The Role of the Stringent Response and Spx in Stress Response and Thermotolerance Development

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Abstract

All organisms are frequently exposed to changing environmental conditions in their natural habitat, which can impose stress and threatens basic biological functions. To cope with such adverse conditions, cells have developed complex stress response systems which allow the sensing and integration of environmental stress signals and the regulation of appropriate responses. For example, a sudden temperature upshift and other proteotoxic conditions which facilitate the misfolding and aggregation of cellular proteins, activate the heat shock response. This global transcriptional response mediates a concerted up-regulation and accumulation of conserved chaperones and proteases, the protein quality control system, to restore and maintain protein homeostasis during stress. Another fast-acting bacterial stress response program, the stringent response, is activated upon amino acid starvation and many other stress signals. It is regulated by the second messenger nucleotide (p)ppGpp, which mediates the transcriptional repression of ribosomal genes while activating stress response- and amino acid synthesis genes, but also causes the inhibition of translation, replication and interferes with other processes.

In this thesis, the heat shock response of the Gram positive model organism *Bacillus subtilis* was studied. It could be demonstrated that the transcriptional regulator Spx is not only a central regulator of many heat shock response genes, but can also participate in the transcriptional down-regulation of rRNA and ribosomal protein genes, which were observed to be strongly down-regulated during many stress conditions. In addition, it could be demonstrated, that the stringent response mediated by (p)ppGpp is activated during the heat shock response. Increased (p)ppGpp levels conferred elevated heat stress resistance while the lack of (p)ppGpp renders cells more sensitive to stress. Remarkably, it appears that both (p)ppGpp and Spx are concurrently involved in the down-regulation of rRNA genes during heat stress. Furthermore, the results suggest that (p)ppGpp is involved in direct adjustments of translation during stress, which appears to be crucial for the protective role of the stringent response in the heat stress response. Together, the results suggest a model by which the heat shock response of *B. subtilis* not only involves the synthesis and accumulation of chaperones and proteases of the protein quality control system but also the concurrent curbing of the protein synthesis rate by (p)ppGpp to support protein homeostasis by reducing the load for the cellular protein quality control system.

Zusammenfassung

Alle Organismen sind in ihrem natürlichen Lebensraum häufig wechselnden Umweltbedingungen ausgesetzt, die Stress hervorrufen und grundlegende biologische Funktionen gefährden können. Um mit solchen widrigen Bedingungen zurechtzukommen, haben Zellen komplexe Stressantwort-Systeme entwickelt, die das Erkennen von Umweltstress-Signalen und die Steuerung geeigneter Maßnahmen ermöglichen. Beispielsweise kann ein plötzlicher Hitzeschock die Fehlfaltung von zellulären Proteinen begünstigen und eine Hitzeschock-Antwort auslösen. Diese aktiviert die Synthese und Akkumulation von Chaperonen und Proteasen, dem Proteinqualitätskontrollsystem, zur Wiederherstellung und Aufrechterhaltung der Proteinhomöostase. Eine weitere und besonders schnell wirkende bakterielle Stressantwort, die *Stringent Response*, wird bei Aminosäuremangel und vielen weiteren Stresssignalen aktiviert. Sie wird durch den *second messenger* (p)ppGpp reguliert, welches die transkriptionelle Herunterregulation von ribosomalen Genen und die Aktivierung von Stressantwort- und Aminosäuresynthese-Genen reguliert und weiterhin auch Translation, Replikation und andere Prozesse beeinflusst.

In dieser Arbeit wurde die Hitzeschock-Antwort des Gram-positiven Modellorganismus *Bacillus subtilis* untersucht. Es konnte gezeigt werden, dass der Transkriptionsfaktor Spx nicht nur ein zentraler Regulator vieler Gene der Hitzeschock-Antwort ist, sondern auch an der Herunterregulation von rRNA und Genen von ribosomalen Proteinen beteiligt sein kann, die bei vielen Stressbedingungen stark herunterreguliert werden. Darüber hinaus konnte gezeigt werden, dass die durch (p)ppGpp vermittelte *Stringent Response* auch während der Hitzeschock-Antwort aktiviert wird. Erhöhte zelluläre (p)ppGpp-Konzentrationen bewirken eine erhöhte Hitzestressresistenz, während das Fehlen von (p)ppGpp die Zellen empfindlicher auf Stress werden ließ. Bemerkenswert ist, dass anscheinend sowohl (p)ppGpp als auch Spx gleichzeitig an der Herunterregulierung von rRNA-Genen unter Hitzestress beteiligt sind. Weiterhin lassen die Ergebnisse vermuten, dass (p)ppGpp bei Stress an der direkten Regulation der Translation beteiligt ist, was vermutlich entscheidend für die Schutzfunktion der *Stringent Response* bei Hitzestress ist. Insgesamt deuten die Ergebnisse darauf hin, dass die Hitzeschock-Antwort von *B. subtilis* nicht nur die Synthese von Chaperonen und Proteasen umfasst, sondern auch die Reduzierung der Proteinsyntheserate durch (p)ppGpp beinhaltet, um die Last auf das Protein-Qualitätskontrollsystem zu reduzieren und die Proteinhomeostase aufrecht zu halten.

Abbreviations

αCTD	C-terminal domain of the RNA polymerase alpha subunit
AAA+	ATPases associated with diverse cellular activities
ACP	acyl carrier protein
ACDQ	6-amino7-chloro-5,8-dioxoquinoline
asRNA	antisense RNA
ATP	adenosine triphosphate
BCAA	branched-chain amino acids
BMM	Belitzkie minimal medium
B. subtilis	*Bacillus subtilis*
CAA	casamino acids
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
c-di-AMP	cyclic 3',5'-di-adenosine monophosphate
CFU	colony forming units
ChIP	chromatin immunoprecipitation
CIRCE	controlling inverted repeat of chaperone expression
CoA	coenzyme A
CTD	carboxy-terminal domain(s)
DNA	desoxyribonucleic acid
E. coli	*Escherichia coli*
e.g.	*exempli gratia;* for example
eIF2α	alpha subunit of the eukaryotic translation initiation factor 2
et al.	and others
GTP	guanosine triphosphate
HrcA	heat regulation at CIRCE; transcriptional repressor of *B. subtilis*
HSP	heat shock protein
HSR	heat shock response
i.e.	*id est*; that is
IF1/2/3	initiation factor 1/2/3
NTD	amino-terminal domain(s)
ORF	open reading frame
ppGpp/pppGpp	guanosine 3'-diphosphate 5'-di- or tri-phosphate
(p)ppGpp	ppGpp or pppGpp
PTS	phosphotransferase system
RF1/2/3	release factor 1/2/3
RNA	ribonucleic acid
RNAP	RNA polymerase
RNA-seq	RNA sequencing
RP	ribosomal protein
RPKM	reads per kilobase per million reads
rRNA	ribosomal RNA
s4U	4-thiouridine
SR	stringent response
TAP	tobacco acid pyrophosphatase
UTR	untranslated region
TSS	transcription start site
UV	ultra violet

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1. Introduction

In their natural environment, bacteria are frequently exposed to changing and stressful environmental conditions, such as rapid temperature up- or downshifts, changes in osmolarity or pH, and exposure to radiation, toxic heavy metal ions or antibiotics. These stressors may inhibit, denature or otherwise damage cellular macromolecules and thus restrict growth and impair survival. Furthermore, bacteria compete for limited nutrients in their environment and often face starvation. To cope with these adverse conditions, they have evolved a magnitude of strategies from fast-acting, transient and specific stress responses to global genetic programs that result in the formation of specialized cell types. These stress responses and developmental processes are governed by complex, interconnected regulatory networks, which sense and integrate stress- and starvation signals and adjust and mediate the appropriate response to assure survival and proliferation [1].

The ubiquitous, soil-dwelling bacterium *Bacillus subtilis* is a well-studied and widely used model organism for Gram positive bacteria. Its ability to differentiate into heat-resistant spores has already been observed in 1877 [2] and studied extensively [3]–[5]. In addition, its natural competence for transformation makes it easily amenable to reverse genetic approaches [6]. The determination of the complete genome sequence of *B. subtilis* in 1997 as one of the first bacterial genomes has strongly facilitated basic research and the application of '–omics' technologies, enabling a more comprehensive understanding of its physiology [7]–[10]. Sophisticated regulatory networks were identified, which control the decision making processes for developmental differentiation programs such as motile or sessile life styles, entry into the sporulation process or the development of competence [11]–[13]. Likewise, a complex architecture of regulatory stress-response networks, which react to internal and external stimuli and fine-tune the appropriate responses has been described [14]–[16].

1.1 The heat shock response

The famous Anfinsen experiment demonstrated that small proteins can spontaneously reach their native fold *in vitro*, which is predetermined only by the amino acid sequence without the need of extrinsic factors [17], [18]. However, folding of proteins *in vivo* is challenged and confounded by the high concentration of biomolecules in the cellular environment that provoke aberrant interactions and thereby

lead to misfolding and aggregation of proteins [19], [20]. Therefore, all living cells maintain a conserved protein quality control system, which supports protein homeostasis by monitoring and maintaining the functional state of cellular proteins. The protein quality control system comprises a set of conserved molecular chaperones and proteases which interact with misfolded protein substrates by recognizing surface-exposed hydrophobic patches and prevent their aggregation, promote the ATP-dependent unfolding and refolding of substrates or catalyze the degradation of damaged proteins [21]–[23].

Heat stress can lead to the misfolding and aggregation of nascent peptides and folded cellular proteins, thus raising the need for the protein quality control system to maintain or restore protein homeostasis and to prevent the accumulation of large, toxic protein aggregates (Figure 1). Accordingly, heat- and other proteotoxic stress activates the heat shock response (HSR), a global transcriptional response to stress which directs the rapid synthesis and accumulation of the chaperones and proteases of the protein quality control system and other stress response proteins to protect or restore cellular functions during stress [24]. The HSR was first observed in *Drosophila*, but similar transcriptional responses were later found in all species and the ubiquitous heat shock proteins show remarkable conservation from bacterial to eukaryotic cells [24], [25].

