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General introduction

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In the first experimental part of this thesis (**Chapters 2** and **3**), we addressed the intra- and interspecies transfer of DNA and protein transfer between *B. subtilis* and members of Lactic Acid Bacteria (LAB). Here, we showed the ubiquity of horizontal gene transfer (HGT) in mixed co-cultures and proposed novel applications for natural strain improvement which are highly desired in producing novel starter cultures for food fermentation

The second part of this dissertation explores the physiological and transcriptional responses of *B. subtilis* to osmotic and antibiotic stress on a population-wide and single-cell level (**Chapters 4**, **5** and **6**). This work sheds new light on how preadaptation to adverse conditions can modulate metabolic activity, affect cell membrane polarization, and finally increase tolerance to certain antibiotics in bacteria. We showed that *B. subtilis*, after being pretreated with osmotic stress, enters the viable but non-culturable state (VBNC) and becomes resistant to aminoglycoside antibiotics. Besides, the observations made in this work draw attention to the typical methods of cell enumeration and their error-prone outcomes and highlight the importance of VBNC bacteria when testing new antibiotic compounds for clinical applications.

1.1. *Bacillus subtilis*: a model organism to study HGT

Bacillus subtilis is one of the most studied Gram-positive bacteria, able to thrive in diverse environments such as soil, plant roots, gastrointestinal tracts of animals or even under extraterrestrial conditions[1–4]. To survive under these ever-changing conditions[5], *B. subtilis* responds to environmental cues by cell differentiation resulting in various specialized phenotypes[6]. For instance, when facing famine, a subpopulation of nutrient-deprived *B. subtilis* cells commits to sporulation and forms highly-resistant dormant endospores able to survive prolonged periods of unfavourable conditions[7]. These airborne spores can be easily transferred across many environments, waiting for the appropriate conditions to germinate and populate a new niche[8]. Besides spore formation, *B. subtilis* induces natural competence for transformation. Essentially, under competence-promoting conditions, a fraction of the *B. subtilis* population enters the so-called competent state and upregulates the DNA-uptake machinery to acquire naked DNA, which can be either recombined into bacteria's genome or utilized as a nutrient source[9–11]. The ability to take up exogenous DNA makes *B. subtilis* accessible for genetic modifications, and since *B. subtilis* is a Generally Recognized as Safe

bacterium (GRAS status), the relatively straightforward gene editing opens a myriad of applications as a microbial cell factory[12].

In its natural environment, *B. subtilis* interacts with many soil-dwelling bacteria and fungi, typically residing in biofilms that form a collective of cells aggregates encapsulated in a self-produced extracellular matrix[13–15]. *B. subtilis* can switch from a motile to a sessile state and activate the expression of genes involved in the production of abundant biofilms, including members of the *epsA-O* operon for exopolysaccharide synthesis[16], the *tapA* operon for the expression of protein fibers[17], and the *blsA* gene whose product confers hydrophobic nature of biofilms[18]. Additionally, the extracellular matrix contains extracellular DNA (eDNA), which is known to modulate the structure of biofilms[19]. Because of their collective nature, biofilms are great hot spots for HGT, facilitating DNA transfer through conjugation, phage transduction, natural transformation and even nanotubes. Therefore, it is expected that *B. subtilis*, as a considerable biofilm producer, will display a high frequency of HGT events when co-cultured with other bacteria.

1.2. Horizontal gene transfer in *Bacillus subtilis* – classical and updated view on DNA transfer mechanisms

Classical HGT mechanisms in *B. subtilis*

Horizontal gene transfer (HGT) plays a crucial role in bacterial evolution and ecology[20–22]. In adaptation to ever-changing environments, bacteria are able to acquire foreign DNA that may be advantageous for survival under various selection pressures[20,23]. In the laboratory strain *Bacillus subtilis* 168, the early analyses of the DNA sequence revealed that approximately 15% of the genome consists of atypical regions representing evolutionary signatures for horizontally transferred DNA fragments[24,25]. The biggest hallmarks of HGT events in *B. subtilis* 168 are 10 AT-rich islands representing SPβ and PBSX prophages and prophage-like regions[26–29], and a region encoding integrative and conjugative element, ICE*Bs1*[30]. The assortment of HGT events discovered just in the genome sequence of the laboratory strain of *B. subtilis* suggests how robust the DNA exchange is in wild types strains occupying natural environments.

