Proteostasis in Bacillus subtilis

chaperones and stress-response mechanisms

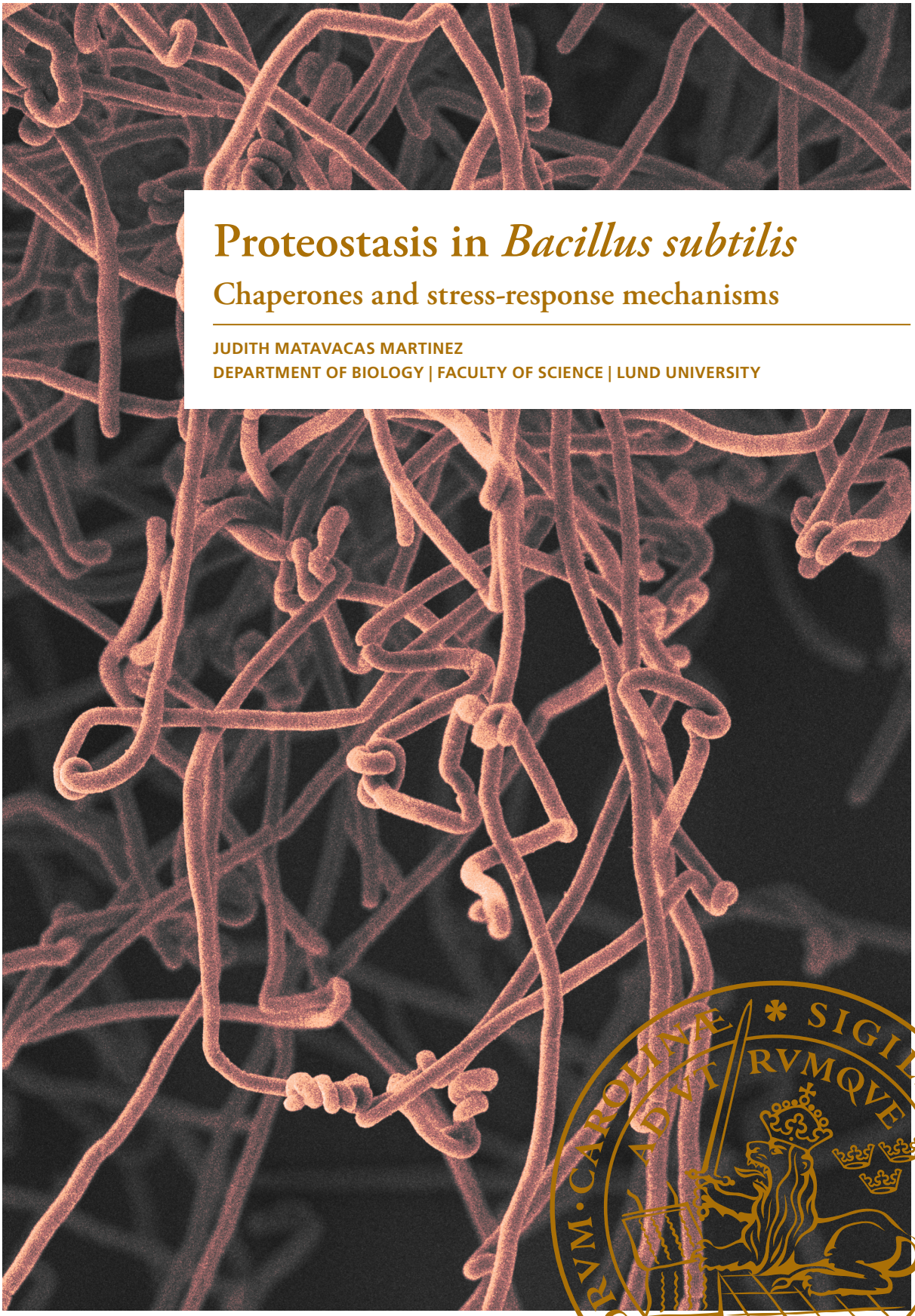
Matavacas, Judith

2023

Proteostasis in *Bacillus subtilis*

Chaperones and stress-response mechanisms

JUDITH MATAVACAS MARTINEZ
DEPARTMENT OF BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY



Proteostasis in *Bacillus subtilis*

Chaperones and stress-response mechanisms

Judith Matavacas Martinez

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Science at Lund University to be publicly defended on the 16th of June 2023 at 09.00 in the Lecture Hall, Department of Biology, Sölvegatan 35, Lund, Sweden.

Faculty opponent
Dr. Kristina Jonas, Stockholm University, Sweden

Organization: LUND UNIVERSITY

Document name: Doctoral dissertation **Date of issue:** 2023-06-16

Author: Judith Matavacas Martinez

Title and subtitle: Proteostasis in *Bacillus subtilis* – Chaperones and stress-response mechanisms

Abstract:

The maintenance of protein homeostasis, also referred to as proteostasis, is essential for preserving the structural and functional stability of the proteome in living organisms. This involves the precise control of protein synthesis, folding, and degradation. As proteostasis disruption can cause growth and survival deficiencies in bacteria, the investigation of proteostasis has rapidly emerged as a growing field of study, and targeting proteostasis has become an attractive strategy for combating bacterial infections.

In this thesis proteostasis was studied in the Gram-positive model organism *Bacillus subtilis*. The research primarily concentrated on two distinct aspects of proteostasis: chaperones (which are highly conserved protein-folding "machines" that play a central role in maintaining proteostasis) and response mechanisms to proteotoxic stress.

Previous studies have extensively examined the role of chaperones and their effects on cell function upon removal in the Gram-negative bacterial model *Escherichia coli*. Research in other kinds of bacteria is needed. The findings presented in Paper I build on our current knowledge about proteostasis in bacteria by showing the pleotropic phenotype caused by the combined removal of the DnaK and trigger factor chaperones in *B. subtilis*. The effects include altered cell morphology, thermotolerance, and cell wall integrity. Paper I also shows how *B. subtilis* can partially adapt to chaperone deficiency by acquiring second-site suppressor mutations in genes required for different biological processes. Paper II shows that chaperone removal not only affects planktonic cells, but also influences *B. subtilis* multicellularity (i.e., formation of biofilm communities). Mutant biofilms are architecturally aberrant and display an altered presence of cell types.

The second part of my study was on the function of the Spx-YjbH system in coping with disulfide stress (a proteotoxic stress condition). Spx is an important transcriptional regulator of the proteotoxic stress response; YjbH is an adaptor protein for Spx, whose levels are regulated by stress-induced protein aggregation. Paper III provides insights into this system by studies at a single cell level, with special focus on the localization, dynamics and inheritance of YjbH aggregates. It also concerns the contribution of the different YjbH protein domains to aggregation function and YjbH orthologs of the Gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes*.

Key words: protein homeostasis, chaperones, DnaK, trigger factor, Spx, YjbH, *Bacillus subtilis*

Language: English

ISBN: 978-91-8039-669-1 (print), 978-91-8039-670-7 (electronic)

Number of pages: 76

Signature Date 2023-04-26

Proteostasis in *Bacillus subtilis*

Chaperones and stress-response mechanisms

Judith Matavacas Martinez

LUND
UNIVERSITY

Coverphoto by Judith Matavacas and Ola Gustafsson. Backphotos by Judith Matavacas

Faculty of Science
Department of Biology

ISBN (print) 978-91-8039-669-1
ISBN (electronic) 978-91-8039-670-7

Printed in Sweden by Media-Tryck, Lund University
Lund 2023

Media-Tryck is a Nordic Swan Ecolabel
certified provider of printed material.
Read more about our environmental
work at www.mediatryck.lu.se
MADE IN SWEDEN

To my family

Table of Contents

Preface

Protein homeostasis (proteostasis) is fundamental to maintain the integrity of the proteome, and involves the proper regulation of synthesis, folding, and degradation of proteins. The study of proteostasis has become a rapidly growing field as proteostasis disruption can result in growth and survival defects in bacteria, making targeting proteostasis an attractive antibacterial strategy.

This thesis explores different aspects of proteostasis in *Bacillus subtilis*, a model organism for Gram-positive bacteria. These aspects include chaperones and response mechanisms to proteotoxic stress.

Chaperones are highly conserved protein folding "machines" that are central to proteostasis. Chaperones and the consequences of chaperone removal for the cell have been extensively studied in the Gram-negative model organism *Escherichia coli*, in contrast to other bacteria. The work I present builds on the current knowledge about proteostasis in bacteria by investigating the effects of chaperone absence (DnaK and trigger factor chaperones) in *B. subtilis*, both in the widely studied domesticated strain and in the biofilm-forming ancestral strain.

Proteotoxic stress conditions challenge the integrity of the proteome. My work also focuses on how *B. subtilis* responds to proteotoxic stress by using the Spx-YjbH response mechanism and provides new insights into this mechanism by performing studies at a single-cell level.

Popular scientific summary

Cells are the smallest unit of life. Some organisms, like plants and animals, consist of billions of cells, while others, like bacteria, are just one cell. Most of the important processes in a cell are carried out by proteins. Proteins in the cell have different shapes and, like tools in a toolbox, each is useful for a different task. While some proteins can acquire their shape on their own, others need to be helped by other proteins. These helping proteins are called chaperones.

In a growing cell, the chaperones are never short of work. The cell is constantly producing new sets of proteins to replace the old and damaged ones. This is a way for the cell to always be prepared for the next situation. To avoid being burdened with unused proteins, the cell recycles them with proteases. Proteases, like chaperones, are also a type of helping proteins. Instead of shaping proteins, proteases break them down. The pieces of the broken-down proteins are usually reused to make new proteins. For a cell to function well, there must be a balance between the making and breaking of proteins. This balance is called proteostasis.

This thesis is about proteostasis in a bacterium called *Bacillus subtilis*. For many years, this bacterium has been used as a model to understand how cells work. *Bacillus subtilis* is important not only in fundamental research, but also in the biotechnological industry for the production of multiple products. However, proteostasis in *Bacillus subtilis* is still quite unexplored.

With the increase of antibiotic resistance, we need to find new ways to defeat bacteria. Damaging proteostasis causes defects in bacteria, so hindering proteostasis could be a possible antibacterial strategy. Proteostasis damage has been extensively studied in one model organism called *Escherichia coli*, but this knowledge has to be complemented with studies in other bacteria like *Bacillus subtilis*.

In the first and second papers of this thesis, the proteostasis system of *Bacillus subtilis* was damaged by removing two important chaperones. This was followed by subjecting the bacterium to different stress conditions, such as high temperature or antibiotics. We observed that damaging proteostasis in *Bacillus subtilis* made the bacterium weaker and less capable of coping with stress. This indicates the potential of this strategy, i.e., damaging proteostasis, in defeating pathogenic bacteria.

However, when the proteostasis system is damaged, *Bacillus subtilis* produces a special protein called Spx, among other proteins. The task of Spx is to repair the damage and restore proteostasis, resulting in bacterial survival. The third paper increases our understanding about how *Bacillus subtilis* uses and regulates Spx under stress, and the effect of Spx repair on bacterial adaptation to stress.

Ultimately, the findings of this thesis could be useful in the fight against bacterial pathogens and antibiotic resistance.

List of papers

Paper I

Judith Matavacas, Joel Hallgren and Claes von Wachenfeldt (2023). *Bacillus subtilis* forms twisted cells with cell wall integrity defects upon removal of the molecular chaperones DnaK and trigger factor. *Frontiers in Microbiology*, 13:988768. DOI: 10.3389/fmicb.2022.988768

Paper II

Judith Matavacas and Claes von Wachenfeldt (2023). Removal of the molecular chaperones DnaK and trigger factor in *Bacillus subtilis* affects biofilm formation. Manuscript.

Paper III

Judith Matavacas, Deepak Anand and Claes von Wachenfeldt (2023). New insights into the disulfide stress response by the *Bacillus subtilis* Spx system at a single-cell level. Manuscript.

Other relevant publications not included in this thesis

Judith Matavacas and Claes von Wachenfeldt (2022). Update on the protein homeostasis network in *Bacillus subtilis*. *Frontiers in Microbiology*, 13:865141. DOI:10.3389/fmicb.2022.865141

My contributions to the papers

Paper I

I took part in the design and planning of the study. I took part in acquiring and analyzing experimental data. I wrote the initial draft of the manuscript and participated in the editing and revision of the manuscript.

Paper II

I took part in the design and planning of the study. I acquired and analyzed experimental data. I wrote the initial draft of the manuscript and participated in the editing and revision of the manuscript.

Paper III

I took part in the design and planning of the study. I took part in acquiring and analyzing experimental data. I wrote the initial draft of the manuscript and participated in the editing and revision of the manuscript.

Chapter 1. Introduction – a balanced proteome

Cells are crowded with thousands of different proteins. Proteins are the workforce of the cell, performing myriad of tasks, from catalyzing chemical reactions to providing cellular scaffolds and transporting substances in and out of the cell.

Even though all proteins are synthesized from the same building blocks (amino acids), proteins differ in amino acid sequence and adopt different shapes. And sequence and shape define function. After being translated by the ribosome, and to be functional in the cell, each protein has to fold into its specific three-dimensional structure, referred to as the native conformation [1]. For the majority of soluble proteins, folding is to a large extent driven by burying amino acid residues with hydrophobic side chains in the core of the protein while exposing those with polar side chains on the surface [1, 2]. Apart from having a hydrophobic core, other forces such as hydrogen bonds, electrostatic interactions, van der Waals interactions, and disulfide bonds contribute to achieve a protein´s native conformation [3].

Many proteins can readily without external support go from an unfolded, energetically expensive conformation, to a thermodynamically favored native conformation [4]. Other proteins have a more complex folding process, going through several folding intermediates before reaching their native state, and this process usually requires the assistance of molecular chaperones [2, 5] (discussed further in Chapter 3).

To ensure that the cellular amount of properly folded and functional proteins is sufficient for essential biological processes, as well as to prevent the accumulation of non-native protein species that can negatively affect these processes, cells possess a complex molecular network for protein homeostasis (proteostasis). The proteostasis network is necessary for the correct functioning of the cell under normal conditions, and it becomes even more crucial under stress conditions that challenge protein stability. Proteostasis is maintaining the balance between synthesis, folding, post-translational modification, transport, and degradation of proteins [6-8] (Figure 1). Key components of the proteostasis network are chaperones, which assist in the correct folding of many proteins and also prevent protein misfolding and formation of aggregates. Very important are also proteases, which eliminate proteins that are no longer necessary for the cell, or that are permanently damaged [9-11]. Although

the proteostasis networks vary among species, their major constituents (chaperones and proteases) are highly conserved and occur in all domains of life [12].

Challenges in protein stability and proteotoxic stress

Proteins are not static structures. In fact, proteins have evolved to be highly dynamic since protein function often entails undergoing conformational changes. For instance, enzymes usually change conformation upon substrate binding. In addition, most proteins are only slightly more stable when they are folded compared to when they are unfolded. This means that even minor changes in a protein's environment can impact the balance between these two states. Protein folding stress or proteotoxic stress challenges all living organisms and refers to the formation and accumulation of non-native protein conformations [13].

The stability of a protein´s native conformation is affected by several conditions, including environmental and cellular stresses. For instance, a change in temperature, pH, osmolarity, ionic strength, or macromolecular crowding can challenge protein stability by disrupting interactions within the polypeptide and lead to protein unfolding or misfolding [4]. Oxidative stress can misfold proteins by the covalent modification of specific amino acid residues, such as incorrect formation of disulfide bonds [14]. Elevated temperatures denature proteins by increasing their molecular motion and by that cause exposure of hydrophobic regions of the protein that are normally buried in the folded state, leading to irreversible aggregation [15]. In addition to environmental stresses, post-translational modifications, mistranslation or genetic mutations change a protein´s primary sequence which can affect folding properties and thus protein function [16].

Accumulated non-native protein species with exposed hydrophobic residues are likely to interact with each other through unspecific hydrophobic bonds, forming stable macromolecular protein clusters called aggregates [2, 17]. Besides that misfolded or aggregated proteins are unable to perform their functions in the cell, they can also bind to and associate with other folding intermediates or functional proteins, disrupting important cellular processes [13, 17]. Accumulation of protein aggregates is associated with aging and diseases such as Parkinson´s and Alzheimer´s in humans, and with defects in growth and survival in prokaryotes [10, 18]. Furthermore, protein aggregates are closely associated with dormant antibiotic-resistant bacteria known as persisters [19-23]. There are cases, however, in which aggregation of specific proteins has beneficial regulatory means [13]. For instance, as will be discussed in Chapter 5, aggregation of the *B. subtilis* protease adaptor protein YjbH under proteotoxic stress conditions results in an increase in Spx transcription factor levels, which in turn change gene expression to cope with stress [24-26].

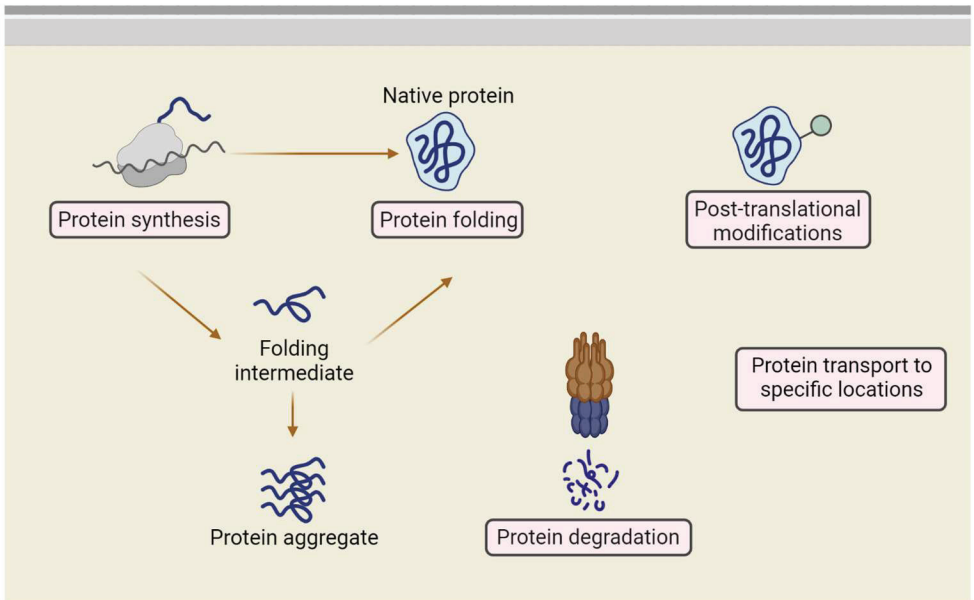


Figure 1. Main cellular processes involved in proteostasis maintenance.
Newly synthesized polypeptide chains can go through several folding intermediates before reaching their native state. Folding can be spontaneous, or be assisted by molecular chaperones. Protein stability is challenged by various stress conditions upon which proteins misfold, and in some cases aggregate. Non-native or not needed protein species are often degraded by proteases. Proteostasis is essential for maintaining the appropriate amout of native proteins in the cell and for preventing protein misfolding and aggregation. Proteostasis is a dynamic operation involving various processes such as synthesis, folding, post-translational modifications, transport, and degradation of proteins. Created with BioRender.com.

Bacterial proteostasis

Although the fundamental principles that control proteostasis are universal across all domains of life, proteostasis maintenance is particularly challenging for bacteria, because they are subjected to frequent changes in their surrounding environment including temperature variations, oxidative stress, osmotic shock, antibiotics, and for pathogens also host defenses (like reactive oxygen and nitrogen species). In contrast to robust communities of bacteria, biofilms, which are more resistant to stress factors, planktonic bacteria are more directly exposed to proteotoxic stressors than multicellular organisms. Moreover, the protein turnover rates are generally higher in bacteria than in eukaryotes [27-29]. In bacteria compared to eukaryotes, and at any given time, there are a greater number of newly synthesized polypeptides in the midst of folding, which are particularly prone to misfolding and aggregation [30].

Sophisticated proteostasis networks and mechanisms to cope with proteotoxic stress allows bacteria to survive and ensures competitiveness with other microorganisms and successful host colonization. Because of the great importance that proteostasis maintenance has for bacterial growth and survival, inducing proteostasis collapse has been proposed as an antibacterial strategy [27]. Increasing our understanding on how bacteria maintain proteostasis under normal and stress conditions can help us in the fight against pathogens, but also in optimizing the performance of bacterial strains used in the biotechnological industry.

In this thesis, proteostasis in the Gram-positive model organism *B. subtilis* will be explored.