1.1.1 Heat shock- and general stress proteins

The molecular chaperones of the protein quality system are also conserved in the HSR of *B. subtilis* (Figure 1). The chaperonin GroEL entraps misfolded substrates within its oligomeric, barrel-like structure and allows their folding in an ATP-dependent cycle by providing a protected, hydrophilic folding environment [26]. GroEL chaperines were found to be essential in *E. coli* and *B. subtilis* at all temperatures [10], [27]. The DnaK chaperone forms a functional system together with its co-chaperone DnaJ and the nucleotide-exchange factor GrpE. It recognizes and binds substrate proteins by exposed hydrophobic peptides, prevents their aggregation and allows ATP-driven the un- and refolding of substrates [23]. In *E. coli,* the synergistic activity of the DnaK system with the AAA+ unfoldase ClpB and small heat shock proteins also allows the disaggregation and unfolding of protein aggregates [28]. However, DnaK appears to have no critical role in stress response and a ClpB homolog is absent in *B. subtilis* [29], [30]. A third family of ubiquitous heat-shock associated chaperones are HSP90/HtpG proteins, which

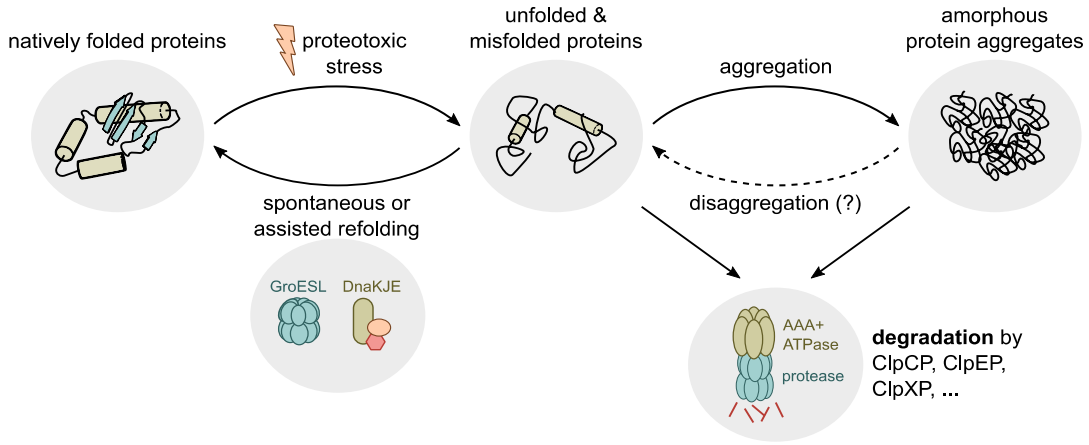

Figure 1: Model of the protein quality control system in *B. subtilis*.

Heat stress and other proteotoxic conditions lead to the unfolding and subsequent misfolding of natively folded and newly synthesized proteins. Misfolded proteins can then form large protein aggregates by hydrophobic interaction. Chaperones such as DnaKJE and GroESL bind misfolded proteins, prevent their aggregation and catalyze their refolding. Misfolded proteins and protein aggregates are also targeted for degradation by proteases such as ClpCP, ClpEP or ClpXP. Partially adopted from [23, 50]. For details, see text.

also possess ATP dependent refolding activity. However, HtpG appears to have no phenotype in the HSR of *B. subtilis* [31].

Damaged and aggregated proteins are also targeted by AAA+ ATPase/protease complexes and removed from the cell by degradation, which represents a second strategy of the protein quality control system (Figure 1) [32]. In *B. subtilis*, the ATPases ClpC, ClpX and ClpE form complexes with the serine protease ClpP [16]. ClpC plays a central role in the protein quality control system and also has important and pleiotropic functions in the control of various developmental processes and stress responses by regulatory proteolysis [33]–[36]. Its activities are guided by adaptor proteins such as MecA, YpbH or McsB [16], [37]–[39]. Interestingly, the ClpC·MecA complex was demonstrated to catalyze the unfolding and refolding of aggregated model substrates *in vitro*, suggesting that ClpC also exhibits chaperone- and disaggregase activity similar to ClpB [38]. The AAA+ proteases Lon and FtsH are also implicated in protein quality control during heat stress and regulatory functions [16], [40], [41].

Furthermore, a large group of heat-inducible proteins, the general stress proteins, accumulate upon heat shock and many other stress- and starvation signals. They are synthesized during the general stress response and provide the cell with multiple and preemptive stress resistance [42], [43]. The general stress response of *B. subtilis* is controlled by the alternative sigma factor σ^B and includes, among others, transporters for the uptake of compatible solutes and multidrug efflux pumps (OpuE, BmrABC) [44],

proteins that protect or repair the chromosome (e.g. RadA, DisA, Dps) [45], enzymes that detoxify reactive oxygen species and maintain redox balance (e.g. KatB, KatX, TrxA, NadE) [46] or modify RNA and protect ribosomes (e.g. *ssrA*/SmpB, Hpf, Ctc) [42], [47]–[49]. However, while stress-sensitive phenotypes have been assigned to many genes of the general stress response, the precise function of a large proportion of general stress proteins is still not known [43].

1.1.2 Acquired thermotolerance and cross-protection

Interestingly, a short activation of the HSR at moderately elevated temperatures and the resulting accumulation of heat shock proteins is sufficient to provide an acquired thermotolerance to more severe temperature stress in many prokaryotic and eukaryotic organisms [24], [50]. In *B. subtilis* cells, acquired thermotolerance to the lethal temperature at 53 °C can typically be induced by a short priming at 48 °C [50]. In contrast, strains with mutations or deletions in the protein quality control system often exhibit severe sensitivity to heat stress and other environmental stresses [34], [51]. These phenotypes emphasize the fundamental importance of heat shock proteins in protein homeostasis and demonstrate that many chaperones have a general role in stress responses and survival. Priming by a mild heat shock can also provide limited tolerance against other biotic or abiotic stressors. Likewise, other treatments that elicit the HSR can also induce thermotolerance. This “cross-protection” phenotype is also conserved in many organisms, indicating that protein unfolding stress and aggregation appears to be a common consequence of many environmental stress stimuli [24], [52].

Notably, the responses to heat stress of *B. subtilis* and other bacteria also include proteins for defense against reactive oxygen species and increasing evidence suggests that cells have to cope with oxidative stress during heat shock and other stress conditions [46], [50], [53]. This “secondary oxidative stress” is thought to be caused by perturbation of the respiratory chain during stress, leading to the release and accumulation of partially reduced oxygen species such as superoxide (O_2^-) or hydroxyl radicals (•OH) [53], [54]. The reactive oxygen species react with cellular macromolecules and can cause oxidative damage to lipids, DNA and proteins [55]. Therefore, the detoxification of reactive oxygen species represents an important part of the *B. subtilis* HSR. The absence of molecular oxygen or the increased expression of genes of the oxidative stress response contributes to survival of heat stress, while their inactivation confers increased sensitivity to stress [46], [50], [53], [56].

1.1.3 Regulation of the heat- and general stress response in *B. subtilis*

While the heat shock proteins and the global transcriptional pattern of the HSR are well-conserved in prokaryotic and eukaryotic organisms, the regulatory networks that govern their expression are very diverse and differ across bacterial phyla or even related species [57]–[59]. Aside from their role in protein homeostasis, many molecular chaperones and proteases are also involved in the feedback control of their own transcription by interacting with their associated transcriptional regulators. Early work on the HSR in *Escherichia coli* led to the discovery of the alternative sigma factor σ^{32} (*rpoH*), which controls the expression of most heat shock genes [60], [61]. In contrast, the expression of the conserved molecular chaperones and proteases in *B. subtilis* is controlled by the transcriptional repressors HrcA and CtsR [58]. In addition, many heat shock- and general stress genes are controlled by σ^{B} and Spx [15], [62]. Historically, the heat shock proteins of *B. subtilis* have been divided into different classes according to their synthesis pattern upon different stress stimuli [15].

The class I heat shock proteins consist of the DnaK/DnaJ/GrpE and GroES/L chaperone systems. Their genes are organized in the *groES-groEL* and the *hrcA-dnaK-dnaJ-grpE-yqeT-yqeU-yqeV* operons, the latter of which also encodes the transcriptional repressor HrcA and three uncharacterized methyltransferases (Figure 2 A) [63], [64]. Transcription of both operons is facilitated by vegetative σ^{A} promoters and regulated by HrcA [65]. HrcA binds inverted repeat sequences termed CIRCE elements (controlling inverted repeat of chaperone expression) downstream of the promoter and represses transcription during non-stress conditions [65], [66]. HrcA is prone to spontaneous aggregation and requires the activity of GroEL to remain active as a repressor [67]. During heat shock, GroEL is titrated by unfolded and misfolded proteins and depleted from HrcA, resulting in the inactivation of HrcA and the de-repression of its regulon (Figure 2 A) [67]. Since the HrcA regulon is directly stimulated by misfolded proteins sensed by GroEL it is particularly strongly activated upon heat stress or puromycin treatment, which also provokes the accumulation of misfolded proteins, but activated to a lesser extent upon salt-, ethanol or hydrogen peroxide treatment [15], [35], [68].

