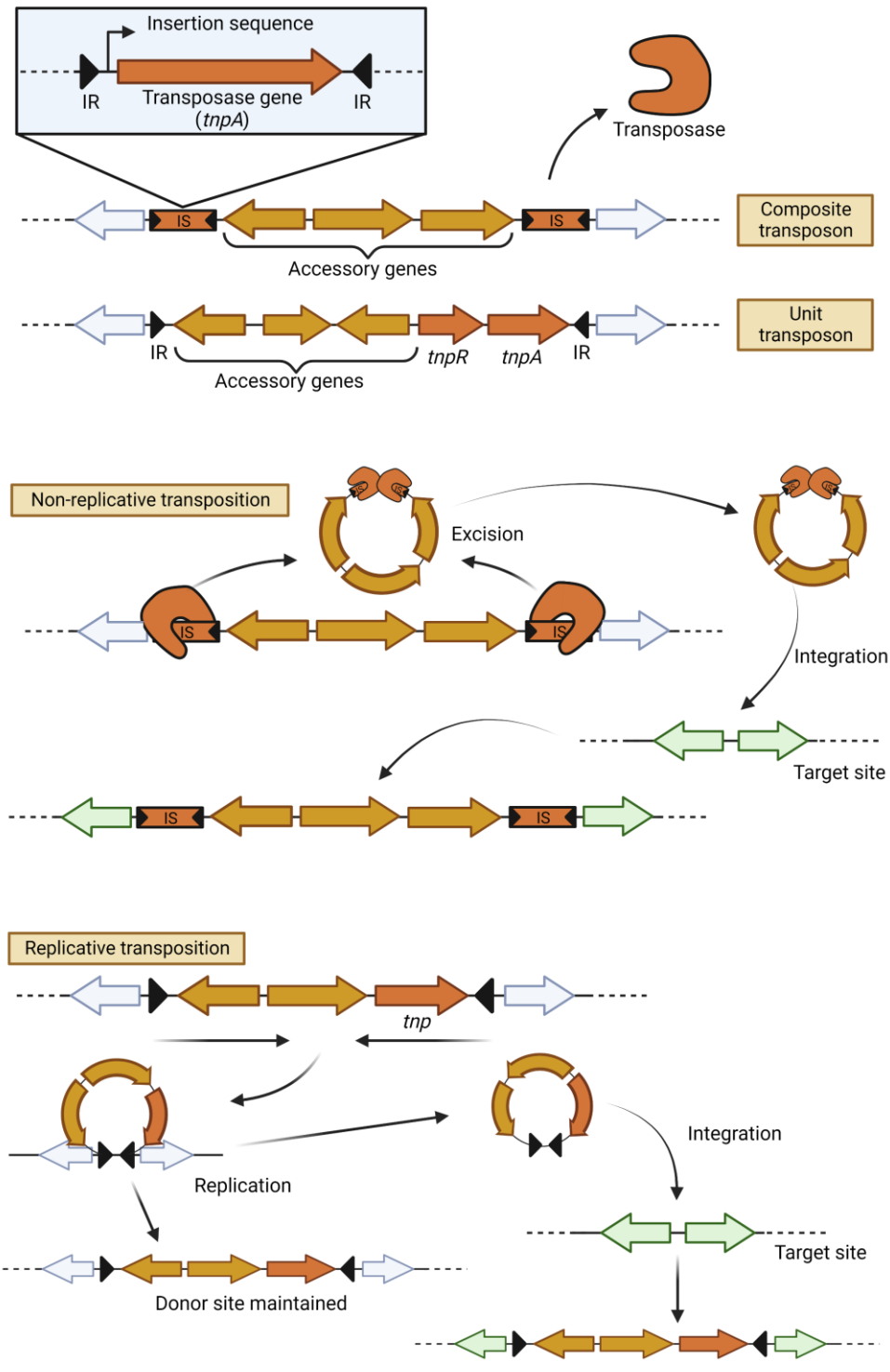

Insertion sequence
IR
Transposase gene
(tnpA)
IR
Transposase
IS
IS
Accessory genes
Composite transposon
IR
Accessory genes
tnpR
tnpA
IR
Unit transposon
Non-replicative transposition
Excision
IS
IS
Integration
Target site
IS
IS
Replicative transposition
tnp
Replication
Integration
Target site
Donor site maintained

Figure 9. Transposable elements and mechanisms of transposition. The simplest kind of transposable element (TE) is an insertion sequence (IS) element. These consists of a gene encoding a transposase protein flanked by inverted repeat (IR) sequences. More advanced TEs are composite and unit transposons. These carry accessory genes flanked by two IS sequences and IR sequences for composite and unit transposons respectively. TEs can be transposed through two main mechanisms. Non-replicative transposition involves a 'cut-and-paste' mechanism where the TE is excised and integrated into another site by the transposase. In contrast, replicative transposition employs a 'copy-and-paste' mechanism where the TE is first replicated and then moved to another site, leaving the original TE intact. Figure created with BioRender.com.

Plasmids

Plasmids are circular, extrachromosomal genetic elements that are replicated independently of the chromosome. Generally, they carry genes involved in their own replication, stability and partitioning as well as accessory genes. The functions of the proteins encoded by these accessory genes vary but are commonly involved in resistance to antibiotics, heavy metals, and other biocides. In addition, these accessory genes can include integrons and transposons that in turn can carry a plethora of resistance genes, making plasmids efficient vehicles of multidrug resistance. Plasmids can be either transmissible or non-transmissible based on their ability to be transferred between bacteria. Transmissible plasmids are further divided into conjugative or mobilizable plasmids. Conjugative plasmids carry genes encoding the machinery necessary for transfer through conjugation and are thus self-transmissible, while mobilizable plasmids do not carry the necessary machinery but are still able to be transferred using a transfer system expressed exogenously. It is estimated that roughly half of all plasmids in bacteria are non-transmissible while conjugative and mobilizable plasmids make up a quarter each (Smillie et al., 2010).

Conjugative and mobilizable plasmids are major players in the spread of antibiotic resistance due to both their association with resistance genes, typically through the presence of integrons and transposons, and their ability to be transferred between bacteria through HGT, especially conjugation (see later section for more details). However, this was not always the case. Analyses

of plasmids from the "pre-antibiotic era", i.e., before the medical use of antibiotics, have shown that conjugative plasmids were common but lacked antibiotic resistance genes (Hughes & Datta, 1983).

Conjugative plasmids are usually grouped into so-called incompatibility (Inc) groups. This grouping is based on the inability of two plasmids using the same replication system to be maintained within the same cell, i.e., they are incompatible with each other. Members of different Inc groups can differ in host range. For example, members of the IncF family of plasmids have a very narrow host range, limited almost exclusively to *Enterobacteriaceae*, while members of the IncP family are broad host range plasmids that can transfer between most bacterial species and some yeast species.

In *Enterobacteriaceae*, genes encoding for aminoglycoside, β-lactam (including ESBLs), macrolide, and quinolone resistance are commonly found on conjugative plasmids, including members of the IncA/C, F, L/M, I1, HI2 and N families (Carattoli, 2009; Rozwandowicz et al., 2018). In particular, the CTX-M family of β-lactamases is widely found on transmissible plasmids. In fact, studies have shown that CTX-M originates from the *Kluyvera* species, found in the human gut, where it was mobilized from the chromosome onto a plasmid which has since spread to various human pathogens (Cantón et al., 2012).

SELECTION OF RESISTANCE AT SUB-INHIBITORY CONCENTRATIONS OF ANTIBIOTICS

The antibacterial activity of antibiotics is not an 'all or nothing' effect. Rather, antibiotics exert their activity over a wide range of concentrations, ranging from the minimum inhibitory concentration (MIC), where bacterial growth is completely inhibited, to extremely low levels where they are speculated to be involved in cell-to-cell communication (Fajardo & Martínez, 2008). An area of research that has garnered increased attention in recent years is that of bacterial growth and selection in the presence of sub-inhibitory levels of antibiotics. These levels more closely reflect the levels that bacteria are

exposed to in the environment, which are typically much lower than those used in clinical research and treatment. Exposure to these sub-inhibitory levels of antibiotics can impair bacterial growth but does not fully inhibit it. In addition, it has other effects on the cell, such as changes to its morphology and virulence (Zhanel et al., 1992). Perhaps most important, these low levels of antibiotics can select for resistance.

Most of the resistance mechanisms discussed earlier in this thesis, confer some sort of fitness cost (Andersson & Hughes, 2010; Melnyk et al., 2015). This fitness cost is typically realized through a reduced functional capacity of a mutated drug target, or the resource cost inferred by the production of resistance factors. In the absence of antibiotics, this fitness cost will allow susceptible bacteria to outcompete those that carry the resistance. In the presence of antibiotics, even at very low levels, the fitness cost can be compensated by the reduced effect that the antibiotic has on growth, conferred by the presence of resistance factors, compared to that of susceptible bacteria. This difference in growth rates leads to a selective pressure in favor of the resistant bacteria, which increases as the concentration approaches the MIC. This has given rise to the term minimum selective concentration (MSC), which is defined as the lowest concentration at which the benefit of resistance outweighs the fitness cost, i.e., the lowest concentration at which selection of resistance occurs (Figure 10). The MSC can be as low as 200-fold lower than the MIC of susceptible bacteria (Gullberg et al., 2011). Antibiotic resistance can also be selected for by the exposure to other biocides, such as heavy metals, through co-selection which is discussed in the next section. This co-selection can also occur at sub-inhibitory concentrations (Gullberg et al., 2014). In addition, it has been suggested that low levels of biocides and some antibiotics can stimulate the spread of resistance through conjugation (Jutkina et al., 2018). Thus, growth at sub-inhibitory levels of antibiotics and other biocides is an important aspect that needs to be considered in research on antibiotic resistance.

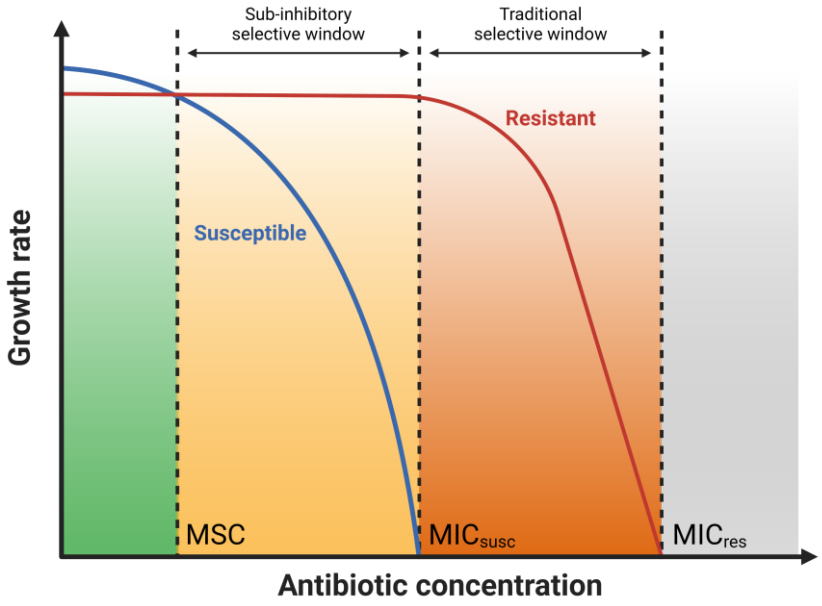


Figure 10. Comparison of the minimal selective concentration and minimal inhibitory concentration. Most antibiotic resistance mechanisms confer a fitness cost. In the absence of antibiotics (green area), bacteria carrying antibiotic resistance factors (red curve) will have a lower growth rate than that of susceptible bacteria (blue curve). However, in the presence of antibiotics the resistant bacteria will be able outcompete the susceptible ones. The lowest concentration at which this occurs is called the minimum selective concentration (MSC). The MSC can be many times lower than the minimum inhibitory concentration of the susceptible bacteria (MIC_{susc}). Above the MIC_{susc}, only the resistant bacteria will grow until the concentration reaches the MIC of the resistant bacteria (MIC_{res}). Adapted from (Gullberg et al., 2011). Figure created with BioRender.com.

HEAVY METALS

Heavy metals are a group of chemical elements characterized by their high atomic densities. They occur naturally as part of the Earth's crust and can contaminate soils and water through various natural processes. The presence of these metals in the environment has driven the development of heavy metal resistance among environmental bacteria. Additionally, human activities such as mining, industry, and the use of heavy metal-containing compounds in agriculture have exacerbated the problem.

