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# Antibiotic interactions and selection for resistance in biofilms

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## Abstract

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The challenges posed by antibiotic-resistant bacteria in treating infections, particularly those associated with biofilms, require a deeper understanding of this lifestyle and its connection to resistance selection. Additionally, gaining insights into drug interactions is crucial for enhancing combination treatment efficacy and mitigating resistance development. This thesis is divided into these two main themes, each consisting of individual papers with specific objectives and aims that tackle these two themes.

The first introduces a proof-of-concept microfluidic chip named Brimor, which demonstrates the selection of ciprofloxacin-resistant mutants in *Escherichia coli* biofilms at concentrations below the minimum inhibitory concentration (sub-MIC). Brimor exhibits potential applications beyond antibiotics and bacteria.

The second explores the emergence of resistance in both planktonic and biofilm lifestyles. Using the FlexiPeg model and uropathogenic *E. coli*, the fitness cost and minimal selective concentrations were assessed for five antibiotics and six resistance-conferring mutations during biofilm and planktonic growth. This analysis revealed resistance development in both lifestyles at sub-MIC.

Furthermore, an assay called CombiANT<sup>®</sup> was developed and validated with three major pathogens, enabling simple quantification and subsequent categorization of antibiotic interactions. This assay demonstrated comparable performance to the gold-standard checkerboard and time-kill assays. CombiANT<sup>®</sup> also shows potential for applications beyond antibiotics and bacteria.

Isolate-specific interaction profiling was emphasized as crucial among five important Gram-negative pathogens for achieving precise and effective combination therapy. Interactions of clinically used antibiotic combinations varied significantly between and within susceptible species, with additive and antagonistic interactions being the most common. Only a small percentage exhibited clinically relevant synergy.

The mutations associated with synergy and loss of synergy for the tetracycline and spectinomycin combination in *E. coli* was elucidated. Genetic changes associated with efflux regulation and metabolic pathways were identified as factors contributing to the loss of synergy in mutants. The bioavailability model was the prevailing mechanism of action accounting for synergy and loss of synergy for the combination.

In summary, the papers presented in this thesis provide valuable insights on antibiotic resistance selection in biofilms, antibiotic interactions, and the development of innovative tools for studying biofilms and combination therapies. Further understanding of these factors is necessary for applying these findings in clinical settings and to optimize combination strategies for effective personalized therapy and antibiotic stewardship.

**Keywords:** antibiotics

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*To my biological & chosen family*

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# List of Papers

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

- I. **Tang, PC.**, Eriksson, O., Sjögren, J., Fatsis-Kavalopoulos, N., Kreuger, J., Andersson, D.I. (2022) A microfluidic chip for studies of the dynamics of antibiotic resistance selection in bacterial biofilms. *Front. Cell. Infect. Microbiol.*, 12:896149
- II. Hjort, K., Fermér, E., **Tang, PC.**, Andersson, D.I. (2022) Antibiotic minimal selective concentrations and fitness costs during biofilm and planktonic growth. *mBio*, 13(3):e014472
- III. Fatsis-Kavalopoulos, N., Roemhild, R., **Tang, PC.**, Kreuger, J., Andersson, D.I. (2020) CombiANT: Antibiotic interaction testing made easy. *PLoS Biol.*, 18(9):e3000856
- IV. **Tang, PC.**, Sánchez-Hevia, D.L.<sup>⊗</sup>, Westhoff, S.<sup>⊗</sup>, Fatsis-Kavalopoulos, N.<sup>⊗</sup>, Andersson, D.I. Low conservation of antibiotic interactions between and within Gram-negative bacterial species. *Under review in PLoS Biology*
- V. **Tang, PC.**, Valdaliso, E., Sánchez-Hevia, D.L., Westhoff, S., Andersson, D.I. Mutations that alter the synergistic interaction of tetracycline and spectinomycin in *Escherichia coli*. *Manuscript*

<sup>⊗</sup>These authors contributed equally.

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Other papers/manuscripts by the author not included in this thesis:

- I. Westhoff, S., **Tang, PC.**, Sánchez-Hevia, D.L., Belliveau, D.J., Andersson, D.I. A genetic approach to understand beta-lactam and aminoglycoside interactions in *Escherichia coli*. *Manuscript*
- II. Sánchez-Hevia, D.L., Westhoff, S., **Tang, PC.**, Andersson, D.I. Decoding the synergistic interaction between trimethoprim and nitrofurantoin in *Escherichia coli*. *Manuscript*
- III. Guðmundsdóttir, J.S., Fredheim, E.G.A., Koumans, C.I.M., Hegstad, J., **Tang, PC.**, Andersson, D.I., Samuelsen, Ø., Johnsen, P.J. (2021) The chemotherapeutic drug methotrexate selects for antibiotic resistance. *EBioMedicine*, 74:103742
- IV. Puértolas-Balint, F., Warsi, O., Linkevicius, M., **Tang, PC.**, Andersson, D.I. (2020) Mutations that increase expression of the EmrAB-TolC efflux pump confer increased resistance to nitroloxline in *Escherichia coli*. *J. Antimicrob. Chemother.*, 75(2):300-308
- V. Lundin, E. **Tang, PC.**, Guy, L., Näsval, J., Andersson, D.I. (2018) Experimental determination and prediction of the fitness effects of random point mutations in the biosynthetic enzyme HisA. *Mol. Biol. Evol.*, 35(3):704-718

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# Abbreviations

AMR	antimicrobial resistance
ABR	antibiotic resistance
$\beta$	beta
COVID	corona virus disease
DNA	deoxyribonucleic acid
ESKAPE	<i><u>E</u>nterococcus faecium, <u>S</u>taphylococcus aureus, <u>K</u>lebsiella pneumoniae, <u>A</u>cinetobacter baumannii, <u>P</u>seudomonas aeruginosa, and <u>E</u>nterobacter species</i>
FICI	fractional inhibitory concentration index
MBEC	minimum biofilm eradication concentration
MDR	multidrug-resistant
MIC	minimum inhibitory concentration
MSC	minimal selective concentration
PBP	penicillin-binding protein
PDMS	polydimethylsiloxane
RNA	ribonucleic acid
sub-MIC	sub-minimal inhibitory concentrations



# Preface

*“Just like the COVID-19 pandemic, antimicrobial resistance (AMR) is no longer a future threat. It is happening here and now and is affecting us all. If AMR is left unchecked, the next pandemic we face could be bacterial and much deadlier if the drugs needed to treat it do not work.”*

Maria Helena Semedo,  
Deputy Director-General of the  
Food and Agriculture Organization of the United Nations

These words illustrate the risk of AMR and emphasize its significance. The discovery of penicillin by Alexander Fleming<sup>1</sup> and its subsequent use in therapy have resulted in remarkable medical advancements, improving the quality and length of life. Life-threatening infectious diseases such as sepsis or pneumonia have become curable with these drugs, often with minimal side effects. We now rely on these medications to manage the risk of infections associated with various medical procedures, including chemotherapy, invasive and replacement surgeries, organ transplants, immunosuppressive therapy, and neonatal care. Recognizing the importance of combating antimicrobial resistance, global efforts were initiated in the early 1940s to prevent its occurrence, particularly by developing new antibiotic compounds for therapeutic purposes. While this undoubtedly remains a central endeavor, the absence of novel drugs for therapeutic use in the last 30 years has necessitated alternative actions. Consequently, numerous public reports<sup>2–4</sup> commissioned to guide world leaders and policy-makers have sought to assess the future implications for global health and the socio-economic consequences of AMR. Despite the complexity of the issue, ongoing initiatives, including international agreements regulating antimicrobial use and stewardship, innovative economic strategies, reimbursement plans for drug development and production, as well as global efforts to enhance sanitation, hygiene, and healthcare infrastructure, are already underway<sup>5,6</sup>. Prior to the COVID-19 pandemic, addressing AMR required urgent and immediate attention. However, with resources redirected toward the pandemic response, evidence suggests substantial pre-emptive antibiotic use, worsening economic conditions, and increased poverty, all of which could impact the levels of AMR<sup>7,8</sup>. AMR remains a significant threat in the challenging and ever-evolving global landscape of the 21st century, with an estimated 4.95

million deaths attributed to bacterial AMR in 2019 alone<sup>9</sup>. To address this pressing issue, a multidisciplinary cross-sectoral approach is essential to develop innovative solutions<sup>10–12</sup>.