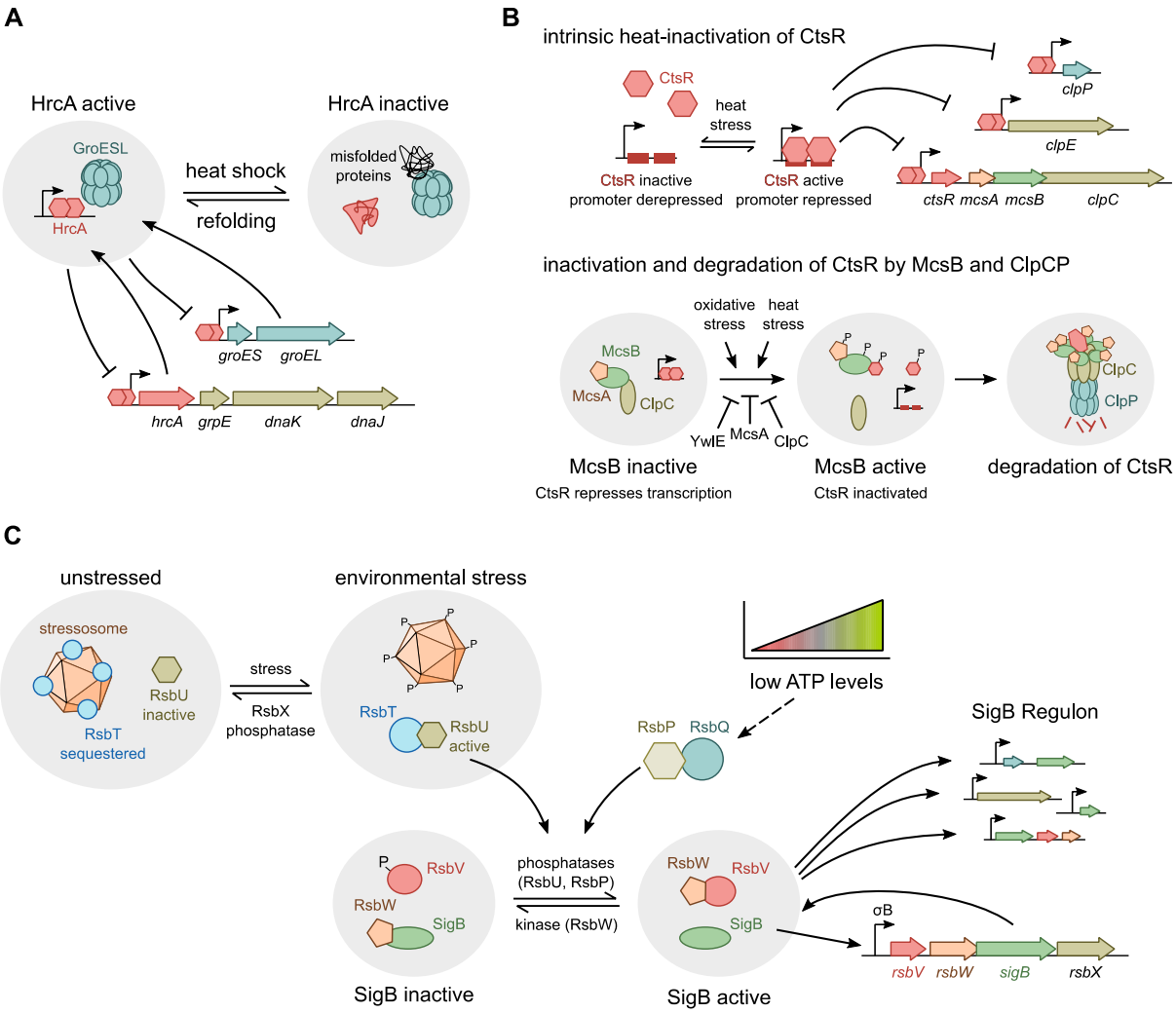

Figure 2: Regulation of the class I, II and III heat shock genes

(A) Regulation of the class I heat shock genes *hrcA-grpE-dnaK-dnaJ* and *groESL* by the repressor HrcA. The repressor activity of HrcA is maintained by interaction with the GroEL chaperone. During heat stress, GroESL is titrated by misfolded proteins and HrcA becomes inactive. For details, see text. (B) Regulation of the class III heat shock genes by CtsR and control of its activity by McsAB and ClpC. CtsR is an intrinsic thermosensor and inactivated during heat stress. In addition, McsB becomes activated by heat- and oxidative stress, inactivates CtsR by phosphorylation on arginine residues and subjects it to ClpCP-dependent degradation. Partially adapted from [15]. For details, see text. (C) The class II heat shock genes are transcribed by SigB. It is inhibited by interaction with the anti-sigma factor RsbW, which also inactivates the anti-anti-sigma factor RsbV by phosphorylation. Environmental stress or energy depletion is sensed by two independent pathways and results in the activation of the phosphatases RsbU or RsbP which mediate a partner switch of RsbV and RsbW and the release of SigB. Partially adapted from [43]. For details, see text.

The class III heat shock genes comprise the *ctsR-mcsA-mcsB-clpC* operon as well as the monocistronic *clpE* and *clpP* genes and are regulated by CtsR (class three stress repressor), a repressor protein which binds direct repeat sequences at the promoter regions of the operons (Figure 2 B) [69]–[71]. The genes of the regulon encode for the AAA+ ATPase/protease complexes ClpEP as well as

ClpCP and its adaptor protein complex McsAB, which participate in the degradation of misfolded proteins and are also implicated in the regulation of CtsR activity [16], [35], [72]. CtsR is an intrinsic thermosensor with impaired DNA binding activity during heat stress, resulting in the de-repression of its regulon. Temperature is sensed via its conserved tetra-glycine loop with a species-specific temperature threshold (Figure 2 B) [73]. Furthermore, CtsR is also regulated by McsB, a global protein-arginine kinase and adaptor protein for ClpCP [37], [74]–[76]. In the absence of proteotoxic stress, McsB is held inactive in a complex with McsA and ClpC and activation of McsB by autophosphorylation is antagonized by the protein arginine phosphatase YwlE. Upon many stress stimuli, the inhibitory interactions between McsA, McsB and ClpC are abolished and McsB becomes activated by auto-phosphorylation (Figure 2 B) [37], [73]–[75], [77]. It then inhibits CtsR activity by direct interaction and impairs its DNA binding by phosphorylation of conserved arginine residues of CtsR [72], [74], [78]. Furthermore, phosphorylated McsB is active as an adaptor protein of ClpCP and targets CtsR for degradation (Figure 2 B) [37], [74], [75]. The McsB-dependent inactivation of CtsR is not required for the de-repression during heat stress but is thought to activate the regulon during other adverse stress conditions and protein folding stress [16], [73], [76].

A second mechanism of McsB activation in response to oxidative stress involves McsA and YwlE. The cysteine residues in the zinc finger motif of McsA act as a sensor for oxidative stress. Their oxidation abolishes the inhibitory interaction with McsB, which then binds to and inhibits CtsR, leading to the de-repression of the operon [76]. In addition, YwlE is inactivated by oxidation of a conserved cysteine in its active site and can no longer prevent autophosphorylation and activation of McsB [79].

The different pathways of CtsR activity control and the independent perception of stress stimuli by CtsR, McsAB and YwlE ensures that the regulon is activated during a wide range of environmental stress conditions [15], [16], [71]. Furthermore, additional promoters recognized by σ^B further enhance transcription of the *ctsR* operon during diverse stress signals [51], [70], [71].

More than 150 class II heat-inducible genes are governed by the alternative sigma factor σ^B, which controls the general stress response [43]. Its activity is regulated by multiple partner switching mechanisms and reversible protein phosphorylation events (Figure 2 C). During non-stress conditions, σ^B is sequestered by direct interaction with its anti-sigma factor RsbW, which is also a protein kinase that

concurrently inactivates the anti-anti-sigma factor RsbV by phosphorylation [80]–[82]. Activation of σ^B is accomplished by dephosphorylation of RsbV~P by the specific phosphatases RsbU and RsbP, which induces a partner switch and results in the sequestration of RsbW by RsbV, the release of σ^B and the transcriptional activation of its regulon [81], [83]–[85]. The expression of the *sigB* operon is under positive auto-regulation by σ^B, which leads to an amplification of the response (Figure 2 C) [15], [86]. The phosphatases RsbU and RsbP are regulated by protein interactions and integrate diverse stress and starvation signals via two independent pathways (Figure 2 C) [85]. RsbU becomes activated upon environmental stress and is regulated by a second partner switch mechanism [83], [85], [87], [88]. For its activation, RsbU requires stimulating interaction with RsbT, which is sequestered by a 1.8 MDa protein complex, termed "stressosome", in the absence of stress. The stressosome is composed of multiple copies of the RsbU antagonist RsbS as well as RsbRA and its paralogs RsbRB, RsbRC and RsbRD, which are thought to sense and integrate different stress signals such as heat- or salt stress, low temperature and exposure to ethanol or heavy metal ions, although the mechanism of stress perception is poorly understood. Upon stress activation, RsbT releases itself from the stressosome by phosphorylation of RsbS and activates RsbU, which results in the release of σ^B (Figure 2 C) [83], [87], [89].

A downshift of glucose, oxygen or phosphate, exposure to metabolic inhibitors such as azide or CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) or entry into the stationary growth phase activates the energy stress pathway via the phosphatase RsbP [85], [90]. The signal of their activation is thought to be a drop in ATP levels sensed by the PAS domain of RsbP in complex with RsbQ (Figure 2 C) [90], [91]. Interestingly, Rel, the ribosome-associated key enzyme of the stringent response (see below) has been demonstrated to be required for the activation of the energy stress pathway [92]. In addition, the ribosomal protein L11 and the ribosome-associated GTPase Obg are involved in the activation of the environmental stress pathway [93]–[95]. However, amino acid starvation, the main signal of the stringent response does not elicit the general stress response [85], [92]. Although the molecular details of their requirements are unknown, these findings indicate an intricate connection between the general stress response, the ribosome and the stringent response.

In addition to the regulators of the class I-III heat-inducible genes, several less-well characterized regulators are involved in the regulation of the HSR in *B. subtilis* [58]. The two-component system

CssRS activates the expression of the genes of the membrane anchored proteases HtrA and HtrB upon heat- and protein secretion stress [96]. The heat-inducible sigma factor σ^I, which directly or indirectly affects transcription of about 130 genes, is regulated by its anti-sigma factor RsgI and appears to be modulated by DnaK and other factors of the protein quality control system [97]–[99]. The ECF-type sigma factor σ^M is responsive to many environmental stresses such heat shock, ethanol, salt or antibiotics that inhibit cell wall synthesis [100]. Other heat-inducible genes, e.g. *htpG*, are regulated by unknown factors [101], [102]. Recently, Runde *et al.* (2014) could demonstrate that Spx, a global stress response regulator which was previously implicated in the thiol-specific oxidative stress response, is an additional critical regulator of the HSR [50], [103]. Its role as a global regulator of stress responses and developmental processes is discussed in the following sections.

1.2 The global regulator Spx

Spx proteins (suppressor of *clpP* and *clpX* mutations) are unusual transcriptional regulators found in low-GC Gram-positive bacteria, which share considerable homology with the ArsC arsenate reductase family. In *B. subtilis*, Spx controls a large regulon of stress-related genes and interferes with many developmental processes such as competence development or sporulation [104]. A second Spx homolog, MgsR, controls a sub-regulon of the general stress response of *B. subtilis* [105].

1.2.1 Mechanisms of Spx-dependent transcriptional control

Spx exerts positive and negative transcriptional regulation via distinct mechanisms. It was first identified as an "anti-alpha" factor [106]: Unlike most transcriptional regulators, Spx has no intrinsic DNA binding activity, but directly modulates RNA polymerase (RNAP) by binding to the C-terminal domain of the RNAP alpha subunit (αCTD) at the same interface which also interacts with transcriptional activators [107], [108]. Thereby, it can interfere with certain activator proteins such as ComK or the phosphorylated response regulators ResD~P and ComA~P [104], [106]. By this mechanism, Spx exerts indirect negative transcriptional control over various genes by interfering with the activity of transcriptional activators.

Spx can also directly stimulate transcription from certain promoters, e.g. of the *trxA* and *trxB* genes [103], [109]. The Spx-stimulated promoters have non-conserved -35 regions and are poorly recognized

by σ^A-RNAP alone. DNase I footprinting, crosslinking and *in vitro* transcription experiments suggested that in the absence of Spx, the σ^A subunit of RNAP holoenzyme mistakenly binds a -35-like element around the -44 region relative to the transcription start site, while only little contact is made with the -35 and -10 region [109]–[111]. While Spx itself does not interact with DNA, the complex of Spx and αCTD can recognize and bind sequences upstream of the core promoter of activated genes [106], [109], [111], [112]. Binding of Spx to αCTD is thought to be required for correct αCTD-guided positioning of RNAP holoenzyme on the promoter and formation of a productive initiation complex [109], [110]. The sequence motif recognized by Spx·αCTD is poorly conserved, but an AGCA consensus motif around the -44 region was identified by crosslinking experiments and mutagenesis [62], [109], [112]. A global chromatin immunoprecipitation (ChIP) study by Rochat *et al.* (2012) revealed more than 280 Spx-RNAP binding sites on the chromosome of *B. subtilis* [62].

