THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Characterization of the *in vivo* mechanisms of action of cell envelope-targeting antibiotics

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Cover:
Microscopy image of *B. subtilis* stained with the membrane dye Nile red and the DNA dye DAPI. The scale bar is 5 µm

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Abstract

Multidrug-resistant bacteria pose a major threat to society. Once easily treatable diseases are now challenging to cure. With the golden age of antibiotics being over, the discovery of new antibiotics has been stagnant, and no new antibiotic class has been introduced into the clinic since 2003. Therefore, it is crucial to discover new antibiotics and to optimize existing antimicrobial compounds.

This thesis aims to provide more information on the mechanisms of antibiotics on living bacteria and can be divided into two parts. One part provides detailed information on techniques that can be utilized to study the mode of action of new antibiotic compounds. Here, methods are discussed in detail and curated to a method set that can be easily utilized by other researchers. The curated assays were then applied, and a phenotypic reference set was created. In the second part, these methods were used to investigate the mechanisms of new antimicrobial compounds and lead structures to provide important information for their further development.

The fluorescence dyes laurdan, Nile red, and DiIC12 that are able to track fluidity changes in the bacterial membranes in response to antibiotic treatment were described. In addition to the detailed protocols and their evaluation, the methods were applied to investigate the effects of daptomycin. These methods were able to detect that daptomycin changes the overall membrane fluidity differently in *B. subtilis* and *S. aureus*. Furthermore, I have successfully applied the curated methods, consisting of BCP, membrane potential ($DiSC_3(5)$, and cell wall synthesis assays (MreB mobility and *PliaI*) to study several new compounds, e.g., antimicrobial peptides from marine *streptomycetes*. Two of these new antimicrobial peptides led to the consecutive permeabilization of the outer and inner membrane in *E. coli*. Interestingly, they also affected DNA packing. Further, an in-depth study of a new promising drug candidate, ES24, was conducted, showing its unique target of the Sec-secretion pathway and an additional DNA-damaging effect.

Overall, this thesis provides important insights into the techniques used in mode of action studies, describes an easily accessible method set for other researchers, and provides important information on the modes of action of new antimicrobial compounds.

Keywords: Mode of action, cell-envelope targeting, antibiotics, antimicrobial peptides

LIST OF PUBLICATIONS

This thesis is based on the following publications; shared first authorships are indicated with asterisks:

I. Madeleine Humphrey*, Ireny Abdelmesseh Nekhala*, Kathi Scheinpflug*, Oxana Krylova, Ann-Britt Schäfer, Jessica A. Buttress, Michaela Wenzel, and Henrik Strahl, Tracking Global and Local Changes in Membrane Fluidity Through Fluorescence Spectroscopy and Microscopy, Sass, P. (eds) Antibiotics. *Methods in Molecular Biology* 2023, pp. 203–229. Doi:10.1007/978-1-0716-2855-3_11. (2023)

II. Ann-Britt Schäfer, Margareth Sidarta, Ireny Abdelmesseh Nekhala, Gabriela Marinho Righetto, Aysha Arshad, and Michaela Wenzel, Dissecting antibiotic effects on the cell envelope using bacterial cytological profiling: A phenotypic analysis starter kit (in print, Journal: *Microbiology Spectrum*, doi:10.1128/spectrum.03275-23)

III. Luisa I. Beyer, Ann-Britt Schäfer, Agustina Undabarrena, Inger Mattsby-Baltzer, Daniel Tietze, Elin Svensson, Alexandra Stubelius, Michaela Wenzel, Beatriz Cámara, and Alesia A. Tietze, Mimicking Nonribosomal Peptides from the Marine Actinomycete Streptomyces sp. H-KF8 Leads to Antimicrobial Peptides. *ACS Infectious Diseases* 10.79-92, doi:10.1021/acsinfecdis.3c00206, (2023)

IV. Ann-Britt Schäfer*, Maurice Steenhuis*, Kin Ki Jim, Jolanda Neef, Sarah O'Keefe, Roger C. Whitehead, Eileithyia Swanton, Biwen Wang, Sven Halbedel, Stephen High, Jan Maarten van Dijl, Joen Luirink, and Michaela Wenzel, Eeyarestatin 24 inhibits Sec-dependent protein secretion and induces DNA damage in *Bacillus subtilis*, ACS Infectious Diseases 9, 253–269, Feb. 2023, doi:10.1021/acsinfecdis.2c00404, (2022)

Additional publications originating from the PhD work not included in the thesis:

V. Ann-Britt Schäfer, Michaela Wenzel, A How-To Guide for Mode of Action Analysis of Antimicrobial Peptides. *Frontiers in Cellular and Infection Microbiology*, doi:10.3389/fcimb.2020.540898, (2020)

VI. Ahmed M. Kamal El-sagheir, Ireny Abdelmesseh Nekhala, Mohammed K. Abd El-Gaber, Ahmed S. Aboraia, Jonatan Persson, Ann-Britt Schäfer, Michaela Wenzel, and Farghaly A. Omar, *N*4-Substituted Piperazinyl Norfloxacin Derivatives with Broad-Spectrum Activity and Multiple Mechanisms on Gyrase, Topoisomerase IV, and Bacterial Cell Wall Synthesis. *ACS Bio & Med Chem Au* 30, 494-506, doi:10.1021/acsbiomedchemau.3c00038, (2023)

VII. Ahmed M. Kamal El-sagheir, Ireny Abdelmesseh Nekhala, Mohammed K. Abd El-Gaber, Ahmed S. Aboraia, Jonatan Persson, Ann-Britt Schäfer, Michaela Wenzel and Farghaly A. Omar, Rational design, synthesis, molecular modeling, biological activity, and mechanism of action of polypharmacological norfloxacin hydroxamic acid derivatives. *RSC Medicinal Chemistry* 14, 2593-2610, doi:10.1039/D3MD00309D, (2023)

VIII. Ahmed M. Kamal El-sagheir, Ireny Abdelmesseh Nekhala, Mohammed K. Abd El-Gaber, Ahmed S. Aboraia, Jonatan Persson, Ann-Britt Schäfer, Michaela Wenzel, and Farghaly A. Omar, Design, Synthesis, Molecular Modeling, Biological Activity, and Mechanism of Action of Novel Amino Acid Derivatives of Norfloxacin. *ACS Omega* 8, 43271–43284, doi:10.1021/acsomega.3c07221 (2023)

Contribution report for the included publications in this PhD thesis:

I. I optimized the method, supervised IAN, and performed, planned, and visualized parts of the experiments.

II. I conceptualized the idea together with MW, performed experiments, analyzed, validated, visualized, and curated data. I wrote the paper together with MW.

III. I planned and performed all bacterial studies, with the exception of the MMC, together with MW. Data derived from these experiments was analyzed, validated, visualized, and curated by me. The section about the bacterial mode of action studies was written by me together with MW.

IV. I planned and performed experiments, analyzed, validated, visualized, and curated data, and wrote the final paper draft together with MW.

Preface

This dissertation was submitted for the partial fulfilment of the degree of Doctor of Philosophy. The original work presented in this dissertation was carried out between September 2019 and February 2024 at Chalmers University of Technology in the Department of Life Sciences (previously Department of Biology and Biological Engineering). This work was supervised by Associate Professor Michaela Wenzel and co-supervised by Professor Fredrik Westerlund.

Abbreviations

ADEP	acyldepsipeptides
AMR	antimicrobial resistance
AMP	antimicrobial peptide
B. subtilis	*Bacillus subtilis*
BCP	bacterial cytological profiling
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
CCCP	Carbonyl Cyanide 3-Chlorophenylhydrazone
CDC	Center for Disease Control
DAPI	4′,6-diamidino-2-phenylindole
DiIC12	Didodecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate
DHF	dihydrofolic acid
$DiSC_3(5)$	3,3′-dipropylthiadicarbocyanine iodide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ES24	eeyarestatin 24
GP	general polarization
GFP	green-fluorescent protein
GlcNAc	N-acetylglucosamine
laurdan	2-dimethylamino-6-lauroylnaphtalene
LPS	lipopolysaccharides
LTA	lipoteichoic acid
MurNAc	N-acetylmuramic acid
MTG	MitoTracker Green
Nile red	9-diethylamino-5H-benzo[α]phenoxazine-5-one
NPN	1-N-phenylnaphtylamine
OD	optical density
OM	outer membrane
OMPs	outer membrane proteins
ONPG	ortho-Nitrophenyl-β-galactoside
OSC	optimal stressor concentration
PBP	penicillin-binding proteins
PE	phosphatidylethanolamine

PG	phosphatidylglycerol
RIF	regions of increased fluidity
RNA	ribonucleic acid
ROS	reactive oxygen species
THF	tetrahydrofolic acid
S. aureus	*Staphylococcus aureus*
Van-FL	BODIPY-vancomycin
WTA	wall teichoic acid

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1. Introduction

Treatments for bacterial infections have been sought-after for more than 2000 years[1]. However, the systematic search, active synthesis, and regulated approval of antibacterial compounds to the market only began approximately 100 years ago with the discovery of pioneering compounds like salvarsan and penicillin[2,3,4]. The discovery of these compounds led to what is now called the golden age of antibiotics[4]. Formerly deadly diseases became treatable and the average life expectancy improved[1]. Unfortunately, the discovery of antibiotics has stagnated to such a degree that no new antibiotic classes have been introduced to the clinic since 2003[5,6,7].

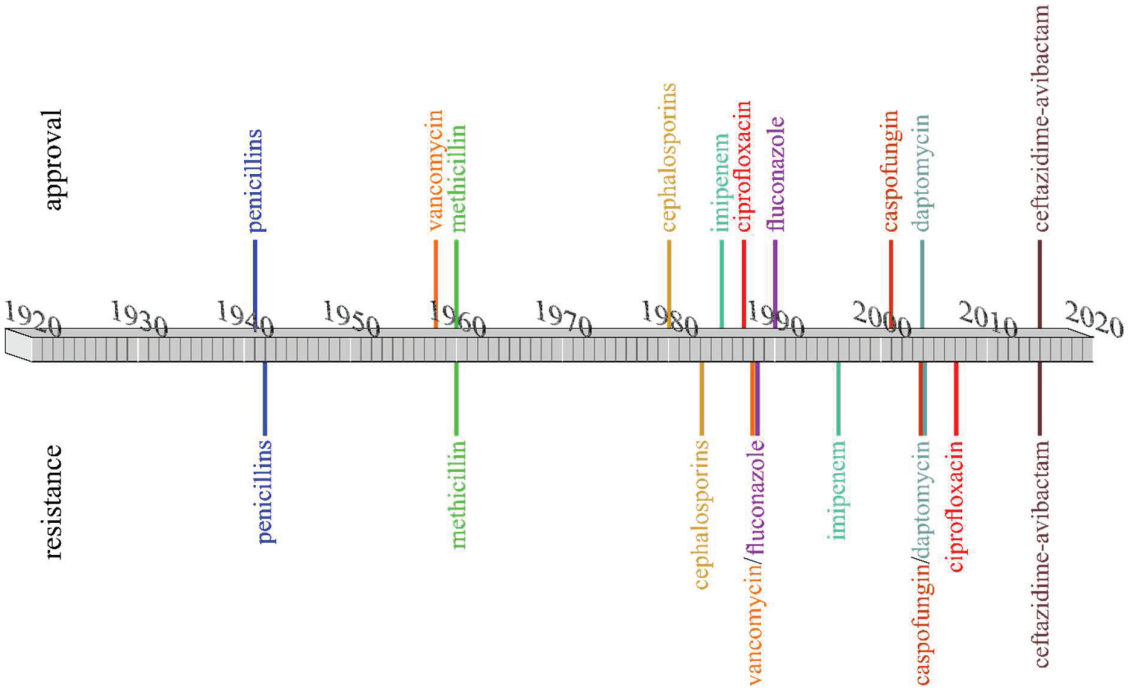

Figure 1 Timeline of selected antibiotics showing their approval/release (top) and identification of resistant strains (bottom) against them according to data published by the Center for Disease Control (CDC) (modified from Acharya and Wilson, 2019)[8,9].

Concurrently with the discovery of antibiotics, resistance against them emerged threatening the continuously successful treatment of bacterial infections (Figure 1)[6]. It has been predicted that without action, up to 10 million people will die from bacterial infections annually by 2050, more deaths than those caused by cancer[10]. According to statistical data, nearly, 5 million deaths in 2019 were caused by or associated with antibiotic-resistant bacterial infections[11].

Some bacteria are intrinsically resistant against certain antibiotics due to, e.g., the presence of an outer membrane (OM), which prevents antibiotics from entering the cells[12]. Resistance can also be acquired. Bacteria can gain resistance through, e.g., mutations in the gene encoding the drug target[13]. This can be problematic, especially for the use of single targeting antibiotics. While these antibiotics were for a long time favored in the clinic, due to their perceived high specificity, one single point-mutation can already render them entirely ineffective[14,15]. In contrast, resistance rates against antibiotics with multiple targets are considerably slower. In fact, clinically successful antibiotics often have multiple targets, thus making multifunctional antibiotics particularly interesting for further development[14,16]. Additionally, bacteria can also share genetic information through horizontal gene transfer, subsequently spreading their resistance genes even across different species[17].

While antibiotic resistance is now widely recognized as a threat to global health, it remains a complex issue to tackle[18]. To fight antimicrobial resistance (AMR), several interdisciplinary issues have to be addressed, such as the regulation of the production and distribution of antibiotics as well as infection control and better hygiene standards, to name a few[18,19].

My thesis addresses the AMR threat by detailed description of techniques used to study antibiotic mechanisms (**paper I** and **II**) as well as providing insights into the mechanisms of action of several antibiotic compounds (**paper II** and **III**).

