THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Contribution of *Bacillus subtilis* cell envelope stress responses to antibiotic survival

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Cover:
"Breaking down the walls: bacterial defense systems as a new target for antibiotic potentiators." This schematic illustration shows the bacterial cell envelope as a brick wall that defends bacteria from antibiotic stress. Breaking the defense mechanism of bacteria will make them vulnerable and restore antibiotics' effectiveness.

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To my dearest late father, my rock and superhero, Mr. Antony Sidarta,
who gave me the freedom to chase my dreams

Preface

This dissertation was submitted for partial fulfillment of the Doctor of Philosophy degree. The original work presented in this dissertation was carried out between June 2020 and November 2024 at Chalmers University of Technology in the Department of Life Sciences (previously the Department of Biology and Biological Engineering). This work was supervised by Associate Professor Michaela Wenzel and co-supervised by Professor Fredrik Westerlund.

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Abstract

Antibiotic potentiators, molecules that increase the activity of antibiotics by inhibiting bacterial defenses, are an emerging strategy to combat antimicrobial resistance. Cell envelope (cell wall and membrane) stress responses are particularly promising as they are the first defense against antibiotics. This thesis focuses on four systems that appeared crucial to cell envelope stress: osmoadaptation (i.e., adaptation to changing water environments) through mechanosensitive channels (MSCs) (**I**), membrane fluidity adaptations (i.e., adaptation related to the mobility of membrane components) through the lipid desaturase Des (**II**), the putative ATP-binding cassette transporter YtrBCDEF (**III**), and reactive oxygen species-mediated killing by the ionophore valinomycin (**IV**). Using *Bacillus subtilis* as model, I studied how and to which extent these cell envelope stress responses contribute to bacterial survival during antibiotic exposure.

When *B. subtilis* is exposed to membrane-targeting antibiotics, it releases amino acids through MSCs, a process coined antibiotic-induced amino acid release (AIAAR). I found that AIAAR is a widely conserved osmoprotection mechanism that is important for survival of antibiotic-induced membrane stress. (**I**). Postulating that AIAAR is mediated by antibiotic-induced membrane stretch, I aimed to develop a membrane thickness sensor based on the Des system. This system senses membrane thickness to adjust membrane fluidity. While no suitable reporter was obtained, new insights related to its function and mechanism were proposed (**II**). I then investigated the role of YtrBCDEF transporter, that is induced by cell wall synthesis inhibitors. I could confirm that this transporter plays a role in cell wall synthesis and sporulation, but its contribution to antibiotic survival was limited (**III**). Characterizing how the potassium ionophore valinomycin kills non-growing cells, we found that depolarization of the cell membrane causes mislocalization of the respiratory chain protein QcrA, leading to lethal accumulation of superoxide radicals. This finding explains why depolarization often induces an oxidative stress response (**IV**).

Based on my results, I propose AIAAR as promising target for antibiotic potentiators and suggest that oxidative stress responses should be assessed for this purpose in future research. The development of molecules interfering with AIAAR and oxidative stress responses as new antibiotic potentiators will help tackle the problem of antimicrobial resistance.

Keywords: cell envelope stress responses; osmotic stress; mechanosensitive channels; membrane fluidity; Des system; cell wall synthesis; ABC transporter; ROS; membrane potential.

List of publications

This thesis is based on the following publications.

I. **Margareth Sidarta**, Kathrin Barlog, Pascal Dietze, Caroline May, Julia E. Bandow, Michaela Wenzel. Antibiotic-induced amino acid release (AIAAR): A bacterial emergency response to membrane-targeting antibiotics. Manuscript.

II. **Margareth Sidarta**, Ana I. Lorente Martín*, Anuntxi Monsalve*, Gabriela Marinho Righetto, Ann-Britt Schäfer, Michaela Wenzel. Lipid phase separation impairs membrane thickness sensing by the *Bacillus subtilis* sensor kinase DesK. *Microbiol Spectr* 12 (6), pp. 1-20, (2024). https://doi.org/10.1128/spectrum.03925-23.

III. Luna Baruah*, **Margareth Sidarta***, Pauline Hammer úr Skúoy, Olivia Johnsson, Emma Frisk, Paula Didelot, Ann-Britt Schäfer, Aysha Arshad, Michaela Wenzel. The *ytrGABCDEF* operon is involved in growth arrest and cell lysis of distinct *Bacillus subtilis* subpopulations. Submitted manuscript.

IV. Declan A. Gray*, Biwen Wang*, **Margareth Sidarta**, Fabián A Cornejo, Jurian Wijnheijmer, Rupa Rani, Pamela Gamba, Kürşad Turgay, Michaela Wenzel, Henrik Strahl, Leendert W. Hamoen. Membrane depolarization kills dormant *Bacillus subtilis* cells by generating a lethal dose of ROS. *Nat Commun* 15 (6877), pp. 1–13, (2024). https://doi.org/10.1038/s41467-024-51347-0.

*These authors contributed equally.

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

V. **Margareth Sidarta**, Luna Baruah, Michaela Wenzel. Roles of bacterial mechanosensitive channels in infection and antibiotic susceptibility. *Pharmaceuticals* 15 (770), pp. 1-18, (2022). https://doi.org/10.3390/ph15070770.

VI. Ann-Britt Schäfer, **Margareth Sidarta**, Ireny Abdelmesseh Nekhala, Gabriela Marinho Righetto, Aysha Arshad, Michaela Wenzel. Dissecting antibiotic effects on the cell envelope using bacterial cytological profiling: a phenotypic analysis starter kit. *Microbiol Spectr* 12 (3), pp. 1-18, (2024). https://doi.org/10.1128/spectrum.03275-23.

Contribution summary

Contribution report for the included publications in this PhD thesis:

I. I constructed strains, performed experiments, analyzed, validated, visualized, and curated data. I wrote the manuscript together with MW.

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

III. I supervised students and performed experiments together with LB and MW. Then, all data obtained from these experiments was analyzed, validated, visualized, and curated by MW and me. I wrote the manuscript together with MW.

IV. I generated part of the experimental data including data analysis, validation, visualization, and curation. I also contributed to writing the sections of the paper pertaining to my own data.

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Abbreviations

ABC transporter	ATP-binding cassette transporter
AIAAR	antibiotic-induced amino acid release
AMP	antimicrobial peptide
ATP	adenosine triphosphate
BA	benzyl alcohol (only used in figure legends)
BCP	bacterial cytological profiling
bocillin	BODIPY FL penicillin
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CESR	cell envelope stress responses
cWFW	a cyclic hexapeptide antibiotic with the sequence cRRRWFW
dap	daptomycin (only used in figure legends)
DAPI	4’,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
ECF	extracytoplasmic function
ETC	electron transport chain
ery	erythromycin (only used in figure legends)
fp	forespore (only used in figure legends)
GFP	green fluorescence protein
GP	general polarization
H2DCFDA	2’,7’-dichlorodihydrofluorescein diacetate
HPLC	high-performance liquid chromatography
laurdan	2-dimethylamino-6-lauroyl naphthalene
MIC	minimal inhibitory concentration
mRNA	messenger ribonucleic acid
MSC	mechanosensitive channel
MscM	mechanosensitive channel mini conductance
MscL	mechanosensitive channel large conductance
MscS	mechanosensitive channel small conductance
msfGFP	monomeric superfolder green-fluorescent protein
Nile red	9-diethylamino-5H-benzo[α]phenoxazine-5-one

nis	nisin (only used in figure legends)
OD	optical density
ONPG	*o*-nitrophenol-β-D-galactoside
PBPs	penicillin-binding proteins
RBS	ribosomal-binding sequence
RNA	ribonucleic acid
RNAP	ribonucleic acid polymerase
ROS	reactive oxygen species
SP	*subtilis* phage
TCA cycle	the citric acid cycle
TCS	two component system
UDP-MurNac	uridine diphosphate N-acteylmuramic acid
van	vancomycin (only used in figure legends)
Van-FL	BODIPY FL vancomycin
val	valinomycin (only used in figure legends)

1. Aims and scope

The failure of antibiotic therapy in curing infections caused by resistant pathogens has become a major global health risk[1]. The alarming emergence of resistant pathogens simply surpass the slower development of new antibiotics[1]. This problem stresses the importance of finding new antimicrobial therapeutics and alternative strategies to classical antibiotics[2,3]. One such alternative strategy is the development of antibiotic potentiators, sometimes also referred to as antibiotic adjuvants. Potentiators are molecules that do not directly kill bacteria but increase the activity of an antibiotic compound, for example by interfering with a bacterial resistance mechanism. A prominent example for this strategy is the combination of β-lactam antibiotics with β-lactamase inhibitors that prevent the enzymatic degradation of the antibiotic[4]. Similar strategies focus on, for example, efflux pump inhibitors or outer membrane permeabilizers that increase the intracellular antibiotic concentration[5,6]. A newer strategy is the inhibition of bacterial stress response systems. Bacteria possess a range of different defense mechanisms to cope with antibiotic stress[7–10] and the proof-of-principle that such mechanisms can serve as potentiator targets has been established for the DNA damage repair protein RecA[11,12].

In my thesis, I focused on cell envelope stress responses in the Gram-positive model organism *Bacillus subtilis*. The bacterial cell envelope, which in Gram-positive bacteria consists of the peptidoglycan cell wall and the cytoplasmic membrane, acts as the first line of defense that prevents the entrance of antibiotics into the cell[13–15]. In addition to their function as permeability barrier, the cell wall confers structural integrity and the cell membrane harbors essential cellular processes, for example, respiration, signaling, transport, and cell wall synthesis[16,17]. Due to these important functions, the cell envelope has become one of the most important antibiotic targets[18–20], cell wall synthesis inhibitors being the most prescribed antibiotic class by far[21,22].

Bacillus subtilis is the most important and best-characterized Gram-positive model bacterium, having been extensively studied with respect to its genetics, biochemistry, and cell biology. It has been particularly well-studied with respect to its highly differentiated cell envelope stress response (CESR) systems[23–25] and has frequently served as model for antibiotic mode of action studies[26–29]. A plethora of studies have used *B. subtilis* as model for proteomic stress response profiling, not only but extensively for antibiotic stress responses. Thus, proteome response libraries provide insight into the specific proteins that are upregulated in response to acute antibiotic stress. These proteins are referred to as proteomic markers and are highly specific for the respective stress conditions. Due to this specificity, they have been successfully applied in antibiotic mode of action elucidation[23,24,30–39]. Marker proteins do not have to be, but often are, part of CESR systems. While some of them are part of well-known CESR systems, proteomic studies have revealed several potential CESR proteins and systems that are yet poorly or not at all characterized. This makes these proteomic datasets a valuable resource for revealing new candidate CESR systems. In my thesis, I have investigated such leads derived from proteomic datasets.

Since these extensive proteomics profiles were generated in *B. subtilis,* I used this species as my model organism. *B. subtilis* is not only extremely well-characterized, but the available data is also organized in an outstanding manner, compiled into one excellent database, SubtiWiki[40], which is by far the most exhaustive, accessible, and intuitive microbe-specific database available. Additionally, *B. subtilis* is easy to genetically modify and strain libraries of non-essential gene deletions as well as essential gene knock-downs are commercially available[41,42]. Furthermore, *B. subtilis* can serve as safe model for many important Gram-positive pathogens such as *Bacillus anthracis*, *Staphylococcus aureus*, and *Listeria monocytogenes*, making it a very useful and versatile model organism[43].

In my thesis, I focused on four stress responses that appeared to be of importance to bacterial survival upon exposure to cell envelope-targeting antibiotics (**Figure 1**). For each of these (potential) CESR systems, I aimed to (i) gain insight into their functions and (ii) evaluate their suitability as new potentiator targets.

Based on proteomic data of *B. subtilis* treated with the antimicrobial peptide (AMP) MP196, a new antibiotic survival mechanism, coined antibiotic-induced amino acid release (AIAAR), was identified[30]. This mechanism is mediated by mechanosensitive channels (MSCs), which are well known for their role in osmoadaptation. In **paper I,** I aimed to further characterize the mechanism behind AIAAR and its role in antibiotic survival, and to confirm its potential as a new potentiator target. Since AIAAR turned out to be a promising potentiator target lead, it was further assessed regarding conservation among bacterial species and breadth of antibiotic classes that induce this CESR.

Studying the mechanism by which antibiotics trigger AIAAR led me to work with a well-known membrane fluidity adaptation system, the lipid desaturase Des, which was previously reported to impact susceptibility to the lipopeptide antibiotic daptomycin[44]. Originally, I intended to develop the Des system into a biosensor for studying AIAAR. However, the system did not react as expected, leading to new insights into the mechanism of this CESR system and its role during membrane stress (**paper II**).

In **paper III**, I investigated a different system, the *ytrGABCDEF* operon which encodes the putative ATP-binding cassette (ABC) transporter YtrBCDEF. YtrB and YtrE have previously been identified as marker proteins for cell wall-targeting antibiotics[23,24,30–32,34–39] and were reported to affect nisin susceptibility[35]. However, the physiological role of this transporter is unknown. In my work, I aimed to further characterize its role as possible CESR system, especially pertaining to antibiotic stress adaptation and cell wall homeostasis.

Several membrane-targeting antibiotics have been shown to induce oxidative stress responses in proteomic profiling studies[35,45]. One example for that is valinomycin, which induces the expression of the superoxide dismutase SodA that detoxifies superoxide anions[45,46]. However, the reason behind this induction, especially why only SodA and not other related detoxification systems are activated, is unknown. In **paper IV**, my collaborators and I studied the killing mechanism of the membrane-depolarizing potassium ionophore valinomycin using stationary phase *B. subtilis* cells. We tested the hypothesis that reactive oxygen species (ROS),

such as superoxide, may underlie the bactericidal activity of this ionophore against non-growing cells, and studied the respiratory electron transport chain as source of ROS.

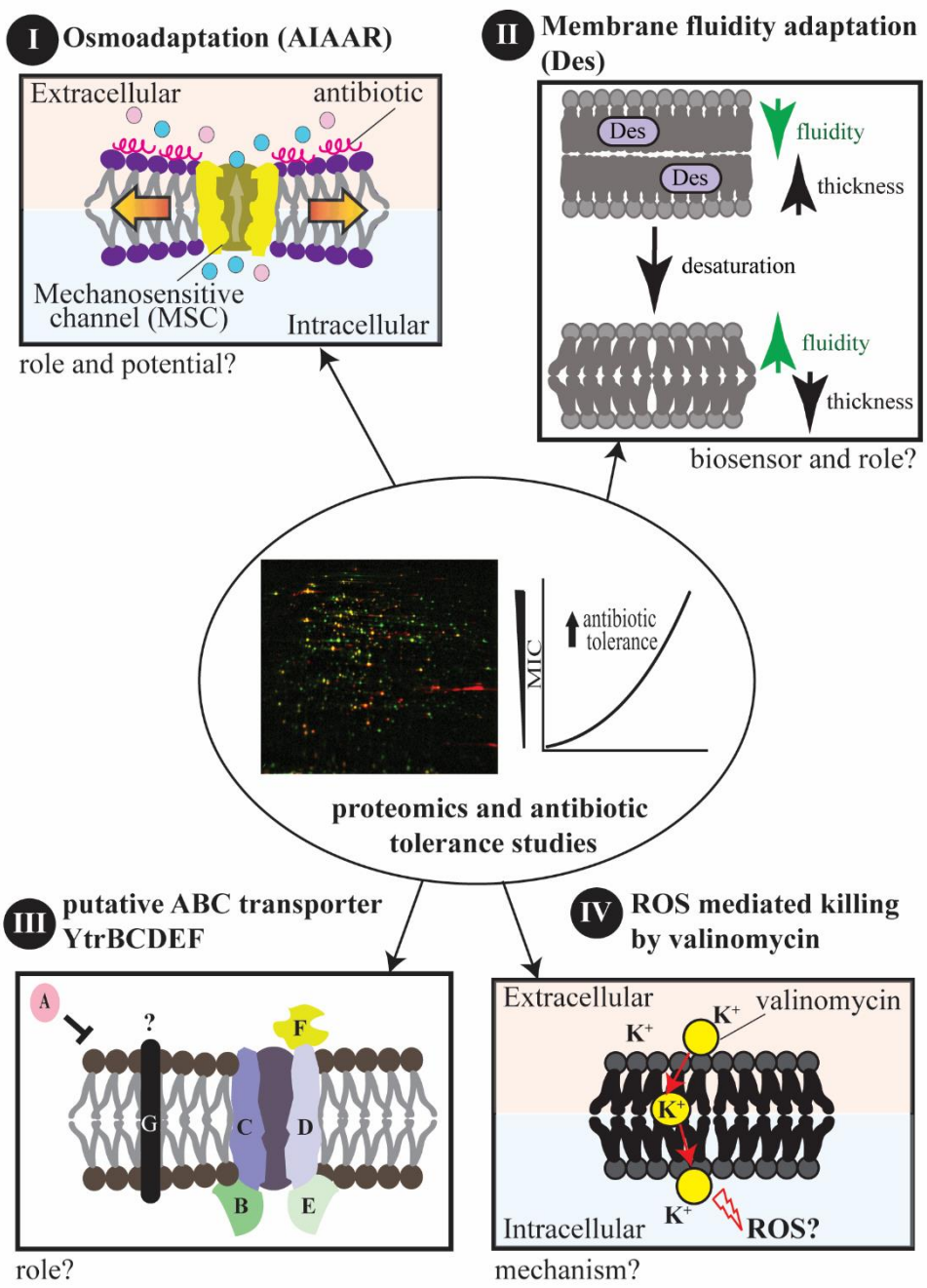

Figure 1: Schematic illustration of projects studied in this work and their aims. Projects were chosen based on proteomics and/or antibiotic tolerance studies. Here, the 2D gel-based proteome response pattern was false-colored to illustrate antibiotic-induced proteins (red), repressed proteins (green), and proteins synthesized equally (yellow). (I) AIAAR is mediated by the well-known osmoadaptation machinery, MSCs. Its role in antibiotic adaptation and potential as new potentiator target were characterized. (II) membrane fluidity adaptation through the *des* operon. To study AIAAR, an attempt to develop a biosensor based on this system was done and a new insight into its role was explored. (III) the *ytrGABCDEF* operon encodes the repressor YtrA, the putative ABC transporter YtrBCDEF, and small unknown function membrane protein YtrG[40]. The physiological roles of this operon, especially in antibiotic adaptation and cell wall homeostasis were characterized. (IV) the ROS mediated killing mechanism of the membrane potential dissipating potassium ionophore valinomycin was studied. Images are adapted from [45,47,48].