The main HGT mechanisms in bacteria are conjugation, phage transduction and natural transformation[31]. The first two strictly depend on the presence of a donor cell or a donor phage near the recipient cell, and both require specific apparatuses for the DNA exchange. In the case of conjugation, after the mating pair formation between donor and recipient cell, the genetic material is transferred through a conjugative pilus, typically encoded on conjugative elements[30,32]. *B. subtilis* harbours one of the best-studied integrative and conjugative elements (ICE), ICE*Bs1*. Excision of this 20-kb ICE is activated by the RapI protein and regulated by the PhrI secreted peptide, both encoded by the conjugative element itself[33]. The cells initiate the expression of *rapI* and *phrI* under conditions of low nutrient availability, high cell density and during the SOS response triggered by global DNA damage[33]. Importantly, ICE*Bs1* can self-transfer from distressed donor to recipient cell, but it can also mobilize plasmids that lack mobilization functions, indicating that ICE*Bs1* may play a significant role in HGT in *B. subtilis*[34].

Bacteriophages, or phages, can mobilize DNA via specialized or generalized transduction modes. During transduction, genetic information is carried through phage virions that acquired fragments of the host's DNA and were able to infect both donor and recipient[35–37]. The specialized transduction results from the defective excision of the prophage and packing of the adjacent fragments of the host's chromosomal DNA[38], whereas in the case of generalized transduction, phage virions erroneously pack random fragments of the host's genetic material, including plasmids, instead of their viral DNA[39,40]. Successful transduction of genetic material to *B. subtilis* was first documented in 1961 with bacteriophage SP-10 isolated from the soil[41]. The study showed curing of indole, arginine, histidine and adenine auxotrophs by phage propagation on a wild type strain and infection of mutant strains. Moreover, another virulent *B. subtilis* phage, SPP1, has been shown to carry out generalized transduction of plasmids with a significant frequency[40].

In the transformation process, bacteria enter the state of competence and acquire naked DNA directly from the environment using complex DNA-uptake machinery[42–44]. Unlike conjugation and phage transduction, this process is genetically encoded in the recipient's cell, and it is regulated by many physiological and environmental factors such as growth phase, nutrient availability, starvation, cell density, and even antibiotic stress[45–49]. In *B. subtilis,* the competent state for transformation is induced at the onset of the stationary phase and in response to high cell densities[9,50,51]. The activation of the competence state (K-state) is controlled by the ComK master regulator[52,53], a multimeric transcription factor that binds to a consensus motif on the promoter regions of competence-related genes[54]. It activates the expression of more than 150 genes, generally related to DNA uptake and homologous recombination[55,56]. Importantly, *comK*-deficient cells are not transformable, highlighting

the essential role of ComK in competence development[57]. However, not all the cells enter the K-state under competence-inducing conditions, and only a fraction of the population becomes competent for transformation. This phenotypic differentiation arises from a positive feedback loop generated by the auto-stimulation of ComK expression[47,52,58].

The induction of competence in *B. subtilis* is also controlled by its population density, and it is linked with the initiation of surfactin production. The ComX pheromone is sensed by the two-component sensor kinase ComP, which activates the transcriptional regulator ComA by phosphorylation. The phosphorylated ComA induces the expression of the surfactin operon together with a *comS* gene which encodes for a peptide, ComS, directly activating the ComK master regulator[57,59,60]. Interestingly, knowing that competence induction is triggered in dense populations, where part of the population triggers the surfactin production for cell lysis and DNA release, this suggests that genetic transfer via natural transformation is meant to share genetic material with kin cells[61].

Cells in the K-state assemble a DNA-uptake machinery complex to translocate the exogenous genetic material across the cell envelope[44,45,47]. The central part of the DNA translocation complex consists of an extendable type IV pilus encoded on the *comG* operon[62]. Before translocation, the double-stranded DNA is cleaved by the membrane-associated nuclease NucA[63], resulting in ~20 kb linear fragments that are further taken up in a single-stranded form (ssDNA). After the pilus retraction, ssDNA is translocated through a thick peptidoglycan layer to the membrane-bound ComEA receptor protein, and the complementary strand is released to the medium[64]. Next, the ssDNA is transported into the cell via the ComEC polytopic transmembrane protein[65], supported by the ATP-dependent helicase-like ComFA[66]. After internalization into the cytoplasm, several proteins, such as SsbB and DprA ssDNA-binding proteins, are recruited to protect the incoming ssDNA from degradation[67,68]. The integration of ssDNA into the recipient's chromosome is catalyzed by the general DNA recombinase RecA, accompanied by AddAB DNA helicase[69]. It seems that *B. subtilis* does not discriminate among sequences of the incoming DNA. Rather, the successful integration depends on the coverage of homology between the foreign DNA and the recipient's chromosomal DNA.