One of the most successful antibiotic targets is cell wall synthesis, which is closely connected with the cytoplasmic membrane, another structure of the bacterial cell that has gained more and more attention for potential drug discovery in recent years[20,21,22]. Thus, the whole cell envelope as an antibiotic target still holds great promise. However, due to its complexity, it can be difficult to elucidate the modes of action of antibiotic compounds on this target[23]. This thesis provides detailed protocols to enable reliable readouts of techniques used for investigating cell-envelope targeting antibiotics and curates a method set that can successfully categorize compounds into cell wall, cytoplasmic membrane, and dual mode of action inhibitors (**paper I** and **II**).

In the second part of my thesis, these methods were applied to investigate antibiotic compounds of several interesting antibiotic classes (chapters 5.1.1, 5.2.1, 5.3.1, **paper III**, and **IV**). Here, the modes of action of metal complexes, antimicrobial peptides (AMPs), and the small inhibitor molecule eeyarestatin 24 (ES24) were investigated, either for quick mode of action categorization or, in case of the latter, elucidated in-depth. Important insights have

been gained and unique modes of action have been discovered, which makes these compounds promising new drug candidates in the fight against AMR.

2. Bacteria

Bacteria are microscopically small, single cell organisms that are omnipresent. These organisms have important functions within our ecosystem, including the decomposition of organic matter and cycling nutrients back into the environment, e.g., by driving geochemical cycles like the nitrogen cycle[24].

Bacteria cannot only be found in our environment, they also make up approximately half of the cells in the human body, in form of our microbiome, the majority of which is located in the gastrointestinal tract, where commensal bacteria help to digest food.[25]. Their presence and function are important for the human body.

Bacteria possess diverse shapes and forms, and can be classed according to their into different categories shape: rods, spheres, and spirals (bacilli, cocci, spirilla) (Figure 2 A)[26].

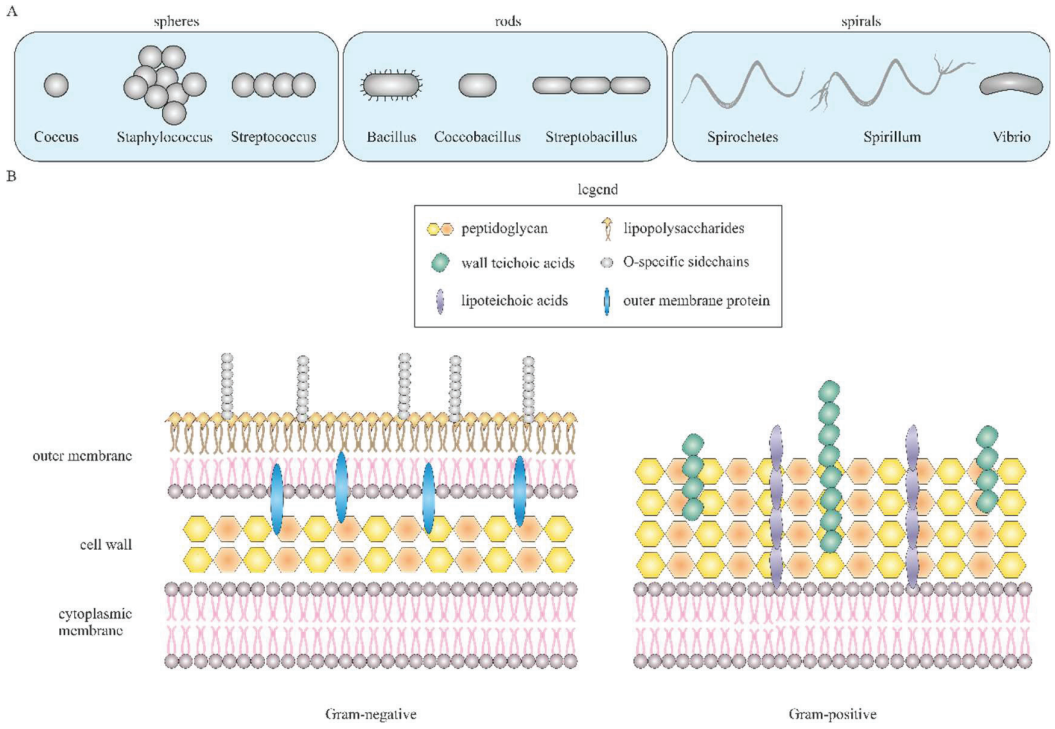

Figure 2 Schematic overview of (A) different bacterial shapes and (B) the Gram-negative and Gram-positive cell envelope[24,26].

In general, bacteria can be grouped into two classes: Gram-positive and Gram-negative (Figure 2 B). This classification stems from a staining technique based on the retention of crystal violet in the thicker layer of the cell wall of Gram-positive bacteria, resulting in a

positive (purple) stain. Gram-negative bacteria, which possess a much thinner cell wall do not retain the dye after washing and are, subsequently, not stained in this assay [27]. Only possessing a thin cell wall layer, Gram-negative cells do possess an extra protective layer, the OM. The Gram-classification is widely used, in particular in medical contexts, as the different cell envelope properties result in vastly different susceptibility to antibiotics.

3. Antibiotic targets

One part of the strategy against AMR is the discovery and development of new antibiotics. Several structures can be targeted within the bacterial cell, as can be seen in Figure 3. In this chapter, each cell component will be described, and examples will be given of already existing antibiotics targeting these structures.

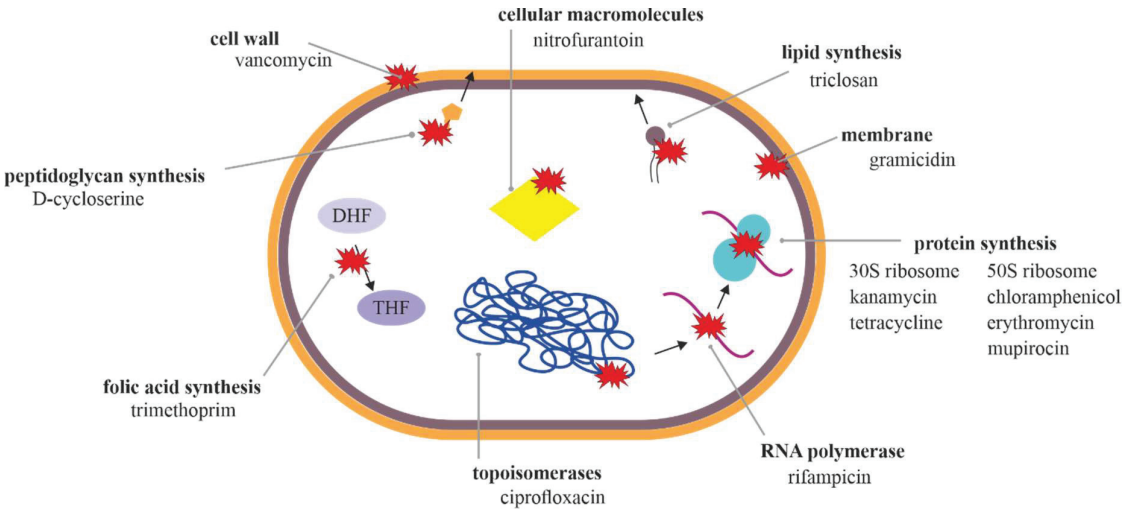

Figure 3 Schematic overview of antibiotic targets in a Gram-positive cell (modified from Sanseverino *et al*. 2018)[28].

3.1. Outer membrane

Gram-negative bacteria contain an extra line of defense against environmental stressors, the OM. It is an asymmetric bilayer, consisting of phospholipids in the inner leaflet and lipopolysaccharide (LPS) glycolipids in the outer leaflet of the membrane[29].This layer plays a major role in the intrinsic resistance of Gram-negative bacteria against several antibiotics, as many antibiotics, e.g. vancomycin or nisin, cannot cross it and therefore not reach their target destination[12]. However, there are antimicrobial compounds that target this structure directly[30]. Polymyxin is one last resort antibiotic, that targets the OM by formation of LPS-polymyxin complexes, which subsequently disrupt the membrane. Furthermore, the OM contains several proteins, which are important to uphold a wide range of functions, e.g., cell adhesion, signaling, and protein secretion[29,31]. These outer membrane proteins (OMPs) can also be targeted by antibiotic compounds. Darobactin, for example targets the OMP BamA, which is

responsible for the folding and correct insertion of OMPs. This compound binds to the closed stage of BamA and prevents insertion of other OMPs[32].

3.2. Cell wall

The cell wall poses one of the first lines of defenses against environmental and mechanical stressors[33]. Its thick mesh-like structure is built up of multiple layers of peptidoglycan. Peptidoglycan consists of crosslinked N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc)[34]. Additionally, the cell wall contains phosphate-rich polymers, also called teichoic acids[33]. Teichoic acids can be divided into two distinct species: wall teichoic acid (WTA) and lipoteichoic acid (LTA). WTAs are linked to the peptidoglycan network while LTAs are anchored to the membrane[35]. The composition of the cell wall varies depending on the organism[33].

The synthesis of the cell wall is localized in the cytosol as well as on both the inner and the outer membrane leaflets. The precursors of peptidoglycan and wall teichoic acid are synthesized in the cytosol, while the later steps are located at the membrane[36]. Both precursors bind to the same carrier molecule, bactoprenol phosphate, to cross the membrane with the help of flippases[37,38,39]. In contrast, LTA is synthesized via a different pathway[40]. Figure 4 shows a schematic overview of the peptidoglycan and WTA synthesis in the Gram-positive model organism *Bacillus subtilis* (*B. subtilis*) and highlights exemplative antibiotics that target the peptidoglycan synthesis.

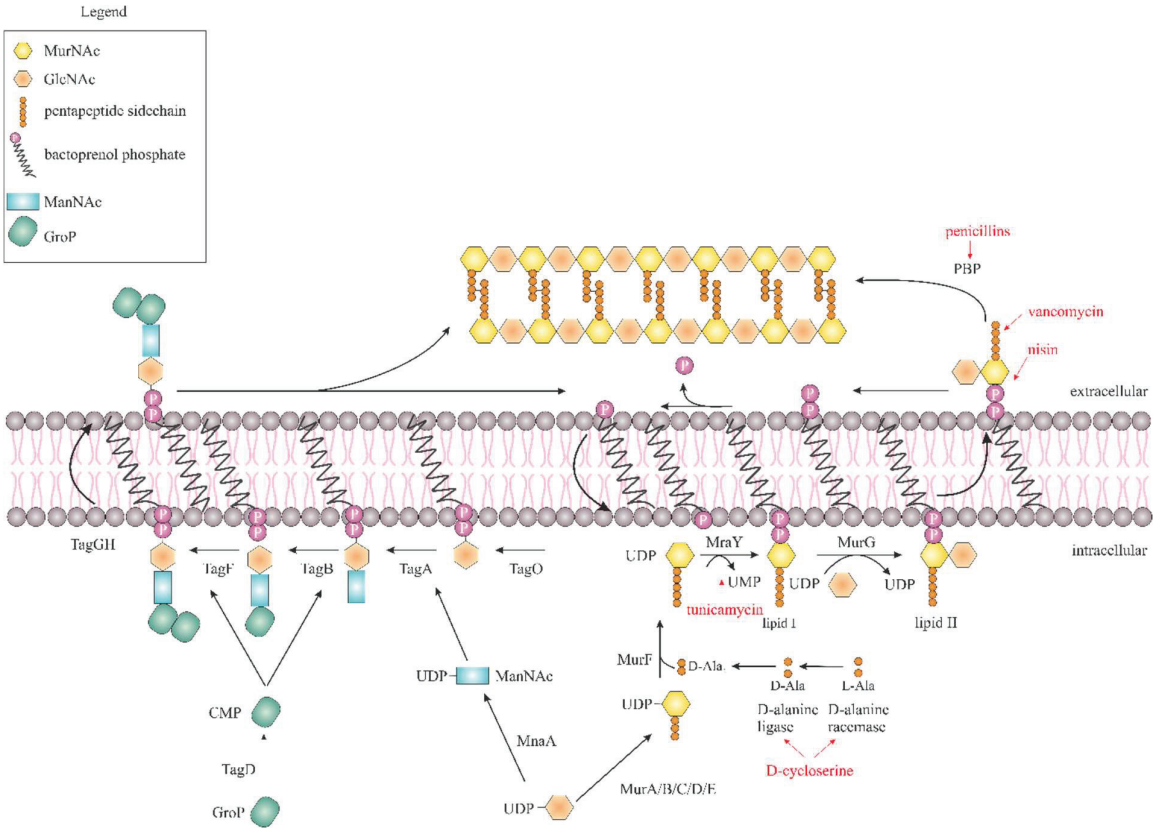

Figure 4 Teichoic acid and peptidoglycan synthesis pathways of *B. subtilis* (modified from Schneider and Sahl 2010 and Bhavsar and Brown 2006)[33,41].

Cell wall synthesis is an established antibiotic target (Figure 4)[20]. An early step of cell wall synthesis can be targeted by, among others, fosfomycin and D-cycloserine. Fosfomycin inhibits MurA, the enzyme catalyzing the first step of the peptidoglycan synthesis, thus preventing the synthesis of the precursor UDP-MurNAc and subsequently disrupting the peptidoglycan synthesis[42,43]. D-cycloserine inhibits the alanine racemase as well as the D-alanine:D-alanine ligase. These two enzymes are necessary for the synthesis of D-alanine:D-alanine, which is the last moiety in the peptide stem of the peptidoglycan precursor, and is important for cross-linking of the glycan chains[34]. Consequently, an inhibition of these enzymes leads to a production of abnormal peptidoglycan building blocks that can no longer be cross-linked[44,45].

In addition, membrane-bound steps of cell wall synthesis can be targeted. One such compound is tunicamycin, which inhibits the MraY-catalyzed step of transferring UDP-MurNAc to bactoprenol phosphate, subsequently blocking the formation of lipid I[46].