# Background

## Antibiotics

Antibiotics are a group of antimicrobial chemical substances that inhibit the progression of bacterial growth and replication. The term encompasses a diverse range of molecules that exhibit significant variations in their chemical structure, size, and properties. These substances can be classified based on various criteria, such as their activity spectrum, cellular target, mode of action, origin, or chemical structure. The latter is commonly employed in standard classifications. In general, chemical substances sharing the same structure exhibit similarities in their characteristics.

## Classes

The  $\beta$ -lactam class of antibiotics, including but not limited to, penicillins, carbapenems, cephalosporins and monobactams, all share a  $\beta$ -lactam ring. This class is commonly used to treat various bacterial infections caused by both Gram-negative (cephalosporins, carbapenems, monobactams) and Gram-positive (penicillins) bacteria. It has a broad-spectrum activity against bacteria and is widely utilized in clinical settings due to its minimal side effects<sup>13</sup>. The aminoglycoside class of antibiotics all share a structure comprising an amino sugar linked to an inositol derivative. This class includes but is not limited to gentamicin, tobramycin, streptomycin, amikacin, and kanamycin. It targets mainly Gram-negative and some Gram-positive aerobic bacteria but can lead to severe side effects such as nephrotoxicity and ototoxicity. Consequently, the clinical use of this class is limited but significant<sup>14</sup>. The quinolone class of antibiotics all have a central bicyclic structure, with many containing an additional fluorine atom (fluoroquinolones). This class exhibits broad-spectrum activity against both Gram-negative and Gram-positive bacteria. Ciprofloxacin, the most prominent representative of this class, is commonly used to treat a wide range of infections, including urinary and respiratory tract infections<sup>15</sup>. The polymyxins class of antibiotics all share a cyclic heptapeptide structure with a fatty acid tail acylated at the N-terminus. This class is unique and has a similar chemical structure to cationic antimicrobial peptides, which are the first line of defence against bacterial colonization in eukaryotic cells<sup>16</sup>. Polymyxins have toxic side effects and are primarily reserved as last resort antibiotics for the treatment of multidrug-resistant (MDR) infections, particularly

against Gram-negative bacteria<sup>17</sup>. Other important antibiotic classes and examples include, but are not limited to, glycopeptides (such as vancomycin), pyrimidines (such as trimethoprim), tetracyclines (such as tigecycline), rifamycins (such as rifampin), and nitrofurantoin derivatives (such as nitrofurantoin). These classes have recently been extensively described and I refer the reader to the reviews discussing them<sup>18,19</sup>.

## Modes of action

Generally, bactericidal antibiotics lead to cell death, while bacteriostatic antibiotics prevent the progression of cell division.  $\beta$ -lactam antibiotics, quinolones, and nitrofurantoin are typical examples of bactericidal antibiotics. Chloramphenicol, tetracyclines, and macrolides, on the other hand, are typical examples of bacteriostatic antibiotics. It is rare for an antibiotic to be exclusively bacteriostatic or bactericidal. Disturbance in cellular processes is essential to exert inhibitory effects on bacteria. Factors such as growth conditions, bacterial density, and antibiotic concentration can influence the effectiveness of the treatment outcome<sup>20</sup>. The targets of cellular processes vary within bacteria (Fig. 1). Antibiotics that target the same pathway or molecule in a cell can still have different modes of action and belong to different classes. The peptidoglycan in the bacterial cell wall is unique to this domain of life and absent in eukaryotic cells, making it an attractive target for antibiotics. For the majority of antibiotics, the primary target is the bacterial cell wall, as is the case with all  $\beta$ -lactams. The cell wall comprises an inner membrane, a periplasmic space, a thin peptidoglycan layer, and an outer membrane in Gram-negative bacteria, or an inner membrane, a periplasmic space, and a thick peptidoglycan layer in Gram-positive bacteria. The peptidoglycan layer is composed of N-acetylglucosamine and N-acetylmuramic acid disaccharides crosslinked via pentapeptides<sup>21</sup>. The cell membrane is another target, as seen with colistin (also known as polymyxin E). Alternatively, antibiotics can target proteins involved in transcription, translation, and replication. Fluoroquinolones inhibit DNA synthesis by targeting two essential enzymes, topoisomerase IV and DNA gyrase, which are crucial for bacterial growth<sup>22</sup>. Trimethoprim and sulfonamides interfere with the synthesis of tetrahydrofolic acid by inhibition of dihydrofolate reductase or dihydropteroate synthetase, respectively. Tetrahydrofolic acid is a precursor for the essential amino acid thymidine<sup>23</sup>. Rifampicin binds to bacterial RNA polymerase and inhibits elongation of newly synthesized transcripts<sup>24</sup>. Macrolides target the large ribosome subunit and block the exit tunnel, while aminoglycosides target the small ribosome subunit and disrupt the elongation of newly synthesized amino acid chains, leading to the accumulation of mis-translated proteins. Both classes ultimately disrupt protein synthesis and inhibit growth<sup>14,25</sup>. Fusidic acid targets an essential step during protein synthesis by preventing the translocation of elongation factor G. Quinolone antibiotics directly target DNA replication by

inhibiting DNA gyrases and topoisomerase IV enzymes, resulting in erroneous unwinding of DNA, introduction of double-strand breaks, and cell death<sup>26</sup>.

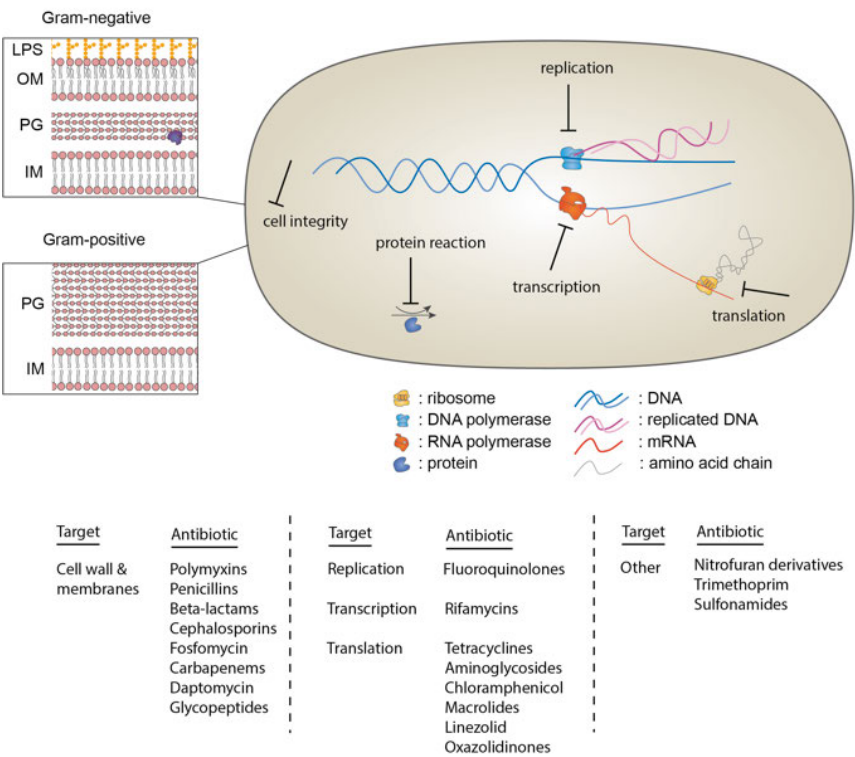


Fig. 1: Major mode of action of antibiotics is revolve around cell growth (replication, transcription, translation) and integrity (membranes of Gram-negative and -positive shown). The targets and corresponding antibiotics are depicted. LPS: Lipopolysaccharide, OM: outer membrane, PG: peptidoglycan, IM: inner membrane, PBP: penicillin binding protein.

## Antibiotic Resistance

Antibiotic resistance (ABR) emerged as a serious global issue in the 1940s and triggered a response from the pharmaceutical industry in the 1950s. During this period, new classes of antibiotics were discovered (known as the golden age of antibiotic discovery) and introduced into the market for therapeutic use (Fig. 2). This gave hope that the observed antibiotic resistance could be overcome through continuous discovery and the use of novel drug classes, especially when resistance to penicillin was already observed<sup>27</sup>. However, resistance began to emerge for all drug classes, sometimes even before their

introduction into clinical practice (Fig. 2). This seemingly paradoxical situation can be explained by the fact that natural microbes had long encountered antibiotics before their medical use<sup>28</sup>. Many of the resistance mechanisms we observe today are ancient and have evolved in bacteria as a means of communication, protection, or destruction<sup>29,30</sup>. With the end of the golden age, pharmaceutical industries gradually withdrew from drug discovery due to a “discovery void”<sup>31</sup>.

