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Unravelling the mechanisms of multifunctional antibiotic drugs

Bachelor's thesis in Biotechnology BBTX01-20-03

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Abstract

In this study we reinvestigated a number of antibiotics with known intracellular modes of action in living cells of the Gram-positive bacterial species *Bacillus subtilis*. The purpose was to look for potential secondary targets using bacterial cytological profiling and protein localization. For this, fluorescence microscopy was used, after determining the minimum inhibitory concentration (MIC) and optimal stress concentration (OSC) of each antibiotic in *B. subtilis*. The intracellular mechanisms included in this study are from well-established classes of antibiotics and cause DNA- and RNA damage, impaired protein synthesis, disturbed cell division site regulation and stress to the cell envelope. Finally, the membrane stain technique DiIC12 was performed, to further investigate antibiotic effects on membrane fluidity.

Notably, we found promising evidence for several of the tested antibiotics, including nitrofurantoin, kanamycin, trimethoprim, chloramphenicol, rifampicin, triclosan, mupirocin, erythromycin, spectinomycin, and ciprofloxacin, having more than one bacterial target. Our results thus imply that these antibiotics, and perhaps others as well, have not yet been adequately characterized and therefore should not be presumed to have only one specific mode of action in living bacteria. However, further *in vivo* research is required for looking into the exact functional pathways of each antibiotic and establishing whether multifunctional antibiotics are the solution to the current antibiotic resistance crisis.

Sammanfattning

I denna studie har vi undersökt ett antal antibiotika med kända intracellulära verkningsmekanismer i levande celler av den Grampositiva bakterien *Bacillus subtilis*. Syftet var att leta efter potentiella sekundära mekanismer med hjälp av bakteriell cytologisk profilering samt proteinlokalisering. För detta ändamål användes fluorescensmikroskopi, efter att ha bestämt 'minimum inhibitory concentration' (MIC) samt 'optimal stress concentration' (OSC) för varje antibiotika i *B. subtilis*. De intracellulära mekanismerna som inkluderas i studien tillhör väletablerade antibiotikaklasser och orsakar skador på DNA och RNA, förhindrad proteinsyntes, störd celldelningsreglering samt stress på cellmembran och cellvägg. Slutligen användes membranfärgningsmetoden DiIC12 för att ytterligare undersöka antibiotikans effekt på membranfluiditet.

Vi fann lovande tecken på att flertalet av de testade antibiotika, däribland nitrofurantoin, kanamycin, trimethoprim, chloramphenicol, rifampicin, triclosan, mupirocin, erythromycin, spectinomycin samt ciprofloxacin har fler än en antimikrobiell verkningsmekanism. Våra resultat visar sålunda att dessa antibiotika, och möjligen även andra, inte har karaktäriserats tillräckligt för att kunna antas ha endast en verkningsmekanism i levande bakterier. Ytterligare *in vivo* forskning krävs dock för att studera de exakta funktionella vägarna för varje antibiotika, samt för att etablera huruvida multifunktionella antibiotika kan vara lösningen på den nuvarande antibiotikaresistenskrisen.

Keywords

GFP – Green Fluorescent Protein, makes the bacterial cells possible to visualize using fluorescent microscopy

In vivo – In the field of microbiology defined as an experiment performed on or taking place in a living organism, in this study in living bacterial cells

In vitro – Generally regarded as an experiment taking place in a test tube, culture dish, or elsewhere outside a living organism. In this context it is referred to as experiments on purified proteins or membranes

LB – Lysogeny broth, a medium providing essential nutrients for bacterial growth

Mechanism/Mode of action – The exact way, on a molecular level, in which a drug affects or kills an organism, in this case bacteria

Nucleoids – Regions within the bacterial cell with a high concentration of supercoiled DNA

OD – Optical Density, a measure of the concentration of bacterial cells in a solution. Performed by measuring the light absorption value of the solution using a spectrophotometer

Contents

1 Introduction

1.1 Preface

According to the World Health Organization *"Without urgent action, we are heading for a post-antibiotic era, in which common infections and minor injuries can once again kill."* Deadly, yet treatable bacterial diseases such as pneumonia, tuberculosis, blood poisoning and gonorrhoea are becoming more difficult and sometimes impossible to treat, resulting in an increasing number of deaths globally [1]. Today, the most common causes of death in developed countries are cardiovascular diseases, followed by cancer. By 2050, a report by the World Bank [2], estimated that about ten million people world-wide would die from infectious diseases caused by antibiotic resistant bacteria, rendering it the number one cause of death globally. The need for new and smarter antibiotics, potentially with a multifunctional mode of action, is therefore urgent.

Multifunctionality in this report means that the antibiotic attack several targets in parallel and/or that their targets are encoded by multiple genes, which drastically slows down the evolutionary machinery towards acquiring antibiotic resistance. This is due to the fact that antibiotics in general, and "single-target" ones especially, act as selection pressure on bacteria towards developing antibiotic resistance. Bacteria, alike us, are subject to constant random genetic mutations and the mutations that proves beneficial for survival eventually out-compete the previous genotype through the Darwinian principles of "survival of the fittest" [3]. Eventually this will manifest in the bacterial population and result in what we call mass spread of drug resistance. Yet if the drug has different targets, the selection pressure on each single target reduces and the likelihood of developing resistance drastically decreases [4]. Before investing in the research for bringing these new multifunctional drugs to market however, the full mechanism of known antibiotics must first be outlined.

1.2 Antibiotic mechanism

The established idea in the scientific community is that antibiotics act by inhibiting or interfering with one specific cellular target and thus impair cell growth (bacteriostatic), kill the bacteria (bactericidal) or cause cells to undergo cell lysis (bacteriolytic). Mode of action-studies are often carried out *in vitro*, on purified proteins and/or membranes, which mimics the natural biologic system but is often a simplified model of it. Recent studies carried out in living bacteria [5] [6], or *in vivo* as we refer to it here, have however challenged the accepted belief that antibiotics only having one specific target. These studies have presented promising results for several common antibiotics to have a multifunctional mode of action.

In one of these studies, tyrocidine was shown to form defined membrane pores, induce lipid phase separation and thus strongly reduce membrane fluidity, cause DNA damage and interfere with DNA-binding proteins [6]. Furthermore, it has been shown that the established ribosome-inhibitor tetracycline also targets the cell membrane [5]. Tetracyclines are a class of broad-spectrum antibiotics, with many derivatives, that have been used for over 70 years to treat infectious diseases in both humans and animals [7]. Until last year it was thought that tetracyclines only acts by inhibiting the 30s ribosomal subunit in bacteria, thus impairing protein synthesis and causing cell death. Yet the new study from 2019 shows that it in fact also targets the cell envelope by causing severe membrane deformations and delocalizing vital peripheral membrane proteins. This ultimately makes us question whether

other well-established "single-target" antibiotics are multifunctional as well.

1.3 Cell envelope

The bacterial cell envelope has many functions, not only is it important for withstanding stress and maintaining cell structure, it is also crucial for metabolism and other vital cellular processes. The composition of the cell envelope of most bacteria fall into two categories. Gram-negative bacteria are surrounded by a thin peptidoglycan structure in between two layers of phospholipid, an inner layer and an outer layer which also has lipopolysaccharides attached to it [8]. The outer membrane highly protects the cell from antibiotics and thereby serve as a natural resistance mechanism. Gram-positive bacteria lack an outer phospholipid membrane and consists instead of an inner membrane and a thick outer peptidoglycan layer [8] which makes Gram positive bacteria more susceptible to antibiotics in general. These two rather simplistic models are not fully accurate in reality however. In fact, the phospholipid membrane is a very heterogeneous and complex mosaic of proteins and lipids that interact dynamically in regions of varying fluidity/rigidity and together fulfills vital cellular functions [9]. Also, since it is a multilayered structure with many intrinsic targets, encoded by many genes, it is less likely to be the subject of acquired antibiotic resistance. This makes the cell membrane and the entire cell envelope an especially interesting target for novel antibiotics and our study on multifunctionality.

1.4 Model organism

Gram positives are suitable model organisms in mode of action-studies especially due to their cell envelope structure with an outer cell wall, as mentioned earlier (1.3). In this study the Gram-positive bacteria *Bacillus subtilis* is used as the model organism. *B. subtilis* is a bacterial species with GRAS-status (Generally Regarded As Safe) and with a strong ability to adapt to different environments and growth media. It is a standard model organism in bacterial cell biology hence, there are extensive genetic and bioinformatic resources and strain collections available [10]. Another reason for using it is that it is a member of the same phylogenetic class as the very pathogenic gram-positive-bacterial species *Streptococcus pneumoniae* and *Staphylococcus aureus*, which renders it a safe alternative model bacterium. *B. subtilis* is however capable of forming highly resilient dormant endospores in response to nutrient deprivation and other environmental stresses. Therefore, biological waste and laboratory equipment must be handled with caution [10]. It is furthermore a strict aerobic obligate- and cold sensitive species, so during the experimental assays one must ensure that each sample is of limited volume (maximum 1:10 of total flask volume), constantly shaken and kept at stable growth temperature [5] [6] [11].

1.5 Aim

Since the discovery of multifunctional mechanisms amongst antibiotics earlier known for only one antimicrobal mechanism, such as for tetracyline, it is of interest to examine other antibiotics with long established "single-target" mechanisms as well. Knowledge about multifunctional antimicrobial mechanisms facilitates the process of developing antibiotics that are more persistent to bacterial resistance, which is necessary as there is an constant

increase of resistance for a wide spectra of antibiotics amongst bacteria today. Thus, this project aims to examine representatives of the major intracellular antibiotic classes currently used in clinics, known to have one single intracellular mechanism, and analyze whether this is true or if there are other yet unknown targets as well. The examination will be conducted in living cells, with a special emphasis to the cell envelope. This is based on the fact that the chosen antibiotics have intracellular functions and therefore must cross the membrane, hence, are likely to affect that part of the cell as well. Some antibiotics have already been studied similarly and will be revisited meanwhile others have never before been examined in living bacteria and will provide new information as of today.

1.6 Scope

This section will outline the project and address the topics included and not included in our research, focusing on the antibiotics and experimental methods used.

1.6.1 Antibiotics

Our part in the ongoing antibiotic research project in the Wenzel lab at the division of Chemical Biology, Chalmers, is to study the *in vivo* mechanisms of selected antibiotics from different classes and with established intracellular targets. Hence, antibiotics that are known to target the membrane and/or cell wall (cell envelope) are not considered, as these will be in focus of a future project. Tetracycline is one of few antibiotics that already has been investigated in living cells with the purpose of unravelling an additional mode of action. In this study tetracycline is included, partly for confirmation of the additional mechanisms found in previous studies and partly for finding possible additional targets. With respect to earlier studies with tetracycline, there will be a special emphasis on finding secondary mechanisms related to the cell envelope. This is of certain interest since none of the antibiotics included in this study are currently known to primarily target the cell envelope, yet all must interact with it to reach the inside of the cells. See Table 1 for the list of antibiotics with respectively known intracellular mechanism.

The specific questions we would like to address concerning each antibiotic are if the established mode of action is correct and/or if there are unexplored additional mechanisms suggesting that the antibiotic is multifunctional?

Table 1: Antibiotics used in this study, their known target and mechanism of action in the bacterial cell

Antibiotic	**Target**	**Intracellular mechanism**
Ciprofloxacin (Cip)	DNA gyrase A	Inhibits bacterial DNA gyrase A, which introduces double-stranded breaks in the DNA strand [12]
Spectinomycin (Spc)	Protein synthesis (30s ribosomal subunit)	Inhibits protein synthesis by blocking the translocation of tRNA and mRNA on the ribosome [13]
Rifampicin (Rif)	RNA polymerase	Inhibits bacterial RNA-polymerase by blocking the DNA/RNA channel of the RNA-polymerase β subunit [12]
Tetracycline (Tet)	Protein synthesis (30s ribosomal subunit)	Inhibits protein synthesis by blocking the attachment of tRNA to the A site on the ribosome [12]
Kanamycin (Kan)	Protein synthesis (30s ribosomal subunit)	Impairs protein synthesis by promoting mistranslation [12]
Sulfonamide (Sul)	Folic acid synthesis	Acts as a structural analog of paraaminobenzoic acid (PABA) and is therefore a competitive antagonist in the folic acid synthesis [13]
Triclosan (Trc)	Fatty acid synthesis	Inhibits fatty acid synthesis by binding to bacterial enoyl-acyl carrier protein reductase (FabI) [12]
Mupirocin (Mup)	Protein synthesis (isoleucyl t-RNA synthetase)	Inhibits protein synthesis by reversibly binding to isoleucyl-tRNA and thus prevents isoleucine from being incorporated into the growing peptide chain [12]
Erythromycin (Ery)	Protein synthesis (50s ribosomal subunit)	Inhibits protein synthesis by interfering with aminoacyl translocation [12]
Platensimycin (Pla)	Fatty acid synthesis (β-ketoacyl synthases I/II (FabF/B))	Inhibits fatty acid synthesis by blocking essential enzymes FabF/B in the fatty acid synthesis process [13]
Trimethoprim (Trim)	Folic acid synthesis	Inhibits the reduction of dihydrofolate (DHF) to trihydrofolate (THF) which in turn inhibits the folic acid synthesis and the synthesis of nucleotides (THF is an essential precursor of folic acid) [12]
Chloramphenicol (Chl)	Protein synthesis (50s ribosomal subunit)	Inhibits the 50s-subunit of the ribosome, hindering the incorporation of new amino acids into the growing peptide chain [12]
D-cycloserine (D-cyc)	Peptidoglycan synthesis	Inhibits alanine racemase and D-alanine, necessary for synthesis of lipid II. Lipid II is an important component in peptidoglycan, thus, important component of proper cell membrane structure [14]
Nitrofurantoin (Nit)	DNA, RNA, proteins and other macromolecules	It's mechanism is not fully understood, but most likely it acts by a mechanism involving oxidative damage [5]

1.6.2 Experimental assays

To fully understand the exact effect of antibiotics on living bacteria, which is crucial for understanding how antibiotic resistance emerges in nature and for enabling correct treatment of bacterial infections, one must find methods to analyze the antibiotic's impact on each intracellular pathway and cell compartment. To get an overview of the most important pathways and molecular targets we focus this study on key pathways and molecular targets including DNA- and RNA-regulation, protein synthesis, cell division, respiration, cell shape maintenance and cell wall synthesis.

Each experiment will be replicated twice for scientific validity. First amongst the experimental assays is the *Minimal inhibitory concentration* (MIC) assay, to get a sense of which concentration of each antibiotic inhibits visible cell growth. To then determine the optimal stress concentration (OSC), implying viable yet negatively affected cells, a growth curve for each antibiotic must be performed. As negative control for these two first set of assays, the wild type strain *B. subtilis*-168 will be used. When the OSC is established, the mode of action of each antibiotic in live bacteria will be studied using fluorescence microscopy, in two different sets of experiments.

Fluorescence microscopy was chosen since it is an established, accurate and non-invasive *in vivo* method for visualizing live bacterial cells. The first procedure, *Bacterial Cytological Profiling* (BCP), is used to observe any direct changes in cell morphology post antibiotic treatment. Here, a *B. subtilis* strain that expresses cytosolic GFP, will be used as a negative control. To then detect other specific antibiotic targets, protein localization experiments will be performed. As negative controls in these experiments, untreated cells of each strain will be used. Furthermore, each antibiotic treated sample and corresponding negative control will be compared to cells treated with a positive control, known to effect that specific target. Thus, for some of the assays concerning membrane targets, gramicidin will be used [6]. Other positive controls include mitomycin C (causes DNA damage) [15], daptomycin (disrupts cell wall structure) [11], tyrocidin C (disrupts cell wall structure) [6], and selected antibiotics from Table 1. All GFP-tagged reporter proteins with their respective positive controls and details regarding the genotype of the different strains are shown in Table A1 and A2 in Appendix A, respectively. The protein localization approach was chosen based on the fact that it provides a direct visualization of proteins in the cell. Also because successful protocols using the selected dyes and strains for antibiotic mode of action purpouses, have been established ([6], [5], [11], [16], [17]).

Lastly, to further disclose whether the antibiotics have an effect on membrane fluidity, a final stain-based assay using the fluorescent membrane dye DilC12 on *B. subtilis*-168 will be performed. Tyrocidine C will be used as positive control.

2 Theoretical background of experimental assays

In the following sections, the theoretical background of the different experimental assays used in this study is presented. They are divided into sections according to the order of implementing the methods, starting with MIC and then growth experiments. These are followed by bacterial cytological profiling, protein localization, and the membrane domain stain DiIC12.

2.1 MIC

MIC, minimum inhibitory concentration, is a standardized assay and it is used to compare antibiotic activities [18]. It is defined as the lowest concentration that inhibits visible growth of bacteria after 16h of incubation in otherwise normal growth conditions [19]. MIC can also be used to monitor the development of resistance of different antibiotic compounds, and to find effective concentrations to treat bacteria with [20].

2.2 Growth experiments

Growth curves are a method for quantifying bacterial growth, and is done by measuring the optical density (OD) of a bacterial culture over time. Bacterial growth is commonly divided into different stages: *lag-phase* where the cells are adjusting to the environment and into finding sufficient amount of nutrition; *log-phase* where protein synthesis and other metabolic activity reaches a peak and the cells multiply exponentially; and lastly, *stationary-phase* where cell growth arrests and is then followed by cell death [21]. Growth curves are used to determine the antibiotic concentrations to use in BCP- and GFP-experiments, the optimal stress concentrations (OSC). These concentrations should inhibit bacterial growth by 30-50%, to ensure effective but not bactericidal treatment.

2.3 Bacterial cytological profiling

Bacterial cytological profiling was developed by Pogliano *et al.* [12] and is used to get an overview of how the antibiotic affects the cell and what cellular processes are targeted [11]. In these experiments, a *B. subtilis* strain that expresses cytosolic GFP (PrpsD), is used to give information on pore formation, membrane- and DNA morphology. If the GFP signal is lower than that of the negative control, the GFP is leaking out of the cell, which indicates pore formation. A phase contrast will also be made to check for cell lysis. Furthermore, cells will be stained with NileRed, which is a membrane dye used to note any irregularities with the membrane when cells are treated with the antibiotic [22]. Cells will also be stained with DAPI which is a blue-fluorescent DNA dye [23]. This enables the visualisation of antibiotic effects on the nucleoids.

2.4 Protein localization

The localization of proteins in the cell occurs in a specific pattern according to their function. Certain antibiotics can cause proteins to delocalize from their normal sites and thus inactivate them. This indicates that the antibiotic's mechanism is related to the pathway or process where that protein is involved. By using fluorescence microscopy it is possible to visualize the delocalization of certain so called reporter proteins that are fused with GFP-tags. These GFP-tagged reporters are expressed by genetically recombinant *B. subtilis* strains and serves as a visible signal for impairment of a designated cellular function or pathway. GFP-tagged reporters are non-invasive and have been successfully used in similar studies (Appendix A). This method allows for quantification of specific antibiotic-induced effects in living cells, which ultimately can provide insight into the full mechanism of an antibiotic [11].

2.5 Membrane domain stain: DiIC12

To investigate if antibiotic treatment affects the fluidity of the bacterial membrane or creates membrane invaginations, staining with the fluorescent membrane dye DiIC12 can be performed. The dye localizes to regions of increased fluidity (RIFs), which consists of fluid lipid species, e.g. with short, branched or unsaturated fatty acid chains. RIFs play a role in spatial organization of cell envelope synthesis [5]. DiIC12 thus enables visual detection of changes in membrane fluidity, such as an accumulation of RIFs post treatment with a specific antibiotic, using fluorescence microscopy according to established DiIC12-protocols [17].