Tunicamycin also inhibits TarO, an enzyme involved in the early stage of wall teichoic acid synthesis, which, like MraY, catalyzes the transfer of the precursor UDP-GlcNAc to bactoprenol phosphate[47,48]. After the formation of lipid I, MurG catalyzes the formation of lipid II, by transferring GlcNAc to the MurNAc residue of lipid I[33]. Lipid II is then flipped across the membrane, and the peptidoglycan precursor is subsequently incorporated into the peptidoglycan layer by penicillin-binding proteins (PBP)[33]. These extracellular steps of cell wall synthesis can also be targeted by antibiotics. The crosslinking of peptidoglycan precursors by cell wall transpeptidases is inhibited by the well-known antibiotic penicillin[49]. Another antibiotic, vancomycin, binds to lipid II and prevents the incorporation of new peptidoglycan precursors into the extracellular mesh[50]. Similarly to vancomycin, nisin also binds to lipid II, but uses it as a docking molecule to form large transmembrane porse[51]. Since several steps of cell wall synthesis are membrane-bound, it can also be disrupted as a result of changes in membrane architecture. A prime example for this process is the peptide MP196, which integrates into the cellular membrane. The subsequent disturbance of the membrane architecture affects several cellular processes, including cell wall synthesis[52].

3.3. Cytoplasmic membrane

The membrane of bacterial cells is another protective layer of the cell. Its composition not only varies between bacterial species, but also changes during different growth phases and environmental conditions[53,54]. The membrane of *B. subtilis* consists of mainly phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), with a ratio of polar to neutral lipids of 70% to 30%. Other lipid species, such as cardiolipin, which are not as prevalent, are also in the membrane[55]. In addition, *Bacillus* is also able to synthesize different types of fatty acids: unsaturated/saturated, anteiso/iso, and different chain length[56]. The prevalence of each fatty acid type varies even within the *Bacillus* species[56,57]. *B. subtilis* generally has a high percentage (95%) of branched chains fatty acids with an average chain length between 14 – 17 carbon atoms[58,59].The variety of membrane components makes the membrane architecture complex with differences in fluidity as well as microdomains. These are in turn crucial for upholding the associated membrane processes as well as for its permeability to, for example, protons[60,61].

There are several compounds that interfere with the bacterial cell membrane, among them nisin and daptomycin[51,60]. Not only, the membrane as a structure but also the synthesis of its lipids as well as membrane-bound proteins can be targeted. Bedaquiline, for example, targets

the ATP synthesis of mycobacteria, which in turn interferes with the electron transport chain. Triclosan, interferes with fatty acid synthesis by binding to FabI[62].

3.4. DNA

The maintenance and replication of deoxyribonucleic acid (DNA) is an intricate system. Several proteins are involved in its replication as well as in its repair mechanisms. Two prominent members of this family are DNA gyrase and topoisomerase IV. While the DNA gyrase controls negative supercoiling of the DNA, which is important for maintaining processes on the DNA, such as replication, topoisomerase IV plays a role in DNA segregation by unlinking newly replicated DNA[63,64]. Ciprofloxacin can target both proteins and bind their active cleavage-ligation sites, consequently inducing double-stranded DNA breaks[65,66]. Another process that can be targeted is the synthesis of the building blocks of DNA, nucleic acids. By binding the dihydrofolate reductase, the antibiotic trimethoprim inhibits the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF), an important co-factor of nucleotide synthesis, and subsequently interferes with the synthesis of pyrimidines and purins[67].

3.5. RNA synthesis

Another important antibiotic target is the synthesis of ribonucleic acid (RNA). The RNA polymerase consists of five conserved subunits ($\alpha_2\beta\beta`\omega$). It is essential and is highly conserved throughout bacterial species[68,69]. One clinically successful antibiotic, especially for treatment of tuberculosis, targeting this machinery is rifampicin[70]. Rifampicin blocks the elongation of the RNA by binding within the DNA/RNA channel of the RNA polymerase[71].

3.6. Protein synthesis

One prominent group of antibiotics are the protein synthesis inhibitors (Figure 5). Protein synthesis is a highly regulated process that is essential for the growth and survival of the bacterial cell. The bacterial ribosome consists of two big subunits, 30S and 50S, as well as three ribosomal RNA chains (5S, 16S, and 23S)[72,73]. Inhibition of protein synthesis can result in either complete inhibition of translation or in mistranslation. Kanamycin, for example, inhibits protein synthesis by binding to the A site of the ribosomal 30S subunit[74,75]. The 50S subunit is the target of, for instance, chloramphenicol which targets the peptidyl transferase, interfering with the transfer of amino acids to the growing peptide chain[76].

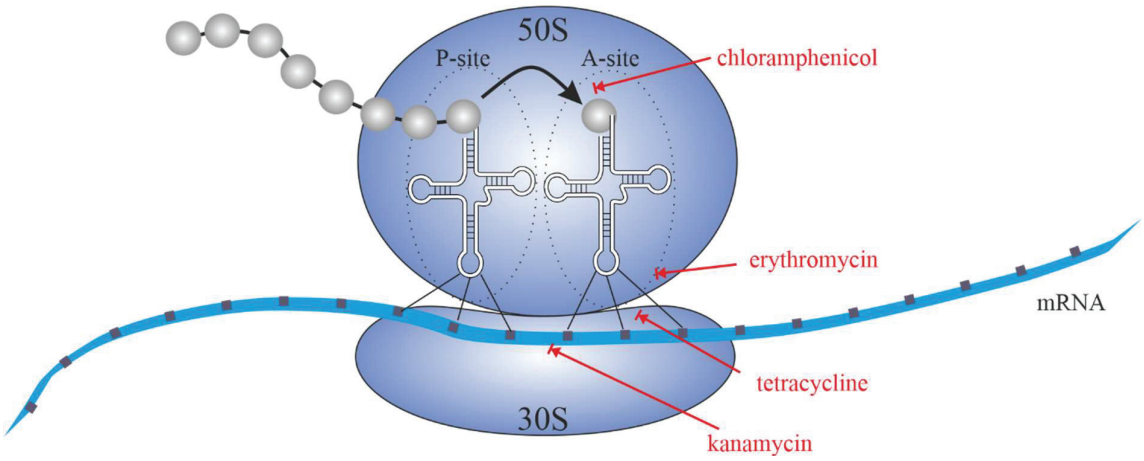

Figure 5 Protein synthesis as a target for antibiotics (modified from McDermott *et al*. 2003)[77] (transfer RNAs were obtained from ChemDraw)[78].

3.7. Cellular macromolecules

Some antibiotics also damage the bacterial cell in several different areas, for example, by the production of reactive species. One such antibiotic is nitrofurantoin. After its activation through nitroreductases, the reactive species damage mainly DNA, but likely also other macromolecules[79,80,81].

3.8. Antibiotics

Several different classes of antibiotics exist, which target a variety of different structures within the bacterial cell. Some of the here studied antibiotics have known targets, which are listed in Table 1 (Figure 6 for structures). In addition, my thesis investigated several new antibiotic compounds whose modes of action are summarized in chapter 5.

Table 1 Antibiotics used in this study, their known targets, and mechanisms of action.

antibiotics	project	target	mechanism
vancomycin	paper II	cell wall synthesis	binds the D-ala-D-ala moiety of peptidoglycan and prevents its incorporation into the cell wall[50]
nisin	paper II	cell membrane cell wall	binds lipid II and forms large pores[51]
daptomycin	chapter 5.1.1	cell membrane cell wall	binds PG, lipid II and targets lipid RIFs, subsequently interfering with cell wall and lipid synthesis[60]
valinomycin	paper II	cell membrane	selectively transports potassium across membrane (ionophore)[82]
gramicidin	paper II	cell membrane	forms ion channels when bound to the membrane (ionophore)[83]
carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP)	paper II	cell membrane	proton carrier that disrupts the electrochemical gradient of the membrane[84]
D-cycloserine	paper II & chapter 5.2.1	cell wall	targets the peptidoglycan synthesis by inhibiting alanine racemase and D-alanine:D-alanine ligase[44]
tunicamycin	paper II	cell wall	binds to MraY inhibiting lipid I synthesis[47]
ciprofloxacin	paper II & chapter 5.2.1	DNA	inhibits topoisomerases[85]
rifampicin	paper II & chapter 5.2.1	RNA	inhibits RNA polymerase[86]
tetracycline	paper II & chapter 5.2.1	protein synthesis	inhibits the 30S ribosomal subunit and prevents aminoacyl-tRNA binding[87]
kanamycin	paper II & chapter 5.2.1	protein synthesis	binds to the 30S subunit and interferes with the correct translation of RNA[88]
chloramphenicol	paper II & chapter 5.2.1	protein synthesis	binds to the 50S subunit and inhibits peptidyl transferase[76]
erythromycin	chapter 5.2.1	protein synthesis	binds close to the peptidyl transferase center in the 50S subunit and prevents the elongation of the peptide chain[89]
mupirocin	chapter 5.2.1	protein synthesis	interferes with the translation process by binding isoleucyl tRNA synthase[90]
triclosan	chapter 5.2.1	lipid biosynthesis	inhibits fatty acid synthesis by binding to the enoyl-acyl carrier protein reductase enzyme[62]
trimethoprim	chapter 5.2.1	nucleic acid synthesis	inhibits folic acid synthesis by binding to dihydrofolate reductase[67]
nitrofurantoin	paper II, IV & chapter 5.2.1	macromolecules	forms reactive derivates that damage the bacterial cell[81]
eeyarestatin 24	paper IV	secretion and macromolecules	inhibits the Sec machinery and forms reactive derivates[81]

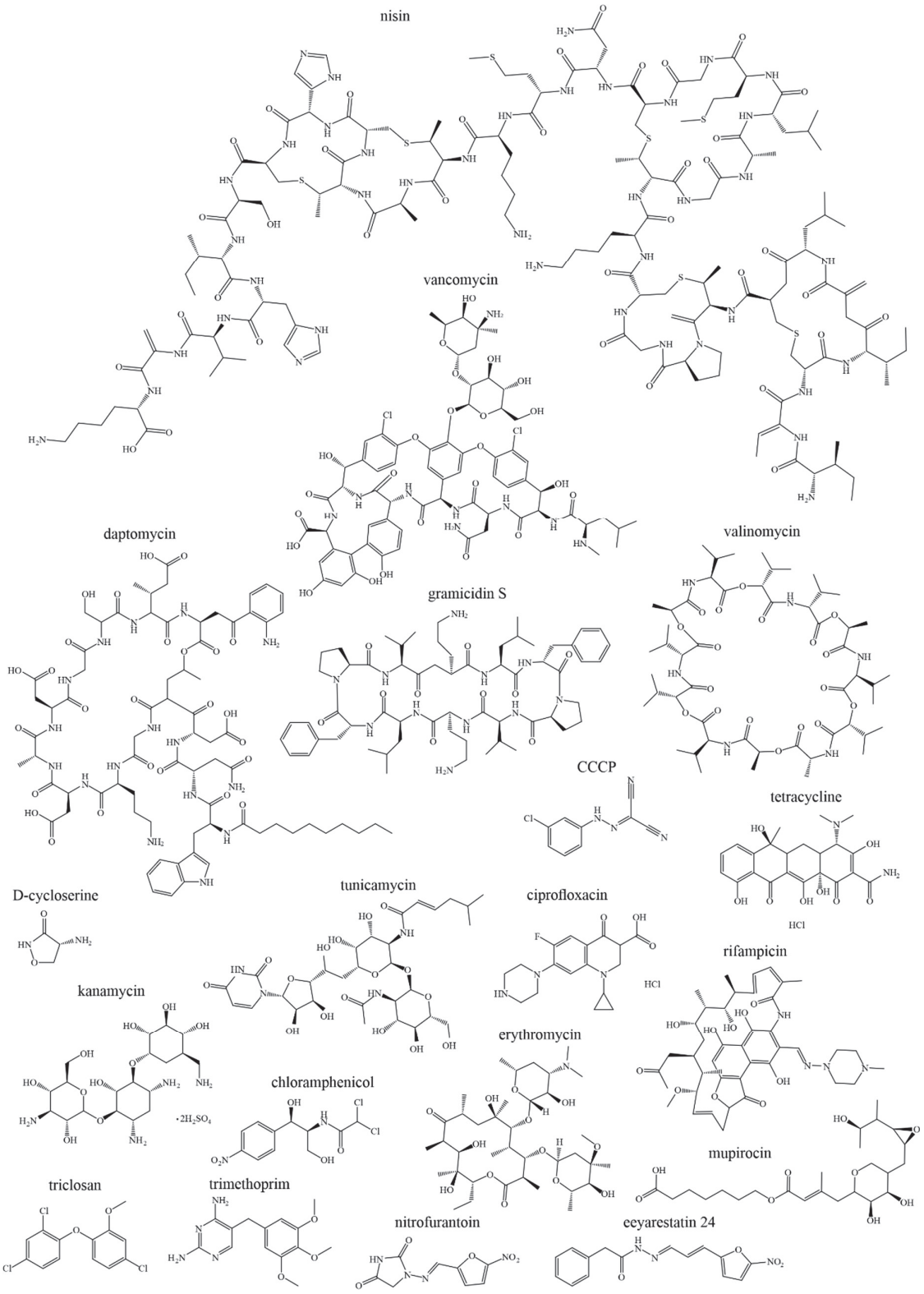

Figure 6 Structures of the antibiotics used in this thesis (Chem Draw)[78].

4. Methodology

4.1. Fluorescence in cell biology

The ability of a molecule to emit light after entering an excited state, e.g., through absorption of light, is called fluorescence. This phenomenon has been widely exploited in cell biology and methods based on fluorescent probes or proteins have become a crucial tool in studying cellular processes. The discovery of green-fluorescent protein (GFP) from jelly fish has fundamentally changed the way in how cell biology can be investigated[91]. After decoding its sequence, researchers were able to engineer this protein for use in many different organisms and change its spectral properties to suit different applications. Nowadays, genetically encoded fluorescent proteins have become an essential tool in cell biology[92]. In this thesis, fluorescent probes and fluorophores are utilized to investigate changes in bacterial cell biology upon antibiotic treatment.