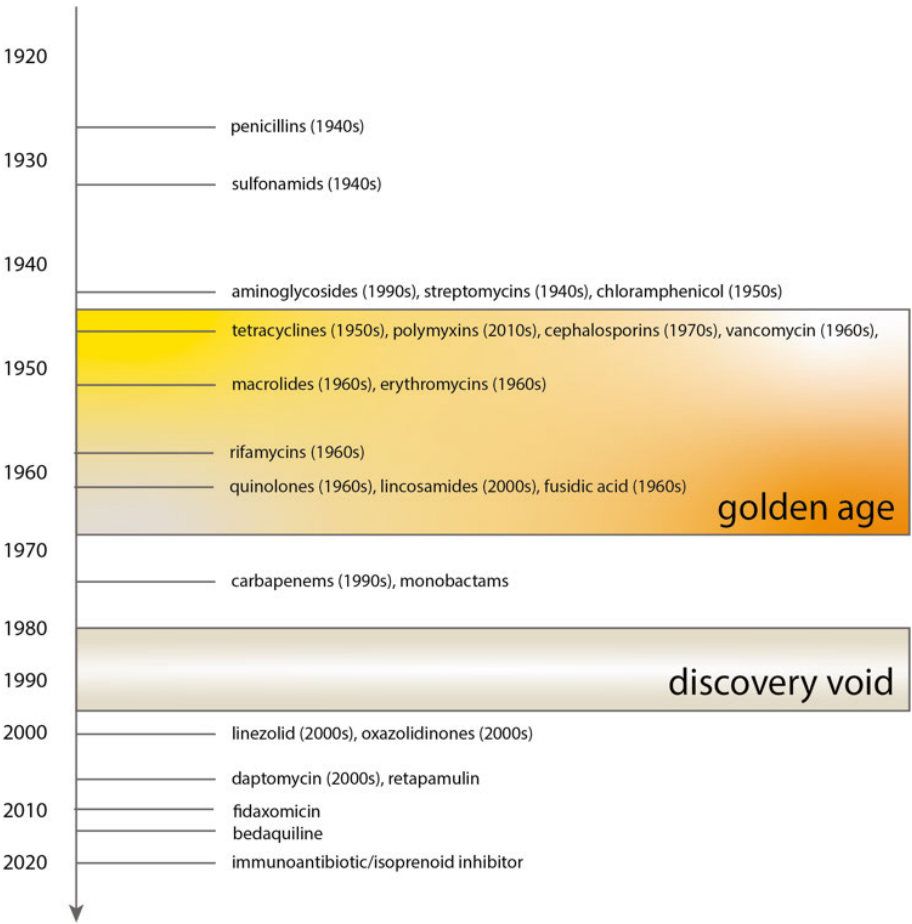


Fig. 2: Major classes of antibiotic discovery and the corresponding reported clinical resistance (years in brackets) from the 1920s to 2020. The period from the mid-1940s to the 1960s is considered the “golden age” of discovery, while the period from the mid-1980s to the 1990s is known as the “discovery void”.

## Mechanisms of resistance

Decreasing intracellular antibiotic concentration, evading antibiotic activity or functional inactivation, and target site alteration, protection or bypass are the molecular mechanisms of resistance employed by bacteria (Fig. 3)<sup>19,32</sup>. The diversity of resistance mechanisms is not surprising given the varied modes of action of antibiotics.

Reduced permeability into the cell and increased antibiotic efflux both contribute to preventing antibiotic access to its target. The lower permeability of the outer membrane in Gram-negative bacteria, compared to Gram-positive bacteria, forms an intrinsic physical barrier. Hydrophilic antibiotics cross the barrier by diffusing through outer membrane porin proteins, such as OmpF and OmpC in *Escherichia coli*<sup>33</sup>. Replacement of porins with selective channels or downregulation of porins has been observed in both Gram-negative and Gram-positive bacteria to reduce the influx of antibiotics, thereby providing phenotypic resistance<sup>34</sup>. When efflux pumps are overexpressed, high levels of resistance to previously useful antibiotics have been observed<sup>35</sup>. Well-studied examples of multidrug efflux pumps include AcrAB in *E. coli*, MexAB in *Pseudomonas aeruginosa*, KexD in *Klebsiella pneumoniae*, and LmrS in *Staphylococcus aureus*<sup>36–40</sup>.

Evading antibiotic activity by functionally inactivating an antibiotic through hydrolysis or transfer of a chemical group is an effective pathway to antibiotic resistance. The most prominent example of this mechanism is  $\beta$ -lactamases. For example, penicillinase enzymes capable of modifying penicillin were already observed in 1940<sup>27</sup>. Diverse enzymes that degrade and modify antibiotics of different classes, including aminoglycosides,  $\beta$ -lactams, and macrolides, have been identified since then. The emergence of enzymes with altered hydrolytic activity spectra has paralleled the development of antibiotic classes. One example is extended-spectrum  $\beta$ -lactamases, which have activity against all three generations of cephalosporins<sup>41</sup>. Notably, antibiotic resistance is not limited to the bacterium producing the enzyme needed to inactivate the antibiotic. Instead, indirect resistance can arise where the bacterium producing the enzyme protects other bacteria<sup>42</sup>.

Most antibiotics specifically bind to their targets with high affinity to exert their antibacterial effects. Bacteria can make changes to antibiotic targets by accumulating mutations that decrease the affinity to the antibiotic without disrupting its original cellular function. This resistance mechanism typically arises from chromosomal mutations and is not horizontally transferred. Examples of this include mutations in genes *gyrA*, *rpoB*, and *rpsL*, which can increase resistance towards ciprofloxacin, rifampicin, and streptomycin, respectively. In addition to direct alteration of the antibiotic target, horizontal transfer of homologous genes with low affinity for the antibiotic, which can functionally replace the inhibited cellular target, can result in resistance. An example of this is methicillin-resistant *S. aureus*, where acquisition of the

*mecA* gene within the chromosomal *mec* element enables cell wall biosynthesis to occur despite the inhibition of native penicillin-binding protein (PBP) in the presence of the antibiotic<sup>43</sup>. The MecA protein encodes a functional  $\beta$ -lactam-incentive PBP 2a. For some antibiotics, several modes of resistance mechanisms exist. For example, resistance to tetracycline can be mediated by genes coding for ribosomal protection by *tet(M)*, reduced intracellular antibiotic concentration by *tet(A)* or *acrAB*, or drug inactivation by *tet(X)*<sup>44</sup>.

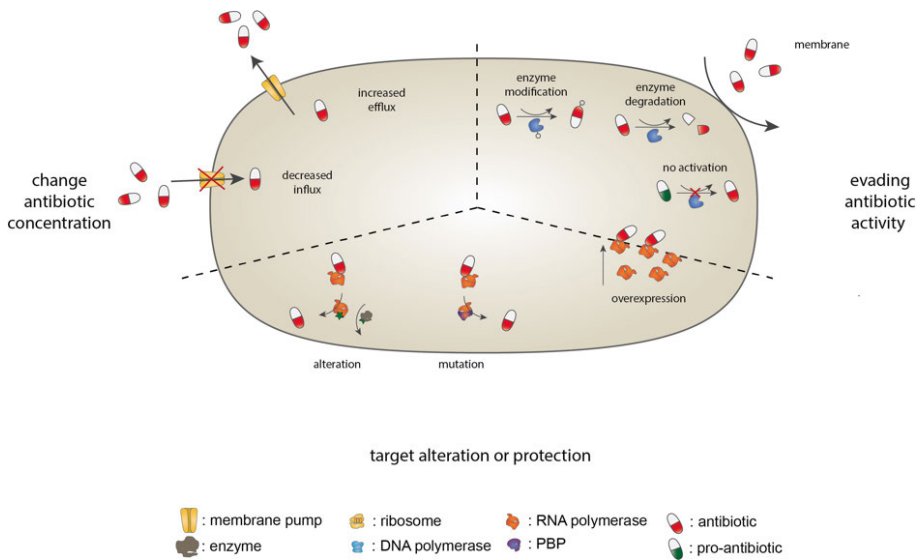


Fig. 3: Overview of antibiotic resistance mechanisms. Bacteria have evolved diverse ways to mitigate the effects of antibiotics, including changes in antibiotic concentration, evading the activity of antibiotics, and modifying or protecting the antibiotic target.