The impact of heavy metals on biological systems ranges from essential to highly toxic and they can be divided into two groups: essential and non-essential. Essential heavy metals, such as copper (Cu), manganese (Mn), and zinc (Zn), play an important role as co-factors for various enzymes needed for cell growth and metabolism while most non-essential heavy metals, such as arsenic (As), cadmium (Cd), and mercury (Hg), do not have an established biological function and are mostly associated with toxicity (Lemire et al., 2013). However, both essential and non-essential heavy metals can be toxic to most forms of life at excessive concentrations. The toxic properties of heavy metals have been used against microorganisms for millennia (Borkow & Gabbay, 2009). However, since the toxicity for humans is also high, the use of heavy metals as treatment has been phased out by alternative treatments, such as antibiotics. In clinical settings, they are instead used in other ways. For example, copper has been used to create antibacterial surfaces used in hospitals to prevent the spread of bacteria among its patients (Grass et al., 2011).

MECHANISMS OF HEAVY METAL TOXICITY

The exact mechanism of toxicity of heavy metals will differ depending on the chemistry of the heavy metal. However, most heavy metal-induced toxicity is through one or more of the following mechanisms. First, a major mechanism is the disruption of protein function. This can occur through the disruption of important functional groups on the protein. For example, heavy metals such as Cu can disrupt the iron-sulfur clusters in the active site of metalloproteins

(Macomber & Imlay, 2009). Other metals can compete with and displace structural and catalytic ions or cause the oxidation of sulfhydryl groups in cysteine residues that are important for protein function (Lemire et al., 2013). A second general mechanism of heavy metal toxicity is the production of reactive oxygen species (ROS) as a result of redox reactions within the cell. ROS can cause damage to both DNA and proteins leading to growth inhibition and cell death. Lastly, the cytoplasmic membrane or membrane-associated proteins and complexes have been suggested to be a major target for heavy metal toxicity. For example, silver (Ag) has been shown to exert its toxic effect by interfering with components of the electron transport chain (Gordon et al., 2010). In addition, peroxidation of lipids in the cytoplasmic membrane has been linked to Cu toxicity in copper-alloy surfaces (Hong et al., 2012).

HEAVY METAL RESISTANCE

Much like antibiotics and antibiotic resistance, bacteria have developed resistance mechanisms to heavy metal-induced toxicity. The mechanisms deployed against heavy metal toxicity are similar to that of resistance mechanisms against antibiotics, including reducing uptake, efflux, and pathway bypass by alternative enzymes (Lemire et al., 2010, 2013). Out of these, the most common mechanism of resistance is efflux. Many metal ions, such as Cd^{2+} and Zn^{2+}, are simply transported out of the cell through efflux pumps like the Czc efflux system (Silver, 1996), while in some cases it is more complicated. Two such cases are that of arsenic and copper. Arsenic can occur either in its trivalent form, arsenite, or its pentavalent form, arsenate. In *E. coli*, arsenic resistance is mediated through the *arsRDABC* operon. This operon includes the ArsA and ArsB proteins which form an inner membrane efflux pump that is able to bind and pump out arsenite, but not arsenate. In order for the cell to effectively remove intracellular arsenate, it first needs to be reduced to arsenite so that ArsA and ArsB can transport it out. This is done by a third member of the operon, ArsC. The remaining members of the operon are ArsD which is an arsenite chaperone that binds to and delivers arsenite to the ArsA protein, and ArsR which regulates the expression of the operon (Y.-F. Lin et al., 2006).

Copper is an essential metal that is needed for the function of many enzymes. However, excessive amounts of intracellular copper are toxic to the cell. Maintaining a proper copper homeostasis is therefore essential for the cell's survival. In biological systems, copper exists in its monovalent or divalent forms, Cu(I) and Cu(II) respectively. However, once imported into the cell, Cu(II) is reduced to Cu(I) (Rapisarda et al., 1999). The efflux of Cu(I) occurs in two steps. First, the inner membrane transporter CopA transports Cu(I) to the periplasmic space. Once in the periplasm, the ions can be oxidized from Cu(I) to the less toxic Cu(II) by the CueO protein (Grass & Rensing, 2001), or transported out into the extracellular environment by the CusCBA efflux system (Munson et al., 2000).

Finally, many bacteria can also produce and secrete extracellular polymers or siderophores capable of interacting with metal ions. These molecules can bind and sequester the ions, which will prevent them from accumulating in the cell. In addition, siderophores that are bound to metal ions can also be transported out of the cell by efflux systems (Hannauer et al., 2012).

CO-SELECTION OF ANTIBIOTIC AND HEAVY METAL RESISTANCE

Antibiotic and heavy metal resistance are both believed to be ancient (D'Costa et al., 2011; Jackson & Dugas, 2003). Today, the two are commonly found to be associated with each other, and the exposure to antibiotics or heavy metals can select for resistance to them both through co-selection. This has allowed for the selection and maintenance of antibiotic resistance genes in the environment, even in the absence of antibiotics, and has led to an increased co-occurrence of the two types of resistance genes. In fact, bacteria carrying biocide or metal resistance genes have been shown to also carry antibiotic resistance genes more often than those that do not (Pal et al., 2015). It has been suggested that the co-occurrence of antibiotic and heavy metal resistance genes on MGEs emerged relatively recently as a result of antibiotic use in humans and animals (Mindlin et al., 2005). For example, *Pseudomonas* derived from permafrost contained transposons carrying mercury resistance genes that are

closely related to transposons found in present-day bacteria. Today, however, those transposons are also associated with antibiotic resistance genes (Mindlin et al., 2005).

There are two main mechanisms of co-selection: co-resistance and cross-resistance. First, co-resistance is when two or more genes conferring resistance to antimicrobial compounds are physically linked, for example if they are located on the same plasmid or other mobile genetic elements (Baker-Austin et al., 2006). This genetic linkage means that the genes are inherited together during cell division and their presence on plasmids also allows the genes to be easily transferred between bacteria through HGT. Plasmids carrying both antibiotic and heavy metal resistance genes have been isolated in many clinically relevant bacterial species, including *E. coli*, *Klebsiella* and *Salmonella* (Fang et al., 2016; Sandegren et al., 2012; Campos et al., 2016) and co-selection through co-resistance has been shown occur even at sublethal levels of antibiotics or heavy metals (Gullberg et al., 2014).

Cross-resistance is when a single mechanism confers resistance to several compounds. This usually happens for compounds that share a mechanism of action or target structure (Baker-Austin et al., 2006). Any mutation or physiological changes that would prevent one compound from exerting its antibacterial effect would thus also prevent the other. In addition, efflux pumps, especially multidrug resistance pumps, are commonly found to confer resistance to many structurally dissimilar compounds, such as antibiotics and heavy metals. Finally, antibiotic and heavy metal resistance genes can be transcriptionally linked where the induction of a bacterial response to one of them also triggers a response to the other, either directly or indirectly (Baker-Austin et al., 2006). This means that treating bacteria with one set of compounds can confer resistance to the other, leading to cross-resistance. This co-regulation is commonly regulated by a single regulatory protein. An example of this is the CzcR protein that increases the expression of the CzcCBA efflux system in response to sublethal level of zinc while at the same time decreasing the expression of OprD, a membrane porin that facilitates the

diffusion of carbapenem antibiotics, leading to carbapenem resistance (Perron et al., 2004).

ESCHERICHIA COLI

Escherichia coli is a rod-shaped Gram-negative bacterium that belongs to the Enterobacteriaceae family. They exist as commensal bacteria in the normal intestinal microflora of humans and animals. They are also found in the environment where they are excreted from their hosts through defecation. While *E. coli* is an integral part of the gut microflora, it is also an important pathogen. Some subgroups, or pathotypes, of *E. coli* have acquired various virulence factors that have allowed them to cause disease in humans. Due to its niche in the human intestine, *E. coli* is commonly associated with enteric disease, in particular diarrheal disease. The pathotypes that cause this type of disease include enteropathogenic, enterotoxigenic, enteroaggregative, enteroinvasive, diffusely adherent, and enterohaemorrhagic *E. coli* (EPEC, ETEC, EAEC, EIEC, DAEC, and EHEC respectively) (Nataro & Kaper, 1998). However, *E. coli* can also cause disease outside of its niche in the intestine and the pathotypes that do this are collectively called extraintestinal pathogenic *E. coli* (ExPEC) (Russo & Johnson, 2000). In fact, one of the most common types of *E. coli* infection is that of urinary tract infections, typically caused by uropathogenic *E. coli* (UPEC). In addition, *E. coli* is also an important veterinary pathogen where many of the pathotypes involved in human disease also occur. Avian pathogenic *E. coli* (APEC) also cause extraintestinal infections in poultry, including respiratory infections and septicemia (Dziva & Stevens, 2008).

E. COLI AS A MODEL ORGANISM

Due to its prevalence in human disease, *E. coli* has been extensively studied for the better part of a century. Because of this, it has become a representative species of both the *Escherichia* genus and the Enterobacteriaceae family of Gram-negative bacteria in research about clinically relevant properties, such as virulence and antibiotic resistance. In addition, *E. coli* has been the workhorse species of bacterial genetics and has been involved in many discoveries that has laid the foundation for modern molecular microbiology. These discoveries include, but are not limited to, the organization of genes in

operons, bacterial conjugation, and that mutations are spontaneous rather than induced (Jacob et al., 2005; Lederberg & Tatum, 1946; Luria & Delbrück, 1943). The success of *E. coli* as a model organism can be partly attributed to the ease at which it can be manipulated genetically, for which many genetic tools have been developed. This has led to the development of resources such as the KEIO and ASKA collections of single-deletion mutants and overexpression plasmids respectively (Baba et al., 2006; Kitagawa et al., 2005), which have allowed researchers to investigate bacterial characteristics at a genome-wide scale.

GENOMICS OF *E. COLI*

The genome of *E. coli* was first sequenced in 1997 (Blattner et al., 1997), just two years after the genome sequence of *Haemophilus influenzae*, the first whole-genome sequenced organism, was finished (Fleischmann et al., 1995). Since then, advances in sequencing technology and sequence analysis have caused the number of available whole-genome sequences to skyrocket. This has led to the emergence of the concept of a pangenome of a species. In genomics, a pangenome is defined as the entire set of genes within a species. The pangenome is made up of the core and accessory genomes where the core genome includes gene families that are present in all isolates of a species while the accessory genome includes gene families that are not. Some gene families in the accessory genome can even be unique to individual strains. Some authors divide the accessory genome further into the "soft core", "shell" and "cloud" genomes corresponding to gene families that are present in more than 95%, between 10 and 95%, and fewer than 10% of the analyzed genomes respectively (Blaustein et al., 2019). *E. coli* has what is called an "open" pangenome (Figure 11). An open pangenome means that the number of gene families found will steadily increase as additional genome sequences are included in the pangenome analysis. In contrast, in a "closed" pangenome the number of gene families initially increase with the number of genomes included but will eventually plateau as more genomes are added. Estimates of the number of gene families in the global *E. coli* pangenome range from 60,000 all the way to 128,000 with between 2,600 and 3,100 genes in the core genome

(Land et al., 2015; Park et al., 2019). Meanwhile the number of genes found in *Staphylococcus lugdunensis*, which is reported to have a closed pangenome, plateaus at under 3,000 genes (Argemi et al., 2018), which shows the vast potential for diversity within species with an open pangenome.

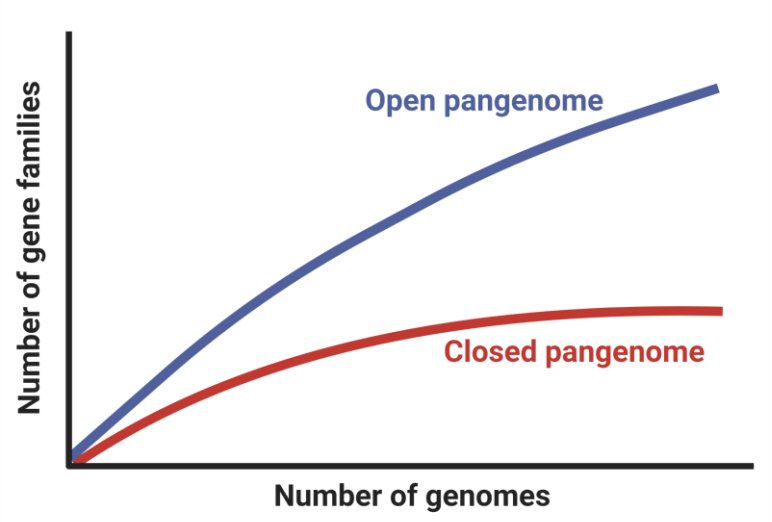


Figure 11. The difference between open and closed pangenomes. In open pangenomes (blue), the number of gene families found steadily increases as the number of additional genomes included increase. In contrast, in closed pangenomes (red) the number of gene families initially increase but eventually plateaus. Figure created with BioRender.com.