2. *Bacillus subtilis*

B. subtilis is a fast-growing, rod-shaped, Gram-positive bacterium which grows optimally between 30-35 °C[49]. It is isolated from diverse natural environments (soil, water, and rhizosphere) and an important part of the soil microbiome[50,51]. Recently, it is found to be part of human gut microbiota that may play role in promoting the gut health[52–54]. Additionally, *B. subtilis* has been utilized in food fermentation such as natto[52,53]. As a facultative anaerobe, *B. subtilis* can grow in aerobic and anaerobic conditions. Under strict anaerobic conditions, *B. subtilis* grows slower and uses nitrate or nitrite as its electron acceptor or by fermentation[55,56]. The anaerobic growth-adapted cells have been shown to change their morphology from rods to longer filament-like structures[55].

Its ability to efficiently convert the organic substrates into valuable biotechnological products during fermentation cycles and to secrete large quantities of proteins into the culture medium has made *B. subtilis* an essential workhouse for industrial applications[43,49,57].

B. subtilis has become the reference point in defining bacterial phylogeny of the order *Bacilllales*[49]. Described initially as *Vibrio subtilis* (coined from the motility or vibration of the thin cells)[58], Ferdinand Julius Cohn renamed it to Bacillus subtilis (the subtle rod) in 1872[59]. *B. subtilis* is related to many important Gram-positive pathogens such as *B. anthracis*, *S. aureus*, or *L. monocytogenes*. Its nonpathogenic nature and ease of culturing make it a model organism for these pathogens and all other *Firmicutes*[43,49].

Additionally, *B. subtilis* was one of the first bacterial species, whose genome has been sequenced[60], and it remains one of the best annotated genomes until now[57,61]. *B. subtilis* is also the subject of many intensive proteomics studies to obtain the protein regulation patterns during different growth or stress conditions, including antibiotic stress[32,34,35,62–64]. One application of these studies, the antibiotic stress response library, has become an important reference to determine the mode of action of novel antimicrobial candidates and is still being expanded[32,33,35]. Consequently, *B. subtilis* has advanced to an interesting model for different antibiotic studies. As one of the best characterized bacterial species next to the Gram-negative *Escherichia coli*, *B. subtilis* has an open access organism-specific database (SubtiWiki) that integrates a plethora of information, including data on genome sequence and organization, gene regulation, protein structure, interaction, localization, mutant phenotype and fitness, as well as available biological materials and literature for every gene/open reading frame[40]. While other organism-specific databases are available, SubtiWiki is by far the most extensive and accessible organism specific knowledge hub.

B. subtilis can form heat and chemical-resistant endospores (**Figure 2**). It switches from a classical binary fission of the vegetative stage to an asymmetrical division between the mother and forespore compartment during the sporulation stage[49,59]. In addition to sporulation[65–68], *B. subtilis* can assume other differentiated states related to motility[69,70], cannibalism[71,72], biofilms[71,73–75], competence[70,76], enzyme secretion[77], autolysis[78], and oligotrophic growth state (extreme slow growth)[79].

Since *B. subtilis* in its natural habitat is constantly exposed to fluctuating stress conditions, it develops diverse stress responses, including the formation of different cellular

states to survive these conditions[80]. All these cellular states are subject to population heterogeneity and bimodal regulation[69,71,73,76]. For example, *B. subtilis* in its vegetative stage forms two types of cells, motile and sessile[69,70]. Sessile cells are usually filamentous. Autolysins (enzymes that hydrolyze peptidoglycan in bacterial cell wall) are responsible to separate these cells into non-filamentous, motile cells[81]. Motile cells can explore new environments that may offer more favorable conditions than their previous habitat. Under dire stress conditions such as nutrient starvation, *B. subtilis* cells undergo a complex regulatory system to initiate the sporulation and to form endospores that will be released upon lysing the surrounding mother cells[65–67]. The resulting spores can remain dormant for long periods of time. Upon improved condition, spores can germinate to resume vegetative growth[68]. To delay entry into the sporulation stage, a subpopulation of *B. subtilis cells* can become cannibalistic, killing their siblings. This subpopulation releases toxins that induce the autolysis of sister cells, thus providing nutrients for the toxin-producing population[71,72]. Alternatively, *B. subtilis* can form a biofilm with distinct localization of activities and division of labor within the biofilm[71,73–75]. *B. subtilis* can also become genetically competent taking up foreign DNA, which can be degraded or integrated into its own genome[70,76]. This natural competence allow easy genetic manipulation and has aided the discovery of many important cellular processes, such as cell wall synthesis, cytokinesis, chromosome organization, membrane organization, and membrane fluidity[43]. Due to these properties, *B. subtilis* has become the major model organism for studying fundamental aspects of cellular development and differentiation as well as other cellular biology processes, including stress responses.

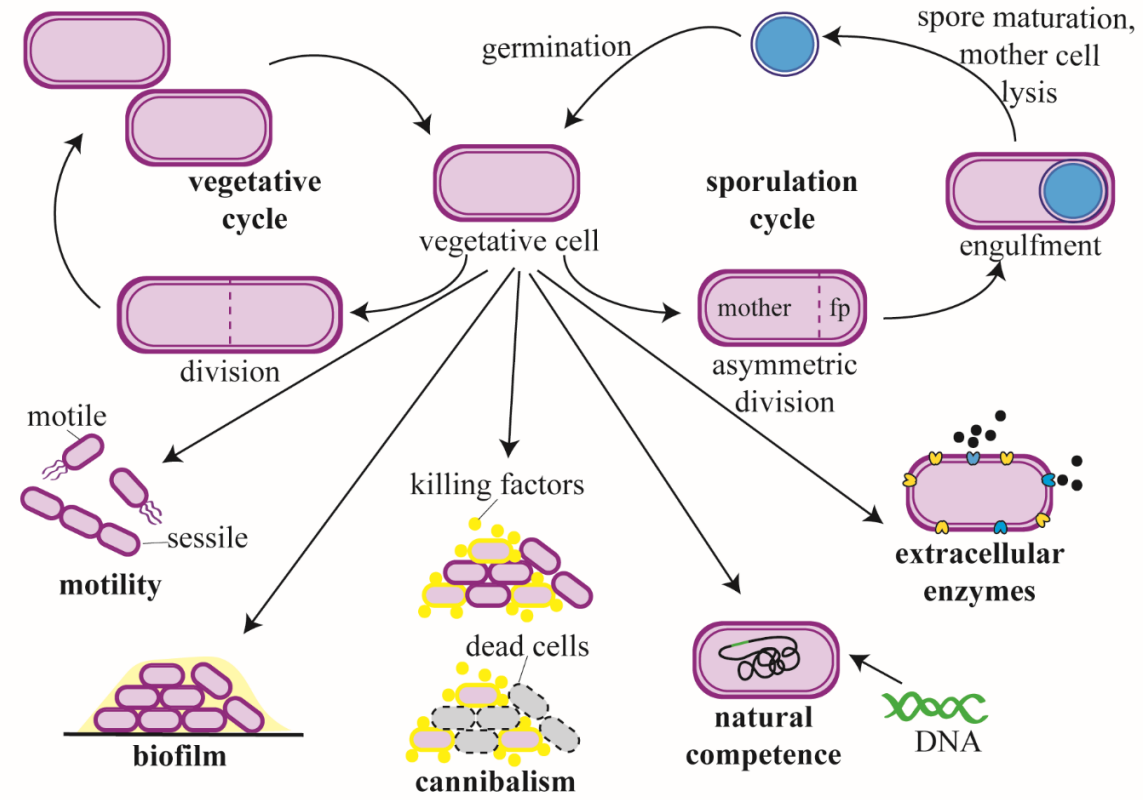

Figure 2: Important properties of *B. subtilis* as the model organism for cell biology and the industrial workhorse. See text for full description. Abbreviation: forespore (fp). Figure is adapted from[49].

3. Gene regulation in bacteria

Bacteria are constantly exposed to diverse environmental challenges, e.g., nutrient limitation or antibiotic stress. To survive these challenges, bacteria carefully regulate the expression of their genes to adapt to their needs under different conditions.

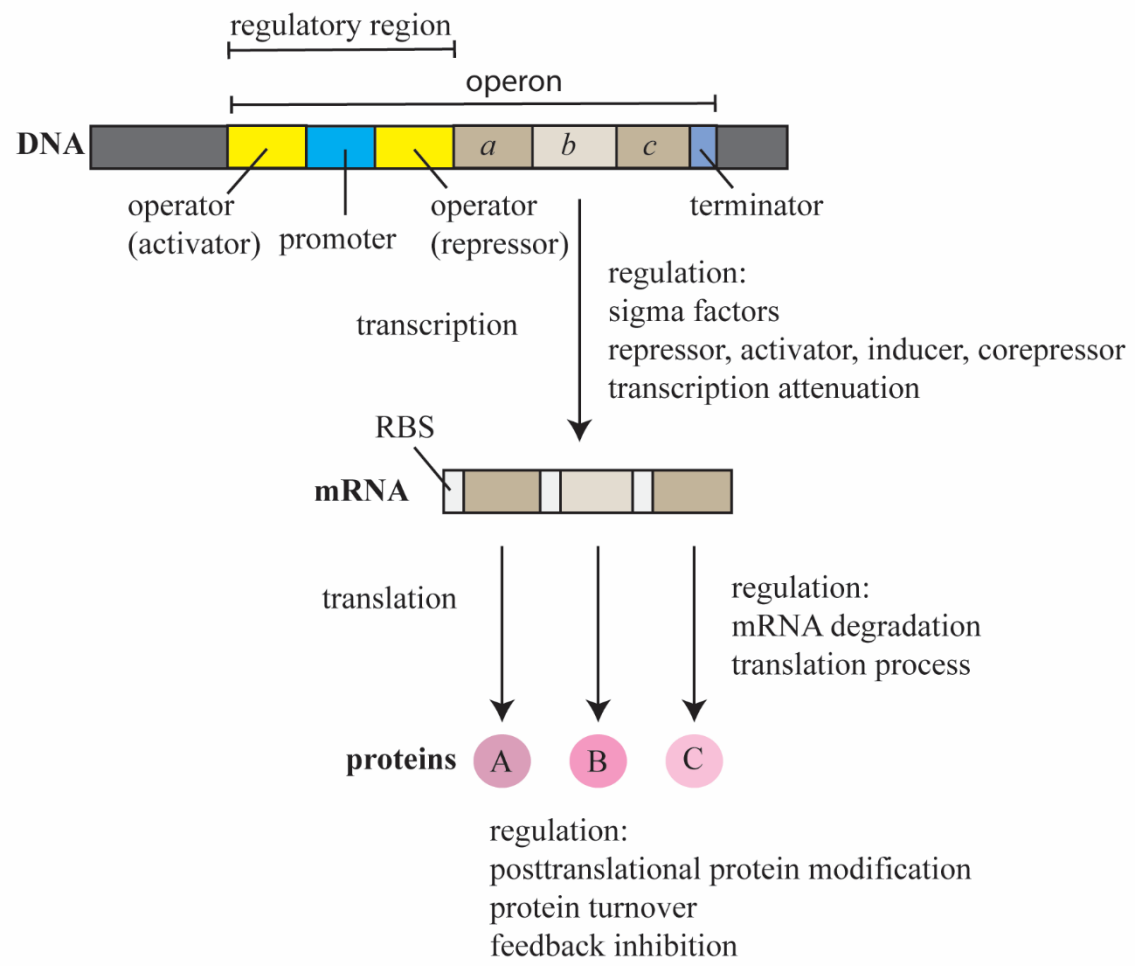

Figure 3: Central dogma of molecular biology with schematic illustration of an operon. In bacteria, genes of related functions are often organized into one operon and transcribed together under the control of a single promoter. Gene regulation can occur in all stages, from transcription, translation, to posttranslational stages. Figure adapted from [82].

According to the central dogma of molecular biology (**Figure 3**), the genetic information flows from DNA to RNA (transcription) and RNA to a functional protein (translation)[83]. Transcription initiation begins when the sigma factor of the RNA polymerase holoenzyme recognizes the promoter sequence. This interaction enables the specific binding of RNA polymerase to the DNA and formation of an open complex that allows transcription to proceed[84]. During transcription, DNA's genetic information is transcribed into messenger RNA (mRNA). Subsequently, the mRNA code is then translated into a protein sequence by the ribosome.

While bacterial cells strive to only express genes that are needed under a specific condition, some genes are required perpetually. These genes are constitutively expressed and usually encode essential enzymes required for housekeeping functions such as core metabolism or cellular maintenance[82]. The sigma factor that allows transcription of housekeeping genes is called housekeeping sigma factor[85]. Sigma factor 70 and Sigma A (SigA) are the housekeeping sigma factors in *E. coli* and *B. subtilis*, respectively[86]. Expression of all other genes is controlled by so-called alternative sigma factors, which recognize different promoter sequences. This system allows cells to initiate the expression of specific sets of genes in response to different types of stress[85].

Bacterial genes with related functions are often clustered into an operon that is controlled by the same regulatory mechanism (**Figure 3**)[87]. An operon is a sequence of genes that is read from the same promoter and transcribed into one polycistronic mRNA that encodes multiple proteins. Each coding sequence in a polycistronic mRNA has a separate translational initiation region containing a ribosomal-binding sequence (RBS) and its own start codon, to ensure the correct and simultaneous translation of each coding sequence[83]. As illustrated in **Figure 3**, an operon contains a promoter, a number of structural genes (*a*, *b*, *c*) that encode proteins (A, B, C) needed for a given process, and one or two operator sequences up and/or downstream of the promoter sequence. The expression of genes in this operon can be regulated in many ways.

Operators allow additional levels of gene regulation, e.g., in response to metabolic substrate or product concentrations. This regulation occurs through transcriptional regulators, that often possess a helix-turn-helix motif and bind to DNA[83]. These regulators can be repressors or activators and are modulated by small molecules, called inducers or corepressors[83]. Repressors bind to the operator region downstream of the promoter and prevent the initiation of transcription, while activators bind to the upstream of the promoter and activate the transcription process. Inducers can inactivate a repressor or activate an activator, resulting in de-repression or activation of gene expression, respectively. Corepressors do the opposite, activating a repressor or inactivating an activator. The example in **Figure 4** shows a repressor (O) that is inactivated by the inducer molecule P (de-repression, **Figure 4A**) and a repressor Y that is activated by its corepressor Z (**Figure 4B**). Induction of gene expression by de-repression (A) is common for catabolic processes where P is the substrate, while corepression (B) is common for anabolic processes, Y being the end product.

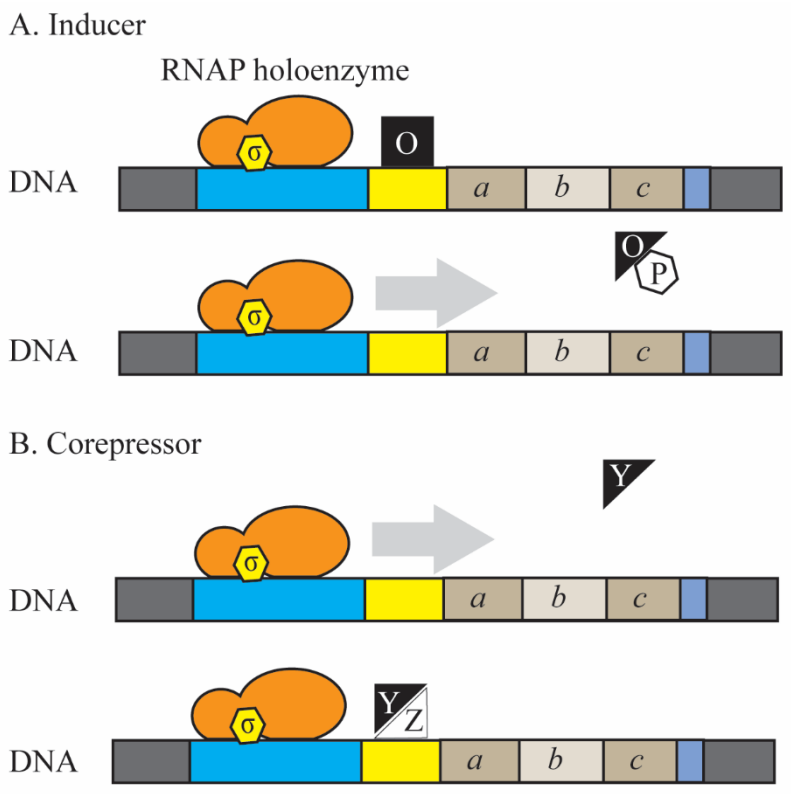

Figure 4: Schematic illustration of inducer and corepressor. The RNAP holoenzyme contain RNA polymerase and sigma factor (σ).

For adaptations that need exceptionally quick responses, it can be beneficial for cells to already possess an adequate amount of mRNA to allow rapid protein synthesis in response to physiological changes[83]. In such cases, the mRNA is kept 'inactive' through its secondary structure, usually a stem-loop structure, that prevents the ribosome from accessing its RBS. For example, *E. coli* utilizes RNA thermometers that directly sense a temperature increase through temperature-dependent destabilization of a stem-loop structure, allowing immediate access to the RBS upon temperature shock, and thus rapid synthesis of heat shock proteins. Such mechanisms that occur on RNA level after transcription is completed are called posttranscriptional regulation. Analogously, protein activities can be regulated through posttranslational protein modification (e.g. phosphorylation), protein turnover, and feedback inhibition. One common example for such a regulation mechanism are two-component systems (TCS), consisting of a sensor kinase and a response regulator. The sensor kinase senses a specific signal and responds by phosphorylating the response regulator, a transcriptional regulator that adapts gene expression to the present condition. For example, in *B. subtilis*, the TCS DesKR modulates the expression of the lipid desaturase Des in response to membrane fluidity changes[88–92].

4. Stress responses in *B. subtilis*

Fluctuating environmental challenges in its natural habitat have caused *B. subtilis* to evolve various survival strategies[80,93]. These survival strategies can be categorized into three major groups: (i) survival strategies with small-scale genetic changes, (ii) survival strategies with a large-scale genetic change, and (iii) survival strategies that require an immediate response.