## Evolution and selection

Evolution is a broad and complex subject that cannot be comprehensively addressed here. Instead, important aspects relevant ABR and to this thesis is discussed. Understanding the emergence of genetic diversity is key to understanding the evolution of ABR. Broadly speaking, genetic information is maintained and transmitted in two mechanisms: vertical and horizontal<sup>45</sup>. Vertical evolution involves mutations that are selected and passed on to the progeny, whilst horizontal evolutions refer to the acquisition of resistance genes from other bacteria through conjugation, transduction, or transformation, followed by transmitting to the progeny. The development of ABR can occur through one or both of these mechanisms. In vertical evolution, errors during DNA replication in genes encoding the antibiotic target give rise to *de novo* mutation. These mutations can, for example, reduce the affinity towards the

drug. Alternatively, erroneous DNA replication can also lead to loss-of-function mutations in negative regulators, which results in de-regulation of gene(s) due to increased export or decreased import of antibiotics. The levels of resistance caused by this type of mutation can vary significantly for different antibiotics. In general, the contribution of vertical or horizontal evolutionary paths to the development of clinical ABR is not yet fully elucidated, but comprehensive genomic characterization of human pathogens over the past decades has shown horizontal evolution is the primary contributor to the selection of ABR human pathogens<sup>45</sup>.

It is easy to comprehend the advantages provided by resistance genes or mutations in relation to lethal concentrations of antibiotics, as bacteria lacking resistance mechanisms are unable to survive and grow, and ultimately die. However, bacteria often inhabit dynamic environments and are exposed to various concentrations of antimicrobial agents that vary in time and space. Consequently, assessing the strength of selection in the resistance conferring mechanism becomes challenging. Instead, the outcome of evolution changes depending on the antibiotic concentrations, whether the concentrations inhibit the growth of the pathogen (above the minimum inhibitory concentration,  $> \text{MIC}$ ) or allow for the growth of both susceptible and resistant bacteria ( $< \text{MIC}$ ). The MIC of an antibiotic refers to the lowest concentration that inhibits the growth of bacteria. The MIC of a resistant strain ( $\text{MIC}_{\text{res}}$ ) would thus be higher than the MIC of a susceptible strain ( $\text{MIC}_{\text{susc}}$ ). Many resistance genes or mutations carry a fitness cost (described in the following section) in the absence of antibiotics and may be selected against in absence of antibiotic pressure<sup>46,47</sup>. Conversely, this fitness cost can be reduced or reversed by compensatory mutations either in the mutated gene or in a different gene entirely<sup>46</sup>. Therefore, selection of ABR is dependent on not only the concentration of the antibiotic but also fitness cost.

The mutant selective window hypothesis is a prominent theory that explains the selection of resistant mutants. According to this hypothesis, the selection process occurs within a concentration range that extends from the MIC of the susceptible strain to the MIC of the resistant mutant<sup>48,49</sup> (Fig. 4). Therefore, majority of literature has focused on how high levels of antibiotics are needed to avoid the enrichment of resistance, known as the mutant preventative concentration. However, experimental evidence suggests that sub-minimal inhibitory concentrations (sub-MIC) also play a crucial role in selection of resistance mutants, known as the sub-MIC selection window. Furthermore, when exposed to mixtures of drugs, resistance can be selected at even lower concentrations, known as the co-selective concentration window<sup>50-54</sup>.

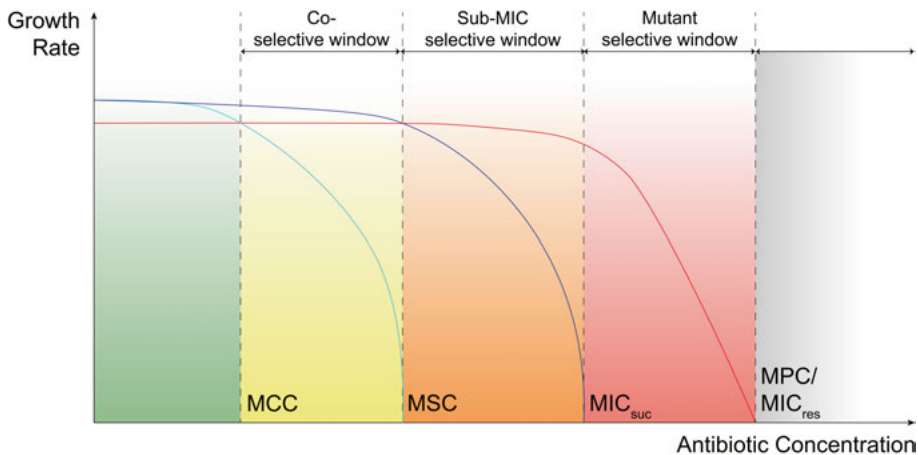


Fig. 4: Theoretical graph depicting growth rates as a function of antibiotic concentration. The resistance strain (red line) exhibits a lower initial growth rate and will be outcompeted by the susceptible strain in the presence (light blue line) and absence (dark blue line) of a combination of antibiotics. Green field indicates the lowest concentration interval where the susceptible strain will outcompete the resistant strain. Orange and red fields indicate the lowest concentration in which resistant strain will outcompete the susceptible strain. Yellow field indicates an even lower concentration interval where the resistant strain will outcompete susceptible strain in the presence of combinations of antibiotics.  $MPC/MIC_{res}$  = mutant preventative concentration/minimal inhibitory concentration of the resistant strain,  $MIC_{sus}$  = minimal inhibitory concentration of the susceptible strain,  $MSC$  = minimal selective concentration, and  $MCC$  = minimal co-selective concentration.

## Bacterial fitness

Fitness is a context-dependent term that describes the ability of a bacterium to survive, proliferate, and transmit between hosts or the environment. Bacteria have naturally evolved to increase their fitness and competitiveness in order to ensure survival, growth, and transmission. This definition suggests that fitness is not constant or dichotomous, depending solely on whether lethal antibiotic selection is present or not. The fitness effects of specific traits on bacteria vary depending on the environmental conditions<sup>46</sup>. Most antibiotic resistance mechanisms are associated with a fitness cost, which influences the development of resistance<sup>55,56</sup>. The magnitude of the fitness cost is a key driver that determines the stability of resistance mechanisms at the bacterial community level, the rate of resistance development, and the rate at which resistance might decrease if antibiotics were absent from the environment.

Various methods have been developed to assess bacterial fitness, but due to the variability in fitness determinants, no single method can comprehensively determine absolute fitness across different hosts and environments. Instead, multiple isolated fitness components can be measured with high

accuracy. The fastest, cheapest, and easiest method is comparing the maximum exponential growth rate between bacteria, where a lower growth rate indicates lower fitness. Other methods include direct competitions, the ability to withstand different stresses (such as bile salts, temperature, osmolarity), and animal or colonization infection models. Five notable effects have been observed in studies on fitness cost: epistatic effects that can influence fitness cost, the impact of specific environmental conditions on fitness costs, the absence of fitness costs for certain mutations, the potential reduction of fitness costs through regulation of resistance mechanisms, and the connection between fitness cost and compensation for resistance<sup>46</sup>.

Resistance mechanisms are tightly regulated and expressed only when bacteria are exposed to antibiotics. Naturally, resistant bacteria have a clear fitness advantage over susceptible bacteria. For instance, the VanRS two-component system in vancomycin-resistant *Enterococcus* triggers the transcription of *vanHAXY* genes only in the presence of the glycopeptide. In the absence of the drug, the *vanHAXY* genes are switched off<sup>57</sup>. Generally, the acquisition of resistance mechanisms in the absence of antibiotics often comes with a fitness cost. The strong selective pressure for rapid growth favors further mutations in bacteria to compensate for the fitness cost imposed by the resistance mechanism<sup>46</sup>. This process has been observed both *in vitro* and *in vivo*<sup>46</sup>. In some cases, these mutations restore fitness to its original level. Compensatory mechanisms can occur within the same gene that causes the resistance phenotype (intragenic compensation) or in other genes (extragenic compensation). In *Mycobacterium*, the fitness cost of *rpoB* mutations that confer rifampicin resistance is alleviated by secondary mutations in *rpoA*, *rpoB*, or *rpoC*<sup>58–60</sup>. Compensation for growth defects caused by antibiotics can result not only from specific intragenic gene mutations but also from gene duplications, amplification of resistant genes, and regulation of gene dosage<sup>61</sup>.