Since bacteria are very small, within the µm range, and their cellular structures even smaller, the Gram-positive cell wall being 30-100 nm thick, advanced instruments are required to visualize the bacterial cell components with fluorescent probes[34]. In this thesis, fluorescence was detected and visualized with epi-fluorescence microscopy.

However, not all cell biology methods require single cell imaging. In some cases, the change of fluorescence intensity within the whole cell population can provide important information, e.g., changes in membrane potential and fluidity [93,94]. In this thesis, fluorescence spectroscopy was used for fluorescent batch studies.

4.2. Mode of action studies

The mode of action of an antibiotic indicates the rate of resistance development against it. Importantly, the mechanism of an antibiotic must normally be characterized before clinical approval is granted. To elucidate the mode of action of antibiotics is therefore important[23,95]. There are many methods available that can be utilized to study this. A global approach to investigate an antibiotic are, for example, omics methods, which among others can provide information about stress-response profiles and posttranslational modifications[95]. These approaches are not specific to a certain pathway but provide a general insight into the bacterial response. While not part of this thesis, they are further described in the review Schäfer and Wenzel (2020)[95]. In this thesis, live cell assays are utilized, including fluorescent protein fusions and specialized reporter dyes, that can be studied with fluorescence microscopy and spectroscopy.

This chapter will describe several different techniques that were used throughout my thesis and will provide information about additional techniques that can be used for either more in-depth studies or as alternatives.

4.2.1. Outer membrane

Effects of antibiotics on the outer membrane can be investigated by several different means. The integrity of the OM in response to antibiotics can be analyzed by utilizing 1-N-phenylnaphtylamine (NPN). This fluorescent probe is not able to cross the intact OM. However, if the OM is permeabilized NPN binds to the inner membrane, leading to an increased fluorescent signal[96].

4.2.2. Cell wall

Cell wall synthesis can be investigated by several methods. Here, *in vivo* (in the living bacterial cell) assays are described that elucidate different aspects of the cell wall synthesis. Effects on the incorporation of peptidoglycan, as well as the integrity of the cell wall, can be visualized by fixation with acetic acid and methanol. This treatment causes the protoplast to shrink, and in case of cell wall breaches, protrudes through these breaches, resulting in a bright “bubble” in phase contrast microscopy (Figure 7 A). However, this assay also depends on autolysin activity and can be unspecific, if compounds affect cell wall synthesis indirectly through targeting the membrane[97,98,95]. In contrast, fluorescently labeled D-amino acids (FDAAS) can be used for live cell imaging. FDAAs label cell wall precursors and are

incorporated into the cell wall[99]. Inhibition of peptidoglycan incorporation subsequently leads to a loss of signal or to a different localization pattern compared to the untreated control[100,101,102]. However, FDAAs can be low in signal or sensitive for photo-bleaching[103]. In addition, it is possible to label some antibiotics directly with 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY). The conjugation of BODIPY to vancomycin (Van-FL) can, for example, be utilized to investigate lipid II localization, since vancomycin binds to its D-ala-D-ala motif[95,104].

In addition to the integrity of the cell wall and the extracellular cell wall synthesis steps, several proteins involved in this pathway can be fluorescently labeled and utilized as reporters (Figure 4). This provides the opportunity to investigate several individual steps of cell wall synthesis[105]. This method is also easily accessible. One such reporter is the cell shape-determining protein MreB, which coordinates the lateral cell wall synthesis. Its movement is dependent on the presence of lipid-linked cell wall precursors and it has been observed that cell wall synthesis inhibitors specifically arrest its movement[106,107,108,109]. To investigate the arrest of movement, images of MreB are taken 30 seconds apart. The overlay of these images can then be used to assess the mobility of MreB. An arrest of MreB movement will result in an overlay of MreB foci (Figure 7 B)[23,95]. However, it is important to keep in mind that cellular proteins are often affected by several factors. MreB localization, for example, is also sensitive to the dissipation of the membrane potential[21]. Also, some changes in protein localization of these proteins may be much more subtle than stalled movement and require more in-depth analysis or an expertly trained eye.

Furthermore, reporter gene fusions can be utilized to investigate cell wall synthesis inhibition. The *PliaI* promotor for example, reports on cell wall stress[110]. Coupling *PliaI* with LacZ results in an induction of β-galactosidase, if the antibiotic interferes with membrane-bound steps of cell wall synthesis. This effect is easily visible as a blue ring surrounding the antibiotic inhibition zone on X-Gal plates, or can be measured with ortho-Nitrophenyl-β-galactoside (ONPG) (Figure 7 C)[111].

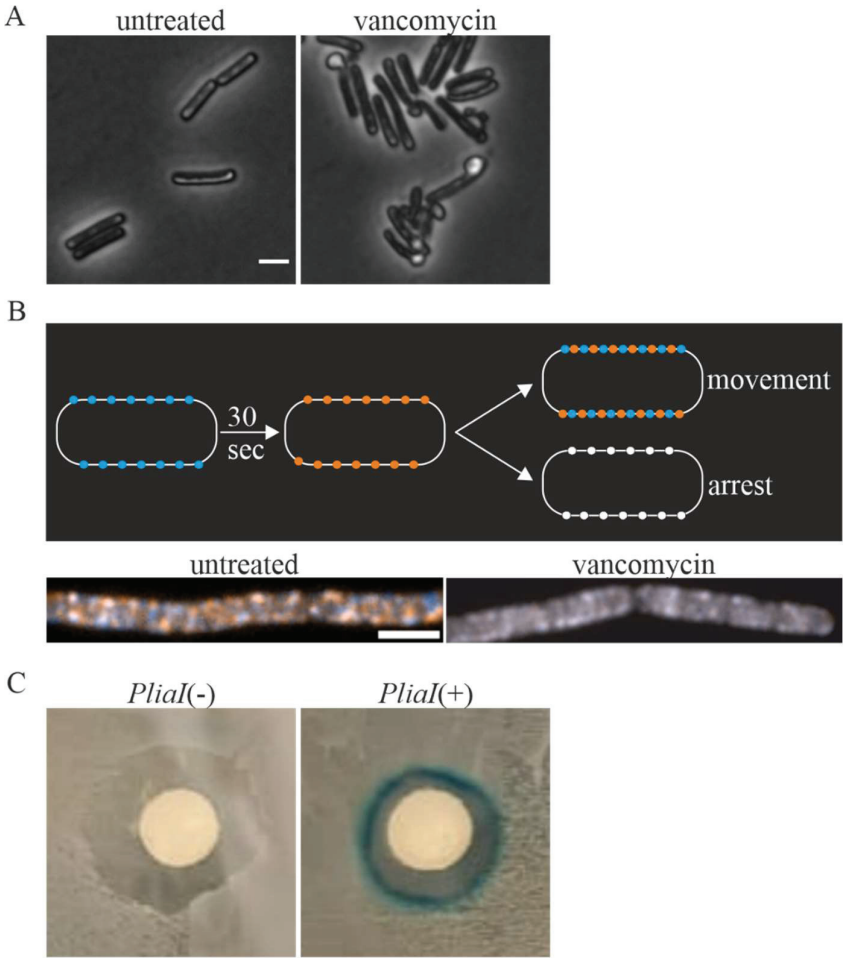

Figure 7 Selected assays to assess effects on cell wall synthesis. (A) Acetic acid/methanol fixation. Protrusion of protoplasts are visible when the cell wall is compromised (*B. subtilis* 168CA). Scale bar 2 µm. (B) Schematic representation of the MreB mobility analysis and representative microscopy images. Scale bar 2 µm. (C) Examples of the cell wall stress induced reporter fusion *PliaI*.

4.2.3. Cytoplasmic membrane

Effects of antimicrobial agents on the cytoplasmic membrane of bacteria have often been assessed in *in vitro* model membrane systems. These methods offer the benefit of a controlled environment, allowing assessment of compounds with, e.g., specific lipid species, can be studied[112]. However, the disadvantage of these methods is that they are unable to capture the full complexity of the bacterial cell membrane. Biological membranes can not only vary considerably with regard to their composition, but they are also constantly adapting to the environmental conditions[113]. When looking into the mechanisms of antibiotics on the cell membrane, it has become apparent that *in vitro* methods do not always translate well to the *in*

vivo mechanism[60,114]. The surge of interest in biological membrane architecture led to the development of techniques targeted toward the investigation of membrane properties in living bacteria[115,113].

General membrane morphology

An easy way to study changes in the morphology of the membrane as a response to treatment with antimicrobial compounds is to stain the bacteria with membrane dyes[23,116]. These membrane dyes produce smooth stains in healthy cells, but also tend to migrate to more fluid phases when phase separation occurs. This makes them a good tool to investigate phase separation induced by antimicrobial compounds[117]. In addition, an accumulation of the dye in the membrane can be indicative for lipid II accumulations, which could be investigated by utilizing Van-FL (chapter 4.1.2). Membrane invaginations can also cause an accumulation of dye, which can be analyzed further by structured illumination or electron microscopy[117,118,22] Such membrane dyes can be combined with fluorescently-tagged proteins for co-localization studies[117]. A bright and relatively photostable red-fluorescent dye which provides good contrast is 9-diethylamino-5H-benzo[α]phenoxazine-5-one, in short, also called Nile red. This dye has an excitation maximum between 552-565 nm and an emission maximum of 636 nm[119]. However, it is prone to bleed into other channels[120]. Moreover, glass coverslips need to be pre-treated with poly-L-dopamine to prevent absorption of the dye to the glass, which will lead to an increased background signal and subsequent obstruction of cell imaging[117]. Another dye that has an excellent signal-to-noise ratio and does not require additional coating of the coverslip is MitoTracker Green (MTG)[117].

Membrane depolarization

While general membrane dyes are useful, they are not able to report on more specific features of membrane architecture or organization. It is, for example, crucial for bacteria to maintain a membrane potential to uphold membrane-associated processes and supply the cells with energy[21]. One prominent dye that is frequently used to assess changes in the membrane potential is 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$). $DiSC_3(5)$ inserts into polarized membranes, where its fluorescence is then reduced due to its self-quenching properties. A depolarization of the membrane leads to a release of the dye and an increase in spectroscopic assays (Figure 8 B)[103]. The sensitivity of this dye even allows for quantitative readouts in combination with calibration with the potassium ionophore valinomycin[103]. While this dye is not suitable for timelapse microscopy due to its cytotoxicity, it can be utilized for endpoint

imaging allowing for single cell analysis[103,121]. In addition to fluorescent dyes, some membrane proteins can also be used as proxy to investigate membrane potential effects. The cell division regulation protein MinD is one such protein. This peripheral membrane protein is part of the MinCD system, which controls Z-ring positioning. In rod-shaped bacteria like *B. subtilis*, MinD localizes to the cell septum and cell poles[122]. Depolarization of bacterial cells leads to a partial dissociation of MinD into the cytosol and/or clustering at the membrane[21]. In *Escherichia coli* (*E. coli*), MinD oscillates from cell pole to cell pole and this oscillation is arrested when depolarization occurs[123,117].

Pore formation

Depolarization can indicate pore formation. Therefore, it is important to test for pore formation when investigating a new antimicrobial compound. This can be accomplished with different assays. An easy way to test for pore formation is to analyze the leakage of cytosolic GFP. However, with a size of 27 kDa, GFP is a relatively large molecule[23,124]. To test for smaller pores, fluorescent dyes like propidium iodide or SYTOX Green can be utilized. Both dyes have an affinity to bind DNA but cannot cross intact membranes. In the case of pore formation, the membrane is compromised and the dye can bind to the DNA[125,126].

Fluidity changes

Another aspect of the membrane is its fluidity. Membrane fluidity is affected by a number of different factors, e.g., lipid species, temperature, and proteins[127]. While there is no assay that can investigate all these aspects at once, there is a fluorescent dye that can be used to report on general changes in membrane fluidity: 2-dimethylamino-6-lauroylnaphtalene (laurdan)[128,93]. This solvatochromic dye intercalates in the membrane and the emission peak of laurdan shifts, depending on how much water molecules are surrounding the dye, indicating head group and fatty acid chain spreading. This shift can be detected, and the general polarization (GP) can be calculated as: $GP = (I_{460} - I_{500})/(I_{460} + I_{500})$ (Figure 8 C)[93,129].

Membrane microdomain effects

The membrane contains different types of lipid species, which lead to the formation of microdomains[130]. These microdomains are important, for example, for the lateral cell wall synthesis machinery, which is localized in so-called regions of increased fluidity (RIFs)[60,22,131,132]. Microdomains also appear to be sensitive to the treatment of membrane-active compounds, often causing fusions of RIFs[105,22]. These membrane domains can be

visualized by the selective dye didodecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate (DiIC12). DiIC12 preferentially localizes to thinner membrane regions due to its short (12C) hydrocarbon tail[133,134]. While larger rigid membrane domains can be visualized with laurdan, there are currently no methods available to study smaller rigid domains[135]. In addition to these dyes, effects on membrane domains can also be studied by utilizing proteins that localize to such domains, e.g., MurG[60].