For example, nutrient limitation will impair growth and cause cultures to enter stationary phase[94,95]. In some cases, small changes are sufficient to adapt, e.g., expression of a limited set of genes to exploit an alternative food source. If such a small-scale change in gene expression is not enough, larger regulons controlled by alternative sigma factors are activated. One example is the *B. subtilis* general stress response that is induced by a wide range of conditions comprising energy and nutritional stress (e.g. carbon, phosphate, oxygen starvation) as well as environmental and physical stress factors (e.g. salt, ethanol, heat, acid shock)[62,80,96]. In *B. subtilis*, the housekeeping sigma factor is SigA[97]. However, a range of alternative sigma factors are known to respond to different stress conditions (**Table 1**)[40,97].

The activity of alternative sigma factors is tightly controlled, most prominently by so-called anti-sigma factors. These are proteins that interact with alternative sigma factors and keep them in an inactive state. Upon sensing stress, a signaling cascade leads to the release of the alternative sigma factor to activate transcription. This posttranslational control allows a rapid activation of the appropriate stress response. One example for this regulation mechanism is the general stress sigma factor SigB[62,80,96]. Under unstressed conditions, SigB forms a complex with its anti-sigma-factor RsbW to prevent the activation of the regulon[98–101]. Additionally, the kinase activity of RsbW keeps the anti-anti-sigma-factor RsbV in its phosphorylated (inactive) state (RsbV-P)[98,102]. Upon stress, RsbV-P is dephosphorylated and the active anti-anti-sigma factor RsbV displaces SigB from the RsbW-SigB complex, leading to the activation of the general stress responses[103]. Depending on the type of stress, RsbV-P can be dephosphorylated by two different phosphatases, RsbU in response to environmental stresses[103–105] and by RsbP in response to energy stresses[106].

In addition to specific stress responses, cells in a *B. subtilis* population can differentiate into subpopulations of distinct cell types (‘differentiation states’). Such differentiation states include subpopulations that are, for example, genetically competent, motile, produce toxins to cannibalize their neighbors, form biofilms or spores[70,95] (**Figure 2**). Differentiation into such states can occur as a direct response to an environmental cue (e.g., biofilm) or randomly as part of a bet-hedging strategy (i.e., the expression of different phenotypes within one population even before the onset of stress conditions, as in motility), or sometimes a combination of the two (e.g., competence, cannibalism, sporulation)[70]. These different cell fates tend to be mutually exclusive and controlled by intricate regulatory networks that rely primarily on the activity of three major transcriptional regulators: Spo0A, DegU, and ComK (reviewed by Lopez *et al.*[95]).

Some stress responses require extremely quick adaptation and are therefore not activated by transcriptional regulation. One example is osmoadaptation during hypoosmotic

shock[107–109]. This response is mediated by MSCs, which are always present in the cell membrane[110,111]. These channels that are sensitive to mechanical-membrane stretch are closed under normal conditions. During a sudden shift from high to low osmolarity, water rapidly enters the cells and increases the turgor pressure threatening osmotic burst. MSCs detect these changes in turgor pressure through membrane stretch and allow the release of ions and osmolytes to restore osmotic balance[107–110]. This adaptation process occurs on a millisecond scale, allowing a much faster response than any transcriptionally regulated stress response could achieve.

Table 1: Summary of sigma factors in *B. subtilis*[40]. Abbreviations: ECF (extracytoplasmic function), AMP (antimicrobial peptide), SP (*subtilis* phage). #Asai *et al*. showed that *gsiB* is induced by both SigB and SigZ[112].

Sigma factors	**functions**	**ref**
SigA	major sigma factor controls expression of housekeeping genes, e.g., respiration genes *qcrABC*	113,114
SigB	general stress responses, e.g., general stress (*gsiB*#) or oxidative stress (*sodA*)	62,115,116
SigD	Flagella (e.g., *fla* operon), chemotaxis (e.g., *che* operon), motility, autolysis (e.g., *lytABC*)	117,118
SigE	early mother cell-specific sporulation, e.g., *spoVD*	119–121
SigF	early forespore-specific sporulation, e.g. *pbpG*	122,123
SigG	late forespore-specific sporulation, e.g. *spoIVB*	122
SigH	controls genes of the transition phase (sporulation, competence), e.g., *spo0A;* not fully active in lab strains due to a mutation	124
SigI	controls a class of heat shock genes, e.g., *mreBH, lytE*	125–127
SigK	late mother cell-specific sporulation, e.g., *cotA*	121,128,129
SigL	cold adaptation, utilization of arginine (e.g., *rocABC*), acetoin, and fructose	130–132
SigM	ECF sigma factor, responds to cell wall antibiotics, heat shock, osmotic, ethanol, acid, and superoxide stresses, e.g., *mreBCD-minCD* operon	133–135
SigN (ZpdN)	plasmid pBS32-encoded, available in ancestral *B. subtilis* strain 3610, unavailable in lab strains, induced cell death, e.g., *zpaABCD*	136–138
SigV	ECF sigma factor, resistance to lysozyme, e.g., *oatA*	139–141
SigW	ECF sigma factor, adaptation to membrane active compounds (e.g., *pspA*), synthesis and/or secretion of bacteriocins	23,134,142
SigX	ECF sigma factor, controls cell envelope modification processes (e.g., *pbpX*), responds to heat shock and cationic AMP, temporary phage resistance	134,143,144
SigY	ECF sigma factor, maintenance of the SP prophage that encodes sublancin, e.g., *ybgB*	134,145,146
SigZ	ECF sigma factor, role is unknown, proposed to control expression of general stress gene *gsiB*# and unknown function gene *yrpG*	112,134,146
Xpf	PBSX phage sigma factor, controls transcription of PBSX prophage genes, e.g., *xlyA*	147
YlaC	ECF sigma factor, responds to oxidative stress, e.g., *ylaABCD*	146,148,149
SigO-RsoA	composite sigma factor, responds to acid stress, e.g., *oxdC-rsiO* operon	150,151

5. Stress responses studied in this work

The bacterial cell envelope, i.e., the cell wall and cytoplasmic membrane in *B. subtilis*, is an essential structure that acts as the first line of defense against any external challenges[13–15]. It has a complex multilayered structure that gives shape to the cell, protects the cellular content from its environment, and keeps the cell intact. In nature, bacteria must compete with other species for survival[152]. Often, bacteria will kill their competitors by releasing antimicrobial compounds that target the cell envelope[7]. To survive threats against their envelopes, bacteria have evolved different strategies[7]. For instance, bacteria modify their cell wall and membrane structures to restrict the access of antimicrobials[8,9]. Gram-positive bacteria can change their overall cell envelope charge by D-alanylation of lipoteichoic acids in their cell wall, a strategy to repel positively charged compounds like cationic antimicrobial peptides (AMPs)[8]. Similarly, lysinylation of phosphatidylglycerol membrane lipids converts a negative to a positive charge, which is a key resistance mechanism to the lipopeptide antibiotic daptomycin[10]. These responses and other stress responses that enable cells to protect the integrity of their envelopes are referred as CESR[15,18].

B. subtilis has a highly differentiated and well-studied CESR[15,18]. The different CESR systems were initially identified based on -omics studies when *B. subtilis* was challenged by different cell wall antibiotics[23–25]. Thus, the CESR in *B. subtilis* was originally defined as all the regulatory systems that are involved in sensing and responding to the presence of cell wall antibiotics and other envelope perturbating conditions[18]. However, later studies have revealed connections of *B. subtilis* CESR to its cellular differentiation processes[134,153], leading to a broader definition of CESR that is not limited to antibiotics-induced stress responses anymore[15,18].

The *B. subtilis* CESR is orchestrated by extracytoplasmic function (ECF) sigma factors (**Table 1**) and two component systems (TCS), both systems containing a membrane-anchored sensory component and a cytoplasmic transcriptional regulator[18]. The sensory component senses the stress condition, leading to the activation of the regulator, thus enabling the expression of their target genes. In the case of ECF sigma factors, the anti-sigma factor is the membrane-anchored sensory component and the ECF sigma factor is the regulator[154]. In normal (unstressed) conditions, the ECF sigma factor is bound to the anti-sigma factor and thus inactive. Upon stress, the ECF sigma factor is released from the anti-sigma factor by a conformational change or proteolytic cascade[146], leading to gene expression. In case of TCSs, the sensor histidine kinases activate their respective transcriptional regulators through phosphorylation[155].

B. subtilis has seven ECF sigma factors: SigM, SigV, SigW, SigX, SigY, SigZ, and YlaC (**Table 1**)[146]. Three of them (SigM, SigW, SigX) are known as major ECF sigma factors involved in CESR[156]. The SigM regulon contains genes related to cell envelope homeostasis[133], SigW controls antibiotic resistance-related genes especially against membrane-active agents[157], and SigX plays a crucial role in modulating the overall net charge of the cell envelope[18,158]. Interestingly, a significant amount of genes are regulated by two or more ECF sigma factors[134], indicating that target gene expression by ECF sigma factors is not solely based on promoter

selectivity but that the expression timing of individual ECF sigma factor upon specific inducing conditions plays a crucial role[18].

In addition to these alternative sigma factors, four TCSs have been identified as part of the *B. subtilis* CESR[18]. Three of them (BceRS-BceAB, PsdSR-PsdAB, and YxdKJ-YxdLM) are classified as Bce-like systems, typically composed of a TCS (BceRS/ PsdSR/ YxdKJ) controlling expression of ABC transporters (BceAB/ PsdAB/ YxdLM)[15,18]. Each of these systems responds to AMPs, such as bacitracin (BceRS-BceAB, PsdSR-PsdAB)[15,18,159], lantibiotics (PsdSR-PsdAB)[159], and the human AMP LL-37 (YxdKJ-YxdLM)[25,159]. In contrast, the LiaRS system responds to a wider range of inducers including various types of AMPs that interfere with the lipid II cycle of cell wall synthesis, alkaline shock, and detergents[25,160]. The LiaRS system regulates the expression of the *liaIH* operon, which is thought to stabilize the cell membrane under stress[160]. Taken together, these systems have been proposed to be involved in the resistance to peptide-based antibiotics such as bacitracin[15].

In addition to these known CESR systems, the antibiotic stress response profiling studies of *B. subtilis* have identified several other marker proteins that appear to be commonly induced by cell envelope-targeting compounds[23,24,30–32,34–39]. Some of these marker proteins have unclear roles in CESR, but seems to be involved in antibiotic resistance[30,35,44]. In my thesis, I studied the biological contribution of four different stress responses to bacterial survival during antibiotic exposure: osmoadaptation by MSCs (**paper I, chapter 5.1**), membrane fluidity adaptation through the lipid desaturase Des (**paper II, chapter 5.2**), the putative ABC transporter of unknown function YtrBCDEF (**paper III, chapter 5.3**), and ROS-mediated killing by the potassium ionophore valinomycin (**paper IV, chapter 5.4**).

5.1. Osmoadaptation through MSCs

In nature, bacteria need to survive various challenges. For example, bacteria accumulate osmolytes such as amino acids, sugars, polyols, and many other soluble organic compounds to prevent cell dehydration during the dry season[109]. Upon heavy rainfall, a sudden shift from high to low osmolarity, causing rapid water influx, will increase turgor pressure and membrane tension. The increasing membrane tension triggers MSC gating, thus allowing the release of osmolytes to prevent cell lysis[161,162]. These channels are activated in response to mechanical membrane stretch and act as emergency-release valves to allow immediate relieve of turgor pressure[107,108,111].

Based on their conductance and sensitivity to membrane tension, bacterial MSCs are classified into three classes: mini (MscM), small (MscS), and large conductance (MscL)[111]. Structurally, MSCs are grouped into two categories, MscL and MscS-type channels[110]. MscL channels have moderately to highly conserved structure by forming homopentamers with a small cytoplasmic domain[161,163]. MscL channels are known to act as emergency release valves for potassium ions under high pressure conditions[164]. In contrast, MscS-type channels exhibit a broader structural diversity but typically form homoheptamers with a large cytoplasmic domain, which undergoes profound structural changes during channel opening[161,162,165,166]. This large cytoplasmic domain, also known as cytoplasmic chamber, acts as a molecular sieve that

balances the passage of positive and negative osmolytes, ensuring a net-neutral efflux to maintain the cellular membrane potential and retain valuable solutes[167,168]. Due to this larger domain, MscS channels are able to release larger osmolytes[162,164]. In bacteria, multiple MSCs with different properties are working together to allow a degree of temporal response to various level of hypoosmotic stress[110].

In addition to their role in hypoosmotic shock, MSCs appear to be crucial for the survival of pathogenic bacteria by facilitating the osmoadaptation of pathogens during the environment/host transition and during host colonization[169–173]. Hence, suggesting that these channels play an important role as virulence factors[110].

Furthermore, MSCs impact antibiotic susceptibility, whereby two opposite roles have been described[110]. Thus, MSCs may act as entrance gate for certain antibiotics, e.g., aminoglycosides, tetracycline, viomycin, and nifuroxazide[174,175]. Supporting this idea, these antibiotics have been shown to be more potent in the presence of MSCs[174–176]. In contrast, observations of *B. subtilis* treated with the membrane-targeting AMP MP196 suggested that MSCs may also play a role in antibiotic-stress adaptation to membrane-targeting antibiotics. In line, a MSCs deletion mutant was more sensitive to MP196[30]. This second phenomenon was named antibiotic-induced amino acid release (AIAAR) and is one focus of my work.

B. subtilis actively and selectively synthesized and released glutamate and aspartate through MSCs when exposed to MP196, showing that this is a dedicated stress responses and not simple leakage[30] (**Figure 5**). *B. subtilis* possesses one MscL-type (MscL, formerly YwpC) and three MscS-type channels (MscT formerly YkuT, MscY formerly YhdY, and MscC formerly YfkC)[109]. A quadruple mutant impaired in glutamate release was more sensitive to MP196, while supplementation of exogenous glutamate or salts (NaCl and KCl) reduced susceptibility to MP196[30]. This observation suggested that AIAAR involves osmotic stabilization rather than a specific effect of glutamate and aspartate[30]. The same response was also induced by gramicidin S, gramicidin A, aurein 2.2, and nisin[30], indicating that AIAAR is a common mechanism in response to membrane-active AMPs.

A similar response was also observed in other microorganisms[110]. Becker *et al.* and Wang *et al.* demonstrated that the industrial amino acid producer *Corynebacterium glutamicum* excretes glutamate through MscS-like channels (MscCG and MscCG2) in response to penicillin or other conditions that increase membrane tension and weaken the cell wall[177–179]. Despite its ability to transport aspartate and phenylalanine, MscCG prefers to transport glutamate[180,181], hence showing similar preferences like AIAAR in *B. subtilis*[30]. Interestingly, *E. coli* MscS reacts in a similar manner to treatment with penicillin[178] and the activity of ampicillin is reduced by D-glutamate supplementation[182]. These observations supported the idea that AIAAR is not limited to *B. subtilis* and AMPs.

While the role of MSCs during hypoosmotic stress has been studied extensively, the mechanisms underlying antibiotic-induced MSCs gating and amino acid release remain unclear. Moreover, their role in AIAAR raises the interest to explore their potential as novel-drug targets for antimicrobial combination therapy.

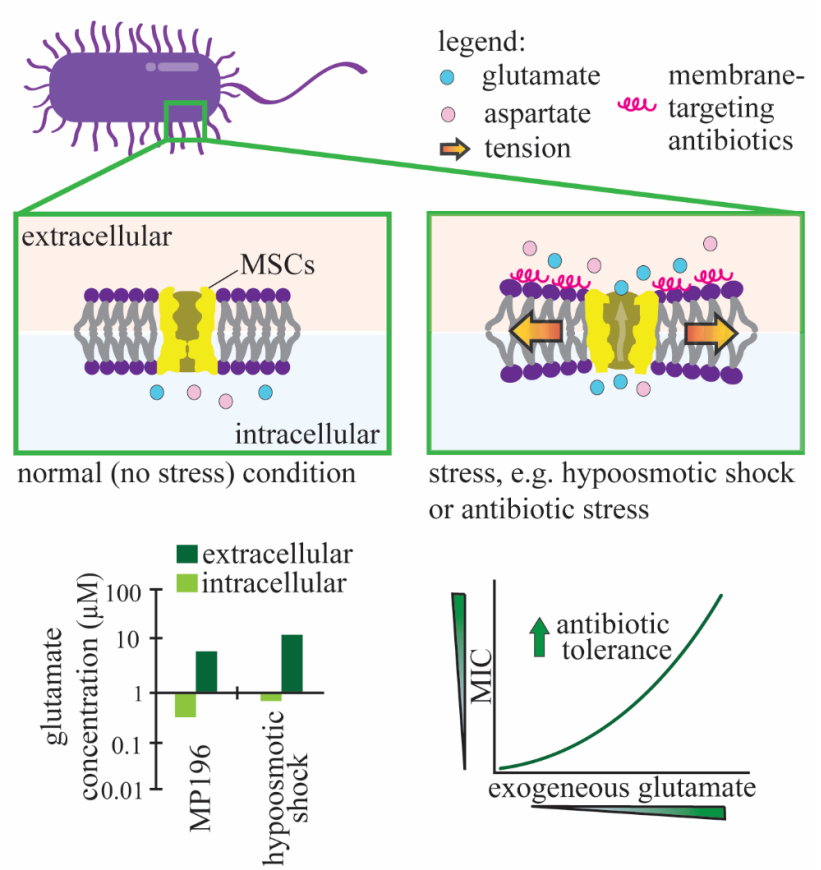

Figure 5: Illustration of AIAAR and its implications in antibiotic tolerance. Upon membrane-targeting antibiotic stress, *B. subtilis* synthesizes and releases glutamate to protect cells against antibiotics. Figures were drawn based on the findings in [30]. See text for full description.

5.2. Membrane fluidity adaptation through the lipid desaturase Des

Membrane fluidity describes the freedom of movement of constituents within the cell membrane[183]. It depends on lipid head group composition, fatty acyl chain length, saturation, branching, membrane protein content, as well as environmental factors, and correlates with membrane thickness[184–186]. For instance, a temperature decrease causes membrane rigidification and thickening[88,187]. Rigidification of the cell membrane limits the movement of constituents within the bilayer and disturbs cellular functions[188,189]. To maintain its membrane fluidity, *B. subtilis* possesses two major mechanisms: (i) the slower long-term adaptation of the overall content of fatty acids by *de novo* synthesis and (ii) the faster adaptation of the saturation level of fatty acids by desaturation of the fatty acyl chains of existing membrane lipids[88,89].