## Antibiotic combinations

During the golden era of novel antibiotic discoveries, there was a popular trend of attempting to combine new drugs. Many ad-hoc combinations, typically involving two or even higher-order combinations, were used without a deep understanding of the molecular mechanisms or drug efficacy. These attempts were opportunistic and aimed to create patentable medicines<sup>62</sup>. Examples of such combinations include streptomycin combined with penicillin in 1950 and trimethoprim combined with sulfonamides in 1968. The latter combination is still used today<sup>63,64</sup>. The main criteria for these combinations were improved efficacy and a broader antibacterial spectrum for clinical treatment. Although controversial, the application of certain combinations with existing antibiotics has now become widespread in order to maintain clinical efficacy, combat the evolution of resistance, and reduce mortality rates<sup>65–69</sup>. Combining

several antibiotics can be done as simultaneous mixtures or in a sequential order, and both approaches can yield distinct or similar outcomes.

## Classifications

Combinations of antibiotics can be classified into different types based on their targets: congruous, syncretic, or coalistic (Fig. 5). Congruous combinations are characterized by the individual inhibition of cell growth through essential targets. Existing antibiotic combination therapies, such as trimethoprim-sulfonamide, are based on this concept. Syncretic combinations involve at least one component that does not have an essential target for cell growth. An example is the combination of  $\beta$ -lactam antibiotics with  $\beta$ -lactamase inhibitors. Coalism combinations consist of compounds that, on their own, do not inhibit cell growth, but when combined, they result in cell lethality by synthesizing lethal gene products<sup>69</sup>. This concept has not been extensively explored in bacteria and is mostly limited to the *S. cerevisiae* model system, which utilizes a combination of proteomic, chemical-genetic, and machine learning approaches<sup>69</sup>. A modern ongoing approach involves combining antibiotics with non-antibiotic compounds that enhance their activity. This coupling is used as a strategy to prolong the effectiveness of current therapeutic antibiotics and achieve clinically significant levels of synergy<sup>69</sup>.

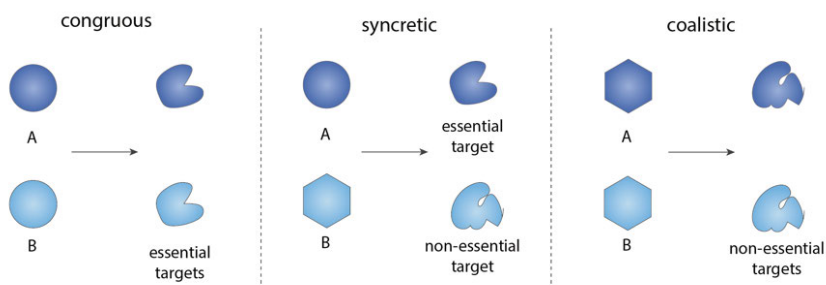


Fig. 5: Classification of antibiotic combinations and combinations with other therapeutic compounds into congruous, syncretic, and coalistic types. Congruous combinations involve the essential target of the compound, while syncretic and coalistic combinations involve non-essential targets of the compound.

Combinations can also be defined based on the response of bacteria, where the effect can be stronger or weaker than expected, known as synergistic, additive, or antagonistic interactions<sup>70</sup>. It is important to consider that these interactions must be defined depending on the context. *In vitro* synergy refers to the administration of two or more bioactive compounds resulting in enhanced activity compared to the expected sum of the individual compounds. On the other hand, diminished activity compared to the expected sum is defined as *in vitro* antagonism. The expected sum of the individual compounds

is defined *in vitro* as additive. *In vitro* screening campaigns typically identify systematic categorical combinations of antibiotics by assessing growth inhibition in the presence of susceptible bacteria. Generally, sublethal concentrations of antibiotic ‘A’ are combined with a candidate antibiotic ‘B’ and assayed. Sublethal concentrations can be, for example, one-quarter of the MIC of a susceptible bacterium.

Several approaches are used for the analysis of antibiotic combinations<sup>71</sup> and can be categorized into effect-based (Fig. 6) or dose-effect-based comparisons (Fig. 7). For effect-based comparisons, the decision process for determining a positive (antagonistic and suppressive), negative (synergy), or null (additive) effect varies among four main strategies, which include combination sub-thresholding, highest single agent, effect additivity or linear interaction effect, and the Bliss-independence model. Suppressive combinations can be directional or reciprocal. Directional combinations indicate that the combined effect is lower than that of one compound, while reciprocal combinations indicate that the combined effect is less than the inhibitory effect of either individual compound<sup>72</sup>. Overall, the effect-based approach compares the antibiotic combination against the measured effect of the individual antibiotics. In dose-effect-based comparisons, the expected additive effect depends on the individual dose-effect curves. This approach provides a more defined definition of synergism, additivity, and antagonism compared to the effect-based approach. Dose-effect-based approaches largely rely on the Loewe additivity mathematical model.

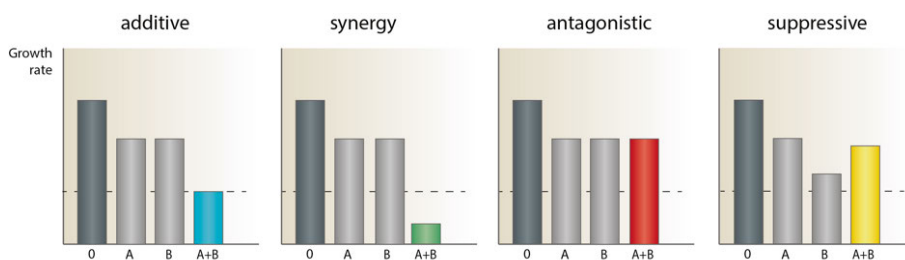


Fig. 6: General effect-based analysis of antibiotic combinations can be categorized as additive, synergistic, antagonistic, or suppressive. The axes represent the increase in response (in this example, growth rate) for different drug combinations (A+B drug), individual drugs (A or B drug), or in the absence of the drug (0). Dotted lines indicate the additive criteria used to determine these categorizations.

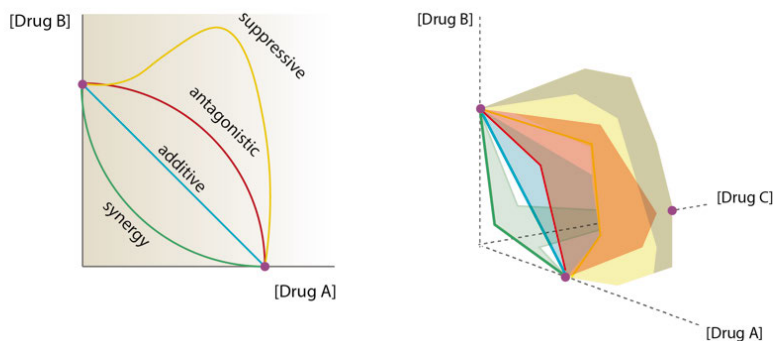


Fig. 7: General dose-effect-based isobolograms of antibiotic combinations for two (left graph) or three (right graph) drug combinations. The axes represent the doses of individual agents. The lines and the area within them represent the combination of concentrations of the two or three drugs required to achieve a particular effect. Purple dots indicate the minimal inhibitory concentration of a drug for a specific bacterial species.

The fractional inhibitory concentration index (FICI) is a microbiological calculation used to determine the synergistic, additive, antagonistic, or suppressive effects of compounds. It utilizes the traditional MIC determination against a specific bacterial isolate for a particular antibiotic. The MIC is measured in liquid culture using a series of antibiotic dilutions. Systematic categorization of combinations is achieved by quantifying the FICI, either through a checkerboard array when two antibiotics are combined or by using a finer gradient isobologram approach with data fitted to the Bliss-independence or Loewe models<sup>73</sup>. While the FICI approach is crude, it offers simplicity and speed, making it attractive for clinical microbiology applications that require high-volume testing. However, FICI does not capture the refined dose-dependence effect achieved through smaller drug interval analyses due to the inherent limitations of the dilution technique. In a typical dilution series, the intervals are always two-fold (e.g., 1, 2, 4, 8, 16, 32, 64, 128  $\mu\text{g/mL}$ ). Expanding the dilution intervals allows for data fitting to the Bliss or Loewe models and subsequent graphical analysis using isobolograms. The Bliss independence model assumes a null hypothesis where two compounds do not interact, while the Loewe additivity model assumes a null hypothesis where the active compound cannot positively or negatively interact with itself<sup>74,75</sup>.