Chapter 2. *Bacillus subtilis*

Bacillus subtilis is a Gram-positive bacterium of the phylum *Firmicutes* (now called *Bacillota* [31]), found in soil, water sources and in association with plants. Among other features, *B. subtilis* is non-pathogenic, rod-shaped, naturally competent, and forms endospores [32, 33].

B. subtilis was first described by Ferdinand Cohn in 1872 [34] - although it was discovered already in 1835 as *Vibrio subtilis* by Christian Gottfried Ehrenberg [35]. Since then, much effort has been directed towards the study of its physiology, genetics and biochemistry. At present, it is one of the most characterized organisms and it is used as a model for Gram-positive bacteria, with plenty of accumulated knowledge and with many genetic and molecular tools available for use in further studies.

The long-term interest in studying *B. subtilis* is due to several reasons, one of them being its capability to differentiate into an endospore. Investigation of sporulation in *B. subtilis* provides fundamental knowledge about the cellular development and differentiation processes. The fact that *B. subtilis* is highly amenable to genetic manipulations was another reason for its attractiveness in research. Its ability to secrete enzymes (such as amylases and proteases) at high yields, and the absence of toxic by-products make *B. subtilis* also interesting for industrial applications [32, 33].

B. subtilis as a model to study proteostasis

When it comes to understanding the fundamental principles of proteostasis in bacteria, *B. subtilis* is a suitable study organism because it survives a wide range of environmental settings and proteotoxic challenges, making it one of the most versatile model organisms. As R. Losick wrote in year 2020, *B. subtilis* is “a bacterium for all seasons” [36].

Importantly, most of the knowledge on chaperones and proteases in bacteria comes from research on the Gram-negative model organism *E. coli*. *B. subtilis* provides us with very valuable knowledge on basic biological processes which is complementary to that gained from studies with *E. coli*. There is approximately two billion years of evolutionary separation between *E. coli* and *B. subtilis*, suggesting

that knowledge obtained with *B. subtilis* can be extrapolated and used as a base to better understand other related Gram-positive bacteria, which include pathogens such as *Bacillus anthracis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, and *Clostridium difficile*. Also, many Gram-positive bacteria, for example, various lactic acid bacteria and *Bacillus licheniformis* are important for biotechnology.

A bacterium with a complex lifestyle

The fact that *B. subtilis* is a very versatile bacterium, able to survive under a broad range of stress conditions, is mainly due to its lifestyle, characterized by several differentiation processes, such as sporulation, induction of competence, motility, and formation of biofilms [37-40]. While these processes are also found in other bacterial species, *B. subtilis* distinguishes itself by possessing an extensive collection of cell types. Here below, I will introduce the main cell types that *B. subtilis* can differentiate into, as well as the key regulators involved in each of these differentiation processes. Note that the processes described not only are regulated in a highly complex manner, but on many occasions partly overlap with each other, sharing key regulators.

Sporulation

B. subtilis has the ability to differentiate into endspores when exposed to prolonged nutrient starvation conditions. Spores are partially dehydrated, metabolically inactive, and highly resilient cells. Compared to the vegetative cell, they resist UV radiation, extreme heat, desiccation, and other challenges [41]. The formation of spores is one survival strategy employed by only certain bacteria to preserve their genetic material and endure harsh environmental circumstances until better conditions arise.

During sporulation, the *B. subtilis* cell divides asymmetrically, resulting in two distinct compartments: the mother cell, which is larger, and the forespore. The mother cell membrane then migrates around the forespore to fully engulf it. The next stage involves the formation of two protective layers around the forespore: the cortex layer made of peptidoglycan and the coat which is composed of many different proteins. Once the endospore reaches maturity, the mother cell lyses, releasing the spore into the environment (Figure 2). When the environment improves, spores can rapidly germinate and revert to the vegetative state [41].

The process of sporulation in *B. subtilis* is complex, taking many hours to complete, and is initially controlled by the master regulator Spo0A. Nutrient limitation is detected by several protein kinases, which eventually lead to activation of Spo0A

by phosphorylation [42]. High levels of Spo0A~P trigger sporulation [43], inducing the transcription of hundreds of genes, including those responsible for asymmetric cell division and the activation of sporulation-specific sigma factors [44]. The first sporulation-specific sigma factor F (σ^F) is activated in the forespore. A cascade of events occurs that result in activation of the alternative sigma factors σ^E, σ^G, and σ^K, each of which regulates different sets of genes involved in specific stages of sporulation, either in the mother cell or the forespore [44].

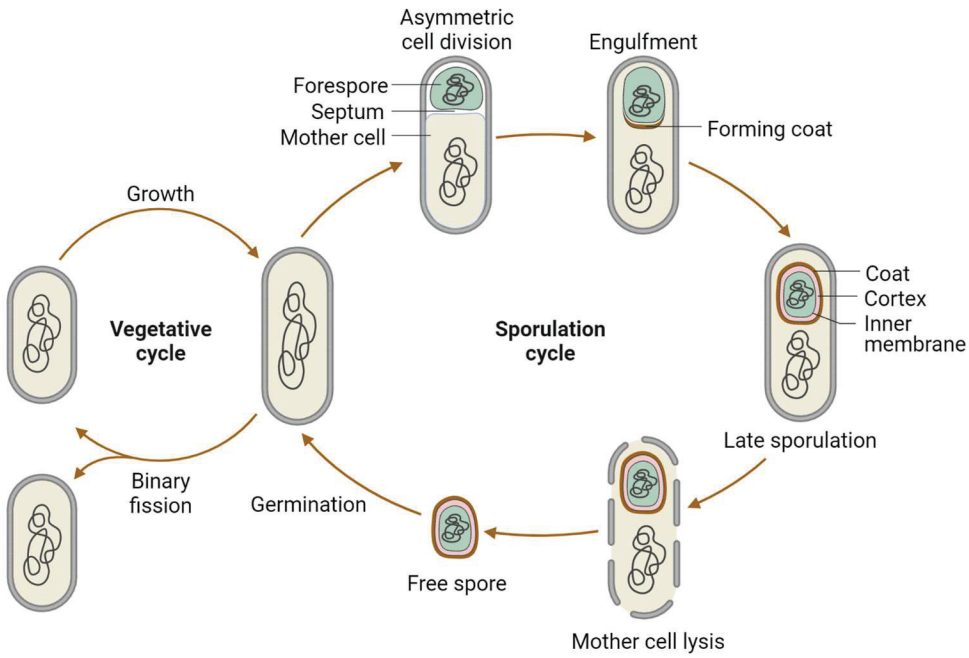


Figure 2. The vegetative and sporulation cycles in *B. subtilis*.
Nutrient limitation triggers sporulation, which is initiated with asymetric cell division, generating a mother cell and a forespore separated by a septum. The forespore is engulfed by the mother cell, and two protective layers (a peptidoglycan cortex and a proteinaceous coat layer) form around the forespore. After lysis of the mother cell, the mature spore is released. With nutrient availability, the spore can germinate and enters the vegetative cycle in which growth is driven by binary fission. Adapted from [41] and created with BioRender.com.

Given that spore differentiation is a costly process and, once started, an irreversible process, the decision to sporulate is elaborate for the cell. There is heterogeneity in the cellular levels of activated Spo0A among the population [45]; cells with sufficient Spo0A~P induce the expression of two operons: the *skf* (sporulation killing factor) operon and the *sdp* (sporulation delaying factor) operon, which lyse surrounding cells (with lower levels of Spo0A~P) to access their nutrients and

postpone sporulation. If nutrients from the lysed cells are insufficient, Spo0A~P levels will continue to rise, ultimately triggering sporulation to occur [46].

Swimming motility and matrix production

B. subtilis can move using flagellum-based swimming motility, enabling the bacterium to explore and colonize various environments. This type of motility relies on the expression of the *fla/che* operon, comprised of 31 genes. These genes encode various proteins, such as those involved in constituting the basal body of the flagellum, chemotaxis, and production of the alternative sigma factor σ^D. σ^D induces the expression of the *hag* locus, which is responsible for flagellin production (the protein that makes up the flagellar filament). Additionally, σ^D facilitates cell separation by inducing the expression of genes encoding autolysins (*lytA*, *lytD*, *lytF*) and flagellar motor proteins (*motA*, *motB*) [47, 48]. The induction of the *fla/che* operon is regulated by the master regulator DegU, which directly binds to the promoter region, and whose binding is enhanced by the SwrA protein [49-51].

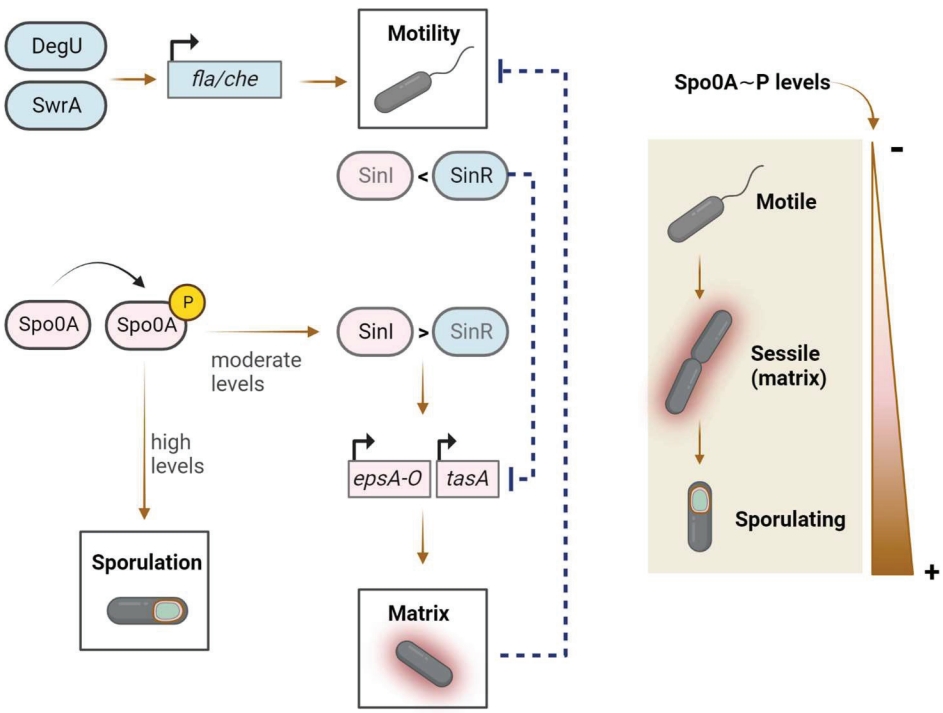


Figure 3. Simplified scheme of the regulatory network controlling cell differentiation in *B. subtilis*. Swimming motility requires expression of the *fla/che* operon and is mainly regulated by the DegU and SwrA regulators. In motile cells, the levels of the SinR repressor are higher than the levels of the SinR

anti-repressor SinI. The SinR repressor inhibits expression of the matrix operons *epsA-O* and *tapA-sipW-tasA*. Moderate starvation conditions lead to an increase in the levels of phosphorylated Spo0A, resulting in the levels of SinI being higher that those of SinR, subsequently de-repressing the matrix operons (and causing the differentiation into a matrix-producer cell). The switch from motile to sessile can also occur spontaneously for a subset of cells under favourable growth conditions. This switch is driven by stochastic fluctuations in the SinI levels. Prolongued nutrient starvation causes the levels of Spo0A~P to increase further, inducing the differentiation into a sporulating cell. Matrix production and motility are two mutually exclusive cell types. Dashed lines indicate inhibition. The illustration on the right depicts that different levels of Spo0A~P determine cell differentiation. Adapted from [52]. Created with BioRender.com.

B. subtilis is unique in that, despite most cells being motile during constant growth conditions, a subset of cells can spontaneously transition to a sessile cell type, characterized by chaining and production of extracellular matrix components. The switch from motile to sessile relies on a stochastic mechanism that involves fluctuations in the levels of the SinR and SinI proteins (Figure 3). SinR functions as a repressor of the sessile state [53, 54], while SinI acts as a SinR anti-repressor [55]. Though SinR primarily represses under favourable growth conditions, stochastic fluctuations can boost SinI levels and trigger the switch to the sessile state [36, 50].

In the sessile state the cells produce extracellular matrix, which requires the induction of two main operons: the 15-gene *epsA-O* operon (responsible for the production of the EPS exopolysaccharide), and the *tapA-sipW-tasA* operon (responsible for the production of TasA, the main matrix protein component that forms fibers) [53, 54]. The EpsE glycosyltransferase, involved in EPS synthesis, inhibits flagellar rotation [56]. Thus, motility is inhibited as a result of matrix production, making these two cell types mutually exclusive (Figure 3).

Under conditions that are favourable for the population, what can be the benefit of switching from a motile to a sessile state? While motile cells can explore new niches, sessile cells produce a matrix that enables them to attach to surfaces, facilitating the exploitation of their environment [36].

Group motility: swarming and sliding

Swimming (described above) is a behaviour of the individual cell, where the bacterium uses flagella to move through a liquid environment. There are other types of motility, however, that *B. subtilis* exhibits when grown on soft surfaces: swarming and sliding (Figure 4). In these two types of multicellular motility, bacteria migrate together to overcome high surface tension.

In the case of swarming, bacteria cluster together, become hyperflagellated, and secrete the lipopeptide surfactin (surfactant that reduces the surface tension) [57]. Swarming is mainly regulated by SwrA which, as mentioned before, is a key regulator of the *fla/che* motility operon and controls the density of flagellar basal bodies [58]. In liquid environments, SwrA is degraded by the AAA+ protease LonA, and this degradation is mediated by the SmiA adaptor protein. Upon contact with a

solid surface, proteolysis of SwrA is inhibited, which increases SwrA levels and activity, ultimately leading to hyperflagellation and swarming [59].

In sliding motility, flagella are not required. Instead, the bacterial colony expands by cell growth, and this expansion requires the secretion of surfactin, as well as EPS and the BslA hydrophobin protein [60-62]. The main regulator of sliding motility is Spo0A. The levels of Spo0A~P needed to trigger sliding are lower than those needed to induce sporulation and biofilm formation (high and moderate Spo0A~P levels, respectively) [61].

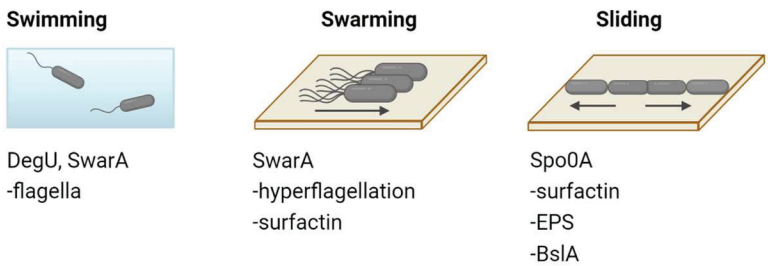


Figure 4. *B. subtilis* main types of motility.
Swimming in liquid is an individual type of motility, powered by flagella rotation and mainly regulated by DegU and SwrA. Swarming, which is also flagella-driven, is a type of multicellular motility that occurs on a surface. Swarming depends on the SwrA regulator, which induces hyperflagellation and the production of surfactin to reduce the surface tension. Sliding motility (also a type of multicellular motility) is driven by bacterial growth and requires surfactin and the production of other extracellular compounds such as EPS and the hydrophobin BslA. Sliding is mainly regulated by Spo0A. Created with BioRender.com.

Natural competence

B. subtilis cells can also undergo a transient state of cellular differentiation called competence. In the competent state, cells stop growing and can take up DNA from the environment. There are two primary hypotheses that have been proposed to explain the advantages of natural competence. The first suggests that competent bacteria make use of DNA as a source of nutrients, while the second proposes that competence is a means of enhancing genetic diversity [63, 64].

The process of genetic competence in *B. subtilis* is complex and tightly regulated and occurs in response to nutrient limitation. Development of competence not only requires a high cell population density [65, 66]. It also depends on the levels in the cell of the major competence regulator ComK [67, 68]. Transcription of *comK* is stochastic, and only a subpopulation of cells (10-20%) will reach a ComK level that is sufficient to trigger competence development [69]. Competence and sporulation are also mutually exclusive events, since in a cell in a competent state the sporulation process is blocked (*spo0A* gene expression is turned off) [70, 71].

Multicellular communities: biofilms

B. subtilis can form multicellular communities called biofilms. These biofilms can be formed both in liquid and on solid surfaces. In the case of solid surfaces, *B. subtilis* biofilms are characterized by a complex architecture with prominent wrinkles (Figure 5) that are believed to enhance the surface-to-volume ratio, thereby improving access to oxygen.

Biofilm formation depends on the production of the extracellular matrix (with EPS and TasA as main components), which is synthesized by the sessile, matrix-producing, cells. The matrix plays a crucial role in holding the cells together within a biofilm and also confers the biofilm with several of its characteristic features: adherence to surfaces, enhanced mechanical rigidity, water retention and uptake of nutrients, communication between cells, and increased resistance to stress conditions such as the presence of antibiotics [72]. Although the matrix is produced by a subpopulation of cells, all cells within the biofilm benefit from it. Therefore, the matrix is often referred to as a "public good."

Within *B. subtilis* biofilms, the various cell types coexist (motile cells, matrix producers, endospores, and competent cells; Figure 5). This phenotypic heterogeneity occurs both in space and time. At the early stage of biofilm formation, a subpopulation of motile cells switches to sessile cells (matrix-producers) [73, 74]. As mentioned earlier, the SinR/I stochastic mechanism results in a spontaneous switch from motile to cell chaining and matrix production during favourable growth conditions. In the case of biofilm formation, the switch to chaining and matrix production is not stochastic but triggered by conditions of moderate nutrient limitation. As explained above, nutrient limitation leads to activation of Spo0A by phosphorylation [42]. Moderate levels of Spo0A~P can upregulate *sinI* transcription and when SinI levels exceed SinR levels, the sessile state is derepressed [75]. Moderate levels of Spo0A~P induce the expression of SlrR, another SinR antagonist, enhancing matrix production [74, 76]. The subpopulation of matrix-producers appears to localize at the propagating front of the biofilm, and as the biofilm colony expands, these cells gradually transition into spores [74, 77]. Note that the cellular levels of Spo0A~P differ among the cells in a biofilm community, with some cells exhibiting more elevated Spo0A~P levels than others. This heterogeneity favours the appearance of different subpopulations (such as matrix-producers) within the biofilm [78].

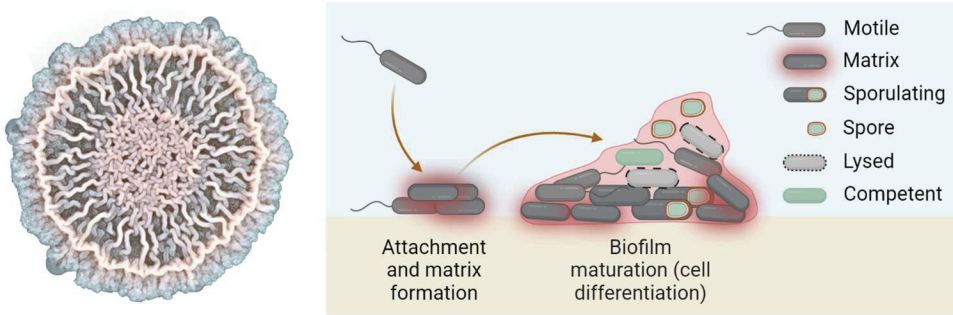


Figure 5. Structure and phenotypic heterogeneity of *B. subtilis* biofilm communities.
On the left is shown a mature colony biofilm formed by *B. subtilis* cells. Cells were grown on biofilm-inducing agar plates (LB agar (1.5%) supplemented with 0.1 mM $MnSO_4$ and 1% glycerol) for 72 hours at 30°C. The shown biofilm is 1-2 cm in diameter. The image was acquired by Claes von Wachenfeldt. On the right is a schematic drawing illustrating the main steps of biofilm formation: a motile cell attaches to a surface; after cell division, a subpopulation of cells differentiate to produce extracellular matrix, which holds the cells together within the biofilm. Biofilm maturation involves the differentiation of cells into different cell types, which coexist within the community. Created with BioRender.com.

Ancestral and domesticated *B. subtilis* strains

Most model bacteria have been domesticated in laboratory conditions. However, the laboratory environment is vastly different from the natural environment in which these bacteria evolved. In their natural habitats, bacteria typically encounter nutrient-poor and oxygen-limited conditions. Consequently, when these bacteria are sampled from their diverse natural habitats and cultured in the laboratory, they adapt by eliminating under the new conditions unnecessary genetic systems and modifying their original phenotypes. Domestication can thus result in increased fitness in the laboratory but give loss of systems that are essential for survival in the natural habitats.