3 Materials and methods

All experiments were performed according to the safety guidelines for the department of Biology and Biological engineering at Chalmers. All antibiotic-treated bacterial cultures were collected and discarded in the chemical waste for destruction in a safe way. Laboratory gear was carefully washed after use to avoid contamination. All antibiotics were purchased from Fisher Scientific in the highest available purity. Information about errors in the assays and possible future improvements of methods can be found in Appendix G. The MIC and growth curve experiments are preparatory assays for our study so the procedure for these are not included in this section. The MICs were determined according to [18]. Experimental details on MICs are provided in Appendix B. The growth curves were performed as described by Wenzel *et al.* [24], experimental details are provided in Appendix C.

3.1 Bacterial cytological profiling

In these experiments, *B. subtilis* strain bSS82 (PrpsD-gfp) was used (see Appendix A for details). *B. subtilis*-bSS82 was inoculated from frozen stock in 2 ml LB in a 50 ml falcon tube and incubated overnight at 30 °C whilst continuously shaking. The following morning, the culture was diluted to an OD_{600} of 0.2-0.5 and 200 µl of the culture was added to 2 ml Eppendorf tubes, kept on a shaking heating block at 30 °C and 800 rpm. The antibiotics were then added to the tubes according to the OSCs in Table C1. During incubation, 12-well microscopy slides were prepared according to Winkel *et al.* [25], using 800 µl of 1.2-1.5% agarose. After treating the cells with antibiotic for 30 min, 1 µl DAPI and 1 µl NileRed were added to the Eppendorf tubes and 0.5 µl of each sample was pipetted onto the microscopy slides.

Four light channels were used in a Nikon Eclipse Ti inverted epi-fluorescence (wide-field) microscope to image the cells: a phase contrast channel to primarily check for cell lysis, GFP channel to visualize the intracellular GFP, mCherry channel to detect NileRed and thus check for any irregularities in the membranes post antibiotic treatment and finally a DAPI channel to visualize the intracellular DAPI and see how the DNA is affected by the antibiotic. Two biological replicates were made for each antibiotic and untreated cells were used as negative controls.

3.2 Protein localization

The protein localization assays were carried out using the same protocol as for the BCP, with the exception that no dye (NileRed or DAPI) was added to the samples after 30 min incubation and that different strains were used for each GFP-fused reporter (Appendix A). To image the cells, GFP and phase contrast light channels were used. The reporter proteins and their positive controls used in these experiments are presented in Table A1 (Appendix A). Some strains required induction to promote expression of the GFP-tagged proteins, which was done before over-night incubation using a 20% xylose stock solution (Table A2, Appendix A).

3.3 Membrane domain stain: DiIC12

The protocol for treating the cells with antibiotics and visualizing the effects using fluorescence microscopy is almost identical to that of BCP and protein localization and is described by Wenzel *et al.* [17]. With the exception being that the Cy3 light channel was used instead of the GFP channel as earlier, together with phase contrast. A washing procedure also had to be performed before the antibiotic incubation, to minimize background fluorescence from the dye. Details on the DiIC12 experiment are provided in Appendix D. Note further that the antibiotics included in this assay were only trimethoprim, triclosan, rifampicin, D-cycloserine, and gramicidin since they had shown signs of either membrane invaginations or fluid domain formation in earlier experiments.

4 Results

The MIC results are shown in Appendix B and the growth curves as well as the chosen OSCs are listed in Appendix C. Successful MIC results was produced for all antibiotics, except for sulfonamide and platensimycin. After evaluating the MIC assays it was decided that sulfonamide was to be replaced with nitrofurantoin, and platensimycin with cerulenin. Later, D-cycloserine replaced cerulenin after evaluating the growth curves. For the rest of the antibiotics, representative and reliable growth curves were produced. It was furthermore decided later during the protein localization assays, to use an OSC for rifampicin that was not tested in the growth experiments (see details in Appendix B and C).

4.1 Bacterial cytological profiling

In Figure 1 the results of the Bacterial cytological profiling assay are presented. For the negative control, the GFP signal was bright and visible in the whole cytosol of the cells, indicating intact membranes. If the signal would have been fainter, it would suggest cell lysis. A regular pattern of DAPI-stained nucleoids was also visible, as single intracellular blue blobs. This means intact and normally packaged DNA. For the NileRed-stained membranes note the absence of any bright red spots, except for at the septum in the negative control. This indicates healthy cells without membrane stress. Lastly, in the phase contrast, the cells appeared black which implies intact cells, in comparison to lysed cells which would be lighter gray. Intact cells was observed for all the antibiotic treated samples as well. A minimum of 50 cells were counted for each replicate to generate the percentage of affected

cells. As positive control, gramicidin was used and an effect on the DNA was clearly visible with the DAPI stain. The nucleoids seemed to be fully relaxed and covering whole cells, suggesting packaging damage and possibly DNA fragmentation. A similar effect could be seen for the cells treated with rifampicin (100%) and mupirocin (75%). Here the nucleoids were relaxed, not to the same extent as for gramicidin but still covering most of the cells. For rifampicin, unusual spots were observed throughout the cell membranes in the NileRed stain (100%), which indicated that the membrane also might be stressed. The same was observed for triclosan where 35% of the cells showed signs of membrane damage, with several distinct blobs visible in the NileRed stain.

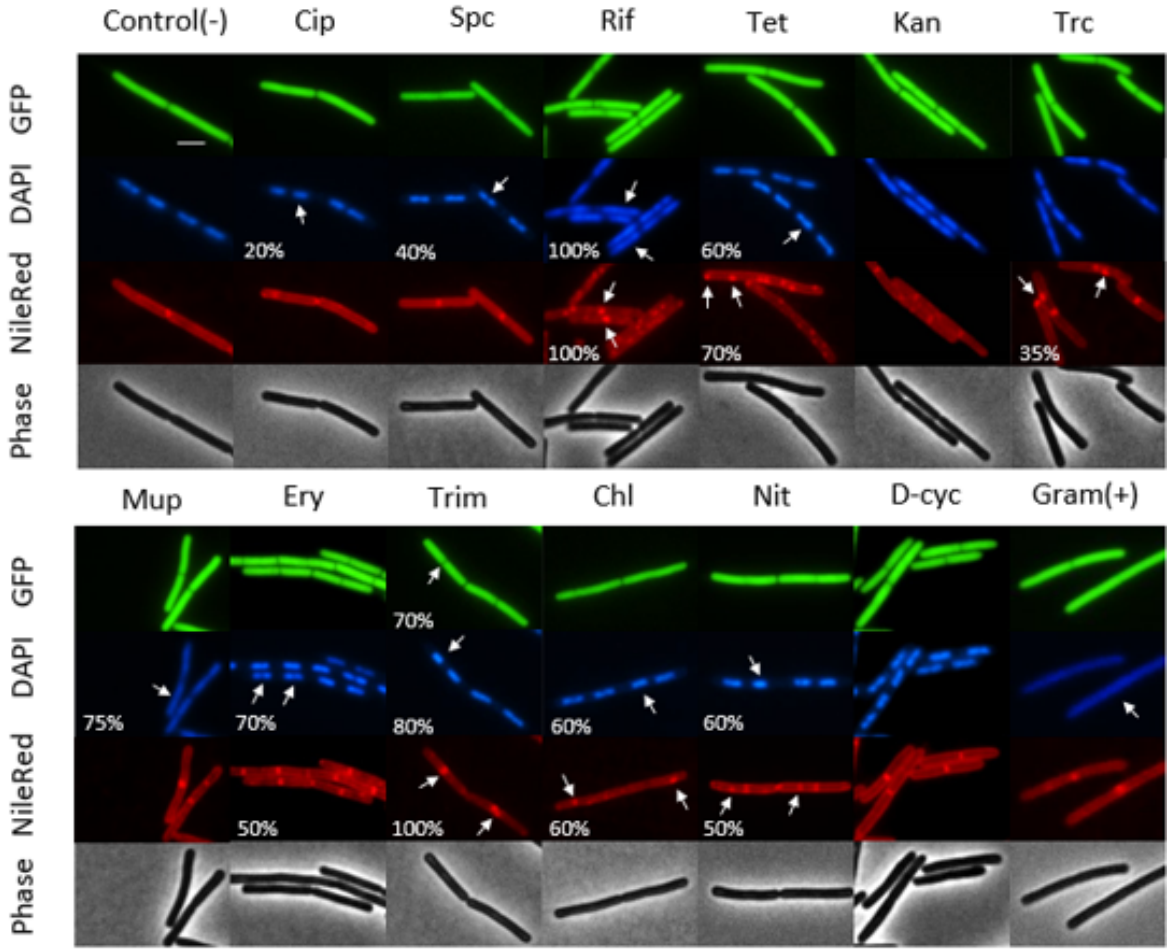

Figure 1: *B. subtilis* strain bSS82 (PrpsD-GFP, see Appendix A for details) stained with the membrane dye NileRed and the DNA dye DAPI was grown until OD_{600} of 0.2-0.5 and subsequently treated with antibiotic for 30 min. Effects on the cytosol, DNA, and membrane are marked with arrows and the number of cells with the same phenotype are displayed as percentages. Negative control (-) was untreated cells and positive control (+) was gramicidin. Scale bar 3 µm.

The antibiotics tetracycline, chloramphenicol, and nitrofurantoin seemed to cause slightly more condensed nucleoids in 60% of the cells. For tetracycline, the ends of the nucleoids were also looking somewhat rounded. An additional effect was observed with the NileRed stain for these three antibiotics. Bright blobs located near the membrane of the cells could be seen, indicating some kind of stress or damage on the membrane (70%, 60%, and 50% respectively).

Condensed nucleoids was also observed for ciprofloxacin, spectinomycin, and erythromycin. For 20% of the ciprofloxacin treated cells the nucleoids seemed to be more condensed compared to the negative control, yet still rounded in shape whereas for spectinomycin (40%) and erythromycin (70%) the nucleoids appeared condensed and somewhat irregular. When looking at the NileRed stain for erythromycin (50%), the signal seemed blurry and the stain spread out in the treated cells, which indicates a disturbing effect on the membrane.

For trimethoprim, the GFP-signal seemed more spaced into segments, where the cell division sites were darker and clearly outlined compared to the corresponding control (70%). The nucleoids looked condensed and irregularly spaced in 80% of the cells. Additional membrane effects could be observed with the NileRed stain, with big blobs at the division sites for all treated cells. This could be an indication of antibiotic induced membrane invaginations or rigid membrane domains.

Furthermore, kanamycin and D-cycloserine showed a high and evenly spread GFP-signal indicating intact cells. No signs of an effect on the packaging of nucleoids (DAPI) nor the membrane (NileRed) could be observed either. So in conclusion, no effect could be seen for kanamycin nor D-cycloserine in the BCP.

4.2 Protein localization assays

In the results of the protein localization assays, only the images of the antibiotic treated samples that had an effect on each strain are shown. Complete figures with all images for every antibiotic can be found in Appendix E. Each section focuses on a specific intracellular pathway or function. Since some of the reporters are involved in the same, yet with different specific targets, each section has been divided into subsections according to the specific GFP-reporter target. Table E1 (Appendix E) summarizes if the respective antibiotics had an effect on the different GFP-fusions in *B. subtilis* or not, X implies an effect and - means no effect. A minimum of 50 cells were counted for each replicate to generate the percentage of affected cells.

4.2.1 DNA, RNA, and protein synthesis

In this section the protein localization of reporters for DNA replication (ParB, DnaN), DNA repair (RecA), RNA synthesis (RpoC), and protein synthesis (RpsB) are evaluated. These are the very fundamental cellular processes necessary for cell survival and are thus important antibiotic targets.

ParB Given that ParB is involved in chromosome segregation prior to cell division [6], the ParB-GFP signal is expected to localize to the nucleoids in each cell. Indeed, in the negative control bright GFP-spots was observed at these sites. For the antibiotics with a delocalizing effect on ParB, the spots were either smaller, indicating more condensed DNA, or more smudged out, implying DNA relaxation/ packaging defects. For the positive control tyrocidine, ParB-GFP was completely delocalized and spread out in the cytoplasm and thus strong DNA damage. See Figure 2 for the controls together with the antibiotic treated samples displaying an effect on the localization of ParB.

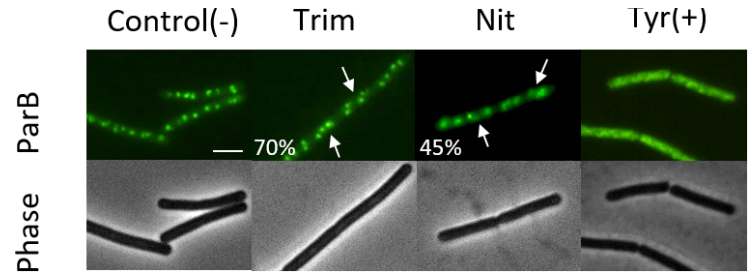

Figure 2: *B. subtilis* strain HM160 (ParB-GFP) was grown until OD_{600} of 0.2-0.5 and subsequently treated with antibiotic for 30 min. Cells treated with tyrocidine C for 10 min was used as positive control (images obtained from [26]). Antibiotic-induced effects on DNA replication and chromosome segregation are marked with arrows. Quantification of observed phenotypes are displayed as percentages. Scale bar 3 µm.

A disturbed spacing or packaging of the nucleoids was seen for trimethoprim (70%), where the nucleoids formed clustered dots with more space in between them, compared to the negative control. This is consistent with its known mechanism of action [12]. For nitrofurantoin, the GFP-spots were larger and less defined than in the control which indicates delocalization of ParB due to DNA relaxation. Note however that the sample was rather heterogeneous, especially the first replicate where the effect could only be seen in 40-50% of the cells in comparison to the second replicate were about 80% showed a clear effect. in conclusion, these results indicate that the antibiotics nitrofurantoin and trimethoprim partly delocalizes ParB and thus possibly impairs chromosome packaging and segregation prior to cell division.

RecA RecA is a protein involved with DNA repair and accumulates at sites of DNA damage in the nucleoids [27]. RecA-GFP was in fact localized all over the cells in the negative control and unaffected samples, yet with a slightly brighter signal from the nucleoids, see Figure 3. The antibiotic treated samples with an accumulating effect on RecA are also presented in Figure 3, and most likely indicates excessive DNA damage caused by the antibiotics, which would recruit RecA for DNA repair. In general they have a similar phenotype with RecA-GFP all over the cells, yet with deviating bright irregular spots in the nucleoids. This was for example clearly visible in the positve control mitomycin C.

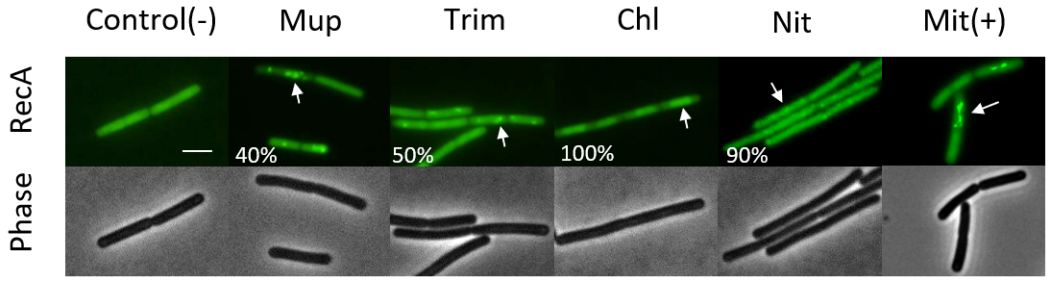

Figure 3: *B. subtilis* strain UG-10 (RecA-GFP) was grown until OD_{600} of 0.2-0.5 and subsequently treated with antibiotic for 30 min. Cells treated with mitomycin C for 10 min was used as positive control (image obtained from [26] and adapted with permission). Antibiotic-induced DNA damage are marked with arrows. Quantification of observed phenotypes are displayed as percentages. Scale bar 3 µm.

For mupirocin (40%), the cells showed bright, irregular spots. The same could be seen for 50% of cells treated with trimethoprim. For 95% of the cells treated with chloramphenicol

the effect was instead that the nucleoids appeared to be smaller and thus more condensed than for the negative control. Nitrofurantoin displayed similar bright spots as described above in 90% of treated cells. In conclusion trimethoprim, mupirocin and nitrofurantoin seems to accumulate RecA-GFP in bright green spots within the nucleoids, indicating DNA damage. Chloramphenicol instead seems to condense the nucleoids.

DnaN Given that DnaN is involved in DNA replication it should be localized to the nucleoids [27]. This was confirmed by the negative control, where the DnaN-GFP signal appears in small spots within each nucleoid, these spots are most likely loosely packed DNA that is being replicated. The antibiotics that had an effect on the DNA polymerase subunit DnaN are displayed in Figure 4 together with an untreated control. Now, an effect on DnaN-localization would indicate some kind of inhibition of DNA replication caused by the antibiotics.

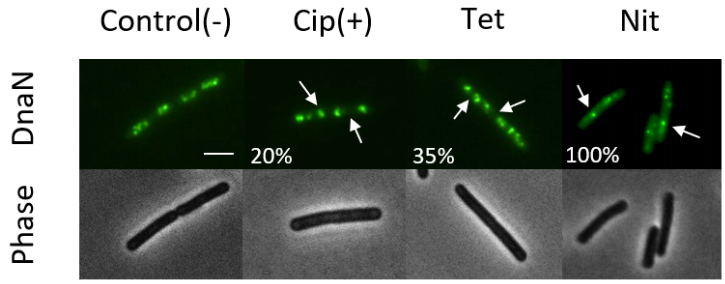

Figure 4: *B. subtilis* strain HM771 (DnaN-GFP) was grown until OD_{600} of 0.2-0.5 and subsequently treated with antibiotic for 30 min. Ciprofloxacin was used as positive control. Antibiotic-induced effects on DNA replication are marked with arrows. Quantification of observed phenotypes are displayed as percentages. Scale bar 3 µm.

For ciprofloxacin, which is the positive control for DnaN, the nucleoids are more condensed compared to the negative control in approximately 20% of the cells. Tetracycline treated cells seems to have the same phenotype as ciprofloxacin in 35% of the cells. This implies that the cells have reduced ability for DNA processes, such as DNA replication, after treatment with these two antibiotics. For nitrofurantoin on the other hand, the DnaN-GFP signal appears to be smeared out and weaker in each cell, compared to the negative control, indicating strong delocalization of the protein to unspecific parts of the cytosol. In conclusion, ciprofloxacin and tetracycline appears to condense the nucleoids and nitrofurantoin instead delocalizes DnaN completely.

RpoC Since the RNA polymerase subunit RpoC is involved with RNA synthesis, it localizes to regions with loosely packed DNA (enabling transcription) in the nucleoids [27]. This was also the confirmed phenotype for the untreated control and unaffected antibiotic samples (Figure E4, Appendix E). If RpoC would delocalize from the nucleoids, it could be interpreted as the RNA transcription being impaired to a certain degree. The antibiotic treated samples that did in fact effect RpoC localization are shown in Figure 5 together with the untreated control.

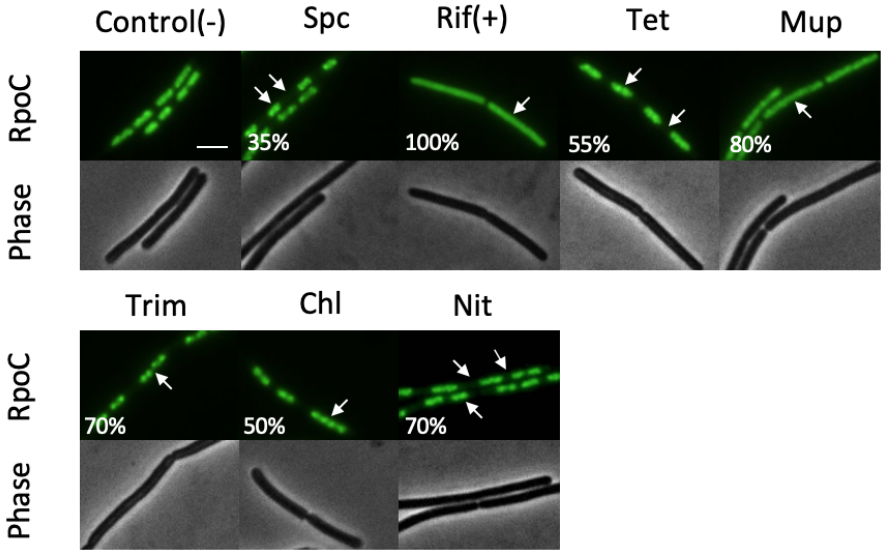

Figure 5: *B. subtilis* strain 1048 (RpoC-GFP) was grown until OD_{600} of 0.2-0.5 and subsequently treated with antibiotic for 30 min. Rifampicin was used as positive control. Antibiotic-induced effects on RNA synthesis are marked with arrows. Quantification of observed phenotypes are displayed as percentages. Scale bar 3 µm.