HORIZONTAL GENE TRANSFER

The transmission of genetic material between cells normally occurs during cell division where the daughter cell inherits the genetic material of the mother cell. This is referred to as vertical gene transfer. In bacteria, however, genetic material can also be transferred horizontally between neighbors through horizontal gene transfer (HGT). HGT can occur through three main mechanisms: transformation, transduction, and conjugation. While genes encoding antibiotic resistance can be spread through all these mechanisms, conjugation has been attributed as the main driving force for the spread of antibiotic resistance we see today, both in the clinic and the environment (von Wintersdorff et al., 2016). The fact that resistance genes can be mobilized in these ways also means that all bacteria can act as a reservoir for resistance, including commensal and environmental bacteria, who rarely encounter human pathogens.

TRANSFORMATION

Transformation is defined as the direct active uptake and genomic integration of extracellular DNA from the surrounding environment. Bacteria that can do this are referred to as "competent". Many species, like *Vibrio cholerae*, *Neisseria gonorrhoeae*, and various *Streptococcus* species, are naturally competent while others, such as *E. coli*, can be made artificially competent through various laboratory techniques (Johnsborg et al., 2007). While taking up foreign DNA can be beneficial for the cell, it can also be detrimental. The uptake and integration of exogenous DNA is thus a tightly regulated process and is typically only induced under specific conditions such as stationary phase, DNA damage, and nutrient starvation (Blokesch, 2016). An exception to this is that of *N. gonorrhoeae* and *N. meningitidis* which express the genes involved in competence constitutively.

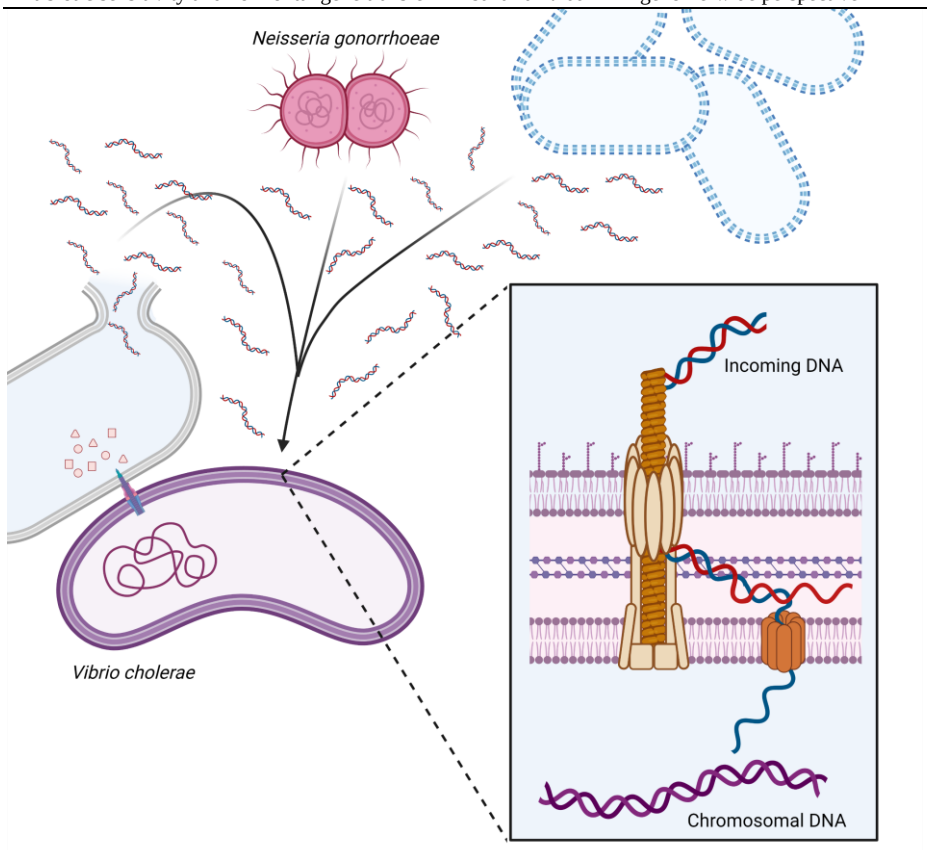


Figure 12. Mechanism of natural transformation. Some bacteria, like *Vibrio cholerae*, are naturally capable of picking up extracellular DNA through transformation. These bacteria express a type IV pilus-like structure and a translocation machinery that allows them to bind DNA, transport it across their cell wall and integrate it into their chromosome in a RecA-dependent manner. The extracellular DNA can be free DNA from cells that have died or lysed of natural causes or released by other bacteria such as *Neisseria gonorrhoeae*. In addition, *V. cholerae* is able to kill neighboring bacteria, using a type VI secretion system, and then pick up its DNA. Figure created with BioRender.com.

Natural competence is mediated through the production of membrane-associated proteins, involved in the uptake of DNA, and proteins involved in the integration of the DNA once inside the cell. During DNA uptake, extracellular DNA somehow binds to a pseudopilus, a type IV pilus-like structure. This pseudopilus retracts to bring the DNA to the translocation machinery where it crosses into the cell (Figure 12). In Gram-positive bacteria, only one of the strands of the DNA molecule enters the cytoplasm while the

other is degraded during uptake. This has also been seen in the Gram-negative species *Haemophilus influenzae* (Barany et al., 1983), but if this occurs in other Gram-negative bacteria is still unclear. Once inside the cell, the imported DNA can be integrated into the recipient's genome through RecA-dependent homologous recombination.

The extracellular DNA that is picked up through transformation is most commonly coming from cells that have lysed or died through natural causes. However, this is not always the case. Some bacteria, like *N. gonorrhoeae*, have evolved ways of actively releasing DNA into their surroundings, which can then be taken up and integrated by neighboring closely related bacteria. Some bacteria even express systems that allow them to kill other competing cells and take their DNA. An example of this is *Vibrio cholerae* which produces a type VI secretion system that it uses to inject toxins into neighboring cells. This system has been shown to be co-regulated with the operon regulating genes involved in competence of *V. cholerae* (Borgeaud et al., 2015), indicating that this has evolved as a way to both compete with other bacteria and at the same time acquire new potentially useful genetic material.

TRANSDUCTION

Transmission of genetic material via transduction is mediated through the action, or rather mistakes, of bacteriophages, also called phages. Phages are bacterial viruses that can infect and kill bacteria. They are widely found in the environment where there are estimates that there are 10^{31} phage particles, making them the most abundant biological entity on Earth (Suttle, 2005). They are made up of a nucleic acid genome that is encased in a protein shell called the phage capsid which both protects the genetic material and mediates its delivery to a recipient cell.

Upon phage infection, the phage attaches to a potential host cell and injects its genome into the cytoplasm of the host. Once in the cytoplasm, the phage can replicate through either the lytic or the lysogenic cycle. Phages that replicate through the lytic cycle are commonly referred to as virulent phages while phages which can switch between the lysogenic and lytic cycle are called

temperate phages. In the lytic cycle, the phage will hijack the replication, transcription, and translation machinery of the host to replicate its genome and produce new capsid proteins. The phage genome is then packaged into the capsid proteins to create new phage particles. The newly produced phage particles are then released into the environment through the lysis of the host cell, either passively or induced by the phage. In contrast, during the lysogenic cycle, the phage genome is integrated into the bacterial chromosome as a prophage. Here they are replicated as part of the bacterial genome and are inherited to daughter cells. Upon stress, the prophage can induce a lytic cycle where it is excised, replicated, and packed into viral particles after which the host cell is lysed, allowing the release of the newly produced phages.

There are three major ways that phages can transfer bacterial genetic material between bacteria: generalized, specialized, and lateral transduction (Figure 13) (Chiang et al., 2019). Generalized transduction occurs when chromosomal DNA is, by mistake, packaged into the protein capsid during viral assembly. This will lead to viral particles that carry chromosomal instead of viral DNA, which can in turn infect other cells where the piece of chromosomal DNA can be integrated into the chromosome through recombination. Any part of the host's genome can be transferred this way, including plasmid DNA. In comparison, specialized transduction is limited in what DNA can be transferred. It occurs when a prophage is excised in an incorrect manner and some flanking chromosomal DNA is included in the excised molecule. This piece of chromosomal DNA is then integrated together with the phage in subsequent host cells. Finally, lateral transduction is a recently discovered mode of transduction capable of transferring host DNA at very high frequencies (Chen et al., 2018). It is, in contrast to generalized and specialized transduction, believed to not be a mistake made by the phage, but instead a natural part of the phage's life cycle. Phages involved in lateral transduction deviate in the usual excision-replication-packaging pathway of other phages by delaying its excision until a late stage in their lytic cycle. In addition, the phage initiates replication and packaging of the viral genome while it is still integrated in the host chromosome. During DNA packaging, the viral genome and spans of adjacent chromosomal DNA is packed into a capsid until the

capsid is full. This continues for several capsids worth of chromosomal DNA, meaning that a large portion of the newly produced phage particles will only carry chromosomal DNA which, like before, can either integrate or recombine into a new host's genome (Chiang et al., 2019).

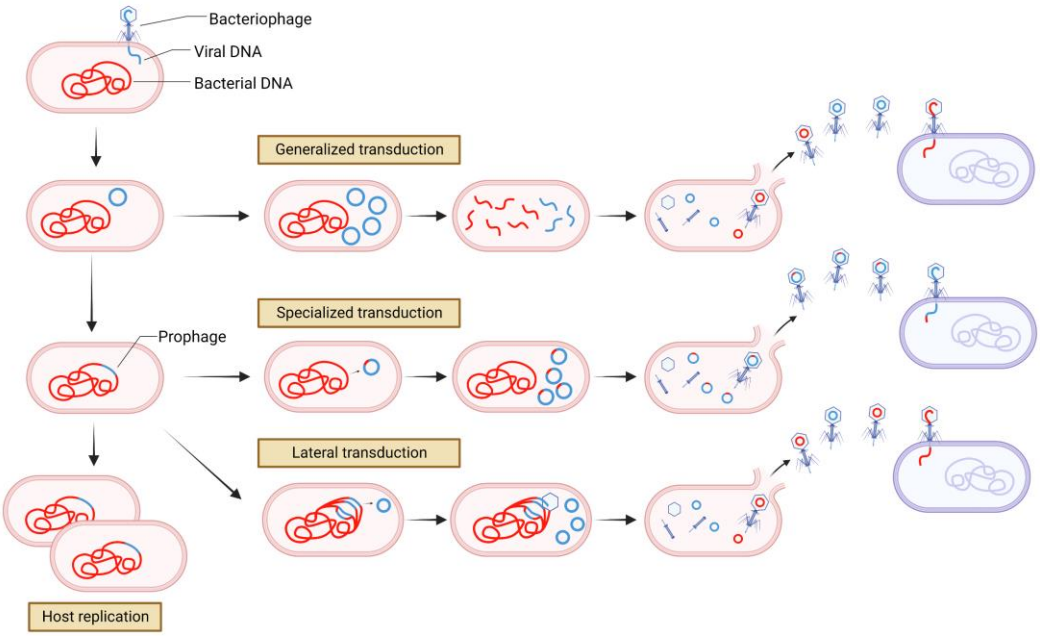


Figure 13. The three main mechanisms of transduction. Bacteriophages can transfer chromosomal genes through three main mechanisms. They all result in the transfer of chromosomal genes to a recipient cell where they can be recombined into the chromosome. Generalized transduction is when chromosomal DNA instead of viral DNA is, by mistake, packaged into protein capsids during virus assembly. Specialized transduction occurs when a prophage excises from the chromosome in an incorrect manner and some flanking chromosomal DNA is included. This chromosomal fragment is subsequently replicated and packaged into the protein capsid together with the phage genome. During lateral transduction, a prophage replicates while still integrated in the chromosome. This way large portions of the bacterial chromosome are included with the phage DNA when packaged into the protein capsid. Figure created with BioRender.com.