Nanotubes as conduits for HGT in *B. subtilis*

Recent advances on HGT in bacteria revealed promising evidence for noncanonical means of genetic transfer, including membrane vesicles[70],

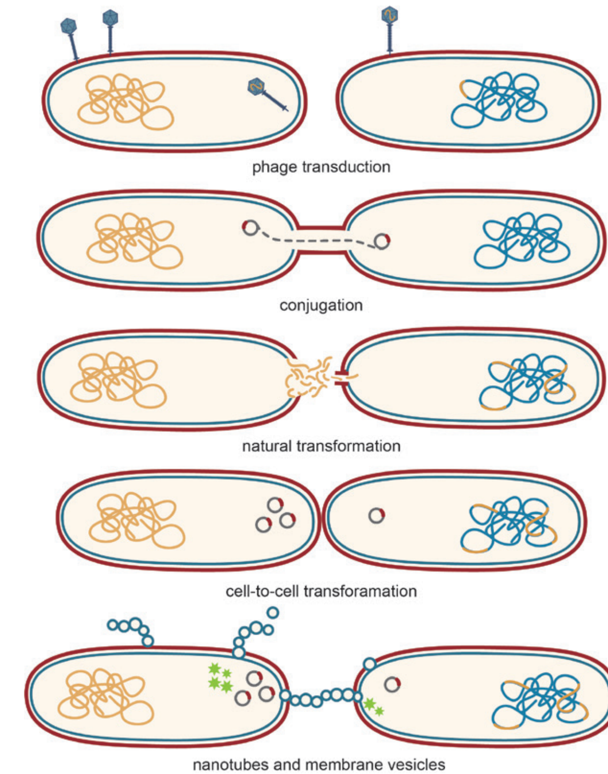

Figure 1. Horizontal gene transfer mechanisms characterized in *B. subtilis*
The schematic representation of HGT events shaping the evolution and ecology documented in *B. subtilis*, including three generally recognized HGT mechanisms: phage transduction, conjugation and natural transformation, and recently studied: cell-to-cell transformation and DNA delivery via membrane-derived structures. In this thesis, we explored the cell-to-cell intra- and interspecies plasmid and protein transfer that is likely mediated via cell-to-cell nanotube transfer.

cell-to-cell natural transformation[71–74], and bacterial nanotubes[75]. To date, *B. subtilis* has been shown to transfer genetic material in a donor-recipient manner not only via conjugation or phage transduction but also through cell-to-cell non-conjugative transfer[73], presumably mediated via nanotubes[75].

Intercellular tubular conduits have been long known to occur in higher organisms (e.g. plants and mammalian cells), providing the routes for intracellular transfer of various cellular components, including nutrients, signalling molecules, proteins, organelles and even viral particles[76–79]. In the bacterial world, the presence of nanotubes was reported in several different species. To date, a list of microorganisms known to establish these structures as either donors or recipients includes *B. subtilis* and *Staphylococcus aureus*[75], *Escherichia coli*[80], *Bacillus megaterium*[81],

Acinetobacter baylyi[82], *Clostridium acetobutylicum* and *Desulfovibrio vulgaris*[83], *Thermococcus gammatolerans* and *T. kodakaraensis*[84].