In the case of *B. subtilis*, the domesticated laboratory strain 168 has been widely used for over half a century. It has increased competence for genetic transformation, which was originally specifically selected for and allowed researchers to use this strain as a tool for genetic manipulation [79, 80]. During domestication, however, various multicellular behaviours were lost, such as the capacity to form architecturally complex and robust biofilms and the swarming motility [57, 81]. The wild-type strain NCIB3610 (3610), which is a closely related ancestor of the 168 strain, forms robust and phenotypically heterogeneous biofilms; 168 forms thin and undifferentiated biofilms [81].

While the use of the 168 strain has allowed for many important advancements in our understanding of *B. subtilis* genetics, physiology, and behaviour, it is important to be cautious when extrapolating laboratory-based results to natural settings.

My research work presented in **Paper I** and **Paper III** was performed with the *B. subtilis* strain 168. The studies in **Paper II**, that focus on biofilm communities, were done with strain 3A38, a derivative of the NCIB3610 ancestral strain that exhibits increased competence due to a single mutation (in *comI*) [82].

Chapter 3. The major classes of molecular chaperones

Molecular chaperones are crucial for maintaining proteostasis. Chaperones assist in the folding of newly synthesized and preexisting proteins, unfolding and refolding of misfolded proteins, and disaggregation of protein aggregates. Thus, chaperones function against misfolding and aggregation of proteins [10, 83]. Generally, chaperones are house-keeping proteins and are important under optimal conditions to maintain proteostasis. Chaperones are further induced upon proteotoxic stress conditions, such as heat shock or oxidative stress, in which protein misfolding is increased [84]. In prokaryotes, the main cytosolic chaperones are the ancient and evolutionary conserved DnaK (Hsp70 homolog), GroEL (Hsp60 homolog), and trigger factor (TF) [85].

The contributions and roles of chaperones in proteostasis are relatively well studied in *E. coli*. Fewer studies on chaperones have been done in other bacteria, such as *B. subtilis*. It is important to study the roles of chaperones in bacteria other than *E. coli* to find out which are their conserved biological functions in prokaryotes. In addition, although chaperones usually possess widely conserved roles in assisting protein folding, they can also have specific functions which vary between organisms. And this might be the reason why proteostasis networks slightly differ between bacteria. Thus, investigating conserved and specific roles of chaperones in *B. subtilis* can provide us with information not only on conserved chaperone functions, but also on particular features of proteostasis in this organism (and other in Gram-positives, which include several important pathogens).

A common experimental approach to get more insight about the role of chaperones is to study the phenotype of mutants. Removing genes for chaperones and observing the resulting consequences can provide information about the general pathways in which individual chaperones are involved. However, because chaperones are involved in the folding of many client proteins, their absence usually gives rise to pleiotropic phenotypes and it can be hard to distinguish which consequences arise from a direct effect of chaperone absence, and which are the cause of indirect effects. To get more information about the cellular role of chaperones, suppressor mutations can be valuable.

This chapter summarizes the conserved mechanisms of action and structures of TF, DnaK, and GroEL (knowledge that mostly has come from studies in *E. coli*). The known roles of these chaperones in *E. coli* and *B. subtilis* will be discussed, as well as some important contrasts between chaperone mutants in these two organisms. Since *groES* and *groEL* are essential genes at all temperatures in both bacteria [86, 87], more focus will be put on single and double deletions of *dnaK* and *tig*.

Trigger factor (TF)

The first chaperone that newly synthesized polypeptides encounter is TF. In its monomeric form, TF is a ribosome-associated chaperone that interacts with nascent polypeptide chains as translation proceeds and stabilizes them for subsequent folding by slowing down their folding rate. This prevents premature protein misfolding and aggregation [88]. In its dimeric form, TF is cytosolic (not bound to the ribosome) and stabilizes and prevents aggregation of partially folded proteins after they are released from the ribosome in the cytosol [89] (Figure 6). In *E. coli*, TF provides co-translational folding assistance to around 70% of the proteome [10].

TF has an overall elongated shape and is comprised of an N-terminal ribosome-binding domain, a middle peptidyl-prolyl isomerase domain, and a C-terminal substrate-binding domain [90, 91]. The domains are united by linkers, which provide the TF with great structural flexibility. The substrate-binding domain adopts a clamp-like structure with its two helical arms. The clamp contains both hydrophobic and hydrophilic residues on its surface [92], which together with its structural flexibility allow the TF to adapt to a wide variety of nascent polypeptide chain substrates from unfolded to folded [93, 94]. In contrast to the GroEL and DnaK systems, TF does not require ATP for binding and releasing protein substrates [95, 96].

Deletion of the gene encoding TF, *tig*, in *E. coli* does not result in any apparent growth defect when cells are grown between 15°C and 42°C [97, 98] and does not affect protein folding at 30°C and 37°C [97]. However, deletion of *tig* reduces the outer membrane integrity [99] and induces the heat shock response [100]. Also, *tig* depletion or overproduction leads to increased filamentation, and the filamentation caused by *tig* overproduction can be suppressed by overexpressing the essential cell division gene *ftsZ* [101].

In *B. subtilis*, it is known that the cell viability in rich growth medium or under heat shock, osmotic shock, or oxidative stress is not compromised by the deletion of *tig* [102]. In **Paper I**, we show that deletion of *tig* increases sensitivity to D-cycloserine and to vancomycin (antibiotics that inhibit synthesis of the cell wall) and affects sporulation.

While production of most molecular chaperones (like DnaK and GroEL) is induced upon heat-shock, TF does not appear to be a heat-shock protein. In fact, expression of *tig* can be induced by cold-shock both in *E. coli* and *B. subtilis* [101, 103]. It has also been shown that in *B. subtilis*, the (p)ppGpp synthetase RelA downregulates transcription of *tig* during the stringent response (which includes amino acid, glucose, and oxygen starvation) [104].

Interestingly, there exists a link between TF and spore germination in *B. subtilis*. During sporulation, and before entering dormancy, TF appears to be phosphorylated on Arg45 by the kinase McsB. This negatively affects the association of TF with the ribosomes. Spore germination requires dephosphorylation of Arg45 by the phosphatase YwlE. This enables TF to properly bind to ribosomes and thus facilitates the translation of proteins [105]. These findings support the idea that chaperones have host-specific roles, apart from their conserved functions.

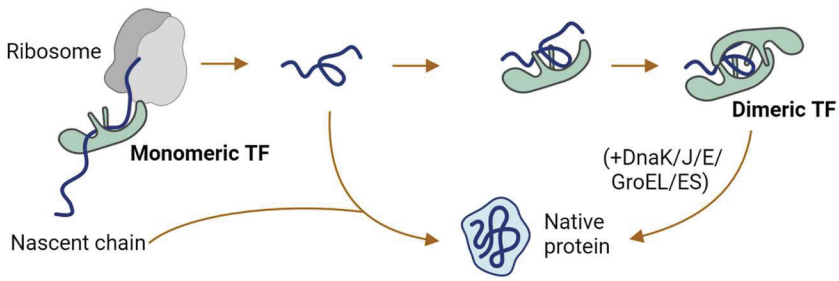


Figure 6. Monomeric and dimeric functions of trigger factor (TF) in protein folding.
Monomeric TF is associated with the ribosome and assists in the folding of newly synthesized polypeptides by stabilizing them and preventing misfolding and aggregation. Unfolded polypeptides may also reach the native protein conformation with the assistance of TF in its dimeric form and/or other chaperone complexes. Created with BioRender.com.

DnaK

If folding assistance from TF is not sufficient, unfolded or misfolded proteins can bind to DnaK (Hsp70), which can function co- and post- translationally [106]. DnaK operates as a monomer and consists of two main domains: a N-terminal ATPase domain and a C-terminal substrate-binding domain that has a β-sandwich and an α-helical lid [107, 108]. DnaK typically recognizes unfolded aggregation-prone proteins by binding to their exposed hydrophobic peptide segments (~5–7 residues)[109-111]. Tight binding of the client substrates to DnaK is enabled by the α-helical lid closing over the β-sandwich, and this requires ATP hydrolysis [107, 112] (Figure 7). Several cycles of DnaK binding and release prevent substrates from

misfolding and aggregation by stabilizing them in the non-native conformation and decreasing the folding rate [113]. DnaK works in cooperation with its co-chaperone DnaJ (Hsp40) and the nucleotide exchange factor GrpE [114, 115]. DnaJ, apart from recognizing and attracting clients to DnaK, also induces the ATPase activity of DnaK [116]. GrpE is a nucleotide exchange factor that facilitates the release of ADP from DnaK, thus regenerating ATP [117, 118]. DnaK is involved in the folding of approximately 20% of the *E. coli* proteome [111].

In *E. coli*, DnaK has a crucial role in the heat-shock response, since it regulates the heat-shock sigma factor σ^{32} [119, 120]; also, the folding of a large amount of *E. coli* "thermolabile" proteins depends on DnaK [110]. DnaK regulates the levels of σ^{32} under normal conditions by interacting with it and enhancing its degradation by the FtsH protease. Upon heat shock stress, DnaK is depleted by binding to many denatured proteins, and therefore σ^{32} levels increase [121].

While DnaK is not essential for growth or protein folding at normal conditions in *E. coli*, its absence causes major cell growth and cell division defects and dysregulation of the heat-shock regulon [122-124]. Importantly, DnaK becomes essential at high or low temperatures [123, 125, 126].

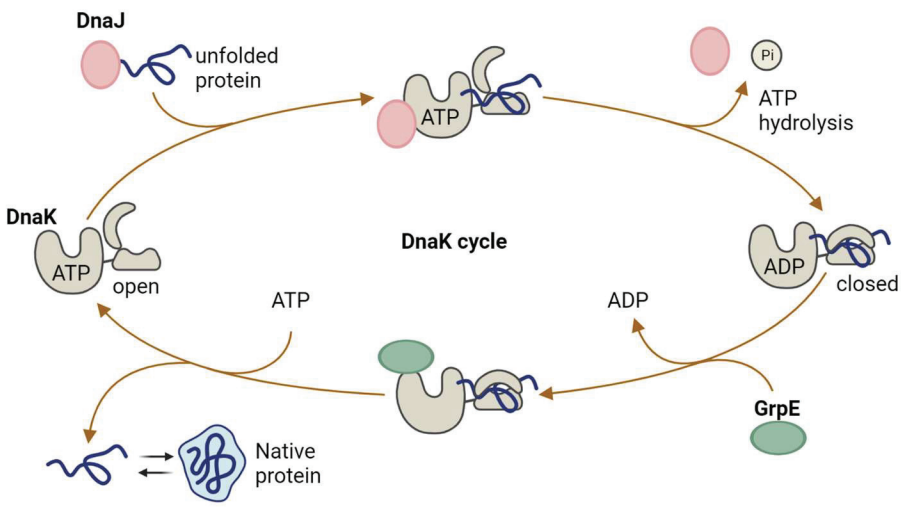


Figure 7. DnaK/J/E cycle of substrate binding and release.
The co-chaperone DnaJ attracts unfolded clients to DnaK and induces its ATPase activity. ATP hydrolysis results in the α-helical lid closing over the β-sandwich, enabling tight substrate binding (closed conformation of DnaK). The nucleotide exchange factor GrpE facilitates the release of ADP from DnaK. ATP binding leads to the DnaK open conformation, and thus causes the release of the substrate, which can either enter another DnaK cycle or fold into its native conformation (with or without further chaperone assistance). Created with BioRender.com.

Of important note, while the heat-shock response in *E. coli* is mediated primarily by the σ^{32} regulon (and requires DnaK), the *B. subtilis* response to heat (as well as other

proteotoxic stressors) is far more complex, being controlled by at least six different mechanisms (some of them reviewed in Chapter 5) [127]. Thus, even though *dnaK* is a heat-shock gene in *B. subtilis*, it is not surprising that in stark contrast to the observations in *E. coli*, *B. subtilis dnaK* operon mutants do not show cell viability defects at temperatures ranging from 16°C to 52°C [128]. At 52°C, these mutants do not exhibit growth on agar plates, are reduced in motility, and show increased filamentation [128].

B. subtilis DnaK is known to be subjected to regulation by phosphorylation. The PtkA kinase and PtpZ phosphatase can phosphorylate and dephosphorylate, respectively, the tyrosine residue 601 (present in the C-terminal DnaK domain), affecting DnaK activity as well as cellular viability under heat-shock conditions [129].

GroEL

While TF and DnaK have important roles in co-translational folding, GroEL (Hsp60) seems to be more of a downstream chaperone, assisting polypeptides that have already been translated [10]. GroEL participates in the folding of 10% of the *E. coli* proteome [130, 131], and within the GroEL obligate substrates are metabolic enzymes that are essential for *E. coli* viability [132].

The GroEL chaperone complex uses ATP hydrolysis as a source of energy to actively refold unfolded or misfolded proteins (Figure 8). Oligomeric GroEL is comprised of two stacked heptameric rings that form two respective large chambers to enclose substrates [133, 134]. GroEL (*cis*-ring) interacts with hydrophobic residues from unfolded proteins and, by a conformational shift driven by ATP and GroES binding, tunnels the unfolded protein to the chamber [135, 136]. GroES (Hsp10) is an heptameric co-chaperonin which serves as a cap for the GroEL chamber [134]. After complete encapsulation of the protein by the GroES, several GroEL conformational changes occur, which turn the hydrophobic chamber into hydrophilic. The hydrophilic interior provides a perfect environment for protein folding, stimulating the burial of hydrophobic residues in the core of the protein substrate [135, 136]. During the period of ATP hydrolysis, proteins can undergo folding in the GroEL chamber. ATP binding to the *trans*-ring of GroEL triggers the dissociation of ADP and GroES, as well as the release of the folded protein. If the released protein is not properly folded yet, it can undergo another GroEL cycle [137]. Because of its delimited size, the GroEL-GroES complex typically assists small protein substrates (less than 60 kDa) [136]. GroEL-GroES is essential in almost all studied bacteria, including *E. coli* and *B. subtilis* [138].

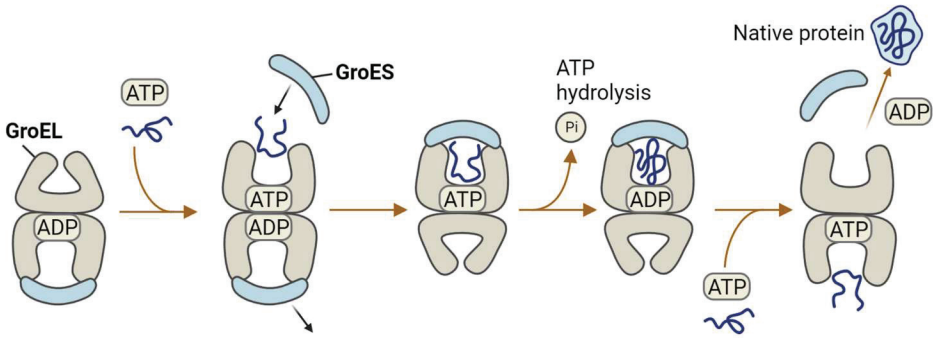


Figure 8. Reaction cycle of the GroEL-GroES chaperone complex.
An unfolded protein interacts with the *cis*-ring of GroEL. ATP binding tunnels the unfolded protein to the GroEL chamber. ATP binding is also accompanied by GroES encapsulation. The encapsulated protein can undergo folding during the time it takes for GroEL to hydrolize ATP. The folded protein is released upon binding of ATP to the GroEL *trans*-ring. Released proteins can undergo additional GroEL folding cycles in case their folding is still incomplete [137]. Created with BioRender.com.

The study of the *dnaK* and *tig* double deletion mutant

Several studies in *E. coli* have shown that DnaK and TF display overlapping roles in protein folding, and that many client substrates are shared between the two chaperones [97, 98, 100, 111, 139]. This overlap could account for the observation that the proportion of newly synthesized polypeptides interacting with DnaK nearly doubles in the absence of TF [97, 98, 111]. Additionally, in a *dnaK tig* double deletion mutant many proteins aggregate at 30°C [140, 141], and the double deletion is synthetically lethal at temperatures above 30°C [97, 98, 139]. These findings indicate that both DnaK and TF chaperones have crucial roles in maintaining proteostasis even at typical growth temperatures.

Multiple mutations have been found to partially suppress the growth defect of an *E. coli* Δ*dnaK* Δ*tig* mutant [140-144]. For instance, overexpression of GroEL, the export chaperone SecB, or the redox-regulated chaperone Hsp33 can compensate for the absence of both DnaK and TF [139, 141, 143, 145]. The suppression resulting from GroEL and SecB overexpression is likely caused by their compensatory roles in protein folding, while the suppression by Hsp33 overexpression is because of different, more specific reasons. Hsp33 interacts with the essential elongation factor Tu (EF-Tu) and directs it for degradation by the AAA+ protease Lon. Hsp33 overexpression in the absence of DnaK and TF increases EF-Tu degradation, likely reducing the rate of protein translation and enabling the cell to restore its folding capacity [143]. Other mutations that suppress the growth defect of *E. coli* Δ*dnaK* Δ*tig* were found in genes involved in various cellular processes such as

transcription, translation, oxidative stress, or metabolism. This suggests that an unbalance in proteostasis can be compensated in multiple ways and provides new players that can be involved in the proteostasis network [144].

The Δ*dnaK* Δ*tig E. coli* strain has proven to be a useful genetic tool to investigate the proteostasis network in this bacterium. However, not much research is reported on such a double mutation in other bacteria.

In *B. subtilis*, a concise study of a *dnaK dnaJ tig* triple deletion mutant showed that it exhibits increased sensitivity to temperature and fails to grow at above 53°C [146]. In stark contrast to the *E. coli* Δ*dnaK* Δ*tig* mutant, the *B. subtilis* triple mutant cells are viable at typical growth temperatures [146]. However, before our study, there were no information available on the effects of the *dnaK* and *tig* double deletion in *B. subtilis*.

In **Paper I**, we investigated the effects of the *dnaK tig* double deletion in *B. subtilis* (168 strain). We found that the absence of both DnaK and TF resulted in reduced viability and thermotolerance, as well as increased protein aggregation already at normal growth temperatures (30 and 37°C), suggesting imbalances in proteostasis maintenance. However, despite these defects, we found it remarkable how the absence of two crucial and conserved chaperones in *B. subtilis* did not hinder significant and complex cellular processes such as cell growth, division, and sporulation from occurring at a population level.

When contrasted with *E. coli*, which cannot survive the combined absence of DnaK and TF at standard growth temperatures, *B. subtilis* proteostasis does not seem to reach a collapse upon both DnaK and TF absence. This suggests that there are additional players of the proteostasis network that can compensate for the absence of these two chaperones. Our results revealed notable distinctions in the proteostasis networks between *E. coli* and *B. subtilis*.

The *dnaK tig* double deletion caused additional defects in *B. subtilis*, such as a reduced colony size, decreased motility (indicated by a reduced expression from the promoter of the flagellin-encoding gene *hag*), and a filamentous and twisted cell morphology (Figure 9). Interestingly, we found that the twisted cell morphology was linked to a compromised cell wall integrity, shown by a reduced tolerance to lysozyme and to cell wall-active antibiotics. Even though further research is needed, simultaneously inducing proteostasis imbalance and administering cell wall-active antibiotics presents a promising combined therapeutic strategy for enhancing treatment against bacterial pathogens.