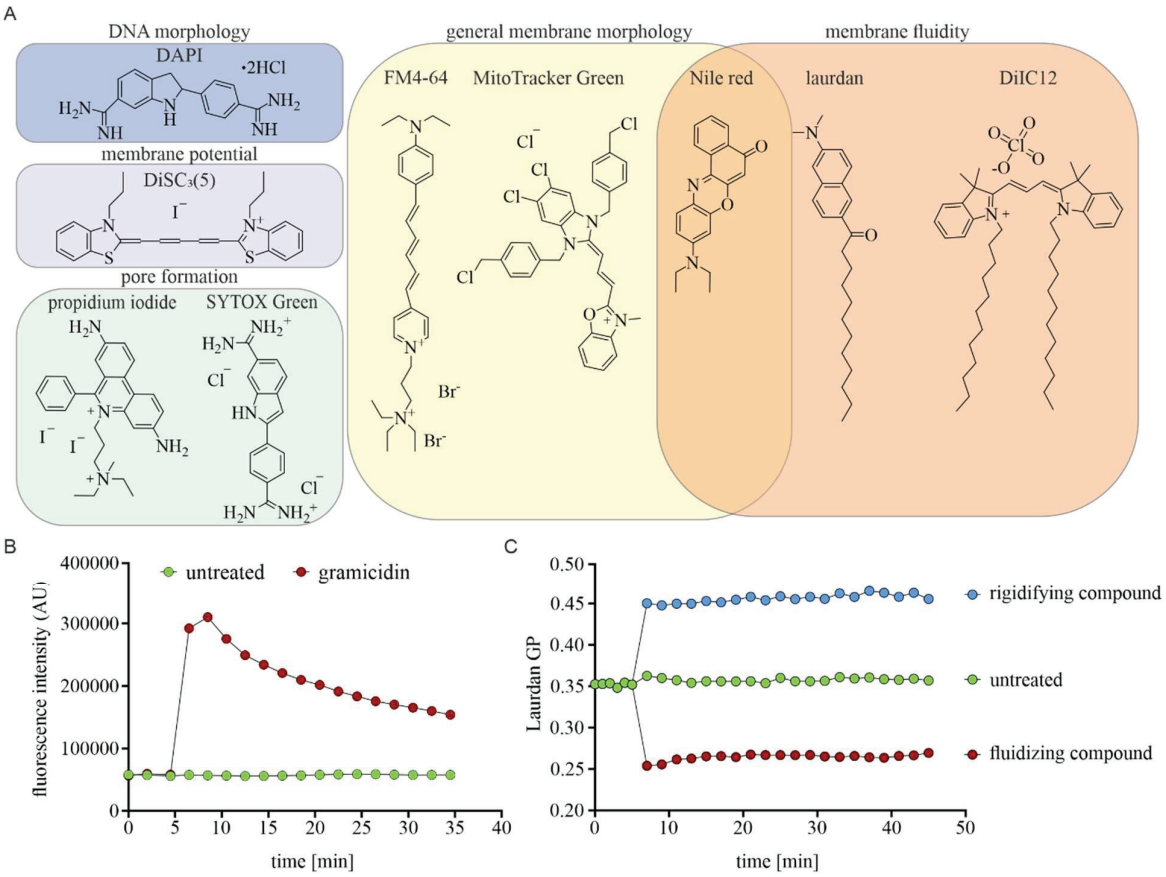

Figure 8 Fluorescent probes and assays used to investigate effects of antibiotics on membranes *in vivo*. (A) Structures of the fluorescent probes utilized in this study. (B) The membrane depolarization-sensitive dye ($DiSC_3(5)$ accumulates in the membrane, which leads to self-quenching. Depolarization causes a disassociation of the dye, subsequently resulting in a higher fluorescence signal in spectroscopic measurements[103]. (C) Membrane fluidity measurements of rigidifying and fluidizing compounds.

4.2.4. Intracellular targets

There are many antibiotics available on the market that target intracellular processes. Although their mechanisms can certainly be further investigated with a plethora of *in vitro*

assays, there are also several *in vivo* methods available. While not exclusively targeted towards intracellular targets, bacterial cytological profiling is a powerful screening method for different modes of action. This method, in its original form, employs the general membrane stain FM4-64, the DNA stain 4′,6-diamidino-2-phenylindole (DAPI), and the pore formation reporter SYTOX Green[116]. The assay provides first insights into the modes of action of antibiotics. The changes in cell morphology can be derived from the microscopy images and principal component analysis leads to clustering of antibiotics targeting the same pathway[116]. Bacterial cytological profiling (BCP) has since been utilized to screen antibiotics in multiple different bacterial species, highlighting its versatility[136,137]. One drawback of this method is that it cannot identify the specific target, especially when it comes to antibiotics targeting the cell envelope[116]. However, this assay is very versatile, and can be modified by combining it with different protein reporters to get further insight. In this thesis, BCP refers to the assay utilizing a DNA and membrane stain, as well as a reporter for pore formation.

In some cases, antimicrobial compounds also cause DNA damage, which can lead to several downstream effects[138]. This can be investigated by utilizing the DNA damage reporter RecA. RecA is part of the DNA repair machinery and is recruited to sites of double strand breaks of the DNA, resulting in bright foci, if DNA damage has occured[22,139,140].

Several compounds also target DNA packing or DNA related processes[141]. There are many proteins with GFP fusions that are available and can be utilized to investigate changes in localization patterns following antibiotic treatment (Figure 9)[95]. However, the localization of these protein fusions, e.g. DnaN (DNA polymerase), can also be affected by changes in DNA morphology[142]. It is therefore advisable to also investigate DNA morphology itself. The visualization of the DNA morphology (through, e.g., DAPI) makes it possible to analyze nucleoid fragmentation as well as the effects on its compaction (Figure 9)[22,143].

A specific target of antibiotics is the RNA polymerase. There are reporter fusions available, for example, of the beta RNA polymerase subunit RpoC in *B. subtilis*[68,69,95]. Similarly to the DNA reporters, its localization can be influenced by DNA morphology changes and a combination of with a DNA stain is advantouges[22].

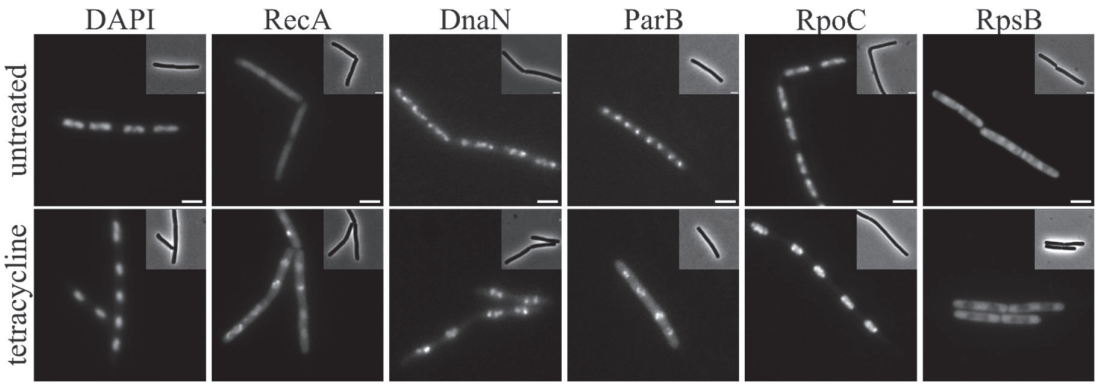

Figure 9 Microscopic assays for investigating intracellular effects using the ribosome inhibitor tetracycline as example. DAPI is used to visualize morphological changes, while RecA is recruited to sites of DNA damage. Impaired DNA replication can be investigated by utilizing the DNA-binding proteins DnaN and ParB. RpoC is a subunit of the RNA polymerase and RpsB is a ribosomal protein[95].

The production of reactive oxygen species (ROS) can affect several structures of the cell, from the DNA to the membrane[81,144]. While bacterial cells encounter ROS in natural environments, treatment with antibiotics can also lead to oxidative stress[79]. It has also been shown that even after the removal of stressors, ROS species can still accumulate to a lethal degree for bacteria; thus highlighting how important it is to include ROS production in the mode of action investigation of antimicrobial compounds[145]. The presence of oxidative stress can be investigated by performing susceptibility assays in the presence and absence of ROS scavangers[81,146]. Additionally, there fluorescent dyes are available, which penetrate the cells and are oxidized in the presence of ROS, e.g., the free radical sensor Oxyburst green[81,147].

5. Original work

This chapter provides a summary of the results found of the four papers included in my thesis as well as additional unpublished work resulting from my PhD. Their key findings will be briefly discussed.

The overall aim of my PhD thesis was to provide an understanding of how antibiotics work. This has been tackled in two ways (a) by providing detailed knowledge on how to use techniques to elucidate the modes of action of antibiotics and make them more accessible for non-expert researchers (**papers I** and **II**) and (b) by investigating new potential antibiotic drug candidates (**paper III**, **paper IV**, chapters 5.1.1, 5.2.1, 5.3.1, and 5.3.2).

Chapter 5.1. discusses phenotypic analysis assays targeted towards elucidating membrane fluidity on a local and global scale and provides an overview of important aspects that need to be considered when using them for mode of action studies (**paper I**). A practical application of these methods will be discussed in Chapter 5.1.1, highlighting the importance of understanding fluidity changes in response to antibiotic treatment across different bacterial organisms.

Paper II presents a curated method set that can provide first insights into cell envelope effects, further expanding the membrane-related methods (**paper I**) by adding techniques to assess cell wall synthesis, allowing distinction between cell envelope-targeting antibiotics (chapter 5.2). The acquired knowledge and curated method kit from **paper II** are then utilized to further investigate the modes of action of antibiotic compounds. Chapter 5.2.1 connects the findings of **paper II** with further in-depth screening of antibiotics with intracellular targets. Additionally, chapter 5.3.1 shows how the curated methods can be applied to study antibiotic compounds with unknown mechanisms of action. Furthermore, it discusses which findings from the initial screen require further follow-up assays. Chapter 5.3.2 highlights the mode of action of novel AMPs (**paper III**), adding to the overall aim of my thesis to gather information for further drug development.

Chapter 5.3.3 provides an example of how the presented phenotypic analysis assays can be expanded to allow a more in-depth studies (**paper IV**) expanding on the initial method tool kit (**paper II**) and presents new insights into the mode of action of a promising antibiotic drug candidate.

5.1. Methods for phenotyping antimicrobial compounds

Many antimicrobial compounds interact with the cell membrane, which can disrupt cellular processes. A wide spectrum of modes of action can underlie these effects, e.g., compounds can induce various types of pores, affect the membrane potential, disorganize membrane lipids, or induce membrane invaginations[51,60,117,143]. To fully understand their effects, suitable techniques are needed to investigate different aspects of the cell membrane. **Paper I** describes methods specifically tailored to measure membrane fluidity, namely laurdan, Nile red, and DiIC12 assays, and provides detailed protocols for their practical application.

Changes of overall membrane fluidity (spectroscopy) as well as local membrane fluidity (microscopy) can be assessed with laurdan[93,129]. Yet, it requires specific filter sets and much care during the handling of these samples. Here we showed that the key factor for fluidity measurements is a tight temperature control, which requires prewarming of all materials used for this assay.

Additionally, the membrane dye Nile red, often used as a general membrane dye, can be used for microscopically studying local membrane fluidity changes, as it migrates to more fluid regions of the membrane upon phase separation. We could show that this dye colocalizes with RIFs by co-staining *B. subtilis* with laurdan and Nile red. Local changes of membrane fluidity induced by CCCP were detected in laurdan colocalized to Nile red foci.

The fluorescent dye DiIC12 is a more sensitive microscopic fluidity sensor and can be used to visualize RIFs. We have made several important observations that impact the quality of the results, for example, that additional washing steps help to reduce background signal and that the optical density (OD) of the overnight culture has an impact on the staining (Figure 10 B). Additionally, we demonstrated the effects of antibiotics on RIFs, which is of special importance for this thesis work. Here, *B. subtilis* cells were treated with nisin and daptomycin, which led to a clustering of RIFs, indicating that they are affecting membrane domain organization.

Detailed step-by-step protocols for these methods are now available to the scientific community in **paper I**.

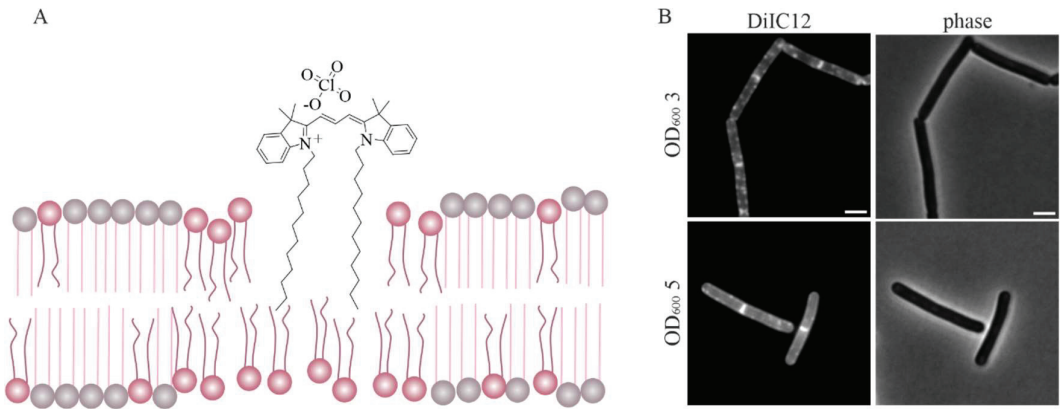

Figure 10 Structure of the membrane dye DiIC12 (A) DiIC12 inserts into regions of increased fluidity (modified from Schäfer and Wenzel, 2020)[95]. (B) Impact of the OD of the overnight culture on its staining quality. Scale bar is 2 µm.

5.1.1. Application of membrane fluidity measurements

In **paper I** we showed that daptomycin led to membrane domain clustering in *B. subtilis*. In this organism, the lateral cell wall synthesis machinery is localized in these domains[131]. However, this is not the case for *Staphylococcus aureus* (*S. aureus*), which, as a coccus, does not possess lateral but only septal cell wall synthesis and is one the main targets of daptomycin treatment[60,131,148].