1.2.2 The role of Spx in stress responses and protein homeostasis

Spx (YjbD) was identified as a member of the general stress regulon by Petersohn *et al.* (1999) and later associated with a salt-sensitive phenotype, but its function was unknown at that time [113], [114]. Using microarrays, Nakano *et al.* (2003) could demonstrate that Spx is a direct regulator of many genes of the thiol-specific oxidative stress response [103]. Additional microarray experiments and ChIP analyses further increased the known members of the Spx regulon to several hundred genes and suggest that Spx is a central regulator of many stress related genes [62], [115]. Conversely, deletion of the *spx* gene renders cells highly sensitive to many stress conditions, such as heat-, salt- or oxidative stress as well as ethanol treatment [42], [46], [50], [103], [113]. A detailed characterization by Runde *et al.* (2014) revealed, that Spx is also a critical regulator of the HSR [50]. Strains with a deletion of *spx* exhibit strongly increased sensitivity to heat stress and do not develop thermotolerance. Conversely, accumulation of Spx conferred strongly increased heat resistance [50].

Many genes of the Spx regulon have functions in the response to oxidative stress. Two of the most up-regulated members of the Spx regulon are the *trxA* and *trxB* genes, which encode for an essential, NADPH-dependent thioredoxin system [56], [103]. Thioredoxins provide reduction equivalents for different metabolic redox reactions and play an important role in the response to oxidative stress [116]. They can act as scavengers and directly detoxify reactive oxygen species and also provide the reduction

equivalent for enzymes which repair oxidative damages such as methionine sulfoxide reductases or hydroperoxide peroxidases [116]–[118]. Furthermore, they can reduce intra- or intermolecular disulfide bonds of misfolded, oxidized proteins and thereby prevent their aggregation and allow their refolding [50], [119]. Thioredoxins also have an important role in the HSR of *B. subtilis* [50], [56]. Overexpression of thioredoxin strongly decreased the accumulation of heat induced protein aggregates, indicating that the formation of disulfide bonds is an important aspect of misfolding and aggregation of cellular proteins during heat stress under aerobic conditions (see above) [50]. In addition to the thioredoxin system, Spx also up-regulates other genes of the oxidative stress defense and genes for the synthesis of cysteine and bacillithiol, a low molecular-weight thiol antioxidant which serves as a substitute for glutathione in Gram-positive bacteria [62], [120], [121].

Transcriptomic and physiological studies suggest that many heat-inducible genes with previously unknown regulatory mechanism (class IV) are controlled by Spx. This includes for example the thioredoxin system *trxAB*, (see above) or the *clpX* and *mecA* genes [56], [62], [106]. Furthermore, Spx also contributes to the induction of heat shock genes which are primarily controlled by other regulators, such as the genes of the *ctsR-mcsA-mcsB-clpC* operon [62].

1.2.3 Control of metabolism and development by Spx

Spx is also implicated in the regulation of many other processes in *B. subtilis*; however the regulatory mechanisms are often not fully understood. Many of the Spx-associated pleiotropic phenotypes can be observed in *clpX* or *clpP* deletion strains in which Spx levels are increased (see section 1.2.4). Mutations in *spx* (*yjbD*) were identified as suppressors, which restored competence development in a *clpX* deletion strain [122]. Expression of competence genes in *B. subtilis* is controlled by the master regulator ComK, which is subject to complex transcriptional and post-translational regulation [123]. During the exponential growth phase, ComK is rapidly inhibited and degraded by MecA/ClpCP-dependent regulatory proteolysis [36], [124]. Competence development requires stimulation of the two-component system ComPA by the extracellular quorum-sensing peptide ComX [125], [126]. The phosphorylated response regulator ComA~P is a transcriptional activator of *comS*, which encodes the anti-adaptor peptide ComS [127]–[129]. ComS in turn binds MecA and results in the release and accumulation of ComK which then activates the expression of competence genes [36], [124]. Spx was demonstrated to inhibit this

process at multiple stages. First, Spx abolishes the transcriptional activation of *comS* by interfering with ComA~P activity at αCTD of RNAP [106], [108]. In addition, Spx can also interfere directly with ComK and thus block its activity [104]. Furthermore, *in vitro* experiments suggested that Spx directly interacts with the ComK·MecA·ClpC complex and may act as an antagonist of ComS by increasing binding of ComK to the complex [130]. In addition, Spx appears to negatively affect motility and biofilm formation by down-regulating genes for the synthesis of matrix polysaccharides [131], [132] and was reported to interfere with sporulation [115], [122].

1.2.4 Control of Spx activity

As a global stress response-regulator, Spx is quickly activated by different stress stimuli such as heat- or oxidative stress and cell-wall active antibiotics. Its activity is precisely controlled at the transcriptional and post-translational level (Figure 3). Spx levels and activity are low during non-stressed growth, but rise quickly when encountering stressful situations [50], [103].

Transcription of the *spx* gene is governed by multiple promoters directly upstream of the *spx* gene or the *yjbC-spx* operon, which are recognized by the major sigma factor σ^A, the general stress-sigma factor σ^B and the ECF-type sigma factors σ^M, σ^W and σ^X and modulated by the redox-sensitive transcriptional repressors PerR and YodB (Figure 3). Thereby, the transcription of *spx* is facilitated under a variety of abiotic and biotic stress conditions such as heat-, salt- or oxidative stress, cell wall-active antibiotics and phosphate starvation [114], [133]–[135].

However, Spx is primarily controlled on the post-translational level by regulated proteolysis (Figure 3). In the absence of stress, Spx is subject to constant degradation by the ClpXP protease and maintained at low levels [122], [136]. The adaptor protein YjbH strongly accelerates degradation of Spx by binding to its C-terminus and exposing a degradation motif [137], [138]. Notably, Spx is also rapidly degraded by ClpCP and the adaptor protein MecA *in vitro* [136], but a deletion of *clpC* does not stabilize Spx *in vivo*, while deletions of *clpX*, *clpP* or *yjbH* result in the accumulation of Spx to high cellular levels [122], [136], [137]. However, recent data by Rojas-Tapias and Helmann (2019) suggests, that the level and activity of Spx is also modulated by ClpCP and the protein arginine kinase McsB *in vivo* [139].

The N-terminal zinc-finger domain of ClpX is believed to be a sensor of oxidative stress [140]. Oxidation of the Zn-coordinating cysteines was suggested to inactivate ClpX *in vitro* and results in the

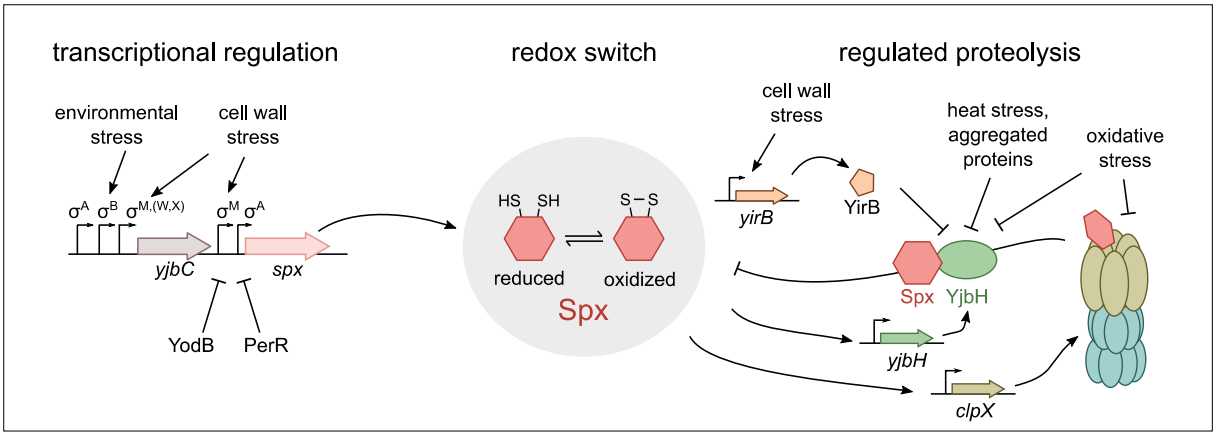

Figure 3: Transcriptional and post-translational regulation of the global regulator Spx

Spx is controlled on the transcriptional and post-translational levels. Multiple stress-responsive promoters direct the transcription of *spx* or the *yjbC-spx* operon. Spx activity is modulated by the cellular redox state via its CxxC motif. During non-stress conditions, Spx is degraded by ClpXP and the adaptor protein YjbH. Upon Stress, YjbH is inactivated by aggregation or interaction with the antiadaptor protein YirB, resulting in the accumulation of Spx. For details see text.

stabilization of Spx [141]. Similarly, the YjbH protein also contains a zinc cofactor and becomes inactivated during oxidative conditions [138]. YjbH appears to be an intrinsically unstable protein that becomes irreversibly aggregated *in vivo* during heat stress and other conditions which perturb protein homeostasis such as treatment with ethanol or diamide as well as overexpression of heterologous, insoluble proteins. Thus, YjbH can be considered a second stress sensor which becomes inactivated upon proteotoxic, environmental stress by regulated aggregation, resulting in the stabilization of Spx [138], [142]. Interestingly, the accumulation of Spx is auto-regulated by a negative feedback loop (Figure 3). Transcription of the *yjbH* and *clpX* genes is positively regulated by Spx [62], [137]. When cellular protein homeostasis is restored, *de novo* synthesized YjbH and ClpX will remain active and again facilitate the degradation of Spx [137], [141], [142]

Recently, a second pathway of Spx activation and stabilization during cell wall stress was described (Figure 3). Cell wall-active antibiotics such as vancomycin or ampicillin induce the transcription of *spx* via a σ^M-dependent promoter [134]. In addition, the transcription of a small ORF encoding the YirB protein is indirectly stimulated [143]. YirB was demonstrated to act as anti-adaptor protein which binds to YjbH and inhibits the interaction with Spx [144]. Together, both mechanisms result in the accumulation of Spx and the activation of its regulon [134], [143].

Furthermore, Spx is also a direct sensor the cellular redox status (Figure 3). It possesses two conserved cysteines within an N-terminal CXXC motif which form a disulfide bond upon oxidation and

modulate Spx activity [109], [112]. Spx-dependent regulation of some genes, e.g. *trxA* and *trxB* was demonstrated to rely only on oxidized Spx, while other genes are activated by Spx independently of its redox state [62], [109], [121].

1.3 The stringent response

The stringent response (SR) is a global and pleiotropic adaptation to stress and starvation. Its investigation started in the 1950s, when the relationship between metabolism and macromolecule synthesis rates was investigated [145]. Auxotrophic mutants starved for certain amino acids were found to be inhibited in rRNA synthesis, a phenomenon which was termed "stringent control" and the first "relaxed" mutants, which continued rRNA synthesis following an amino acid downshift were isolated and mapped [146]–[150]. In 1969, Cashel and Gallant reported the observation of two phosphorylated guanosine molecules on autoradiograms, termed "magic spots", which accumulated in response to amino acid starvation, but were absent in relaxed strains [151]. These nucleotides were identified as ppGpp (guanosine 3',5'-bispyrophosphate) and pppGpp (guanosine 3'-diphosphate 5'-triphosphate), hereafter referred to as (p)ppGpp or alarmones [152], [153]. The authors also hypothesized that these molecules are synthesized by the "stringent factor" upon shortage of activated amino acids during translation, which was confirmed in the following years [151], [153], [154].