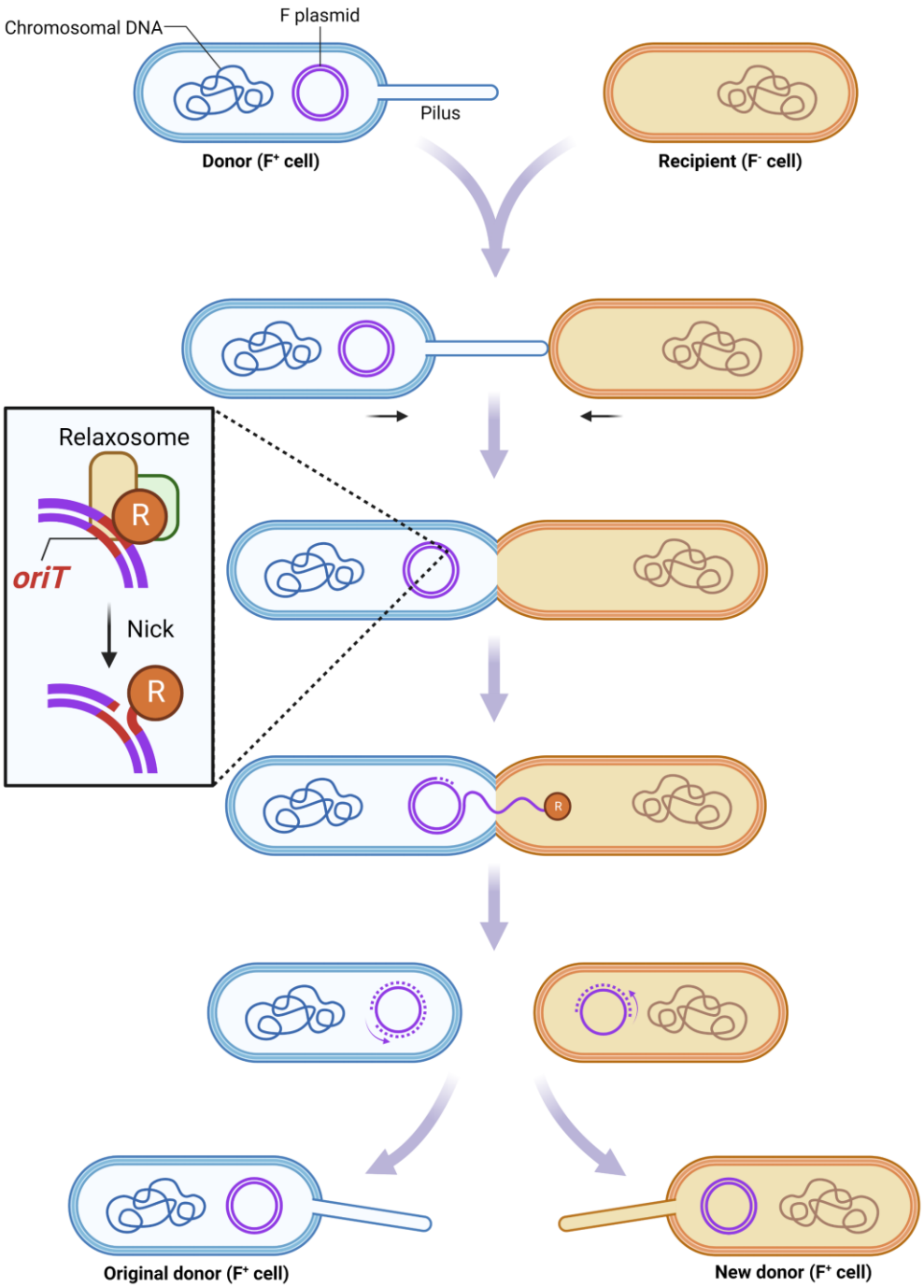

Chromosomal DNA
F plasmid
Pilus
Donor (F+ cell)
Recipient (F- cell)
Relaxosome
R
oriT
Nick
R
R
Original donor (F+ cell)
New donor (F+ cell)

Figure 14. General mechanism of bacterial conjugation. Bacterial conjugation is initiated when a donor cell carrying a conjugative plasmid encounters a recipient cell. The pilus produced by the donor attaches to the recipient and brings the two cells together. Once in close proximity, a mating pore forms between the cells. The relaxosome complex then binds to the oriT site on the plasmid and the relaxase protein (denoted with an R) creates a nick in one of the DNA strands. The DNA-relaxase complex then travels through the mating pore to the recipient, where the plasmid is re-circularized and replicated. Figure created with BioRender.com.

CONJUGATION

Bacterial conjugation, often referred to as bacterial "mating", is the transfer of genetic material through cell-to-cell contact. The machinery needed for conjugation consists of a type IV secretion system (T4SS) and a DNA processing complex called the relaxosome. The T4SS is made up of a pilus, a transport apparatus, and a type IV coupling protein while the relaxosome is made up of a relaxase protein and proteins that bind to a site on the plasmid from which transfer is initiated, called the origin of transfer (*oriT*). These components are all encoded on the conjugative plasmid itself in a transfer (*tra*) operon. Like transformation, conjugation is a well-regulated process. While most of the regulators are encoded on the plasmid, there are some chromosomal regulators that are also involved, such as ArcA and IHF (Strohmaier et al., 1998; Moncalián et al., 1999) and those implicated in **Paper IV**.

The exact mechanism of transfer differs slightly between different Inc groups, but a general mechanism is as follows (Figure 14). The donor cell carrying the conjugative plasmid produces the pilus which, upon encountering a recipient cell, attaches to the cell and brings the donor and recipient cells together. Once the two cells are close together, a pore forms between them through which the DNA transfer will occur. The relaxosome complex then binds to the *oriT* sequence and the relaxase protein creates a nick in one of the DNA strands of the plasmid. By doing so, the relaxase becomes covalently bound to the DNA strand. The DNA-relaxase complex is recruited to the mating pore where the transfer of a single strand of DNA (ssDNA) through the mating pore starts. As the plasmid is being transferred it is also replicated so that the plasmid is

maintained in the donor. Once in the recipient cell, the relaxase performs a reverse nicking reaction, which re-circularizes the ssDNA, after which it is replicated. After having received and replicated the plasmid, the recipient cell is referred to as a transconjugant. The transconjugant can then act as a donor as well and transfer the conjugative plasmid to other recipient cells.

Mobilizable plasmids can also be transferred via conjugation. Most mobilizable plasmids carry all or some of the genes encoding the relaxosome complex and an *oriT* sequence but lack the genes encoding the T4SS (Smillie et al., 2010). Because of this, they are dependent on other plasmids to encode the T4SS. The most common way a mobilizable plasmid is transferred through conjugation is by exploiting the mating pore formed by other conjugative plasmids in various ways. For example, in *Staphylococcus* mobilizable plasmids are transferred by encoding a similar *oriT* sequence, or relaxase that is compatible with the conjugative plasmid's type IV coupling protein (Ramsay et al., 2016).

While the vectors of conjugation are typically conjugative or mobilizable plasmids, the chromosome can also be transferred via conjugation in so called high frequency of recombination (Hfr) strains. In these strains, a conjugative plasmid has integrated into the chromosome. One of the most studied examples of this is that of the fertility factor (F factor or F plasmid), a member of the IncF family of plasmids. The F factor will initiate transfer while still integrated into the chromosome. Since it is integrated, the F factor will drag the chromosome along with it during transfer to a recipient cell that does not carry an F factor (F^-). Given enough time, the entire chromosome will be transferred this way. However, due to the size of the chromosome and the inability to maintain cell-to-cell contact with the recipient cell over time the transfer never finishes. This leads to the transfer of portions of the chromosome without transferring the entire plasmid to the recipient which remains F^- but has acquired new chromosomal genes that can be recombined into its chromosome. In addition, the F factor can also be excised and exist in the cell as a plasmid. The excision of the F factor sometimes results in the inclusion of chromosomal genes flanking the integration site (Rosenberg & Hastings, 2001). These

chromosomal genes will be transferred to other cells as part of the F factor, now referred to as an F´ factor.

SUMMARY OF PAPERS

PAPER I

In recent years, the potential of sub-inhibitory levels of antibiotics and heavy metals to select for antibiotic resistance has become increasingly evident. These concentrations better reflect the levels of antibiotics that bacteria are exposed to in the environment and some clinical settings and are thus an important factor to consider to understand the evolution of antibiotic resistance. In addition, the open pangenome of *E. coli* allows for vast diversity within the global population. However, the pangenome is relatively unexplored when it comes to growth on sub-inhibitory levels of antibiotics and heavy metals. Therefore, in **Paper I**, we set out to investigate the genetic and phenotypic landscape of *E. coli* exposed to sub-inhibitory concentrations of antibiotics and heavy metals.

To accomplish this, we compiled a collection of more than 2,000 *E. coli* isolates. These isolates were originally isolated from a wide range of hosts, broadly grouped into animals, environment, and human. The animal samples come from both mammal and bird hosts while the environmental samples are from wastewater and sewage as well as natural environmental sources such as soils and streams. Most of the isolates from human hosts were clinical isolates and a large portion of them were previously identified to produce ESBLs. In addition, a subset of the isolates was isolated in the 1930s and 1940s, i.e., the 'pre-antibiotic era'.

To measure the growth characteristics of our collection, we used large-scale pinning robotics which we use to transfer bacteria onto plates containing sub-inhibitory levels of antibiotics or heavy metals in a high-throughput manner. These plates are subsequently placed in photo scanners that take an image of the plate at regular intervals. The images are in turn analyzed using a software originally used for growth analysis of yeast called Scan-o-matic (Zackrisson et al., 2016), which we have adapted to be used with *E. coli*. This software will analyze each image and calculate the population size of each colony, based on

the pixel intensity, and construct growth curves. From these growth curves, we can extract and estimate various growth phenotypes, such as generation time and yield, for each isolate. Using this system, we determined the growth phenotypes of our collection on three sub-inhibitory concentrations of ten antibiotics, belonging to clinically relevant classes, and six heavy metals.

To explore the genotypic and phenotypic landscape of our collection, we also sequenced and assembled the genomes of most of the isolates in the collection. The main findings of the paper were:

a) We found 44,026 gene families in the pangenome distributed among the isolates in a clade-specific manner.
b) The pangenome contains known antibiotic resistance genes, as well genes and gene variants that confer a smaller fitness advantage at sub-inhibitory concentrations.
c) Isolates carrying resistance genes exhibited a wide variation in growth at sub-inhibitory concentrations. This suggests that there are other genetic factors that influence growth at these concentrations.
d) Interestingly, and perhaps somewhat surprising, we could not see a correlation between growth on antibiotics and heavy metals on a pangenome level.

PAPER II

Clinical diagnostics is a central part of modern healthcare. An approach that has seen increased potential use in a clinical setting in recent years is machine learning. Machine learning, which is a subfield of artificial intelligence, allows systems to learn and improve without being explicitly programmed. Of particular use in a clinical setting is the ability to train computer systems to make predictions that can help medical workers make decisions, such as the identification of antibiotic resistant bacteria. This is done by supplying the model with data with a known outcome, for example the genotypes or phenotypes of bacteria that are known to be susceptible or resistant to a certain

antibiotic. Thus, by parsing this data the model can 'learn' and become better at predicting outcomes.

In **Paper II**, we used machine learning to train models to be able to predict the generation time and growth yield based on the genotype of *E. coli* strains. The models were trained using genotypic and phenotypic data of a subset of isolates that were obtained through the work described in **Paper I**. The main findings of the paper were:

a) The models were able to predict both generation time and growth yield with a moderate to strong correlation between the predicted and actual value.
b) Models trained on whole genome sequences are better at predicting growth characteristics than those that use only known antibiotic resistance genes. This indicates that the presence of antibiotic resistance does not tell the whole story when it comes to growth on sub-inhibitory concentrations.
c) We detected synonymous, non-synonymous, and intergenic mutations that were predictive of generation time and growth yield in the presence of sub-inhibitory levels of antibiotics.

PAPER III

Most ways of dealing with the problem of increasing antibiotic resistance levels involve two broad approaches. First, reducing the exposure of bacteria to antibiotics to prevent the development of resistance through better diagnostics and prescription practices, as well as decreased environmental pollution. Second, developing new or expanded strategies to prevent or treat bacterial infection including vaccinations, novel antibiotics, improved hygiene, and alternative treatment, e.g., phage therapy.

A promising approach that has gained some traction in recent years is decreasing the rate at which resistance spreads, particularly through conjugation. Conjugation inhibitors (COINs) could help reduce the prevalence of antibiotic resistance genes in environments where the potential for them

spreading is high, such as in water treatment and the livestock industry. Reducing the spread of novel antibiotic resistance determinants to pathogens would thus extend the lifespan of clinically relevant antibiotics. In **Paper III** we briefly discuss some strategies that could be used to inhibit conjugation.