Rifampicin, which is a positive control for this GFP-fusion, showed a complete delocalization of RpoC in 100% of the treated cells. A similar phenotype was observed by Saeloh *et al.* [27] for *B. subtilis* and was thus expected. For spectinomycin the nucleoids seemed to be more condensed and further apart, but still evenly spaced. Quantification of the phenotype showed that approximately 35% of the cells where affected. For tetracycline, 55% of the treated cells showed a phenotype where the nucleoids were of equal length yet further apart than in the control. Mupirocin (80%) showed the same type of delocalization pattern as rifampicin, with the protein being found all over the cells. For trimethoprim (70%) and chloramphenicol (50%) the nucleoids were instead condensed and more out spread than in the control. For nitrofurantoin (70%), the nucleoids were more irregularly spaced and of different lengths, some more relaxed and some more condensed than the negative control. In conclusion, rifampicin and mupirocin appears to completely delocalize RpoC, whereas trimethoprim, chloramphenicol, spectinomycin, nitrofurantoin, and tetracycline instead have an impact on the packaging and spacing of the nucleoids.

RpsB The ribosome 30s-subunit protein RpsB is involved in protein synthesis [27], which takes place everywhere in the cytosol. In normal cells RpsB should localize everywhere in the cytosol but less where the nucleoids are, since condensed DNA occupy a lot of space. This could in fact be seen for the negative control (Figure 6) and for the unaffected samples (Figure E5, Appendix E) as a regular pattern of brighter and weaker RpsB-GFP signal. The patches of weaker GFP-signal are nucleoids and the brighter patches are regions with a high concentration of ribosomes. The antibiotics that did show an effect on RpsB-localization are also displayed in Figure 6.

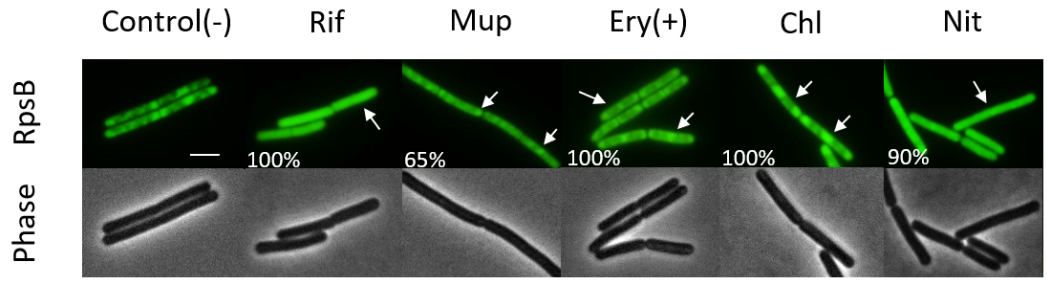

Figure 6: *B. subtilis* strain 1049 (RpsB-GFP) was grown until OD_{600} of 0.2-0.5 and subsequently treated with antibiotic for 30 min. Erythromycin was used as positive control. Antibiotic-induced effects on protein synthesis are marked with arrows. Quantification of observed phenotypes are displayed as percentages. Scale bar 3 µm.

Ribosome inhibitors hinders the protein synthesis by binding to either the 30s- (RpsB) or the 50s ribosomal subunit, so an effect on RpsB localization is likely for at least the known 30s inhibitors. Yet this could not be experimentally confirmed, since none of kanamycin, tetracycline or spectinomycin seemed to delocalize RpsB. On the other hand, both of the 50s subunit inhibitors chloramphenicol and erythromycin (positive control) displayed a clear delocalizing effect in all of the treated cells, where RpsB seemed to be more accumulated to the end and in the middle of the cells than in the negative control. The nucleoids also appeared to be more condensed in all cells. Mupirocin (isoleucyl-tRNA inhibitor) furthermore showed a similar effect as the 50s subunit inhibitors in 65% of treated cells. Treatment with rifampicin instead had the opposite effect as the 50s ribosome inhibitors, where the nucleoids were expanded/relaxed and had formed a diffuse structure all over the cells, which in turn seemed to cause RpsB to disperse throughout the cytoplasm in 100% of the cells. Nitrofurantoin also displayed a clear delocalizing effect in 90% of the cells, similar to that of rifampicin, with a weaker and less organized intracellular RpsB-GFP signal compared to the negative control. In conclusion, neither of the established ribosome-inhibitors (kanamycin, tetracycline or spectinomycin seem to delocalize this target, in contrast to chloramphenicol, erythromycin, and mupirocin. Rifampicin and nitrofurantoin instead appear to interfere with the packing of nucleoids resulting in a more relaxed structure, in turn causing RpsB to delocalize.

4.2.2 Cell division site regulation

MinD and DivIVA are two cell division site reporters. Before looking into the results of their respective protein localization assays, it is important to understand the concept of partial- and complete protein delocalization. The localization of MinD and DivIVA (and MreB, see 4.4.3) depends on the maintenance of an ion membrane potential and thus partial- or complete delocalization of these proteins most likely implies that the membrane has been depolarized to a certain degree [16]. A partial depolarized phenotype means that these proteins that are normally located at the septum and cell poles accumulate in dots all over the membrane. Fully depolarized phenotype instead implies that the proteins completely fall off the membrane and accumulates in the cytosol.

MinD MinD interacts with MinC in the cell membrane to form a complex that inhibits initiation of cell division at the cell poles and is thus a reporter for cell division [28]. Due to its sensitivity towards the membrane potential, it is also a reporter for depolarization. MinD

should normally localize at the septum and cell poles [28], as can be seen in the untreated control sample in Figure 7 and unaffected antibiotic treated samples (Figure E6, Appendix E). The antibiotics that had an effect on this protein is also displayed in Figure 7 together with the negative and positive controls.

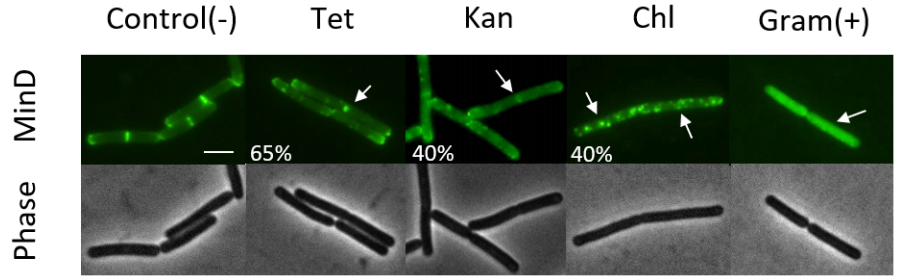

Figure 7: *B. subtilis* strain LH131 (MinD-GFP) was grown until OD_{600} of 0.2-0.5 and subsequently treated with antibiotic for 30 min. Cells treated with gramicidin for 30 min was used as positive control. Antibiotic-induced effects on cell division are marked with arrows. Quantification of observed phenotypes are displayed as percentages. Scale bar 3 µm.

For the positive control gramicidin, MinD was completely delocalized and localized in the cytosol, implying full membrane depolarization. Tetracycline instead showed a delocalizing effect with bright spots on the cell membranes (65%). Kanamycin also revealed a strong delocalization effect, with the GFP signal irregularly spaced and weakly localized at the septum and cell poles . For chloramphenicol a similar effect was seen with bright dots localized irregularly over the whole cells (40%). This indicates that the last three antibiotics may partially depolarize the membrane. In conclusion, chloramphenicol, kanamycin, and tetracycline seems to influence cell division site regulation and possibly membrane polarization, and should be evaluated further in this regard.

DivIVA DivIVA, which is the second reporter for cell division site regulation and membrane depolarization, normally localizes to the septum and cell poles [6] and should give a stronger GFP-signal at these sites. Studies have also shown that it binds specifically to negatively curved (concave) membrane areas. This also makes it a reporter for membrane invaginations [11], which instead should appear as bright blobs. The antibiotics that had an effect on the cell division-protein DivIVA are shown together with negative and positive controls in Figure 8, the rest are presented in Figure E7, Appendix E.

For the positive control gramicidin, DivIVA is completely delocalized in every cell and can be detected in the cytosol instead of at the cell poles and in the septum, thus indicating full membrane depolarization. For ciprofloxacin and rifampicin, the DivIVA protein delocalized and is accumulated in smaller spots. A quantification showed that 50-90% for ciprofloxacin (due to the difference between the two replicates) and 90% for rifampicin had this phenotype. A partial delocalization effect could be seen for tetracycline (75%) and triclosan (60%), which both had small spots scattered all over the cell membrane, beyond the normal phenotype. For 50 % of the cells treated with erythromycin the delocalization effect is stronger and thus indicating full membrane depolarization. Here, DivIVA is clearly displaced into dots all over the cell membrane instead of at the septum and cell poles as it should be. For trimethoprim (40%), there is a partial delocalization, similar to the effects of tetracycline and triclosan. had a strong delocalization effect, with large bright blobs.

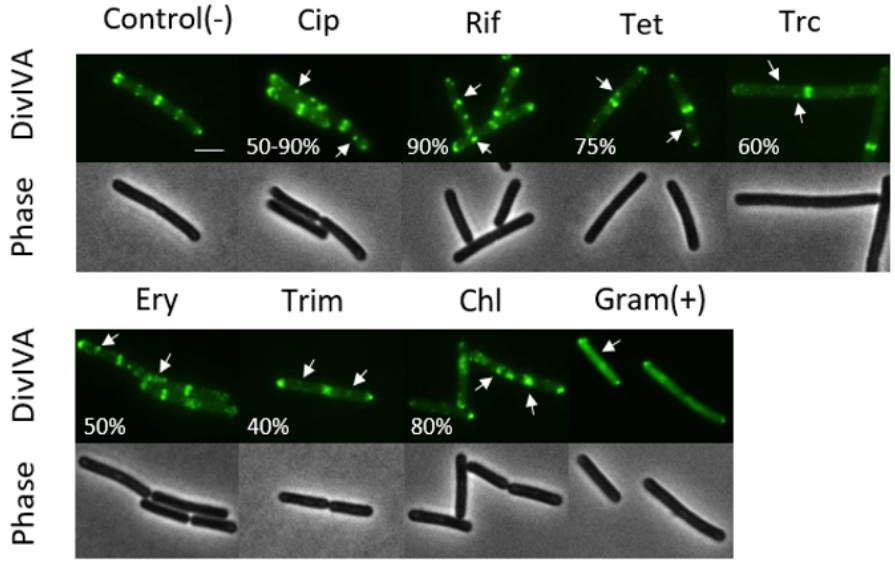

Figure 8: *B. subtilis* strain BMK21 (DivIVA-GFP) was grown until OD_{600} of 0.2-0.5 and subsequently treated with antibiotic for 30 min. Cells treated with gramicidin for 30 min was used as positive control. Antibiotic-induced effects on cell division are marked with arrows. Quantification of observed phenotypes are displayed as percentages. Scale bar 3 µm.

In conclusion, trimethoprim, triclosan, chloramphenicol, erythromycin, ciprofloxacin, rifampicin, and tetracycline partially delocalized DivIVA and can therefore be believed to have depolarized the cell membranes to different degrees. The positive control gramicidin completely delocalized DivIVA and is therefore believed to have fully depolarized the cell membranes. In either case, they are all candidates for further research on membrane depolarization (see Discussion).

4.2.3 Cell membrane organisation

As earlier mentioned, the bacterial cell envelope is a complex and heterogeneous multilayered structure of proteins and lipids with many potential antibiotic targets [8]. In this section, organisation of the cell membrane is in focus and localization of GFP-tagged reporters for cytoskeleton and membrane depolarization (MreB), peptidoglycan (cell wall) synthesis (MurG), rigid membrane domains (FloA), and phospholipid synthesis (PlsX) are performed. These are together reporters for impaired membrane functions and structural defects. For example changes in membrane fluidity, membrane depolarization or areas of negatively curved membrane (membrane invaginations).

MreB The actin homologue protein MreB is involved with the formation of the cytoskeleton that organizes lateral cell wall synthesis in rod-shaped bacteria. Thus it is a reporter for cytoskeleton damage and impaired cell wall synthesis, but since it is sensitive to the membrane potential it can also function as a reporter for depolarization. The cytoskeleton and thereby MreB moves across the inside of the cell wall in a horizontal spiralling pattern, which is hinted in the negative control in Figure 9. One can also observe subtle pairs of brighter dots along the inside of the membrane, one on each side of the cell. These are the anchor sites for the cytoskeleton, where MreB also localizes [6]. The spiralling pattern only arises however if the cell is correctly polarized, so the antibiotics that delocalizes MreB could possibly

depolarize the membrane to a certain degree. The antibiotic treated samples that had this effect on MreB are presented in Figure 7 together with the negative control.

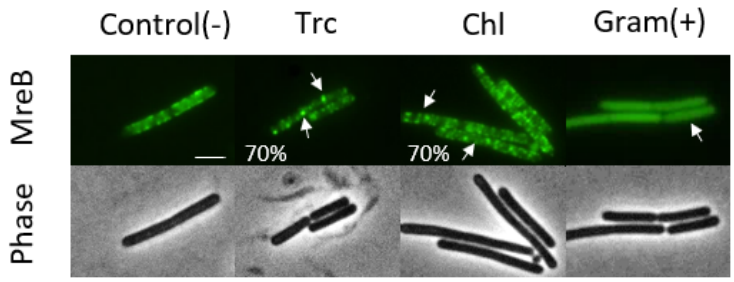

Figure 9: *B. subtilis* strain MW10 (MreB-GFP) was grown until OD_{600} of 0.2-0.5 and subsequently treated with antibiotic for 30 min. Gramicidin was used as positive control. Antibiotic-induced effects on cell wall synthesis are marked with arrows. Quantification of observed phenotypes are displayed as percentages. Scale bar 3 µm.

For the positive control gramicidin, the GFP-signal was as expected completely delocalized from the membrane into the cytosol [6], which causes full membrane depolarization. Triclosan instead formed large and bright blobs in an irregular pattern across the membrane (70%), which could suggest the formation of membrane invaginations or RIFs (see 5.1.5). For 70% of the cells treated with chloramphenicol the protein does not form a regular spiralling pattern, which could mean that MreB has "fallen off" the membrane due to complete depolarization. The antibiotic treated samples that did not show an effect on MreB are displayed in Figure E8, Appendix E. In summary, chloramphenicol seems to delocalize the membrane causing the MreB protein to "fall off" into the cytosol, while triclosan made the protein accumulate in regions that could possibly be membrane invaginations.

MurG Since the protein MurG is involved in the synthesis of peptidoglycan, which is a crucial part of the cell wall, MurG is a suitable reporter for impaired cell wall synthesis [6]. As Figure 10 displays, MurG-GFP is located in the membrane of the untreated control, with some brighter irregular spots. Like MreB, MurG accumulates at regions of increased fluidity (RIFs) which gives rise to the visible spots of brighter MurG-GFP signal [11].

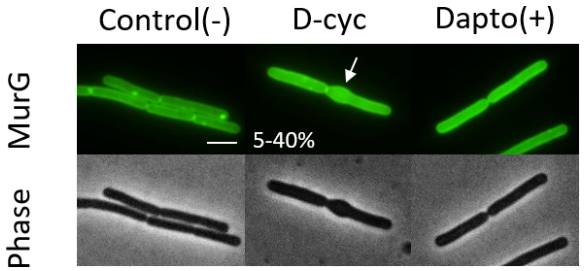

Figure 10: *B. subtilis* strain TNVS175 (MurG-GFP) was grown until OD_{600} of 0.2-0.5 and subsequently treated with antibiotic for 30 min. Daptomycin was used as positive control. Antibiotic-induced effects on cell wall synthesis and fluid lipid domains are marked with arrows. Quantification of observed phenotypes are displayed as percentages. Scale bar 3 µm.

A typical phenotype, where the antibiotic has an effect, is that the MurG-GFP protein is delocalized from the membrane which can be observed as a homogeneous signal throughout

the entire cytoplasm. This phenotype was clearly visualized for the positive control daptomycin. For D-cycloserine however, the observed phenotype was rather different. Some of of the cells (40% of the cells for one replicate meanwhile less than 5% for the other replicate) were deformed and appeared bloated as the upper cell in figure 10. Whether MurG is delocalized can not be confidently stated for D-cycloserine and more research has to be done in order to do so. The antibiotic treated samples that did not show an effect on MurG are included in Figure E9, Appendix E. In conclusion, D-cycloserine seemed to cause a structural deformation in the shape of the cell.

FloA Flotillin proteins, including FloA, are rigid membrane proteins responsible for lateral segregation of membrane domains, so called lipid rafts. FloA forms large, dynamic membrane assemblies that are able to influence membrane fluidity and organization, hence it is used as a reporter for this effect. FloA-GFP should be visible as patches of brighter signal (rigid domains) in the membrane when localized correctly [6]. When delocalized it is believed that the membrane loses its structure and organisation [9] and FloA will then be partly or completely excluded from the membrane or accumulated in an unusual pattern. Both the negative control and the twelve antibiotic treated samples had similar and expected FloA-GFP phenotypes, so none of the antibiotics tested seemed to have a delocalizing effect on FloA, see Figure E10 Appendix E.

PlsX PlsX is a peripheral membrane protein involved in the phospholipid synthesis, coupling phospholipid synthesis in the membrane along with fatty acid synthesis in the cytosol [29]. Thus, the PlsX-GFP signal for untreated cells is localized in the membrane. More specifically, a previously conducted study [30] suggests that the location of PlsX in mid-log phase of *B. subtilis* is homogenous throughout the membrane, with a slightly more intense signal at the septum due to double membrane bilayer next to each other. This localization could be seen for the negative control as well as for all twelve treated samples, indicating that the antibiotics had no clear effect, see Figure E11 in Appendix E. The positive control used, daptomycin, is displayed in the figure as well. For daptomycin, PlsX was completely delocalized from the membrane, causing a homogeneous signal throughout the cytosol.

4.2.4 Respiratory chain and ATPase

In contrast to eukaryotic cells, bacterial cells lack mitochondria or correspondent organelles. Instead their metabolism, including the respiratory chain and ATP synthesis, is linked to the bacterial cell envelope. Thus, the reporters we used for studying antibiotic effects on the respiratory chain (SdhA) and ATP-synthesis (AtpA) are also indirect reporters for structural defects on the cell envelope, such as membrane invaginations [8].

AtpA AtpA is a reporter for inhibition of ATP synthesis and abnormal membrane shapes such as membrane invaginations. A previous study [31] has shown that AtpA is not homogeneously distributed in the membrane but rather localizes in discrete spotted domains. For the negative control as well as the twelve antibiotic treated samples, AtpA-GFP was in fact visible in the whole cell membrane with some randomly distributed brighter spots (Figure E12 in Appendix E). Antibiotic treated cells, in which AtpA has been partly or completely delocalized to the cytosol, would instead show membrane patches of weaker GFP-signal.

This could however also indicate regions where the protein AtpA-GFP has been excluded due to membrane invaginations, as shown by Wenzel *et al.* [6]. No antibiotic in this study showed any effect on the AtpA protein. All antibiotic treated samples are presented in Figure E12 in Appendix E. Note further that there was no positive control used for AtpA.