To date, the SR is known as the global physiological response mediated by (p)ppGpp, which serves as a second messenger and directly or indirectly modulates transcription, translation and many additional enzyme activities [155], [156]. The SR is conserved in all eubacteria with the only exception of some obligate intracellular pathogens and also found in chloroplasts [157], [158]. The accumulation of alarmones upon amino acid starvation and the resulting transcriptional down-regulation of rRNA as well as the up-regulation of amino acid synthesis pathways are the conserved and well-studied hallmarks of the SR. However, the SR was also demonstrated to respond to multiple additional signals and mediate adaptation to different environmental niches and stresses beyond this paradigm [156], [159], [160].

1.3.1 Synthesis and hydrolysis of (p)ppGpp

The cellular (p)ppGpp levels are primarily controlled by conserved multi-domain RSH-type (RelA, SpoT homolog) enzymes which bear distinct synthetase- and hydrolase domains located on the N-

terminus (NTD; Figure 4 A) [157]. They catalyze the transfer of pyrophosphate from ATP to the 3'-OH group of GTP or GDP to create pppGpp or ppGpp [153], [161] and the hydrolysis of alarmones by removal of the pyrophosphate from the 3' position, yielding GTP or GDP [162]. The C-terminal domains (CTD), most commonly TGS domains (homology to ThrRS, Obg family GTPases and SpoT), ACT-domains (homology to aspartate kinase, chorismate mutase and TyrA) and additional alpha-helical- and zinc-finger domains are involved in the control of these opposing activities [157], [163], [164]. Several orthologs of RSH-type enzymes with different properties have been identified, designated RelA, SpoT and Rel [157], [165]. Like most bacteria, *B. subtilis* encodes a single, bifunctional Rel homolog, which possesses mutually exclusive synthetase- and hydrolase activities [157], [166], [167]. In contrast, β- and γ-proteobacteria commonly encode both RelA and SpoT homologs [157]. RelA is the main alarmone synthetase in *E. coli* upon amino acid starvation, but possesses an inactive hydrolase domain [154]. SpoT exhibits a weaker synthetase activity, which appears to respond to additional stress signals and is the main alarmone hydrolase in *E. coli* [168], [169].

In addition to long RSH-type enzymes, monofunctional small alarmone synthetases (SAS) and -hydrolases (SAH) with a single catalytic domain are common among bacteria (Figure 4 A) [157]. Two SAS proteins, SasA (YwaC) and SasB (YjbM), but no SAH homologs are encoded in the *B. subtilis* genome [166]. Several additional enzymes have been suggested to participate in the alarmone metabolism. The exopolyphosphatase GppA of *E. coli* hydrolyzes pppGpp to ppGpp, but has no homolog in *B. subtilis* [170]. Therefore, ppGpp is the dominant alarmone in *E. coli*, while pppGpp can be more abundant in *B. subtilis* [167], [171], [172]. Recent studies suggest that additional enzymes of the nudix hydrolase-family and other enzymes can hydrolyze (p)ppGpp, but their role in the regulation of the SR is unknown to date [173], [174].

1.3.2 Activation of RSH in response to amino acid starvation

The synthesis of alarmones upon amino acid deprivation is the best-studied signaling pathway of the SR and appears to be well-conserved in bacteria [159], [175]. The molecular details are mostly studied for RelA of *E. coli* but appear to be similar for Rel of *B. subtilis* [167], [176], [177]. RelA becomes activated in the presence of translating ribosomes and uncharged tRNA corresponding to the codon displayed in the ribosomal acceptor site (Figure 4 B) [154]. The ribosomal protein L11 (*rplK*;*relC*)

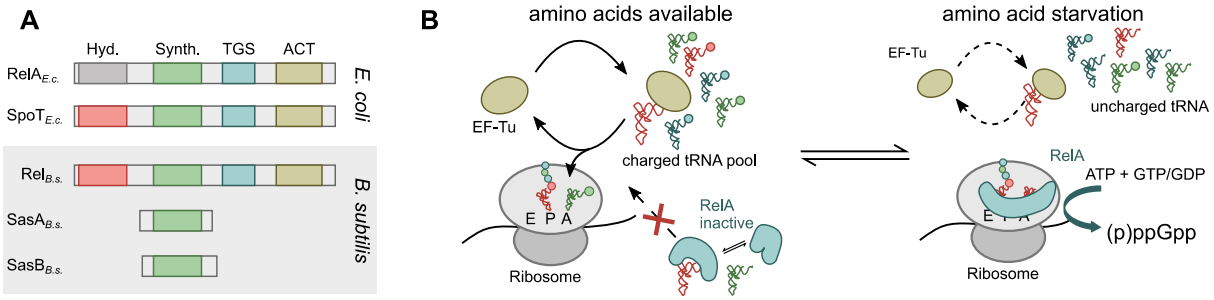

Figure 4: Activation of the SR during amino acid starvation.

(A) Domain organization of (p)ppGpp synthetases and hydrolases of *E. coli* and *B. subtilis*. Hyd.: (p)ppGpp hydrolase domain (inactive in RelA). Synth.: (p)ppGpp synthetase domain. TGS: homology to ThrRS, GTPases and SpoT. ACT: homology to aspartate kinase, chorismate mutase and TyrA. Domains are not to scale. Adapted from [164]. (B) Model of the activation of RelA during amino acid starvation. Under optimal growth conditions, EF-Tu delivers charged tRNAs to the ribosomal acceptor site. RelA interacts with the uncharged tRNA fraction, but is blocked from interacting with the ribosome by EF-Tu and does not synthesize (p)ppGpp. During amino acid starvation, EF-Tu cannot deliver tRNAs to the vacant acceptor site, thereby allowing the RelA·tRNA complex to bind the ribosome and synthesizes (p)ppGpp. For details, see text. Adapted from [187,188].

appears to have an essential role in the SR as mutations in L11, which confer thiostrepton resistance strongly impair activation of RelA [178]–[180]. Likewise, chloramphenicol and other antibiotics which interfere with translation, completely inhibit (p)ppGpp synthesis by stalling translation and therefore preventing the consumption of charged tRNA [181]. In the absence of ribosomes and tRNA, RelA exhibits no alarmone synthesis activity and the synthetase domain is thought to be inhibited by intra- and intermolecular interaction with the C-terminus [154], [182], [183]. Studies on bifunctional Rel homologs suggested that the synthetase and hydrolase activities are reciprocally regulated by the C-terminal domains and that without stimulation, Rel enzymes are in a hydrolase-ON/synthetase-OFF state [184], [185].

Different models for the activation of RelA and the formation of the active RelA·tRNA·ribosome complex have been developed. Based on the *in vitro* observation, that the affinity of RelA for the ribosome decreases upon (p)ppGpp synthesis and considering the low cellular RelA concentration relative to the high number of ribosomes, a ‘hopping’ model was proposed. By this model, ribosomes stalled by uncharged tRNA in the acceptor site are detected by RelA, which synthesizes a small amount of (p)ppGpp, then dissociates from the ribosome and ‘hops’ to the next blocked ribosome [178]. Thereby, the relatively few molecules of RelA in the cell could accurately monitor the starved ribosome population and respond accordingly [178]. Further investigations by single molecule fluorescence microscopy yielded contradictory results which either supported or rejected this model [186], [187].

In 2016, three independent cryo-electron microscopy studies of the RelA·ribosome complex were published [188]–[190]. These studies demonstrate, in good agreement with each other that the C-terminal domains of RelA wrap around the acceptor site loaded with an uncharged tRNA, while the TGS domain interacts with its 3'-CCA end. The intricate contact of RelA·CTD with ribosomal proteins (RP) and rRNA is thought to relieve the auto-inhibition from the catalytic NTD, which is flexible and extends into the solvent [188]–[190]. However, the complex interaction between RelA, tRNA and the ribosome argues against the 'hopping' model, which implicated transient interaction and instead suggests a two-step model (Figure 4 B) by which RelA interacts with uncharged tRNA in the absence of ribosomes without becoming activated in alarmone synthesis [154], [188], [191], [192]. This preformed complex may already induce conformational changes in RelA, which allow the subsequent binding of the complex to ribosomes stalled by the lack of aminoacylated tRNA in the acceptor site [188], [192]. This model is supported by quantitative analyses of the interactions of RelA with tRNA or rRNA during exponential growth or isoleucine starvation. The data suggests that RelA transiently interacts with the sub-population of uncharged tRNA already in the absence of starvation *in vivo.* During active translation, the RelA·tRNA complex is likely outcompeted from binding to the translating ribosome by the much more abundant EF-Tu·tRNA complex. Upon amino acid shortage, the ribosomal acceptor site is vacant due to the lack of charged tRNA, while the cognate pool of uncharged tRNA bound to RelA increases, thus allowing the RelA·tRNA complex to bind the ribosome and become activated (Figure 4 B) [154], [189], [191]. However, other models have also been proposed [193]. The mechanism of Rel activation in *B. subtilis* is not studied in detail but is most likely similar to RelA of *E. coli*.

The role of the bifunctional RSH-homolog SpoT of *E. coli* during amino acid starvation is less well-understood. SpoT associates only weakly with ribosomes and interacts with the ribosome-associated GTPase Obg (CgtA) [194], [195]. SpoT was also demonstrated to bind uncharged tRNAs, which do not stimulate alarmone synthesis, but inhibit its hydrolase activity [169], [196].