Ideally, a COIN should exert its function without reducing the fitness of the bacteria, as this will generate a selective pressure against it. We envisage three major ways of inhibiting conjugation: targeting the recipient, targeting the plasmid, and targeting the donor. The three approaches present both advantages and disadvantages. First, targeting the recipient could include the inhibition of the receptor that the pilus attaches to. However, for plasmids (and thus pili) with a broad host-range this is unlikely to work since those receptors are likely not specific. Second, targeting the plasmid itself would target the factors encoded on the plasmid, such as the T4SS and accessory systems. This would allow selective inhibition of conjugation, but the diversity of plasmids and plasmid families would complicate this. Finally, there are chromosomal determinants that, when disrupted, severely impairs the cell's ability to act as a conjugative donor, e.g., ArcA and the ones implicated in **Paper IV**. Thus, targeting the donor can be an appealing approach to stop the spread at its source. However, the efficiency of this will depend on the plasmid family as some plasmids are less dependent on chromosomal factors than others.

PAPER IV

While some chromosomal factors are known to be important for conjugation of some Inc groups of plasmids, e.g., ArcA for IncF, there has not been a systematic effort to find all chromosomal factors. The traditional way of measuring conjugation efficiency is through liquid or solid mating assays, where donor and recipient cells are allowed to mate in liquid or solid media respectively, after which they are diluted and plated to count colony forming units. Doing this on a genome-wide scale would be both very time-consuming and laborious. Therefore, in **Paper IV**, we developed a system that allows for the high-throughput genome-wide screening for chromosomal determinants involved in conjugation.

This system utilizes a large-scale pinning robot to construct a donor library by mating a conjugative plasmid into the *E. coli* single-deletion mutant KEIO collection (Baba et al., 2006). This library is pinned together with a recipient strain to allow the formation of transconjugants. To measure the efficiency at which the mutant donor can transfer the plasmid we utilized an adapted version of the Scan-o-matic system described in **Paper I**. In contrast to **Paper I**, we do not measure growth rate or yield of the mating. Instead, we measure the period of growth lag that occurs when the donor and recipient are pinned together on plates selecting for the transconjugant.

This lag time is mainly due to three factors: First, the plasmid needs to be transferred from the donor to the recipient. Second, the newly formed transconjugant needs to express the resistance gene, that it has just received, to allow for growth on the selective medium. Finally, the transconjugant needs to grow above the detection limit of the system. Since the same recipient strain is used for all matings, the resulting transconjugant will be identical in all cases and thus the ability to express the resistance gene and to grow above the detection limit should be constant. Therefore, any differences in lag time between strains will be due to differences in the donor's ability to transfer the plasmid, allowing us to evaluate the effect of the deletion mutation on conjugation.

To demonstrate the system and identify chromosomal mutants which alter conjugation efficiency, we constructed a deletion mutant donor library by introducing the F plasmid into the KEIO collection. By measuring the conjugation efficiency of the mutant donor library, we could identify many novel chromosomal deletion mutants that are deficient in conjugation, as well as all seven previously known ones. The functions of the novel mutants span a wide range but were enriched in genes involved in DNA replication, chaperone or protein folding, and lipopolysaccharide core biosynthesis. The effect of many of these novel mutants were verified and work has started to further elucidate what causes the observed effects. This work highlights the potential of this system as a robust tool for measuring the effect that various environmental or genetic factors have on conjugation.

PAPER V

In addition to chromosomal factors, we also began to look at environmental factors that could affect conjugation efficiency. In the environment, bacteria are exposed to low levels of both heavy metals and antibiotics occurring naturally or through contamination. Additionally, heavy metal surfaces are used in hospital settings to prevent the spread of pathogenic bacteria. It is well established that such low levels of antibiotics and heavy metals can select for antibiotic and heavy metal resistance through co-selection. This allows resistance genes to be maintained and act as a reservoir of resistance that can be spread to other bacteria.

How heavy metals affect the rate of transfer through conjugation is thus important. Previous studies on this topic have yielded contradictory results, most likely due to differences in experimental setup and the plasmid investigated. In **Paper V**, we systematically investigated the effect that sub-inhibitory levels of the heavy metals arsenic (As), cadmium (Cd), copper (Cu), manganese (Mn), and zinc (Zn) have on the conjugation efficiency of an IncF family plasmid. The main findings of the paper were:

a) Cu drastically decreases conjugation efficiency while As, Cd, Mn, and Zn have a mild to no effect.
b) Further investigation revealed that Cu needs to be present during mating to impair conjugation efficiency.
c) The decrease in conjugation efficiency in the presence of Cu is not due to changes in transcription of the *tra* operon of the plasmid.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

When antibiotics were first introduced to treat human disease, they were hailed as miracle drugs. Indeed, they have changed human medicine, and by extension the world, for the better by allowing the treatment of previously untreatable infections. Resistance to antibiotics has existed as a defense mechanism among bacteria for millennia. However, inappropriate use over the last decades has driven the selection of resistance in both human and animal pathogens as well as the environment. This has resulted in a situation where the use of these miracle drugs should be limited to extend their potential future use. Today, the spread of antibiotic resistance has reached a critical point where many types of infections are becoming untreatable once again.

In this thesis we have investigated various aspects of antibiotic resistance. In the first part, we explored the genotype-phenotype landscape of *E. coli* exposed to sub-inhibitory levels of antibiotics and heavy metals (**Paper I**). In addition, we successfully used genetic and phenotype data to train machine learning models able to predict growth characteristics based on genomic information (**Paper II**). In the second part of the thesis, we turned our attention to the spread of antibiotic resistance through conjugation. We discussed inhibiting conjugation as an alternative approach in the fight against antibiotic resistance (**Paper III**). We also evaluated the effect of genetic and environmental perturbations on the conjugation efficiency of an IncF plasmid in *E. coli* (**Papers IV** and **V**).

In both **Paper I** and **II**, we have demonstrated that the presence of known antibiotic resistance genes does not always correlate with good growth on sub-inhibitory levels of antibiotics. This indicates that growth, and thus selection, at these concentrations is driven by other factors. To further dissect the underlying genetic factors, one could perform genome-wide association analysis (GWAA). The genotypic and phenotypic data generated in **Paper I** opens the door for this kind of further analyses, and it is our hope that this genetically diverse collection can be used as a resource for future research.

As discussed in **Paper III**, inhibiting conjugation can be a promising approach to prevent the spread of antibiotic resistance to pathogens. However, finding suitable targets for potential drugs is both time-consuming and expensive. The system that we have developed in **Paper IV** could be instrumental in alleviating this, where donor libraries of various Inc groups can easily be constructed and screened. The chromosomal determinants found to affect IncF conjugation could also act as a starting point for further investigation of the underlying mechanisms of conjugation.

In **Paper V**, we found that exposure to copper during mating decreased conjugation efficiency of an IncF plasmid in *E. coli*. In addition, it has been suggested that sub-inhibitory levels of biocides and antibiotics can promote the spread of antibiotic resistance through conjugation. However, the mechanism through which this occurs is not known. Given that both antibiotics and other biocides exist at low levels in the environment, where they maintain a reservoir of resistance, this is an important aspect to study. Again, the system described in **Paper IV** would be ideal for elucidating the mechanisms behind this, where the effects of low levels of antibiotics or biocides on the KEIO collection's ability to act as donors could be evaluated. In addition, the collection described in **Paper I** could be used for this purpose to perform GWAA to find genetic determinants involved. While this would be more complicated, due to the high prevalence of conjugative plasmids in natural isolates, it could also be much more informative.

In closing, the adaptability and 'cleverness' of bacteria likely mean that we will never win the war against antibiotic resistance. Introducing new antibiotics would certainly alleviate the problem but, unfortunately, it would only be a matter of time until resistance emerges if it does not already exist. To this end, stopping the spread of antibiotic resistance could be a more appropriate approach to stop resistance at its source. Taken together, the results shown in this thesis emphasizes that studying what happens at sub-inhibitory concentrations is a crucial piece of the puzzle to fully understand the emergence, selection and spread of antibiotic resistance. A puzzle that needs to be solved to save the precious miracle drugs we have left.

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REFERENCES

Abraham, E. P., & Chain, E. (1940). An Enzyme from Bacteria able to Destroy Penicillin. *Nature*, *146*(3713), Article 3713. https://doi.org/10.1038/146837a0

Aldred, K. J., Kerns, R. J., & Osheroff, N. (2014). Mechanism of Quinolone Action and Resistance. *Biochemistry*, *53*(10), 1565–1574. https://doi.org/10.1021/bi5000564

Ambler, R. P. (1980). The structure of beta-lactamases. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *289*(1036), 321–331. https://doi.org/10.1098/rstb.1980.0049

Aminov, R. I. (2009). The role of antibiotics and antibiotic resistance in nature. *Environmental Microbiology*, *11*(12), 2970–2988. https://doi.org/10.1111/j.1462-2920.2009.01972.x

Andersson, D. I., & Hughes, D. (2010). Antibiotic resistance and its cost: Is it possible to reverse resistance? *Nature Reviews Microbiology*, *8*(4), Article 4. https://doi.org/10.1038/nrmicro2319

Argemi, X., Matelska, D., Ginalski, K., Riegel, P., Hansmann, Y., Bloom, J., Pestel-Caron, M., Dahyot, S., Lebeurre, J., & Prévost, G. (2018). Comparative genomic analysis of Staphylococcus lugdunensis shows a closed pan-genome and multiple barriers to horizontal gene transfer. *BMC Genomics*, *19*(1), 621. https://doi.org/10.1186/s12864-018-4978-1

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., & Mori, H. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: The Keio collection. *Molecular Systems Biology*, *2*, 2006.0008. https://doi.org/10.1038/msb4100050

Baker-Austin, C., Wright, M. S., Stepanauskas, R., & McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends in Microbiology*, *14*(4), 176–182. https://doi.org/10.1016/j.tim.2006.02.006

Barany, F., Kahn, M. E., & Smith, H. O. (1983). Directional transport and integration of donor DNA in Haemophilus influenzae transformation. *Proceedings of the National Academy of Sciences*, *80*(23), 7274–7278. https://doi.org/10.1073/pnas.80.23.7274

Barber, M. (1947). Staphylococcal Infection due to Penicillin-resistant Strains. *Br Med J*, *2*(4534), 863–865. https://doi.org/10.1136/bmj.2.4534.863

Bentley, R. (2009). Different roads to discovery; Prontosil (hence sulfa drugs) and penicillin (hence β-lactams). *Journal of Industrial Microbiology and Biotechnology*, *36*(6), 775–786. https://doi.org/10.1007/s10295-009-0553-8

Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B., & Shao, Y. (1997). The Complete Genome Sequence of Escherichia coli K-12. *Science*, *277*(5331), 1453–1462. https://doi.org/10.1126/science.277.5331.1453

Blaustein, R. A., McFarland, A. G., Ben Maamar, S., Lopez, A., Castro-Wallace, S., & Hartmann, E. M. (2019). Pangenomic Approach To Understanding Microbial Adaptations within a Model Built Environment, the International Space Station, Relative to Human Hosts and Soil. *MSystems*, *4*(1), e00281-18. https://doi.org/10.1128/mSystems.00281-18

Blokesch, M. (2016). Natural competence for transformation. *Current Biology: CB*, *26*(21), R1126–R1130. https://doi.org/10.1016/j.cub.2016.08.058

Borgeaud, S., Metzger, L. C., Scrignari, T., & Blokesch, M. (2015). The type VI secretion system of Vibrio cholerae fosters horizontal gene transfer. *Science*, *347*(6217), 63–67. https://doi.org/10.1126/science.1260064

Borkow, G., & Gabbay, J. (2009). Copper, An Ancient Remedy Returning to Fight Microbial, Fungal and Viral Infections. *Current Chemical Biology*, *3*(3), 272–278. https://doi.org/10.2174/187231309789054887

Brewer, N. S., & Hellinger, W. C. (1991). The Monobactams. *Mayo Clinic Proceedings*, *66*(11), 1152–1157. https://doi.org/10.1016/S0025-6196(12)65797-8

Burman, L. G. (1986). The Antimicrobial Activities of Trimethoprim and Sulfonamides. *Scandinavian Journal of Infectious Diseases*, *18*(1), 3–13. https://doi.org/10.3109/00365548609032299

Burns, D. J. W., & Cundliffe, E. (1973). Bacterial-Protein Synthesis. *European Journal of Biochemistry*, *37*(3), 570–574. https://doi.org/10.1111/j.1432-1033.1973.tb03020.x

Bush, K. (1988). Beta-lactamase inhibitors from laboratory to clinic. *Clinical Microbiology Reviews*, *1*(1), 109–123. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC358033/

Cambray, G., Guerout, A.-M., & Mazel, D. (2010). Integrons. *Annual Review of Genetics*, *44*(1), 141–166. https://doi.org/10.1146/annurev-genet-102209-163504