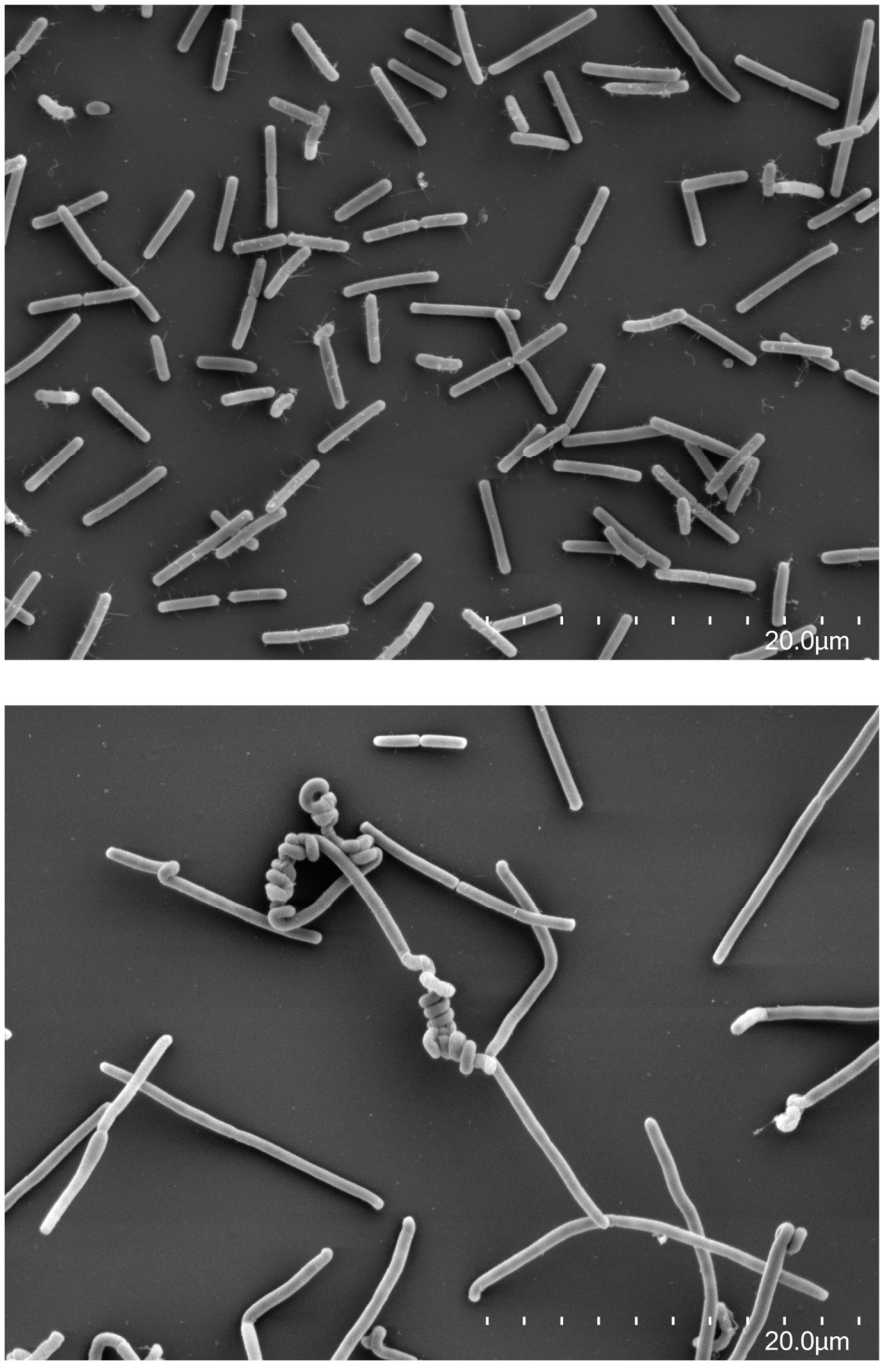


Figure 9. The combined absence of DnaK and TF in *B. subtilis* results in morphology defects. Scanning electron microscopy images of *B. subtilis* strains. Top panel, wild-type (168 strain). Lower panel, *dnaK tig* double deletion mutant.

We found that second-site suppressor mutations in genes for enzymes in metabolism, regulation of gene expression, RNA synthesis or degradation, and stress response could partially restore the cell morphology, colony size, and thermotolerance in DnaK and TF deficient *B. subtilis*. These findings suggest that *B. subtilis* can fine-tune multiple biological pathways to counteract an imbalance in proteostasis resulting from the absence of DnaK and TF, highlighting the complexity of the proteostasis network. Hence, maintaining a "healthy" proteome in *B. subtilis* seems to involve multiple enzymes and pathways that work together to ensure proper protein folding and function.

Taken together, the findings in **Paper I** not only contribute to our understanding of proteostasis maintenance in Gram-positive bacteria, but they also highlight the potential of the *dnaK tig* double mutant as a model to investigate proteostasis, cell wall homeostasis, and specific roles of DnaK and TF in *B. subtilis*.

In **Paper II**, we show that the double *dnaK tig* deletion in the *B. subtilis* ancestral strain causes phenotypic defects similar to the ones observed in the domesticated strain 168, for example, reduced colony size, aberrant morphology, and reduced heat tolerance. As discussed in Chapter 2, the *B. subtilis* ancestral strain retains social traits such as the ability to form multicellular communities (biofilms) and to swarm. Thus, the ancestral strain allowed us to study effects of chaperone deficiency in *B. subtilis* multicellularity (see next subsection).

Effects of chaperone absence in biofilm formation

A bacterial biofilm is a major factor of some chronic infections. The biofilm can form on medical devices, implants, and tissues, and thereby poses significant challenges within medicine. Biofilms are generally highly resistant to antibiotics and host immune responses, making infections difficult to treat. In industrial settings, biofilms cause equipment failure, product contamination, and decreased efficiency. They are difficult to remove and can lead to increased use of harsh chemicals and water resources. Therefore, many research efforts work towards finding strategies to prevent and combat biofilms.

Studies in *E. coli* suggest that DnaK is an attractive target for antibiotics against biofilms. Deletion of *dnaK* in this bacterium negatively impacts biofilm formation [147, 148], as DnaK plays an important role in the biogenesis of curli amyloids (which are key biofilm matrix components). One the one hand, DnaK influences the levels and activity of the transcriptional regulators RpoS and CsgD, thereby regulating expression of CsgA and CsgB, which are the structural components of curli. On the other hand, DnaK binds to and prevents aggregation of CsgA and CsgB, allowing their transport across the cytoplasmic membrane [148].

Deletion of *dnaK* also reduces biofilm formation in *S. aureus*. It was found that the absence of *dnaK* affects the regulation of many genes, including those involved in general stress tolerance, cell wall synthesis, metabolism, and virulence. This suggests that the proteins encoded by these genes directly or indirectly are responsible for the defects in biofilm formation [149]. Deletion of *tig* in *S. aureus* also reduces the capacity to form biofilms, but the reason is still unknown [150].

Defects in biofilm formation upon removal or down-regulation of DnaK have been reported also in *L. monocytogenes* and *Streptococcus mutants* [151, 152]. The deletion of *dnaK* in *Clostridium difficile*, on the contrary, enhances biofilm formation, and this may be partly explained by the upregulation of GroEL (known to enhance surface adhesion) in this mutant [153]. GroEL is known to impact biofilm formation in *various* bacteria, including *Bacillus anthracis* [154], *Haemophilus influenzae* [155], *Campylobacter jejuni* [156], *S. mutans* [152], and mycobacteria [157]. Further research is needed to define the roles of chaperones in biofilm formation.

Turning our attention to *B. subtilis*, even though molecular chaperones have been reported to affect biofilm formation in several bacteria, it is surprising that as far as we are aware, no direct study of the effects of chaperone deletions in *B. subtilis* had been done before this present thesis work. To our knowledge, the only study with *B. subtilis* linking chaperones to biofilm features is the one by Stubbendieck and Straight (2017). Through transposon mutagenesis experiments, that study identified that *dnaK*, *dnaJ* and *tig* (as well as the protein quality control genes *hprT*, *ftsH*) are important for development of rough and wrinkly biofilm-like colonies. However, these experiments were not performed in the *B. subtilis* wild-type genetic background (they were done with a mutant with a point mutation in the *yfiJK* operon, which encodes for a two-component signalling system necessary for linearmycin resistance) [158]. Moreover, the agar medium used was MYM agar, which is not a biofilm-inducing medium [159].

In **Paper II** we explored links between chaperones and biofilm formation in *B. subtilis* (ancestral strain) and show that the combined removal of DnaK and TF results in biofilms with an aberrant architecture. Moreover, the relative proportion of the different cell types that coexist in the biofilm was found different from the wild-type biofilms. The *dnaK tig* double mutant biofilms contain a larger number of spores and chains. They also contain a subpopulation of matrix overproducers and motility seems to be downregulated. The observations suggest that the absence of the two chaperones is linked to defects in the regulation between the different cell types within *B. subtilis* biofilm communities, and this might be either a consequence or a cause of the aberrant macrocolony architecture. In addition, swarming (multicellular motility) was impaired by the double deletion.

Paper II shows that formation of aberrant biofilms can be caused by elevated growth temperatures (which causes imbalances in proteostasis as it increases protein

misfolding) (Figure 10). In addition, we observed that the lack of DnaK and TF correlates with greater defects in the biofilm architecture at elevated temperatures when compared to the wild-type, presumably due to a higher extent of proteostasis perturbation. The findings suggest that conditions that affect proteostasis (like the absence of chaperones or environmentally induced) are linked to defects in biofilm formation in this organism. It remains to be tested whether the observed changes in *B. subtilis* biofilm architecture are linked to decreased tolerance to stress conditions such as antibiotics.

Figure 10. Biofilm formed by the *B. subtilis* Δ*tig* mutant.
To obtain the shown biofilm, 5 µl cell suspension was spotted on a biofilm-inducing medium plate containing Congo red and Coomassie blue. The plate was grown at 42°C for 24 hours. The shown biofilm's diameter is 3.3 cm. Images of wild-type, Δ*tig* and Δ*dnaK* single and double mutant strains grown at different temperatures can be found in Paper II.

Chapter 4. Key players in the elimination of proteins

Protein degradation is a critical process in maintaining proteostasis. The cell needs to eliminate incomplete, damaged, or misfolded proteins, as well as protein aggregates. The amino acids generated from degradation of proteins are usually reused in the cell [160, 161]. Protein degradation is not only important for protein quality control. It also contributes to modulate the abundance of proteins in the cell.

AAA+ proteases

Most cytoplasmic proteins in bacteria are degraded by the conserved AAA+ family of intracellular proteases (AAA+: ATPases associated with a variety of cellular activities). These proteases identify, unfold, and eliminate specific protein substrates [162]. *B. subtilis* contains the proteases ClpCP, ClpEP, ClpXP, ClpYQ, LonA, LonB, and FtsH (Figure 11) [163]. Interestingly, the AAA+ unfoldase ClpB, which is present in most bacteria and has a disaggregase activity independent of ClpP, is not found in *B. subtilis* [17, 164].

The Clp complexes typically consist of an ATP-dependent serine protease (such as ClpP) forming a proteolytic chamber, coupled to an AAA+ unfoldase (such as ClpC, ClpE, and ClpX) [165]. The unfoldase domain recognizes the protein substrate to be degraded and by going through several conformational changes driven by ATP hydrolysis it translocates and unfolds the substrate towards the proteolytic chamber. Translocated proteins are cleaved in the proteolytic chamber into peptides of 7-8 residues, which are then released and degraded to amino acids by various peptidases [163, 166]. In the case of LonA, LonB, and FtsH, the unfoldase and protease domains are present in a single polypeptide [167].

ClpXP is the most characterized protease complex in bacteria and it degrades unfinished peptides, originating from stalled ribosomes, which are C-terminally marked with the SsrA degradation tag [168, 169]. Proteolysis of SsrA-tagged proteins is important in maintaining proteostasis [168, 170].

Similar to chaperones, many proteases are stress-induced. This is the case for ClpC and ClpE in *B. subtilis* [161, 171, 172], and absence of these proteins can disrupt proteostasis. For example, *clpC*, *clpP*, and *clpX* defective mutants display accumulation of misfolded proteins [161].

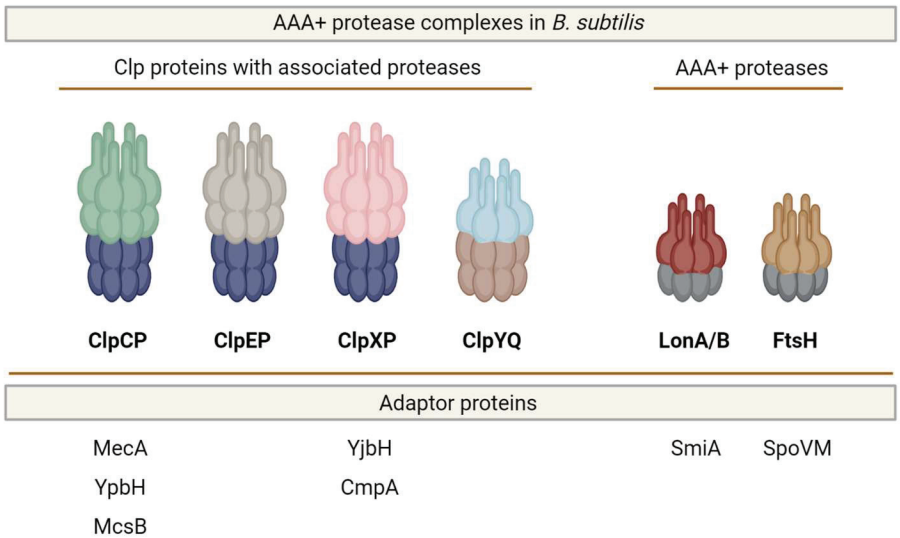


Figure 11. AAA+ protease complexes present in *B. subtilis*.
ClpCP, ClpEP, ClpXP, and ClpYQ consist of an AAA+ unfoldase domain coupled to a protease domain. The unfoldase and protease domains of LonA, LonB, and FtsH are present in a single polypeptide. The identified adaptor proteins for each protease complex are indicated. Adapted from [167]. Created with BioRender.com.

Adaptor proteins

Proteolysis needs to be regulated so that only specific protein substrates are degraded and at the appropriate time. For this, cells make use of adaptor proteins that confer proteases with substrate specificity. Adaptors typically interact with both substrate and protease, which increases the local substrate concentration and thus promotes degradation [173].

Examples of adaptor proteins in *B. subtilis* are CmpA and YjbH, which are ClpXP adaptors, and MecA, YpbH, and McsB, which are ClpCP adaptors (Figure 11) [167]. The expression or activity of adaptor proteins is usually regulated. The cellular level of an adaptor protein can be modulated by degradation, anti-adaptor proteins, post-translational modifications, aggregation, or sequestration [163, 174,

175]. Later in this thesis, focus is put on the adaptor protein YjbH, which is a conditional aggregation protein that controls Spx proteolysis by the ClpXP protease complex.

Degradation tags

AAA+ proteases or their respective adaptor proteins recognize short amino acid sequences contained in protein substrates. These sequences, which are called degrons, can be located at the N-terminal, C-terminal or at an internal position in the substrate [176].

As mentioned earlier, the SsrA degradation tag is a degron that plays a role in maintaining cellular proteostasis, as it helps eliminate unfinished polypeptides originated from stalled ribosomes. The tag is added to the C-terminus of truncated polypeptides by the transfer-messenger RNA (tmRNA) system [177, 178]. Although the ClpXP complex is the main protease responsible for degradation of SsrA-tagged polypeptides [162], it is known that also ClpAP and FtsH degrade these proteins in *E. coli* [179, 180]. Details on the molecular mechanism by which SsrA-tagged polypeptides are recognized and degraded by ClpXP have been shown by means of cryo-EM. The SsrA degron specifically binds to ClpX triggering a ClpX conformational change which is followed by substrate translocation through the channel [181].

A novel mechanism of ClpXP-mediated degradation of truncated polypeptides in *B. subtilis* was recently discovered [182]. This mechanism involves $tRNA^{Ala}$, which is recruited to stalled ribosomes by RqcH and tags the truncated polypeptides with C-terminal poly-alanine tails. The poly-alanine tail displays similarity to the SsrA ALAA motif and is recognized by ClpXP [182].

Relevant for *B. subtilis* proteostasis is also the phospho-arginine (pArg) degradation tag introduced by the arginine kinase and ClpCP adaptor McsB [183, 184]. By phosphorylating arginine residues McsB marks proteins for degradation by ClpCP [184]. McsB is a conserved protein among Gram-positive bacteria and plays an important role in the removal of damaged cytosolic proteins, especially upon proteotoxic stress. McsB forms octamers stabilized by self-phosphorylation, creating a chamber-like structure with a narrow entrance and the kinase active site inside [185]. Octamerization occurs under proteotoxic stress, when McsB levels rise. pArg tagging is highly selective for unfolded proteins, since only those can fit the narrow entrance and access the buried kinase site of the chamber [185]. pArg-tagged proteins include members of the proteostasis network such as CtsR, HrcA, GroEL, TF, ClpC, and ClpP [186]. Phosphorylation of residues in the DNA-binding domains of the transcriptional regulators CtsR and HrcA triggers the activation of the proteotoxic stress response [186-188].

Chapter 5. Response mechanisms to proteotoxic stress

When a bacterium is faced with proteotoxic stress, response mechanisms are activated to assist the adaptation of the cell to the condition while safeguarding its proteome. In this chapter, the main mechanisms used by *B. subtilis* to cope with proteotoxic stress will be reviewed. Special attention is paid to the Spx-YjbH system, as it is a main topic of my research.

HrcA

Upon proteotoxic stress, the production of chaperones is increased above the basal level to avoid accumulation of misfolded proteins or aggregates. One way to induce synthesis of chaperones in *B. subtilis* is the use of a feedback mechanism involving the transcriptional repressor of class I heat-shock genes HrcA. HrcA controls transcription of the *hrcA-grpE-dnaK-dnaJ-yqeT-yqeU-yqeV* and the *groES-groEL* operons [189]. To be folded properly, HrcA needs assistance of the GroEL-GroES chaperone complex. Under proteotoxic stress conditions, such as heat, the accumulation of misfolded proteins depletes the number of GroEL-GroES available for the folding of HrcA and this reduces the activity of the repressor, leading to induction of chaperone expression (Figure 12). Higher levels of chaperones protect the proteome and avoid further accumulation of misfolded proteins, including HrcA proper folding (that will again be able to repress the expression of chaperones) [189, 190].

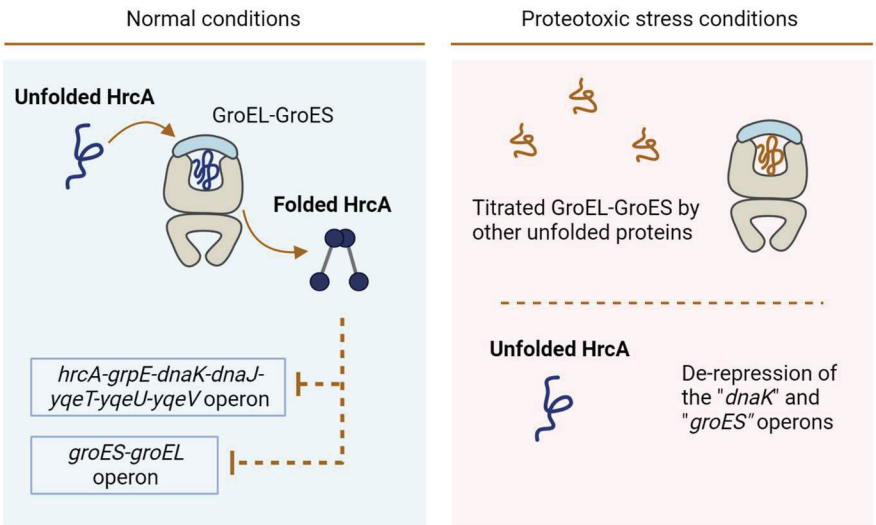


Figure 12. Mechanism of HrcA in controlling chaperone gene expression.
The repressor HrcA controls transcription of the *dnaK* and *groES-groEL* operons. To be active, HrcA requires folding assistance by the GroEL-GroES chaperone complex. Upon proteotoxic stress conditions, the number of free GroEL-GroES is reduced by unfolded and misfolded proteins, increasing the amounts of unfolded HrcA. Therefore, chaperones like DnaK and GroEL-GroES are induced under proteotoxic stress. Created with BioRender.com.

CtsR

Apart from increasing chaperone production, response to proteotoxic stress also involves the increased production of proteases to degrade damaged proteins. In *B. subtilis*, the CtsR repressor is known as the master regulator of protein degradation. It negatively controls expression of the *clpC* (*ctsR-mcsA-mcsB-clpC-radA-disA* operon), and the *clpP* and *clpE* genes (Figure 13) [191-193].

CtsR repression involves a complex regulatory network, comprising the arginine kinase and ClpCP adaptor protein McsB, which can phosphorylate CtsR and adapt it for ClpCP degradation [184, 193, 194]. Under non-stress conditions, the interaction between ClpCP and McsB causes the inactivation of McsB kinase activity. Activation of McsB occurs under various proteotoxic stress conditions, such as heat stress and disulfide stress, and partly depends on the activator McsA, which contributes to the release of McsB from ClpCP [192, 195]. Interestingly, McsA activity might be regulated by the reduction of critical thiol groups which function as a molecular redox switch [195]. CtsR can also regulate its own activity through a glycine-rich loop that serves as an intrinsic heat sensor [196]. McsB

phosphorylates hundreds of different proteins *in vivo,* including the HrcA repressor, and they are subsequently degraded by ClpCP [183, 197].