SdhA The protein SdhA is the subunit of the succinate dehydrogenase (complex II) active in the electron transport chain and thus used as a reporter for impaired mambrane-coupled respiration. According to the study [31] previously mentioned for AtpA, the protein SdhA has a very similar localization pattern. Hence the phenotype for the negative control would be GFP all over the membrane, with small brighter spots at random locations [6]. This was in fact the phenotype observed for the negative control as well as all twelve antibiotic treated samples (Figure E13 in Appendix E). No antibiotic appeared to have a delocalizing effect on SdhA, which would manifest similarly as for AtpA, as regions of significantly weaker SdhA-GFP signal. Note further that no positive control was used for SdhA.

4.3 Membrane domain stain: DiIC12

Since DiIC12 is a fluorescent dye which accumulates in regions of high fluidity (RIFs) it is used to detect changes in membrane fluidity post treatment with an antibiotic. The results of the DiIC12 assay is displayed in Figure 11, with an untreated negative control and antibiotic treated samples including a positive control. In the negative control the DiIC12-signal was observed as a yellow transparent stain on the membranes with some random brighter spots occurring, these corresponds to the localization of RIFs [27]. When treated with D-cycloserine however, the stain was accumulated into bigger and more intense blobs, suggesting a fusion of the otherwise smaller RIFs. This phenotype was present in 55% of the total cell count. A similar phenotype was observed for triclosan, where the signal also implied larger dots on each cell. Approximately 70% of the cells demonstrated the presented phenotype. Tyrocidine, which was used as a positive control, displayed a similar phenotype where the signal was remarkably accumulated into bigger domains. In conclusion, both D-cycloserine and triclosan showed evidence of creating larger regions of increased fluidity.

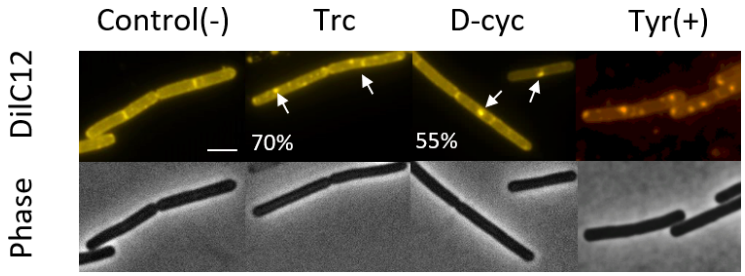

Figure 11: *B. subtilis*-168 stained with the fluid lipid dye DilC12. Antibiotic-induced accumulation of fluid lipids are marked with arrows. Tyrocidine C was used as positive control and images were obtained from [26]. Scale bar 3 µm.

5 Discussion

5.1 BCP and Protein localization assays

The outline of this section is based on the known mode of action of each antibiotic, divided into subgroups of their known target. Here, we will discuss the results of the bacterial cytological profiling and the protein localization assays, with emphasis on the antibiotics that showed new antimicrobial effects on the bacteria.

5.1.1 50s ribosome- and isoleucyl t-RNA synthase inhibitors

Chloramphenicol Chloramphenicol inhibits the 50s subunit of the ribosome, more specifically by inhibiting peptidyl transferase. This in turn inhibits the elongation of the peptide chain, disabling correct synthesis of proteins [12]. It is therefore expected to see an effect on RpsB since it is a reporter for inhibited protein synthesis. In this study it showed a much more distinct localization of RpsB, avoiding the nucleoids, compared to the negative control. What could also be seen was that the nucleoids were condensed in RpsB, and this could be seen in the RpoC strain, the RecA strain and in the DAPI stain in the BCP as well. This effect has not been seen before and could be the effect of a new secondary mechanism. Another explanation is that the condensed nucleoids are a result of the already known ribosome-inhibiting mechanism and thus not a newly discovered secondary mechanism, see Discussion on ribosome inhibitors.

Chloramphenicol also had an effect on the membrane depolarization reporters MinD, MreB and DivIVA, and possible membrane damage could be seen for the stain NileRed in BCP. This is interesting since it cannot be explained by its known mechanism of action as a ribosome inhibitor. These results strongly indicate that membrane depolarization could be a secondary mechanism for chloramphenicol, but more replicates need to be made for all reporters for membrane depolarization to confirm this hypothesis. It would also be interesting to perform a staining assay using the membrane potentiometric dye Disc3(5), to see if membrane depolarization could be confirmed, see Further research. The conclusion from this study of chloramphenicol is that its known mechanism as a ribosome inhibitor is confirmed by our results, but it also shows signs of having a secondary mechanism that leads to condensed nucleoids and membrane depolarization.

Erythromycin Based on what is known as the mode of action related to erythromycin, a reasonable expectation would be that there is an effect for RpsB, indicating an impaired protein synthesis. Indeed, erythromycin had an effect on the proteins localization, which confirms what is already stated concerning its mechanism. This effect has also been shown for *B. subtilis* in a previous *in vivo* study [27].

Another effect which, on the contrary, was not as expected was for DivIVA. This effect suggests membrane depolarization but on the other hand no effects were observed for other reporter proteins such as MinD or MreB. It could also be that there is a potential secondary mechanism related to erythromycin which affects the DivIVA protein specifically, which is perhaps not as likely. It would be of interest to perform a DisC3(5) assay for further research, which also reports on membrane depolarization. Erythromycin also had an effect on the BCP in the NileRed stain, suggesting that there might be formation of membrane invaginations or an effect on the lipid domains in the membrane. No effect was observed for AtpA which

is a reporter for membrane invaginations. Furthermore, no effect was observed for either FloA which reports on rigid membrane domains or for MurG and PlsX which are reporters for fluid membrane domains. Thus, no conclusion about mechanism or target within the membrane can be determined without further research. It could be that the antibiotic in fact targets another structure within the membrane.

Erythromycin also seemed to have an effect on the packaging of nucleiods, which could be interpret from the DAPI stain of the BCP where the nucleoids appear irregular and condensed. Unfortunately, no other effect was observed for any DNA binding reporter proteins such as RecA, ParB and DnaN. Thus, any specific target can not be stated without further research.

Consequently, no conclusion about the specific target of erythromycin on either the nucleoid nor the membrane can be stated. However, an impact of erythromycin was observed and would be of interest to examine further through other assays such as DisC3(5) and by using other GFP-proteins with other reporter properties related to the affected areas of the cell. Perhaps, it is possible that the observed effects could be related to a secondary mechanism of erythromycin. Nevertheless, it can be stated that erythromycin do have an effect on the protein synthesis, which aligns with the research as of today.

Mupirocin As mupirocin is a known and documented protein- and RNA synthesis inhibitor [12], it comes as no surprise that an effect can be seen on both RpsB and RpoC, which report for impaired protein and RNA synthesis, respectively. In the RpoC strain, it was evident that the nucleoids were very much relaxed and elongated, which is in line with the elongated nucleoids clearly spotted in the DAPI stains in the BCP. Furthermore, there was a noticeable effect for RecA, reporting for DNA damage. These results combined suggest that mupirocin may have an effect on the nucleoid (DNA). That mupirocin affects the DNA has been hinted at in another study, conducted some 40 years ago for *Staphylococcus Aureus* [32], but no recent *in vivo* studies have been done for *B. subtilis*. There was, however, no major or reproducible effect on either DnaN or ParB, so the replication of the cells appears to be unimpaired under the conditions studied.

The membrane also seemed mostly unaffected by mupirocin, with no clear or reproducible effects for any of the related GFP fusions. The membrane stain in the BCP did show a few blobs in a few of the cells, but not enough to suggest a distinctive effect. In conclusion, mupirocin showed signs of a secondary mechanism, DNA damage, apart from its previously established modes of action.

5.1.2 30s ribosome inhibitors

Kanamycin One might argue that kanamycin should have displayed an effect on the ribosomal 30s protein subunit RpsB, since it is its known target. The mode of action of kanamycin involves binding to the rRNA in the 30s subunit, resulting in misalignment of the incoming mRNA and thereby incorporation of the wrong amino acid into the growing peptide [12]. So, delocalization would not be expected for kanamycin since the ribosomes are still fully functional, only expressing the wrong polypeptides. This may provide an explanation as to why we did not observe a delocalizing effect on RpsB for kanamycin but furthermore raises the question weather RpsB is actually a good reporter for impaired protein synthesis.

The only delocalized reporter noted for kanamycin was the cell-division protein MinD, which it caused to almost completely delocalize from the septum and cell poles (in 40% of

cells), where it is normally accumulated. This could imply that kanamycin has membrane depolarizing abilities, but since it did not have an effect on neither DivIVA nor MreB which are also reporters for this effect, it is unlikely. It may well affect membrane fluidity and/or cell division, yet the reporters tested are not sensible enough to know for sure. It can thus be said that kanamycin has a potential secondary mechanism involving the cell membrane, as shown with MinD. Yet the nature of which remains unclear and must be investigated further, for example with a DisC3(5) assay. In conclusion, kanamycin did not show signs of delocalizing RpsB, which is it's known target. The only strain it had an effect on was MinD, where the protein was completely delocalized in affected cells. This indicates that kanamycin may target the cell membrane as a secondary mechanism, how still remains unestablished.

Spectinomycin Spectinomycin showed an effect for the DAPI stain in the BCP and the reporter for impaired RNA synthesis, RpoC. Both effects stated were some form of condensed nucleoids. For the BCP the nucleoids seemed to be irregular in shape and for RpoC they were clearly more spaced. No effect was seen for the reporters for DNA damage or impaired DNA replication (RecA, DnaN, and ParB) and this leads to the conclusion that the condensed nucleoids seen for the BCP were not caused by damage to the DNA. Thus, this is a possible effect of the known mode of action of spectinomycin which is inhibition of the translocation of tRNA and mRNA on the ribosome during translation [13]. The condensed nucleoids could be a result of the ribosomes being affected in the cytosol (see Discussion on ribosome inhibitors). The effect on RNA synthesis (RpoC) is new which suggests that this is potentially a secondary mechanism for spectinomycin. However, since inhibition of ribosomes has been shown to condense nucleoids [33], it is likely that this is the explanation for the effect shown on RNA synthesis as well. To be able to confidently state that the condensed nucleoids are in fact a result of a new secondary mechanism, further experiments need to be conducted. Here, a possible next step would be the use of mutant strains (see Discussion on ribosome inhibitors).

Since RpsB is a reporter for impaired protein synthesis it would be expected to see an effect on this protein when the cells are treated with spectinomycin. However, no effect could be clearly stated since only a few cells showed any effect at all, and the result is considered to be inconclusive. Inconclusive results are also seen for the strain MurG, where only one replicate showed any effect. This could be due to the high MIC (128µg/ml) used for spectinomycin, *B. subtilis* is not very sensitive to the antibiotic and it is possible that the concentration is too low and therefore not very effective. The cells could be affected differently depending on fitness and state of growth at this concentration which is a plausible explanation for the inconclusive results.

Both MurG and PlsX are reporters for fluid lipid domains, but since no effect could be stated for either protein, a conclusion that spectinomycin does not affect these proteins was made. But due to the inconclusive results for MurG and possible usage of a too low concentration, more replicates are needed. To be able to confidently state that there is no effect on fluid lipid domains, a DiIC12 can be performed.

In this study, it is shown that RNA synthesis could be a target and thus a secondary mechanism for spectinomycin. However, the known mode of action, impaired protein synthesis was not clearly seen. With further research, it could be investigated whether spectinomycin also affects fluid lipid domains.

Tetracycline Tetracycline is a protein synthesis inhibitor that blocks the attachment of tRNA to the A site on the ribosome. In previous studies, tetracycline has shown an effect for RpsB and has caused nucleoids to condense [33] [34]. In Wenzel *et al.* [5], it was found that tetracycline had a secondary mechanism that was independent from ribosome inhibition. Tetracycline caused blobs in the NileRed stain, which was determined to be caused by membrane invaginations. It also caused the peripheral membrane proteins MreB, MinD and MinC to delocalized, but without dissipating the membrane potential. Furthermore, the delocalization of MreB, which is associated with RIFs, indicated that tetracycline affects fluid lipid domains. This was tested with a DiIC12 assay, which showed that the delocalization of MreB, MinD, and MinC was a result of the delocalized RIFs caused by tetracycline [5].

The results from this project showed that tetracycline caused the nucleoids to condense and caused an irregular NileRed stain. No effect, however, could be seen for AtpA, which implies that no membrane invaginations were formed. RpsB showed no effect as well, which is surprising considering it is a known target for tetracycline. Furthermore, DnaN appeared more concentrated in certain areas in the cells, and the localization of RpoC was more condensed than the control. Based on the positive controls for DnaN and RpoC, the effects shown for tetracycline for DnaN and RpoC are not consistent with the expected effects for these reporters, meaning the effects shown are most likely a result from the condensed nucleoids rather than impaired DNA replication and impaired RNA synthesis.

Both MinD and DivIVA showed an effect, and the phenotype seen for MreB has been seen before in previous studies [5]. However, the result for MreB was not reproducible so no effect could be confidently stated. In order to confirm that the delocalization of these proteins are not due to membrane depolarization, a DisC(3)5 assay should be performed.

A big part of the conclusion that tetracycline has a secondary mechanism was the results that showed delocalized RIFs [5]. In this study, MurG and PlsX are used as fluid membrane domain reporters and both were expected to show an effect since they co-localizes in the cell [11]. However, the results for these strains were not reproducible enough to confidently state an effect, and are therefore key experiments to repeat in order to confirm the hypothesis that tetracycline delocalizes RIFs. To further study fluid domains, a Laurdan assay can also be preformed.

To summarise, no new mechanism was found for tetracycline and experiments focused on fluid membrane domains need to be repeated in order to confirm previous results. In future studies, it can be interesting to investigate the effect tetracycline, and other ribosome inhibitors, have on the nucleoids since the majority of them show similar effects. It can also be interesting to investigate the molecular mechanism of the tetracycline-membrane interaction.

5.1.3 Discussion on ribosome inhibitors

All ribosome inhibitors, except mupirocin and kanamycin, showed an effect on the DAPI stain, where the nucleoids were condensed. It is known for *E. coli* that the structure of the nucleoid is determined by multiple compaction forces and one major expansion force. The expansion force is regulated by transertion, which is the coupling of transcription, translation, and translocation of membrane proteins [35]. Ribosome inhibitors block transertion, which causes the remaining compaction forces to condense the nucleoids [33]. A possible reason for this is described in previous studies [36], [37]. Under normal conditions, DNA is coupling with the membrane by transertion causing the DNA to stretch. When transertion is blocked,

the DNA relaxes which leads to more condensed nucleoids. Thus, the condensed nucleoids are likely a result of the inhibition of the ribosomes.

To investigate whether the condensed nucleoids are indeed an effect of ribosome inhibition or a new secondary mechanism for the antibiotics, mutant strains can be used. By changing the structure of the ribosomes in such a way that it hinders the antibiotics to bind, it is possible to observe if the nucleoids still condense.

Another interesting observation of the ribosome inhibitors was that only the 50s inhibitors showed an effect on RpsB localization and not the 30s as expected. This raises the question whether RpsB is actually an adequate reporter for impaired protein synthesis or not. Perhaps there is a need for several reporters involved with translation in different ways, for example the associated rRNA and tRNA.

5.1.4 DNA gyrase inhibitor

Ciprofloxacin Ciprofloxacin inhibits bacterial DNA gyrase A and introduces double stranded breaks in the DNA strand, which leads to a packing defect [12]. This could be confirmed by the effects shown on the BCP and DnaN, a reporter for impaired DNA replication. Condensed nucleoids could be observed for 20% of the cells in both experiments. For the strain DnaN, the nucleoids were clearly condensed and only visible as small spots in the cells. An effect on DnaN was expected based on the known mode of action and ciprofloxacin was used as a control for this strain. The effect on DAPI was not as clear as for DnaN but an effect could still be detected. This effect was also seen in a previous study [38] where *B. subtilis* cells treated with ciprofloxacin and stained with DAPI showed an abnormal DNA condensation pattern. It could, therefore, be concluded that ciprofloxacin, used with the concentration seen in Table C2 in Appendix C, causes the DNA to condense. Note that no effect was observed for RecA, a reporter for DNA damage, which indicates that the condensed nucleoids are not a result of damaged DNA.

A possible secondary mechanism was discovered by studying the effects of ciprofloxacin on the membrane depolarization and cell division reporter DivIVA. The cells had clearly, delocalized protein that had accumulated into bright spots. Even if the two replicates showed an effect, there was a difference in the proportion of cells affected. The phenotype was present in 90% of the cells in one replicate and around 50% in the other. This could be due to different growth conditions for the two replicates. No effect could be seen for the strains MinD and MreB which are also reporters for membrane depolarization. This suggests that the effect seen for DivIVA is not caused by depolarization, but rather that ciprofloxacin affects a specific target in cell division, delocalizing the DivIVA protein. To further investigate whether ciprofloxacin causes membrane depolarization a DisC3(5) assay can be performed.

Since ParB is a reporter for impaired DNA replication, it was expected to see an effect when treated with ciprofloxacin. An effect on ParB would also indicate problems with chromosome segregation. However, no effect was observed for ParB, which could be due to the low concentration used, since incomplete segregation of chromosomes would lead to cell death.

In conclusion, this study confirms that inhibition of DNA processes, such as the known target DNA gyrase, is indeed the mode of action for ciprofloxacin. There is also a possibility of a secondary mechanism that affects the protein DivIVA by depolarizing the membrane or targeting cell division.

5.1.5 RNA polymerase inhibitor

Rifampicin In regards to the known mechanism of rifampicin and from previous studies, a delocalization of RpoC was expected [27]. Rifampicin was also expected to delocalize RpsB and expand the nucleoids [34]. Our results showed a delocalization of RpoC, RpsB, and a relaxation of the nucleoids, which is in agreement with the literature. RpoC, in untreated cells, localizes to the nucleoids, were the protein binds to DNA via RNA polymerase. According to Jin *et al.* [33], RNA polymerase and transcription play a role in the dynamic structure of the nucleoid, and transcription was found to be important for nucleoid condensation in *E.coli* [39]. This gives a possible explanation for why the inhibition of transcription by rifampicin, causes the nucleoids to expand and can also explain the delocalization of RpoC and RpsB. RpsB predominately localizes to areas not occupied by the nucleoids, and when the nucleoids expand, RpsB will diffuse all over the cell. Furthermore, there was an effect for RecA and DnaN. However, the observed effects were not consistent with the expected effect for DNA damage and impaired DNA replication, and are possibly a result of the uncondensed nucleoids. Moreover, the results for ParB and SdhA were not reproducible, as only one replicate for each fusion showed an effect.

An unexpected result was the effect on the NileRed stain. The result showed an irregular staining with spots all over the cell, which indicates that the membrane domains might be affected, or that rifampicin causes invaginations. However, there was no effect for AtpA, which is a reporter for impaired ATP synthesis and membrane invaginations, meaning that the spots in the NileRed might not be caused by membrane invaginations. Moreover, the result for FloA was insufficient for establishing if there was an effect or not since only one replicate was made. Further replicates are therefore needed to determine if the rigid membrane domains are affected. No effect could be seen for either MurG, PlsX or DiIC12, indicating that the fluid membrane domains are unaffected.

Irregularities in the NileRed stain can also be caused by membrane depolarization [40]. There was an effect for DivIVA, which could indicate membrane depolarization. However, the results for MinD and MreB were inconclusive, meaning that the results are not enough to state with confidence that rifampicin causes depolarization of the membrane. More replicates of these GFP-fusions need to be made, along with a DisC(3)5 assay to better understand if the membrane potential is affected.

Wenzel [41], provides another explanation for the delocalization of DivIVA, where it was shown that rifampicin causes rigidification of the membrane all over the cell. This can make it harder for peripheral membrane protein, such as DivIVA, to bind to the membrane, which can cause them to delocalize. How this is connected with the NileRed stain, however, is unclear and requires further research.

In conclusion, this study confirms the known mechanism and suggests that the membrane is a possible second target for rifampicin. However, the effect on the membrane cannot be explained by membrane invaginations or membrane domains. Further research is therefore required in order to determine if rifampicin causes membrane depolarization and how rigidification of the membrane affects the cell.