Campbell, E. A., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfarb, A., & Darst, S. A. (2001). Structural Mechanism for Rifampicin Inhibition of Bacterial RNA Polymerase. *Cell*, *104*(6), 901–912. https://doi.org/10.1016/S0092-8674(01)00286-0

Campos, J., Cristino, L., Peixe, L., & Antunes, P. (2016). MCR-1 in multidrug-resistant and copper-tolerant clinically relevant Salmonella 1,4,[5],12:i:- and S. Rissen clones in Portugal, 2011 to 2015. *Eurosurveillance*, *21*(26), 30270. https://doi.org/10.2807/1560-7917.ES.2016.21.26.30270

Cantón, R., González-Alba, J. M., & Galán, J. C. (2012). CTX-M Enzymes: Origin and Diffusion. *Frontiers in Microbiology*, *3*, 110. https://doi.org/10.3389/fmicb.2012.00110

Carattoli, A. (2009). Resistance Plasmid Families in Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy*, *53*(6), 2227–2238. https://doi.org/10.1128/AAC.01707-08

Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., & Sanders, A. G. (1940). PENICILLIN AS A CHEMOTHERAPEUTIC AGENT. *The Lancet*, *236*(6104), 226–228. https://doi.org/10.1016/S0140-6736(01)08728-1

Chen, J., Quiles-Puchalt, N., Chiang, Y. N., Bacigalupe, R., Fillol-Salom, A., Chee, M. S. J., Fitzgerald, J. R., & Penadés, J. R. (2018). Genome hypermobility by lateral transduction. *Science*, *362*(6411), 207–212. https://doi.org/10.1126/science.aat5867

Chiang, Y. N., Penadés, J. R., & Chen, J. (2019). Genetic transduction by phages and chromosomal islands: The new and noncanonical. *PLoS Pathogens*, *15*(8), e1007878. https://doi.org/10.1371/journal.ppat.1007878

Coates, A. R., Halls, G., & Hu, Y. (2011). Novel classes of antibiotics or more of the same? *British Journal of Pharmacology*, *163*(1), 184–194. https://doi.org/10.1111/j.1476-5381.2011.01250.x

Cohen, M. L. (2000). Changing patterns of infectious disease. *Nature*, *406*(6797), Article 6797. https://doi.org/10.1038/35021206

Colebrook, L., & Kenny, M. (1936). TREATMENT OF HUMAN PUERPERAL INFECTIONS, AND OF EXPERIMENTAL INFECTIONS IN MICE, WITH PRONTOSIL. *The Lancet*, *227*(5884), 1279–1281. https://doi.org/10.1016/S0140-6736(01)20734-X

Cornaglia, G., Mazzariol, A., Fontana, R., & Satta, G. (1996). Diffusion of Carbapenems Through the Outer Membrane of Enterobacteriaceae and Correlation of Their Activities with Their Periplasmic Concentrations. *Microbial Drug Resistance*, *2*(2), 273–276. https://doi.org/10.1089/mdr.1996.2.273

Corona, F., & Martinez, J. L. (2013). Phenotypic Resistance to Antibiotics. *Antibiotics*, *2*(2), 237–255. https://doi.org/10.3390/antibiotics2020237

Davis, B. D., Chen, L. L., & Tai, P. C. (1986). Misread protein creates membrane channels: An essential step in the bactericidal action of aminoglycosides. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(16), 6164–6168. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC386460/

D'Costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W. L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H. N., & Wright, G. D. (2011). Antibiotic resistance is ancient. *Nature*, *477*(7365), Article 7365. https://doi.org/10.1038/nature10388

Domingues, S., da Silva, G. J., & Nielsen, K. M. (2012). Integrons. *Mobile Genetic Elements*, *2*(5), 211–223. https://doi.org/10.4161/mge.22967

Dziva, F., & Stevens, M. P. (2008). Colibacillosis in poultry: Unravelling the molecular basis of virulence of avian pathogenic Escherichia coli in their natural hosts. *Avian Pathology*, *37*(4), 355–366. https://doi.org/10.1080/03079450802216652

Eagle, H. (1952). Experimental approach to the problem of treatment failure with penicillin: I. Group a streptococcal infection in mice. *The American Journal of Medicine*, *13*(4), 389–399. https://doi.org/10.1016/0002-9343(52)90293-3

Fajardo, A., & Martínez, J. L. (2008). Antibiotics as signals that trigger specific bacterial responses. *Current Opinion in Microbiology*, *11*(2), 161–167. https://doi.org/10.1016/j.mib.2008.02.006

Fang, L., Li, X., Li, L., Li, S., Liao, X., Sun, J., & Liu, Y. (2016). Co-spread of metal and antibiotic resistance within ST3-IncHI2 plasmids from E. coli isolates of food-producing animals. *Scientific Reports*, *6*(1), Article 1. https://doi.org/10.1038/srep25312

Ferretti, J. J., Gilmore, K. S., & Courvalin, P. (1986). Nucleotide sequence analysis of the gene specifying the bifunctional 6'-aminoglycoside acetyltransferase 2"-aminoglycoside phosphotransferase enzyme in Streptococcus faecalis and identification and cloning of gene regions specifying the two activities. *Journal of Bacteriology*, *167*(2), 631–638. https://doi.org/10.1128/jb.167.2.631-638.1986

Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J.-F., Dougherty, B. A., Merrick, J. M., McKenney, K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J. D., Scott, J., Shirley, R., Liu, L., Glodek, A., … Venter, J. C. (1995). Whole-Genome Random Sequencing and Assembly of Haemophilus influenzae Rd. *Science*, *269*(5223), 496–512. https://doi.org/10.1126/science.7542800

Fleming, A. (1929). On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ. *British Journal of Experimental Pathology*, *10*(3), 226–236. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2048009/

Flensburg, J., & Sköld, O. (1987). Massive overproduction of dihydrofolate reductase in bacteria as a response to the use of trimethoprim. *European Journal of Biochemistry*, *162*(3), 473–476. https://doi.org/10.1111/j.1432-1033.1987.tb10664.x

Fritsche, T. R., Castanheira, M., Miller, G. H., Jones, R. N., & Armstrong, E. S. (2008). Detection of Methyltransferases Conferring High-Level Resistance to Aminoglycosides in Enterobacteriaceae from Europe, North America, and Latin America. *Antimicrobial Agents and Chemotherapy*, *52*(5), 1843–1845. https://doi.org/10.1128/AAC.01477-07

Garrod, L. P., & Waterworth, P. M. (1968). ACTION OF THREE DRUG COMBINATIONS ON GONOCOCCI*. *British Journal of Venereal Diseases*, *44*(1), 75–79. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1047919/

Gillings, M. R. (2014). Integrons: Past, Present, and Future. *Microbiology and Molecular Biology Reviews*, *78*(2), 257–277. https://doi.org/10.1128/MMBR.00056-13

Gordon, O., Vig Slenters, T., Brunetto, P. S., Villaruz, A. E., Sturdevant, D. E., Otto, M., Landmann, R., & Fromm, K. M. (2010). Silver Coordination Polymers for Prevention of Implant Infection: Thiol Interaction, Impact on Respiratory Chain Enzymes, and Hydroxyl Radical Induction. *Antimicrobial Agents and Chemotherapy*, *54*(10), 4208–4218. https://doi.org/10.1128/AAC.01830-09

Grass, G., & Rensing, C. (2001). CueO Is a Multi-copper Oxidase That Confers Copper Tolerance in Escherichia coli. *Biochemical and Biophysical Research Communications*, *286*(5), 902–908. https://doi.org/10.1006/bbrc.2001.5474

Grass, G., Rensing, C., & Solioz, M. (2011). Metallic Copper as an Antimicrobial Surface. *Applied and Environmental Microbiology*, *77*(5), 1541–1547. https://doi.org/10.1128/AEM.02766-10

Gullberg, E., Albrecht, L. M., Karlsson, C., Sandegren, L., & Andersson, D. I. (2014). Selection of a Multidrug Resistance Plasmid by Sublethal Levels of Antibiotics and Heavy Metals. *MBio*, *5*(5), e01918-14. https://doi.org/10.1128/mBio.01918-14

Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D., & Andersson, D. I. (2011). Selection of Resistant Bacteria at Very Low Antibiotic Concentrations. *PLOS Pathogens*, *7*(7), e1002158. https://doi.org/10.1371/journal.ppat.1002158

Hallet, B., & Sherratt, D. J. (1997). Transposition and site-specific recombination: Adapting DNA cut-and-paste mechanisms to a variety of genetic rearrangements. *FEMS Microbiology Reviews*, *21*(2), 157–178. https://doi.org/10.1111/j.1574-6976.1997.tb00349.x

Hannauer, M., Braud, A., Hoegy, F., Ronot, P., Boos, A., & Schalk, I. J. (2012). The PvdRT-OpmQ efflux pump controls the metal selectivity of the iron uptake pathway mediated by the siderophore pyoverdine in Pseudomonas aeruginosa. *Environmental Microbiology*, *14*(7), 1696–1708. https://doi.org/10.1111/j.1462-2920.2011.02674.x

Henderson, G. B., & Huennekens, F. M. (1986). [42] Membrane-Associated folate transport proteins. In *Methods in Enzymology* (Vol. 122, pp. 260–269). Academic Press. https://doi.org/10.1016/0076-6879(86)22180-1

Hernando-Amado, S., Blanco, P., Alcalde-Rico, M., Corona, F., Reales-Calderón, J. A., Sánchez, M. B., & Martínez, J. L. (2016). Multidrug efflux pumps as main players in intrinsic and acquired resistance to antimicrobials. *Drug Resistance Updates*, *28*, 13–27. https://doi.org/10.1016/j.drup.2016.06.007

Heth, D. A., & Bird, H. R. (1962). Growth Response of Chicks to Antibiotics from 1950 to 1961. *Poultry Science*, *41*(3), 755–760. https://doi.org/10.3382/ps.0410755

Hewitt, W. L. (1967). Penicillin-Historical Impact on Infection Control. *Annals of the New York Academy of Sciences*, *145*(2), 212–215. https://doi.org/10.1111/j.1749-6632.1967.tb50219.x

Hong, R., Kang, T. Y., Michels, C. A., & Gadura, N. (2012). Membrane Lipid Peroxidation in Copper Alloy-Mediated Contact Killing of Escherichia coli. *Applied and Environmental Microbiology*, *78*(6), 1776–1784. https://doi.org/10.1128/AEM.07068-11

Hughes, V. M., & Datta, N. (1983). Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature*, *302*(5910), Article 5910. https://doi.org/10.1038/302725a0

Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: Past, present and future. *Current Opinion in Microbiology*, *51*, 72–80. https://doi.org/10.1016/j.mib.2019.10.008

Jackson, C. R., & Dugas, S. L. (2003). Phylogenetic analysis of bacterial and archaeal arsC gene sequences suggests an ancient, common origin for arsenate reductase. *BMC Evolutionary Biology*, *3*(1), 18. https://doi.org/10.1186/1471-2148-3-18

Jacob, F., Perrin, D., Sánchez, C., & Monod, J. (2005). L'opéron: Groupe de gènes à expression coordonnée par un opérateur [C. R. Acad. Sci. Paris 250 (1960) 1727–1729]. *Comptes Rendus Biologies*, *328*(6), 514–520. https://doi.org/10.1016/j.crvi.2005.04.005

Johnsborg, O., Eldholm, V., & Håvarstein, L. S. (2007). Natural genetic transformation: Prevalence, mechanisms and function. *Research in Microbiology*, *158*(10), 767–778. https://doi.org/10.1016/j.resmic.2007.09.004

Jung, D., Rozek, A., Okon, M., & Hancock, R. E. W. (2004). Structural Transitions as Determinants of the Action of the Calcium-Dependent Antibiotic

Daptomycin. *Chemistry & Biology*, *11*(7), 949–957. https://doi.org/10.1016/j.chembiol.2004.04.020

Jutkina, J., Marathe, N. P., Flach, C.-F., & Larsson, D. G. J. (2018). Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. *Science of The Total Environment*, *616–617*, 172–178. https://doi.org/10.1016/j.scitotenv.2017.10.312

King, D. E., Malone, R., & Lilley, S. H. (2000). New Classification and Update on the Quinolone Antibiotics. *American Family Physician*, *61*(9), 2741–2748. https://www.aafp.org/pubs/afp/issues/2000/0501/p2741.html

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H., & Mori, H. (2005). Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): Unique resources for biological research. *DNA Research: An International Journal for Rapid Publication of Reports on Genes and Genomes*, *12*(5), 291–299. https://doi.org/10.1093/dnares/dsi012

Krause, K. M., Serio, A. W., Kane, T. R., & Connolly, L. E. (2016). Aminoglycosides: An Overview. *Cold Spring Harbor Perspectives in Medicine*, *6*(6), a027029. https://doi.org/10.1101/cshperspect.a027029

Kumon, H., Tomochika, K., Matunaga, T., Ogawa, M., & Ohmori, H. (1994). A Sandwich Cup Method for the Penetration Assay of Antimicrobial Agents through Pseudomonas Exopolysaccharides. *Microbiology and Immunology*, *38*(8), 615–619. https://doi.org/10.1111/j.1348-0421.1994.tb01831.x

Lam, R. F., Lai, J. S. M., Ng, J. S. K., Rao, S. K., Law, R. W. K., & Lam, D. S. C. (2002). Topical chloramphenicol for eye infections. *Hong Kong Medical Journal = Xianggang Yi Xue Za Zhi*, *8*(1), 44–47.