In this work, I focused on the regulation of lipid desaturation by the TCS DesKR, which controls expression of the lipid desaturase Des. DesK is the sensor histidine kinase. It comprises five transmembrane domains and a cytoplasmic kinase/phosphatase domain[90,188,190–194]. *In vitro*, DesK has been demonstrated to detect membrane thickness changes[191,195] and its autokinase activity increases in membranes with longer fatty acyl chains (increased rigidity/thickness)[90,191]. The transcriptional activator for desaturase expression, DesR[92,194], is phosphorylated and dephosphorylated by DesK in a temperature-dependent manner[192–194]. The *desKR* operon is located directly downstream of the desaturase gene *des* (**Figure 6A**).

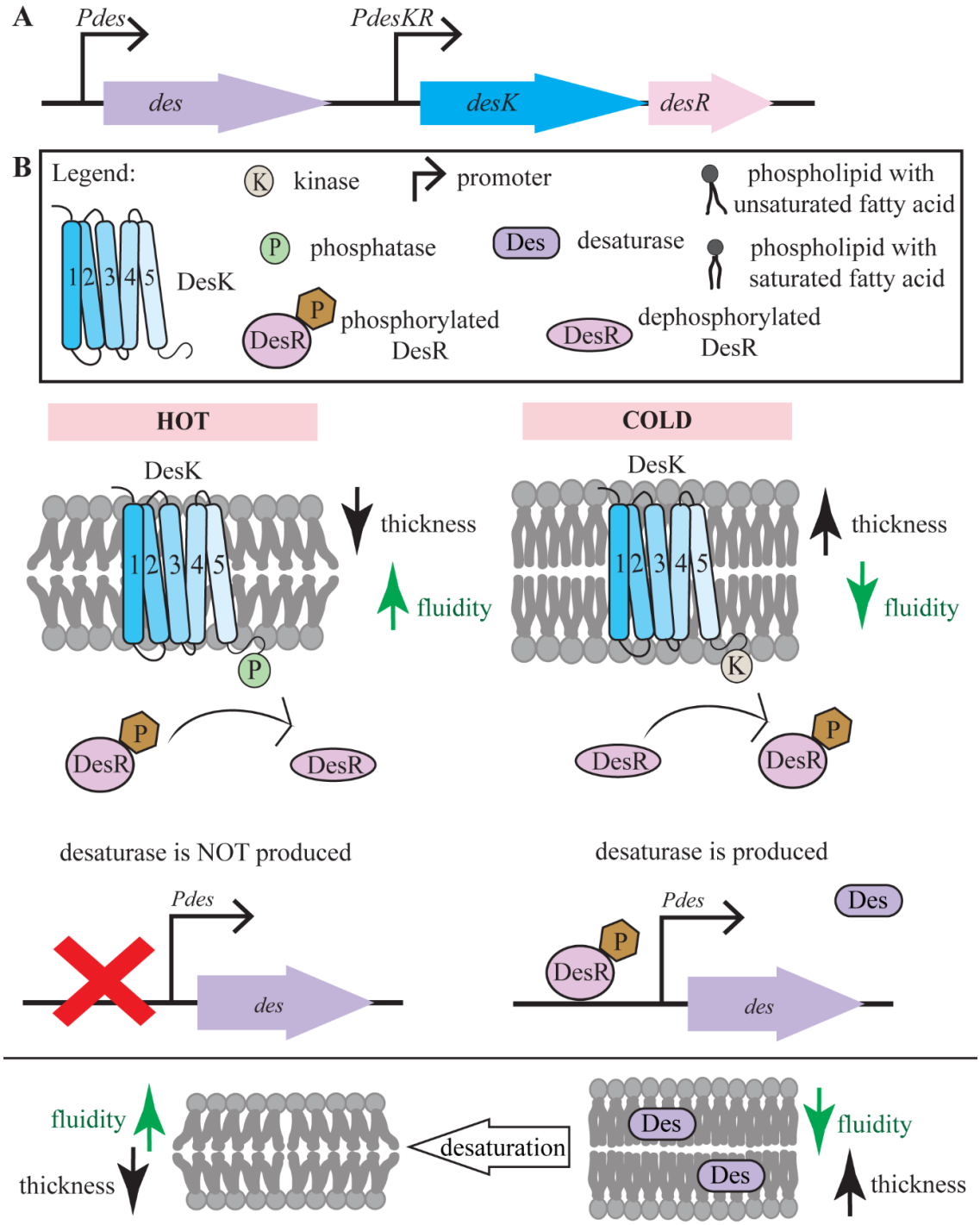

Figure 6: The *B. subtilis* Des system. **(A)** Genetic organization of the *desKR* operon. **(B)** Current model of cold sensing by DesK. Figure adapted from Sidarta *et al.*[196].

The current model of membrane thickness sensing by this system is as follows (**Figure 6B**)[92,192–194,197,198]. Cold shock rigidifies and thickens the cell membrane, causing DesK to phosphorylate DesR (P-DesR)[193,198]. P-DesR binds to the *des* promoter (*Pdes*), activating expression of the *des* gene[92]. Des then desaturates the fatty acyl chains of membrane lipids, resulting in membrane fluidization. As fluidity increases, the resulting unsaturated fatty acids decrease rigidity and concomitantly bilayer thickness. Once sufficient fluidization has been

achieved, a negative feedback loop activates the phosphatase-dominant state of DesK[192,194,197]. DesK then dephosphorylates P-DesR, and *des* transcription is stopped.

In addition to cold shock[199], the lipid desaturase Des has been reported to affect the activity of the lipopeptide antibiotic daptomycin[44]. This correlates with this compound's mechanism of action, which includes rigidification of the cell membrane[37]. Several other membrane-targeting antibiotics including AMPs have been shown to affect membrane fluidity[29,37,200–202] and membrane thickness[203–205], and for some they have been proposed to be crucial factors for their activity. Since membrane fluidity and thickness are correlated, these parameters are difficult to differentiate and currently there are no tools to do so in living bacterial cells. The proposed sensing mechanism of Des system[191,195] could form the basis for a new biosensor for membrane thickness.

5.3. The putative ABC transporter YtrBCDEF

Proteomic profiling studies have revealed many marker proteins that appear to be involved in CESR[30–32,34,35,37,39]. While the role of some of these markers is known, several others were poorly or not at all characterized. In this work, I focused on the *ytrGABCDEF* operon that encodes the putative ABC transporter of unknown function YtrBCDEF.

This operon is under SigA regulon[47] and contains seven genes that encode the unknown function transmembrane protein YtrG[38,40], the transcriptional repressor YtrA[38,47], and the putative ABC transporter YtrBCDEF[38,47,48,206] (**Figure 7**). YtrG has a single transmembrane domain that shares about 42.5% amino acid similarity with the first transmembrane segment of YtrF ($E=8^{-10}$), yet it is not predicted to be part of the ABC transporter[38,207–210]. YtrA belongs to the GntR family of transcriptional repressors that bind small molecule compounds. Its inactivation results in the constitutive expression of the *ytrGABCDEF* operon[38,47]. The putative ABC transporter YtrBCDEF consists of the peripheral ATP-binding proteins YtrB and YtrE, the transmembrane subunits YtrC and YtrD, and the extracytoplasmic substrate-binding lipoprotein YtrF[38,47,48,206]. While the biological role of this operon remains enigmatic, several hypotheses have been proposed.

-Antibiotic stress adaptation

The peripheral ABC transporter subunits YtrB and YtrE were found to be upregulated in *B. subtilis* treated with compounds that interfere with cell wall biosynthesis[30–32,34–37]. A similar induction pattern was observed on transcript level[38]. Several other studies have confirmed the induction of *ytr* genes in response to vancomycin[23], bacitracin[24], and plectasin[39] and the *ytr* promoter (*Pytr*) has been used as reporter for glycopeptides, including vancomycin[211,212] and ristocetin[211]. Taking together all published data, it seems that compounds binding to lipid-linked cell wall precursors (bactoprenol phosphate/pyrophosphate, lipid I/II) induce *ytr,* whereas compounds that interfere with cytosolic or extracellular steps of cell wall biosynthesis have no effect on the operon[23,24,30–32,34–39]. Thus, YtrBCDEF may play a role related to the lipid II cycle.

The notion that this transporter may be involved in antibiotic resistance stems from the finding that a Δ*ytrA* mutant, constitutively expressing *ytrGABCDEF*, is more tolerant to the acute effects of nisin[35]. This was supported by the presence of a MacB-like domain in the N-terminus of YtrF[48]. MacB domains are known for binding antibiotics[213], inspiring the hypothesis that YtrBCDEF could be an antibiotic exporter. However, antibiotics that induce the operon are structurally diverse (glycopeptides, lipopeptides, lantibiotics, defensins, and an atypical tetracycline), reducing the likelihood of this hypothesis.

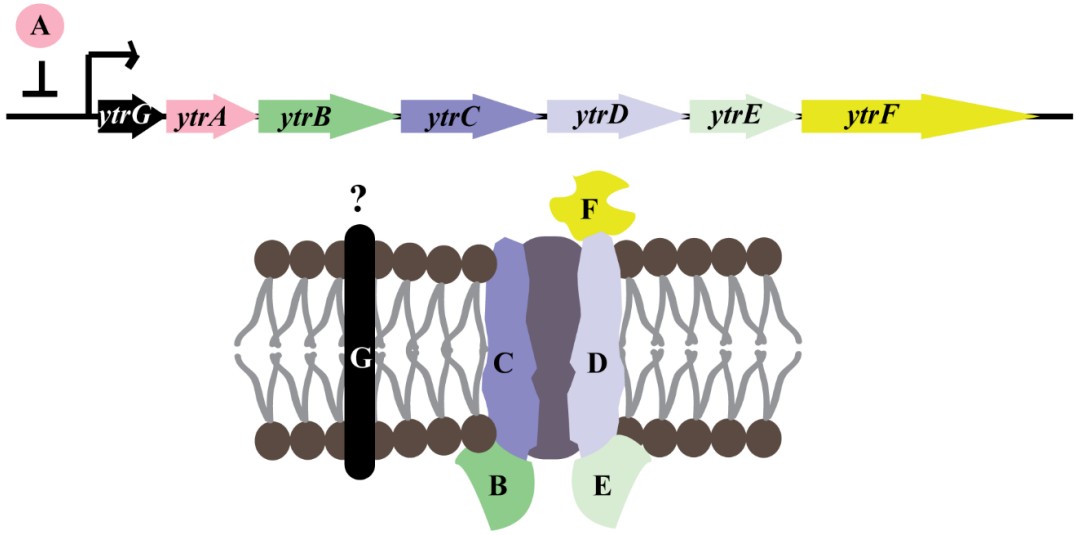

Figure 7: Genetic organization of the *ytr* operon and predicted subunits of the encoded ABC transporter. The function of the transmembrane protein YtrG is not known[40]. Image is adapted from [47,48]

-Cell wall synthesis and homeostasis

An alternative explanation for induction of the operon by lipid II-binding antibiotics could be that it is involved in or otherwise affects cell wall synthesis or homeostasis[41,47,48]. This hypothesis was explored by Benda *et al.*, who investigated the reasons behind the loss of genetic competence of a Δ*ytrA* mutant[41] by characterizing different *ytr* deletion mutants[48]. In addition to the constitutive expression of the operon (Δ*ytrA*), the constitutive expression of incomplete ABC transporter variants (Δ*ytrAB,* Δ*ytrAC*, Δ*ytrAD,* Δ*ytrAE,* Δ*ytrABE,* and Δ*ytrACD,* see also **Figure 14**) drastically reduced genetic competence[48]. This effect was attributed to decreased DNA uptake due to strongly increased cell wall thickness, which was observed for Δ*ytrA*, Δ*ytrAB*, and Δ*ytrAE* mutants using transmission electron microscopy. Additionally, the Δ*ytrA* mutant also showed altered biofilm morphology. None of these phenotypes was observed in the Δ*ytrGABCDEF* mutant[48].

The *ytr* operon also affects sporulation. Yoshida *et al*. observed that strains carrying a pMUTIN2 integration in either the *ytrA* promoter region (comparable to inactivation of the whole operon) or in *ytrF* sporulated less efficiently[47]. Koo *et al*. were unable to confirm this effect for a Δ*ytrF* deletion, but confirmed reduced sporulation in Δ*ytrA*[41].

Competence, biofilm formation, and sporulation can all be affected by a disruption of cell wall homeostasis[48,214–216]. It has been proposed that YtrBCDEF could be responsible for the import of a cell wall precursor or a signaling molecule involved in cell wall regulation. This

notion is supported by the presence of the extracellular substrate-binding protein YtrF, a feature typical for importers[48]. However, YtrF belongs to the ABC-4 integral membrane protein family, the same family as FtsX[40,48,217]. FtsX is part of the ABC transporter FtsEX that is essential for the autolysin activity of the peptidoglycan hydrolase CwlO during cell wall elongation[218]. Thus, it has been proposed that the YtrBCDEF could possibly play a role in autolysin regulation affecting cell wall turnover[48].

-Acetoin utilization

Early studies on the *ytr* operon have put forward the hypothesis that YtrBCDEF could be an acetoin importer[47]. Yoshida *et al.* observed that the *ytr* operon is expressed in early stationary phase and that its expression decreases in late stationary phase[47]. A mutant not expressing the *ytr* operon gave a decreased cell yield[47]. These two observations prompted the hypothesis that the *ytr* operon may be involved in the maintenance of stationary growth in a glucose-rich medium[47]. Exponentially growing *B. subtilis* cultures produce and secrete acetoin as a metabolic by-product of glucose consumption. To extend the active growth period after glucose has been exhausted, acetoin is reused as alternative carbon source[219]. Inactivation of the *ytr* operon did not affect acetoin production, but significantly slowed down its consumption[47]. Based on this observation, it was suggested that the *ytr* operon is involved in acetoin import during transition and early stationary phase[47].

-Cold shock

In addition to cell wall synthesis inhibitors, cold shock (from 37 °C to 18 or 15 °C) is a major inducer of the *ytr* operon[199,220]. Additionally, a *ytrA* deletion mutant shows reduced fitness when grown at 16 ºC[41]. These observations indicate a possible role of the *ytr* operon during cold shock. Interestingly, it has been reported that acetoin might act as a precursor for different cryoprotectants[220–222] and that cold shock activates cell wall autolysis in *B. subtilis*[223–225], tying this notion together with the hypotheses that the *ytr* operon may be involved in acetoin uptake and/or in cell wall homeostasis.

5.4. ROS-mediated killing by the potassium ionophore valinomycin

A bacterial cell membrane consists of lipids and proteins that together enclose the cytoplasm of the cell, protecting its cellular contents from the environment[16]. The hydrophobic lipid bilayer makes the cell membrane impermeable by preventing the diffusion of hydrophilic (water-soluble) molecules across the membrane. The presence of membrane proteins then allows the transport of specific molecules across this impermeable membrane. These properties make cell membranes act as a permeability barrier that controls the transport and distribution of molecules (such as ions) between the intra and extracellular space[16,226], thus contributing to the maintenance of the transmembrane electrochemical gradient (membrane potential), which powers many crucial cellular functions[16,17]. One of these functions is respiration (**Figure 8**).

Under aerobic conditions, *B. subtilis* has a branched respiratory chain comprising various cytochromes and terminal oxidases (**Figure 8**), thus allowing the cells to cope with variations in the environment[227–230]. *B. subtilis* membranes contain menaquinone-7[231] which can be reduced to menaquinol by three dehydrogenases: glycerol-3-phosphate dehydrogenase (Gdh), type II NADH dehydrogenase (Ndh), and succinate dehydrogenase (Sdh, complex II). The electron from menaquinol pool (Q) can then be transported to oxygen via two pathways, the cytochrome oxidase or the quinol oxidase. Following the cytochrome oxidase pathway, electrons from the menaquinol pool are transferred to the cytochrome *bc* complex (complex III) and then to the terminal oxidase cytochrome *caa₃* (complex IV). It has been hypothesized that the small *c*-type cytochromes c_{550} and c_{551} may mediate the electron transfer from cytochrome *bc* complex to *caa₃*, yet so far no conclusive evidence has been found supporting this hypothesis[227,229,232]. In the quinol oxidase pathway, menaquinol can also transfer the electrons to the quinol oxidases, cytochrome *aa₃* and *bd*.

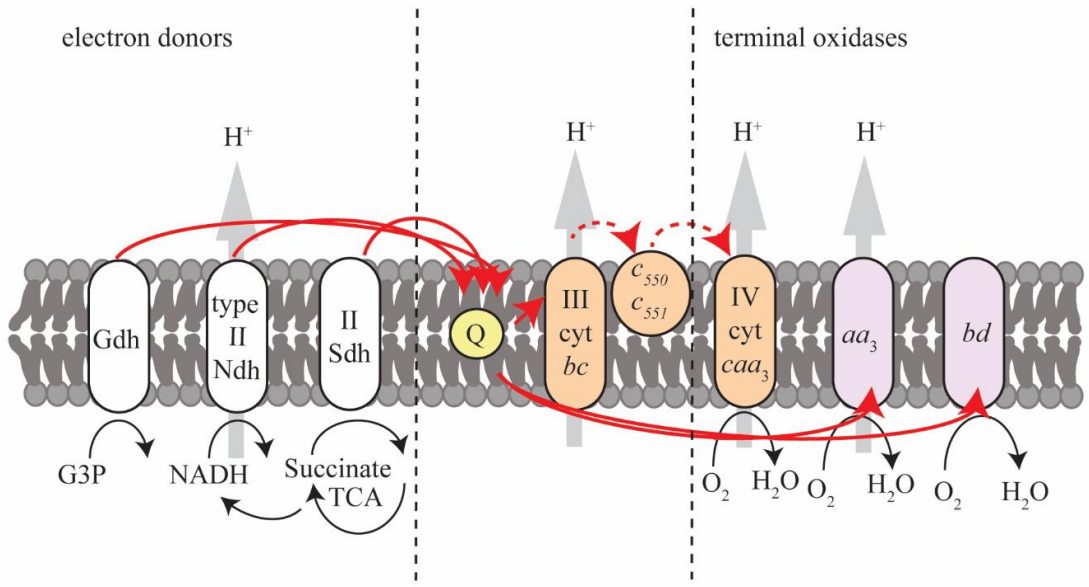

Figure 8: Schematic illustration of the respiratory chain in *B. subtilis*. The respiratory chain is divided into electron donors and terminal oxidases (marked by dashed line). The components of respiratory chain are colored based on the pathways: cytochrome oxidase (tan) and quinol oxidase (light purple). The components: the electron donors are glycerol-3-phosphate (G3P) dehydrogenase (Gdh), type II NADH dehydrogenase (type II Ndh), and succinate dehydrogenase (complex II, Sdh); menaquinol pool (Q); cytochrome *bc* complex (complex III, cyt *bc*); cytochrome c_{550} and c_{551}; the terminal oxidases are cytochrome *caa₃* (complex IV, cyt *caa₃*), cytochrome *aa₃* quinol oxidase (*aa₃*), and cytochrome *bd* quinol oxidase (*bd*). Electron transport is indicated by red arrows. Putative electron transport is indicated by dashed red arrows. Figure adapted based on the information from[28,227].