Many studies of the mode of action of daptomycin have been conducted in *B. subtilis*, including most data from our own lab[117,60]. Due to the differences observed between this model organism and *S. aureus*, we decided to investigate the effects of daptomycin on this clinically relevant *Staphylococcus* species with a similar methodology as previously used for *B. subtilis* (**paper I**)[60]. Here, these methods were practically applied and their transferability to different bacterial species was tested.

The effect of daptomycin on fluid membrane domains was investigated with DiIC12, which is one of the key techniques described in **paper I**[93]. *S. aureus* does not possess RIFs and accordingly does not produce a spotty DiIC12 pattern[117,93]. However, it does possess membrane domain organization, which is visible as very subtle intensity differences of the DiIC12 dye[117].

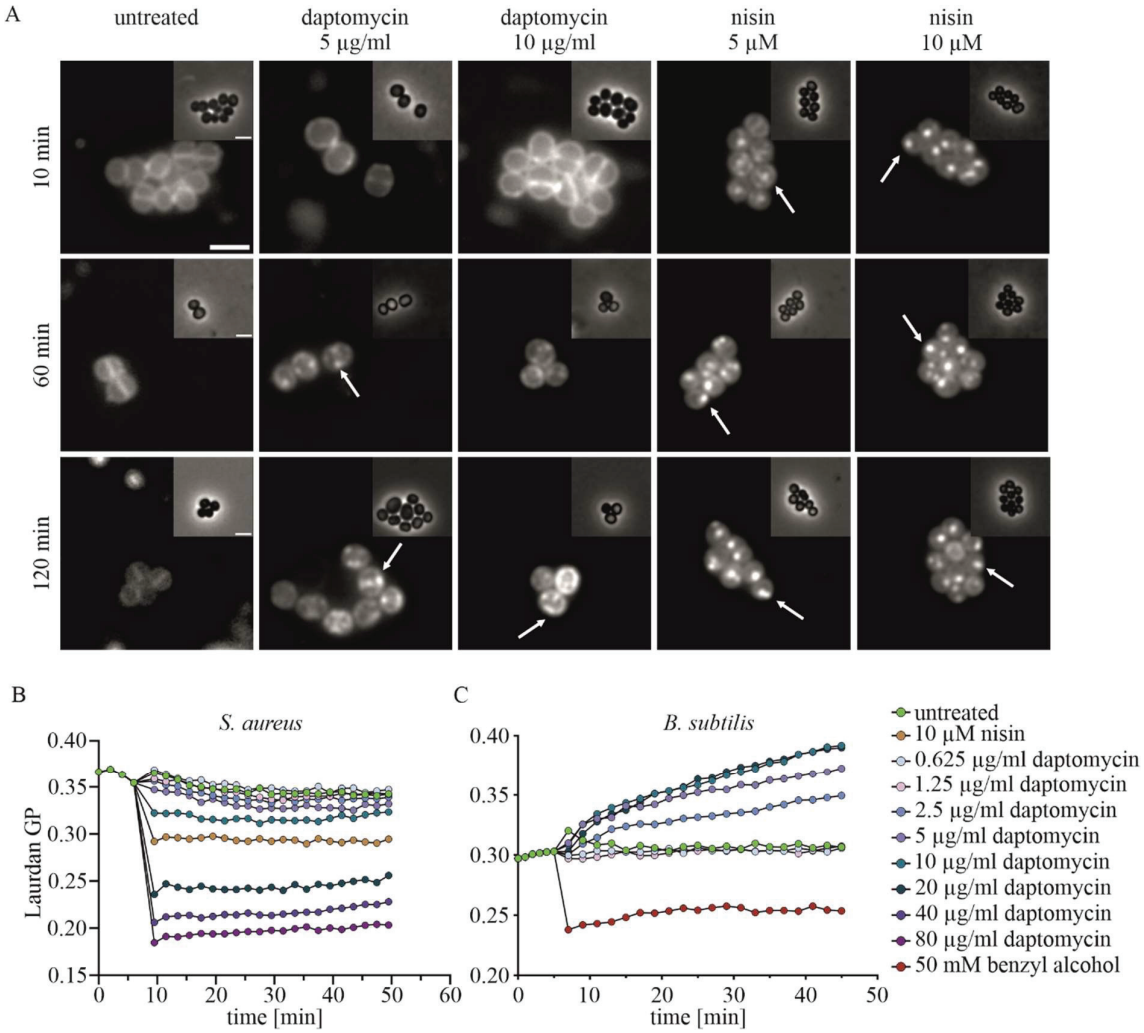

Figure 11 DiIC12 membrane domains stains of *S. aureus* SH1000 after 10 min, 60 min, and 120 min of antibiotic treatment. Scale bar is 2 µm. Kinetic laurdan measurements of *S. aureus* SH1000 and *B. subtilis* 168CA were performed for 50 min. Antibiotics were added after recording the baseline for 5 min.

After 60 min of treatment with 5 µg/ml daptomycin, a clear clustering of the dye could be observed. Similar clustering could also be observed after 2 h for cells treated with 10 µg/ml daptomycin. The same concentration-dependent effect was previously observed in *B. subtilis*[60,104,114]. Treatment with different nisin concentrations immediately resulted in one big membrane domain independent of the concentration (Figure 11 A), demonstrating the differences in the mechanisms of these two compounds.

Next, we used the spectroscopic laurdan assay described in **paper I** to gain insight into overall membrane fluidity changes. Interestingly, daptomycin had the exact opposite effect in

the two organisms. In *B. subtilis*, a clearly concentration-dependent membrane rigidification was observed, which is in line with reported data (Figure 11 C)[60]. In contrast, an equally clearly concentration-dependent membrane fluidization was observed in *S. aureus* (Figure 11 B).

Here, we utilized the methods discussed in **paper I** to investigate the mechanism of action of daptomycin in *S. aureus* and compared them with key findings previously made in *B. subtilis*. These methods enabled us to show that daptomycin affects the membrane domain formation phenotypically similarly in both organisms[60,114,104]. Interestingly, laurdan measurements showed that daptomycin has the opposite effects on membrane fluidity, acting in a fluidizing manner in *S. aureus* while rigidifying *B. subtilis*. What exactly causes this difference remains unclear. However, it could be speculated that the specific membrane domain organization of *B. subtilis*, in particular, MreB-organized RIFs may play a role. Further investigations will be needed to unravel these differences, e.g., fluidity could be assessed in *B. subtilis* strains devoid of MreB.

Overall, this study showed that our membrane fluidity methods can provide important insights into the modes of action of antibiotics and can elucidate mechanistic differences between bacterial species.

5.2. Expanded methods for phenotyping

Paper I provided methods to analyze changes in membrane fluidity, which is one important aspect to study when investigating the modes of action of antibiotic compounds (chapter 5.1.1). However, many other phenotypic analysis assays exist that assess various additional aspects of the bacterial cell[95]. Yet, non-expert researchers might struggle to decide which assays are suitable, and not all of them can be easily implemented, either due to technical limitations or considerable expertise being required for their correct analysis and interpretation.

In **paper II**, we curated a set of assays that can be used to distinguish between different cell envelope-targeting compound classes, that are easy to implement and interpret. We propose a combination of three core techniques consisting of an initial assessment with BCP and specific reporters for the membrane potential ($DiSC_3(5)$ and MinD), and cell wall synthesis (MreB mobility and *PliaI* induction). Additionally, we propose using laurdan-based fluidity measurements in cases, where it is indicated, and the equipment is accessible (see **paper II** FIG7)[23].

In this paper, each step of phenotypic mode of action studies is described including determining the compound activity and deciding on suitable concentrations for further experiments, assay selection, workflows, protocols, and data analysis, which will be helpful for other researchers, including non-experts outside of the field or labs with limited resources.

The suitability and specificity of this tool kit were initially assessed with 3 specific and well-characterized cell envelope-targeting antibiotics, targeting either the cell wall (vancomycin), the cell membrane (valinomycin), or both (nisin). We showed that the curated method set was sufficient to categorize these three antibiotics accordingly. To further validate this tool kit, we additionally analyzed 10 antibiotics with various modes of action. We could show that the assay kit could reliably identify and distinguish between cell wall and cell membrane antibiotics as well as dual-action inhibitors.

Taken together, **paper II** provided a phenotypic analysis tool kit that is easy to implement and accessible to non-expert researchers. It can also serve as a good starting point for mode of action analysis, even for more experienced researchers.

Interestingly, some of the tested comparator antibiotics gave unexpected results in some assays, leading us to the hypothesis that they may possess more than just their originally

described targets. These findings were intriguing and prompted us to further evaluate these compounds and investigate whether the modes of action of these antibiotics are indeed more complex than currently assumed.

5.2.1. Multifunctional antibiotics

In this chapter, the findings from **paper II** are investigated further. In addition, we aimed to establish a reference set of already characterized antibiotics to be able to compare the phenotypes of new antimicrobial compounds with the phenotypic patterns of known inhibitors. Here, only a fraction of the project is presented, focusing on data collected during the supervision of my Master students Hashlin Utami and Priyanka Rawat.

Prompted by the observations made in **paper II**, our data on daptomycin, and previous work on tetracycline and gramicidin S, we aimed to elucidate how antibiotics that belong to long-established clinically used classes and are commonly known as single-target compounds, really affect bacterial cell biology[60,143,22,52]. To this end, we used the curated methods described in **paper I** and **II** and expanded them where necessary.

First, we subjected 11 antibiotics with known intracellular targets (Table 1) to our modified BCP assay, following the workflow proposed in **paper II**. Data analysis revealed that nine antibiotics affected nucleoid morphology, none caused visible leakage of GFP, and three showed a significant increase in membrane stress, signified by blobs in the Nile red membrane stain (Figure 12 B). While D-cycloserine only induced membrane stress to a small degree, it has been shown to be more prominent under different culturing conditions (**paper II**)[23].

Since many antibiotics showed an effect on the nucleoid, we expanded the tool kit of **paper II** to include further DNA reporters, which can be used to get more detailed insights into the effects underlying DNA morphology changes observed in the BCP. To visualize if the compounds induced DNA damage, a RecA-GFP fusion was used. Four out of the nine antibiotics affecting DNA compaction caused accumulation of the DNA damage reporter (Figure 12 C). The observations for trimethoprim and ciprofloxacin are in line with the known modes of action of both compounds (Table 1)[85,67].

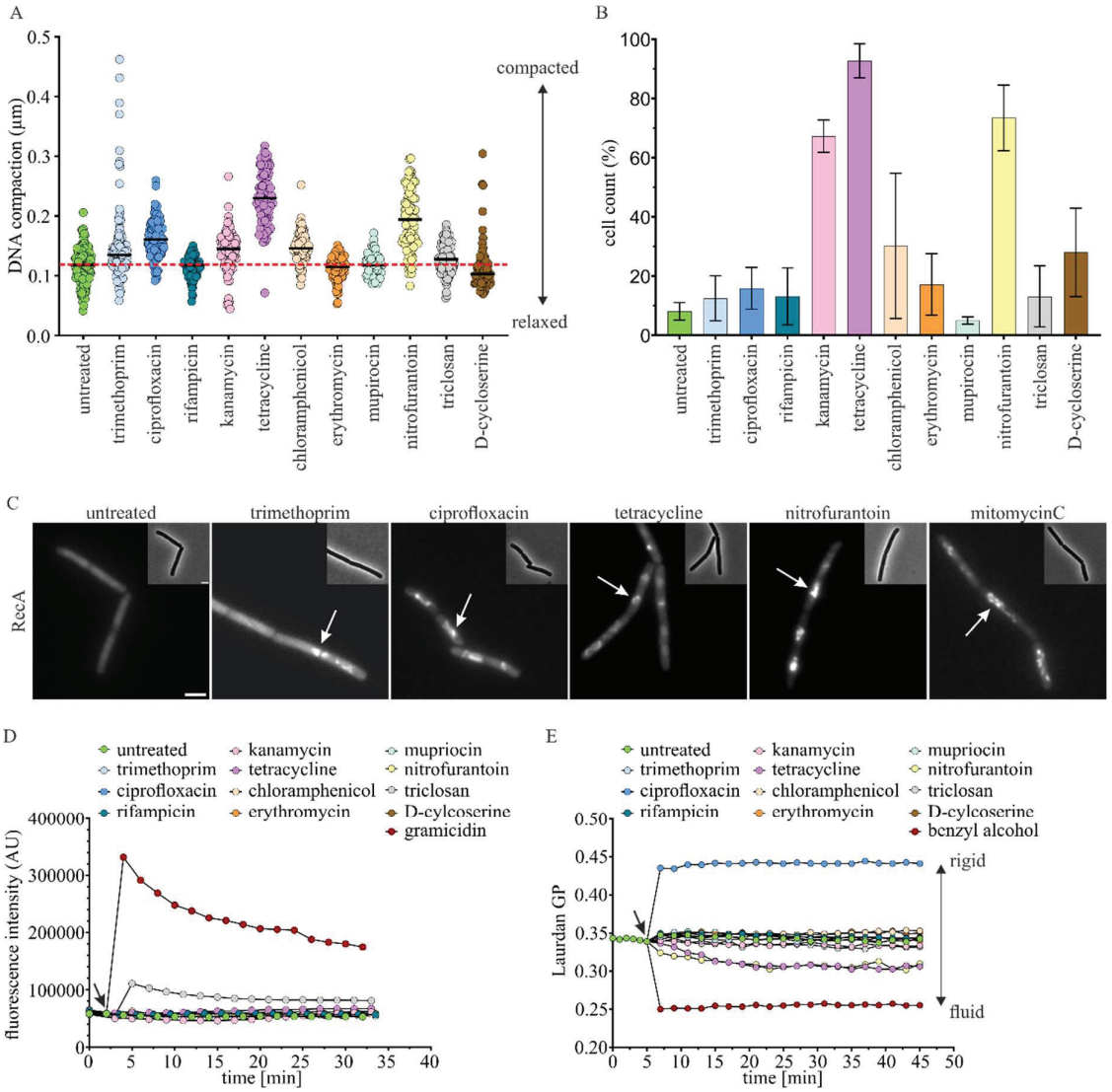

Figure 12 Image analysis of nucleoid compaction (A) and membrane stress (B) observed after 30 min of antibiotic treatment, (C) DNA damage, (D) membrane potential effects, and (E) fluidity changes caused by antibiotic treatment. (A) Nucleoid compaction values were calculated with ImageJ[149]. Cells from three biological replicates were merged for analysis and a minimum of 55 cells per condition were analyzed. The black line indicates the median of each sample, while the red dotted line indicates the median of the untreated control. (B) Quantification of cells displaying membrane defects in the Nile red stain. Error bars represent the standard deviation of the mean of three biological replicates. A minimum of 271 cells were counted per antibiotic condition. (C) Recruitment of RecA-GFP caused by antibiotic treatment in *B. subtilis* UG10 (*recA-gfp*). Mitomycin C (0.05 µg/ml) was used as a positive control. Arrows indicate accumulation of RecA in foci. Scale bar is 2 µm. (D) Membrane potential measurements with $DiSC_3(5)$ of *B. subtilis* 168 CA. (E) Kinetic laurdan measurements *B. subtilis* 168CA. Black arrow indicates the star of antibiotic treatment (modified from H. Utami, 2022)[142].