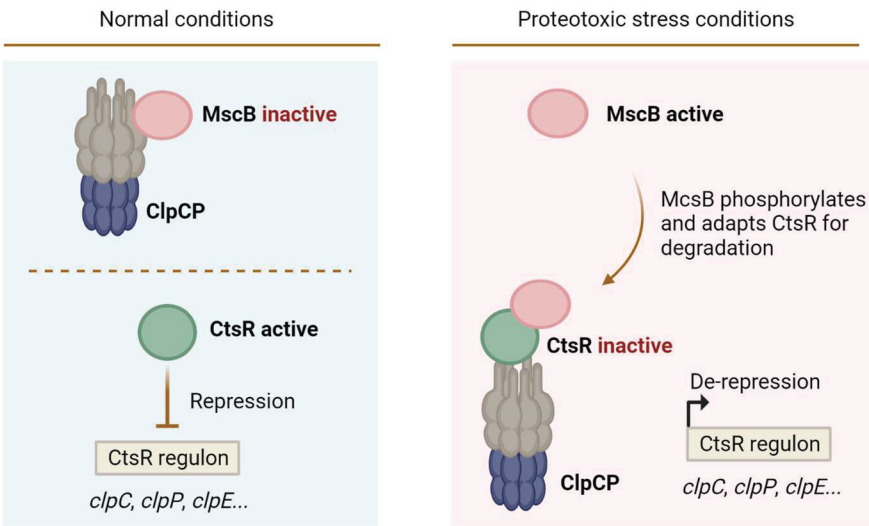


Figure 13. Simplified model of the regulation of the CtsR activity under different conditions. Under non-stress conditions, CtsR represses its regulon which includes genes encoding the ClpC, ClpP, and ClpE proteases as well as CtsR itself. Proteotoxic stress can inactivate CtsR. Moreover, the arginine kinase McsB becomes active, phosphorylates CtsR (contributing to its inactivation) and adapts CtsR for ClpCP degradation. Thus, the CtsR regulon is de-repressed upon proteotoxic stress conditions. Created with BioRender.com.

The general stress response by σ^B

Proteotoxic stress activates the *B. subtilis* general stress response, which is a major non-specific stress response mechanism controlled by the alternative sigma factor σ^B. Sigma factors are required for the initiation of transcription by interacting with the RNA polymerase core enzyme and thereby influence promoter specificity. The general stress response enhances resistance to many different stresses such as starvation, elevated or lowered temperature, ethanol, salt, and oxidative stress [198]. The general stress transcription factor σ^B regulates more than 200 genes [199, 200], including genes related to proteolysis, like *clpP* and *clpC*, and genes important for defence against oxidative stress, such as peroxidase (*ohrA*), superoxide dismutase (*sodA*), and thioredoxin (*trxA*) [201-204]. Induction of the σ^B regulon requires activation of σ^B itself through a sophisticated signal transduction cascade mainly regulated by the Rsb proteins [198].

Under non-stress conditions, σ^B is inactive as is bound to the anti-sigma factor RsbW. Environmental stress conditions are sensed by a cytoplasmic multiprotein complex called the stressosome. The stressosome contains several Rsb proteins including RsbR, RsbS, and RsbT. Stress results in phosphorylation of RsbR and RsbS and in subsequent release of RsbT from the stressosome. RsbT binds to and activates the RsbU phosphatase; RsbU dephosphorylates the RsbV anti-anti-sigma factor. Dephosphorylated RsbV binds to the anti-sigma factor RsbW. This releases σ^B, which can now bind to RNAP and induce expression of the general stress response genes [205].

The Spx-YjbH system

Another important response mechanism that *B. subtilis* uses to cope with proteotoxic stress conditions is the Spx-YjbH system.

The transcriptional regulator Spx

Spx is a 15-kDa protein that belongs to the thioredoxin-like superfamily of proteins. In *B. subtilis*, Spx was originally identified as a transcriptional regulator of the thiol-specific oxidative (disulfide) stress response [206], affecting ~275 genes (~144 transcriptional units) [207]. *B. subtilis* cells lacking Spx show increased sensitivity to the disulfide-stress inducer diamide and other conditions, including heat shock and compounds targeting the cell wall [206, 208, 209]. Recent studies have revealed that Spx plays a role in the response to various stressors other than disulfide stress [208-210]. The significance of Spx as a regulator of the proteotoxic stress response is therefore gaining increased recognition [211-213].

Spx is an atypical transcriptional regulator, since it does not directly bind to DNA. Instead, it interacts with the C-terminal domain of the α-subunit (α-CTD) of the RNA polymerase (RNAP) and enhances its promoter binding [207, 214-218]. Spx can act both as a transcriptional activator and as a repressor. Spx stimulates transcription of many genes that help the cell to cope with stress, including *clpC*, *clpE*, *clpX, trxA* (thioredoxin), and *trxB* (thioredoxin reductase) [206, 207]. The *ctsR* operon is also stimulated by Spx [211]. As a repressor, Spx inhibits the binding of transcriptional activators to promoter regions. A well-known example of Spx as repressor is the case of expression of *comS* (which encodes a regulatory peptide that plays a role in induction of competence) from the *srf* promoter. Spx in complex with the α-CTD of RNAP binds to the *srf* promoter, preventing the binding of the transcriptional activator ComA. Thus, high Spx levels inhibit competence development [219]. Accumulation of Spx also affects other physiological processes, such as growth, and sporulation [220, 221]. Because a high level of Spx is only

beneficial under stress conditions, the cellular concentration of Spx needs to be tightly regulated.

Regulation of the Spx levels

Regulation of the amounts of Spx in the cell occurs at multiple levels, including transcription and post-translational control. The *spx* gene is constitutively expressed and it is further induced upon stress conditions [222-225]. The transcription of the gene involves five promoters and two repressors, YodB and PerR (Figure 14A) [226]. The activity of the Spx protein can be regulated via its redox-sensing switch involving the residues Cys10 and Cys13, that are located in its N-terminal part [227]. Certain stress conditions, such as disulfide stress, lead to oxidation of these two cysteines (that form an intramolecular disulfide bridge) which enhance the activity of Spx as a transcriptional regulator [227]. Some genes in the Spx regulon are only induced by the oxidized form of Spx [223, 228]. As shown by recent structural studies, the redox-activated form of Spx interacts with both the α-CTD and σ^A in the holo-RNAP. This interaction leads to the binding at the −44 position of promoter DNA, thereby increasing transcription activation [229]. The -44 position appears to be highly conserved in genes belonging to the Spx regulon [230].

The primary mechanism for regulating the level of Spx appears to be proteolysis. Under non-stress conditions, Spx levels are kept low through ClpXP-mediated degradation (Figure 14B) [231, 232]. Efficient ClpXP degradation of Spx requires interaction with the adaptor protein YjbH [24, 220].

The interaction surface area between YjbH and Spx involves 25 residues of Spx and 33 residues of YjbH [233]. There is an overlap between the Spx surface that interacts with YjbH and that with the α-CTD domain of the RNAP, suggesting that there is competition for Spx-binding [234]. Unlike most adaptor proteins, YjbH does not seem to directly interact with the protease complex ClpXP [235]. The C-terminus of Spx (its last 28 residues) is important for recognition by ClpXP [236] and, when bound to YjbH, the C-terminal sequence displays reduced conformational flexibility. This makes it more easily recognized by ClpXP for degradation [25, 234, 236]. Usually, adaptor proteins facilitate proteolysis of several clients. However, up to this date, YjbH seems be specific for Spx [24].

Importantly, proteotoxic stress conditions cause YjbH to aggregate. The YjbH aggregation associated with a decrease in its solubility appears to correlate with increased Spx levels at the population level [26]. In **Paper III**, we confirm by fluorescence microscopy that this correlation holds true also at the single-cell level.

High Spx levels help the cell to cope with stressful conditions (Figure 14B). While *B. subtilis yjbH* null mutant cells show increased Spx levels, the *spx* gene transcription is not affected in such cells, indicating that Spx regulation by YjbH is only at the post-translational level [24, 25]. Transcription of the *yjbH* gene, which is part of the *yjbIH* operon, is activated by Spx creating a negative feedback loop

[223]. Induction of *yjbH* expression might be useful to rapidly degrade Spx and avoid its accumulation once the cell is back to normal conditions.

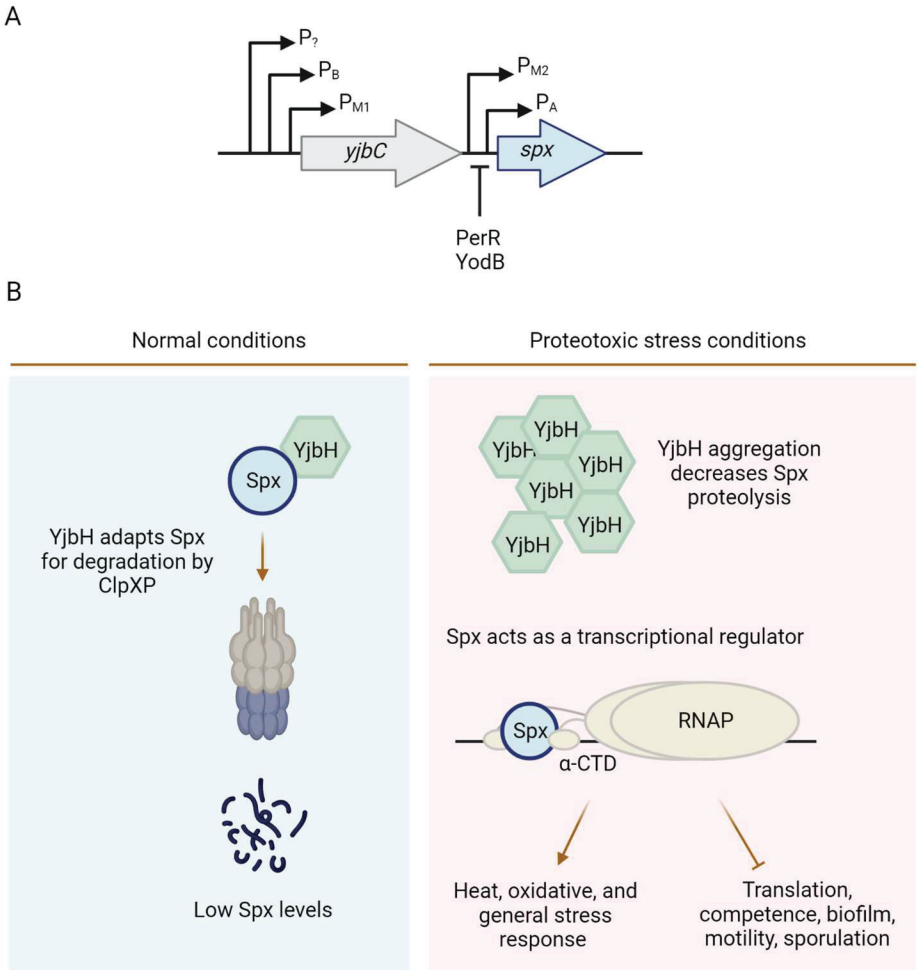


Figure 14. Regulation of Spx at the transcriptional and post-translational levels.
spx transcription depends on five promoters and two repressors. The *spx* gene is present in the same operon as the *yjbC* gene, which encodes for a putative acetyltransferase. Different stress conditions dictate the contribution of the different promoters to the transcription of *spx* (**A**). Model showing the regulated YjbH aggregation mechanism, which controls Spx proteolysis. Under non-stress conditions, the ClpXP protease efficiently degrades Spx with the aid of the adaptor protein YjbH. During proteotoxic stress, YjbH aggregates resulting in lower rate of Spx degradation. Spx functions as a transcriptional regulator by binding to the α-CTD domain of the RNAP. Spx in complex with RNAP can both induce and inhibit the expression of several target genes (**B**). Created with BioRender.com.

Although to a lesser extent, ClpCP and its adaptor McsB also contribute to the degradation of Spx [211] and McsB-dependent arginine phosphorylation might play a role in modulating the stability and activity of Spx [237].

The anti-adaptor protein YirB (of only 6.6. kDa) appears to interact with YjbH, resulting in the release of Spx from YjbH. It has been suggested that the interaction between YirB and YjbH may prevent Spx proteolysis through a partner switching mechanism or by allosteric effects that hinder the Spx-YjbH interaction [233]. *yirB* expression is induced under cell-wall stress conditions, such as exposure to vancomycin, but not under ethanol or diamide treatments, and therefore it does not seem very relevant under proteotoxic stress conditions [238].

Aggregation of the YjbH adaptor protein

The YjbH protein is aggregation-prone. It aggregates under proteotoxic stress conditions, such as disulfide, heat, and ethanol induced stress [26]. In all cases, YjbH aggregation correlates with increased cellular Spx levels. YjbH apparently aggregates when the inclusion-body forming PorA protein from *Neisseria meningitidis* is produced in *B. subtilis* [26].

Stress-induced YjbH aggregates adopt a polar and midcell localization within the bacterium and this distribution seems to be driven by the purely passive mechanism of nuceloid occlusion (**Paper III**). This subcellular localization has been documented previously for other proteins in *E. coli* [239-243] and *B. subtilis* [208, 244-246] under proteotoxic stress. This suggests that under stress conditions, YjbH may potentially co-aggregate with numerous other misfolded proteins via non-specific hydrophobic interactions, but further research is required to confirm this.

From the structural point of view, YjbH contains a N-terminal thioredoxin-like domain with an alpha-helical insertion (together it makes a DsbA-like fold) (DsbAD) and a C-terminal winged helix-turn-helix domain (WHD), as revealed by the crystal structure of *Geobacillus kaustophilus* YjbH bound to *B. subtilis* Spx. The DsbAD and WHD domains are connected by a rigid proline-rich linker [234]. In **Paper III**, we show that the DsbAD and the WHD domains of YjbH individually display stress-induced aggregation, similar to the aggregation behavior of the full-length YjbH.

<u>Population heterogeneity and impact of YjbH aggregation on the cellular fitness</u>

In **Paper III**, we studied YjbH aggregation in the single-cell perspective in order to analyze heterogeneity in the population in response to stress. We found that the cell population is heterogenous in terms of YjbH aggregate carriage induced by stress (i.e., the aggregate load differs between cells). Notably, a higher extent of aggregation correlated with reduced cellular fitness. This suggests that, following the period of stress, faster-growing cells that have small or none YjbH aggregates

might eventually dominate the population. Conversely, slow-growing cells containing bigger YjbH aggregates may be more resistant to stress, providing an advantage to the population in case of re-exposure to stress. While the Spx-YjbH system requires further investigation, research conducted in *E. coli* suggests that the presence of protein aggregates is associated with increased tolerance to subsequent stresses [35, 36]. Therefore, the observed heterogeneity in the population in terms of YjbH aggregate load may be a bacterial survival strategy.

Spx and YjbH in other *Firmicutes*

Spx was originally described in *B. subtilis* [247]. Later on, genes encoding Spx orthologs were found in other low GC Gram-positive bacteria from the phylum *Firmicutes* [248]. For instance, Spx orthologs are found in *S. aureus* and *L. monocytogenes*. They show approximately 80% identity with *B. subtilis* Spx, and appear to be essential proteins (only under aerobic conditions in the case of *L. monocytogenes*) [249-254].

Spx orthologs usually have similar functions as those of *B. subtilis* Spx, especially when it comes to their roles in the oxidative stress response [255]. This is why *B. subtilis* Spx, which is very well-characterized, serves as a study prototype for the other orthologs. Some bacteria, including *B. subtilis*, contain multiple Spx paralogs. MgsR and YusI are Spx paralogs in *B. subtilis*. MgsR has a 46% identity to Spx and controls expression of some genes in the σ^B regulon. Similarly to Spx, MgsR is proteolysed by ClpXP [256]. The function of YusI, which has a 29% identity to Spx, is not known.

S. aureus has only one Spx protein whereas *Lactococccus lactis* and *L. garvieae* contain six and eleven Spx orthologs, respectively [255]. This indicates that in some species, Spx-like proteins might have evolved specialized other functions.

YjbH is also widely conserved among *Firmicutes*, but is little studied compared to Spx. In *S. aureus*, YjbH also has a role in adapting Spx for ClpXP proteolysis [249, 257] and its activity seems to be regulated by aggregation under stress conditions. Recently, YjbH was found to regulate Spx abundance in *L. monocytogenes*, and in the same study, *B. subtilis* YjbH was shown to complement a *L. monocytogenes yjbH* null mutant [258].

YjbH has been reported to play a role in the virulence of some bacterial pathogens. As revealed by two genetic screens in *L. monocytogenes*, *yjbH* is needed for synthesis of virulence factors [253, 259] and the growth of *L. monocytogenes* in host cells requires the presence of YjbH [260]. However, it is not known if the observed implications in virulence are directly dependent on YjbH or on the accumulation of Spx due to the absence of YjbH.

Complementation studies clearly indicate that YjbH orthologs function similarly despite low sequence identity. *G. kaustophilus*, *S. aureus* and *L. monocytogenes* YjbH can all complement a *B. subtilis* Δ*yjbH* strain [26, 257].

In **Paper III**, we uncovered conserved YjbH aggregation properties by showing that *S. aureus* and *L. monocytogenes* YjbH (and their respective DsbAD domains) aggregate in a similar way as the *B. subtilis* protein and without requiring host-specific factors. Interestingly, the WHD from *S. aureus* and *L. monocytogenes* did not aggregate on their own, contrary to the aggregation-prone *B. subtilis* WHD. This suggests that further comparison of WHD orthologs could provide valuable insight into the aggregation mechanism, as some critical residues might exist only in the *B. subtilis* WHD which make this domain to aggregate under stress.

The studied YjbH orthologs and DsbAD domains seem to adapt to *B. subtilis* Spx for degradation. The inter-species Spx and YjbH interactions suggest a conserved mechanism of recognition. Evolution likely favored YjbH sequences that are aggregation-prone, while retaining the residues that allow specific interaction with Spx.

Summary of papers

Paper I: *Bacillus subtilis* forms twisted cells with cell wall integrity defects upon removal of the molecular chaperones DnaK and trigger factor

In Paper I, we investigated the impact on the cell physiology of removing DnaK and TF in *B. subtilis*. Our findings indicate that both chaperones are crucial for maintaining cellular viability and thermotolerance, as well as preventing protein aggregation even under typical growth temperatures. Notably, the *dnaK tig* double deletion mutant exhibited a twisted and filamentous morphology, along with a significantly reduced colony size seemingly due to motility defects.

We observed that the aberrant morphology of the Δ*dnaK* Δ*tig* mutant is associated with compromised cell wall integrity. The double mutant showed an increased sensitivity to compounds that target the cell wall and a rough and irregular cell wall surface, as observed under TEM. Nevertheless, the absence of both chaperones still allowed important and complex cellular processes such as cell growth, division, and spore formation to occur.

Finally, we found that the cell morphology and other phenotypic traits of the Δ*dnaK* Δ*tig* mutant can be partially restored by second-site suppressor mutations. Unexpectedly, these mutations were not found in genes directly involved in the cell wall synthesis but in genes associated with metabolism or with other basic cellular processes (regulation of gene expression, RNA synthesis or degradation, and coping with stress). This suggests that *B. subtilis* can partly overcome the defect caused by the absence of DnaK and TF by adjusting multiple cellular processes.

These findings enhance our comprehension of proteostasis in *B. subtilis*. Additionally, they emphasize the potential of the *dnaK tig* double deletion mutant as a model for investigating cell wall homeostasis and the specific roles of DnaK and TF in *B. subtilis*, similar to the *E. coli* double mutant.

Paper II: Removal of the molecular chaperones DnaK and trigger factor in *Bacillus subtilis* affects biofilm formation

The research described in Paper II aimed to investigate the effects of the absence of DnaK and TF chaperones on biofilm formation in the ancestral strain of *B. subtilis*. We validated that the lack of both chaperones in the *B. subtilis* ancestral isolate led

to a similar phenotype as the one observed in the *dnaK* and *tig* double deletion mutant of strain 168, highlighting the important role of these chaperones in thermotolerance and cell morphology maintenance.