Land, M., Hauser, L., Jun, S.-R., Nookaew, I., Leuze, M. R., Ahn, T.-H., Karpinets, T., Lund, O., Kora, G., Wassenaar, T., Poudel, S., & Ussery, D. W. (2015). Insights from 20 years of bacterial genome sequencing. *Functional & Integrative Genomics*, *15*(2), 141–161. https://doi.org/10.1007/s10142-015-0433-4

Leclercq, R. (2002). Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications. *Clinical Infectious Diseases*, *34*(4), 482–492. https://doi.org/10.1086/324626

Lederberg, J. (2000). Infectious History. *Science*, *288*(5464), 287–293. https://doi.org/10.1126/science.288.5464.287

Lederberg, J., & Tatum, E. L. (1946). Gene Recombination in Escherichia Coli. *Nature*, *158*(4016), Article 4016. https://doi.org/10.1038/158558a0

Lee, S. W., Foley, E. J., & Epstein, J. A. (1944). Mode of Action of Penicillin. *Journal of Bacteriology*, *48*(4), 393–399. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC373984/

Lemire, J., Harrison, J. J., & Turner, R. J. (2013). Antimicrobial activity of metals: Mechanisms, molecular targets and applications. *Nature Reviews Microbiology*, *11*(6), Article 6. https://doi.org/10.1038/nrmicro3028

Lemire, J., Mailloux, R., Auger, C., Whalen, D., & Appanna, V. D. (2010). Pseudomonas fluorescens orchestrates a fine metabolic-balancing act to counter aluminium toxicity. *Environmental Microbiology*, *12*(6), 1384–1390. https://doi.org/10.1111/j.1462-2920.2010.02200.x

Levin, B. R., & Rozen, D. E. (2006). Non-inherited antibiotic resistance. *Nature Reviews Microbiology*, *4*(7), Article 7. https://doi.org/10.1038/nrmicro1445

Lin, A. H., Murray, R. W., Vidmar, T. J., & Marotti, K. R. (1997). The oxazolidinone eperezolid binds to the 50S ribosomal subunit and competes with binding of chloramphenicol and lincomycin. *Antimicrobial Agents and Chemotherapy*, *41*(10), 2127–2131. https://doi.org/10.1128/AAC.41.10.2127

Lin, Y.-F., Walmsley, A. R., & Rosen, B. P. (2006). An arsenic metallochaperone for an arsenic detoxification pump. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(42), 15617–15622. https://doi.org/10.1073/pnas.0603974103

Livermore, D. M. (1998). Beta-lactamase-mediated resistance and opportunities for its control. *The Journal of Antimicrobial Chemotherapy*, *41 Suppl D*, 25–41. https://doi.org/10.1093/jac/41.suppl_4.25

Llano-Sotelo, B., Azucena, E. F., Kotra, L. P., Mobashery, S., & Chow, C. S. (2002). Aminoglycosides Modified by Resistance Enzymes Display Diminished Binding to the Bacterial Ribosomal Aminoacyl-tRNA Site. *Chemistry & Biology*, *9*(4), 455–463. https://doi.org/10.1016/S1074-5521(02)00125-4

Long, K. S., Poehlsgaard, J., Kehrenberg, C., Schwarz, S., & Vester, B. (2006). The Cfr rRNA Methyltransferase Confers Resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A Antibiotics.

Antimicrobial Agents and Chemotherapy, *50*(7), 2500–2505. https://doi.org/10.1128/AAC.00131-06

Long, T. E., & Williams, J. T. (2014). Cephalosporins currently in early clinical trials for the treatment of bacterial infections. *Expert Opinion on Investigational Drugs*, *23*(10), 1375–1387. https://doi.org/10.1517/13543784.2014.930127

Luria, S. E., & Delbrück, M. (1943). MUTATIONS OF BACTERIA FROM VIRUS SENSITIVITY TO VIRUS RESISTANCE. *Genetics*, *28*(6), 491–511. https://doi.org/10.1093/genetics/28.6.491

Macomber, L., & Imlay, J. A. (2009). The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proceedings of the National Academy of Sciences*, *106*(20), 8344–8349. https://doi.org/10.1073/pnas.0812808106

Mathew, R., & Chatterji, D. (2006). The evolving story of the omega subunit of bacterial RNA polymerase. *Trends in Microbiology*, *14*(10), 450–455. https://doi.org/10.1016/j.tim.2006.08.002

McDermott, W. (1958). Microbial persistence. *The Yale Journal of Biology and Medicine*, *30*(4), 257–291.

Melnyk, A. H., Wong, A., & Kassen, R. (2015). The fitness costs of antibiotic resistance mutations. *Evolutionary Applications*, *8*(3), 273–283. https://doi.org/10.1111/eva.12196

Menninger, J. R., & Otto, D. P. (1982). Erythromycin, carbomycin, and spiramycin inhibit protein synthesis by stimulating the dissociation of peptidyl-tRNA from ribosomes. *Antimicrobial Agents and Chemotherapy*, *21*(5), 811–818. https://doi.org/10.1128/AAC.21.5.811

Miller, W. R., Munita, J. M., & Arias, C. A. (2014). Mechanisms of antibiotic resistance in enterococci. *Expert Review of Anti-Infective Therapy*, *12*(10), 1221–1236. https://doi.org/10.1586/14787210.2014.956092

Mindlin, S., Minakhin, L., Petrova, M., Kholodii, G., Minakhina, S., Gorlenko, Z., & Nikiforov, V. (2005). Present-day mercury resistance transposons are common in bacteria preserved in permafrost grounds since the Upper Pleistocene. *Research in Microbiology*, *156*(10), 994–1004. https://doi.org/10.1016/j.resmic.2005.05.011

Moncalián, G., Valle, M., Valpuesta, J. M., & De La Cruz, F. (1999). IHF protein inhibits cleavage but not assembly of plasmid R388 relaxosomes. *Molecular*

Microbiology, *31*(6), 1643–1652. https://doi.org/10.1046/j.1365-2958.1999.01288.x

Munita, J. M., & Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiology Spectrum*, *4*(2), 10.1128/microbiolspec.VMBF-0016–2015. https://doi.org/10.1128/microbiolspec.VMBF-0016-2015

Munson, G. P., Lam, D. L., Outten, F. W., & O'Halloran, T. V. (2000). Identification of a Copper-Responsive Two-Component System on the Chromosome of Escherichia coli K-12. *Journal of Bacteriology*, *182*(20), 5864–5871. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC94710/

Murray, C. J., Ikuta, K. S., Sharara, F., Swetschinski, L., Aguilar, G. R., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S. C., Browne, A. J., Chipeta, M. G., Fell, F., Hackett, S., Haines-Woodhouse, G., Hamadani, B. H. K., Kumaran, E. A. P., McManigal, B., … Naghavi, M. (2022). Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. *The Lancet*, *399*(10325), 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0

Naas, T., Mikami, Y., Imai, T., Poirel, L., & Nordmann, P. (2001). Characterization of In53, a Class 1 Plasmid- and Composite Transposon-Located Integron of Escherichia coli Which Carries an Unusual Array of Gene Cassettes. *Journal of Bacteriology*, *183*(1), 235–249. https://doi.org/10.1128/JB.183.1.235-249.2001

Nagy, Z., & Chandler, M. (2004). Regulation of transposition in bacteria. *Research in Microbiology*, *155*(5), 387–398. https://doi.org/10.1016/j.resmic.2004.01.008

Nataro, J. P., & Kaper, J. B. (1998). Diarrheagenic Escherichia coli. *Clinical Microbiology Reviews*, *11*(1), 142–201. https://doi.org/10.1128/CMR.11.1.142

Nikaido, H. (1994). Prevention of drug access to bacterial targets: Permeability barriers and active efflux. *Science (New York, N.Y.)*, *264*(5157), 382–388. https://doi.org/10.1126/science.8153625

Nikaido, H., & Zgurskaya, H. I. (2001). AcrAB and related multidrug efflux pumps of Escherichia coli. *Journal of Molecular Microbiology and Biotechnology*, *3*(2), 215–218.

Oliva, B., Gordon, G., McNicholas, P., Ellestad, G., & Chopra, I. (1992). Evidence that tetracycline analogs whose primary target is not the bacterial ribosome cause lysis of Escherichia coli. *Antimicrobial Agents and Chemotherapy*, *36*(5), 913–919. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC188751/

Pal, C., Bengtsson-Palme, J., Kristiansson, E., & Larsson, D. G. J. (2015). Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genomics*, *16*(1), 964. https://doi.org/10.1186/s12864-015-2153-5

Park, S.-C., Lee, K., Kim, Y. O., Won, S., & Chun, J. (2019). Large-Scale Genomics Reveals the Genetic Characteristics of Seven Species and Importance of Phylogenetic Distance for Estimating Pan-Genome Size. *Frontiers in Microbiology*, *10*. https://www.frontiersin.org/articles/10.3389/fmicb.2019.00834

Partridge, S. R., Kwong, S. M., Firth, N., & Jensen, S. O. (2018). Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clinical Microbiology Reviews*, *31*(4), e00088-17. https://doi.org/10.1128/CMR.00088-17

Perron, K., Caille, O., Rossier, C., van Delden, C., Dumas, J.-L., & Köhler, T. (2004). CzcR-CzcS, a Two-component System Involved in Heavy Metal and Carbapenem Resistance in Pseudomonas aeruginosa*. *Journal of Biological Chemistry*, *279*(10), 8761–8768. https://doi.org/10.1074/jbc.M312080200

Piddock, L. J. V. (2006). Multidrug-resistance efflux pumps—Not just for resistance. *Nature Reviews Microbiology*, *4*(8), Article 8. https://doi.org/10.1038/nrmicro1464

Poole, K., Krebes, K., McNally, C., & Neshat, S. (1993). Multiple antibiotic resistance in Pseudomonas aeruginosa: Evidence for involvement of an efflux operon. *Journal of Bacteriology*, *175*(22), 7363–7372. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC206881/

Poulsen, S. M., Kofoed, C., & Vester, B. (2000). Inhibition of the ribosomal peptidyl transferase reaction by the mycarose moiety of the antibiotics carbomycin, spiramycin and tylosin1 1Edited by D. E. Draper. *Journal of Molecular Biology*, *304*(3), 471–481. https://doi.org/10.1006/jmbi.2000.4229

Ramirez, M. S., & Tolmasky, M. E. (2010). Aminoglycoside modifying enzymes. *Drug Resistance Updates*, *13*(6), 151–171. https://doi.org/10.1016/j.drup.2010.08.003

Ramsay, J. P., Kwong, S. M., Murphy, R. J. T., Yui Eto, K., Price, K. J., Nguyen, Q. T., O'Brien, F. G., Grubb, W. B., Coombs, G. W., & Firth, N. (2016). An updated view of plasmid conjugation and mobilization in Staphylococcus. *Mobile Genetic Elements*, *6*(4), e1208317. https://doi.org/10.1080/2159256X.2016.1208317

Rapisarda, V. A., Montelongo, L. R., Farías, R. N., & Massa, E. M. (1999). Characterization of an NADH-Linked Cupric Reductase Activity from the Escherichia coli Respiratory Chain. *Archives of Biochemistry and Biophysics*, *370*(2), 143–150. https://doi.org/10.1006/abbi.1999.1398

Robicsek, A., Strahilevitz, J., Jacoby, G. A., Macielag, M., Abbanat, D., Hye Park, C., Bush, K., & Hooper, D. C. (2006). Fluoroquinolone-modifying enzyme: A new adaptation of a common aminoglycoside acetyltransferase. *Nature Medicine*, *12*(1), Article 1. https://doi.org/10.1038/nm1347

Rosenberg, S. M., & Hastings, P. J. (2001). F Factor. In S. Brenner & J. H. Miller (Eds.), *Encyclopedia of Genetics* (pp. 677–680). Academic Press. https://doi.org/10.1006/rwgn.2001.0454