B. subtilis only needs one active quinol oxidase (cytochrome *aa₃* or *bd*) to grow aerobically and cytochrome *aa₃* acts as the main terminal oxidase during exponential growth[233,234]. Cells lacking cytochrome *aa₃,* grown in low oxygen content, or with a decreased ratio of NAD^+/NADH have a higher concentration of cytochrome *bd*[235]. Currently, the importance of cytochrome *bc-caa₃* complex (contain cytochrome *c*) in *B. subtilis* cell physiology is not fully understood. Cytochrome *c* appears not to be essential for growth or

sporulation as mutants lacking cytochrome *c* show no distinct phenotypes[229]. However, cytochrome *c* is repressed by glucose and induced at early stationary phase[233].

The electron transport chain (ETC) contributes to the formation of the transmembrane electrochemical gradient that determines the membrane potential and the proton motive force. Membrane potential and proton motive force are crucial for many cellular processes including adenosine triphosphate (ATP) synthesis[17,45]. Thus, disruption of the ETC will hamper multiple cellular processes. Additionally, incomplete reduction of oxygen to water results in the formation of ROS such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$)[46,236]. ROS can attack the metal centers, e.g., iron (Fe), zinc (Zn), and iron-sulfur (Fe-S) clusters of crucial metabolic enzymes[46]. In the case of Fe-S clusters, ROS oxidize the cluster to an unstable intermediate state which, will be degraded by the cell[237]. This degradation process releases Fe^{2+}, which together with hydrogen peroxide undergoes the Fenton reaction to produce hydroxyl radicals[46,237]. This highly reactive ROS then interacts with and causes damage to DNA, RNA, and proteins[238]. *B. subtilis* encodes several ROS detoxification enzymes to eliminate ROS[46]. For example, superoxide dismutase converts superoxide to hydrogen peroxide, which will be further converted into water and oxygen by catalase[239,240]. SodA and KatA are the major superoxide dismutase and catalase enzymes in *B. subtilis*[46,241]. To counter oxidative DNA damage, *B. subtilis* activates the SOS response, which is regulated by RecA and LexA, and induces many genes related to DNA damage repair mechanisms[242,243].

Several bactericidal antibiotics have been shown to generate lethal levels of ROS, mainly hydroxyl radicals generated via the Fenton reaction. This ROS production has been postulated as common antibiotic killing mechanism[244–247]. However, other studies could not confirm this correlation as bactericidal antibiotics are still killing bacteria under anaerobic conditions[248,249]. Further, mutants that are hypersensitive to ROS display similar antibiotic sensitivity as wild type cells[249,250]. While the broad hypothesis that ROS underlie killing by bactericidal antibiotics as a general mechanism can be seen as disproven, the contribution of ROS to killing by different antibiotics is still an open debate.

Proteomic profiling revealed that the superoxide dismutase SodA is induced in *B. subtilis* cells treated with the potassium ionophore valinomycin[45]. However, the reason behind this induction is unknown. Valinomycin has a highly specific mechanism of action with no known off-target activity and is thus often used as a tool to study the effects of membrane depolarization[27]. It is not clear how this specific mechanism would be bactericidal as removal of the ionophore should allow eventual recovery of the proton gradient. However, this is not the case[28]. Furthermore, valinomycin kills non-growing cells with lower metabolism that require less energy than growing cells. Generation of ROS through the electron transport chain could explain these observations. However, it has been proposed that membrane depolarization is not expected to induce ROS as it reduces electron transfer during respiration[251].

6. Original work

Using *B. subtilis* as model, my PhD projects were focused on four systems that were chosen based on the previous proteomic profiling studies of antibiotic-stressed *B. subtilis*[30–32,34,35,37,39] and previous reports related to their antibiotic susceptibility[30,35,44].

Thus, the proteomic stress response to MP196 revealed upregulation of proteins involved in amino acid synthesis, especially of glutamate and aspartate[30]. Further investigation demonstrated that cells actively synthesized and released these amino acids through MSCs when exposed to MP196[30]. These amino acids appeared to provide osmotic stabilization, mitigating the effects of MP196 and other membrane-active AMPs[30]. These findings formed the basis of my study on the role of this mechanism (AIAAR) in antibiotic survival (**paper I**).

In the current model of MSC gating, the force from membrane lipids (membrane stretch) pulls the channel apart to open[252,253]. This gating mechanism depends on the direct interaction of membrane lipids with the channel proteins as well as the overall membrane composition and organization, including membrane fluidity and thickness[252,254–258]. Postulating that AIAAR is mediated by an antibiotic-induced membrane stretch resulting in thinner membranes, I tried to develop a membrane thickness sensor using the DesKR system. The observations that DesK senses the temperature-induced changes of membrane thickness *in vitro*[191,195] and a *des* deletion mutant was described to be more sensitive to daptomycin[44], formed the idea that the Des system could be used as such a sensor (**paper II)**.

Proteomic profiling studies also revealed upregulation of the *ytrGABCDEF* after treatment with cell wall synthesis inhibitors[30–32,34,35,37,39]. Yet, the function of this putative ABC transporter remains unclear[38,47,48,206]. Following the observation that the constitutive expression of the operon affects cell wall thickness[48] and nisin sensitivity[35], I aimed to characterize its biological role with regard to antibiotic stress adaptation as well as cell wall synthesis and homeostasis (**paper III**).

The potassium ionophore valinomycin is known for its specific and singular mechanism of action, namely membrane depolarization[259–261], making it a common control to distinguish membrane potential effects from other mechanisms[27]. Yet, it is capable of killing metabolically inactive non-growing cells[28]. Proteomic profiling studies identified the superoxide dismutase SodA as marker protein for valinomycin[45]. These observations prompted the hypothesis that valinomycin-induced membrane depolarization promotes the formation of ROS, possibly superoxide. In **paper IV** my collaborators and I investigated the connection between membrane depolarization by valinomycin and generation of ROS.

In all four projects, the respective stress responses were assessed for their potential as antibiotic potentiator targets in addition to the basic research questions regarding their functions.

6.1. Antibiotic-induced amino acid release (AIAAR)

In this work, I aimed to further characterize the biological role of AIAAR for antibiotic survival and evaluate its suitability as an antibiotic potentiator target.

One major fundamental question was which MSCs contribute to AIAAR. It has been previously shown that AIAAR is limited in a *B. subtilis* mutant devoid all four known MSCs (SMB80)[30], but whether that effect is due to one specific MSC or all four channels was unclear. Using amino acid analysis of single MSC deletion mutants, we could confirm that indeed each of the known MSCs contributes AIAAR (**paper I Figure 2**). Intriguingly, AIAAR was not completely abolished in the quadruple MSCs mutant and could be further suppressed by addition of gadolinium chloride, a known inhibitor of MSC opening[262,263] (**Figure 9A**). These results suggested the presence of an additional MSC in *B. subtilis*. Using bioinformatic analyses, I identified the protein YgxB as a new potential MSC in *B. subtilis*. Its role as MSC and its involvement in AIAAR were then experimentally confirmed by assessing MP196-triggered glutamate release in a quintuple mutant devoid of all MSCs including YgxB (MS6). This strain released even lower levels of glutamate, which could be entirely suppressed by gadolinium chloride (**Figure 9B**), suggesting a possibility of yet another unknown MSC contributes in AIAAR. The idea that MSCs are the sole channels involved in AIAAR is in line with studies done on *C. glutamicum* reporting that glutamate is solely excreted through MSCs[177–179].

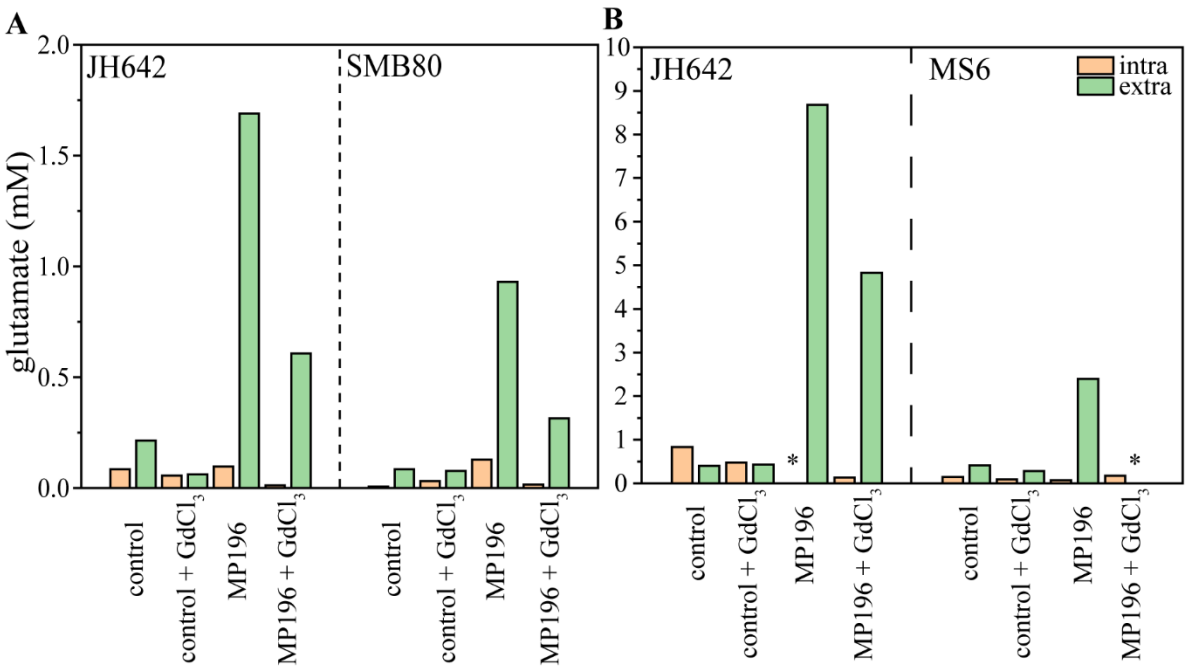

Figure 9: Intra- and extracellular concentrations of glutamate in the wild type JH642, quadruple (SMB80), and quintuplet MSC mutant (MS6) with and without pre-incubation with 20 mM mechanosensitive channel inhibitor gadolinium chloride ($GdCl_3$). Asterisk (*) indicates below detection limit. The glutamate was measured by HPLC in graph (A) and by commercial glutamate assay kit in graph (B).

The question remains how antibiotics trigger MSC opening. Previously, it has been shown that AMPs induce AIAAR[30]. Membrane-active AMPs are known to delocalize membrane proteins, either by depolarization or domain formation[30,37,202,264–267]. We hypothesized that such effects could mimic the membrane stretch that is required for MSC gating. To assess if antibiotic treatment affects the localization of MSCs, we constructed different *B. subtilis* strains expressing MSCs (MscL, MscT, MscY, MscC, YgxB) fused to GFP

under a xylose-inducible promoter. Indeed, all five MSCs changed their localization patterns after treatment with MP196. Specifically, they accumulated in GFP foci, indicative of transition into fluid membrane domains (**Figure 10**). Such fluid domains are also thinner as they are preferentially labeled by fluorescent reporters with short fatty acyl anchors[37,268,269]. Thus, transitioning into more fluid and thinner membrane regions may mimic the membrane stretch that underlies channel opening under hypotonic shock[110,252,254,255].

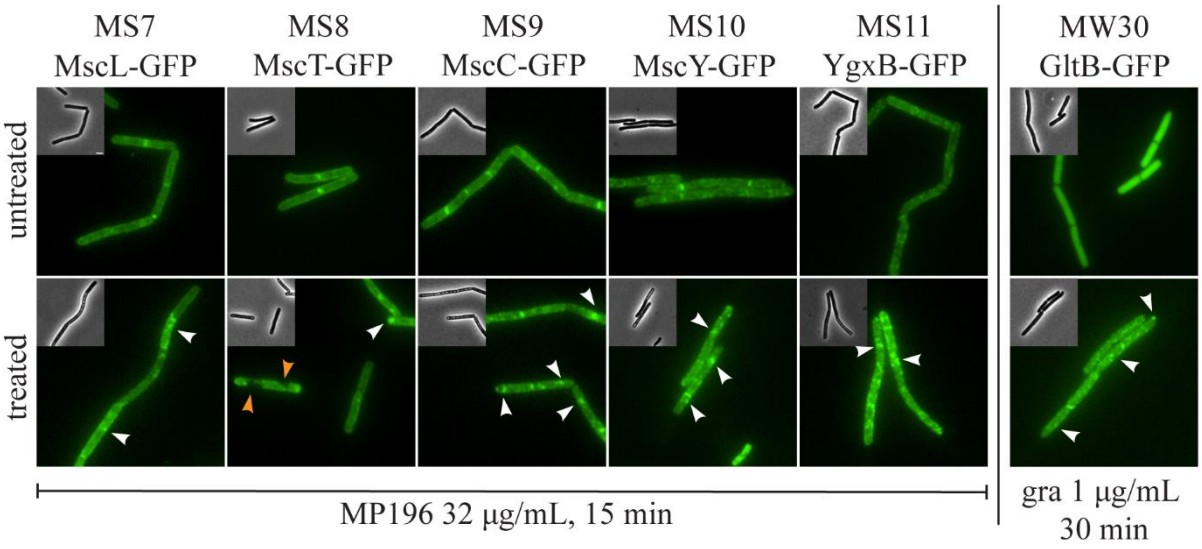

Figure 10: Localization of MSCs and GltB under antibiotic stress. *B. subtilis* MS7 (MscL-GFP), MS8 (MscT-GFP), MS9 (MscC-GFP), MS10 (MscY-GFP), MS11 (YgxB-GFP), and MW30 (GltB-GFP) were grown at 37 ˚C until an OD_{600} 0.3 and treated with 32 µg/mL MP196 or 1 µg/mL gramicidin (gra). Scale bar represents 2 µm. White arrowheads indicate GFP clusters. Orange arrowheads indicate domains that are void of GFP.

Previously, the normally cytosolic glutamate synthase accumulated in membrane fractions of MP196-treated cell[30], suggesting the possibility of these proteins being tethered to the membrane under stress conditions. In my work, the glutamate synthase GltB was shown to be localized in membrane-associated foci after treatment with gramicidin (**Figure 10**). Interestingly, this effect seemed to be specific for glutamate synthase as both cytosolic GFP and the proline synthase ProA exhibited normal cytosolic localization under similar stress (**paper I Figure 8**). While we cannot yet explain how this observation is functionally connected to MSCs and AIAAR, this specificity certainly warrants closer inspection of this phenomenon.

It was shown previously that the MSC quadruple mutant is more sensitive to MP196 and that addition of exogenous glutamate reduces MP196 activity[30]. These observations prompted the hypothesis that AIAAR could be a possible target for antibiotic potentiators. To further evaluate this, we first analyzed the amino acid profiles of different pathogenic species treated with the membrane-active antibiotic squalamine. Indeed, AIAAR was conserved among both Gram-positive and Gram-negative bacteria (*S. aureus*, *E. coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*) as well as yeast (*Candida albicans*) (**paper I Table 1**). While Gram-positive bacteria predominantly released glutamate, Gram-negative bacteria released high amounts of a non-proteinogenic amino acid. Based on the HPLC retention time[270], this non-proteinogenic amino acid was narrowed down to be citrulline. Citrulline and glutamate

metabolism are closely linked, since glutamate can be metabolized to ornithine and further to citrulline. Thus, it is likely that a similar process is taking place in Gram-negative bacteria, but that either citrulline is preferred for release or that it is produced as overflow metabolite after upregulation of glutamate production and is simply more abundant at the timepoint of sampling. Despite this difference, the broad conservation of AIAAR suggests that it could be used as a potentiator target in various microbial pathogens.

AIAAR has previously been shown to be induced by cationic AMPs and beta-lactam antibiotics[30,177–181]. Here, an extended list of antibiotics was tested, including different structural and mechanistic antibiotic classes. Regardless of their specific target or mechanism, AIAAR was induced by a broad range of membrane-targeting compounds that destabilize and/or deform the cell membrane (**Table 2,** see **paper I Tables 2** and **S1**). This broad range of AIAAR inducers suggests that MSCs can be used as potentiator targets for combination with most membrane and at least some cell wall-active compounds. Interestingly, cells were less susceptible to compounds that trigger AIAAR by several orders of magnitude or even became entirely resistant, on agar plates (**paper I Figure 1**). It was then hypothesized that the limited diffusion of released amino acids in solid medium creates a much higher local concentration surrounding the bacterial cell, which osmotically protects the colony from antibiotics. Supporting this hypothesis, the glutamate-auxotroph *E. coli* strain PA340 experiment (**paper I Figure S5**) proved that (i) AIAAR indeed happens on solid growth medium and (ii) diffusion of glutamate released from AIAAR is indeed limited.