5.1.6 Fatty acid synthesis inhibitor

Triclosan At low concentrations, triclosan inhibits the synthesis of fatty acids [12] and could be expected to have an effect for PlsX, which is vital in phospholipid synthesis and a

reporter for the localization of phospholipids in the membrane. It did, however, not show an effect for this strain. This could perhaps be due to triclosan inhibiting the synthesis of new fatty acids in an intracellular step, but not affecting the localization of the lipids in the membrane.

A clear phenotype could be seen on the membrane stain in BCP, where large blobs were scattered over the cells, indicating damage to the membrane. Triclosan also showed an effect for MreB, where similar blobs were seen, and DivIVA, both of which are reporters for membrane depolarization. These blobs may be caused by either membrane invaginations; areas where the cell membrane is strongly negatively curved and where excess membrane accumulates, or by fluid lipid domains. No effect was seen for AtpA, a reporter for membrane invaginations, which suggests that invaginations are not the cause of the blobs. In the DilC12 assay conducted to check for fluid lipid domains, a prominent effect was seen.

This suggests that triclosan has an effect on the cellular membrane, which is not necessarily unexpected given that the membrane consists mainly of lipids. In previous studies triclosan has been found to have multiple mechanisms, including an effect on the membrane [24]. This has mostly been observed for higher concentrations[24] [42], but that effect would according to this study also be displayed, to an extent, at a low concentration. However, the results also suggest that triclosan creates fluid lipid domains, which is new information. From these results, it is not possible to determine if these domains are caused directly by triclosan. Another possible explanation is that the imbalance of lipids in the cells, following treatment, affects the structure of the membrane as a side effect. The impact of impaired fatty acid synthesis on the membrane can be investigated by using a mutant strain, where the FabI gene is repressed. The domains can further be investigated with an assay of the membrane fluidity-sensitive dye Laurdan [11]. To conclude; triclosan did not show an effect for the localization of phospholipids, but did have a noticeable impact on the membrane and especially the formation of fluid lipid domains.

5.1.7 Peptidoglycan synthesis inhibitor

D-cycloserine D-cycloserine is known to inhibit the synthesis of lipid II, an important component in the peptidoglycan structure in the cell wall. Another antibiotic related to lipid II is daptomycin, which interferes with fluid membrane domains causing peripheral proteins to detach from the lipid structures. [11]. Thus, D-cycloserine might interfere with fluid membrane domains as well, which if so should be seen as effects for reporters such as MreB, MurG and PlsX along with the NileRed stain for BCP. D-cycloserine however demonstrated no effect for neither BCP nor for any of the reporter proteins apart from MurG.

It is difficult to state whether MurG itself is effected by D-cycloserine. The signal of MurG in the positive control is fully delocalized throughout the cytosol, a phenotype which is not observed for d-cycloserine. Thus, the protein is not necessarily removed from the membrane. On the other hand, the cells turned bloated and deformed with prominent bulbs in the cell structure. A possible explanation could be that the structure keeping the cell wall together is weakened. Since D-cycloserine inhibits the two enzymes D-alanine racemase and D-alanine ligas the structure of lipid II will not be completed and instead of a pentapeptide sidechain the lipid will only consist of a tripeptide sidechain. This will inhibit the formation of the interpeptide bridge between the lipids, and the peptidoglycan structure is thus kept together solely through its backbone, see appendix F for illustrative figure. This would affect how strongly the cell wall is kept together and is a possible explanation of the observed

phenotype.

The phenotype was only markedly observed for the MurG fusion, hence, the effect could be related to the overexpression of MurG. This would be of interest to examine further, using cells that overexpress the MurG protein but without the GFP-tag to see if the same phenotype is demonstrated. Furthermore, the prominence of the phenotype varied from 40% in one replicate respectively 5% in the other replicate. An explanation to the heterogeneous results would be that the concentration of antibiotic is too low and the phenotype will only occur in some cells that are more susceptible.

D-cycloserine also had an effect on the DilC12 assay, where the signal can be seen as cluster within the cells. This phenotype was observed for 55% of the cells. Since D-cycloserine showed no effect for AtpA, which would report possible membrane invaginations, the signal is most likely clustered in fluid membrane domains. This would not be too unusual since lipid II prefers higher fluidity and therefore is likely to accumulate in RIFs and D-cycloserine is known to affect lipid II [43] [44]. However, since the antibiotic demonstrated no effect for MreB, which would indicate an effect on the fluid lipid regions, the specificity can not be stated without further research. It would be interesting to examine treated cells using Laurdan dye, which would enable measurements of the membrane fluidity [11]. This would indicate whether the regions observed in the DilC12 assay are in fact regions with a higher fluidity. It would also visualise an increase or decrease of the fluidity of the treated cell compared with the untreated cell, thus, indicate the possibility of D-cycloserine restricting the flexibility of the membrane domain. If the regions turn out to be less fluid and consequently less flexible MreB will not accumulate.

Conclusively, this study suggests that D-cycloserine does not depolarize the membrane nor in an remarkable extent delocalize the examined reporter proteins, but rather prevents the correct formation of the peptidoglycan structure and thus, weakens the cell wall. It is possible for D-cycloserine to possess several mechanisms, but no secondary mechanism was observed in this study.

5.1.8 Folic acid synthesis inhibitor

Trimethoprim Trimethoprim inhibits the folic acid production in the bacterial cell by disabling the formation of tetrahydrofolic acid (THF) from dihydrodilic acid (DHF). THF is essential for the production of nucleotides, which means that treatment with trimethoprim leads to a lack in nucleotides that are necessary to build DNA and RNA [12]. From the results it can be concluded that trimethoprim affects the composition, size and regularity in spacing of the nucleoids. This can be seen in the DAPI in BCP, in the RNA polymerase protein RpoC and in the chromosome segregation protein ParB. This is consistent with the known mechanism of trimethoprim. DNA damage can be seen in RecA and this result is also consistent with the known mechanism. Based on these results our study thus confirm the known mode of action for trimethoprim [12].

In the NileRed staining in the BCP large dots could be seen in the septum. This result could either mean that trimethoprim form membrane invaginations or that it affects the fluid lipid domains of the membrane. No effect could be seen on AtpA, which is a reporter for membrane invaginations, so there is no evidence that the dots seen are membrane invaginations. The DiIC12 staining assay would show if there are fluid lipid domains in the membrane, but since no effect could be seen here, fluid lipid domains is not a probable explanation either. In line, trimethoprim also did not have an effect on PlsX and MurG which

are reporters for fluid lipid domains. The lack of effect on DiIC12 could mean that the dots are in fact the opposite of fluid lipid domains, namely rigid lipid domains. If so, trimethoprim should have an effect on FloA, a protein organizing rigid membrane domains. However, that was also not the case. Thus, no evidence could not be found to determine whether the dots seen in BCP could be explained by either membrane invaginations, fluid lipid domains or rigid lipid domains. The conclusion is that that they must be a different type of membrane defect, which has not been reported before and could be an indication of a new secondary mechanism.

Another interesting effect for trimethoprim is that it delocalized DivIVA, which could indicate that it depolarizes the membrane. However, no effect could be seen on any of the other reporters for membrane depolarization (MinD and MreB), so it is possible that trimethoprim has a secondary mechanism that specifically affects DivIVA instead of depolarizing the membrane.

The conclusion for this study of trimethoprim is that its known mode of action as a folic acid synthesis inhibitor seems to be correct. There is an effect on the membrane that has not been seen before, that could not be explained by either membrane invaginations, fluid lipid domains or rigid lipid domains. Lastly, it might also have a secondary mechanism that affects DivIVA specifically.

5.1.9 Oxidative damage

Nitrofurantoin Nitrofurantoin, which is one of the antibiotics with least known *in vivo* mechanisms, showed signs of protein delocalization for many of the reporter proteins, however only clearly for the DNA-, RNA- and protein synthesis reporters: RecA, DnaN, RpoC, ParB. The current belief is that its mode of action involves bacterial nitroreductases, which converts nitrofurantoin into highly reactive electrophilic species that causes oxidative damage to sensitive cellular targets [45]. Using the GFP-tagged proteins RecA, DnaN and ParB, it was shown that nitrofurantoin damages the DNA and maybe also RNA. Yet in contrast to the DAPI stain, where the nucleoids were more condensed and round in shape, the nucleoids seemed more relaxed and undefined in these assays. However, the samples were rather heterogeneous, in terms of the displayed phenotype. For ParB especially, which made it difficult to state a uniform delocalizing effect in this strain.

In an earlier mode of action-study, similar nucleoid effects were observed with DAPI. After a short period of treatment with nitrofurantoin, the nucleoids were condensed, yet after longer treatment time the nucleoids were clearly relaxed and even fragmented [16]. Our findings supports these results and the same tiny membrane spots observed with NileRed during BCP, indicating oxidative stress, had also been observed in this study [16]. Oxidative stress could also explain the observed delocalization effect on RpsB in our study, where the GFP-signal was clearly weaker and less defined, implying strong ribosomal damage.

The RecA assay shows that nitrofurantoin most likely induces DNA damage and possibly even DNA fragmentation, resulting in an accumulation of this DNA repairing enzyme at the damage sites. This may also provide a possible explanation as to why the DAPI-stained nucleoids looked more condensed. It might be that the cell initially responds to the oxidative stress by condensing/tightly packing the DNA for protective purposes, resulting in the phenotype observed with DAPI. Eventually however the cells succumbs to the antibiotic and loses its ability to package the DNA in a supercoiled structrure and eventually fragments. This hypothesis is supported by the results of the DnaN assay where we saw complete protein

delocalization (weak and smudged GFP signal).

In conclusion, nitrofurantoin seems to attack several targets, including nucleic acids, ribosomes and the cell membrane. In this sense it can be regarded as having a multifunctional mode of action, yet through the unspecific mechanism of oxidative damage, as previous studies argue [45], [16]. The concentration tested however (0.5xMIC) seems to be slightly too low to see a definite and homogeneous effect in treated strains, which makes further replicates at a higher concentration necessary to be certain of the effects.

5.2 Importance of results

At a time when the world is facing the greatest pandemic since the Spanish flu, in the beginning of the 20th century, it is easy to forget about the emerging global threat of antibiotic resistance. It is an issue that eventually will become even more severe and lethal than the current Covid-19 pandemic, if the World Bank forecast [2] is accurate. Such a crisis will have catastrophic impact on our healthcare system and society at large, if drastic and urgent preventative measures are not taken. These measures include smarter and reduced use of current antibiotics, especially within the agricultural sector. In parallel, private investors and governments must fund research for the development of new and better antibiotics that are more resilient to antibiotic resistance [46]. As addressed in this report, there is a theory that multifunctional antibiotics are more beneficial than single-target ones, in terms of less occurrence of antibiotic resistance. We have shown that many of the established single-target antibiotics have not been fully characterized and may in fact be multifunctional, which could explain why resistance against some of them is not yet recognized in nature. These findings will hopefully help researchers understand the full *in vivo* mechanisms of some of the current antibiotics in use and ultimately shed a light on how multifunctionality can be used for creating smarter antibiotics.

5.3 Outlook

In this section we share our thoughts on both general research and more specific experimental assays that could complement the research carried out in this study.

5.3.1 Antibiotic concentrations

A generally noted concern during both BCP and protein locaization was that the OSC for some antibiotics seemed too low for yielding a homogeneous result, in terms of visible phenotype. This can for example be observed for RpoC treated with nitrofurantoin, where the protein only delocalized in 50% of the cells. The same goes for MurG treated with D-cycloserine, where only 40% and 5% for the two replicates, respectively, demonstrated an effect. An explanation to the observed heterogeneity of cells in the replicates could be that the concentrations of antibiotic were borderline effective. It would be interesting to examine higher concentrations for each antibiotic in this study and see whether the effect is stronger. However, a higher concentration could also increase the rate of cell death, making it difficult to determine whether the observed phenotype is caused by a secondary mechanism or is the result of the cells dying. Nevertheless, it would be necessary in order to state whether antibiotics with a low percentage of effect truly have additional targets and thus a possible secondary mechanism.

5.3.2 Membrane potential assay: DisC3(5)

When assessing antimicrobial effects in the membrane of bacterial cells most methods do not consider changes in ion permeability. Changes in ion permeability could cause damage to the membrane structure and even kill the cell. In order to monitor whether the antibiotics affect the membrane ion potential, a DiSC3(5) assay can be used. DiSC3(5) is a dye that accumulates on hyperpolarized membranes and thus, provides an overview over the membrane's level of polarization when examined through fluorescence microsopy. The method was described by Derk *et al.* [25]. Antibiotics that would be suitable for this assay are chloramphenicol, trimethoprim, kanamycin, tetracycline, rifampicin, triclosan, ciprofloxacin, and erythromycin, since these antibiotics have shown signs of membrane depolarization effects in protein localization assays.

5.3.3 Membrane fluidity assay: Laurdan

To examine the membrane fluidity of *B. subtilis* it is possible to use a dye called Laurdan. This dye changes the wavelength of its fluorescence emission as a result of changes in the constitution of the lipid membrane it is attached to. It is sensitive to the amount of water molecules associated to the membrane and can therefore be used as a measure of how fluid the membrane is. From these results it is also possible to examine the flexibility of the fatty acid chains in the membrane as well as the lipid head group density [11]. The procedure of staining and vizualising cells was described by H. Strahl *et al.* [40]. Antibiotics suitable for this assay would be D-cycloserine and triclosan since they showed clear signs of potential fluid membrane domains in the DiIC12 assay. Tetracycline is also a suitable antiobitoic to test since it has been shown to delocalize RIFs in previous studies.

6 Conclusion

Using bacterial cytological profiling and protein localization approaches in *B. subtilis*, we have found promising evidence that several well established single-target antibiotics have in fact more than one bacterial target and could thus be multifunctional. These antibiotics are nitrofurantoin, kanamycin, trimethoprim, chlorampheicol, triclosan, rifampicin, mupirocin, spectinomycin, ciprofloxacin, and erythromycin. In addition to the intracellular targets, we put a special emphasis on the cell envelope when evaluating the mode-of-action of each antibiotic in living bacteria. Using the GFP-tagged membrane associated proteins MreB, MurG, and MinD we have seen that some of these antibiotics likely causes both functional- and structural defects to the cell membrane. This includes changes in membrane fluidity, membrane depolarization and membrane invaginations, which defies the current belief that these antibiotics only attack intracellular targets. Our findings suggest that most antibiotics have not been studied enough in living bacteria and future mode-of-action research would strongly benefit from being carried out this way, mimicking the natural processes to a higher degree than *in vitro* experiments.

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Appendix A Bacterial strains and reporter proteins

The GFP-tagged reporter proteins used in this study are presented in Table A1, together with their reporter functions and positive controls.

Table A1: GFP-tagged reporter proteins and their functions together with the positive control used for each strain.

Protein	Function	Reporter for	Positive control
RecA	DNA-repairing enzyme [27]	DNA damage [27]	Mitomycin C [27]
DnaN	DNA polymerase subunit [27]	Impaired DNA replication [27]	Ciprofloxacin [27]
ParB	DNA replication regulator [6]	Impaired DNA replication and thus incomplete chromosome segregation [6]	Tyrocidine C [6]
RpoC	RNA polymerase subunit [27]	Impaired RNA synthesis [27]	Rifampicin [27]
RpsB	Ribosomal 30s subunit [27]	Impaired protein synthesis [27]	Erythromycin [27]
MinD	Cell division protein [6]	Membrane depolarization and inhibition of cell division [6]	Gramicidin [11]
MreB	Actin homologue protein important for cytoskeleton synthesis and thus cell wall structure in rod-shaped bacteria [6]	Membrane depolarization and impaired cell wall synthesis [6]	Gramicidin [11]
PlsX	Phospholipid synthase [6]	Impaired phospholipid synthesis and disruption of fluid lipid domains [6]	Daptomycin [27]
MurG	Membrane-associated lipid II synthase (peptidoglycan synthesis) [6]	Fluid lipid domains and inhibition of cell wall synthesis [6]	Daptomycin [27]
DivIVA	Cell division protein that directs MinD to the polar septation sites [6]	Membrane depolarization and cell division inhibition [6]	Gramicidin [11]
FloA	Integral flotillin membrane protein [9]	Rigid membrane domains (lipid rafts in eukaryotes) [9]	Tyrocidine C [6]
AtpA	Regulatory subunit of ATP synthase [6]	ATP synthesis inhibition and membrane invaginations [11]	No known control
SdhA	Subunit of the succinate dehydrogenase (complex II) in the respiratory chain [6]	Inhibition of electron transport in respiratory chain	No known control

Bacterial strains used for the protein localization assays and inducers used for each strain are presented in Table A2 below.

Table A2: *B. subtilis* strains, and their genotype, used for protein localization assays together with the specific inducer concentrations.

Protein	**Strain**	**Genotype**	**Inducer**	**Reference**
RecA	UG-10	*amy::spc Pxyl-recA-mgfp*	0.5% xylose	[27]
DnaN	HM771	*dnaN::gfp-dnaN-cat (MS104 into 168)*	-	[47]
ParB	HM160	*kan spo0J-gfp (spo0J = ParB)*	-	[48]
RpoC	1048	*cat rpoC-gfp Pxyl-'rpoC*	1% xylose	[49]
RpsB	1049	*amyE::sps Pxyl-rpsB-gfp*	1% xylose	[49]
MinD	LH131	*amyE::spc Pxyl-gfp-minD*	0.1% xylose	[11]
MreB	MW10	*amyE::spc Pxyl-gfp-mreB*	0.1% xylose	[11]
PlsX	TNVS29D	*amyE::spc-Pxyl-mgfp-plsX*	0.5% xylose	[11]
MurG	TNVS175	*amyE::spc-Pxyl-murG-msfgfp*	0.05% xylose	[11]
DivIVA	BMK21	*amyE::scp Pxyl-divIVA-sfgfp*	0.1% xylose	[11]
FloA	DL1367	*amyE::spc-yqfA-gfp (yqfA=floA)*	-	[50]
AtpA	BS23	*atpA-gfp Pxyl-'atpA cat*	0.1% xylose	[51]
SdhA	BS121	*sdhA-gfp Pxyl-'sdhA ca*	0.1% xylose	[51]
PrpsD	bSS82	*amyE:: spc PrpsD-gfp*	-	[52]

Appendix B Antibiotic solvents and Minimum Inhibitory Concentrations

B.1 Antibiotic solvents

Before carrying out the MIC experiments all antibiotics used in this study was first diluted to individual stock-solutions (c:10mg/ml). The antibiotics were purchased from Fisher Scientific in the highest available purity (solid form) and was diluted using the solvent prescribed by the manufacturer. The solvents used to dilute the antibiotics from pure, solid form are presented in Table B1.

Table B1: Antibiotic solvents used for dilutions.

Antibiotic	**Solvent**
Ciprofloxacin	H_2O
Spectinomycin	H_2O
Rifampicin	DMSO
Tetracycline	DMSO
Kanamycin	H_2O
Sulfonamide	DMSO
Triclosan	DMSO
Mupirocin	DMSO
Erythromycin	EtOH
Platensimycin	DMSO
Trimethoprim	DMSO
Chloramphenicol	EtOH
Nitrofurantoin	H_2O
D-cycloserine	DMSO

B.2 Minimum Inhibitory Concentration

Method

In order to determine the MIC of each antibiotic, a serial two-fold dilution was made in a 96-well plate. 100 µl sterile LB was added in every well, and an additional 100 µl LB was added to the sterile control (SC) and the second well (column 11 in Table B2). Antibiotics from 10 mg/ml stocks (see Table B1 for the solvent used for each antibiotic) were then added to each well, except for the growth control and the sterile control, so that the first well after the dilution had a concentration of 512 µg/ml. After this series-twofold dilutions were made from well 11 down to well 2. See Table B2, for a schematic view of the 96-well plate and the concentrations in each well. Finally, 100 µl of *B. subtilis*-168 inoculated overnight at 30 °C in LB was added to every well (except for the sterile control), rendering a cell count of $5x10^5$ cells/ml in each well. The plates were then sealed and incubated for 16h in 30 °C, after which the MIC was determined. The range tested for was 1 µg/ml - 512 µg/ml. Two technical and two biological replicates were made for the MIC determination. For the second biological replicate, the maximum concentration was lowered to 16 µg/ml antibiotic, to be able to detect MIC's in the range of 0.03125 µg/ml - 16 µg/ml. With exception for spectinomycin where the maximum concentration was 128 µg/ml for the second replicate.