With the advancement of modern high-throughput technology, accurate classification and screening of antibiotic combinations can be achieved through a systems network approach<sup>76-78</sup>. Observations include, but are not limited to, synergistic combinations that can accelerate the evolution of resistance compared to individual antibiotics, antagonistic drug pairs that can suppress resistance evolution, alternating antibiotic treatment that can slow the evolution of resistance by constraining the mutational path, and the

challenges of modeling higher-order combinations (involving more than two compounds) with unexpected interactions<sup>79–85</sup>.

## Bacterial biofilms

Biofilm formation, although recently recognized, is a prevalent lifestyle in clinical bacterial infections. Examples of such infections include chronic lung, wound, and bone infections, among others<sup>86,87</sup>. Biofilm refers to a cluster of microbes that attach to a surface and are surrounded by an extracellular matrix<sup>88,89</sup>. The surface can be composed of various materials, including tissues, abiotic materials, other cell types, and daughter cells. This definition encompasses a wide range of emerging properties that contribute to the evolutionary success of this lifestyle. These properties include social cooperation, resource capture, and enhanced survival against antimicrobials. It is important to note that our understanding of this lifestyle cannot be fully comprehended by studying free-living bacterial cells alone<sup>90</sup>. The presence of *Pseudomonas aeruginosa* aggregates in the lungs of cystic fibrosis patients was first described by Niels Højby<sup>91</sup>, and the term “biofilm” was introduced clinically by John William Costerton<sup>92</sup>. While acute infections have been associated with planktonic bacteria, the dispersion of planktonic cells from biofilms in chronic infections serves as a connecting point and can lead to systemic infections such as bacteremia<sup>93,94</sup>.

## Lifecycle

A variety of environmental and genetic factors influence biofilm formation, growth, composition, and structure<sup>90,95</sup>. The life cycle of a biofilm encompasses several distinct stages, including aggregation and attachment (1), followed by growth and accumulation (2), and finally disaggregation and detachment (3) (Fig. 8). Each stage can be further divided into multiple steps. Attachment begins with reversible adhesion, which can have two outcomes: either weakly attached cells that can return to a planktonic lifestyle, or initial interactions between the cell and the surface leading to irreversible attachment<sup>96</sup>. Under specific conditions, growth occurs, resulting in the formation of multicellular microcolonies that eventually develop into a mature biofilm. Factors such as limited nutrient availability or reduced oxygen levels can trigger detachment or dispersion during the maturation stage. Dispersion refers to the release of cells from the biofilm into the surrounding liquid environment. These dispersed planktonic cells can then initiate a new life cycle<sup>95,97</sup>.

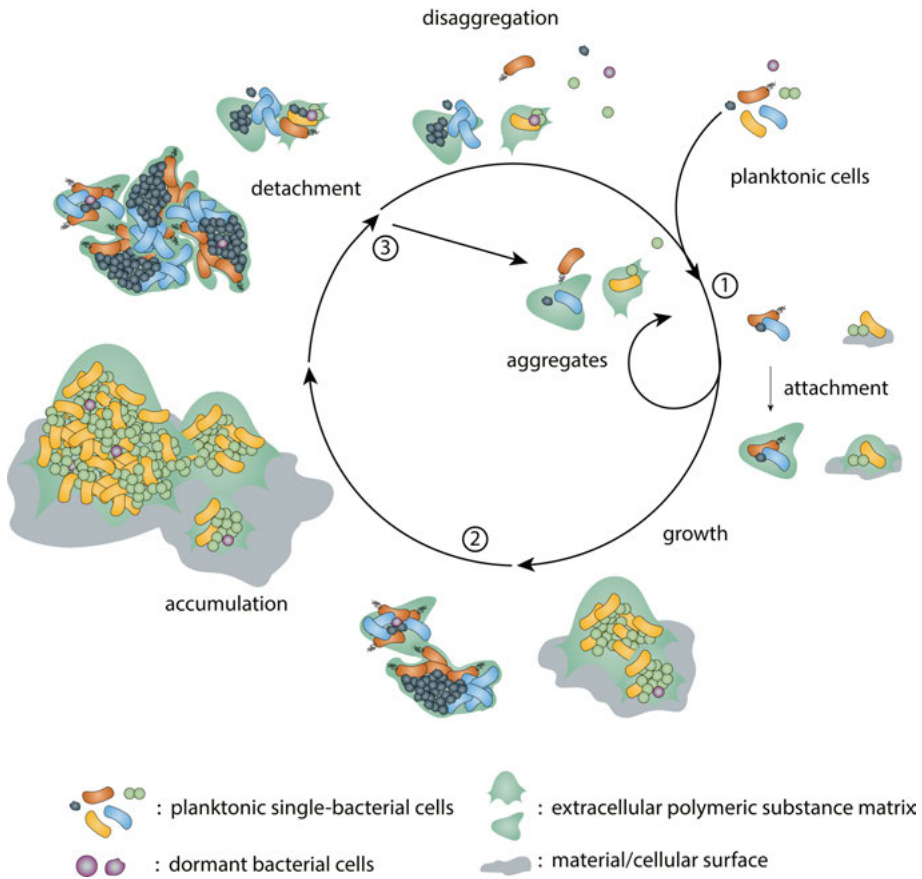


Fig. 8: Bacterial biofilm lifecycle. The cyclic process occurs in a stage-specific and progressive manner and can initiate from planktonic cells, whether single or multiple cells. The cells undergo sequential stages, including attachment (with reversible and irreversible sub-stages to surfaces or other cells and can return to planktonic form as aggregates), growth (cellular proliferation and production of extracellular components), accumulation (where cell clusters mature, resulting in a biomass several cells thick and embedded in an extracellular polymeric substance matrix), and detachment (where cells evacuate from the interior portions of cell clusters, forming void spaces as aggregates, multiple cells, or planktonic cells and then further disaggregate into planktonic cells).

## Model systems

Due to the multistage lifecycle of biofilms, there is no standard method for studying them. To investigate specific research questions, several methodologies have been adapted or developed<sup>98</sup>, which can be broadly categorized as *in vitro* or *in vivo* models. *In vitro* models involve simple artificial systems using material surfaces. They can be grouped based on nutrient availability: closed or static, and open or dynamic. These can be further classified as air-

liquid interface, colony, drip-fed, and flow-cell systems. Commonly used systems include the standard 96-well microtiter plate, introduced to laboratories in the 1980s, accompanied by crystal violet staining for biomass quantification<sup>99</sup>. The MBEC<sup>TM</sup> assay (formerly known as the Calgary device) extends the format by cultivating biofilms on plastic pegs attached to a lid submerged into wells in the 96-well plate<sup>100</sup>. These models allow for biofilm quantification not only through staining but also by enumerating viable bacteria in the biofilm using colony forming units. Moreover, different treatments can be easily applied to biofilms by transferring the lid with biofilms on pegs into a new plate. With the advancement of live imaging and image analysis, flow-based systems have become widely used<sup>101</sup>. In these systems, biofilms are cultured over a long period in a channel with a constant supply of fresh growth medium and removal of waste. Microfluidic-based approaches have gained attention for better experimental control and high-throughput capabilities, although they are not widely adopted due to the technical skillset required<sup>102–104</sup>. The major limitation of *in vitro* models is the absence of infection in host materials and the human immune response<sup>105</sup>.