BCP indicated membrane damage for several antibiotics (Figure 12 B). Therefore, membrane potential and fluidity were assessed. Only triclosan showed a slight loss of membrane potential, which indicates a partial or heterogenous depolarization (Figure 12 D). Indeed, it is known that triclosan affects the cell membrane directly at higher concentrations, so this result is unsurprising[150,151]. Membrane fluidity was assessed with laurdan following the proposed protocols of **paper I** and **II**. Interestingly, ciprofloxacin seemed to rigidify the membrane, while nitrofurantoin and tetracycline had fluidizing effects, which were also visible under different media compositions in **paper II** (FIG S13, SI **paper II**)[23]. Nitrofurantoin was known from previous studies to cause membrane damage and it can be speculated that this may be due to lipid peroxidation by reactive species formed after reduction of the pro-drug[81,143]. Similarly, tetracycline was shown to affect the cell membrane, in particular RIFs, so the observed effect on overall fluidity fits well with these previous observations[143]. For ciprofloxacin, no effects on membrane fluidity have been reported before, but in our other work, not included in this thesis, we have indeed observed membrane defects and effects on membrane protein localization, suggesting that it has additional or secondary effects on the cell membrane[152,153,154]. No membrane stress or fluidity changes could be detected for trimethoprim, which is known to target folic acid synthesis[67].

Overall, seven of the 11 investigated antibiotics affected other pathways in addition to their known targets (Table 2).

Table 2 Phenotypic patterns of the reference set assessed in this study. Positive results are indicated with a + (modified from H. Utami, 2022)[142].

antibiotics	**DNA effects**				**membrane effects**		
	DAPI	RecA	DnaN	ParB	Nile red	$DiSC_3(5)$	laurdan
trimethoprim	+	+	+	+			
ciprofloxacin	+	+	+	+			+
rifampicin			+				
kanamycin	+		+		+		
tetracycline	+	+	+	+	+		+
chloramphenicol	+						
erythromycin	+						
mupirocin			+				
nitrofurantoin	+	+	+	+	+		+
triclosan	+			+		+	
D-cycloserine	+		+				

Taken together, this study shows that the modes of action of commercially available antibiotics are not fully understood and provides further insights into their effects on the bacterial cell. However, except for tetracycline, it is not yet clear if the observed effects are due to independent secondary targets or caused by downstream effects of known target inhibition[143]. Furthermore, the effects of these antibiotics on membrane-related processes and the possible generation of oxidative stress should be investigated. Assays for ROS detection have been used in **paper IV** and will be discussed in chapter 5.3.

5.3. Mode of action studies of novel antibiotic compounds

A successful method platform was curated with the work presented in chapters 5.1 and 5.2. Furthermore, this platform has been validated regarding its usability, and a reference data set using antibiotics with known mode of actions has been generated. In the following chapter, the curated method set was used to analyze the modes of actions of new compounds.

5.3.1. Categorization of the modes of action of metal complexes

The methods presented in **paper II** were used to categorize the mode of action of new metal complexes. This study was conducted in collaboration with Dr. Angelo Freis' group (University of Bern). Since this work is unpublished, the compound names and structures will not be disclosed in this thesis.

Metal complexes have been used to treat various diseases for a long time[155]. In fact one of the first compounds used to treat bacterial infection, salvarsan, is a metal complex[3]. Yet, they are rarely used in the antibiotic field, even though they offer unique new structural properties that can be utilized for antibiotic development[156]. This chapter discusses the main findings of an initial mode of action assessment of three new metal complexes (MA, MB, MC) and their *in vivo* effects on bacteria.

Both MA and MB show DNA relaxation (Figure 13 A). MC treatment resulted in a heterogeneous population of condensed and relaxed nucleoids. Furthermore, the bacterial cytological profiling showed a slight decrease in GFP signal with MC, which can be indicative for pore formation[23]. In contrast to MC, MB did not cause a decrease in the GFP signal, but small areas adjacent to the cell membrane seemed to have no detectable GFP. MB also caused membrane deformations which co-localized with the exclusions of GFP. These findings suggest that MB could cause membrane invaginations. MA and MC also caused changes in membrane morphology (Figure 13 A).

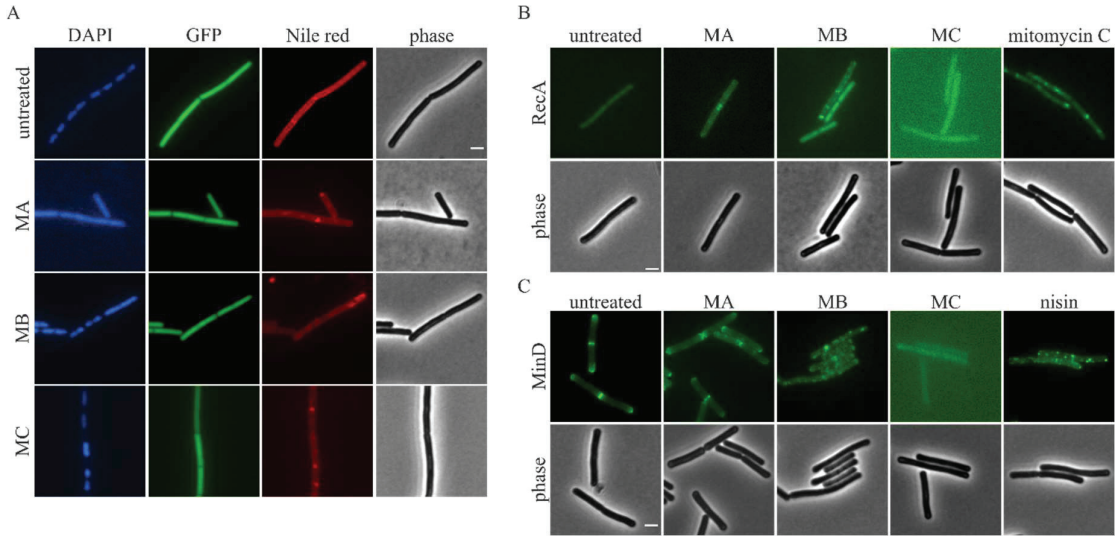

Figure 13 Bacterial cytological profiling of *B. subtilis* MW54 (*PrpsD-gfp*) exposed to metal complexes for 30 min in *B. subtilis* (*PrpsD-gfp*). DNA was stained with DAPI, while the membrane was visualized with Nile red. Scale bar is 2 µm. (B) Localization of the DNA damage reporter RecA after 30 min of antibiotic treatment (*B. subtilis* UG10 (*recA-gfp*)). (C) Localization of the membrane potential-sensitive protein MinD (*B. subtilis* TB35). Scale bar is 2 µm.

Since both MA and MB showed very prominent nucleoid relaxation in the BCP (Figure 13 A), we investigated if these complexes caused DNA damage. Interestingly, none of the tested compounds showed the classical DNA damage phenotype as seen with the positive control compound mitomycin C treatment[157]. Instead, RecA seemed to localize at the septa of the cells when treated with MA. MB treatment resulted in a small subpopulation that localized to the membrane. The most striking effect could be seen with MC treatment, which strongly diminished the GFP signal (Figure 13 B).

Using the membrane potential-sensitive reporter protein MinD (as proposed in **paper II**), a clear delocalization of the protein could be detected for MB (Figure 13 C). Interestingly, treatment with MC strongly reduced GFP signal also in this strain. To assess whether this may be spectral interference, we stained MC-treated cells with a green membrane dye, MitoTracker Green, but did not observe any reduction of fluorescence. Likewise, a spectral scan of the compound did not reveal any spectral interference. We then treated a strain expressing mCherry-MreB and observed the same reduction in fluorescence signal as our GFP fusions. Using an mCherry antibody, we could show that the protein was indeed entirely absent on the Western blots of MC-treated cells expressing mCherry-MreB (data not shown).

Based on these observations, we hypothesized that MC promotes protein degradation. To test this, the effect of MC on the whole protein content was tested. The cytosolic content was extracted after treatment with MC, and the obtained samples were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently stained with Coomassie blue, a dye that stains proteins by intercalation. This follow-up assay revealed that treatment with MC strongly reduced the overall cytosolic protein content (Figure 14)[51]. Culture supernatants were analyzed as well but did not show an increased protein content, showing that proteins are not leaking out of the cells (Figure 14)

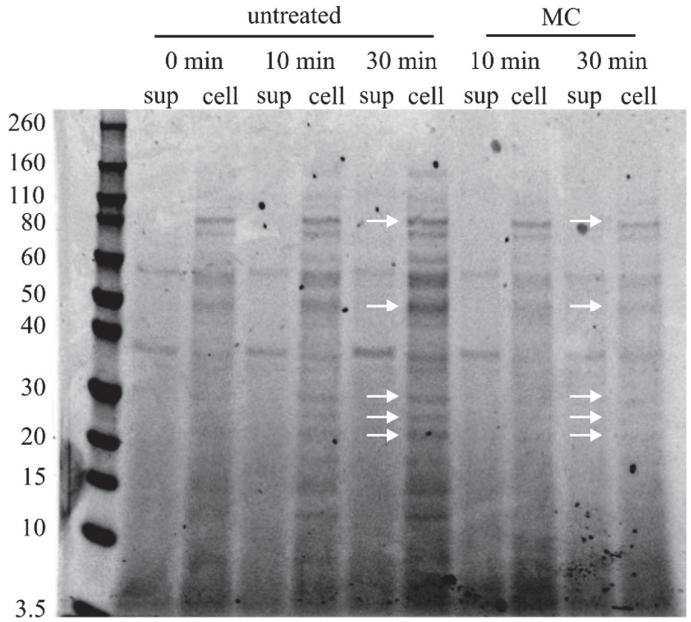

Figure 14 Coomassie-stained SDS-PAGE of the cytosolic content of *B. subtilis* 168CA with and without treatment of MC. Samples were normalized to OD. Sup refers to the supernatant, while cell refers to the cytosolic content of bacteria. Decreasing bands between untreated and MC-treated cells are indicated by white arrows.

Finally, we investigated why the strain MW54, expressing cytosolic GFP did not lose its fluorescence signal (Figure 13 A). Since the *PrpsD* is an exceptionally strong promoter, we hypothesized that it may simply be that GFP is so abundant in this strain that reduction cannot be seen by eye. Indeed, both quantification of microscopy images and spectroscopic batch measurements showed reduction in GFP intensity. Importantly, such effects were not observed with nisin or chloramphenicol, corroborating our protein degradation hypothesis (data not shown).

Overall, this study shows that the three metal compounds have very different modes of action, which is promising for further development of metal complexes within the antibiotic field. While they all seemed to cause DNA compaction defects, their effects on the DNA damage reporter differed. It is unclear why exactly MA causes a septal accumulation of this reporter and this effect needs to be further investigated. Recruitment of RecA to the membrane by MB may be indicative of protein aggregation, which was confirmed in additional experiments utilizing the YocM reporter (data not shown). MB also delocalized MinD, indicating that it might induce membrane depolarization, while MA only displayed a slight depolarization effect that seemed to not be strong enough to cause delocalization of MinD. Most striking are the findings for the complex MC. This compound does not cause pore formation or significant membrane depolarization. However, our data indicate that MC interferes with the protein degradation process. This process is the unique target of acyldepsipeptides (ADEP), which have been shown to dysregulate the ClpP protease, preventing cell division by degrading FtsZ[158]. The next step for MC will now be to identify the target, i.e. the affected protease(s). A screen of protease deletion mutants is currently being conducted.

This chapter shows how versatile metal complexes are and that they, based on our mode of action analysis, are able to target several different components of the cell. Most strikingly we found indications of a yet unknown mode of action for metal complexes, protein degradation. Furthermore, we showed that the tool kit discussed in **paper II** provides important insight into the mode of action of metal complexes.

5.3.2. Categorization of the modes of action of antimicrobial peptides

In **Paper III**, novel nonribosomal peptides from marine *streptomycetes* were identified based on genome mining predictions, chemically synthesized, and subsequently characterized with respect to their structure, activity, toxicity, and modes of action.

AMPs are an important class of antimicrobials. They can be found in all domains of life and are part of the innate host defense system[159]. One such source of AMPs is the marine environment, which provides an enormous resource for new antimicrobial peptides[160]. Not only is this resource vast, but it also presents a challenging environment wherein peptides have been adapted to extreme temperatures as well as high salt concentrations[161]. The adaptation of these peptides to high salt environments is of special interest considering that certain bacterial infections thrive under high-salt conditions, e.g., in the mucus of patients, rendering many peptides ineffective due to salt sensitivity[161]. However, several challenges remain on the road to clinical application of these AMPs.