In *B. subtilis,* nanotubes were identified when a weak fluorescent signal was gradually delivered from GFP-producing cells to adjacent wild type cells on an agarose pad[75]. These structures were proposed to transfer not only small proteins but also plasmid DNA[75], indicating their potential contribution to HGT in neighbouring bacteria. The analysis of nanotube formation in real-time revealed that these structures are formed in the course of minutes on solid media and display rapid movements on a millisecond scale[85]. Notably, nanotubes seem sensitive to low concentrations of sodium dodecyl-sulfate, suggesting their membranous nature[75]. Further analysis with super-resolution, light and electron microscopy confirmed that these appendages are composed of chains of constricted membranous segments that share continuous lumen[85] (**Fig. 2A**). This membrane compartmentalization has been proposed to be a footprint of continuous membrane budding during nanotube elongation. In the low-density populations, the extending nanotubes reach a length of up to 40 μm. Cryo-EM analysis indicated the nanotube diameter to be approximately 40–60 nm, depending on the width of the constriction sides of each nanotube segment (each approximately 100 nm long). Additionally, the extending nanotubes are typically thinner than those connecting adjacent cells. Because of the presence of long extendable nanotubes, besides bridging neighbouring cells to exchange cytoplasmic content, nanotubes were proposed to assist in investigating near surroundings, plausibly for nutrients or allies.

So far, several molecular determinants have been associated with nanotube biogenesis and mediated transfer. The early study on *B. subtilis* nanotubes suggested a calcineurin-like phosphodiesterase YmdB strongly affecting nanotube formation[85]. The analysis of the *ymdB* mutant revealed that these cells could no longer establish nanotube connections with nearby cells. YmdB controls bistable expression of genes related to flagella and biofilm formation, and the mutation of the YmdB catalytic site, responsible for hydrolyzing cAMP and c-di-GMP signalling molecules, causes defective biofilm formation, strong overexpression of flagella and reduced colony size[86,87]. Thus, it is plausible that nanotube formation in *B. subtilis* could be associated with the capacity to produce biofilm structures and has a regulatory function in the nanotube elongation process.

The work of Bhattacharya et al., 2019, revealed the identity of a molecular platform for nanotube biogenesis[88]. The CORE apparatus is composed of several copies of five integral membrane proteins, FliP,

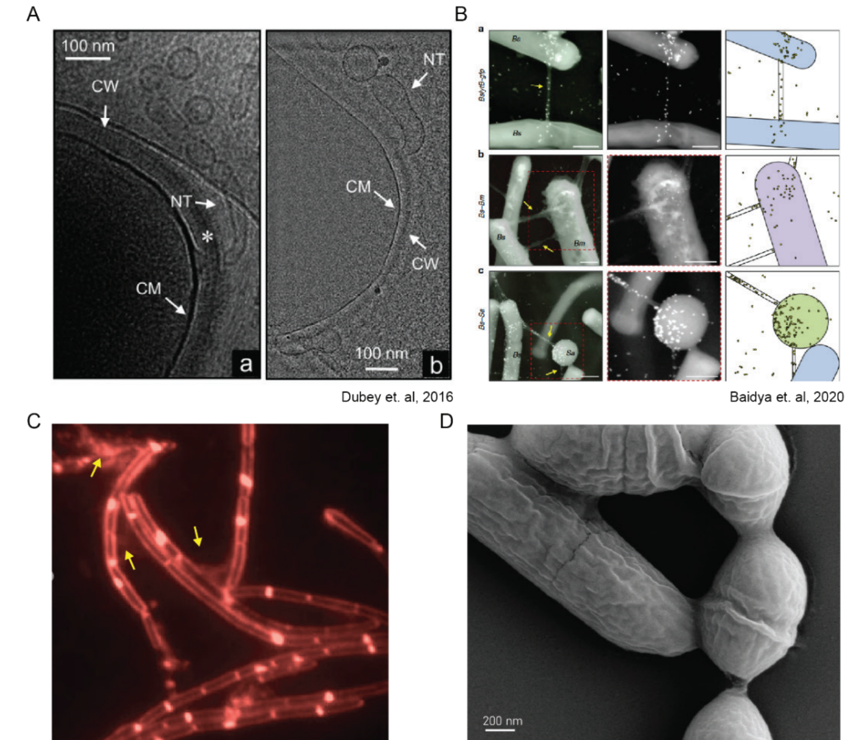

Figure 2. Microscopic pictures of *B. subtilis* nanotubes.
(**A**) Cryo-EM micrographs of *B. subtilis* cells overexpressing *ymdB* emanating nanotubes. Asterisk indicates the place from which nanotubes are released into the environment. (**B**) shows LytB molecules travel along intercellular nanotubes to reach the recipient cell surface. (**C**) membrane staining of *B. subtilis* cells with visible nanotube-like structures (this thesis). (**D**) SEM microscopy of *B. subtilis* and *L. lactis* co-culture, showing merged contact sites for cell-to-cell molecular transfer (**Chapter 2**).