Table B2: MIC plate layout, antibiotic concentrations in (µg/ml) displayed in the second row.

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
SC	1	2	4	8	16	32	64	128	256	512	GC	AB
SC											GC	AB1_1
SC											GC	AB1_2
SC											GC	AB2_1
SC											GC	AB2_2
SC											GC	AB3_1
SC											GC	AB3_2

Results

The MIC concentrations used are presented in Table B3. Important notes from these experiments was that both experimental replicates with sulfonamide resulted in bacterial growth in every well. It was therefore decided to replace sulfonamide with nitrofurantoin in the study. Nitrofurantoin has a MIC of 16 µg/ml [5].

Table B3: Minimum inhibitory concentrations.

Antibiotic	**MIC [µg/ml]**
Ciprofloxacin	1
Spectinomycin	128
Rifampicin	0.125
Tetracycline	2
Kanamycin	4
Triclosan	4
Mupirocin	0.125
Erythromycin	2
Trimethoprim	0.5
Chloramphenicol	16
Nitrofurantoin	16
D-cycloserine	15

Appendix C Growth curves and Optimal Stress Concentrations

Method

B. subtilis-168 from frozen glycerol stock was added to 2 ml LB medium and incubated over night at 30 °C and continuous shaking. The following morning, the culture was grown to an OD_{600} of 0.3 - 0.5 and then for each antibiotic, 200 µl culture was inoculated in 20 ml sterile LB in a 200 ml Erlenmeyer flask (kept in shaking waterbath at 30 °C), followed by separation into five 50 ml falcon tubes (4 ml in each). Antibiotics were added from 10 mg/ml stocks in four of them, yielding concentrations corresponding to 0X- (control), 0.5X-, 1X-, 2X- and 4X MIC for each antibiotic, see Table B3 for MIC. An OD measurement was taken at zero hour for each concentration and the flasks were kept shaking at 30 °C, to ensure proper oxygenation and heating. OD measurements were then taken for each concentration and antibiotic, at an even interval of 30 min during lag-phase and 20 min during log-phase, until reaching stationary phase. To follow the growth of untreated and treated bacteria under different antibiotic concentrations, over time, growth curves with time versus optical density of each sample were generated in *MS Office Excel*. Each growth curve experiment was done in two biological replicates and, if necessary, with slight modifications of the concentration range for the second replicates, see Table C1 for exact concentration ranges tested (in multiples of MIC).

Table C1: Antibiotic concentrations for growth curves, presented in multiples of MIC.

Antibiotic	**Growth curve I [µg/ml]**	**Growth curve II [µg/ml]**
Ciprofloxacin	0, 0.5, 1, 2, 4	0, 0.5, 1, 2, 4
Spectinomycin	0, 64, 128, 256, 512	0, 64, 128, 256, 512
Rifampicin	0, 0.0625, 0.125, 0.25, 0.5	0, 0.0019, 0.00038
Tetracycline	0, 1, 2, 4, 8	0, 0.0625, 0.125, 0.25, 0.5
Kanamycin	0, 2, 4, 8, 16	0, 2, 4, 8, 16
Triclosan	0, 2, 4, 8, 16	0, 0.5, 1, 2, 4
Mupirocin	0, 0.0625, 0.125, 0.25, 0.5	0.0156, 0.0313, 0.0625, 0.125
Erythromycin	0, 1, 2, 4, 8	0, 0.25, 0.5, 1, 2
Platensimycin	0, 8, 16, 32, 64	-
Trimethoprim	0, 0.25, 0.5, 1, 2	0, 0.5, 1, 2, 3
Chloramphenicol	0, 8, 16, 32, 64	0, 2, 4, 8, 16
Nitrofurantoin	0, 8, 16, 32, 64	0, 4, 8, 16, 32
D-cycloserine	0, 7.5, 15, 30, 60	90, 120

Results

The OSCs used for the BCP and protein localization assays are presented in Table C2. A note from these experiments is that the available stock of platensimycin was not enough for the growth curve experiments. Thus, platensimycin was substituted with cerulenin which has a MIC of 5 µg/ml [24]. Cerulenin however did not show any effect on the first growth curve experiment and the available amount was to limited to increase the concentration. Thus, cerulenin was subsituted with yet another antibiotic, D-cycloserine. The growth curve of D-cycloserine is shown in Figure C2 and the MIC of D-cycloserine is 15 µg/ml

[19]. Furthermore, rifampicin showed no effect on the GFP-fusions for the concentration chosen from the growth curve. Therefore, a range of other concentrations were tested until phenotypes described by Saeloh *et al.* [27] and Hunt *et al.* [34] were found. Only 1xMIC showed similar effects and was therefore chosen. However, no growth curve was made for this concentration.

Table C2: Optimal stress concentrations (OSC) chosen for the BCP and protein localization assays.

Antibiotic	**OSC [µg/ml]**
Ciprofloxacin	1
Spectinomycin	128
Rifampicin	0.125
Tetracycline	0.25
Kanamycin	4
Triclosan	2
Mupirocin	0.03125
Erythromycin	0.25
Trimethoprim	1
Chloramphenicol	2
Nitrofurantoin	8
D-cycloserine	90

The growth curves are presented in Figure C1 and C2 below. The OSC is underlined in each plot (for each antibiotic) and was individually determined by comparing the the curves of each antibiotic concentration to the curve of the untreated sample (0xMIC), where the aim is to see 50-70% inhibition of cell growth. This could be read out from the graphs by looking at the height of the curves during stationary phase (last planar section) that should be in between the height of the 0xMIC(control) curve and the highest concentration resulting in no growth.

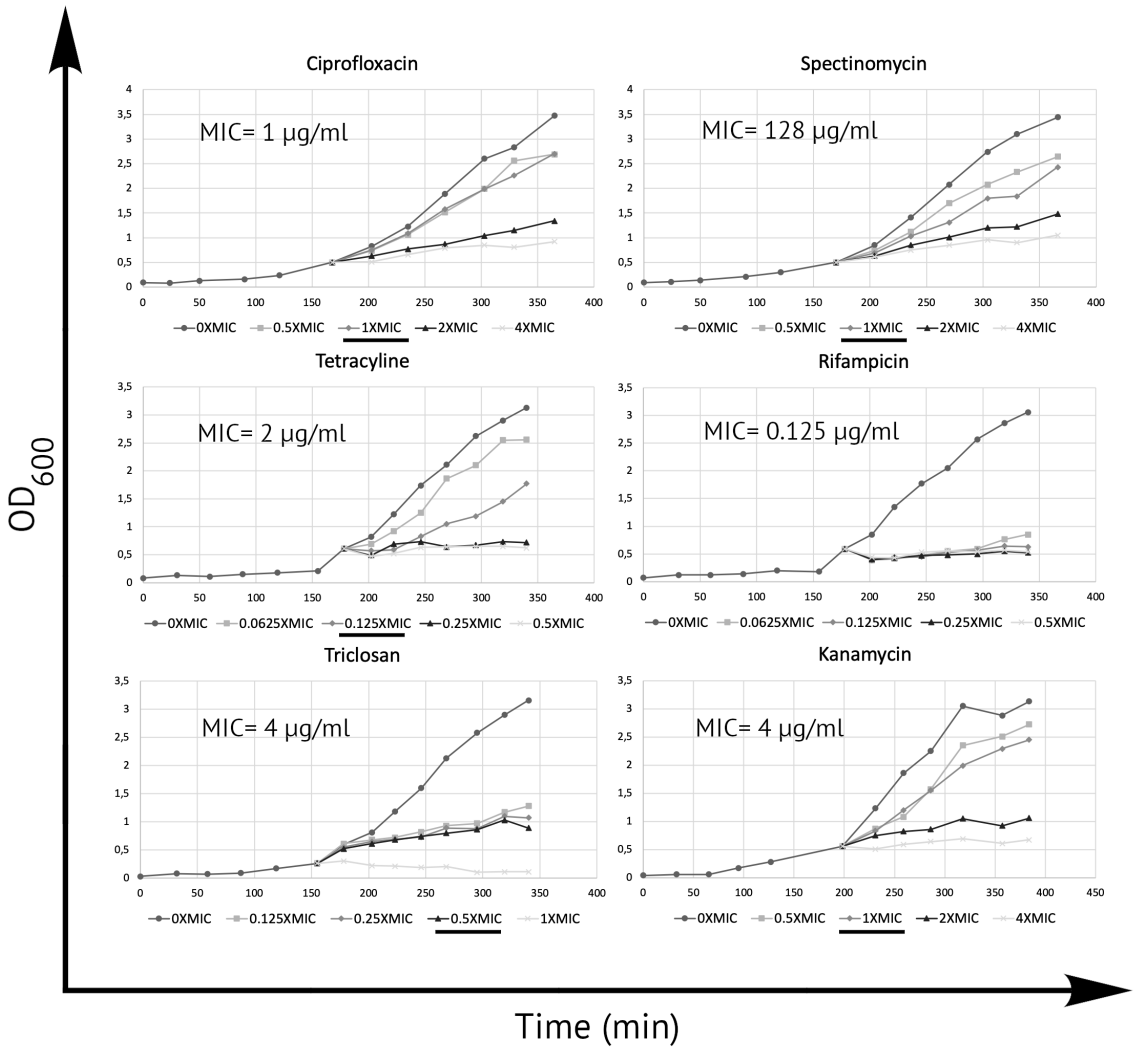

Figure C1: Growth curve of *B. subtilis*-168. Samples were taken every 30 min during lag-phase and every 20 min during log-phase, until reaching stationary phase. Antibiotics were added at an OD_{600} of 0.3-0.5 in four different concentrations determined by the MIC. OCSs chosen for GFP and BCP experiments are underlined. For rifampicin, 1XMIC was chosen, which was not tested in growth curve experiments and is therefore not marked in the figure.

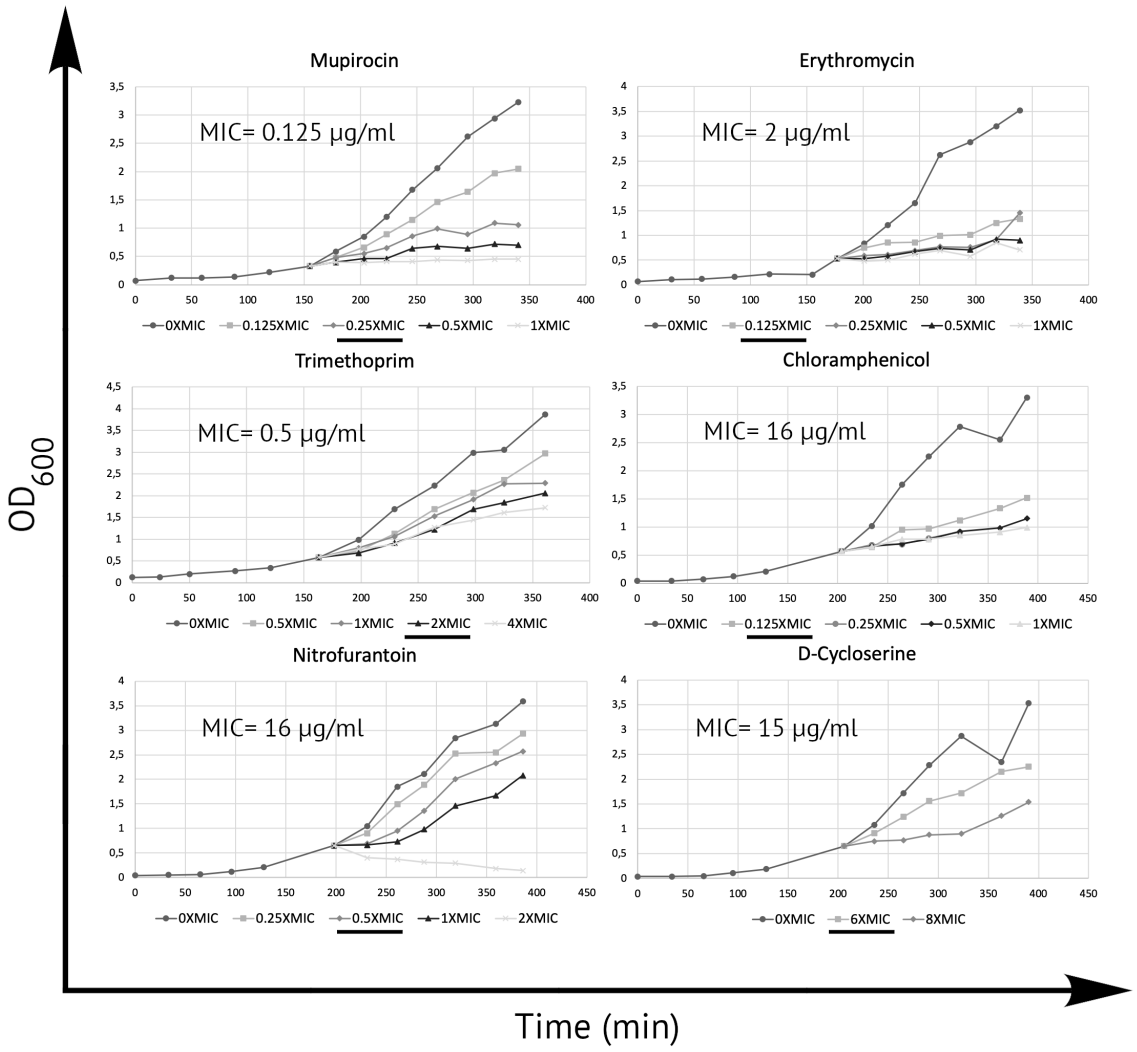

Figure C2: Growth curve of *B. subtilis*-168. Samples were taken every 30 min during lag-phase and every 20 min during log-phase, until reaching stationary phase. Antibiotics were added at an OD_{600} of 0.3-0.5 in four different concentrations determined by the MIC.

Appendix D Membrane domain staining: DiIC12

D.1 Method

The protocol for staining with DiIC12 was described by Wenzel et al. [17]. DilC12 have a very strong pink colour that can give a disturbing background signal in the microscopy images. Thus, the cells had to be washed according to the following protocol before incubation with antibiotic: 20 µl of an over-night culture of *B. subtilis* was diluted in 2 ml of LB, dyed with 20 µl of a 100 M DiIC12 stock solution and grown until early log-phase. 2 ml of this diluted culture was put in a 2 ml eppendorf-tube and placed in a pre-heated 30 °C centrifuge and centrifuged for 30 s at highest speed (20000xg). The supernatant was removed and 1 ml of pre-heated 30 °C LB+1 % DMSO was added. The tube was vortexed and centrifuged with the same settings as before. Be careful not to pipette instead of vortex since the shearing forces may disrupt the cell membrane. The washing with LB+DMSO was repeated 4 times and after the last centrifugation the supernatant was discarded and the cell pellet resuspended in 1.3 ml of LB+DMSO.

Then the experiment proceeded as for the BCP and the protein localization assay, with 200 µl of the washed culture being added to each of the marked, pre-heated 2 ml Eppendorf tubes and then incubated with the OSC concentrations of antibiotics presented in Table 3. for 30 min together with a negative control. During the microscopy the Cy3 and phase channels were used to visualize the antibiotic effects on the cell membrane.

Note further that the antibiotics included in this assay were only trimethoprim, triclosan, rifampicin, D-cycloserine and gramicidin since they had shown signs of either membrane invaginations or fluid membrane domains in earlier experiments.

D.2 Result

Images of all antibiotics included in the DiIC12 assay are presented in Figure D1, together with a negative control of untreated cells and a positive control of the antibiotic tyrocidine C. No abnormal RIFs could be seen for Trim or Rif, and the effects of Trc and D-cyc are evaluated in Results.

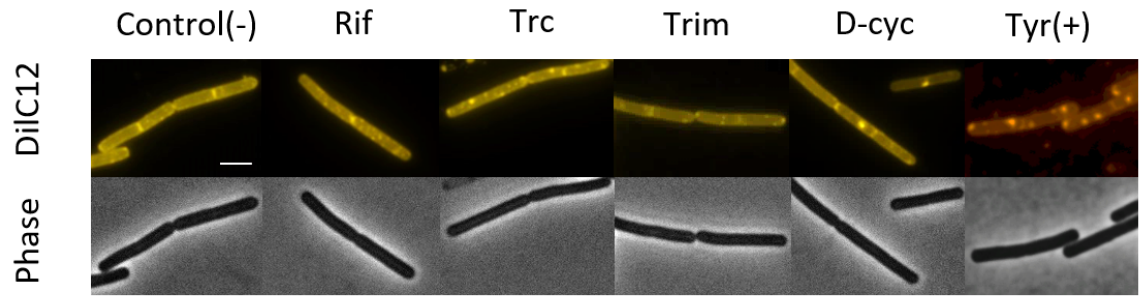

Figure D1: Antibiotic effect on *B. subtilis*-168 stained with DilC12 dye. Positive control retrieved from [26]. Adapted with permission. Scale bar 3 µm.

Appendix E Protein localization assays

Here we present the additional results of the Protein Localization Assays with images of the antibiotic treated samples where no or negligible small differences from the control sample were observed. In Table E1 an overview of the effects seen for each antibiotic on each strain are presented. An observed effect is indicated with "X" and no observed or no reproducible effect is indicated with "-".

Table E1: Overview of antibiotic effects on GFP-fusions in *B. subtilis*. X indicates an effect and - means no effect.

	Cip	Spc	Rif	Tet	Kan	Trc	Mup	Ery	Trim	Chl	Nit	D-cyc
RecA	-	-	-	-	-	-	X	-	X	X	X	-
DnaN	X	-	-	X	-	-	-	-	-	-	X	-
ParB	-	-	-	-	-	-	-	-	X	-	X	-
RpoC	-	X	X	X	-	-	X	-	X	X	X	-
RpsB	-	-	X	-	-	-	X	X	-	X	X	-
MinD	-	-	-	X	X	-	-	-	-	X	-	-
MreB	-	-	-	-	-	X	-	-	-	X	-	-
PlsX	-	-	-	-	-	-	-	-	-	-	-	-
MurG	-	-	-	-	-	-	-	-	-	-	-	X
DivIVA	X	-	X	X	-	X	-	X	X	X	-	-
FloA	-	-	-	-	-	-	-	-	-	-	-	-
AtpA	-	-	-	-	-	-	-	-	-	-	-	-
SdhA	-	-	-	-	-	-	-	-	-	-	-	-

DNA, RNA and protein synthesis

ParB

Images of the effect on ParB for all antibiotics tested are presented in Figure E1, together with a negative control of untreated cells and a positive control of the antibiotic tyrocidine C. No effect could be seen for Cip, Spc, Rif, Tet, Kan, Trc, Mup, Ery, Chl or D-cyc. The effects of Trim and Nit are evaluated in Results.