Table 2: List of compounds that triggered AIAAR in our experiments.

antibiotic	class	target	response
untreated	-	-	no
osmotic downshift	-	-	yes
MP196	cationic AMP	membrane deformation	yes
gramicidin S	cyclic AMP	membrane disruption	yes
gramicidin A	α-helical AMP	K^+/Na^+ transport	yes
aurein 2.2	α-helical AMP	membrane integration	yes
squalamine	aminosterol	unknown, integrates in membranes	yes
monensin	non-peptide ionophore	Na^+/H^+ transport	yes
calcimycin	non-peptide ionophore	$Ca^{2+}/Fe^{2+}/Mn^{2+}$ transport	yes
nisin	lantibiotic	blocks lipid II synthesis, forms pores	yes
lysozyme	protein	cell wall digest	yes

Overall, this study adds new perspective to the AIAAR phenomenon by showing that AIAAR is conserved in many pathogens and renders most membrane-targeting antibiotics ineffective. The fact that MSCs have been shown to be crucial for pathogen infection[169–173] and for antibiotics susceptibility[30,174–176], highlights their potential as antibiotic potentiator targets for combination therapy approaches[110]. Bacterial MSCs are conserved and structurally distinct from mammalian's channels[271,272], thus allowing the development of selective inhibitors. However, there are currently not many studies working on developing MSCs inhibitors[110], making them an attractive new drug target to be explored more thoroughly in the future. AIAAR is a particularly efficient protection strategy on solid medium, suggesting that it may

have particularly impactful applications in the treatment of skin and soft tissue infections as well as biofilms.

6.2. Membrane fluidity adaptation through the lipid desaturase Des

AIAAR has been hypothesized to be mediated by antibiotic-induced membrane stretch, which affects membrane fluidity and thickness. While membrane fluidity can be measured in living bacteria with different tools, the intimate link between fluidity and thickness makes it difficult to distinguish between the two. In this paper, the potential of the Des system as a specific membrane thickness reporter was explored.

We designed three assays based on the Des system (**Figure 11**): (i) activation of *Pdes* as reporter for membrane rigidification/thickening, (ii) localization of DesK as proxy for rigidified/thickened membrane domains, and (iii) antibiotic sensitivity of deletion mutants of *des*, *desK,* and *desR*[41] to indicate whether rigidification/thickening plays a role for the compound's activity. These assays were then tested under different temperature shift and antibiotic stress conditions. For temperature shift, cells were first grown at 37 °C then shifted to 25 °C, 16 °C, or 4 °C. For antibiotic stress, cells were treated with the membrane-rigidifying antibiotics daptomycin, valinomycin, nisin, and cWFW[200,266,273]. As controls, fluidizing conditions such as temperature shift to 50 °C, the fluidizing compounds benzyl alcohol, and the proton ionophore CCCP were included. The cell wall synthesis inhibitor vancomycin, which does not affect membrane fluidity, was used as additional control.

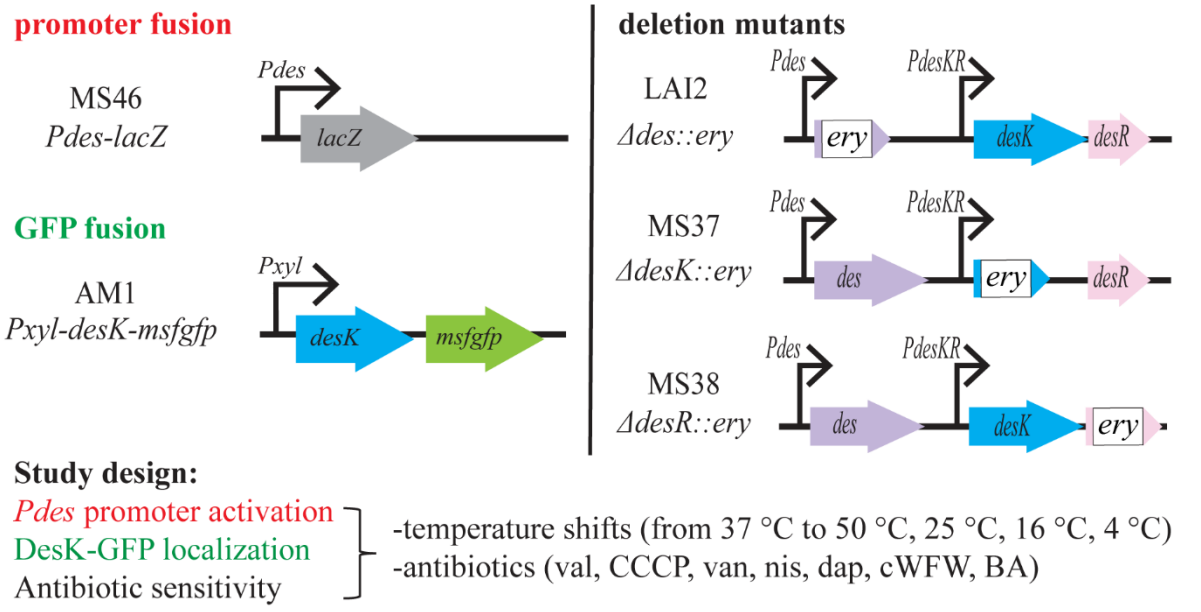

Figure 11: Study design and reporter strains used in this work. Strains are color-coded based on the experiment design: red for promoter activation, green for GFP localization, and black for antibiotic sensitivity. Gene deletions were based on strains published by Koo *et al.*[41], constructed by replacement of the deleted gene with an erythromycin resistance cassette keeping only the start and stop codons of the original gene. Abbreviations: nisin (nis), vancomycin (van), carbonyl cyanide m-chlorophenyl hydrazone (CCCP), cRRRWFW (cWFW), benzyl alcohol (BA), daptomycin (dap), valinomycin (val), erythromycin (ery), monomeric superfolder green-fluorescent protein (*msfgfp*). Figure adapted from Sidarta *et al.*[196]

To ensure the suitability of the chosen conditions to assess the potential of the Des system as reporter, I measured the membrane fluidity changes caused by these conditions using 2-dimethylamino-6-lauroylnaphtalene (laurdan), a fluorescent membrane dye that shifts its emission peak depending on the amount of water molecules surrounding the probe[274]. This shift allows calculation of generalized polarization (GP) values, which are indicative of membrane fluidity in terms of head group and fatty acyl chain spreading[269]. As shown in **Figures 12A-B (**see **Paper II Figure 2** for complete results**)** the selected conditions changed membrane fluidity to different extents, except for temperature shift to 25 ˚C. Thus, the test conditions allowed thorough probing of the capacity of the Des system as reporter for membrane thickness.

Once the test conditions had been established, the activation of *Pdes* as reporter for membrane rigidification/thickening was assessed. Using strain MS46 (*Pdes-lacZ*)[194] (**Figure 11**) and the *o*-nitrophenol-β-D-galactoside (ONPG) assay to measure the beta-galactosidase activity, I only observed a significant activation at 120 min post shifting cultures to 25 ˚C. Yet, there was no significant promoter activation at earlier time points or with harsher cold shock conditions (16 °C and 4 °C). This was surprising considering that (i) a shift to 25 °C did not significantly alter membrane fluidity, not even after 120 min (**Figure 12A**), and (ii) a shift to 16 °C is a commonly used condition to induce cold shock in *B. subtilis* and has been shown to activate *des* expression and DesK autophosphorylation in other studies[191,195,199,220]. Similarly, no antibiotic stress condition led to a significant induction of the *Pdes* promoter (**Figures 12C-D**, see complete result in **paper II Figure 3**). Thus, *Pdes* activation was not a reliable reporter for antibiotic-induced fluidity changes. Furthermore, these observations suggested that the Des system may not be acting as fast as previously assumed and that this system senses much more subtle membrane fluidity changes than those induced by antibiotics that are too small to be detected by laurdan GP measurements.

Next, I examined whether DesK localization could be used to visualize rigidified/thickened membrane domains in *B. subtilis*. Fluorescent membrane dyes typically have a preference for the fluid phase and some of them can serve as reporters to a certain degree, but there is no reliable proxy with a preference for the more rigid phase[274]. Membrane proteins usually display similar behavior[29,37,201,202]. Yet, the proposed sensing mechanism of DesK suggests that this protein should not partition into the fluid phase but rather into the rigid phase, or possibly be unaffected by phase separation. To examine this, strain AM1 (*Pxyl-desK-msfgfp)* (**Figure 11**) was stained with the fluorescent membrane dye 9-diethylamino-5H-benzo[α]phenoxazine-5-one (Nile red), which stains cell membranes uniformly under normal culture conditions but partitions into fluid domains upon phase separation[274].

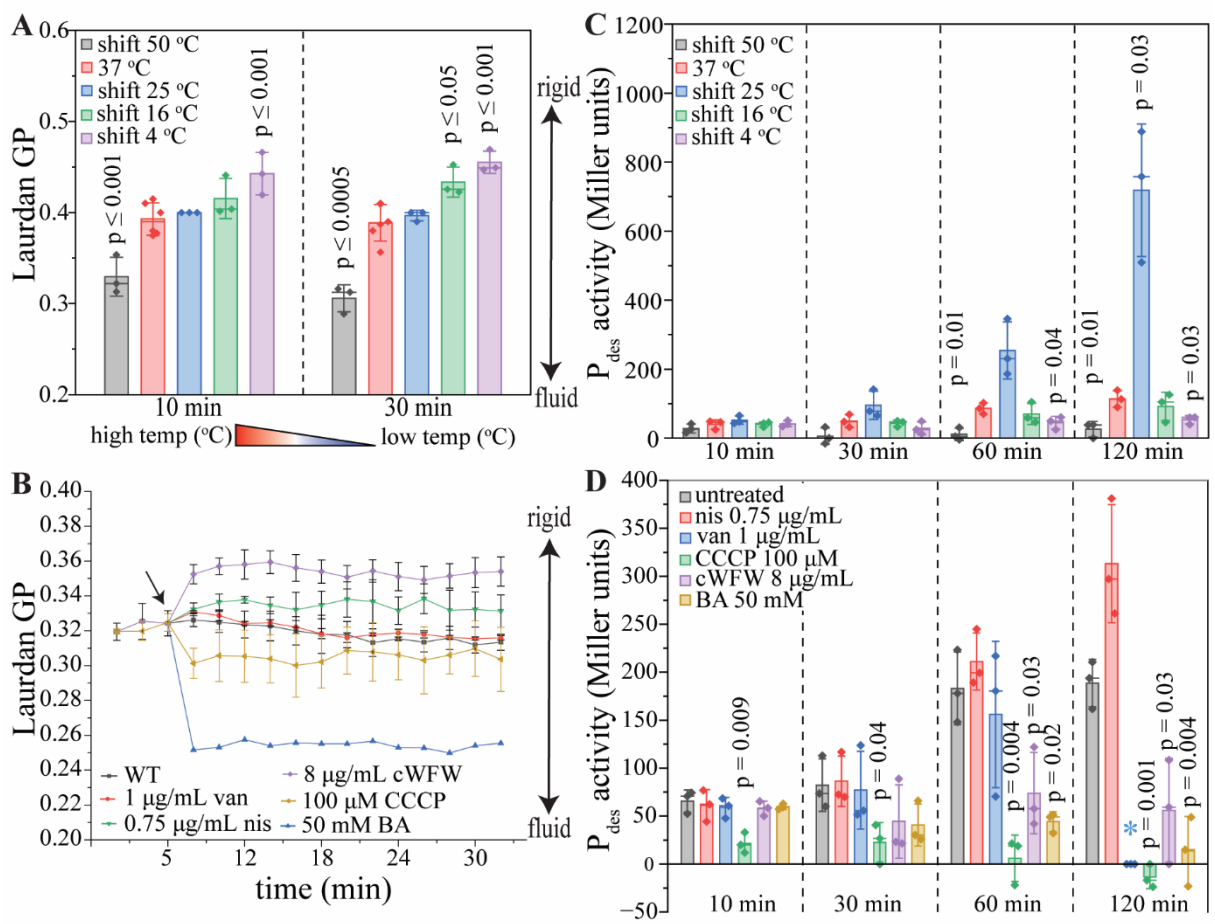

Figure 12: Membrane fluidity measurements using laurdan generalized polarization (GP) **(A-B)** and promoter activation of *Pdes* using ONPG assays **(C-D)**. An increase in GP indicates membrane rigidification, a decrease fluidization. *B. subtilis* 168CA was grown at 37 °C until early log phase (OD_{600}=0.3) prior to **(A)** temperature shift or **(B)** antibiotic addition. Arrows indicate time points of antibiotic addition. *B. subtilis* MS46 (*Pdes-lacZ*) was cultured at 37 °C until early log phase (OD_{600}=0.3) prior to **(C)** temperature shift or **(D)** antibiotic addition. Statistical significance was determined using two-tailed, homoscedastic t-tests **(A)** or heteroscedastic t-tests **(C-D)**. Only significant p values (≤0.05) are indicated. Blue asterisks indicate conditions, for which no data could be obtained due to cell lysis. Significance was tested between the 37 °C or untreated sample and the shifted or antibiotic samples. Abbreviations: nisin (nis), vancomycin (van), carbonyl cyanide m-chlorophenyl hydrazone (CCCP), cRRRWFW (cWFW), benzyl alcohol (BA). Figure adapted from Sidarta *et al.*[196]

Under control conditions, DesK localized in the cell membrane. However, after different temperature shifts, DesK-GFP clustered into membrane foci. All temperature downshifts except 25 °C induced Nile red clusters, indicating the formation of fluid membrane domains due to phase separation (**Figure 13**, full result at **paper II Figure 4**). Surprisingly, DesK-GFP foci overlapped with these domains, suggesting that it partitions into the fluid phase. Supporting this observation, DesK-GFP also preferred fluid regions upon antibiotic treatment (**paper II Figure 5**). Thus, DesK localization was also not a suitable reporter for antibiotic-induced membrane fluidity or thickness changes. Intriguingly, I noticed that all conditions that were expected to induce *Pdes* but failed to do so triggered phase separation (**paper II Figure S24**). This observation suggested that membrane thickness sensing by DesK is impaired by phase separation due to partitioning of DesK into the fluid phase.

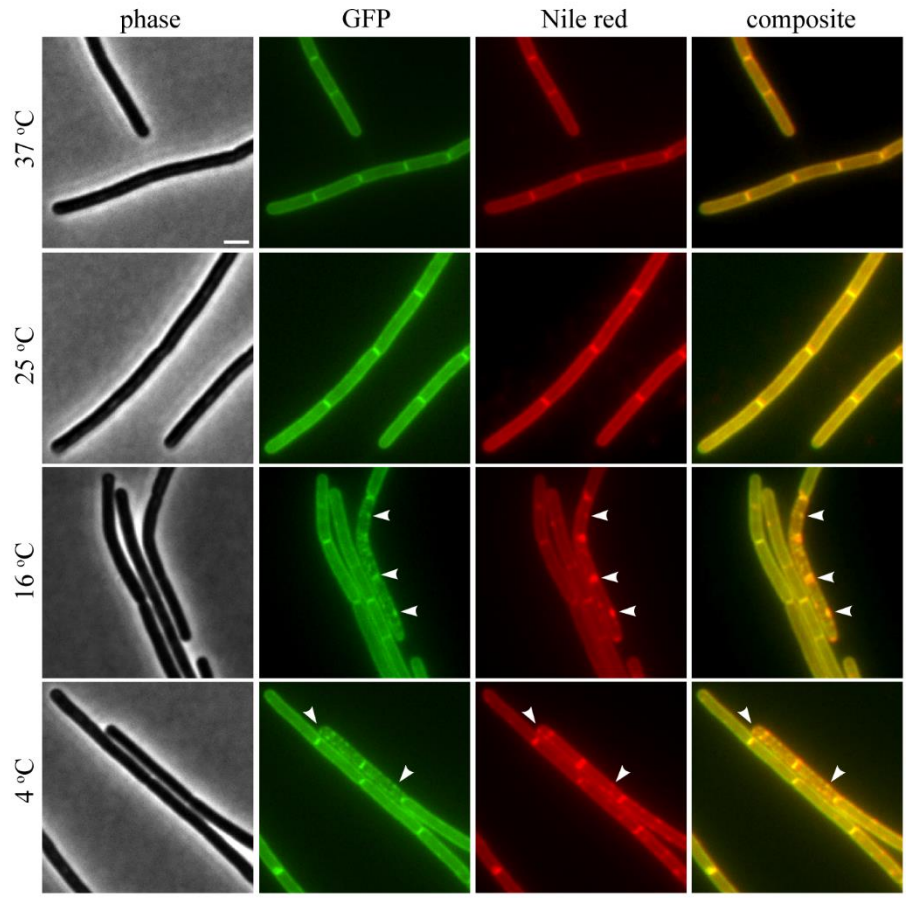

Figure 13: Localization of DesK after temperature shifts. *B. subtilis* AM1 (*Pxyl-desK-msfgfp*) was grown at 37 °C until an OD_{600} of 0.3 and subsequently shifted to the indicated temperatures. Cells were stained with Nile red for 5 min prior to microscopy. Scale bar represents 2 µm. Arrows indicate GFP clusters overlapping with Nile red foci. Figure adapted from Sidarta *et al.*[196]

A previous study showed that a strain lacking the desaturase Des was slightly more susceptible to daptomycin at 24 °C[44]. This study sparked the question if deletion mutants of the Des system are generally antibiotic-sensitive, and thus possible potentiator targets (**paper II Figure 1C**). However, none of the tested deletion mutants displayed increased sensitivity towards any of the tested compounds including daptomycin. Taken together, it appears that the Des system does not play a role in adaptation to antibiotic stress. Consequently, it was dismissed as candidate target for antibiotic potentiators. Additionally, I measured the laurdan GP of the wild type and the deletion mutants and did not observe any notable difference in membrane fluidity at any tested condition (**paper II Figure 7**), suggesting that the fluidity adaptations carried out by Des are too subtle to be detected by laurdan. Similarly, I could not observe any growth defects of the mutants at 37 or 24 °C (**paper II Figure S8-21**).