1.3.3 Control of the stringent response by additional stress signals

The synthetases and hydrolases which control the (p)ppGpp level and thus the SR integrate diverse additional stress- and starvation signals and are regulated by many transcriptional and post-translational events (Figure 5) [160], [175]. However, the mechanisms by which these additional signals are sensed

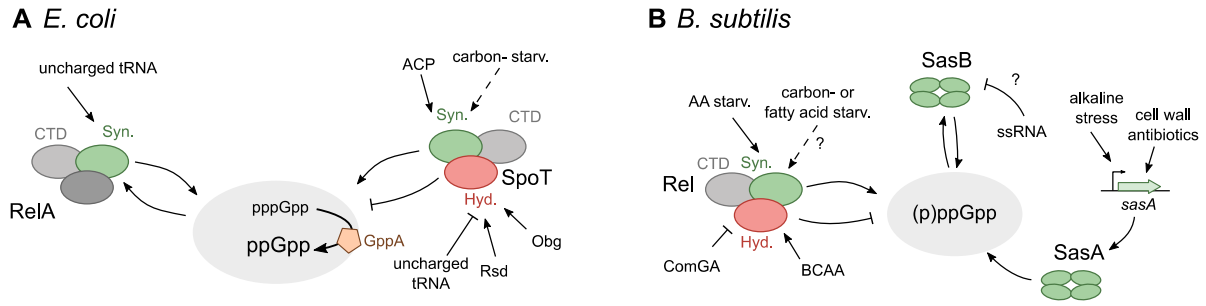

Figure 5: Stimuli that regulate (p)ppGpp synthesis and -hydrolysis in *B. subtilis* and *E. coli*
(A) In *E. coli*, (p)ppGpp synthesis by RelA is activated by amino acid starvation sensed via uncharged tRNAs on the ribosome (see section 1.3.2). Furthermore, RelA is stimulated by its product (p)ppGpp. SpoT responds to additional stimuli and is regulated by interaction with ACP (fatty acid- and carbon-starvation), Rsd (responds to changes in the phosphotransferase system) and Obg (stimulates alarmone hydrolase activity, role unknown) or uncharged tRNA (inhibition of its hydrolase activity). In addition, RelA is under complex transcriptional control (not shown). (B) (p)ppGpp synthesis by Rel of *B. subtilis* is stimulated by amino acid starvation similar to RelA of *E. coli*. Rel does not interact with ACP like SpoT, but becomes activated upon carbon- or fatty acid-starvation by an unknown pathway. Interaction with ComGA inhibits its alarmone hydrolase, while branched chain amino acids (BCAA) stimulate its hydrolase domain. SasB is allosterically activated by pppGpp. SasA is under transcriptional control and up-regulated in response to cell wall stress via σ^M and σ^W. Partially adopted from [164,177]. For details see text.

and transmitted are often not well understood. In addition, many aspects of the signaling pathways of the SR differ between species and appear to exhibit specific adaptations to the requirements of the respective habitats [159], [160].

The SR appears to be modulated by the nutritional status of different metabolic pathways. The acyl carrier protein (ACP), a central factor in lipid biosynthesis, was demonstrated to interact in its deacylated state with the TGS domain of SpoT and activates the SR by stimulation of its (p)ppGpp synthesis activity upon fatty acid starvation in *E. coli* [197], [198]. In contrast, the bifunctional Rel of *B. subtilis* was reported to not interact with ACP [199]. However, an involvement of the SR, dependent on the Rel synthetase, was observed in *B. subtilis* upon fatty acid starvation, suggesting an alternative but unknown activation mechanism [200]. Activation of the SR has also been observed upon downshift of the main carbon source, which was demonstrated to stimulate (p)ppGpp synthesis by Rel in *B. subtilis* and by SpoT and RelA in *E. coli* [201], [202]. Carbon starvation reduces the cellular acetyl-CoA level and thus likely leads to activation of the SR by secondary fatty acid starvation [197]. In addition, glucose deprivation may lower the levels of the amino acid precursors of the citrate cycle and provoke a partial amino acid limitation [203].

An additional feedback regulation between carbon metabolism and SR was described in *E. coli* [204]. The protein Rsd, which was initially characterized as an anti-σ^{70} factor during the stationary phase, was demonstrated to stimulate alarmone hydrolysis by interaction with the TGS domain of SpoT. The Rsd·SpoT interaction is antagonized by Hpr, a central protein of the phosphotransferase system, but not by phosphorylated Hpr~P. This feedback regulation is thought to prevent uncontrolled accumulation of (p)ppGpp during starvation and may shut down the SR after adaptation to a new carbon source during diauxic growth [204]. However, Rsd has no homolog in *B. subtilis*.

The interaction of *B. subtilis* Rel with ComGA, a late competence protein, establishes an interesting connection between the SR and genetic competence. The competent subpopulation of cells exhibits arrested growth known as the K-state and displays increased tolerance to certain antibiotics. The development of the K-state is bypassed in (p)ppGpp^0 cells and is apparently conferred by the ComGA-mediated inhibition of the alarmone hydrolase activity of Rel [205].

Interestingly, RSH and SAS enzymes are also modulated by small molecules. Bifunctional Rel enzymes from *Rhodobacter capsulatus* or *B. subtilis* are allosterically regulated by branched-chain amino acids (BCAA) which bind to the ACT domain and stimulate the alarmone hydrolase activity, thus down-regulating the SR when amino acid levels are reestablished [206]. RelA of *E. coli* is allosterically stimulated by its product ppGpp while still requiring interaction with the ribosome. This positive feedback loop might facilitate the rapid activation of the SR and also promote bistability and heterogeneity [207]. In *B. subtilis*, it is unknown whether the bifunctional Rel is also feedback regulated by alarmones. However, the monofunctional synthetase SasB (YjbM) was found to be activated by allosteric binding of pppGpp but not ppGpp to a central binding pocket and could therefore represent a similar positive feedback loop [208]. A homolog of SasB in *Enterococcus faecalis* is inhibited by binding single stranded RNA at the same allosteric site, which can be outcompeted by pppGpp, but the role of this allosteric regulation is unknown [209].

In addition, (p)ppGpp synthesis is also regulated on the transcriptional level. In *B. subtilis*, *rel* and *sasB/yjbM* are constitutively expressed and regulated by post-translational events, while *sasA/ywaC* is under the control of $\sigma^{M,W}$ dependent promoters and up-regulated in response to cell wall-active

antibiotics and alkaline stress [210]. In *E. coli*, *relA* is under complex transcriptional control and up-regulated upon different stress- and starvation conditions [160].

1.3.4 Direct and indirect regulation of transcription

The SR directly or indirectly controls many cellular processes. (p)ppGpp negatively regulates bacterial growth in a concentration-dependent manner by inhibiting the synthesis of macromolecules such as ribosomal RNA, DNA, proteins and membrane lipids [211], [212]. The probably best studied molecular consequences of the SR are global and pleiotropic alterations in the transcriptome. Most notably, the transcription of rRNA and ribosomal protein (RP) genes is inhibited by the SR in a dose-dependent manner, while amino acid synthesis genes and genes associated with the stationary phase and stress responses are up-regulated [213]–[215]. Aside from the stringent regulation by high alarmone levels during starvation, basal (p)ppGpp levels are important for regulating metabolism and growth rate in the absence of stress [216]. Conversely, *E. coli* or *B. subtilis* strains lacking (p)ppGpp (designated (p)ppGpp^0) exhibit multiple auxotrophies and (p)ppGpp^0 strains of pathogenic organisms often display attenuated virulence [168], [217].

In *E. coli* and related gram-negative bacteria, (p)ppGpp directly binds two distinct, allosteric sites on the RNAP and modulates both transcription initiation and elongation in synergy with the RNAP-associated DksA protein [218], [219]. The details of this mechanisms have recently been reviewed extensively by Gourse *et al.* (2018) [218]. Several hundred genes have been found to be positively or negatively regulated by the SR [213], [220]. Additionally, (p)ppGpp also indirectly regulates transcription by influencing the competition of alternative sigma factors with the 'housekeeping' factor σ^{70} for RNAP [221].

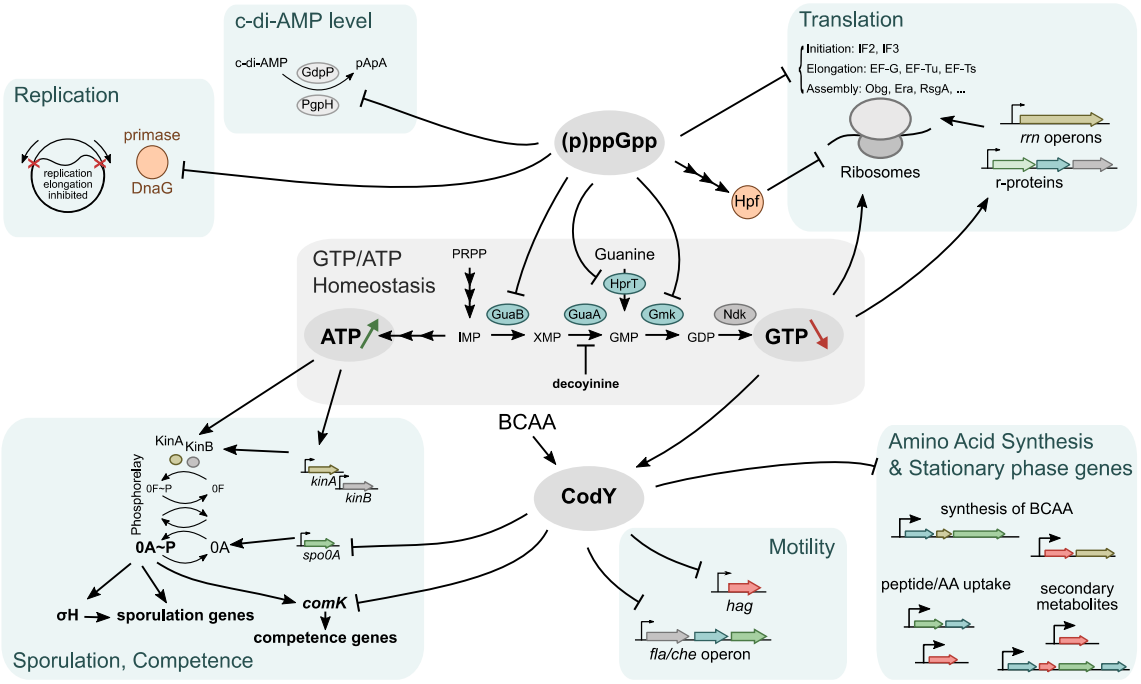

Figure 6: Direct and indirect targets of (p)ppGpp in *B. subtilis*

(p)ppGpp inhibits GTP synthesis at multiple steps, leading to decreased GTP concentration, while the ATP concentration is increased. Changed GTP and ATP levels alter the efficiency of promoters that initiate transcription with the respective nucleotide. Transcription is also modulated by CodY, which required binding of GTP for its repressor activity. (p)ppGpp binds to many ribosome-associated GTPases, thereby slowing down translation. Additionally, (p)ppGpp slows down replication by inhibiting the primase DnaG and leads to an increase of c-di-AMP by inhibition of the phosphatases PgpH and GdpP.. For details, see text.

However, the mechanism of stringent transcriptional regulation differs fundamentally in *B. subtilis* and other *Firmicutes* [222]. These organisms lack a DksA homolog and direct regulation of RNAP by (p)ppGpp is not observed [215]. Instead, (p)ppGpp regulates transcription indirectly by competitively inhibiting GuaB (IMP dehydrogenase), HprT (hypoxanthine-guanine phosphoribosyltransferase) and Gmk (guanylate kinase) of the GTP synthesis pathway, thus lowering the GTP level, while the ATP level is increased (Figure 6) [223]–[225]. Many phenotypes of the SR can also be elicited by treatment with decoyinine, an inhibitor of GMP synthase (GuaA), which causes similar reciprocal changes in GTP and ATP levels [226], [227]. The promoters which control rRNA synthesis and ribosomal protsin (RP) operons initiate transcription with GTP and require high nucleotide concentration for maximal activity. By lowering the cellular GTP level, these promoters are indirectly down-regulated in a concentration-dependent manner (Figure 6). In turn, certain promoters which initiate transcription with ATP exhibit positive stringent regulation [215], [226], [228]. Additionally, GTP binds, together with branched-chain amino acids (BCAA), to allosteric sites of the global repressor protein CodY and stimulates its activity.