FliQ, FliR, FlhB, FlhA, and a soluble protein FliO, and it is settled at the base of the flagellar motor. Interestingly, similar structures with high homology to CORE proteins were found in the type III secretion system (TS33) injectosome of pathogenic *E. coli*, involved in the extraction of cytoplasmic molecules from infected human host cells via nanotubes[80]. The deletion of CORE genes in *B. subtilis* impairs nanotube production and consequently abolishes the molecular exchange. It has been suggested that when in the planktonic state, bacteria use the CORE complex and promote flagella formation, whereas when grown on solid media, they utilize it for nanotube formation[88].

To transfer cytoplasmic molecules between two neighbouring cells, the donor cell must overcome the cell wall barrier and establish a stable connection with the recipient cell. Baidya et al., 2020, demonstrated that nanotubes are provided with enzymes capable of cutting and

disentangling the complex mesh of peptidoglycan, the main component of the cell wall. Two cell wall hydrolases, LytC and LytB, were found to localize within the nanotubes and were proposed to be among the first delivered cargo to the recipient cell[89] (**Fig. 2B**).

Nanotube-mediated transfer of plasmid DNA and presumably chromosomal DNA offers another route for transferring various genetic traits between different bacteria. When appropriate co-culture conditions are established, the nanotube transfer may be a promising tool for genetic modification of non-competent bacteria. Moreover, a well-characterized and naturally competent *B. subtilis* could serve as an intermediate host for transforming wild isolates or other industrially-relevant bacteria.

Sexual isolation and efficiency of HGT

While conjugation, transduction, natural competence and cell-to-cell DNA transfer significantly contribute to HGT, a successful exchange of genetic material and its functionality in the recipient's cell is determined by sexual isolation[90]. The degree of sexual isolation, or incompatibility between genetically divergent microorganisms, depends on several factors. First, the donor cell or naked donor DNA must be within reach of the recipient and the genetic material successfully transported inside the cell. Following the DNA uptake, the donor's DNA is exposed to the recipient's restriction and modification (R/M) systems. In the case of an acquired unmodified or inappropriately modified donor's DNA, the recipient's restriction nuclease will digest the foreign genetic material[91,92]. Second, for successful integration into the recipient's genomic DNA, the incoming DNA must form a stable DNA heteroduplex based on the homology between donor and recipient DNA. The overall divergence of the DNA sequence between donor's and recipient's DNA lowers the stability of the donor-recipient heteroduplex and consequently decreases the probability of recombination[93].

In *B. subtilis,* the R/M systems appear to have a marginal effect on transformation efficiency, and only a minority of *B. subtilis* strains display restriction activity, which reduces the transformation efficiency by only 2–6 fold[92,94,95]. In contrast, the differences in the homology of incoming DNA versus host DNA are mostly responsible for severe reductions in transformation rates[92,93,96]. To create stable DNA heteroduplex during transformation, *B. subtilis* needs 18–24 nearly identical base pairs at both ends of the donor strand[97].

1.3. GMO regulations and natural strain improvement

The use of genetic engineering and microbes as micro-cell factories has given rise to significant advances in biotechnology and synthetic biology. For instance, the possibility of engineering the *B. subtilis* genome enabled improved heterologous production of chemicals, enzymes, and antimicrobial materials for industry, agriculture, and medicine[12]. Nonetheless, the use of genetically engineered microorganisms for food production and direct environmental applications, especially in Europe, is troublesome due to the strict regulations on using genetically engineered strains in food and feed products and biocontrol agents released to the environment. Genetically modified organisms (GMO) are under severe social criticism that influences the legislation steps and guidelines for GMOs to be placed on the market. The main objections to using GMOs consider adverse effects on biodiversity, the possible toxicity of newly engineered strains or negative effects on the economy of developing countries[98].

But what makes a bacterium a GMO? The latest European directive 2001/18/EC defines GMOs as organisms in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. Hence, only several techniques with considerably lower efficiencies of genetic modification are accepted, including UV-induced random mutagenesis, conjugation, phage transduction and natural transformation, provided they do not use recombinant DNA or involve other GMOs[98–100].