In contrast to **papers I** and **II**, which focused on the Gram-positive model organism *B. subtilis*, we used the Gram-negative bacterium *E. coli* in this study. Therefore, the $DiSC_3(5)$ assay was adapted to suit measurements in *E. coli* and an outer-membrane-permeable strain was included to assess whether the AMPs affect both membranes.

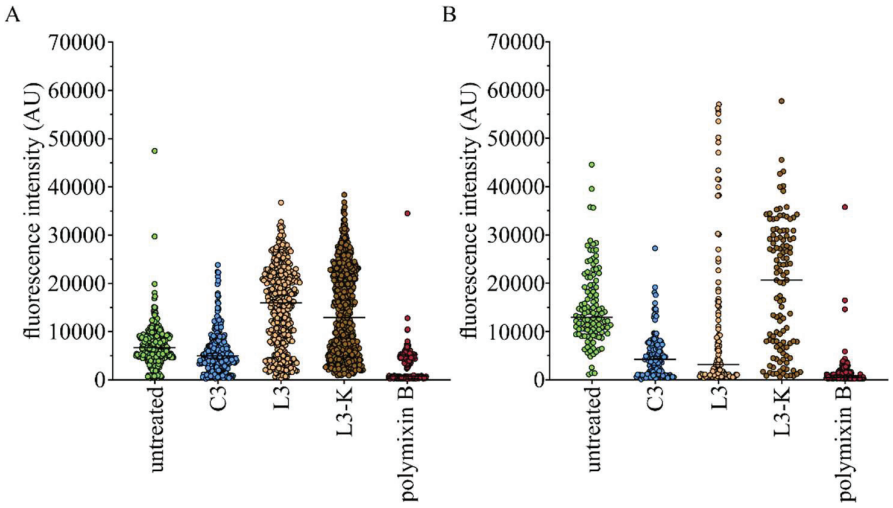

Figure 15 Membrane potential measurements after 10 min of peptide exposure in (A) *E. coli* CCUG31246 and (B) *E. coli* MC4100 pABcon2-fhuA ΔC/Δ4L (outer membrane permeable through overexpression of the porin FhuA). Black lines indicate the median of each sample (modified from Beyer *et al.*, 2023)[162].

Utilizing the $DiSC_3(5)$ way, we could show that the peptides L3 and L3-K first permeabilize the outer and then the inner membrane, while C3 did not show notable effects on the outer membrane. Furthermore, treatment with these peptides caused DNA relaxation, a phenotype that is normally not associated with AMPs and will need to be explored further[22].

Here, we categorized the mode of action of new AMPs and provided the antimicrobial field with useful information for further development of these compounds.

5.3.3. In-depth mechanistic analysis of a promising new drug candidate

Up to this point, the established assays have mainly been employed for initial mode of action testing or fast mode of action categorization, with focus on Gram-positive bacteria. In the last chapter (**paper IV**), an in-depth mechanistic study was conducted on a promising new antibiotic drug candidate, compromising both *B. subtilis* and *E. coli*.

This promising new broad-spectrum antibiotic is the small molecule inhibitor ES24. This compound is structurally similar to nitrofurantoin and likewise requires activation by nitroreductases (Figure 16). Since nitrofurantoin has been used for well over 70 years and still resistance against it is comparably low, ES24 holds great promise as a new drug. Moreover, ES24 has broad-spectrum activity and has been shown to possess a novel mechanism of action in *E. coli*[163], targeting SecYEG-dependent protein translocation. However, Gram-positive bacteria lack a periplasm and inhibiting their secretion systems may affect them differently. We set out to assess the mode of action of ES24 in Gram-positive bacteria and at the same time assess its mechanism in a broader more systematic approach in both groups of bacteria, since the previous study focused solely on the Sec pathway and did not yet assess possible additional targets.

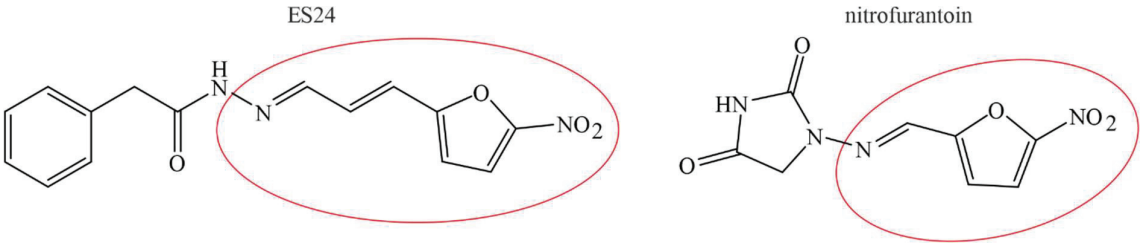

Figure 16 Chemical structures of nitrofurantoin and ES24. The structurally similar nitrofurantoin ring moiety is circled in red. Structures were drawn in ChemDraw[78].

In **paper IV**, we further investigated the mode of action of ES24 in both *B. subtilis* and *E. coli* and compared it to that of nitrofurantoin. To this end, we used a broad panel of assays, including transcriptomics, BCP, ROS probes, $DiSC_3(5)$ assays, MinD oscillation, additional protein localization reporters, and secretion assays.

Transcriptomics can also provide a good starting point for further phenotypic analysis assays[164,165]. This method gives insight into the stress response of bacteria. It is, however, important to note that this technique is sensitive to parameters like profiling and treatment

time and antibiotic concentrations have to be chosen carefully. In **paper IV**, this method provided starting points for further phenotypic profiling, most notably showing that ES24 and nitrofurantoin both induced oxidative stress and DNA damage responses, but differed with respect to membrane and secretion stress responses.

Throughout this thesis, several compounds have induced DNA damage, either through known or as of yet unknown modes of actions (**paper II**, **paper III**, chapters 5.2.2, and 5.3.1). DNA can be damaged through several different means, e.g., direct DNA damage as is the case for bleomycin or mitomycin C, or through targeting DNA-associated proteins causing DNA damage as a downstream effect like ciprofloxacin[85,138,157]. However, DNA damage also commonly occurs through the presence or production of ROS[138]. It is, therefore, important when studying antimicrobial compounds that show DNA damage, to include assays that can detect the presence of ROS. In **paper IV** we used three different ROS probes that are also accessible and present a feasible option for expanding the tool kit of **paper II** when observing DNA damage.

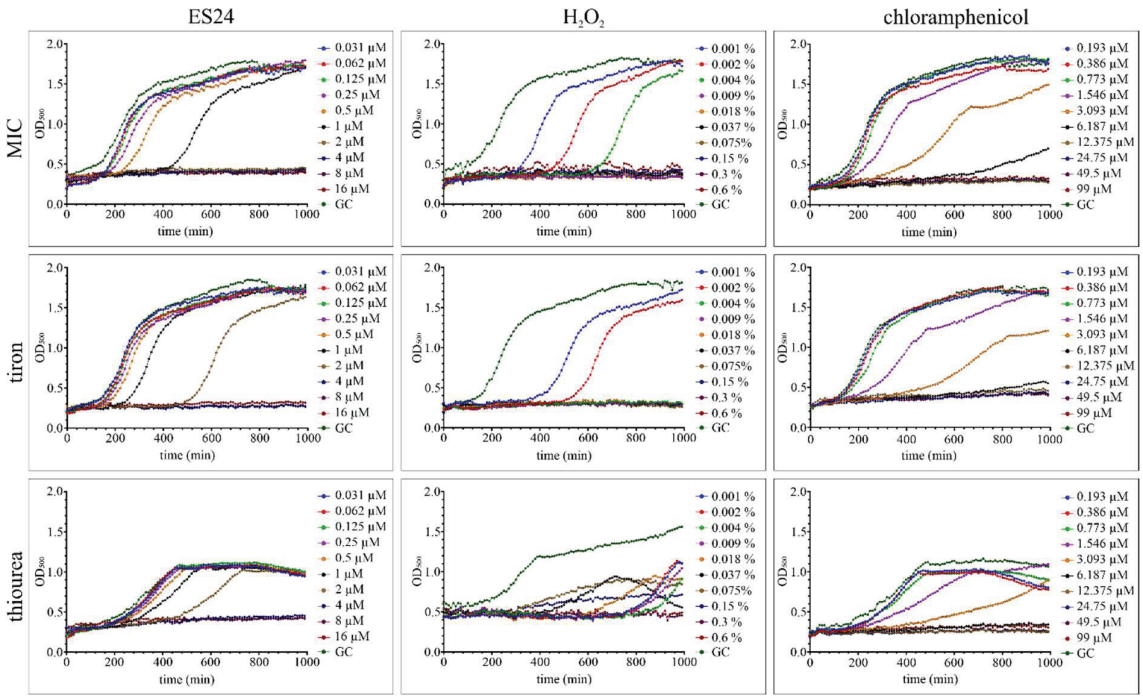

Figure 17 ROS sensitivity assay ES24, H_2O_2, and chloramphenicol in the presence and absence of scavengers for superoxide (tiron) and hydroxyl radicals (thiourea).

Even though clear DNA damage could be seen with the other reporters (RecA) and transcriptomics showed an oxidative stress response, none of the tested ROS probes detected

ROS after antibiotic treatment. Here, the superoxide scavenger tiron, the hydroxyl radical scavenger thiourea, as well as a superoxide-sensitive dye were used. However, when employing these types of assays, it is important to keep in mind that there are other types of reactive species, that are not detectable with these probes. Moreover, we noticed that certain compounds seem to interfere with these scavengers, rendering the results unreliable. Thus, more ROS probes have to be tested to develop a better assay setup for future studies.

ES24 has the rather specific target of inhibiting the Sec secretion pathway. Here, we could show that this also happens in Gram-positive bacteria, by using protein localization studies of SecA and supplementing it with an AmyM secretion assay.

In conclusion, this work showed that ES24, while structurally similar to nitrofurantoin, primarily inhibits the Sec-dependent protein translocation machinery and additionally damages DNA. Furthermore, we could show that the mechanism of action of compounds can vary between bacterial species, since ES24 did not affect the cytoplasmic membrane in *B. subtilis*, but led to gradual depolarization in *E. coli*. We could further show that ES24 outcompeted nitrofurantoin in a zebrafish embryo infection model, highlighting the clinical promise of ES24.

6. Concluding remarks

Antimicrobial resistance is one of the top 10 threats to global health according to the WHO[166]. Several multi-resistant strains of various bacteria have emerged, causing increasing challenges to the successful treatment of bacterial infections[18]. Even though the world is faced with such a global threat, antibiotic discovery has been slow over the last 20 years. While there are new antibiotics in the pipeline, the majority are still derivates of already existing antibiotics[10,167]. It is therefore pivotal to develop antibiotics that have low resistance development rates and new targets to overcome the antibiotic crisis.

Understanding the effects of antibiotics on the complexity of the living bacterial cell is important for the development and evaluation of new, as well as old, antibiotics[60,168,169]. This thesis provides insights into methods that can be used to study *in vivo* modes of action of antibiotic compounds (**paper I** and **II**) [23,94]. Here, a method set up was specifically curated to enable a broad range of researchers to make use of phenotypic mode of action assay without the need for specialized equipment, and we provided detailed protocols and work-flows (**paper II**)[23].

Additionally, the work presented in this thesis showed that not all antibiotic compounds on the market are fully understood (**paper II** and chapter 5.2.1). Seven out of the 11 tested antibiotics showed effects unrelated to their known modes of action. This raises an important issue for the further development of such antibiotics, since it will be difficult to predict how derivates will act on the bacterial cells, if their full mode of action is not understood.

In the future, this method setup can be expanded with more techniques, e.g., ROS detection, DNA damage typing, and direct RNA reporter assays, to enable even more mechanistic studies. Furthermore, the antibiotic reference set can be expanded, e.g., by adding compounds that target teichoic acid synthesis and ionophores that do not cause membrane depolarization like divalent cation ionophores.

Several new compounds have been investigated in this thesis. Interestingly, we could show that the AMPs developed in **paper III** led to DNA relaxation in *E. coli*. This is a curious finding since AMPs are not normally known to cause DNA relaxation. Further studies need to be performed to elucidate how these peptides affect the DNA, by, e.g., looking into DNA damage reporters or conducting direct DNA-AMP interaction studies *in vitro*. Furthermore, these AMPs will be undergoing structural optimization to improve their bioactivity.

One of the most striking findings was the discovery made in the mode of action assessment of metal complexes (chapter 5.3.1). Here, we could not only show that all three metal complexes displayed unique modes of action, but also found indications that one of them caused protein degradation. This process was previously unknown to be the target of metal complexes and opens up a new research line for metal complexes that target proteolysis. In addition, this mode of action indicates great clinical potential, since ADEP, an AMP targeting ClpP, is currently explored to treat difficult to treat infections such as tuberculosis[158,170].
The mode of action of all three metal complexes has to be studied in more detail, by for example using direct DNA damage assays, and, in case of MC screening for affected proteases, to provide deeper insights into their unique modes of action. This is especially important for the compound MC, as it remains unclear how exactly it affects protein degradation.

Furthermore, we investigated the mode of action of the small molecule inhibitor ES24 and found that it targets the Sec-dependent protein translocation in Gram-positive bacteria and causes DNA damage (**paper IV**). This unique mode of action and its *in vivo* efficacy in zebra fish infection model showed that ES24 is a very interesting new lead structure.
At this stage, further studies of how ES24 blocks secretion could be performed, by e.g. crystallization of the protein complex. However, further optimization of this compound to generate competitive candidates for further drug development may be the most important step to take.

Overall, the search for new antimicrobial compounds, in addition to the detailed re-investigation of known antibiotics, is ongoing. Many more techniques can be utilized, and several different research areas are targeted towards antibiotic development. The work presented in this thesis contributes to the development of effective tools and methods needed for further investigations of the modes of action of antibiotics, as well as provides important insights into the mechanisms of action of several promising antibiotic drugs.

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