We found that the absence of both chaperones severely affected the macroscopic architecture of the biofilm and altered the proportion of the different cell types within the colony, with a greater number of spores in the biofilm centre and chained cells in the biofilm periphery. We also observed, with the use of fluorescence reporters, a reduction in motility (indicated by a reduced expression from the *hag* promoter), and an increase in the production of extracellular matrix (indicated by an increased expression from the *tasA* promoter) in the double mutant biofilm communities. The increase of matrix abundance in the double mutant biofilm was also indicated by SEM images showing the presence of an extracellular mesh-like structure. These findings suggest that DnaK and TF are directly or indirectly involved in pathways controlling biofilm formation and phenotypic cell heterogeneity in *B. subtilis* biofilms.

Apart from the absence of chaperones, elevated temperatures (which are known to increase protein misfolding) also altered biofilm appearance. When compared to the wild-type strain, the absence of chaperones (DnaK, or both DnaK and TF) seemed to enhance the effect of elevated temperatures on the biofilm. These findings suggest a link between compromised proteostasis and aberrant biofilm formation.

Paper III: New insights into the disulfide stress response by the *Bacillus subtilis* Spx system at a single-cell level

In Paper III, the Spx-YjbH system was investigated in response to disulfide stress at a single-cell level. By using a double fluorescence reporter strain and time-lapse microscopy, we found a correlation between Spx and YjbH protein levels in individual cells, consistent with understanding obtained by studies at the population level.

With the focus on YjbH aggregation, we observed that YjbH aggregates exhibit polar and midcell subcellular localization, driven by nucleoid molecular crowding and aggregate size. Importantly, time-lapse microscopy and subsequent analysis revealed the existence of population heterogeneity in terms of YjbH aggregate carriage and a strong link between aggregation and cellular fitness.

The contribution of the two YjbH domains to its aggregation properties was clarified. Conserved YjbH aggregation features were found by study of YjbH orthologs from *S. aureus* and *L. monocytogenes*. We show that YjbH orthologs and their respective DsbAD domains appear to share the same stress-induced aggregation behaviour (independently of host-specific factors), as well as their ability to adapt *B. subtilis* Spx for ClpXP degradation (indicating inter-species protein interaction). The WHD domains showed a different pattern. While the *B.*

subtilis WHD aggregated upon stress similar to the full length YjbH protein, the WHD domain of the other orthologs did not.

These studies provide increased understanding of the Spx-YjbH stress response and reveal the importance of single-cell analyses to uncover cell-to-cell variations.

Concluding remarks and future perspectives

In this thesis I have explored various aspects of proteostasis in *B. subtilis*, with a particular emphasis on the chaperones DnaK and TF. While the conserved and specific roles of DnaK and TF have been well characterized in *E. coli*, it is not clear which are the host-specific functions of these two chaperones in *B. subtilis*. In **Paper I**, we took a step forward in understanding the contributions of DnaK and TF in *B. subtilis* proteostasis by examining the phenotype that results from the combined deletion of the *dnaK* and *tig* genes. Our findings highlight significant differences between the *E. coli* and *B. subtilis* proteostasis networks and they could be attributed to the evolutionary divergence of approximately 2 billion years between the two model organisms with their respective adaptation to distinct environments. The differences serve well as a reminder that relying solely on studies with *E. coli* to understand chaperone functions and bacterial proteostasis is not sufficient to capture all aspects of the proteostasis network that may operate in bacteria.

The phenotype of the *B. subtilis* Δ*dnaK* Δ*tig* mutant (**Paper I**), as well as Δ*dnaK* Δ*tig* suppressor mutants, are valuable tools in further research aimed to uncover host-specific functions of DnaK and TF, especially in connection to cell morphology, cell wall integrity, and thermotolerance. The finding that suppressor mutations are found in genes for many different processes, and that the double deletion mutant phenotype was only partially restored, hints about the complexity of the proteostasis network. Future proteomic studies in *B. subtilis* could help to identify substrates of DnaK and TF, including their obligate clients. Studies could also determine the *B. subtilis* proteins that are most prone to aggregation in the absence of DnaK and TF. Other open questions are: What is the extent of overlap in substrates of the different core chaperones? Do these chaperones cooperate in *B. subtilis* proteostasis maintenance (as has been observed in *E. coli*)? In *E. coli*, DnaK clients (including obligate clients) have been identified by quantitative proteomics using SILAC (stable isotope labelling with amino acids in cell culture) [111]. This methodology also allowed to study the consequences of DnaK absence at the proteome level and to show partial functional redundancy of the DnaK and TF chaperones [111]. The contributions of DnaK in maintaining a stable proteome in *E. coli* were recently analysed with pulse proteolysis combined with SILAC-based proteomics (PP-SILAC) [261]. Similar approaches could be used in *B. subtilis*.

In **Paper II**, the Δ*dnaK* Δ*tig* mutation was created in the *B. subtilis* ancestral strain. This double mutant showed less pronounced cell morphology changes than the domesticated double mutant strain studied in **Paper I**. Additionally, no suppressor mutations appeared in the ancestral double mutant, suggesting that the damage caused by the absence of chaperones was mild. How does the proteostasis networks, as well as the contributions of the DnaK and TF in proteostasis, differ between the two *B. subtilis* strains? The ancestral strain is presumably adapted to tolerate frequent stress conditions whereas the domesticated strain has, in the laboratory over the years, adapted to much more stable and favourable conditions. In future studies one could investigate whether the ancestral strain has a better ability to cope with proteotoxic changes and if its proteome relies less on the folding assistance from DnaK and TF.

A main finding in **Paper II** was that the absence of DnaK and TF resulted in an aberrant biofilm architecture with increased extracellular matrix. The reason for the aberrant biofilm architecture remains unclear. Is it the increase in matrix abundance? If this is the case, deletion of some of the genes required for matrix production could potentially counteract the biofilm appearance of the chaperone deficient mutant. A change in matrix composition could also result in an altered biofilm. Possibly DnaK and TF are involved in the folding of key regulators of biofilm formation, some of which have perhaps not yet been uncovered. Biofilm formation in *B. subtilis* is a complex and highly regulated process and, thus, it is likely that the aberrant biofilm is a result of combined protein folding defects (of proteins that directly or indirectly influence biofilm formation). The Δ*dnaK* Δ*tig* mutant can be used for investigating proteostasis and the functions of DnaK and TF in relation to biofilm formation.

The *dnaK tig* double mutation also resulted in notable changes in the subpopulations of cells present within the biofilms. Advanced microscopy techniques, together with the use of fluorescence reporters for different cell types, could provide information about how cell differentiation is regulated in space and time within the mutant biofilm.

We found that elevated temperatures led to aberrant biofilm architectures (**Paper II**), again indicating that protein stability defects can impact biofilm formation. However, the effects of other proteotoxic stress conditions should also be tested. In further investigations one should try to confirm that protein aggregation (indicating proteostasis failure) is associated with (some) aberrant biofilms. A key question that remains to be tackled is whether the aberrant biofilms are less tolerant to stress conditions, such as the presence of antibiotics. If so, is proteostasis disruption a strategy to combat biofilms? And how do these findings relate to the properties of biofilms in nature in general? *B. subtilis* forms biofilms in plant roots and is known to protect plant roots from bacterial pathogens [78]. Do proteotoxic stress conditions that occur in the soil (like temperature fluctuations) influence biofilm formation and their benefits on plant roots?

Another question regarding proteostasis in *B. subtilis* that has also been addressed in this thesis is how *B. subtilis* maintains proteostasis under conditions of protein misfolding and aggregation. **Paper III** provides insights into one of the key mechanisms used by *B. subtilis* to deal with proteotoxic stress, the Spx-YjbH system. A key finding was the observation of population heterogeneity in YjbH aggregate loads in cells, which negatively correlated with cell fitness. We would expect the aggregate load to correlate positively with cellular Spx levels, but this was not tested in our study, mainly because the fluorescence reporter that was used to indirectly measure the Spx levels in single cells did not seem to be sensitive enough to detect only minor differences. Utilizing more sensitive single-cell level techniques seems necessary to determine if there is heterogeneity in the Spx response to proteotoxic stress in the *B. subtilis* population.

Possibly there is stochastic variation between cells regarding the Spx levels under non-stress conditions. If this is the case, it is likely that cells with more Spx would be more resistant to adverse conditions, but they would be less competitive under normal conditions due to the detrimental effect of high Spx levels on bacterial growth [220].

With regards to YjbH aggregation, there are several questions. For example: Does YjbH self-interact? Does YjbH non-specifically bind to other misfolded proteins under stress, leading to the formation of aggregates? Does YjbH require assistance from chaperones to fold correctly? If the answer to this question is yes, general protein misfolding and aggregation could deplete the levels of chaperones available for YjbH folding, leading to YjbH misfolding and aggregation. Are there specific amino acid residues that make YjbH prone to aggregation? It would be interesting to investigate whether certain amino acid substitutions or modifications could increase the stability of YjbH and reduce its tendency to aggregate. The C-terminal WHD domain of YjbH is a promising candidate for such exploration, given its small size and the observed differences between the *B. subtilis* WHD (which exhibits stress-induced aggregation) and the domain from other species, like *S. aureus* and *L. monocytogenes* (which do not aggregate).

The cell localization, dynamics, and inheritance of YjbH aggregation was investigated in **Paper III** by observing relatively large mNG-YjbH fluorescent foci using fluorescence microscopy. Utilizing super resolution microscopy techniques to observe smaller aggregates could provide additional informative data. Exploring the dynamics and inheritance of smaller aggregates and their correlation with cell fitness would enhance our understanding of the YjbH system, as well as how Gram-positive bacteria in general handle protein aggregation.

With this thesis, I hope to have provided significant new knowledge about the proteostasis network of *B. subtilis* and materials useful in future research efforts in the field of bacterial proteostasis. The study of proteostasis both increases our

fundamental understanding of cell biology and have implications in the fight against pathogens and in the design of bacterial strains for use within biotechnology.

Acknowledgements

Claes, I am deeply grateful to you for letting me be a part of your lab, and for all your help and supervision during my PhD journey. I really enjoyed the scientific discussions we had over these years. It was very inspiring to work and learn from you!

Lars, thank you for all your help with reviewing my work. I really appreciated your feedback and that you always gave me advice after my presentations. I have learned a lot from you during my academic journey. **Klas**, thank you for all the work you have done as my examiner and for your efforts in organizing the MRC, which was a very valuable learning experience. **Fredric**, thank you for your input and support as my co-supervisor during the progress meetings.

I am incredibly grateful to all the past and current members of the von Wachenfeldt lab for making it such an exceptional, inspiring, and enjoyable place to work! **Vinardas**, the best lab and office mate I could ever have, thank you for all of the fun times and for all your help. **Rawana**, thank you for your great friendship, for all the help, support and care on so many occasions. You even brought me fudge on my last day of the thesis writing! **Swati,** thanks for the fun times at the office and in the lab. It was great to have you around! **Joel**, thank you for our inspiring discussions about science and for discovering the poor twisted *Bacillus*, for the fun we had and for going with me to pick cherries during our work breaks. **Martin**, thank you for our fun chats and for your patience in helping me learn a bit more Swedish. Oh, and of course, for listening to my complaints during my peaks of stress! **Deepak**, thank you for all the fika times and for collaborating with me in the YjbH project. Also, a big thank you to **Johanna**, **Federico**, **Jorge**, **Svetlana**, **Yasmin**, **Meenakshi**, and **Philipp** for making it a very fun and lively atmosphere in the lab.

I also want to thank my friendly colleagues who I had the opportunity to meet: **Abraham**, **Bir**, **Cuong**, **Dima, Esther, Héloïse, Humberto, Karla, Katie**, **Manuel**, **Markus**, **Teresa**, **Valeria**, **Veronika**, **Vivian**, and **many others,** thank you for all the fun and interesting conversations in the lunchroom, and for the good times spent outside of work. It has been great to have you around!

Elisabeth, thank you for all your amazing help during the course labs. **Ola**, thank you for your assistance with the electron microscope.

I am very happy to have had the opportunity to work in the Biology Department, where I found everyone to be very friendly. To all the **PhD students, post docs, students, PIs, technical and administrative staff**: thank you! Each and every one of you played a role in making my experience as a PhD student very enjoyable.

To my **friends** outside the university: thank you for supporting me during difficult times, for celebrating with me during moments of happiness, and for always helping me gain a fresh perspective on things.

To my **family**: papa, mama, i Uri, gràcies per donar-me la mà durant tot el camí, per creure en mi, i per totes les vegades que m'heu agafat el telèfon!

Jack, thank you for your infinite love, support, and help. For navigating with me through all the highs and lows of completing my thesis. Thank you for being in my team!

References

1. Dill, K.A., et al., *The protein folding problem.* Annu. Rev. Biophys., 2008. 37: p. 289-316.
2. Balchin, D., M. Hayer-Hartl, and F.U. Hartl, *In vivo aspects of protein folding and quality control.* Science, 2016. 353(6294).
3. Pace, C.N., J.M. Scholtz, and G.R. Grimsley, *Forces stabilizing proteins.* FEBS letters, 2014. 588(14): p. 2177-2184.
4. Anfinsen, C. and H. Scheraga, *Experimental and theoretical aspects of protein folding.* Advances in protein chemistry, 1975. 29: p. 205-300.
5. Brockwell, D.J. and S.E. Radford, *Intermediates: ubiquitous species on folding energy landscapes?* Current opinion in structural biology, 2007. 17(1): p. 30-37.
6. Powers, E.T., et al., *Biological and chemical approaches to diseases of proteostasis deficiency.* Annu Rev Biochem, 2009. 78: p. 959-91.
7. Richter, K., M. Haslbeck, and J. Buchner, *The heat shock response: life on the verge of death.* Mol Cell, 2010. 40(2): p. 253-66.
8. Schramm, F.D., K. Schroeder, and K. Jonas, *Protein aggregation in bacteria.* FEMS Microbiol Rev, 2020. 44(1): p. 54-72.
9. Powers, E.T. and W.E. Balch, *Diversity in the origins of proteostasis networks-a driver for protein function in evolution.* Nat Rev Mol Cell Biol, 2013. 14(4): p. 237-48.
10. Balchin, D., M. Hayer-Hartl, and F.U. Hartl, *In vivo aspects of protein folding and quality control.* Science, 2016. 353(6294): p. aac4354.
11. Olivares, A.O., T.A. Baker, and R.T. Sauer, *Mechanistic insights into bacterial AAA+ proteases and protein-remodelling machines.* Nat Rev Microbiol, 2016. 14(1): p. 33-44.
12. Powers, E.T. and W.E. Balch, *Diversity in the origins of proteostasis networks—a driver for protein function in evolution.* Nature reviews Molecular cell biology, 2013. 14(4): p. 237-248.
13. Schramm, F.D., K. Schroeder, and K. Jonas, *Protein aggregation in bacteria.* FEMS microbiology reviews, 2020. 44(1): p. 54-72.
14. Cumming, R.C., et al., *Protein disulfide bond formation in the cytoplasm during oxidative stress.* Journal of biological chemistry, 2004. 279(21): p. 21749-21758.
15. Fitter, J., *The temperature dependence of internal molecular motions in hydrated and dry α-amylase: the role of hydration water in the dynamical transition of proteins.* Biophysical journal, 1999. 76(2): p. 1034-1042.
16. Deller, M.C., L. Kong, and B. Rupp, *Protein stability: a crystallographer's perspective.* Acta Crystallographica Section F: Structural Biology Communications, 2016. 72(2): p. 72-95.

17. Mogk, A., B. Bukau, and H.H. Kampinga, *Cellular handling of protein aggregates by disaggregation machines.* Molecular cell, 2018. 69(2): p. 214-226.
18. Cheng, J., et al., *The emerging roles of protein homeostasis-governing pathways in Alzheimer's disease.* Aging Cell, 2018. 17(5): p. e12801.
19. Leszczynska, D., et al., *The formation of persister cells in stationary-phase cultures of Escherichia coli is associated with the aggregation of endogenous proteins.* PLoS One, 2013. 8(1): p. e54737.
20. Pu, Y., et al., *ATP-dependent dynamic protein aggregation regulates bacterial dormancy depth critical for antibiotic tolerance.* Mol Cell, 2019. 73(1): p. 143-156 e4.
21. Yu, J., et al., *Regrowth-delay body as a bacterial subcellular structure marking multidrug-tolerant persisters.* Cell Discov, 2019. 5: p. 8.
22. Dewachter, L., et al., *The dynamic transition of persistence toward the viable but nonculturable state during stationary phase is driven by protein aggregation.* mBio, 2021. 12(4): p. e0070321.
23. Huemer, M., et al., *Molecular reprogramming and phenotype switching in Staphylococcus aureus lead to high antibiotic persistence and affect therapy success.* Proc Natl Acad Sci U S A, 2021. 118(7).
24. Garg, S.K., et al., *The YjbH protein of Bacillus subtilis enhances ClpXP-catalyzed proteolysis of Spx.* J Bacteriol, 2009. 191(4): p. 1268-77.
25. Larsson, J.T., A. Rogstam, and C. Von Wachenfeldt, *YjbH is a novel negative effector of the disulphide stress regulator, Spx, in Bacillus subtilis.* Molecular microbiology, 2007. 66(3): p. 669-684.
26. Engman, J. and C. von Wachenfeldt, *Regulated protein aggregation: a mechanism to control the activity of the ClpXP adaptor protein YjbH.* Mol Microbiol, 2015. 95(1): p. 51-63.
27. Khodaparast, L., et al., *Bacterial protein homeostasis disruption as a therapeutic intervention.* Front Mol Biosci, 2021. 8: p. 681855.
28. Ramakrishnan, R., et al., *Differential proteostatic regulation of insoluble and abundant proteins.* Bioinformatics, 2019. 35(20): p. 4098-4107.
29. de Groot, N.S. and S. Ventura, *Protein aggregation profile of the bacterial cytosol.* PLoS One, 2010. 5(2): p. e9383.
30. Willmund, F., et al., *The cotranslational function of ribosome-associated Hsp70 in eukaryotic protein homeostasis.* Cell, 2013. 152(1-2): p. 196-209.
31. Oren, A. and G.M. Garrity, *Valid publication of the names of forty-two phyla of prokaryotes.* Int J Syst Evol Microbiol, 2021. 71(10).
32. Errington, J. and L.T. van der Aart, *Microbe Profile: Bacillus subtilis: model organism for cellular development, and industrial workhorse.* Microbiology, 2020. 166(5): p. 425.
33. Arnaouteli, S., et al., *Bacillus subtilis biofilm formation and social interactions.* Nature Reviews Microbiology, 2021: p. 1-15.
34. Cohn, F., *Utersuchungen uber Bakterien.* Beitr Biol Pflanz, 1872. 1: p. 124-224.
35. Ehrenberg, C., *Dritter Beitrag zur Erkenntniss grosser Organisation in der Richtung des kleinsten Raumes.* Berlin: Konigl. Akad. d. Wiss., 1834. 1833: p. 145-336.
36. Losick, R.M., *Bacillus subtilis: a bacterium for all seasons.* Current Biology, 2020. 30(19): p. R1146-R1150.