Overall, we were unable to prove the suitability of the Des system as a reporter for antibiotic studies. One possible reason is simply that we still have a very limited understanding of how this system works. For instance, the Des system has been categorized as a fast reacting "emergency fluidization response" that modifies the existing membrane lipids in response to cold shock. However, our results showed that the Des system is reacting much more slowly (120 min) than expected. Similarly, our initial hypothesis was that the Des system will generally

react to rigidifying conditions. Yet, it appeared to be only activated by a very subtly rigidifying condition (25 °C) and not under harsher conditions. This prompts the question whether the Des system is activated as previously described. Indeed, there seems to be more to the puzzle. For example, DesK has been reported to detect pH changes and is rendered inactive at low pH regardless of temperature[275]. In line with this, the sensing of DesK seems to be impaired by phase separation as it transitions into the fluid phase. Based on our and others' results, we hypothesized that this system is activated by minor changes in membrane fluidity/thickness while harsher conditions will be counteracted by other means of fluidity adaptation, for example, *de novo* synthesis of branched and short-chain fatty acids. In fact, branched-chain fatty acid composition can change considerably within only one doubling time (30 min)[29], suggesting that *de novo* synthesis is acting faster than previously thought and faster than the Des system. None of the deletion mutants showed any fluidity adaptation or growth defects in our assays, challenging the importance of the contribution of this system to membrane fluidity adaptation in *B. subtilis*.

6.3. The putative ABC transporter YtrBCDEF

The *B. subtilis ytrGABCDEF* operon encodes the putative ABC transporter YtrBCDEF, its transcriptional repressor YtrA, and the small transmembrane protein YtrG (**Figure 7**)[38,40,47,48,206]. This operon is induced by inhibitors of the membrane-bound lipid II cycle[23,24,30–32,34–39], cold shock[41,199,220], and during exponential to stationary phase transition[47,199]. It has been implicated in antibiotic stress adaptation[35], cell wall synthesis[41,47,48], sporulation[41,47], biofilm formation[48], competence[41,48], and acetoin utilization[47] (**paper III Table S3**). However, its precise function remains enigmatic. In this work, four aspects of the *ytr* operon, namely its role in (i) antibiotic stress adaptation, (ii) cell wall synthesis and/or homeostasis, (iii) cold shock, and (iv) sporulation, were explored. For this purpose, mutants that either constitutively express or lack the full operon, or parts thereof (**Figure 14**) were phenotypically characterized at two different temperatures (37 °C and 24 °C).

(i) Antibiotic adaptation

Despite being reliably induced by inhibitors of the lipid II cycle[23,24,30–32,34–39], previous susceptibility assay using a wide array of cell wall synthesis inhibitors did not show any altered sensitivity of a Δ*ytrABCDEF* mutant[38]. However, acute shock experiments with Δ*ytrA* showed reduced nisin susceptibility[35], suggesting that phenotypes may be observed in strains constitutively expressing the *ytr* operon, or with acute shock assays. To examine this, susceptibility assays with a range of *ytr* mutants (**Figure 14**) and various cell wall synthesis-inhibiting antibiotics were performed at both 37 and 24 °C. As control, a compound with an unrelated mechanism (ribosome inhibition) was included.

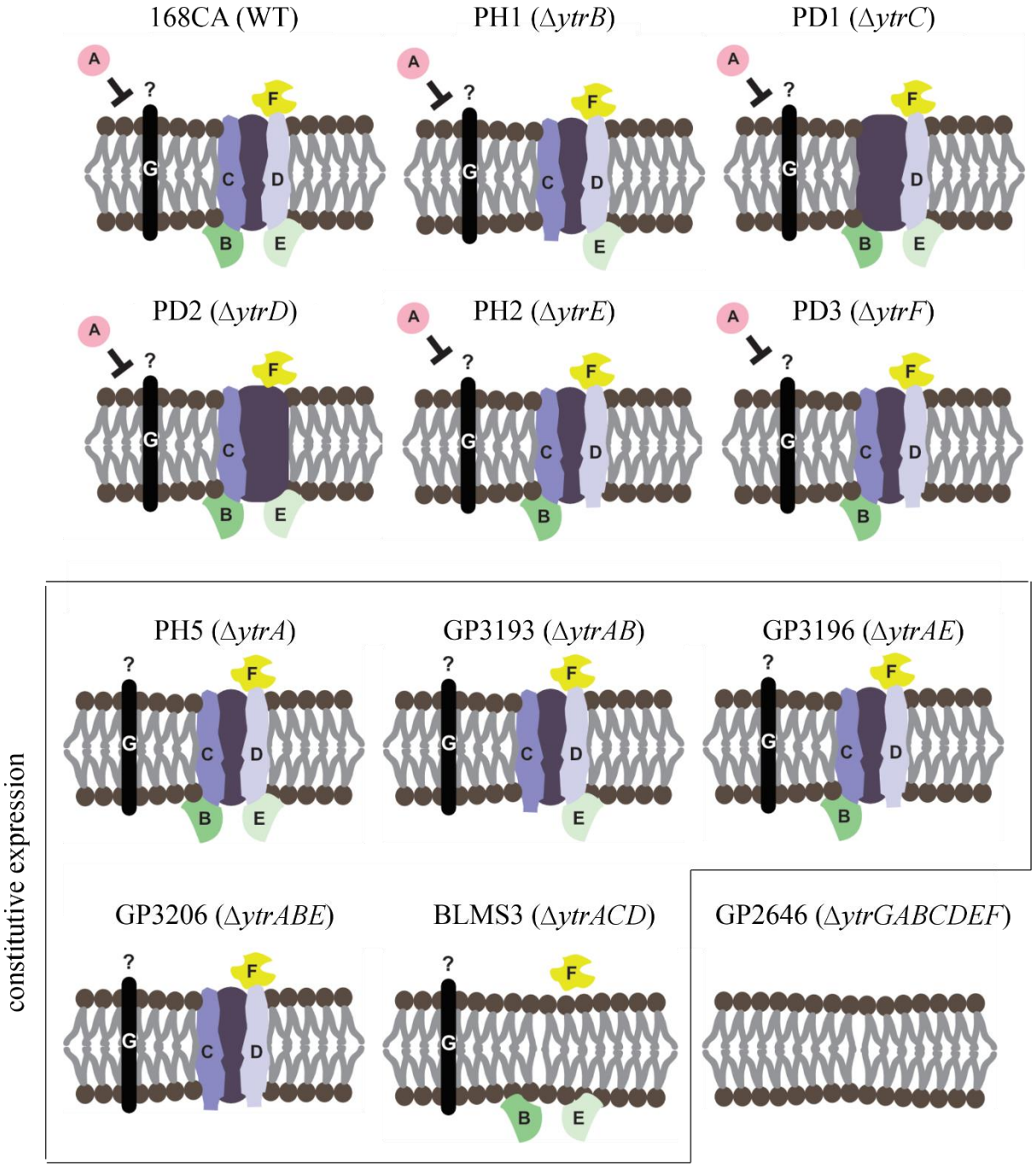

Figure 14: Schematic structure of the putative ABC transporter encoded by the operon (WT) and transporter expression in the different deletion mutants used in this study. Note that deletion of *ytrA* results in constitutive expression of the remaining *ytr* genes (box). The function of YtrG is not known. Structural predictions classify it as small transmembrane protein[40]. Predicted subunits of the transporter is based on [47,48].

The antibiotics used in this work were ampicillin, ertapenem, cefoxitin, meropenem, cloxacillin, nisin, vancomycin, D-cycloserine, and tetracycline. Only ampicillin, nisin, and ertapenem showed particularly interesting results (**paper III Figures S2-10, Tables 1, S5-6**). While there was no clear pattern to antibiotic susceptibility, the effects shown for ampicillin and ertapenem supported a possible role of this operon in cell wall synthesis. Unexpectedly,

my results were unable to reproduce the lower nisin susceptibility phenotype of the Δ*ytrA* mutant[35]. These results also crossed the *ytr* operon off our list of possible antibiotic potentiator candidates.

Taken together, the *ytr* operon does not seem to be part of a protective stress response against antibiotics even though it was reproducibly induced by cell wall inhibitor compounds. Instead, the treatment with these compounds probably mimics the same cellular signal that induces the operon. It is tempting to hypothesize that induction could be related to the accumulation of intracellular cell wall precursors, since the accumulation of uridine diphosphate N-acteylmuramic acid (UDP-MurNac) pentapeptide is a common effect of lipid II-binding antibiotics[31]. Yet, this needs to be assessed in future studies.

(ii) Cell wall synthesis

Different *ytrGABCDEF* deletion mutants (**Figure 14**) were characterized with respect to their cell wall synthesis and turnover phenotypes. First, these strains were stained with BODIPY FL vancomycin (Van-FL), which allows the visualization of lipid II and indicates the localization of active cell wall synthesis. While other mutants showed similar patterns as wild type, fluorescence microscopy revealed distinct phenotypes for the Δ*ytrAB*, Δ*ytrAE*, Δ*ytrABE*, and Δ*ytrACD* mutants (**Figure 15**, see full results **paper III Figures 2**, **S14-15**). These mutants showed a decreased septal or overall fluorescence signal, especially when grown at 24 °C, suggesting reduced cell division activity (**Figure 15**). These observations were further supported by quantification of whole-cell fluorescence (indicating the amount of lipid II in the cell membrane) and line scans through the lateral cell axis (indicating accumulation of lipid II at mid-cell) (**Figure 15, paper III Figures S16-39**). A similar set of experiments was performed to visualize penicillin-binding proteins (PBPs) using the fluorescently labeled penicillin, BODIPY FL penicillin (bocillin). However, no major disruption of PBP localization was observed (**paper III Figures S40-41**), supporting the idea that the *ytr* operon is rather linked to the membrane-bound lipid II cycle.

(iii) Cold shock and autolysis

Cell wall synthesis and autolysis are intimately linked as autolysins need to open up gaps in the peptidoglycan layer for the incorporation of new cell wall material. An imbalance of cell wall homeostasis can lead to cell lysis through autolysis. In *B. subtilis,* such autolysis is induced by cold shock[223]. Cold shock also induces the *ytr* operon[199,220] and the Δ*ytrA* mutant displayed reduced fitness at 16 °C[41]. Moreover, YtrF belongs to the same protein family as FtsX, which regulates the major autolysin CwlO[48]. Therefore, there is a possibility that the *ytr* operon regulates cold-induced autolysis. Growth experiments at different temperatures (**Paper III Figure 3)** demonstrated that the *ytr* operon affects both cell lysis and resumed growth of surviving cells after partial lysis of the population. Δ*ytrA* (constitutive expression of the operon) showed increased lysis, lower fitness, and reduced capacity for regrowth. The opposite effect was observed in the whole operon mutant, suggesting that the *ytr* operon promotes cell lysis.

To gain more insight into cell lysis on single-cell level, bacterial cytological profiling (BCP)[276] was performed at constant 37 °C and 24 °C as well as after shifting cells from 37 °C to 24 °C (**paper III Figures 4**, **S43-44**, **Table S7**). In general, Δ*ytrC*, Δ*ytrD*, Δ*ytrAB*, Δ*ytrAE*, Δ*ytrABE*, and Δ*ytrACD* strains showed membrane aberrations in the Nile red stain and subpopulations of lysing cells, which became more profound upon temperature shift (**Figure 16**). Curiously, a strong heterogenous cell populations with a 'small cell' phenotype was observed (see **Figure 16**, yellow arrowheads). This small cell phenotype only appeared in the exponential phase (**paper III Figures 5, S45-47**). Moreover, small cells showed a typical lysis phenotype (reduced phase darkness, increased and/or granular fluorescence stains), did not actively synthesize cell wall peptidoglycan, and did not grow (**paper III Figures S48-49**).

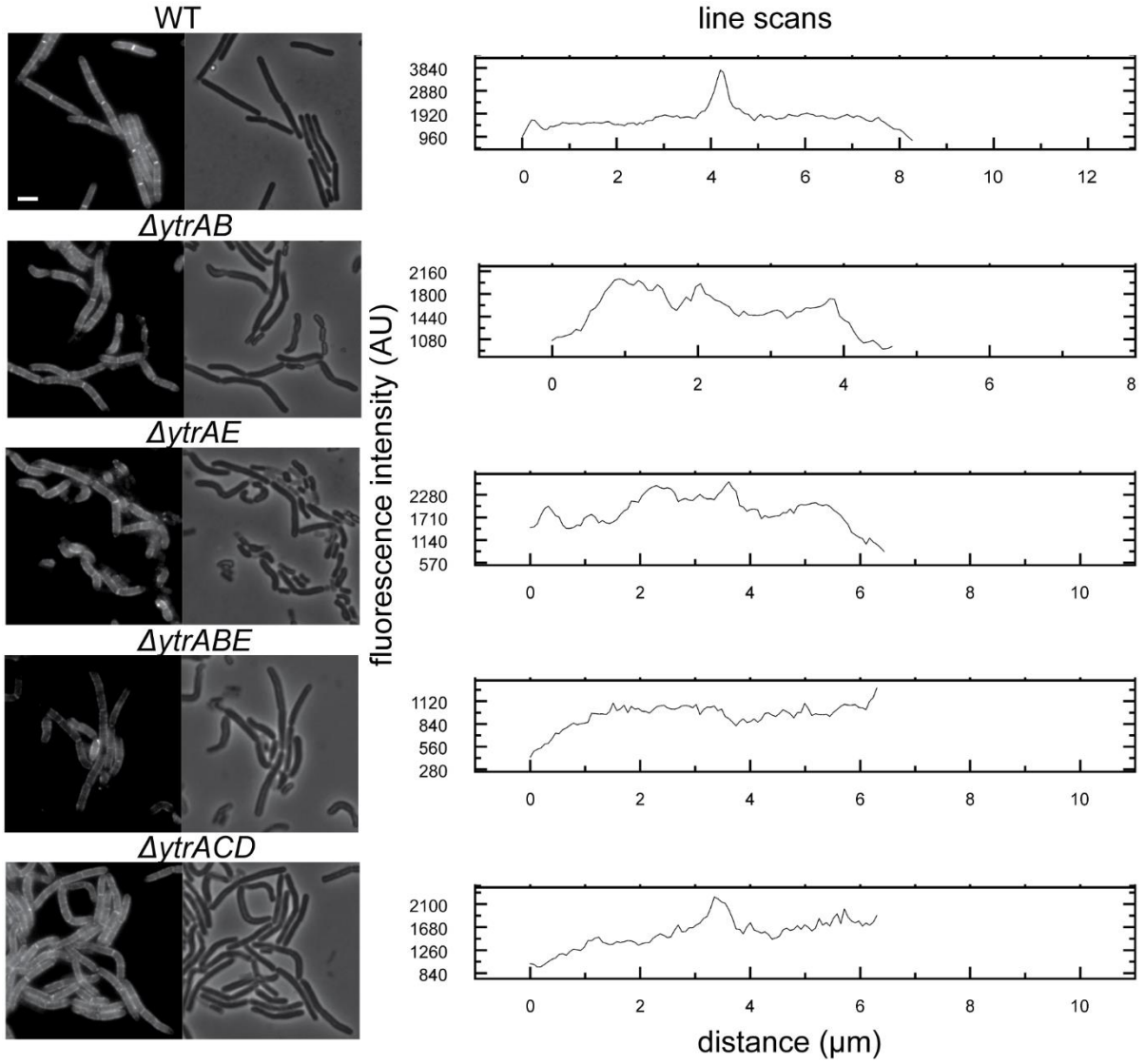

Figure 15: Microcopy images and longitudinal line scans of individual *B. subtilis ytr* mutants labeled with Van-FL. Cells were grown at 24 °C. Exposure times, light intensity, and brightness/contrast settings were identical for all samples. Scale bar 2 μm.

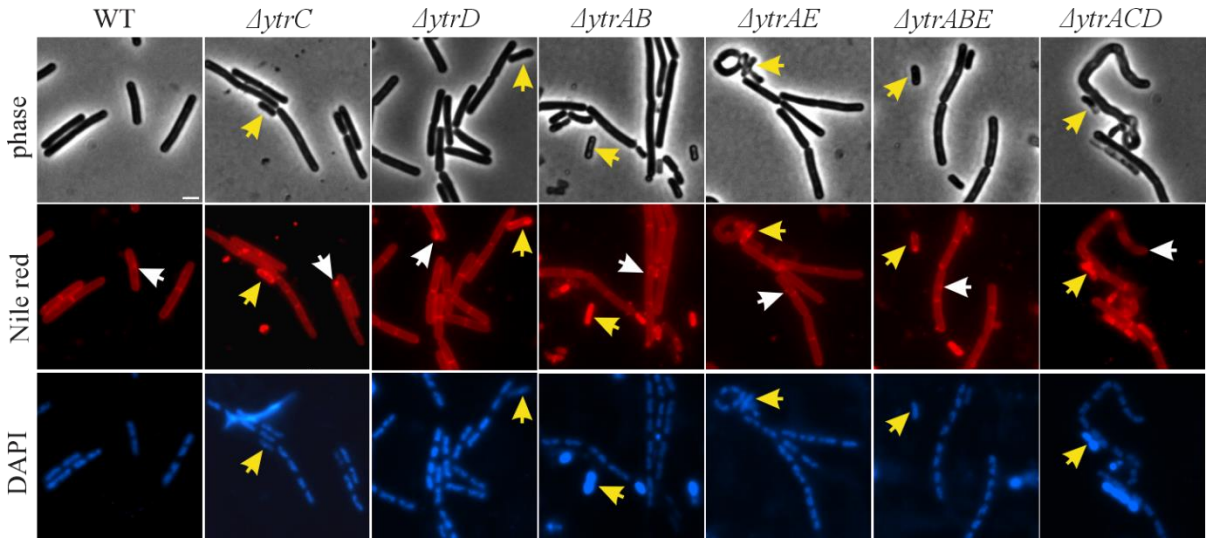

Figure 16: BCP of cultures shifted from 37 °C to 24 °C after dilution of overnight cultures. Membranes were stained with Nile red and DNA with 4',6-diamidino-2-phenylindole (DAPI). White arrows indicate membrane irregularities. Yellow arrows indicate small cells. Scale bars 2 µm.

The small cell phenotype raised two questions: (i) what is the cause of this phenotype? and (ii) why do strains that constitutively express an incomplete *ytr* operon show such a strong small cell phenotype?