The Lactic Acid Bacteria (LAB) are of great importance to the food industry because of their great potential in producing fermented foods, such as dairy products, fermented vegetables and meats, and alcoholic beverages[101], and they have been marketed as health-promoting probiotics[102]. The LAB strains used for fermentation have been isolated from natural habitats like plants or fermented foods. Their use as starter cultures provides desired organoleptic properties to the final product by enhancing taste and texture and preventing spoilage with lactic acid production. However, producing healthier and better-tasting products with long-term shelf life requires genetic adjustments of commonly used starter cultures or modifications of newly acquired wild environmental strains[99,100]. The most desirable features are improved bacteriophage resistance, improved texture forming ability with the production of exopolysaccharides, introduction of genetic determinants for stress resistance, citrate metabolism, improved urease activity and modifications of carbohydrate metabolism and finally, plasmid curing[100]. Recombinant

DNA technology offers efficient and precise ways for genome modifications. Combined with the current sequencing technologies and the development of the "omics" tools, the physiology of newly engineered strains could be thoroughly studied before their release to the market. However, this approach still cannot be adopted due to the current regulations on GMO-derived products.

The current strain improvement methods are mainly based on random mutagenesis, directed evolution, and dominant selection[100]. The first one has been used extensively in industrial strain modifications by producing a set of mutation variants that can be later screened using high-throughput techniques. However, the introduction of random mutations via UV-mutagenesis not only yields cells with desired mutations but also in many accompanying unintended mutations that can hamper a strain's performance[100]. In the case of directed evolution, a strain is slowly adapted to the desired conditions for further applications. This adaptive strategy is time-consuming and, similarly to random mutagenesis, it can generate unwanted mutations over the adaptation period[103]. The last technique, dominant selection, is based on phenotype selection under stringent growth conditions. If the selection regime is powerful enough, the dominant selection of strains with single mutations can be obtained without using mutagens[100]. However, dominant selection requires considerable knowledge of the strain's physiology, and like the previous method, can be time-consuming.

A valuable addition to the classical methods for strain improvement is HGT. Since the use of naturally occurring gene transfer methods is in accordance with current European directives, in recent years, the interest in the HGT-mediated mobilization of genetic traits has been renewed[39,98,104], and the novel HGT method constituted by nanotube transfer is a welcome addition to the non-GMO toolbox.

2.1. Osmostress response in *B. subtilis*

Because of interchanging periods of flooding and drying of the topmost layers of the soil, *B. subtilis* is frequently challenged with osmotic stress[105]. To survive under hypo- and hyperosmotic conditions, this Gram-positive bacterium adapts a step-wise response strategy to maintain turgor and minimize the damage caused by rapid water fluxes[106]. When facing hypoosmotic stress, the cell rapidly jettisons ions and metabolites through the mechanosensitive channels to reduce the driving force for water influx and avoid cell rupture[105,106]. On the other hand, the cell increases the osmotic potential of the cytoplasm when it encounters hyperosmotic conditions to avoid dehydration. In the primary response to hyperosmotic stress, the cell initiates uptake of water-attracting ions such as potassium, involving two potassium uptake systems, KtrAB and KtrCD[107]. The accumulated ions are subsequently replaced, at least partially, by compatible solutes, or osmoprotectants, to prevent the development of a long-lasting high ionic-strength cytoplasm[108].

B. subtilis utilizes a set of transporters from the Opu family to import a variety of compatible solutes[109]. Two potent osmoprotectants, proline and glycine betaine, can be acquired directly from the environment via OpuE and OpuA-D, respectively, but they can also be synthesized by the cell from their precursors: proline from glutamate and glycine betaine from the choline pools[110–113]. Notably, only proline can be synthesized *de novo*[108,112,114]. In members of *Bacillus sp.*, proline is synthesized in three enzymatic steps carried out by γ-glutamyl kinase (ProB/ProJ), γ-glutamyl phosphate reductase (ProA/ProAA) and Δ^1-pyrroline-5-carboxylate reductase (ProI/ProH/ProG/ComER)[115–118]. *B. subtilis* employs two distinct biosynthetic routes: the anabolic and the osmo-adaptive one, and both pathways are interconnected via γ-glutamyl phosphate reductase, ProA[115]. For the anabolic route, the cell activates ProB-ProA-ProI enzymes to sustain basal proline levels of about 10–20 mM[108,113,114]. Under osmotic stress, the cell's demand for proline increases, and the cell employs ProJ-ProA-ProH enzymes to rapidly elevate the intracellular proline pools[112,115]. In the case of a severe osmotic upshift, the upregulation of the osmo-adaptive route yields from 20 mM to 500 mM of proline[108,112,114].