Reduced levels of GTP during the SR or starvation for BCAA lead to the de-repression of the CodY regulon, which comprises of many genes required for the synthesis of amino acids and adaptation to the stationary phase (Figure 6) [229]–[231]. In (p)ppGpp^0 cells, the strong repressor activity of CodY is the cause of the multiple amino acid auxotrophies [217].

It was recognized early on that the SR is a signal for the initiation of sporulation in *B. subtilis*. Mutant strains with inactive Rel displayed poor sporulation during amino acid downshift as well as delayed accumulation and phosphorylation of the master regulator Spo0A~P, which is in turn is required for σ^H activity (Figure 6) [179], [232], [233]. In contrast, sporulation can also be induced in stringent and relaxed strains by treatment with decoyinine, suggesting that a GTP downshift is the trigger for initiation of sporulation [227]. De-repression of CodY, which also controls the *spo0A* gene, is required but not sufficient for sporulation [229], [234], [235]. Additionally, expression of the *kinA* and *kinB* genes, which encode two kinases of the phosphorelay that controls Spo0A activity, is under positive stringent control and induced by increased ATP levels during the SR. Furthermore, the kinase activity of KinA and KinB is stimulated by increased ATP levels (Figure 6) [235], [236]. The SR is also required for competence development by alleviating the CodY-dependent repression of *comK* [234], [237] and was found to regulate swimming motility via CodY-dependent regulation of *sigD*, a regulator of genes required for the development of motility (Figure 6) [238].

1.3.5 Inhibition of ribosome biogenesis and translation

It is established that the SR is an inhibitor of translation at multiple stages (Figure 6). First, biogenesis of new ribosomes is inhibited at the transcriptional level (see above). In addition, (p)ppGpp directly controls proteins synthesis rates by regulating the activity of existing ribosomes [239], [240]. Due to the structural similarities, (p)ppGpp can often compete with GTP and interfere with many steps of protein synthesis, which require GTP hydrolysis [164], [212]. For example, (p)ppGpp was reported to reduce binding of fMet-tRNA to the ribosomal P-site during translation initiation by inhibiting the GTPase IF2 [240], [241]. It was suggested that IF2 acts as a metabolic sensor of both decreased GTP and increased (p)ppGpp levels [242]. Likewise, inhibition of translation elongation by ppGpp was reported via competitive inhibition of EF-Tu, EF-Ts and EF-G [164], [240], [241]. Furthermore, ribosome-recycling is slowed down by (p)ppGpp via competitive inhibition of RF3, which stimulates the release of RF1 and

RF2 after peptide chain release [243]. These mechanisms are observed and studied in *E. coli*, but are widely assumed to apply also for *B. subtilis* and other species due to the conserved nature of the translation apparatus.

Interestingly, (p)ppGpp was also found to bind and inhibit additional, ribosome-associated GTPases, which are universally-conserved, often essential factors in biogenesis and maintenance of the ribosomal subunits [244]–[246]. The inhibition of these enzymes results in decreased formation of mature 70S ribosomes, reduced translation, growth inhibition and was demonstrated to contribute to antibiotic tolerance and stress survival [244]. In addition, these GTPases have been associated with diverse and pleiotropic function such as DNA replication, stress response and –tolerance, sporulation and persister cell formation, but molecular details are often missing [245], [247], [248].

The SR also mediates the dimerization of 70S ribosomes to translationally inactive 100S disomes during stress and starvation by activating the transcription of the responsible factors [249], [250]. These disomes are protected from damage or degradation and contribute to survival during prolonged starvation and environmental stress [46], [251], [252]. However, substantial structural differences exist between the 100S disomes of *E. coli* and *B. subtilis* [252]–[255]. As in in most bacteria, the Hpf protein (hibernation promoting factor) is sufficient for ribosome dimerization in *B. subtilis*, while 100S formation in *E. coli* is mediated by a shorter Hpf homolog together with the ribosome modulation factor (RMF) [250], [253], [256], [257]. The hibernating 100S disomes are translationally inactive but can be monomerized to functional 70S ribosomes during outgrowth after stress, thereby circumventing the cost of synthesizing new ribosomes and allowing a fast recovery of translation [251], [256], [258].

1.3.6 Additional targets of (p)ppGpp

The SR induces arrest of DNA replication in *B. subtilis* and, to a lesser extent, in *E. coli* (Figure 6) [259], [260]. It was demonstrated that (p)ppGpp interferes the activity of DNA primase DnaG. Thereby, replication elongation is gradually inhibited in a dose-dependent manner and adjusted according to the nutritional status of the cell [172], [261]. In *E. coli*, the transcription of fatty acid biosynthesis pathways is down-regulated during the SR. In addition, several enzymes of the fatty acid and phospholipid biosynthesis pathways are directly inhibited by (p)ppGpp [212], [262]. It is currently unknown, whether

the SR modulates fatty acid biosynthesis enzymes in *B. subtilis*, but the SR is implicated in survival upon fatty acid starvation [200].

Interestingly, (p)ppGpp was demonstrated to influence the metabolism of another second messenger nucleotide, c-di-AMP (cyclic di-3',5'-adenosine monophosphate) in different Gram-positive bacteria (Figure 6) [263]–[265]. C-di-AMP is both essential for growth and toxic at high levels. It is a crucial regulator of potassium uptake and -homeostasis and has been implicated in different processes such as DNA repair, cell wall homeostasis and biofilm formation [266]. (p)ppGpp acts as an allosteric inhibitor of the c-di-AMP phosphodiesterase PgpH, while the phosphodiesterase GdpP is competitively inhibited by (p)ppGpp [265]. However, the physiological implications of the cross-talk between the two signaling molecules are not well-understood. In the last years, several studies aimed to expand the set of known (p)ppGpp interacting proteins in *E. coli* or *S. aureus* by screening gene expression libraries for interaction with radiolabelled nucleotides or by affinity-based enrichment of interacting proteins [173], [244], [267]. Besides confirming and validating the known interaction of (p)ppGpp with ribosome-associated GTPases, the studies suggested, that (p)ppGpp is more intricately involved in the regulation of the purine metabolism of *E. coli* than previously known [173], [267]. Additionally, several enzymes, which were found to possess (p)ppGpp hydrolysis activities *in vitro* and *in vivo,* may constitute new pathways for the degradation of (p)ppGpp [173].

1.3.7 (p)ppGpp and other unusual nucleotides during environmental stress

A number of observations have linked the SR to the regulation of the HSR and other stress responses [268], [269]. It was first reported for *E. coli* that during a sudden temperature upshift the (p)ppGpp level is transiently increased while ribosomal RNA synthesis was found to be down-regulated [270]–[272]. Similar observations were made for other stress conditions such as oxidative stress or treatment with alcohols [273], [274]. Furthermore, increased synthesis rates of a number of heat shock- or general stress proteins was observed during the stringent response elicited by amino acid starvation or oxygen limitation [275], [276]. A similar correlation was found between the synthesis of heat shock proteins and the occurrence of many additional unusual adenylated di-nucleotides, such as AppppA, AppppG, ApppGpp, ApppA or ApppG during heat and oxidative stress conditions. In light of these observations, it was proposed that the alarmone (p)ppGpp or unusual adenylated nucleotides may represent important signals

which direct the activation of the HSR upon different stress stimuli. However, later analyses demonstrated that the synthesis of heat shock proteins was independent of (p)ppGpp and other nucleotides [24], [273], [274], [277], [278]. The correlation between heat shock protein synthesis and the SR was also studied in *B. subtilis*, but the induction of the HSR was found to be largely independent of (p)ppGpp as well [276], [279]–[281].

1.4 Aims of this study

In *B. subtilis*, (p)ppGpp was reported to accumulate upon a number of stress conditions such as salt stress, oxidative stress and exposure to antibiotics or zinc oxide [281]–[285]. Furthermore, a participation of the SR in the response to different stresses was hypothesized based on transcriptomic or proteomic data [286], [287]. However, the role of the SR in the HSR and thermotolerance development of *B. subtilis* is largely unknown. A transcriptomic analysis of *B. subtilis* during thermotolerance development by Anja Heinz (Institut für Mikrobiologie, Freie Universität Berlin) revealed a stringent response-like gene expression pattern in thermotolerant cells with many translation-related genes being strongly repressed. This observation argues for an activation and participation of the SR during thermotolerance development [288] (see section 2.1). Therefore, this work aimed to validate the involvement of the (p)ppGpp-mediated SR in the HSR of *B. subtilis* and to determine its function and impact on thermotolerance development.

To do so, mutant strains with artificially increased or decreased (p)ppGpp levels were constructed and analyzed. By employing RNA-sequencing of these mutants during heat stress in collaboration with the group of Prof. Petra Dersch (Institute of Infectiology, University of Münster), it was aimed to gain insights into the regulon of SR-regulated genes and its impact on the transcriptional regulation of ribosomal and stress-related genes during thermotolerance development (section 2.2). Furthermore, it was sought to identify the signals and mechanisms which result in the activation of the SR during heat- and oxidative stress conditions (sections 2.2 & 2.4). The *hpf* (*yvyD*) gene was identified as a marker gene for the activation of the SR during heat stress. In collaboration with the group of Prof. Daniel Wilson (Institute for Biochemistry and Molecular Biology, Universtiy of Hamburg), which could solve a

structure of the hibernating 100S ribosome, the role of Hpf in disome formation and survival during heat stress was investigated (sections 2.2. & 2.2)

Previously, Runde *et al*. (2014) identified the global regulator Spx as an important transcription factor of the HSR [50]. During the studies of this thesis, it has become apparent that Spx is also a negative regulator of many translation-related genes which resembles the SR in this regard. Therefore, the second objective of this work was to characterize this previously unknown activity of Spx, its impact on the HSR and its interplay with the SR (sections 2.1 & 2.3).

2. Results

2.1 Spx, the central regulator of the heat and oxidative stress response in *B. subtilis*, can repress transcription of translation-related genes

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Authorship: First author

Share of work: ~ 70 %

Contributions: The authors HS (i, ii, iii), AH (ii), PS (ii), MV (ii), IH (ii), LK (ii, iii), KT (i, ii, iii) have made major contributions to (i) the conception or design of the study, (ii) the acquisition, analysis, or interpretation of the data; and (iii) writing of the manuscript.