## Tolerance

Biofilms have been demonstrated to exhibit greater tolerance to antibiotic therapy compared to planktonic bacteria<sup>106</sup>. In the case of planktonic bacteria, tolerance refers to the ability of bacteria to survive antibiotic exposure without developing resistance due to dormancy, persistence, or slow growth<sup>107,108</sup>. Recent research has revealed that the molecular mechanisms of tolerance evolve rapidly under intermittent antibiotic exposure<sup>109–111</sup>, suggesting that tolerance typically precedes resistance and can involve the acquisition of mutations from the wild-type bacterium<sup>112</sup>. In a biofilm, tolerance is associated with the growth mode of the biofilm and encompasses various factors such as the inability of antibiotics to penetrate different regions of the biofilm, reduced growth within distinct microenvironments, heterogeneous metabolism, the presence of persister cells, oxygen gradients, and the diverse actions of the extracellular biofilm matrix<sup>113,114</sup>. For instance, in *P. aeruginosa*, heterogeneous subpopulations within a biofilm were found to produce  $\beta$ -lactamases when exposed to imipenem and ceftazidime<sup>115</sup>. Generally, studies on tolerance and resistance mechanisms are limited and predominantly focused on *P. aeruginosa*. It is not possible to generalize the unique tolerance of one species to another due to differences in their lifecycles and the matrix they produce.

# Current Investigations & Future Perspectives

The reduced susceptibility of bacterial biofilms to antibiotics poses a significant challenge in effectively treating various infections, particularly those associated with biofilm-colonized medical devices. While our understanding of the unique growth mode of biofilms has improved through advancements in investigative technologies, the properties of biofilms cannot be simply extrapolated from our knowledge of planktonic bacteria<sup>90</sup>. Extensive research has been conducted on the evolution of antibiotic resistance at concentrations above the MIC, but experimental evidence suggests that sub-MIC (sub-minimal inhibitory concentration) selection may play a crucial role, at least for planktonic cells<sup>50–54</sup>. However, much less is known about sub-MIC exposure in biofilms and how the distinct physiology of biofilms influences the rate and trajectory of selection. Addressing this knowledge gap was the primary focus of our research in Papers I and II.

## Paper I

### A microfluidic chip for studies of the dynamics of antibiotic resistance selection in bacterial biofilms

In this paper, we present a novel microfluidic model designed to address the aforementioned question. The study of biofilms *in vitro* requires diverse model systems due to the wide variety of biofilm structures. However, there is always a trade-off between high-throughput, ease-of-use, and physiological relevance of the model. By combining microfluidic approaches with advanced live imaging, we have developed a platform that allows for *in situ* investigation of biofilms under different hydrodynamic conditions and at high resolutions. This platform enables us to examine the competitive abilities of susceptible and resistant bacteria in a mixed biofilm, both in the absence and presence of antibiotics. We named the chip *Brimor*, where the letter B represents biofilms and “rimor” is derived from the Latin word meaning to probe, search, or explore. The microfluidic chips were designed as single-use disposable devices, which were easily and cost-effectively fabricated using 3D-printed molds for fluidic channels, polydimethylsiloxane (PDMS) casting, and bonding the PDMS replica piece to a glass slide. Along with the essential

components of a microfluidic system, this model allows for controlled cultivation of bacterial biofilms.

We utilized *Escherichia coli* for biofilm formation and confirmed the presence of extracellular cellulose in the biofilms through in situ staining. By employing live imaging techniques and manipulating flow rates, we demonstrated that planktonic cells seeded in the microfluidic chip transitioned into a biofilm state within 16 hours of cultivation. Additionally, we showcased the novel capability of the system to selectively harvest specific layers of the biofilms. This new biofilm model enabled us to measure the growth and death rates of *E. coli* during biofilm formation and determine the minimal selection concentration in biofilms when exposed to ciprofloxacin. Importantly, we found that ciprofloxacin-resistant mutants can be selected in biofilms at concentrations well below the MIC of susceptible planktonic bacteria.

In principle, Brimor is not limited to studying antibiotic compounds but can also be used to investigate the effects of other bioactive compounds. Moreover, while there are limitations associated with the use of microfluidic approaches for biofilm studies<sup>116</sup>, it's worth noting that bacteria are not the only organisms that transition into a biofilm lifestyle, and the microchannels can accommodate various cell types. We anticipate that this approach will find applications in numerous areas where biofilms are prevalent<sup>117,118</sup>.

## Paper II

### Antibiotic minimal selective concentrations and fitness costs during biofilm and planktonic growth

In this paper, our aim was to investigate two crucial parameters that influence the selection of resistant bacteria: the fitness cost and the minimal selective concentration (MSC) of resistance<sup>54</sup>. The fitness cost of resistance refers to the decrease in relative fitness caused by a resistance mechanism and directly impacts the MSC. Specifically, a higher fitness cost associated with a resistance mutation or gene is expected to result in an increased MSC for bacteria in the planktonic growth mode.

To assess these parameters, we utilized the high-throughput model Flex-iPeg<sup>119</sup>, which is a modified version of the widely used MBEC<sup>TM</sup> assay. We examined five antibiotics (fosfomycin, nitrofurantoin, rifampicin, streptomycin, and trimethoprim) and six resistance-conferring mutations (*uhpT* STOP 5aa,  $\Delta$ *nfsAB*, *rpoB* S531L, *rpsL* K42N and K42R, *dfr*) in uropathogenic biofilm-forming *E. coli* strain CFT073. Our results revealed an important finding for the five antibiotics: the selection of resistance occurred at concentrations well below the MIC<sub>suc</sub> bacteria. This emphasizes the emergence and enrichment of resistant bacteria in both planktonic and biofilm lifestyles. This finding contradicts observations from other biofilm models, as we specifically

examined the early phases of biofilm formation, whereas most previous studies focused on later phases. Therefore, it is crucial to consider the different stages of the biofilm life cycle and their impact on the fitness cost of selection<sup>89</sup>, as well as the possibility that resistance selection in biofilms can be dynamic.

While our study primarily focused on determining fitness costs and MSCs in defined biofilms for comparison to planktonic growth, it is important to acknowledge that complex biofilms consisting of multiple bacterial species are predominant in clinical and environmental settings. It has been suggested that MSCs may be higher in complex biofilm communities due to reduced free drug concentrations and higher costs of resistance<sup>120</sup>. However, there is limited research supporting this notion, and further studies are needed to explore this aspect.

Combining multiple antibiotics, either in mixtures or in a sequential order, is proposed to enhance treatment efficacy and counteract resistance<sup>69,77,121</sup>. While the immediate effect of antibiotic action is often considered a linear chain of events, various nonlinear phenomena associated with antibiotic combinations have been observed<sup>122–124</sup>. Our understanding of the physiological responses and genetic mechanisms underlying these antibiotic interactions is generally limited<sup>70</sup>. Only recently, with advancements in technology, have high-throughput approaches been utilized to systematically investigate gene-gene, drug-gene, and drug-drug effects, and their potential mechanisms in both Gram-negative and Gram-positive bacteria<sup>125–127</sup>. Understanding drug-drug and drug-genetic interactions is crucial for antibiotic stewardship, yet an important unanswered question is whether combination strategies have clinical applications. Answering the former was the central focus of Papers III and IV, while exploring the clinical implications of combination strategies was the central focus of Paper V.

## Paper III

### CombiANT<sup>®</sup>: antibiotic interaction testing made easy

In this paper, we developed a simplified drug interaction assay using the Loewe additivity model isoboles and provided proof-of-concept studies for 10 important pairwise combinations against three selected clinical pathogens. Combination therapy is commonly prescribed with the assumption that combination effects are consistent across strains within the same species. However, recent results have revealed extensive and unpredictable variation both between and within bacterial species<sup>126,128,129</sup>. To refine combination therapy, it is necessary to examine antibiotic interactions on a case-by-case basis for each isolate. However, the gold-standard methods for determining antibiotic

combination effects, such as checkerboard assays and time-kill experiments, are complex and labor-intensive<sup>130,131</sup>, limiting their widespread use outside academic settings. Our assay, called CombiANT<sup>®</sup> (for combination of antibiotic testing), is based on the diffusion of three different antibiotics on agar plates, allowing the formation of concentration landscapes on which bacterial samples can be applied and the growth or inhibition can be quantified to establish the effects of drug combinations. The concentration landscapes of the three antibiotics are generated using a custom-designed insert that houses three reservoirs, into which antibiotics are added at high concentrations and placed on a single agar plate.

We validated the method using three major pathogens (*E. coli*, *P. aeruginosa*, and *S. aureus*) and demonstrated its comparable performance to the gold-standard checkerboard assay, with the added benefits of reduced assay complexity and costs, as well as the possibility of integration into established clinical diagnostic pipelines. Importantly, the simplicity and similarity of the assay to antibiotic susceptibility testing with disk diffusion make it suitable for rapid adoption in low-resource settings and warrants further investigation.