In addition to the cellular alterations, the σ^B-controlled general stress response system also contributes to the cell's ability to cope with osmotic stress[119,120]. It has been reported that continuous growth of *B. subtilis* cells in high salt concentrations sustains activation of the general stress response, albeit to a much lower degree than cells subjected to a sudden osmotic upshift[121].

2.2. VBNC state

When exposed to harsh environmental conditions, microorganisms display several phenotypes that can endure long periods of stress. The best-studied examples are highly-resistant endospores, well-studied in Bacilli[122]. These dormant spores are metabolically inactive, waiting for the appropriate conditions to germinate and repopulate the

environment. Besides spore formation, bacteria can enter into persister or viable but non-culturable (VBNC) state[123]. These dormant cell variants can be differentiated from truly dormant endospores since, unlike spores, they maintain basal metabolic rates and do not undergo evident morphological changes[124,126].

Persisters were identified as a subpopulation of nongrowing cell variants highly tolerant to antibiotics due to the extremely slow metabolic rates[127]. These dormant cell variants were observed to form two types of persistent cells. Type I persisters were generated at the stationary phase, and they displayed a significant lag time after antibiotic removal[128,129], whereas type II persisters occurred stochastically through phenotype-switching mechanisms that seemed to be independent of entering the stationary phase[128,130]. Mechanisms governing the formation of persister cells include stringent response to amino acid starvation or even oxidative stress response and phage shock[128,131,132]. Notably, the hallmark of bacteria switching to a persistent state is the biphasic killing curve of an exponentially growing culture exposed to lethal antibiotics (**Fig. 3**)[133]. Initially, the first killing phase shows a rapid drop in culturability, representing the susceptible fraction of the population. In the second phase, the decrease in cell count is milder, and the residual persisting cells (~1% of the population) are still viable and culturable after the antibiotic removal.

VBNC cells share many characteristics with persister cells[123]. Similarly to persisters, the VBNC cells are stochastically present in the population[130,134] but can also be generated by environmental stresses[135–137]. Moreover, similar to persisters, VBNC cells are metabolically active, however, they have lost the ability to regrow on a medium that typically supports proliferation after the antibiotic pressure is removed[134,136,138–140]. While persisters can rapidly regrow on growth-promoting media, the VBNC cells require prolonged treatment to regain full culturability. The similarities and co-occurrences of both phenotypes in the same culture suggested that these survival strategies are part of the dormancy continuum in which VBNC cells are in a deeper dormancy state than persisters[134,141,142].

Cells in the VBNC state can be resuscitated with appropriate stimuli such as amino acids, gas mixture, heat shock, supernatants of growing cells, as well as the resuscitation promoting factor discovered in *Salmonella enterica*[143]. However, so far, the exact mechanism underlying VBNC state induction and resuscitation is not fully explored[138,141,143].