One possible cause could be cannibalism. To delay sporulation, cannibalistic cells release toxins to kill non-cannibalistic cells and obtain new nutrient to grow, resulting in growing and lysing subpopulations. *B. subtilis* cellular differentiation processes (e.g., cannibalism, competence, sporulation, biofilm, motility) involve bimodal gene expression that allows the differentiation of the population into two distinct phenotypes[69,71,73,76]. Interestingly, *ytr* deletion strains have been shown to affect all of these processes[41,47,48], thus explaining their heterogeneous phenotypes and indicating a possible role of this operon in cellular differentiation. The activation of competence, biofilm, cannibalism, and sporulation depends on stochastic variation of the concentration of phosphorylated Spo0A and the ability of the respective promoters to respond to these different concentrations[69,71]. Similarly, YtrA has been shown to exhibit a concentration-dependent shift in promoter binding[38], suggesting a potential bimodal regulation for this operon. Additionally, the bimodal control of motility has been postulated to be influenced by a reduction in cell size caused by slower growth rate[69,277]. Assuming that the *ytr* operon is under bimodal control, there is a possibility that a reduction in cell volume (in small cells) causes the regulator to become more concentrated and easier to reach the specific threshold that triggers the effects of the *ytr* operon.

My observation that a reporter strain carrying the 300-basepair upstream sequence of *ytrA,* containing both the promoter region and the *ytrG* coding sequence, could not be constructed, indicates that YtrG is toxic for *E. coli*. This led us to hypothesize that YtrG could encode a membrane-active toxin. This may explain the lysis phenotype observed in *B. subtilis* strains that force-express *ytrG*.

Assuming that the YtrBCDEF transporter may be involved in either the secretion or membrane insertion of YtrG, the strong phenotypic effects observed in mutants constitutively

expressing non-functional ABC transporter variants (Δ*ytrAB*, Δ*ytrAE*, Δ*ytrABE*, Δ*ytrACD*) would make sense. These strains would overexpress a toxic gene product that would either accumulate inside the cells or undergo faulty membrane insertion. It is also possible that YtrG is not a toxin per se but fulfills an unknown cellular function, possibly related to cell wall synthesis, but that an imbalance of YtrG levels causes growth arrest and cell lysis. This possibility is supported by the bioinformatic analyses, which found some toxin motifs in the YtrG sequence but did not specifically predict the protein to be a toxin (**paper III Figures 6A-C**, **S50-51**). The mixed bioinformatic results suggest that if *ytrG* should encode for a toxic gene product, it is not part of a known type of toxin-antitoxin system.

Supporting the toxin hypothesis, synthetic YtrG inhibited the growth of *B. subtilis* and to a lesser extent of *E. coli* (**paper III Figures 6D**, **S52-54**). The milder growth-inhibitory effect for Δ*ytrF* and Δ*ytrGABCDEF* suggest that the presence of a functional operon may aggravate the peptide's effects. Moreover, the lower growth-inhibitory effect for *E. coli* indicates that the primary target of YtrG peptide is its own producer strain. Considering that *B. subtilis* cells exposed to this synthetic peptide did not show similar small cell phenotypes as observed in *ytr* mutants (**paper III Figures 6E**), there is a possibility that *ytrG* needs to be expressed inside the cells to elicit these phenotypes. Unfortunately, I was unable to overexpress the gene in *B. subtilis*, leaving this question unanswered. An alternative explanation could be that the YtrG peptide is posttranslationally modified into its active form, e.g., by proteolytic cleavage. This possibility needs to be examined in future studies.

(iv) Sporulation

Assuming that YtrG is indeed a toxin, there is a possibility that YtrG is expressed to lyse a subpopulation of cells to delay sporulation. Such a scenario could possibly explain most observations around the *ytr* operon, assuming that this operon is triggered by cell wall synthesis inhibition. If this scenario is true, it would be expected that constitutive expression of the operon (Δ*ytrA*) delays sporulation. Supporting this notion, previous studies showed decreased sporulation efficiency in Δ*ytrA* and a pMUTIN2 insertion in the promoter region that does not express the operon[41,47]. In this work, asymmetric septation of the Δ*ytrA* and Δ*ytrGABCDEF* mutants was followed over time using fluorescence microscopy. While the Δ*ytrGABCDEF* mutant showed delayed sporulation, the *ΔytrA* mutant initiated sporulation around the same time as the wild type (**paper III Figures 8**, **S56-57**). Unexpectedly, there were no differences in sporulation efficiency of the wild type, Δ*ytrA,* and Δ*ytrGABCDEF* strains after 24 h, indicating that sporulation is not defective but merely delayed in the whole operon deletion strain (**paper III Figure S58**). Since sporulation is a medium-dependent processes[278–280], this conflicting result could be caused by the usage of different sporulation media. In future experiments, more extensive studies will be needed to assess the connection between sporulation and the *ytr* operon in more detail and explain the conflicting observations made by different groups.

Taken together, my work explored different hypotheses related to the function of the *ytr* operon. Despite its reliable induction by antibiotics that inhibit the lipid II-cycle, the *ytr* operon

seems to not play a notable role in antibiotic stress adaptation. Consequently, it is not a suitable target for antibiotic potentiators. However, we did find additional evidence for the involvement of this operon in cell wall synthesis and/or homeostasis. While its specific role remains unclear, it is likely linked to the membrane-bound lipid II cycle. Our observation of a pronounced population heterogeneity phenotype indicates a possible new role of this operon in cell differentiation and suggests a bimodal regulation mechanism. Based on our observations, we propose a new hypothetical function of the *ytr* operon. Thus, YtrG could be a secreted or membrane-bound toxin that relies on YtrBCDEF for secretion/membrane insertion. These hypotheses open a new research direction for understanding the role of this system.

6.4.ROS-mediated killing by the ionophore valinomycin

Valinomycin is a potassium carrier ionophore that is commonly used to study the effects of membrane depolarization[27]. While it is clear that depolarization will inhibit actively growing cells due to energy depletion, it is unclear how it exerts it bactericidal effects on both growing and non-growing cells[28]. To investigate this, stationary phase, non-sporulating *B. subtilis* cells, were chosen as a simple model for metabolically inactive, non-growing cells.

Our data suggested that valinomycin affects viability by introducing DNA damage (**paper IV Figure 2**). Since valinomycin is well-established to not possess secondary mechanisms independent of membrane depolarization, this is most likely explained by the accumulation of ROS, which can occur as side products of aerobic respiration and are a major source of endogenous DNA damage in cells. Yet, it sounds unlikely that metabolically inactive cells would produce significant amounts of ROS, especially since membrane potential-dissipating agents have been reported to prevent ROS production[251,281,282]. Moreover, antibiotics known to generate ROS (e.g., norfloxacin, vancomycin, kanamycin) are not active against non-growing cells[245,247,283]. Despite these notions, several membrane-active antibiotics including valinomycin, trigger oxidative stress responses in proteomic profiling studies[35,45]. To explore if membrane depolarization indeed generates ROS, stationary *B. subtilis* cultures were incubated with the cell-permeant ROS probes 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA)[247] or Oxyburst Green H2DCFDA succinimidyl ester (Oxyburst Green) and exposed to valinomycin or the superoxide inducer paraquat[284]. Higher fluorescence signal indicates higher ROS concentration. Surprisingly, valinomycin did generate ROS and its effect even exceeded those of paraquat (**Figure 17A, paper IV Figure S6**).

This observation led us to identify the type of ROS that valinomycin generates. Hydroxyl ($^{\bullet}OH$) and superoxide ($O_2^{\bullet-}$) radicals are the main ROS formed during aerobic growth[285]. Antibiotics that have been shown to generate ROS, like norfloxacin, ampicillin, and kanamycin, primarily generate hydroxyl radicals through the Fenton reaction[245,247,283]. However, our results showed that valinomycin primarily generated superoxide radicals. Thus, addition of the hydroxyl radical scavenger thiourea did not reduce the killing effect of valinomycin but the superoxide scavenger tiron suppressed this effect (**Figure 17B)**. Furthermore, inactivation of SodA, the main superoxide dismutase in *B. subtilis*, made cells become more sensitive to valinomycin (**Figure 17C)**. In contrast, the catalase mutant *ΔkatA*

behaved like the wild type. In line with these observations, SodA has been identified as one of the marker proteins for valinomycin stress[45]. Experiments using the superoxide-specific probe Mitosox Red[286–288] further supported the idea that membrane depolarization triggers production of the superoxide radicals (**paper IV Figures 4d, S6**).

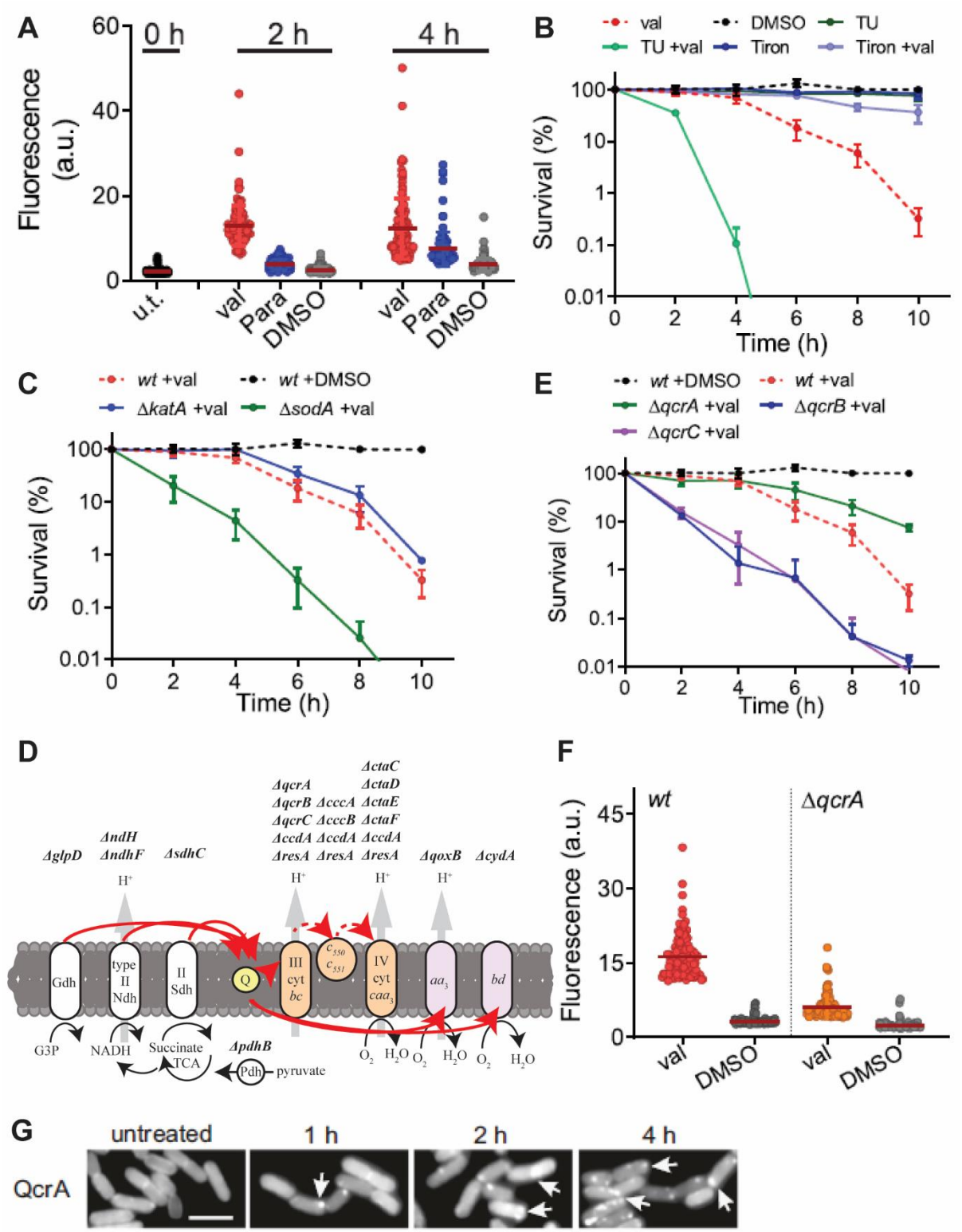

Figure 17: ROS-mediated killing of valinomycin in the stationary phase *B. subtilis* cells. **(A)** ROS production measured by the fluorescent ROS probe H2DCFDA. Cells were treated with 100 µM

valinomycin (val), 1 mM paraquat (para), or 1% DMSO for 2 and 4 h. The fluorescence intensities of 120 cells were measured microscopically and plotted. **(B)** Survival curves of cells incubated with 100 µM val in the presence of the hydroxyl radical scavenger thiourea (TU, 150 mM) or the superoxide scavenger tiron (10 mM). **(C)** Survival curves of the catalase *ΔkatA* and the superoxide dismutase *ΔsodA* mutants incubated with 100 µM val in the presence of the hydroxyl radical scavenger thiourea (TU, 150 mM) or the superoxide scavenger tiron (10 mM). The viable counts of cells in the presence of 1% DMSO were similar to wild type cells and are not indicated. **(D)** Schematic illustration of the key enzymes in the *B. subtilis* TCA cycle and ETC. Deleted genes encoding the different components are shown above the related subunits. The different components shown are pyruvate dehydrogenase (Pdh), glycerol-3-phosphate (G3P) dehydrogenase (Gdh), succinate dehydrogenase (complex II, Sdh), NADH dehydrogenase (Ndh), menaquinol pool (Q), cytochrome *bc* complex (complex III, cyt *bc*), cytochrome *c550* and *c551*, cytochrome-*c* oxidase (complex IV, caa_3), cytochrome aa_3 quinol oxidase (aa_3) and cytochrome *bd* ubiquinol oxidase (*bd*). QcrA is the Rieske factor, menaquinol:cytochrome *c* oxidoreductase (iron-sulfur subunit), component of the cytochrome *bc* complex. (E) Survival curves of the single deletion mutants of the cytochrome *bc* complex components (*qcrABC)* incubated with 100 µM val. **(F)** ROS production in wild type (WT) and *ΔqcrA* after 4 h incubation with 100 µM val or 1% DMSO. ROS was measured with the fluorescent ROS probe H2DCFDA. The fluorescence intensities of 120 cells were measured microscopically and plotted. **(G)** Cellular localization of QcrA-GFP incubated with 100 µM val for 1, 2, and 4 h. Three biological replicates were performed for all experiments. Data shown in **(B)**, **(C),** and **(E)** reflect mean ± SD of three biological replicates. All images were taken or adapted from [28].

The major source of endogenous ROS is the ETC (see **Chapter 5.4**). To explore which of the ETC complexes is responsible for superoxide accumulation, the growth of different deletion mutants was assessed under valinomycin stress (see **Figure 17D** for the list of mutants). While other deletion mutants became more sensitive to valinomycin, Δ*qcrA* showed the opposite (**Figure 17E, paper IV Figure 5**). QcrA is the Rieske-type iron-sulfur subunit of complex III with a non-typical 2Fe-2S cluster, in which one of the two iron atoms is held in place by two histidines[289]. The electron transfer process mediated by this cluster is a well-known source of superoxide radicals in mitochondria[290]. To test whether the same is true in *B. subtilis*, ROS production in the Δ*qcrA* mutant was measured using the ROS probes. Indeed, this mutant showed significantly less ROS after valinomycin treatment, confirming that QcrA is involved in valinomycin-induced superoxide production (**Figure 17F, paper IV Figure S12**). Our localization study using QcrA-GFP revealed that membrane depolarization affects QcrA distribution in both growing and non-growing cells (**Figure 17G, paper IV Figure 6**). We speculate that detachment of QcrA from complex III hampers electron transfer from the 2Fe-2S cluster of QcrA to the heme of QcrB and/or exposes this cluster to oxygen, hence causing the production of lethal levels of superoxide radicals.

Our work supports the idea that membrane potential-dissipating antibiotics can induce lethal levels of ROS through interference with ETC complexes, specifically QcrA. However, the accumulated ROS is superoxide rather than hydroxyl radicals produced by the Fenton reaction. These observations explain why such compounds induce an oxidative stress response, specifically the upregulation of SodA. Interestingly, valinomycin and several other membrane-targeting antibiotics specifically induce SodA[35,45] and not necessarily other ROS-detoxifying enzymes. Importantly, SodA has been reported to play a role in antibiotic tolerance[291–293], suggesting that superoxide may play an important role in the killing mechanism of more

antibiotics. If this is true, SodA can potentially be developed as a new antibiotic potentiator target.

6.5.Concluding remarks

My work has provided novel mechanistic insights into four different antibiotic stress response systems and evaluated their suitability as targets for the development of antibiotic potentiators. I have shown that AIAAR, which is mediated by MSCs, is a broadly conserved and highly effective antibiotic survival strategy against cell envelope-targeting antibiotics. These properties highlight the suitability of MSCs as new drug targets for combination therapy approaches. Bacteria typically have multiple MSCs, which are not essential. Thus, MSC inhibitors are not generally desirable as stand-alone drugs. However, they are of high importance for bacterial survival under stress conditions, making them attractive accessory drug targets to increase antibiotic activity. The fact that AIAAR is highly efficient on solid media suggests that such combination approaches may be particularly well-suited for infections that occur in environments with limited diffusion rates, such as skin or soft tissue infections or for anti-biofilm applications. It can also be worth exploring if MSCs inhibitors could be used as coatings for medical devices, such as catheters, since MSCs play an important role in adapting to environments with fluctuating osmolarity such as the urogenital tract.

My work has also revealed the potential of oxidative stress responses as antibiotic potentiators targets. In **paper IV**, we observed that valinomycin causes lethal levels of ROS and that SodA plays an important role for survival in presence of valinomycin. Regardless of the controversy around ROS being the common killing mechanism of bactericidal antibiotics, it is undisputed that there are many bactericidal antibiotics that trigger ROS. Bacteria do activate their oxidative stress responses to cope with this stress, indicating that ROS production at least contributes to killing, even if it is not the only factor. Thus, it will be interesting to explore the potential of bacterial ROS detoxification systems as potentiator targets.

Both AIAAR and SodA are good examples of how proteomic profiling can reveal new factors that are important for bacterial survival under antibiotic stress conditions. However, not all clues that we obtained from these studies could be confirmed to contribute to survival. Since proteomic profiling detects the global stress, it will pick up everything that is reliably upregulated including secondary effects of antibiotic stress and co-regulated genes. This is likely the case for the *ytr* operon. Despite being a highly specific and reproducible marker for cell wall synthesis-inhibiting compounds, I was unable to confirm any notable contribution to survival under antibiotic stress. Nonetheless, stress profiling data, not being limited to proteomic profiling, constitutes a plentiful resource for identifying new antibiotic survival mechanisms, which can constitute new targets for antimicrobial combination therapy.

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