In principle, CombiANT<sup>®</sup> is not limited to antibiotic compounds alone; it can also be used to investigate the effects of other bioactive compounds. Despite the limitations associated with bacterial growth on agar<sup>132,133</sup>, it is worth noting that bacteria are not the only organisms that can be cultivated. With slight modifications, any cell type, such as HeLa cells, can be incorporated into the assay. We anticipate the application of this assay in various fields where the determination of drug interactions is required.

## Paper IV

### Low conservation of antibiotic interactions between and within Gram-negative bacterial species

In this paper, we employed the method from paper III to systematically determine the effectiveness of 12 pairwise combinations of clinically used antibiotics against five Gram-negative pathogens. These pathogens belong to the ESKAPE group, which consists of priority pathogens<sup>134</sup>. We examined a total of 696 interactions from antibiotic combinations across five classes (aminoglycosides,  $\beta$ -lactams, polymyxins, quinolones, and tetracyclines) using 232 antibiotic-susceptible isolates from a collection of 500 non-duplicate patient-derived isolates.

Within each species, the interactions were often specific to the isolate and the antibiotic combination, ranging from antagonistic to synergistic. Particularly in the case of *E. cloacae*, all types of interactions were observed. The interactions also varied significantly among the five Gram-negative species (*A. baumannii*, *E. cloacae*, *E. coli*, *P. aeruginosa*, and *K. pneumoniae*).

Additive and antagonistic interactions were the most common across the different species and the tested antibiotic combinations. Specifically, 99.7% of all isolate and antibiotic combinations fell into one of these two categories, while only 0.3% exhibited clinically relevant levels of synergy. Our findings emphasize the importance of routinely performing isolate-specific interaction profiling to achieve the highest precision and efficacy in combination therapy.

It remains unclear whether the interactions observed under these *in vitro* conditions can directly correlate with patient outcomes and if the determined interactions can be clinically exploited. Understanding whether these effects lead to improved or reduced treatment outcomes compared to monotherapy is a crucial next question to address in order to ensure effective personalized combination therapy and antibiotic stewardship.

## Paper V

### Mutations that alter the synergistic interaction of tetracycline and spectinomycin in *Escherichia coli*.

In this paper, we investigated the mutations and proposed mechanisms underlying the changes that result in the synergy and loss of synergy from the combination of tetracycline and spectinomycin in the model *E. coli* strain MG1655. We selected 89 spontaneous mutants under different selective conditions, which involved varying the ratio of either antibiotic in the combination.

A total of 89 spontaneous mutants were isolated on plates containing both tetracycline and spectinomycin at low concentrations (near MIC) that prevented growth. For a random sub-set of 41 mutants, we examined the antibiotic interactions and 12 % (5/41) mutants had become more synergistic, 27 % (11/41) mutants retained the same level of synergy as the parental strain, and 61 % (25/41) mutants had lost their synergy and became more additive or antagonistic. The latter type of mutants showed a class of mutations in which no change in individual MICs of either tetracycline or spectinomycin could be observed. Genetic changes in this class of mutants were associated mainly with the efflux regulatory network (*acrR*, *lon*, *nupC*, *ompF*), and to a lesser extent the pentose phosphate pathway (*gnd*, *ptsI*), glycolysis and gluconeogenesis switching (*yggF*), nucleoside transport systems (*nupC*), heat shock protein (*clpP*) or an uncharacterised gene (*yhaC*). Measurements of intracellular tetracycline levels in a sub-set of four mutants revealed that tetracycline levels were reduced, and likely contributed to abolishing the synergistic effect and converting it towards additivity. These findings suggest a diversity of genetic alterations that contribute to the loss of synergy. Notably, a common response among several mutants was a reduction in intracellular levels of tetracycline.

Our results align with the bioavailability model<sup>135</sup>, which posits that two drugs will be synergistic if one antibiotic increases another antibiotic's intracellular concentration, either by increasing the entry or decreasing the degradation or efflux of the second drug. It is important to identify whether the observed genetic changes are also present in clinical isolates where combination treatment fails to achieve the therapeutic objective and whether these alterations are relevant *in vivo*.

## Concluding Remarks

As a modern society, we face numerous challenges: increasing geopolitical instability, ethnic conflicts, climate change, food insecurity, and infectious diseases, including antibiotic resistance, to name just a few. These challenges necessitate a comprehensive approach from all members of society to swiftly mitigate their negative impacts. ABR serves as an excellent example, where research is not only required to deepen our understanding and knowledge for the development of new drugs but also alternative treatment strategies, new economic models and improved non-discriminatory distribution is needed.

Although the majority of new antibiotic candidates in the drug discovery pipeline do not belong to novel antibiotic classes, there are currently 76 antibacterial candidates under development. Among these, 45 are traditional and 31 are non-traditional, with 28 in phase one, 32 in phase two, 12 in phase three, and 4 under regulatory evaluation<sup>136,137</sup>. Alternative therapies have been suggested, including but is not limited to phage therapy, antivirulence therapies, microbiome-modifying therapies, and agents targeting bacterial conjugation<sup>138,139</sup>.

Furthermore, the current economic model of relying on market sales for antibiotic businesses is unsustainable. Two examples of this are the bankruptcies of Achaogen<sup>140</sup> and Melinta Therapeutics<sup>141</sup> in 2019. These were the biopharmaceutical companies behind the plazomicin and the delafloxacin antibiotics, both of which was approved in 2018 and 2019, respectively. Recently, the WHO has proposed alternative “push and pull” incentives<sup>142</sup> as new economic models for antimicrobials. “Push” incentives aim to reduce the early development costs for companies through funding (e.g., grant support, contract funding, tax incentives, and private-public partnerships), while “pull” incentives aim to optimize the late stage of drug development and create a viable market demand for sponsors (e.g., market entry rewards, extended exclusivity period, tradable market voucher, and higher reimbursement). An example of the latter is Sweden and United Kingdom’s pilot subscription model for accessing antibiotics instead of purchasing them based on units sales<sup>143</sup>.

Antimicrobial stewardship, including antibiotic stewardship, is another key aspect in controlling AMR. Simply put, it refers to strategies that promote responsible use of antimicrobials<sup>144,145</sup>. Approximately 50 % of current antimicrobial use is estimated to be unnecessary or inappropriate and can be reduced<sup>146</sup>. Therefore, effective antibiotic stewardship aims to balance the

individual's need with the need of society, namely rapid and efficient treatment versus preserve functioning antibiotics for the future<sup>147</sup>.

All these aspects highlight the multifaceted nature of the universal challenge posed by ABR and AMR. Despite antibiotics being a valuable societal resource that has been irresponsibly managed for many decades, we should resist our natural instinct to ignore, deny, or withdraw from the challenge. The solution simply begins with gaining a better understanding of the problem to facilitate the development of solutions, a path that has driven many global progresses in past centuries.

With this guiding principle in mind, I present five investigations conducted by co-authors and myself during my doctoral studies from 2018 to 2023. It is my hope that the tools and knowledge generated from these innovations and investigations will support and further guide global efforts in preventing AMR as "*the next pandemic*".

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During my time at Uppsala and Stockholm in Sweden over the past eight years, I have had the pleasure of meeting many amazing individuals, many of whom have left a positive impact on me. To be honest, my journey here was driven by my xenophile wanderlust, and I had no intention of pursuing further education. After all, why would a sane Taiwanese-African willingly travel from the southern to the northern tip of the globe? I remember googling if Sweden was even a country back in 2014 with a dear friend, and we wondered, “Do people actually live there?”. I apologize if I have missed mentioning names, but please know that I deeply appreciate your kindness, support, and encouragement. I wouldn’t have completed this journey without all of you.

First and foremost, I would like to express my utmost gratitude to my supervisors and co-authors for their invaluable contributions to the papers presented in this thesis and beyond. There’s a Taiwanese idiom that says “a bunch of chopsticks is stronger than a pair”, and I truly believe that the collective work showcased here embodies this wisdom. It has been an absolute pleasure to collaborate with you, navigating through the ups and downs, from beginning to end (or termination). Let us hope that our work does not gather dust but instead contributes to a better future.

To the current members of the DA group, it is our cosmos fortune that we’ve gathered and embarked on this quest together, venturing where most have not gone before. It has been an exciting journey onboard this ship into the unknown with you. I wish you favourable winds as you continue to explore uncharted waters with your unwavering determination and hard work, leading to tales of successful adventures.

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