37. Errington, J., *Regulation of endospore formation in Bacillus subtilis.* Nature Reviews Microbiology, 2003. 1(2): p. 117-126.
38. Vlamakis, H., et al., *Sticking together: building a biofilm the Bacillus subtilis way.* Nature Reviews Microbiology, 2013. 11(3): p. 157-168.
39. López, D., et al., *Cannibalism enhances biofilm development in Bacillus subtilis.* Molecular microbiology, 2009. 74(3): p. 609-618.
40. Mukherjee, S. and D.B. Kearns, *The structure and regulation of flagella in Bacillus subtilis.* Annual review of genetics, 2014. 48: p. 319-340.
41. McKenney, P.T., A. Driks, and P. Eichenberger, *The Bacillus subtilis endospore: assembly and functions of the multilayered coat.* Nat Rev Microbiol, 2013. 11(1): p. 33-44.
42. Hoch, J.A., *Regulation of the phosphorelay and the initiation of sporulation in Bacillus subtilis.* Annu Rev Microbiol, 1993. 47: p. 441-65.
43. Fujita, M., J.E. Gonzalez-Pastor, and R. Losick, *High- and low-threshold genes in the Spo0A regulon of Bacillus subtilis.* J Bacteriol, 2005. 187(4): p. 1357-68.
44. Molle, V., et al., *The Spo0A regulon of Bacillus subtilis.* Mol Microbiol, 2003. 50(5): p. 1683-701.
45. Veening, J.W., et al., *Bet-hedging and epigenetic inheritance in bacterial cell development.* Proc Natl Acad Sci U S A, 2008. 105(11): p. 4393-8.
46. Gonzalez-Pastor, J.E., E.C. Hobbs, and R. Losick, *Cannibalism by sporulating bacteria.* Science, 2003. 301(5632): p. 510-3.
47. Marquez-Magana, L.M. and M.J. Chamberlin, *Characterization of the sigD transcription unit of Bacillus subtilis.* J Bacteriol, 1994. 176(8): p. 2427-34.
48. Chen, R., et al., *Role of the sigmaD-dependent autolysins in Bacillus subtilis population heterogeneity.* J Bacteriol, 2009. 191(18): p. 5775-84.
49. Tsukahara, K. and M. Ogura, *Promoter selectivity of the Bacillus subtilis response regulator DegU, a positive regulator of the fla/che operon and sacB.* BMC Microbiol, 2008. 8: p. 8.
50. Kearns, D.B. and R. Losick, *Cell population heterogeneity during growth of Bacillus subtilis.* Genes Dev, 2005. 19(24): p. 3083-94.
51. Calvio, C., et al., *Swarming differentiation and swimming motility in Bacillus subtilis are controlled by swrA, a newly identified dicistronic operon.* J Bacteriol, 2005. 187(15): p. 5356-66.
52. Qin, Y., L.L. Angelini, and Y. Chai, *Bacillus subtilis Cell Differentiation, Biofilm Formation and Environmental Prevalence.* Microorganisms, 2022. 10(6).
53. Kearns, D.B., et al., *A master regulator for biofilm formation by Bacillus subtilis.* Mol Microbiol, 2005. 55(3): p. 739-49.
54. Chu, F., et al., *Targets of the master regulator of biofilm formation in Bacillus subtilis.* Mol Microbiol, 2006. 59(4): p. 1216-28.
55. Bai, U., I. Mandic-Mulec, and I. Smith, *SinI modulates the activity of SinR, a developmental switch protein of Bacillus subtilis, by protein-protein interaction.* Genes Dev, 1993. 7(1): p. 139-48.
56. Blair, K.M., et al., *A molecular clutch disables flagella in the Bacillus subtilis biofilm.* Science, 2008. 320(5883): p. 1636-8.
57. Kearns, D.B. and R. Losick, *Swarming motility in undomesticated Bacillus subtilis.* Mol Microbiol, 2003. 49(3): p. 581-90.

58. Guttenplan, S.B., S. Shaw, and D.B. Kearns, *The cell biology of peritrichous flagella in Bacillus subtilis.* Mol Microbiol, 2013. 87(1): p. 211-29.
59. Mukherjee, S., et al., *Adaptor-mediated Lon proteolysis restricts Bacillus subtilis hyperflagellation.* Proc Natl Acad Sci U S A, 2015. 112(1): p. 250-5.
60. Kinsinger, R.F., M.C. Shirk, and R. Fall, *Rapid surface motility in Bacillus subtilis is dependent on extracellular surfactin and potassium ion.* J Bacteriol, 2003. 185(18): p. 5627-31.
61. Grau, R.R., et al., *A Duo of Potassium-Responsive Histidine Kinases Govern the Multicellular Destiny of Bacillus subtilis.* mBio, 2015. 6(4): p. e00581.
62. van Gestel, J., H. Vlamakis, and R. Kolter, *From cell differentiation to cell collectives: Bacillus subtilis uses division of labor to migrate.* PLoS Biol, 2015. 13(4): p. e1002141.
63. Johnsborg, O., V. Eldholm, and L.S. Havarstein, *Natural genetic transformation: prevalence, mechanisms and function.* Res Microbiol, 2007. 158(10): p. 767-78.
64. Mell, J.C. and R.J. Redfield, *Natural competence and the evolution of DNA uptake specificity.* J Bacteriol, 2014. 196(8): p. 1471-83.
65. Magnuson, R., J. Solomon, and A.D. Grossman, *Biochemical and genetic characterization of a competence pheromone from B. subtilis.* Cell, 1994. 77(2): p. 207-16.
66. Ansaldi, M., et al., *Specific activation of the Bacillus quorum-sensing systems by isoprenylated pheromone variants.* Mol Microbiol, 2002. 44(6): p. 1561-73.
67. Dubnau, D., *Genetic competence in Bacillus subtilis.* Microbiol Rev, 1991. 55(3): p. 395-424.
68. van Sinderen, D., et al., *comK encodes the competence transcription factor, the key regulatory protein for competence development in Bacillus subtilis.* Mol Microbiol, 1995. 15(3): p. 455-62.
69. Suel, G.M., et al., *An excitable gene regulatory circuit induces transient cellular differentiation.* Nature, 2006. 440(7083): p. 545-50.
70. Berka, R.M., et al., *Microarray analysis of the Bacillus subtilis K-state: genome-wide expression changes dependent on ComK.* Mol Microbiol, 2002. 43(5): p. 1331-45.
71. Claverys, J.P. and L.S. Havarstein, *Cannibalism and fratricide: mechanisms and raisons d'etre.* Nat Rev Microbiol, 2007. 5(3): p. 219-29.
72. Ciofu, O., et al., *Tolerance and resistance of microbial biofilms.* Nat Rev Microbiol, 2022. 20(10): p. 621-635.
73. Vlamakis, H., et al., *Control of cell fate by the formation of an architecturally complex bacterial community.* Genes Dev, 2008. 22(7): p. 945-53.
74. Chai, Y., et al., *An epigenetic switch governing daughter cell separation in Bacillus subtilis.* Genes Dev, 2010. 24(8): p. 754-65.
75. Chai, Y., et al., *Bistability and biofilm formation in Bacillus subtilis.* Mol Microbiol, 2008. 67(2): p. 254-63.
76. Kobayashi, K., *SlrR/SlrA controls the initiation of biofilm formation in Bacillus subtilis.* Mol Microbiol, 2008. 69(6): p. 1399-410.
77. Srinivasan, S., et al., *Matrix Production and Sporulation in Bacillus subtilis Biofilms Localize to Propagating Wave Fronts.* Biophys J, 2018. 114(6): p. 1490-1498.

78. Arnaouteli, S., et al., *Bacillus subtilis biofilm formation and social interactions.* Nat Rev Microbiol, 2021. 19(9): p. 600-614.
79. Zeigler, D.R., et al., *The origins of 168, W23, and other Bacillus subtilis legacy strains.* J Bacteriol, 2008. 190(21): p. 6983-95.
80. Spizizen, J., *Transformation of Biochemically Deficient Strains of Bacillus Subtilis by Deoxyribonucleate.* Proc Natl Acad Sci U S A, 1958. 44(10): p. 1072-8.
81. Branda, S.S., et al., *Fruiting body formation by Bacillus subtilis.* Proc Natl Acad Sci U S A, 2001. 98(20): p. 11621-6.
82. Konkol, M.A., K.M. Blair, and D.B. Kearns, *Plasmid-encoded ComI inhibits competence in the ancestral 3610 strain of Bacillus subtilis.* J Bacteriol, 2013. 195(18): p. 4085-93.
83. Mogk, A., D. Huber, and B. Bukau, *Integrating protein homeostasis strategies in prokaryotes.* Cold Spring Harb Perspect Biol, 2011. 3(4).
84. Hartl, F.U. and M. Hayer-Hartl, *Molecular chaperones in the cytosol: from nascent chain to folded protein.* Science, 2002. 295(5561): p. 1852-1858.
85. Moliere, N. and K. Turgay, *Chaperone-protease systems in regulation and protein quality control in Bacillus subtilis.* Res Microbiol, 2009. 160(9): p. 637-44.
86. Fayet, O., T. Ziegelhoffer, and C. Georgopoulos, *The groES and groEL heat shock gene products of Escherichia coli are essential for bacterial growth at all temperatures.* J Bacteriol, 1989. 171(3): p. 1379-85.
87. Commichau, F.M., N. Pietack, and J. Stulke, *Essential genes in Bacillus subtilis: a re-evaluation after ten years.* Mol Biosyst, 2013. 9(6): p. 1068-75.
88. Hoffmann, A., et al., *Concerted action of the ribosome and the associated chaperone trigger factor confines nascent polypeptide folding.* Molecular cell, 2012. 48(1): p. 63-74.
89. Saio, T., et al., *Oligomerization of a molecular chaperone modulates its activity.* Elife, 2018. 7.
90. Stoller, G., et al., *A ribosome-associated peptidyl-prolyl cis/trans isomerase identified as the trigger factor.* EMBO J, 1995. 14(20): p. 4939-48.
91. Zarnt, T., et al., *Modular structure of the trigger factor required for high activity in protein folding.* J Mol Biol, 1997. 271(5): p. 827-37.
92. Ferbitz, L., et al., *Trigger factor in complex with the ribosome forms a molecular cradle for nascent proteins.* Nature, 2004. 431(7008): p. 590-596.
93. Martinez-Hackert, E. and W.A. Hendrickson, *Promiscuous substrate recognition in folding and assembly activities of the trigger factor chaperone.* Cell, 2009. 138(5): p. 923-34.
94. Saio, T., et al., *Structural basis for protein antiaggregation activity of the trigger factor chaperone.* Science, 2014. 344(6184): p. 1250494.
95. Mashaghi, A., et al., *Reshaping of the conformational search of a protein by the chaperone trigger factor.* Nature, 2013. 500(7460): p. 98-101.
96. Singhal, K., et al., *The trigger factor chaperone encapsulates and stabilizes partial folds of substrate proteins.* PLoS computational biology, 2015. 11(10): p. e1004444.
97. Deuerling, E., et al., *Trigger factor and DnaK cooperate in folding of newly synthesized proteins.* Nature, 1999. 400(6745): p. 693-6.

98. Teter, S.A., et al., *Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains.* Cell, 1999. 97(6): p. 755-65.
99. Oh, E., et al., *Selective ribosome profiling reveals the cotranslational chaperone action of trigger factor in vivo.* Cell, 2011. 147(6): p. 1295-1308.
100. Deuerling, E., et al., *Trigger Factor and DnaK possess overlapping substrate pools and binding specificities.* Mol Microbiol, 2003. 47(5): p. 1317-28.
101. Guthrie, B. and W. Wickner, *Trigger factor depletion or overproduction causes defective cell division but does not block protein export.* J Bacteriol, 1990. 172(10): p. 5555-62.
102. Gothel, S.F., et al., *Cyclophilin and trigger factor from Bacillus subtilis catalyze in vitro protein folding and are necessary for viability under starvation conditions.* Biochemistry, 1998. 37(38): p. 13392-9.
103. Kandror, O. and A.L. Goldberg, *Trigger factor is induced upon cold shock and enhances viability of Escherichia coli at low temperatures.* Proc Natl Acad Sci U S A, 1997. 94(10): p. 4978-81.
104. Eymann, C., et al., *Bacillus subtilis functional genomics: global characterization of the stringent response by proteome and transcriptome analysis.* Journal of bacteriology, 2002. 184(9): p. 2500-2520.
105. Zhou, B., et al., *Arginine dephosphorylation propels spore germination in bacteria.* Proceedings of the National Academy of Sciences, 2019. 116(28): p. 14228-14237.
106. Winardhi, R.S., et al., *The holdase function of Escherichia coli Hsp70 (DnaK) chaperone.* bioRxiv, 2018: p. 305854.
107. Zhu, X., et al., *Structural analysis of substrate binding by the molecular chaperone DnaK.* Science, 1996. 272(5268): p. 1606-14.
108. Perales-Calvo, J., et al., *The force-dependent mechanism of DnaK-mediated mechanical folding.* Sci Adv, 2018. 4(2): p. eaaq0243.
109. Rudiger, S., A. Buchberger, and B. Bukau, *Interaction of Hsp70 chaperones with substrates.* Nat Struct Biol, 1997. 4(5): p. 342-9.
110. Mogk, A., et al., *Identification of thermolabile Escherichia coli proteins: prevention and reversion of aggregation by DnaK and ClpB.* EMBO J, 1999. 18(24): p. 6934-49.
111. Calloni, G., et al., *DnaK functions as a central hub in the E. coli chaperone network.* Cell Rep, 2012. 1(3): p. 251-64.
112. Liberek, K., et al., *Escherichia coli DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK.* Proc Natl Acad Sci U S A, 1991. 88(7): p. 2874-8.
113. Szabo, A., et al., *The ATP hydrolysis-dependent reaction cycle of the Escherichia coli Hsp70 system DnaK, DnaJ, and GrpE.* Proc Natl Acad Sci U S A, 1994. 91(22): p. 10345-9.
114. Kampinga, H.H. and E.A. Craig, *The HSP70 chaperone machinery: J proteins as drivers of functional specificity.* Nature reviews Molecular cell biology, 2010. 11(8): p. 579-592.
115. Schröder, H., et al., *DnaK, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage.* The EMBO journal, 1993. 12(11): p. 4137-4144.

116. Laufen, T., et al., *Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones.* Proceedings of the National Academy of Sciences, 1999. 96(10): p. 5452-5457.
117. Bukau, B. and A.L. Horwich, *The Hsp70 and Hsp60 chaperone machines.* Cell, 1998. 92(3): p. 351-366.
118. Mally, A. and S.N. Witt, *GrpE accelerates peptide binding and release from the high affinity state of DnaK.* Nature structural biology, 2001. 8(3): p. 254-257.
119. Gamer, J., H. Bujard, and B. Bukau, *Physical interaction between heat shock proteins DnaK, DnaJ, and GrpE and the bacterial heat shock transcription factor sigma 32.* Cell, 1992. 69(5): p. 833-42.
120. Liberek, K., et al., *The DnaK chaperone modulates the heat shock response of Escherichia coli by binding to the sigma 32 transcription factor.* Proc Natl Acad Sci U S A, 1992. 89(8): p. 3516-20.
121. Nonaka, G., et al., *Regulon and promoter analysis of the E. coli heat-shock factor, σ32, reveals a multifaceted cellular response to heat stress.* Genes & development, 2006. 20(13): p. 1776-1789.
122. Paek, K.H. and G.C. Walker, *Escherichia coli dnaK null mutants are inviable at high temperature.* J Bacteriol, 1987. 169(1): p. 283-90.
123. Bukau, B. and G.C. Walker, *Cellular defects caused by deletion of the Escherichia coli dnaK gene indicate roles for heat shock protein in normal metabolism.* J Bacteriol, 1989. 171(5): p. 2337-46.
124. Bukau, B. and G.C. Walker, *Mutations altering heat shock specific subunit of RNA polymerase suppress major cellular defects of E. coli mutants lacking the DnaK chaperone.* EMBO J, 1990. 9(12): p. 4027-36.
125. Paek, K. and G.C. Walker, *Escherichia coli dnaK null mutants are inviable at high temperature.* Journal of bacteriology, 1987. 169(1): p. 283-290.
126. Bukau, B. and G.C. Walker, *Mutations altering heat shock specific subunit of RNA polymerase suppress major cellular defects of E. coli mutants lacking the DnaK chaperone.* The EMBO Journal, 1990. 9(12): p. 4027-4036.
127. Schumann, W., *The Bacillus subtilis heat shock stimulon.* Cell Stress Chaperones, 2003. 8(3): p. 207-17.
128. Schulz, A., B. Tzschaschel, and W. Schumann, *Isolation and analysis of mutants of the dnaK operon of Bacillus subtilis.* Mol Microbiol, 1995. 15(3): p. 421-9.
129. Shi, L., et al., *Tyrosine 601 of Bacillus subtilis DnaK undergoes phosphorylation and is crucial for chaperone activity and heat shock survival.* Front Microbiol, 2016. 7: p. 533.
130. Ewalt, K.L., et al., *In vivo observation of polypeptide flux through the bacterial chaperonin system.* Cell, 1997. 90(3): p. 491-500.
131. Kerner, M.J., et al., *Proteome-wide analysis of chaperonin-dependent protein folding in Escherichia coli.* Cell, 2005. 122(2): p. 209-20.
132. Fujiwara, K., et al., *A systematic survey of in vivo obligate chaperonin-dependent substrates.* EMBO J, 2010. 29(9): p. 1552-64.
133. Langer, T., et al., *Chaperonin-mediated protein folding: GroES binds to one end of the GroEL cylinder, which accommodates the protein substrate within its central cavity.* EMBO J, 1992. 11(13): p. 4757-65.
134. Mayhew, M., et al., *Protein folding in the central cavity of the GroEL-GroES chaperonin complex.* Nature, 1996. 379(6564): p. 420-6.

135. Balchin, D., M. Hayer-Hartl, and F.U. Hartl, *Recent advances in understanding catalysis of protein folding by molecular chaperones.* FEBS letters, 2020. 594(17): p. 2770-2781.
136. Hayer-Hartl, M., A. Bracher, and F.U. Hartl, *The GroEL–GroES chaperonin machine: a nano-cage for protein folding.* Trends in biochemical sciences, 2016. 41(1): p. 62-76.
137. Balchin, D., M. Hayer-Hartl, and F.U. Hartl, *Recent advances in understanding catalysis of protein folding by molecular chaperones.* FEBS Lett, 2020. 594(17): p. 2770-2781.
138. Lund, P.A., *Multiple chaperonins in bacteria–why so many?* FEMS microbiology reviews, 2009. 33(4): p. 785-800.
139. Genevaux, P., et al., *In vivo analysis of the overlapping functions of DnaK and trigger factor.* EMBO Rep, 2004. 5(2): p. 195-200.
140. Genevaux, P., et al., *In vivo analysis of the overlapping functions of DnaK and trigger factor.* EMBO reports, 2004. 5(2): p. 195-200.
141. Vorderwulbecke, S., et al., *Low temperature or GroEL/ES overproduction permits growth of Escherichia coli cells lacking trigger factor and DnaK.* FEBS Lett, 2004. 559(1-3): p. 181-7.
142. Ullers, R.S., et al., *SecB is a bona fide generalized chaperone in Escherichia coli.* Proc Natl Acad Sci U S A, 2004. 101(20): p. 7583-8.
143. Bruel, N., et al., *Hsp33 controls elongation factor-Tu stability and allows Escherichia coli growth in the absence of the major DnaK and trigger factor chaperones.* J Biol Chem, 2012. 287(53): p. 44435-46.
144. Angles, F., et al., *Multilevel interaction of the DnaK/DnaJ(HSP70/HSP40) stress-responsive chaperone machine with the central metabolism.* Sci Rep, 2017. 7: p. 41341.
145. Ullers, R.S., et al., *Trigger Factor can antagonize both SecB and DnaK/DnaJ chaperone functions in Escherichia coli.* Proc Natl Acad Sci U S A, 2007. 104(9): p. 3101-6.
146. Reyes, D.Y. and H. Yoshikawa, *DnaK chaperone machine and trigger factor are only partially required for normal growth of Bacillus subtilis.* Biosci Biotechnol Biochem, 2002. 66(7): p. 1583-6.
147. Arita-Morioka, K., et al., *Novel strategy for biofilm inhibition by using small molecules targeting molecular chaperone DnaK.* Antimicrob Agents Chemother, 2015. 59(1): p. 633-41.
148. Sugimoto, S., et al., *Multitasking of Hsp70 chaperone in the biogenesis of bacterial functional amyloids.* Commun Biol, 2018. 1: p. 52.
149. Singh, V.K., et al., *An insight into the significance of the DnaK heat shock system in Staphylococcus aureus.* Int J Med Microbiol, 2012. 302(6): p. 242-52.
150. Keogh, R.A., et al., *Staphylococcus aureus Trigger Factor Is Involved in Biofilm Formation and Cooperates with the Chaperone PpiB.* J Bacteriol, 2021. 203(7).
151. van der Veen, S. and T. Abee, *HrcA and DnaK are important for static and continuous-flow biofilm formation and disinfectant resistance in Listeria monocytogenes.* Microbiology (Reading), 2010. 156(Pt 12): p. 3782-3790.
152. Lemos, J.A., Y. Luzardo, and R.A. Burne, *Physiologic effects of forced down-regulation of dnaK and groEL expression in Streptococcus mutans.* J Bacteriol, 2007. 189(5): p. 1582-8.