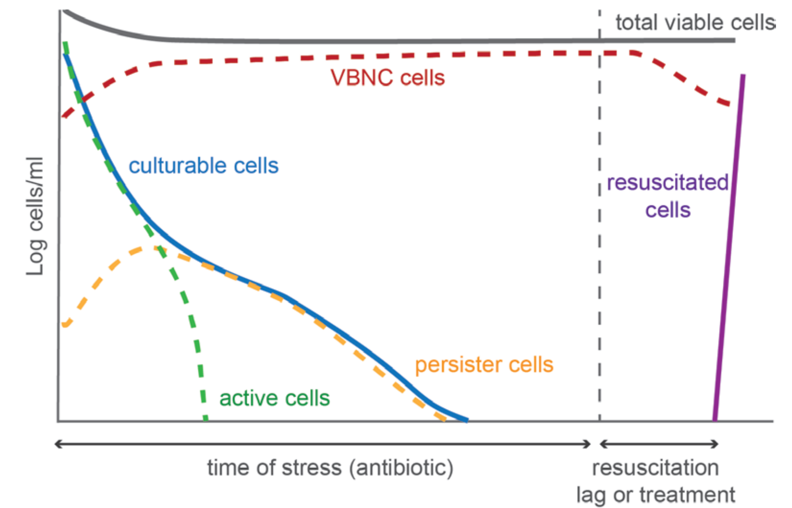

Figure 3. Experimental dormancy dynamics of antibiotic persistence and the VBNC state (adapted from Ayrapetyan et al., 2018)
When an exponentially growing culture is exposed to a lethal concentration of antibiotics, the remaining cells that can be further revived on the growth-promoting media are defined as persisters. The hallmark of the presence of persister cells in the culture is the biphasic killing curve, indicated with the orange dashed line. When the antibiotic is removed from the media (the grey dashed line), after an appropriate lag time, which duration directly correlates with the period of the antibiotic exposure, the persister cells repopulate the environment (the purple line). In the case of the VBNC state indicated with the red dashed line, most of the cells that encounter severe stress stimuli remain viable but cannot be cultured on the standard growth media. However, these cells can be resuscitated upon specific conditions that vary from cell to cell.

3. Scope of this thesis

In this thesis, we explore two independent research questions. In the first part of this work (**Chapters 2** and **3**), we address the importance of the cell-to-cell interactions in the intra- and interspecies HGT in *B. subtilis* and LAB members. We show that molecular transfer between neighbouring cells may greatly contribute to the HGT in natural environments, but also it can be a promising tool in the laboratory setup for the natural strain improvement for the development of new starter cultures. In **Chapter 2**, we developed a co-culturing protocol and provided proof of transfer of a small high copy non-conjugative plasmid from *B. subtilis* to LABs and vice versa. In **Chapter 3**, we performed a case study of cell-to-cell intra- and interspecies protein transfer and explored the possibility of the ComK delivery from a hyper-competent donor strain to the *comK*-deficient strains of *B. subtilis* and induction of natural transformation with plasmid DNA.

General introduction

The second part of the thesis (**Chapters 4**, **5**, **6**) sheds new light on the adaptational transcriptional and physiological responses to osmotic and antibiotic stress in the laboratory strain of *B. subtilis* 168. In **Chapter 4**, we studied the kinetics of the activation of the osmotically-controlled *proHJ* promoter on a single-cell and population-wide level. Under mild osmotic upshift conditions, we observed a switching point and heterogeneous proline biosynthesis gene expression on a solid media, showing that only part of the population will switch to proline synthesis and likely supply it to the neighbouring cells. Additionally, we demonstrate that the osmotically-controlled proline biosynthesis pathway is also involved in the antibiotic-mediated stress response in *B. subtilis*, suggesting a new role of proline in antibiotic resistance. Consequently, this study prompted us to explore further the response to changing environments and how the preadaptation to osmotic stress affect the antibiotic's efficacy in *B. subtilis* cells. In **Chapter 5**, we studied physiological changes caused by pre-exposure to osmotic stress and simultaneous aminoglycoside treatment in *B. subtilis* cells. Using microfluidics combined with time-lapse microscopy, we followed the uptake of fluorescently-labelled kanamycin and examined the metabolic activity of differently preadapted populations at a single-cell level. Combining single-cell studies and population-wide analysis of differently preadapted cultures, we demonstrate for the first time that kanamycin-tolerant *B. subtilis* cells could be entrapped in a viable but VBNC state. This study highlights the importance of the bacterial growth conditions and metabolic state prior to the antibiotic treatment. Using a robust RNA-Seq, the differences in transcriptional profiles of *B. subtilis* VBNC cells, kanamycin-sensitive cells and culturable cells without kanamycin treatment are described and presented in **Chapter 6**. This study's comparative analysis of differently expressed genes and operons indicated high similarities in the transcriptional responses of VBNC and kanamycin-sensitive cells. Moreover, we showed that VBNC cells strongly upregulate genes involved in proline uptake and catabolism, suggesting a putative role of proline as a nutrient in VBNC cells.

The last part of this work summarizes the most recent reports about bet-hedging phenomena in various microorganisms. **Chapter 7** shows how versatile a bet-hedging strategy is within the microbial realms.

Finally, in **Chapter 8**, we summarize the results of all chapters and discuss the future perspectives, presenting new research lines.

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