Rowe-Magnus, D. A., Guerout, A.-M., Biskri, L., Bouige, P., & Mazel, D. (2003). Comparative Analysis of Superintegrons: Engineering Extensive Genetic Diversity in the Vibrionaceae. *Genome Research*, *13*(3), 428–442. https://doi.org/10.1101/gr.617103

Rozwandowicz, M., Brouwer, M. S. M., Fischer, J., Wagenaar, J. A., Gonzalez-Zorn, B., Guerra, B., Mevius, D. J., & Hordijk, J. (2018). Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy*, *73*(5), 1121–1137. https://doi.org/10.1093/jac/dkx488

Russo, T. A., & Johnson, J. R. (2000). Proposal for a New Inclusive Designation for Extraintestinal Pathogenic Isolates of Escherichia coli: ExPEC. *The Journal of Infectious Diseases*, *181*(5), 1753–1754. https://doi.org/10.1086/315418

Sandegren, L., Linkevicius, M., Lytsy, B., Melhus, Å., & Andersson, D. I. (2012). Transfer of an Escherichia coli ST131 multiresistance cassette has created a Klebsiella pneumoniae-specific plasmid associated with a major nosocomial outbreak. *Journal of Antimicrobial Chemotherapy*, *67*(1), 74–83. https://doi.org/10.1093/jac/dkr405

Schwarz, S., Kehrenberg, C., Doublet, B., & Cloeckaert, A. (2004). Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS*

Microbiology Reviews, *28*(5), 519–542. https://doi.org/10.1016/j.femsre.2004.04.001

Silver, S. (1996). Bacterial resistances to toxic metal ions—A review. *Gene*, *179*(1), 9–19. https://doi.org/10.1016/S0378-1119(96)00323-X

Silverman, J. A., Perlmutter, N. G., & Shapiro, H. M. (2003). Correlation of Daptomycin Bactericidal Activity and Membrane Depolarization in Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy*, *47*(8), 2538–2544. https://doi.org/10.1128/AAC.47.8.2538-2544.2003

Smillie, C., Garcillán-Barcia, M. P., Francia, M. V., Rocha, E. P. C., & de la Cruz, F. (2010). Mobility of Plasmids. *Microbiology and Molecular Biology Reviews*, *74*(3), 434–452. https://doi.org/10.1128/MMBR.00020-10

Spanogiannopoulos, P., Waglechner, N., Koteva, K., & Wright, G. D. (2014). A rifamycin inactivating phosphotransferase family shared by environmental and pathogenic bacteria. *Proceedings of the National Academy of Sciences*, *111*(19), 7102–7107. https://doi.org/10.1073/pnas.1402358111

Spoering, A. L., & Lewis, K. (2001). Biofilms and Planktonic Cells of Pseudomonas aeruginosa Have Similar Resistance to Killing by Antimicrobials. *Journal of Bacteriology*, *183*(23), 6746–6751. https://doi.org/10.1128/JB.183.23.6746-6751.2001

Spratt, B. G., & Cromie, K. D. (1988). Penicillin-binding proteins of gram-negative bacteria. *Reviews of Infectious Diseases*, *10*(4), 699–711. https://doi.org/10.1093/clinids/10.4.699

Strahl, H., & Hamoen, L. W. (2010). Membrane potential is important for bacterial cell division. *Proceedings of the National Academy of Sciences*, *107*(27), 12281–12286. https://doi.org/10.1073/pnas.1005485107

Strohmaier, H., Noiges, R., Kotschan, S., Sawers, G., Högenauer, G., Zechner, E. L., & Koraimann, G. (1998). Signal transduction and bacterial conjugation: Characterization of the role of ArcA in regulating conjugative transfer of the resistance plasmid R1. *Journal of Molecular Biology*, *277*(2), 309–316. https://doi.org/10.1006/jmbi.1997.1598

Sutherland, C., & Murakami, K. S. (2018). An Introduction to the Structure and Function of the catalytic core enzyme of Escherichia coli RNA polymerase. *EcoSal Plus*, *8*(1), 10.1128/ecosalplus.ESP-0004–2018. https://doi.org/10.1128/ecosalplus.ESP-0004-2018

Suttle, C. A. (2005). Viruses in the sea. *Nature*, *437*(7057), Article 7057. https://doi.org/10.1038/nature04160

Swaney, S. M., Aoki, H., Ganoza, M. C., & Shinabarger, D. L. (1998). The Oxazolidinone Linezolid Inhibits Initiation of Protein Synthesis in Bacteria. *Antimicrobial Agents and Chemotherapy*, *42*(12), 3251–3255. https://doi.org/10.1128/AAC.42.12.3251

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y., Ouellette, M., Outterson, K., Patel, J., Cavaleri, M., Cox, E. M., Houchens, C. R., Grayson, M. L., Hansen, P., Singh, N., … Zorzet, A. (2018). Discovery, research, and development of new antibiotics: The WHO priority list of antibiotic-resistant bacteria and tuberculosis. *The Lancet Infectious Diseases*, *18*(3), 318–327. https://doi.org/10.1016/S1473-3099(17)30753-3

Terán, W., Felipe, A., Segura, A., Rojas, A., Ramos, J.-L., & Gallegos, M.-T. (2003). Antibiotic-Dependent Induction of Pseudomonas putida DOT-T1E TtgABC Efflux Pump Is Mediated by the Drug Binding Repressor TtgR. *Antimicrobial Agents and Chemotherapy*, *47*(10), 3067–3072. https://doi.org/10.1128/AAC.47.10.3067-3072.2003

The Guardian. (2017, October 13). Antibiotic resistance could spell end of modern medicine, says chief medic. *The Guardian*. https://www.theguardian.com/society/2017/oct/13/antibiotic-resistance-could-spell-end-of-modern-medicine-says-chief-medic

TIME Magazine. (1936, December 28). *Prontosil*. https://web.archive.org/web/20081215084216/http://www.time.com/time/magazine/article/0,9171,771900,00.html

Tipper, D. J., & Strominger, J. L. (1965). Mechanism of action of penicillins: A proposal based on their structural similarity to acyl-D-alanyl-D-alanine. *Proceedings of the National Academy of Sciences of the United States of America*, *54*(4), 1133–1141. https://doi.org/10.1073/pnas.54.4.1133

Tooke, C. L., Hinchliffe, P., Bragginton, E. C., Colenso, C. K., Hirvonen, V. H. A., Takebayashi, Y., & Spencer, J. (2019). β-Lactamases and β-Lactamase Inhibitors in the 21st Century. *Journal of Molecular Biology*, *431*(18), 3472–3500. https://doi.org/10.1016/j.jmb.2019.04.002

Tréfouël, J., Tréfouël, T., Nitti, F., & Bovet, D. (1935). Activate du p-aminophenylsulfamide sur les infections streptococciques. Experimentales de la souris et du lapin. *C. R. Seanc. Soc. Biol.*, *120*, 756–758.

Trimble, M. J., Mlynárčik, P., Kolář, M., & Hancock, R. E. W. (2016). Polymyxin: Alternative Mechanisms of Action and Resistance. *Cold Spring Harbor Perspectives in Medicine*, *6*(10), a025288. https://doi.org/10.1101/cshperspect.a025288

Ubukata, K., Nonoguchi, R., Matsuhashi, M., & Konno, M. (1989). Expression and inducibility in Staphylococcus aureus of the mecA gene, which encodes a methicillin-resistant S. aureus-specific penicillin-binding protein. *Journal of Bacteriology*, *171*(5), 2882–2885. https://doi.org/10.1128/jb.171.5.2882-2885.1989

Vaara, M., Plachy, W. Z., & Nikaido, H. (1990). Partitioning of hydrophobic probes into lipopolysaccharide bilayers. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1024*(1), 152–158. https://doi.org/10.1016/0005-2736(90)90218-D

Van den Bergh, B., Fauvart, M., & Michiels, J. (2017). Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. *FEMS Microbiology Reviews*, *41*(3), 219–251. https://doi.org/10.1093/femsre/fux001

Ventola, C. L. (2015). The Antibiotic Resistance Crisis. *Pharmacy and Therapeutics*, *40*(4), 277–283. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378521/

Vetting, M. W., Hegde, S. S., Wang, M., Jacoby, G. A., Hooper, D. C., & Blanchard, J. S. (2011). Structure of QnrB1, a Plasmid-mediated Fluoroquinolone Resistance Factor*. *Journal of Biological Chemistry*, *286*(28), 25265–25273. https://doi.org/10.1074/jbc.M111.226936

von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., Savelkoul, P. H. M., & Wolffs, P. F. G. (2016). Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in Microbiology*, *7*, 173. https://doi.org/10.3389/fmicb.2016.00173

Waglechner, N., Culp, E. J., & Wright, G. D. (2021). Ancient Antibiotics, Ancient Resistance. *EcoSal Plus*, *9*(2), eESP-0027-2020. https://doi.org/10.1128/ecosalplus.ESP-0027-2020

Wainwright, M., & Kristiansen, J. E. (2011). On the 75th anniversary of Prontosil. *Dyes and Pigments*, *88*(3), 231–234. https://doi.org/10.1016/j.dyepig.2010.08.012

Wehrli, W. (1983). Rifampin: Mechanisms of Action and Resistance. *Reviews of Infectious Diseases*, *5*(Supplement_3), S407–S411. https://doi.org/10.1093/clinids/5.Supplement_3.S407

Westfall, L. W., Carty, N. L., Layland, N., Kuan, P., Colmer-Hamood, J. A., & Hamood, A. N. (2006). MvaT mutation modifies the expression of the Pseudomonas aeruginosa multidrug efflux operon mexEF-oprN. *FEMS Microbiology Letters*, *255*(2), 247–254. https://doi.org/10.1111/j.1574-6968.2005.00075.x

Womble, D. D., & Rownd, R. H. (1988). Genetic and physical map of plasmid NR1: Comparison with other IncFII antibiotic resistance plasmids. *Microbiological Reviews*, *52*(4), 433–451. https://doi.org/10.1128/mr.52.4.433-451.1988

Wurmbach, P., & Nierhaus, K. H. (1983). The Inhibition Pattern of Antibiotics on the Extent and Accuracy of tRNA Binding to the Ribosome, and Their Effect on the Subsequent Steps in Chain Elongation. *European Journal of Biochemistry*, *130*(1), 9–12. https://doi.org/10.1111/j.1432-1033.1983.tb07109.x

Yarlagadda, V., Manjunath, G. B., Sarkar, P., Akkapeddi, P., Paramanandham, K., Shome, B. R., Ravikumar, R., & Haldar, J. (2016). Glycopeptide Antibiotic To Overcome the Intrinsic Resistance of Gram-Negative Bacteria. *ACS Infectious Diseases*, *2*(2), 132–139. https://doi.org/10.1021/acsinfecdis.5b00114

Yoshida, H., Bogaki, M., Nakamura, M., & Nakamura, S. (1990). Quinolone resistance-determining region in the DNA gyrase gyrA gene of Escherichia coli. *Antimicrobial Agents and Chemotherapy*, *34*(6), 1271–1272. https://doi.org/10.1128/AAC.34.6.1271

Zackrisson, M., Hallin, J., Ottosson, L.-G., Dahl, P., Fernandez-Parada, E., Ländström, E., Fernandez-Ricaud, L., Kaferle, P., Skyman, A., Stenberg, S., Omholt, S., Petrovič, U., Warringer, J., & Blomberg, A. (2016). Scan-o-matic: High-Resolution Microbial Phenomics at a Massive Scale. *G3: Genes|Genomes|Genetics*, *6*(9), 3003–3014. https://doi.org/10.1534/g3.116.032342

Zeng, D., Debabov, D., Hartsell, T. L., Cano, R. J., Adams, S., Schuyler, J. A., McMillan, R., & Pace, J. L. (2016). Approved Glycopeptide Antibacterial Drugs: Mechanism of Action and Resistance. *Cold Spring Harbor Perspectives in Medicine*, *6*(12), a026989. https://doi.org/10.1101/cshperspect.a026989

Zhanel, G. G., Hoban, D. J., & Harding, G. K. (1992). Subinhibitory antimicrobial concentrations: A review of in vitro and in vivo data. *The Canadian Journal of Infectious Diseases*, *3*(4), 193–201. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3328040/

Zhang, W.-J., Xu, X.-R., Schwarz, S., Wang, X.-M., Dai, L., Zheng, H.-J., & Liu, S. (2014). Characterization of the IncA/C plasmid pSCEC2 from Escherichia coli of swine origin that harbours the multiresistance gene cfr. *The Journal of Antimicrobial Chemotherapy*, *69*(2), 385–389. https://doi.org/10.1093/jac/dkt355