153. Jain, S., et al., *Inactivation of the dnaK gene in Clostridium difficile 630 Deltaerm yields a temperature-sensitive phenotype and increases biofilm-forming ability.* Sci Rep, 2017. 7(1): p. 17522.
154. Arora, G., et al., *Ser/Thr protein kinase PrkC-mediated regulation of GroEL is critical for biofilm formation in Bacillus anthracis.* NPJ Biofilms Microbiomes, 2017. 3: p. 7.
155. Gallaher, T.K., et al., *Identification of biofilm proteins in non-typeable Haemophilus Influenzae.* BMC Microbiol, 2006. 6: p. 65.
156. Kalmokoff, M., et al., *Proteomic analysis of Campylobacter jejuni 11168 biofilms reveals a role for the motility complex in biofilm formation.* J Bacteriol, 2006. 188(12): p. 4312-20.
157. Ojha, A., et al., *GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria.* Cell, 2005. 123(5): p. 861-73.
158. Stubbendieck, R.M. and P.D. Straight, *Linearmycins Activate a Two-Component Signaling System Involved in Bacterial Competition and Biofilm Morphology.* J Bacteriol, 2017. 199(18).
159. Vlamakis, H., et al., *Sticking together: building a biofilm the Bacillus subtilis way.* Nat Rev Microbiol, 2013. 11(3): p. 157-68.
160. Mogk, A., D. Huber, and B. Bukau, *Integrating protein homeostasis strategies in prokaryotes.* Cold Spring Harbor perspectives in biology, 2011. 3(4): p. a004366.
161. Krüger, E., et al., *The clp proteases of Bacillus subtilis are directly involved in degradation of misfolded proteins.* Journal of Bacteriology, 2000. 182(11): p. 3259-3265.
162. Sauer, R.T. and T.A. Baker, *AAA+ proteases: ATP-fueled machines of protein destruction.* Annu Rev Biochem, 2011. 80: p. 587-612.
163. Sauer, R.T. and T.A. Baker, *AAA+ proteases: ATP-fueled machines of protein destruction.* Annual review of biochemistry, 2011. 80: p. 587-612.
164. Mogk, A., P. Katikaridis, and V. Bohl, *Resisting the heat: bacterial disaggregases rescue cells from devastating protein aggregation.* Frontiers in Molecular Biosciences, 2021. 8: p. 320.
165. Wickner, S., M.R. Maurizi, and S. Gottesman, *Posttranslational quality control: folding, refolding, and degrading proteins.* Science, 1999. 286(5446): p. 1888-1893.
166. Choi, K.-H. and S. Licht, *Control of peptide product sizes by the energy-dependent protease ClpAP.* Biochemistry, 2005. 44(42): p. 13921-13931.
167. Elsholz, A.K.W., et al., *Functional Diversity of AAA+ Protease Complexes in Bacillus subtilis.* Front Mol Biosci, 2017. 4: p. 44.
168. Keiler, K.C., *Biology of trans-translation.* Annu. Rev. Microbiol., 2008. 62: p. 133-151.
169. Himeno, H., D. Kurita, and A. Muto, *tmRNA-mediated trans-translation as the major ribosome rescue system in a bacterial cell.* Frontiers in genetics, 2014. 5: p. 66.
170. Keiler, K.C., P.R. Waller, and R.T. Sauer, *Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA.* Science, 1996. 271(5251): p. 990-993.

171. Krüger, E., U. Völker, and M. Hecker, *Stress induction of clpC in Bacillus subtilis and its involvement in stress tolerance.* Journal of Bacteriology, 1994. 176(11): p. 3360-3367.
172. Derré, I., et al., *ClpE, a novel type of HSP100 ATPase, is part of the CtsR heat shock regulon of Bacillus subtilis.* Molecular microbiology, 1999. 32(3): p. 581-593.
173. Battesti, A. and S. Gottesman, *Roles of adaptor proteins in regulation of bacterial proteolysis.* Curr Opin Microbiol, 2013. 16(2): p. 140-7.
174. Kirstein, J., et al., *Adapting the machine: adaptor proteins for Hsp100/Clp and AAA+ proteases.* Nature Reviews Microbiology, 2009. 7(8): p. 589-599.
175. Battesti, A. and S. Gottesman, *Roles of adaptor proteins in regulation of bacterial proteolysis.* Current opinion in microbiology, 2013. 16(2): p. 140-147.
176. Kirstein, J., et al., *Adapting the machine: adaptor proteins for Hsp100/Clp and AAA+ proteases.* Nat Rev Microbiol, 2009. 7(8): p. 589-99.
177. Keiler, K.C., P.R. Waller, and R.T. Sauer, *Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA.* Science, 1996. 271(5251): p. 990-3.
178. Moore, S.D. and R.T. Sauer, *The tmRNA system for translational surveillance and ribosome rescue.* Annu Rev Biochem, 2007. 76: p. 101-24.
179. Gottesman, S., et al., *The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system.* Genes Dev, 1998. 12(9): p. 1338-47.
180. Herman, C., et al., *Degradation of carboxy-terminal-tagged cytoplasmic proteins by the Escherichia coli protease HflB (FtsH).* Genes Dev, 1998. 12(9): p. 1348-55.
181. Fei, X., et al., *Structural basis of ClpXP recognition and unfolding of ssrA-tagged substrates.* Elife, 2020. 9.
182. Lytvynenko, I., et al., *Alanine tails signal proteolysis in bacterial ribosome-associated quality control.* Cell, 2019. 178(1): p. 76-90 e22.
183. Trentini, D.B., et al., *Arginine phosphorylation marks proteins for degradation by a Clp protease.* Nature, 2016. 539(7627): p. 48-53.
184. Kirstein, J., et al., *The tyrosine kinase McsB is a regulated adaptor protein for ClpCP.* EMBO J, 2007. 26(8): p. 2061-70.
185. Hajdusits, B., et al., *McsB forms a gated kinase chamber to mark aberrant bacterial proteins for degradation.* Elife, 2021. 10.
186. Schmidt, A., et al., *Quantitative phosphoproteomics reveals the role of protein arginine phosphorylation in the bacterial stress response.* Mol Cell Proteomics, 2014. 13(2): p. 537-50.
187. Kirstein, J., et al., *A tyrosine kinase and its activator control the activity of the CtsR heat shock repressor in B. subtilis.* EMBO J, 2005. 24(19): p. 3435-45.
188. Fuhrmann, J., et al., *McsB is a protein arginine kinase that phosphorylates and inhibits the heat-shock regulator CtsR.* Science, 2009. 324(5932): p. 1323-7.
189. Schumann, W., *Regulation of bacterial heat shock stimulons.* Cell Stress Chaperones, 2016. 21(6): p. 959-968.
190. Mogk, A., et al., *The GroE chaperonin machine is a major modulator of the CIRCE heat shock regulon of Bacillus subtilis.* The EMBO Journal, 1997. 16(15): p. 4579-4590.

191. Derre, I., G. Rapoport, and T. Msadek, *The CtsR regulator of stress response is active as a dimer and specifically degraded in vivo at 37 degrees C.* Mol Microbiol, 2000. 38(2): p. 335-47.
192. Elsholz, A.K., U. Gerth, and M. Hecker, *Regulation of CtsR activity in low GC, Gram+ bacteria.* Adv Microb Physiol, 2010. 57: p. 119-44.
193. Krüger, E., et al., *Clp-mediated proteolysis in Gram-positive bacteria is autoregulated by the stability of a repressor.* The EMBO journal, 2001. 20(4): p. 852-863.
194. Fuhrmann, J., et al., *McsB is a protein arginine kinase that phosphorylates and inhibits the heat-shock regulator CtsR.* Science, 2009. 324(5932): p. 1323-1327.
195. Elsholz, A.K., et al., *CtsR inactivation during thiol-specific stress in low GC, Gram+ bacteria.* Molecular microbiology, 2011. 79(3): p. 772-785.
196. Elsholz, A.K., et al., *CtsR, the Gram-positive master regulator of protein quality control, feels the heat.* EMBO J, 2010. 29(21): p. 3621-9.
197. Elsholz, A.K., et al., *Global impact of protein arginine phosphorylation on the physiology of Bacillus subtilis.* Proceedings of the National Academy of Sciences, 2012. 109(19): p. 7451-7456.
198. Hecker, M., J. Pané-Farré, and V. Uwe, *SigB-dependent general stress response in Bacillus subtilis and related gram-positive bacteria.* Annu. Rev. Microbiol., 2007. 61: p. 215-236.
199. Nannapaneni, P., et al., *Defining the structure of the general stress regulon of Bacillus subtilis using targeted microarray analysis and random forest classification.* Microbiology, 2012. 158(3): p. 696-707.
200. Petersohn, A., et al., *Global analysis of the general stress response of Bacillus subtilis.* Journal of bacteriology, 2001. 183(19): p. 5617-5631.
201. Helmann, J.D., et al., *Global transcriptional response of Bacillus subtilis to heat shock.* J Bacteriol, 2001. 183(24): p. 7318-28.
202. Petersohn, A., et al., *Global analysis of the general stress response of Bacillus subtilis.* J Bacteriol, 2001. 183(19): p. 5617-31.
203. Price, C.W., et al., *Genome-wide analysis of the general stress response in Bacillus subtilis.* Mol Microbiol, 2001. 41(4): p. 757-74.
204. Nannapaneni, P., et al., *Defining the structure of the general stress regulon of Bacillus subtilis using targeted microarray analysis and random forest classification.* Microbiology, 2012. 158(Pt 3): p. 696-707.
205. Rodriguez Ayala, F., M. Bartolini, and R. Grau, *The Stress-Responsive Alternative Sigma Factor SigB of Bacillus subtilis and Its Relatives: An Old Friend With New Functions.* Front Microbiol, 2020. 11: p. 1761.
206. Nakano, S., et al., *Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in Bacillus subtilis.* Proc Natl Acad Sci U S A, 2003. 100(23): p. 13603-8.
207. Rochat, T., et al., *Genome-wide identification of genes directly regulated by the pleiotropic transcription factor Spx in Bacillus subtilis.* Nucleic Acids Res, 2012. 40(19): p. 9571-83.
208. Runde, S., et al., *The role of thiol oxidative stress response in heat-induced protein aggregate formation during thermotolerance in Bacillus subtilis.* Mol Microbiol, 2014. 91(5): p. 1036-52.

209. Rojas-Tapias, D.F. and J.D. Helmann, *Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in Bacillus subtilis.* Mol Microbiol, 2018. 107(5): p. 659-674.
210. Schafer, H. and K. Turgay, *Spx, a versatile regulator of the Bacillus subtilis stress response.* Curr Genet, 2019. 65(4): p. 871-876.
211. Rojas-Tapias, D.F. and J.D. Helmann, *Identification of novel Spx regulatory pathways in Bacillus subtilis uncovers a close relationship between the CtsR and Spx regulons.* J Bacteriol, 2019. 201(13).
212. Rojas-Tapias, D.F. and J.D. Helmann, *Roles and regulation of Spx family transcription factors in Bacillus subtilis and related species.* Adv Microb Physiol, 2019. 75: p. 279-323.
213. Schafer, H., et al., *Spx, the central regulator of the heat and oxidative stress response in B. subtilis, can repress transcription of translation-related genes.* Mol Microbiol, 2019. 111(2): p. 514-533.
214. Zuber, P., *Spx-RNA polymerase interaction and global transcriptional control during oxidative stress.* J Bacteriol, 2004. 186(7): p. 1911-8.
215. Newberry, K.J., et al., *Crystal structure of the Bacillus subtilis anti-alpha, global transcriptional regulator, Spx, in complex with the alpha C-terminal domain of RNA polymerase.* Proc Natl Acad Sci U S A, 2005. 102(44): p. 15839-44.
216. Reyes, D.Y. and P. Zuber, *Activation of transcription initiation by Spx: formation of transcription complex and identification of a Cis-acting element required for transcriptional activation.* Mol Microbiol, 2008. 69(3): p. 765-79.
217. Lamour, V., et al., *Crystal structure of the in vivo-assembled Bacillus subtilis Spx/RNA polymerase alpha subunit C-terminal domain complex.* J Struct Biol, 2009. 168(2): p. 352-6.
218. Nakano, M.M., et al., *Promoter recognition by a complex of Spx and the C-terminal domain of the RNA polymerase alpha subunit.* PLoS One, 2010. 5(1): p. e8664.
219. Nakano, S., et al., *A regulatory protein that interferes with activator-stimulated transcription in bacteria.* Proc Natl Acad Sci U S A, 2003. 100(7): p. 4233-8.
220. Larsson, J.T., A. Rogstam, and C. von Wachenfeldt, *YjbH is a novel negative effector of the disulphide stress regulator, Spx, in Bacillus subtilis.* Mol Microbiol, 2007. 66(3): p. 669-84.
221. Nakano, M.M., S. Nakano, and P. Zuber, *Spx (YjbD), a negative effector of competence in Bacillus subtilis, enhances ClpC-MecA-ComK interaction.* Mol Microbiol, 2002. 44(5): p. 1341-9.
222. Eiamphungporn, W. and J.D. Helmann, *The Bacillus subtilis σM regulon and its contribution to cell envelope stress responses.* Molecular microbiology, 2008. 67(4): p. 830-848.
223. Rochat, T., et al., *Genome-wide identification of genes directly regulated by the pleiotropic transcription factor Spx in Bacillus subtilis.* Nucleic acids research, 2012. 40(19): p. 9571-9583.
224. Rojas-Tapias, D.F. and J.D. Helmann, *Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in Bacillus subtilis.* Molecular microbiology, 2018. 107(5): p. 659-674.
225. Schäfer, H. and K. Turgay, *Spx, a versatile regulator of the Bacillus subtilis stress response.* Current genetics, 2019. 65(4): p. 871-876.

226. Leelakriangsak, M., K. Kobayashi, and P. Zuber, *Dual negative control of spx transcription initiation from the P3 promoter by repressors PerR and YodB in Bacillus subtilis.* Journal of bacteriology, 2007. 189(5): p. 1736-1744.
227. Nakano, S., et al., *Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx.* Molecular microbiology, 2005. 55(2): p. 498-510.
228. Gaballa, A., et al., *Regulation of Bacillus subtilis bacillithiol biosynthesis operons by Spx.* Microbiology, 2013. 159(Pt_10): p. 2025-2035.
229. Shi, J., et al., *Structural basis of transcription activation by the global regulator Spx.* Nucleic Acids Res, 2021. 49(18): p. 10756-10769.
230. Lin, A.A., D. Walthers, and P. Zuber, *Residue substitutions near the redox center of Bacillus subtilis Spx affect RNA polymerase interaction, redox control, and Spx-DNA contact at a conserved cis-acting element.* J Bacteriol, 2013. 195(17): p. 3967-78.
231. Nakano, M.M., et al., *Loss-of-function mutations in yjbD result in ClpX- and ClpP-independent competence development of Bacillus subtilis.* Mol Microbiol, 2001. 42(2): p. 383-94.
232. Nakano, S., et al., *Multiple pathways of Spx (YjbD) proteolysis in Bacillus subtilis.* J Bacteriol, 2002. 184(13): p. 3664-70.
233. Al-Eryani, Y., et al., *Exploring structure and interactions of the bacterial adaptor protein YjbH by crosslinking mass spectrometry.* Proteins, 2016. 84(9): p. 1234-45.
234. Awad, W., et al., *Structural basis for YjbH adaptor-mediated recognition of transcription factor Spx.* Structure, 2019. 27(6): p. 923-936 e6.
235. Chan, C.M., et al., *Geobacillus thermodenitrificans YjbH recognizes the C-terminal end of Bacillus subtilis Spx to accelerate Spx proteolysis by ClpXP.* Microbiology, 2012. 158(5): p. 1268-1278.
236. Chan, C.M., E. Hahn, and P. Zuber, *Adaptor bypass mutations of Bacillus subtilis spx suggest a mechanism for YjbH-enhanced proteolysis of the regulator Spx by ClpXP.* Mol Microbiol, 2014. 93(3): p. 426-38.
237. !!! INVALID CITATION !!! [128].
238. Rojas-Tapias, D.F. and J.D. Helmann, *Stabilization of Bacillus subtilis Spx under cell wall stress requires the anti-adaptor protein YirB.* PLoS genetics, 2018. 14(7): p. e1007531.
239. Winkler, J., et al., *Quantitative and spatio-temporal features of protein aggregation in Escherichia coli and consequences on protein quality control and cellular ageing.* EMBO J, 2010. 29(5): p. 910-23.
240. Coquel, A.S., et al., *Localization of protein aggregation in Escherichia coli is governed by diffusion and nucleoid macromolecular crowding effect.* PLoS Comput Biol, 2013. 9(4): p. e1003038.
241. Gupta, A., et al., *In vivo kinetics of segregation and polar retention of MS2-GFP-RNA complexes in Escherichia coli.* Biophys J, 2014. 106(9): p. 1928-37.
242. Neeli-Venkata, R., et al., *Robustness of the process of nucleoid exclusion of protein aggregates in Escherichia coli.* J Bacteriol, 2016. 198(6): p. 898-906.
243. Oliveira, S.M., et al., *Increased cytoplasm viscosity hampers aggregate polar segregation in Escherichia coli.* Mol Microbiol, 2016. 99(4): p. 686-99.

244. Kirstein, J., et al., *Localization of general and regulatory proteolysis in Bacillus subtilis cells.* Mol Microbiol, 2008. 70(3): p. 682-94.
245. Stannek, L., et al., *Factors that mediate and prevent degradation of the inactive and unstable GudB protein in Bacillus subtilis.* Front Microbiol, 2014. 5: p. 758.
246. Hantke, I., et al., *YocM a small heat shock protein can protect Bacillus subtilis cells during salt stress.* Mol Microbiol, 2019. 111(2): p. 423-440.
247. Nakano, S., et al., *Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in Bacillus subtilis.* Proceedings of the National Academy of Sciences, 2003. 100(23): p. 13603-13608.
248. Turlan, C., et al., *SpxA1, a novel transcriptional regulator involved in X-state (competence) development in Streptococcus pneumoniae.* Molecular microbiology, 2009. 73(3): p. 492-506.
249. Pamp, S.n.J., et al., *Spx is a global effector impacting stress tolerance and biofilm formation in Staphylococcus aureus.* Journal of bacteriology, 2006. 188(13): p. 4861-4870.
250. Whiteley, A.T., et al., *A redox-responsive transcription factor is critical for pathogenesis and aerobic growth of Listeria monocytogenes.* Infection and immunity, 2017. 85(5): p. e00978-16.
251. Villanueva, M., et al., *Rifampin resistance rpoB alleles or multicopy thioredoxin/thioredoxin reductase suppresses the lethality of disruption of the global stress regulator spx in Staphylococcus aureus.* Journal of bacteriology, 2016. 198(19): p. 2719-2731.
252. Borezee, E., et al., *Identification in Listeria monocytogenes of MecA, a homologue of the Bacillus subtilis competence regulatory protein.* Journal of Bacteriology, 2000. 182(20): p. 5931-5934.
253. Reniere, M.L., A.T. Whiteley, and D.A. Portnoy, *An in vivo selection identifies Listeria monocytogenes genes required to sense the intracellular environment and activate virulence factor expression.* PLoS pathogens, 2016. 12(7): p. e1005741.
254. Cesinger, M.R., et al., *Listeria monocytogenes SpxA1 is a global regulator required to activate genes encoding catalase and heme biosynthesis enzymes for aerobic growth.* Molecular microbiology, 2020. 114(2): p. 230-243.
255. Rojas-Tapias, D.F. and J.D. Helmann, *Roles and regulation of Spx family transcription factors in Bacillus subtilis and related species.* Advances in microbial physiology, 2019. 75: p. 279-323.
256. Reder, A., et al., *The modulator of the general stress response, MgsR, of Bacillus subtilis is subject to multiple and complex control mechanisms.* Environmental microbiology, 2012. 14(10): p. 2838-2850.
257. Engman, J., et al., *The YjbH adaptor protein enhances proteolysis of the transcriptional regulator Spx in Staphylococcus aureus.* Journal of bacteriology, 2012. 194(5): p. 1186-1194.
258. Ruhland, B.R. and M.L. Reniere, *YjbH Requires Its Thioredoxin Active Motif for the Nitrosative Stress Response, Cell-to-Cell Spread, and Protein-Protein Interactions in Listeria monocytogenes.* J Bacteriol, 2020. 202(12).
259. Zemansky, J., et al., *Development of a mariner-based transposon and identification of Listeria monocytogenes determinants, including the peptidyl-prolyl isomerase PrsA2, that contribute to its hemolytic phenotype.* Journal of bacteriology, 2009. 191(12): p. 3950-3964.

260. Cheng, C., et al., *YjbH mediates the oxidative stress response and infection by regulating SpxA1 and the phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) in Listeria monocytogenes.* Gut microbes, 2021. 13(1): p. 1-19.
261. Zhao, L., et al., *The Hsp70 Chaperone System Stabilizes a Thermo-sensitive Subproteome in E. coli.* Cell Rep, 2019. 28(5): p. 1335-1345 e6.

Printed by Media-Tryck, Lund 2023 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

Department of Biology
Faculty of Science
Lund University
ISBN 978-91-8039-669-1