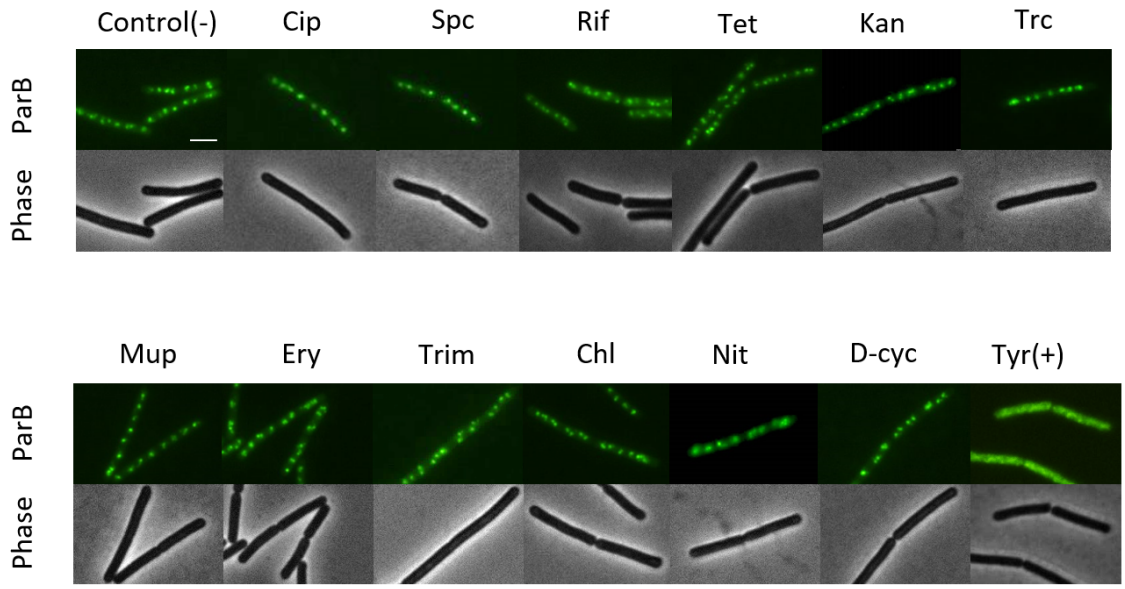

Figure E1: Localization of ParB after antibiotic treatment for 30 min on *B. subtilis* strain HM160. Positive control, marked (+), retrieved from [26]. Adapted with permission. Scale bar 3 µm.

RecA

Images of the effect on RecA for all antibiotics tested are presented in Figure E2, together with a negative control of untreated cells and a positive control of the antibiotic mitomycin C. No effect could be seen for Cip, Spc, Rif, Tet, Kan, Trc, Ery or D-cyc. The effects of Mup, Trim, Chl, and Nit are evaluated in Results.

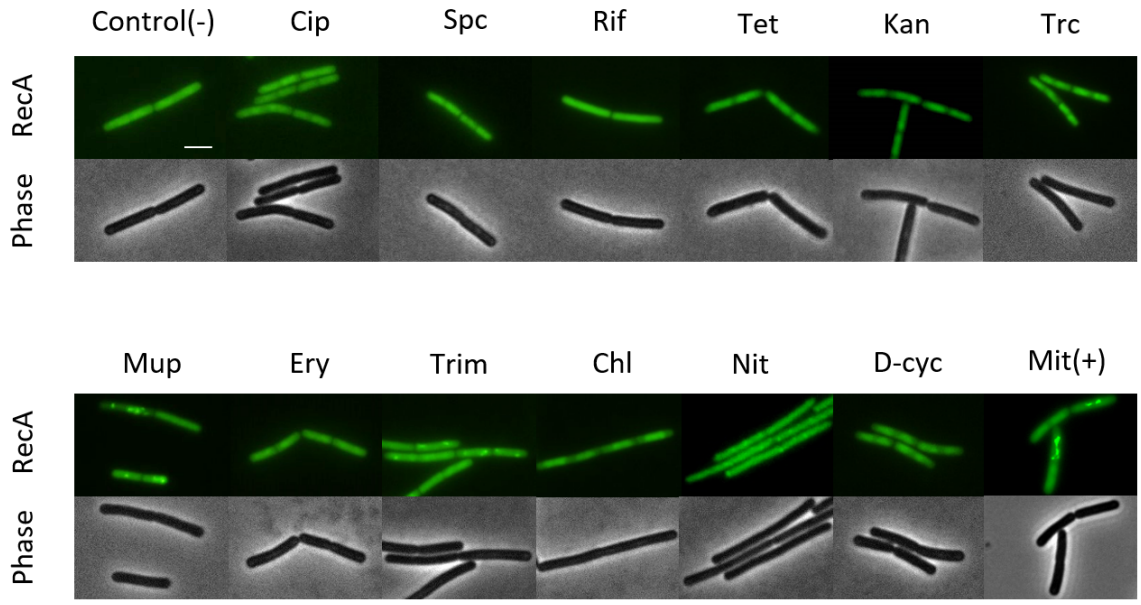

Figure E2: Localization of RecA after antibiotic treatment for 30 min on *B. subtilis* strain UG-10. Positive control retrieved from [26]. Adapted with permission. Scale bar 3 µm.

DnaN

Images of the effect on DnaN for all antibiotics tested are presented in Figure E3, together with a negative control of untreated cells and a positive control of the antibiotic ciprofloxacin. No effect could be seen for Spc, Rif, Kan, Trc, Mup, Ery, Trim, Chl or D-cyc. The effects of Cip, Tet, and Nit are evaluated in Results.

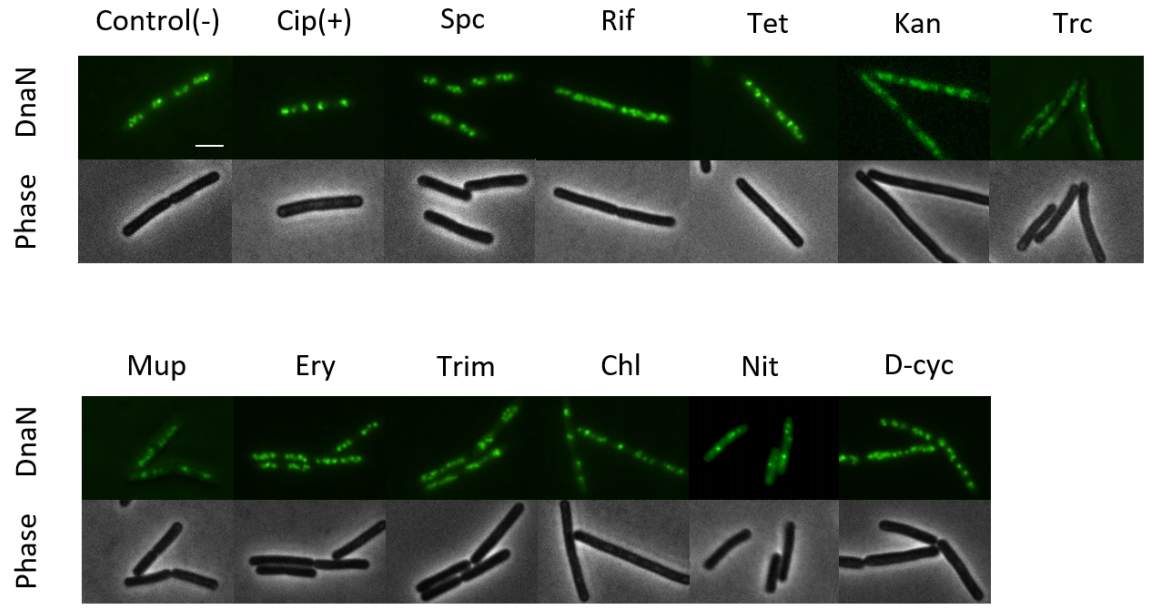

Figure E3: Localization of DnaN after antibiotic treatment for 30 min on *B. subtilis* strain HM771. Scale bar 3 µm.

RpoC

Images of the effect on RpoC for all antibiotics tested are presented in Figure E4, together with a negative control of untreated cells and a positive control of the antibiotic rifampicin. No effect could be seen for Cip, Kan, Trc, Ery or D-cyc. The effects of Spc, Rif, Tet, Mup, Trim, Chl, and Nit are evaluated in Results.

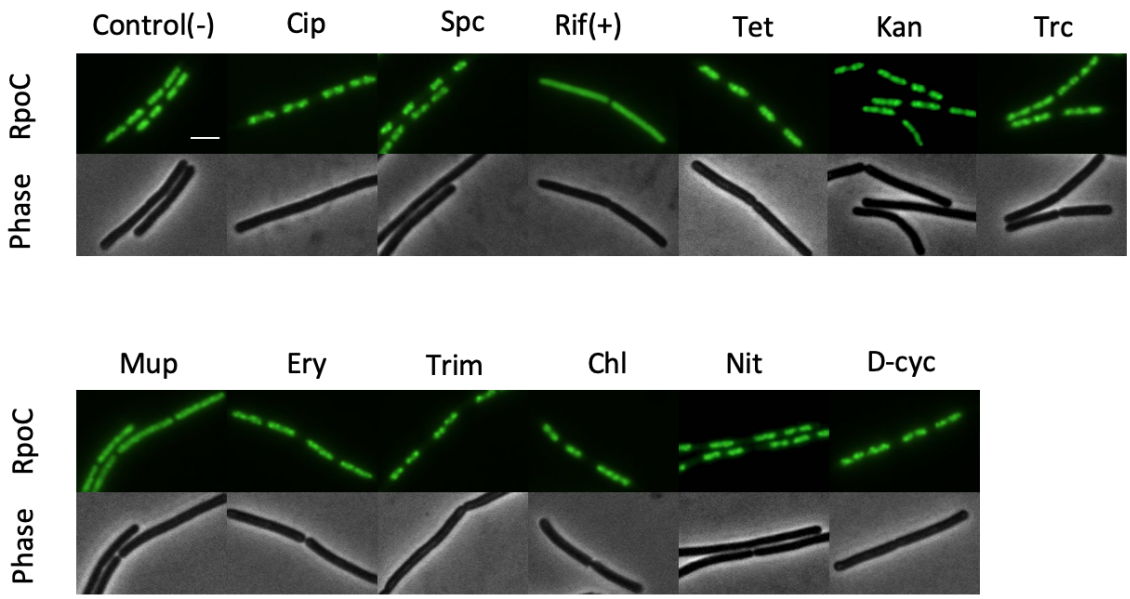

Figure E4: Localization of RpoC after antibiotic treatment for 30 min on *B. subtilis* strain 1048. Scale bar 3 µm.

RpsB

Images of the effect on RpsB for all antibiotics tested are presented in Figure E5, together with a negative control of untreated cells and a positive control of the antibiotic erythromycin. No effect could be seen for Cip, Spc, Tet, Kan, Trc, Trim or D-cyc. The effects of Rif, Mup, Ery, Chl, and Nit are evaluated in Results.

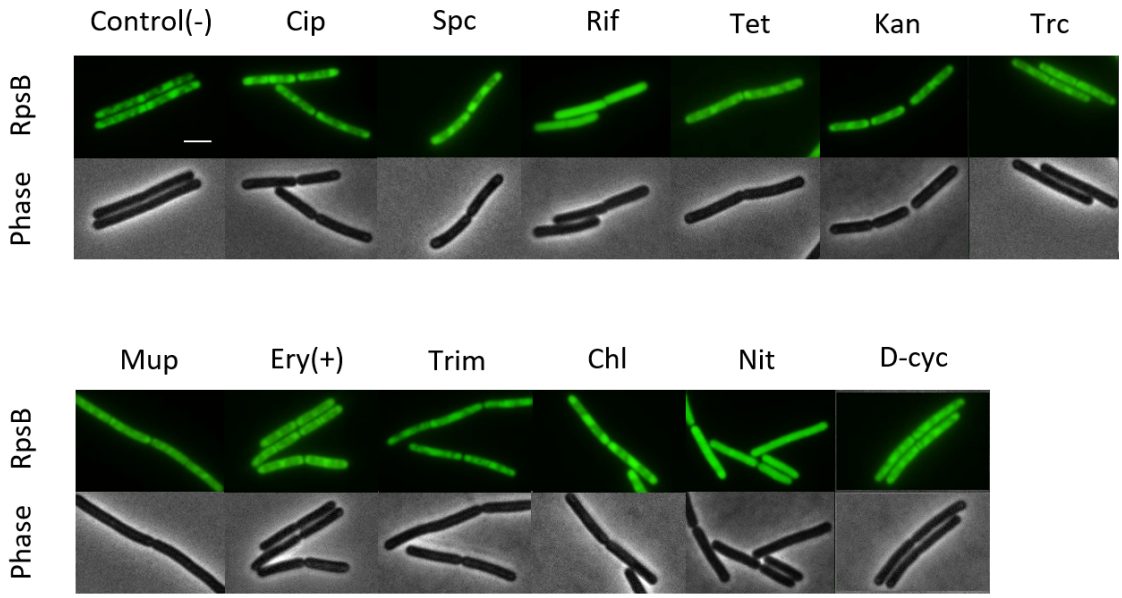

Figure E5: Localization of RpsB after antibiotic treatment for 30 min on *B. subtilis* strain 1049. Scale bar 3 µm.

Cell division site regulation

MinD

Images of the effect on MinD for all antibiotics tested are presented in Figure E7, together with a negative control of untreated cells and a positive control of the antibiotic gramicidin. No effect could be seen for the effects of Cip, Spc, Rif, Trc, Mup, Ery, Trim, Nit or D-cyc. The effects of Tet, Kan, and Chl are evaluated in Results.

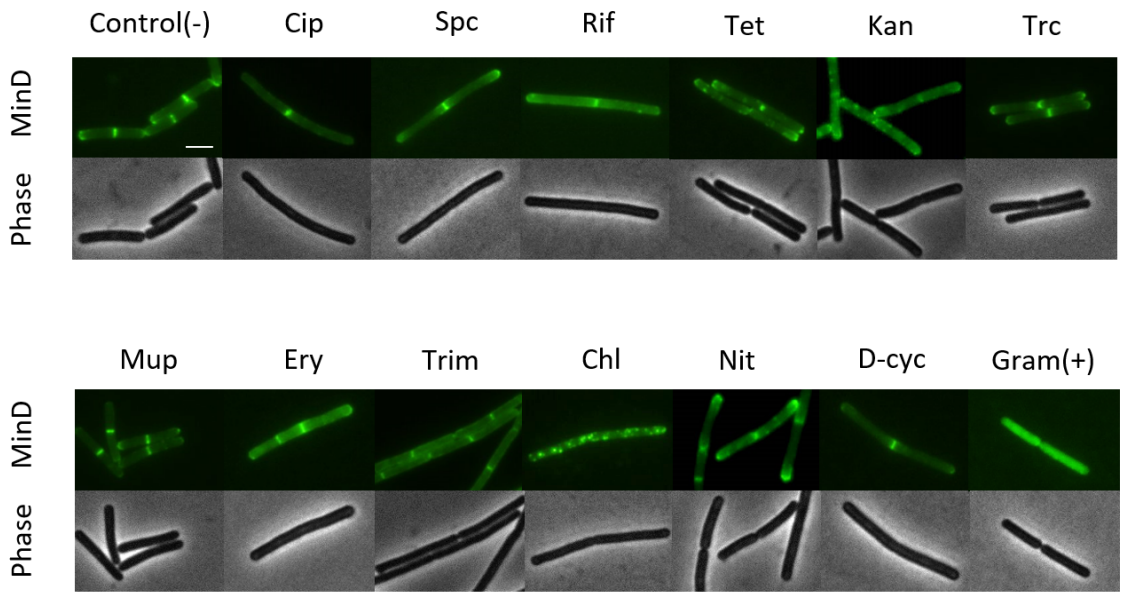

Figure E6: Localization of MinD after antibiotic treatment for 30 min on *B. subtilis* strain LH131. Scale bar 3 μm.

DivIVA

Images of the effect on DivIVA for all antibiotics tested are presented in Figure E8, together with a negative control of untreated cells and a positive control of the antibiotic gramicidin. No effect could be seen for Spc, Kan, Mup, Nit or D-cyc. The effects of Cip, Rif, Tet, Trc, Ery, Trim, and Chl are evaluated in Results.

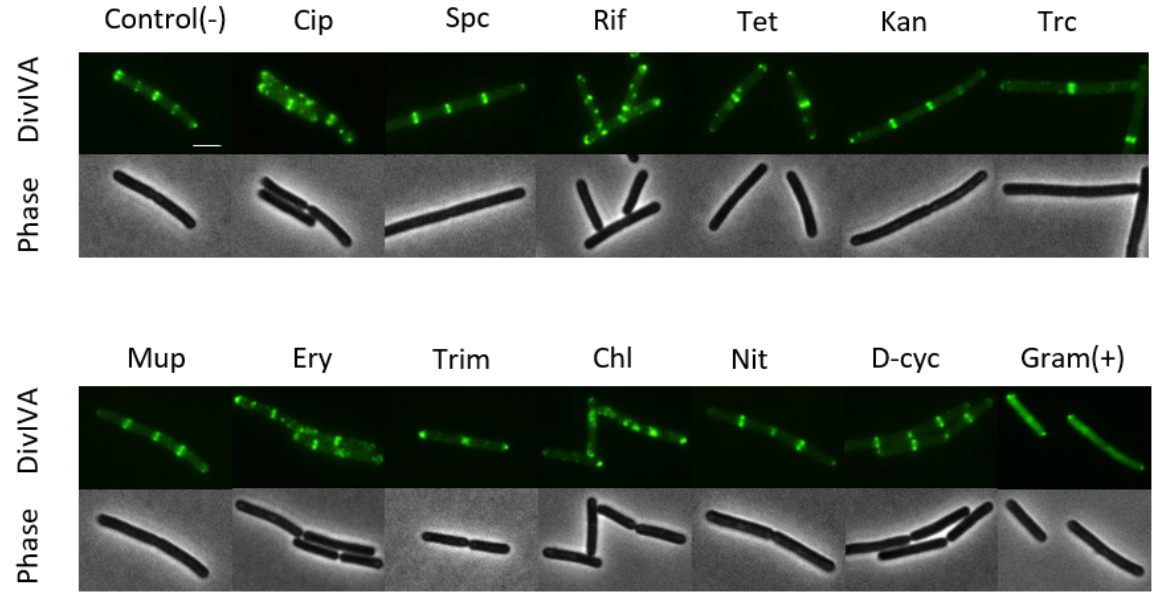

Figure E7: Localization of DivIVA after antibiotic treatment for 30 min on *B. subtilis* strain BMK21. Scale bar 3 µm.

Cell wall and phospholipid synthesis

MreB

Images of the effect on MreB for all antibiotics tested are presented in Figure E6, together with a negative control of untreated cells and a positive control of the antibiotic gramicidin C. No effect could be seen for Cip, Spc, Rif, Tet, Kan, Mup, Ery, Trim, Nit or D-cyc. The effects of Trc and Chl are evaluated in Results.

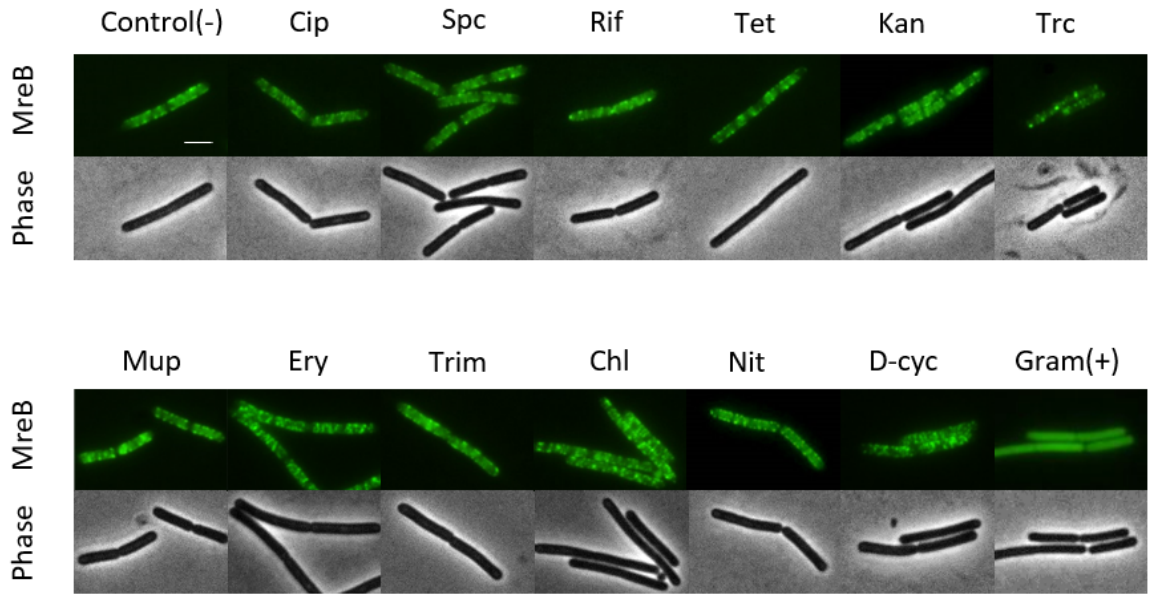

Figure E8: Localization of MreB after antibiotic treatment for 30 min on *B. subtilis* strain MW10. Scale bar 3 µm.

MurG

Images of the effect on MurG for all antibiotics tested are presented in Figure E9, together with a negative control of untreated cells and a positive control of the antibiotic daptomycin. No effect could be seen for Cip, Spc, Rif, Tet, Kan, Trc, Mup, Ery, Trim, Chl or Nit. The effect of D-cyc is evaluated in Results.

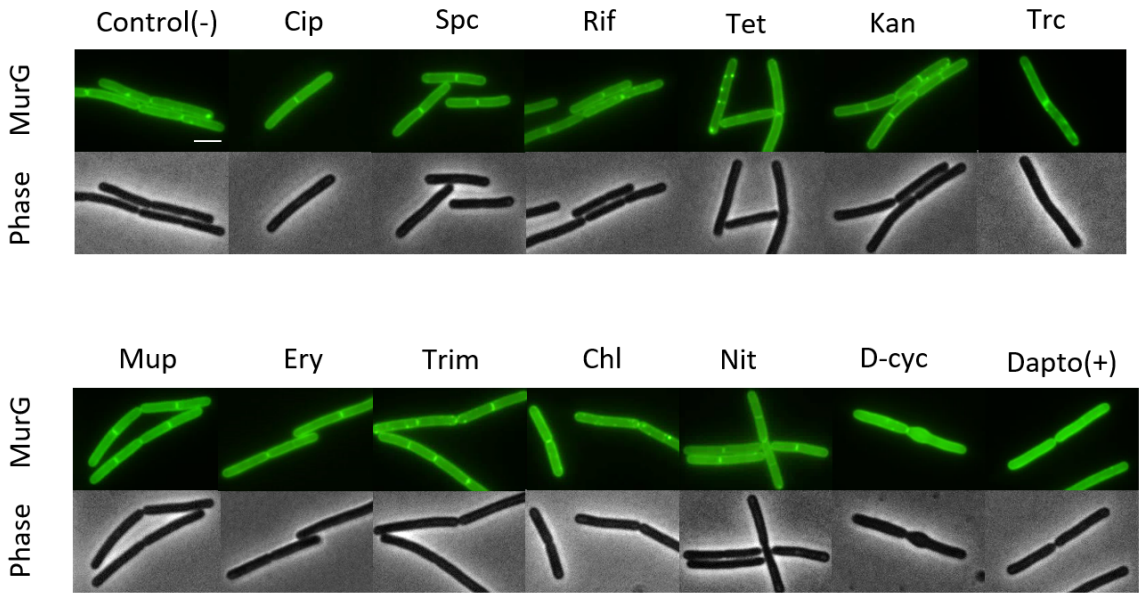

Figure E9: Localization of MurG after antibiotic treatment for 30 min on *B. subtilis* strain TNVS175. Scale bar 3 µm.

FloA

Images of the effect on FloA for all antibiotics tested are presented in Figure E10, together with a negative control of untreated cells and a positive control of the antibiotic tyrocidine C. No effect could be seen for any of the twelve evaluated antibiotics.

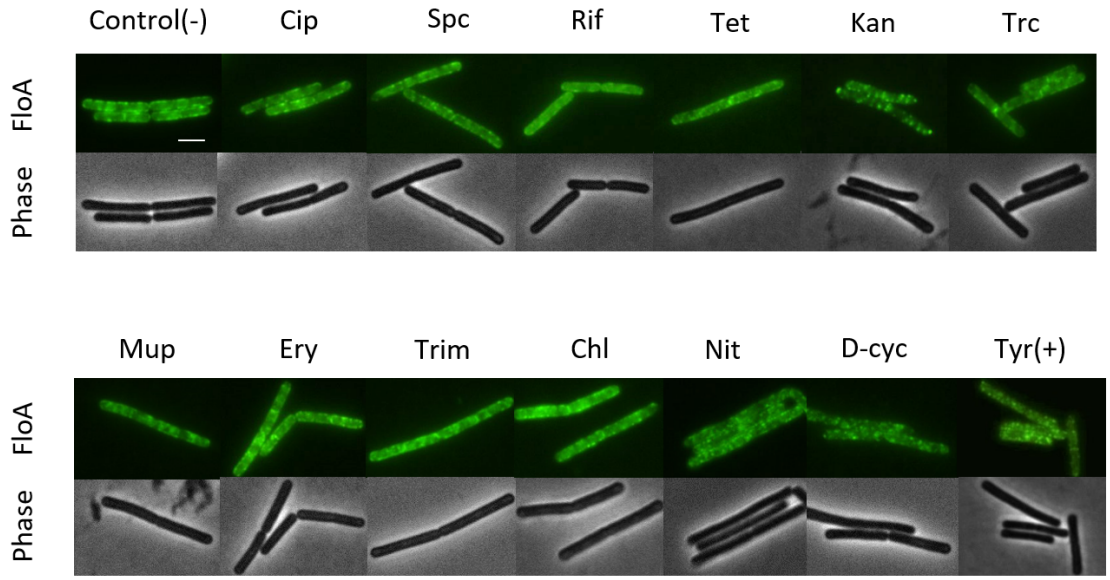

Figure E10: Localization of FloA after antibiotic treatment for 30 min on *B. subtilis* strain DL1367. Positive control retrieved from [26]. Adapted with permission. Scale bar 3 µm.

PlsX

Images of the effect on PlsX for all antibiotics tested are presented in Figure E11, together with a negative control of untreated cells and a positive control of the antibiotic daptomycin. No effect could be seen for any of the twelve evaluated antibiotics.

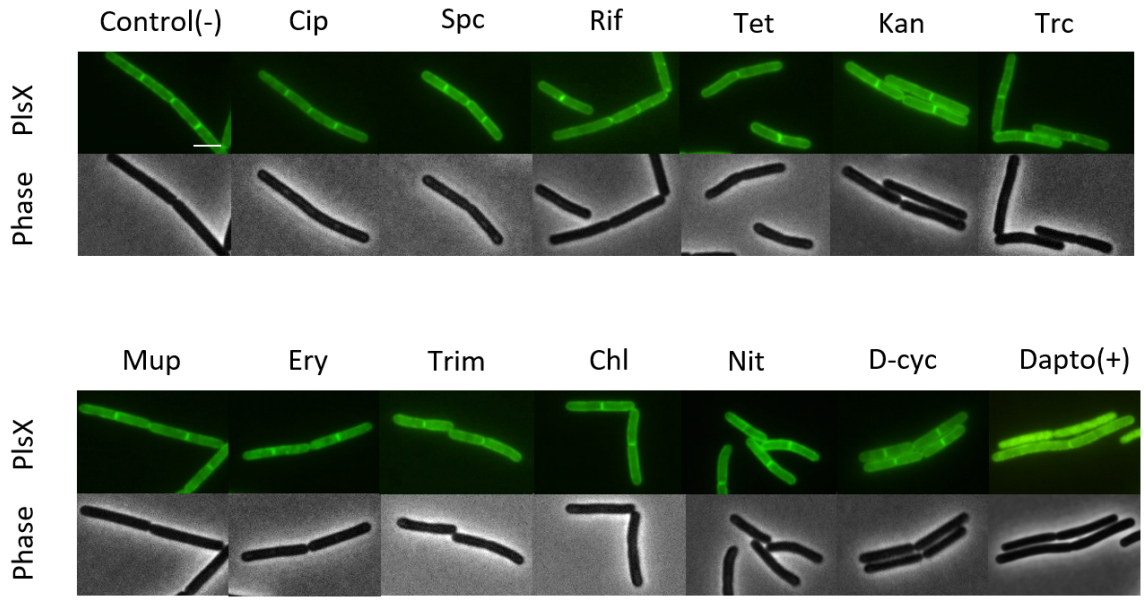

Figure E11: Localization of PlsX after antibiotic treatment for 30 min on *B. subtilis* strain TNVS29D. Positive control retrieved from [26]. Adapted with permission. Scale bar 3 µm.

Respiratory chain and ATPase

AtpA

Images of the effect on AtpA for all antibiotics tested are presented in Figure E12, together with a negative control of untreated cells. No effect could be seen for any of the twelve evaluated antibiotics.

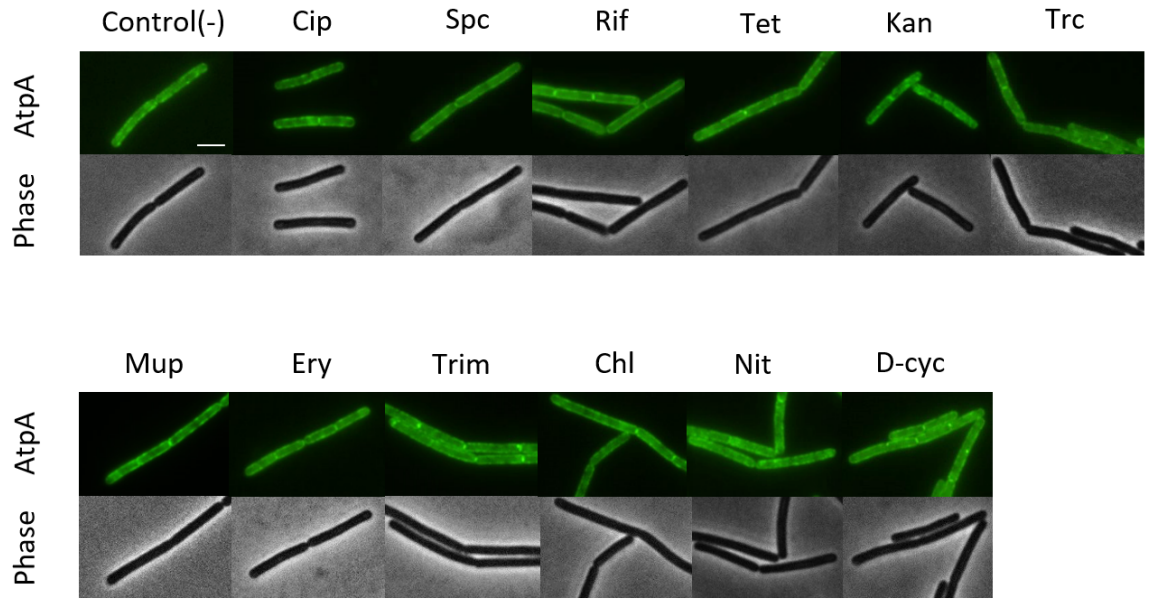

Figure E12: Localization of AtpA after antibiotic treatment for 30 min on *B. subtilis* strain BS23. Scale bar 3 µm.

SdhA

Images of the effect on SdhA for all antibiotics tested are presented in Figure E13, together with a negative control of untreated cells. No effect could be seen for any of the twelve evaluated antibiotics.

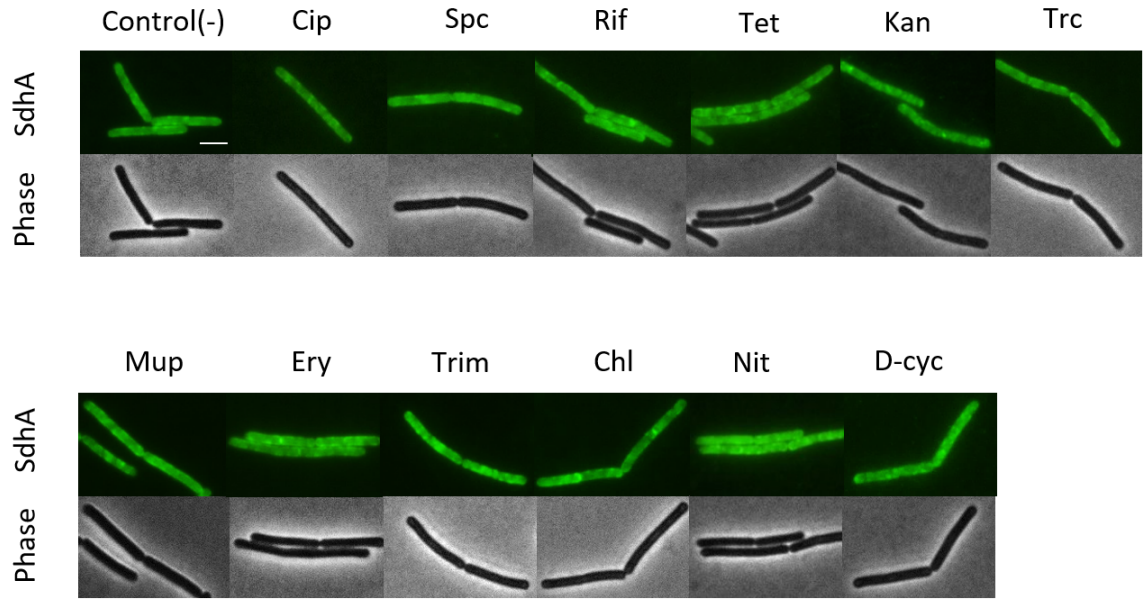

Figure E13: Localization of SdhA after antibiotic treatment for 30 min on *B. subtilis* strain BS121. Scale bar 3 µm.

Appendix F Peptidoglycan synthesis

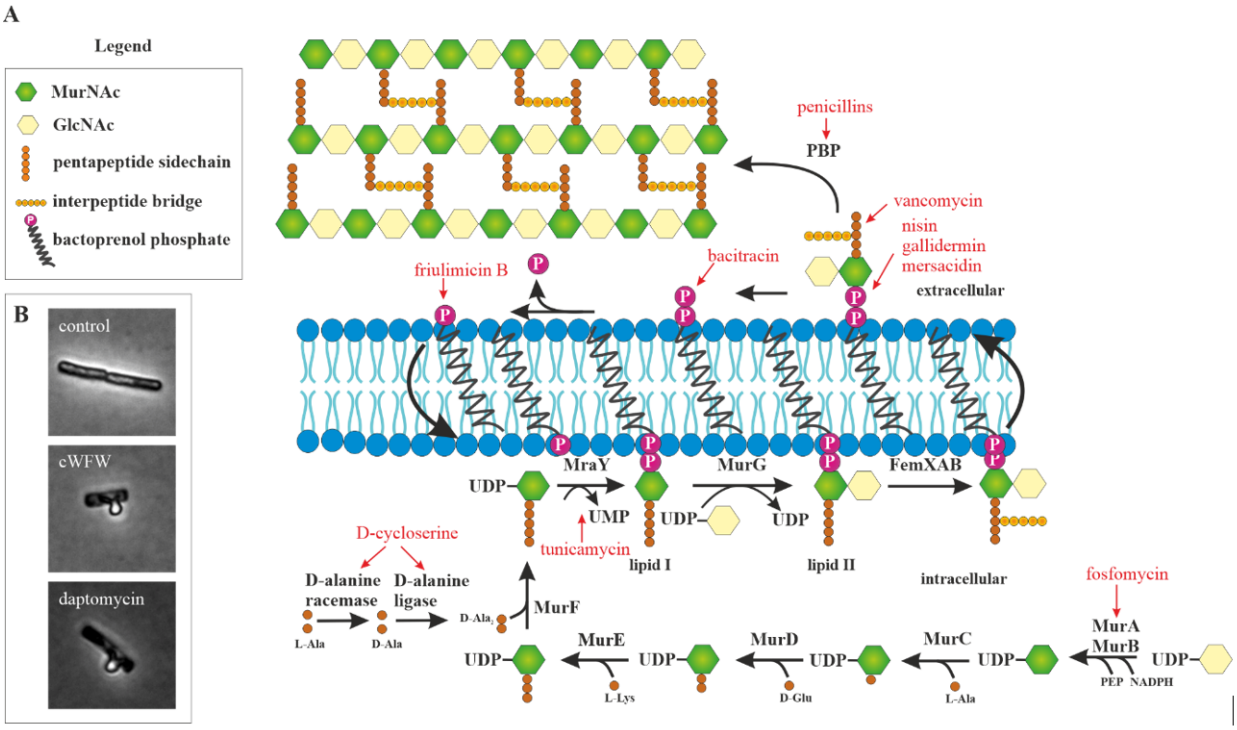

Figure F1: Scheme of synthesis of peptidoglycan with indications of where different antibiotics inhibit important steps in the synthesis. From[53]. Adapted with permission.

Appendix G Sources of error

There are several things that can, and in some cases did, go wrong in experiments such as these, that will be addressed in this section. Much of the laborative work required using sterile technique, meaning wiping every surface in vicinity of the samples with 70% ethanol, working close to an open flame, not touching un-sterile surfaces, using autoclaved pipettes and making sure everything is concealed before turning off the burner. Mistakes were sometimes made however, such as unconsciously working too far away from the flame, using days old growth media or having the flame dying out in lack of gas. In some cases, this resulted in visible contamination with other bacterial species in our bacillus cultures. In first set of experiments the MIC plates were sometimes left open for an extended period of time when pipetting and the plates lacked proper lids, which forced us to create non-concealing improvised lids. These are factors that can explain the contamination that was observed for some MIC-plates in the sterile control, forcing us to redo those replicates. A side note concerning the MIC-experiments is that the MIC value for cerulenin, provided from earlier studies, later turned out to be incorrect. This might explain its lack of bactericidal effect.

In general, the growth curves and GFP assays were not conducted under sterile conditions, which should not have posed a big issue, since the initial number of cells would have vastly outnumbered any contaminating bacteria. An exception to this was the bacillus cultures prepared for use later the same day, which should be diluted to a very low OD using sterile LB. However, sometimes the LB was pipetted directly over the (non-sterile) waterbath and sometimes the wrong, non-sterile, LB-media was used, ultimately risking contamination. This occurred on separate occasions for both RecA and DnaN, where we discovered cocci and rod-shaped bacteria, suspected to be *Staphylococcus aureus* and *Escherichia coli*, during microscopy for protein localization assays, forcing us to redo the entire experiments. Something similar was seen during the first replicates of the ParB-assay, where the frozen stock culture turned out to be contaminated with *E. coli*.

Many of the strains require inducers to help express the GFP signal, such as the transcription inducer xylose. These are supposed to be added to the tubes at the start of inoculation but were sometimes forgotten and added in later. Sometimes as much as 30 minutes to an hour after inoculation. This may have affected the behaviour of the cells during microscopy if they were not given enough time to express the GFP signal. For some strains, we did see a weird looking signal after this type of event, although it is difficult to say for sure that the inducers were the cause. These experiments had to be repeated.

Also, since bacillus is strictly aerobic obligate and thus sensitive to anaerobic conditions, we tried to be careful not to store the bacterial samples in too small tubes or completely vertical in the water bath. Yet on some occasions evaluating the GFP-localization assays (especially first replicates of MinD and MreB) we saw indications of anaerobic stress on the cell membranes (bright unexpected dots in the negative control). Moreover, some of the BCP- and GFP-assays, took longer than what the protocols recommend. It is for example preferable to not spend more than 10 minutes under the microscope, for risk of inducing anaerobic stress, but sometimes the measurement took as long as 25 to 30 minutes. The most sensitive strains are the ones reporting for membrane depolarization, such as MinD, MreB and DivIVA, since anaerobic stress can give misleading results similar to membrane damage. We tried to mitigate this as best as possible by dividing the antibiotics for each strain into smaller groups, of 3 or 4, as to not have to spend too much time on the microscopy. In the initial experiments, the agar plates we used for microscopy were often not completely

flat, which meant that we had to adjust the focus for every single well. This made each microscopy session take an excessive amount of time, but this was reduced by a significant amount when we began to be more critical with the slides.