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Spx, the central regulator of the heat and oxidative stress response in *B. subtilis*, can repress transcription of translation-related genes

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Summary

Spx is a *Bacillus subtilis* transcription factor that interacts with the alpha subunits of RNA polymerase. It can activate the thiol stress response regulon and interfere with the activation of many developmental processes. Here, we show that Spx is a central player orchestrating the heat shock response by up-regulating relevant stress response genes as revealed by comparative transcriptomic experiments. Moreover, these experiments revealed the potential of Spx to inhibit transcription of translation-related genes. By *in vivo* and *in vitro* experiments, we confirmed that Spx can inhibit transcription from rRNA. This inhibition depended mostly on UP elements and the alpha subunits of RNA polymerase. However, the concurrent up-regulation activity of stress genes by Spx, but not the inhibition of translation related genes, was essential for mediating stress response and antibiotic tolerance under the applied stress conditions. The observed inhibitory activity might be compensated *in vivo* by additional stress response processes interfering with translation. Nevertheless, the impact of Spx on limiting translation becomes apparent under conditions with high cellular Spx levels. Interestingly, we observed a subpopulation of stationary phase cells that contains raised Spx levels, which may contribute to growth inhibition and a persister-like behaviour of this subpopulation during outgrowth.

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Introduction

All cells need to monitor and maintain their protein homeostasis, which becomes particularly challenging during adverse environmental conditions that induce unfolding, misfolding or aggregation of cellular proteins. For this purpose, a cellular protein quality control system (PQS) evolved, which is conserved and present in all domains of life (Balchin *et al.*, 2016). This PQS includes chaperones which can prevent protein aggregation or facilitate the refolding of already misfolded proteins. Specific chaperone complexes can disaggregate and refold already aggregated proteins. Furthermore, potentially toxic subcellular protein aggregates can be removed from the cell by AAA+ protease complexes (Wickner *et al.*, 1999; Kirstein *et al.*, 2009; Mogk *et al.*, 2011; Kim *et al.*, 2013). Consequently, the cellular levels of most chaperones and proteases of the PQS, also known as heat shock proteins, are increased in response to a temperature upshift, but also to other stress conditions affecting protein homeostasis. Importantly, a pre-shock at elevated but not lethal temperature can provide cells with an acquired resistance to extreme and otherwise lethal temperatures in a process called priming or thermotolerance. This heat-mediated acquisition of thermotolerance appears to be conserved in all domains of life (Lindquist, 1986).

The heat shock response of the Gram-positive model organism *Bacillus subtilis* is controlled by multiple regulators (Hecker *et al.*, 1996; Elsholz *et al.*, 2017). Two heat-sensitive transcriptional repressors, HrcA and CtsR, control the expression of chaperone systems of the Hsp60 (GroESL) and Hsp40/70 (DnaKJ/GrpE) families, or the Hsp100/Clp unfoldase/protease systems ClpCP, ClpXP and ClpEP respectively (Mogk *et al.*, 1997; Krüger and Hecker, 1998; Wiegert and Schumann, 2001; Elsholz *et al.*, 2010). The membrane anchored proteases HtrA and HtrB are under transcriptional control of the CssRS

two-component system, which can respond to secretion and cell wall stress (Darmon *et al.*, 2002; Rojas-Tapias and Helmann, 2018b). These stress response regulons are augmented by the activation of the general stress response controlled by the alternative sigma factor σ^B also during heat stress (Hecker *et al.*, 2007).

Recently, we identified Spx, the regulator of thiol- and oxidative-stress response, which affects various regulatory pathways such as competence development (Nakano *et al.*, 2001; Petersohn *et al.*, 2001; Zuber, 2004), as a critical regulator for thermotolerance development and heat shock response (Runde *et al.*, 2014). It should be noted that Spx was initially identified and named as suppressor of *clpP* and *clpX* and that the raised levels of Spx in *B. subtilis* strains lacking the active ClpXP protease system result also in decreased growth rate, which can be alleviated by mutations in *spx* (Nakano *et al.*, 2002a; Runde *et al.*, 2014).

Spx is subject to multiple stages of regulation. Its transcription is controlled by several promoters recognized by the sigma factors σ^A, σ^B, σ^M, σ^W and σ^X and regulated by the repressors PerR and YodB, thereby the transcription of *spx* is stimulated under a variety of abiotic and biotic stress conditions, including heat stress (Petersohn *et al.*, 1999; Antelmann *et al.*, 2000; Helmann *et al.*, 2001; Leelakriangsak *et al.*, 2007; Jervis *et al.*, 2007; Nicolas *et al.*, 2012; Rojas-Tapias and Helmann, 2018a). However, the activity of Spx is primarily controlled post-translationally through regulatory proteolysis by the ClpXP protease complex and the adaptor protein YjbH during non-stress conditions (Nakano *et al.*, 2003b; Larsson *et al.*, 2007; Garg *et al.*, 2009).

Proteotoxic conditions such as heat and oxidative stress lead to the aggregation and inactivation of YjbH and thus increase the Spx protein level and activity (Zhang and Zuber, 2007; Engman and von Wachenfeldt, 2015). As a second layer of post-translational activity control, Spx possesses a CXXC motif that can undergo reversible cysteine oxidation and disulphide bond formation, thereby acting as a redox-sensitive switch (Nakano *et al.*, 2005). The expression of some but not all genes of the Spx regulon exclusively depends on the oxidized state of Spx (Rochat *et al.*, 2012; Gaballa *et al.*, 2013; Rojas-Tapias and Helmann, 2018a). Spx also becomes activated upon cell wall stress via an independent mechanism by increased transcription of *spx* from a σ^M-dependent promoter and stabilization of Spx by cell wall and secretion stress dependent synthesis of the anti-adaptor protein YirB (Rojas-Tapias and Helmann, 2018a; 2018b).

Unlike most transcriptional regulators, Spx does not appear to possess DNA-binding activity on its own. Instead it directly interacts with the C-terminal domain of the RNA-polymerase (RNAP) alpha subunit (α-CTD), which itself can interact with specific UP-elements, AT-rich sequences encoded in the DNA upstream of the core promoter region (Zuber, 2004; Newberry *et al.*, 2005; Reyes and Zuber, 2008; Lamour *et al.*, 2009; Nakano *et al.*, 2010; Delumeau *et al.*, 2011; Rochat *et al.*, 2012). In this complex, Spx can modulate RNAP activity and influence gene expression by (1) disrupting the binding of other transcriptional activators, e.g. ResD or ComA (Nakano *et al.*, 2003b; Zhang *et al.*, 2006). However, Spx can also (2) stimulate transcription from certain promoters, e.g. of the *trxA* and *trxB* genes. This stimulatory activity requires the interaction of the Spx/α-CTD complex with a *cis*-acting sequence motif associated with the UP-element upstream of the core promoter (Nakano *et al.*, 2003a; Reyes and Zuber, 2008; Nakano *et al.*, 2010; Rochat *et al.*, 2012; Lin *et al.*, 2013).

Several transcriptomic studies revealed that Spx is a global and pleiotropic regulator of the thiol-stress and oxidative stress response in *B. subtilis* (Leichert *et al.*, 2003; Nakano *et al.*, 2003a; Rochat *et al.*, 2012; Gaballa *et al.*, 2013). In addition, Rochat and co-workers applied global ChIP–chip experiments, which allowed to specifically identify the binding sites of the Spx/RNAP complex on the *B. subtilis* chromosome (Rochat *et al.*, 2012). Upon heat or oxidative stress, i.a. the thioredoxin system encoded by *trxA* and *trxB*, the AAA+ ATPase *clpX*, the genes of the adaptor proteins YjbH, MecA and YpbH or the genes required for the synthesis of bacillithiol *bshA,B1,B2,C* are upregulated by Spx (Leichert *et al.*, 2003; Nakano *et al.*, 2003a; Rochat *et al.*, 2012; Gaballa *et al.*, 2013). Thus, *B. subtilis* cells lacking Spx display high sensitivity to a variety of stress conditions, including heat, oxidative stress caused by exposure to diamide or paraquat, low temperatures, salt and cell wall-active antibiotics (Petersohn *et al.*, 2001; Höper *et al.*, 2005; Reder *et al.*, 2012; Runde *et al.*, 2014; Rojas-Tapias and Helmann, 2018a).

Previously, we studied thermotolerance in *B. subtilis* and could show that the activity of Spx is directly required for the development of thermotolerance. Cells lacking Spx are unable to develop thermotolerance when primed by a mild pre-shock. Additionally, accumulation of Spx prior to stress, either by deletion of *yjbH, clpX* or *clpP* or by expression of Spx^{DD} *in trans*, resulted in a strongly increased thermoresistance phenotype and reduced cell growth (Nakano *et al.*, 2002b; Runde *et al.*, 2014).

By investigating the role of Spx in thermotolerance and heat stress response, we observed a specific ability of Spx to inhibit transcription of genes associated with translation such as genes encoding ribosomal proteins (r-proteins) and ribosomal RNA (rRNA). This inhibitory activity of Spx depended mostly on specific UP elements and the interaction of Spx with the alpha subunits of RNAP. Further experiments demonstrated that this specific ability of Spx is not necessary for Spx-dependent stress response, possibly because other redundant stress induced cellular systems, which also interfere with protein synthesis,

might complement this inhibitory activity. However, during specific environmental conditions such as the stationary phase, Spx reaches relatively high cellular levels in a subpopulation of cells and might contribute to a slower growth of this subpopulation during outgrowth in fresh medium. The raised Spx levels may contribute to a persister-like phenotype and support the survival of this sub-population of cells, when confronted with antibiotics or environmental stress.

Results

Microarray-based characterization of thermotolerance development

In order to characterize the development of thermotolerance in *B. subtilis*, we carried out microarray experiments of cells treated with a lethal heat shock with or without prior priming. A log-phase culture of *B. subtilis* grown at 37°C was divided and either treated with 48°C or left untreated at 37°C. After 15 min, both cultures were transferred to 53°C for another 15 min. Total RNA from all samples was prepared and analysed by microarrays directly comparing the four different conditions (Fig. 1A).

Transcriptional changes important for thermotolerance could be recognized in array 4 that compared primed (48–53°C) and non-primed (37–53°C) cells at 53°C (Fig. 1B, Tables S2 and S3). The transcripts of 334 genes were more than twofold differentially expressed. A significant portion of these differentially regulated genes are known to be under SigB (88 with 87 up-regulated, according to SubtiWiki (Michna *et al.*, 2016)) or Spx (79 with 55 up- and 24 down-regulated, as defined in previous studies (Nakano *et al.*, 2003a; Rochat *et al.*, 2012), including direct and indirect regulation control. In addition, many of the most down-regulated genes are also known to be under the control of stringent response mediated transcriptional down-regulation upon amino acid starvation (Eymann *et al.*, 2002; Kriel *et al.*, 2012; Hauryliuk *et al.*, 2015) (Fig. 1B, Tables S1–S3).

For the mild heat shock condition (37°C vs 48°C; array 3), we observed 529 genes which appear to be

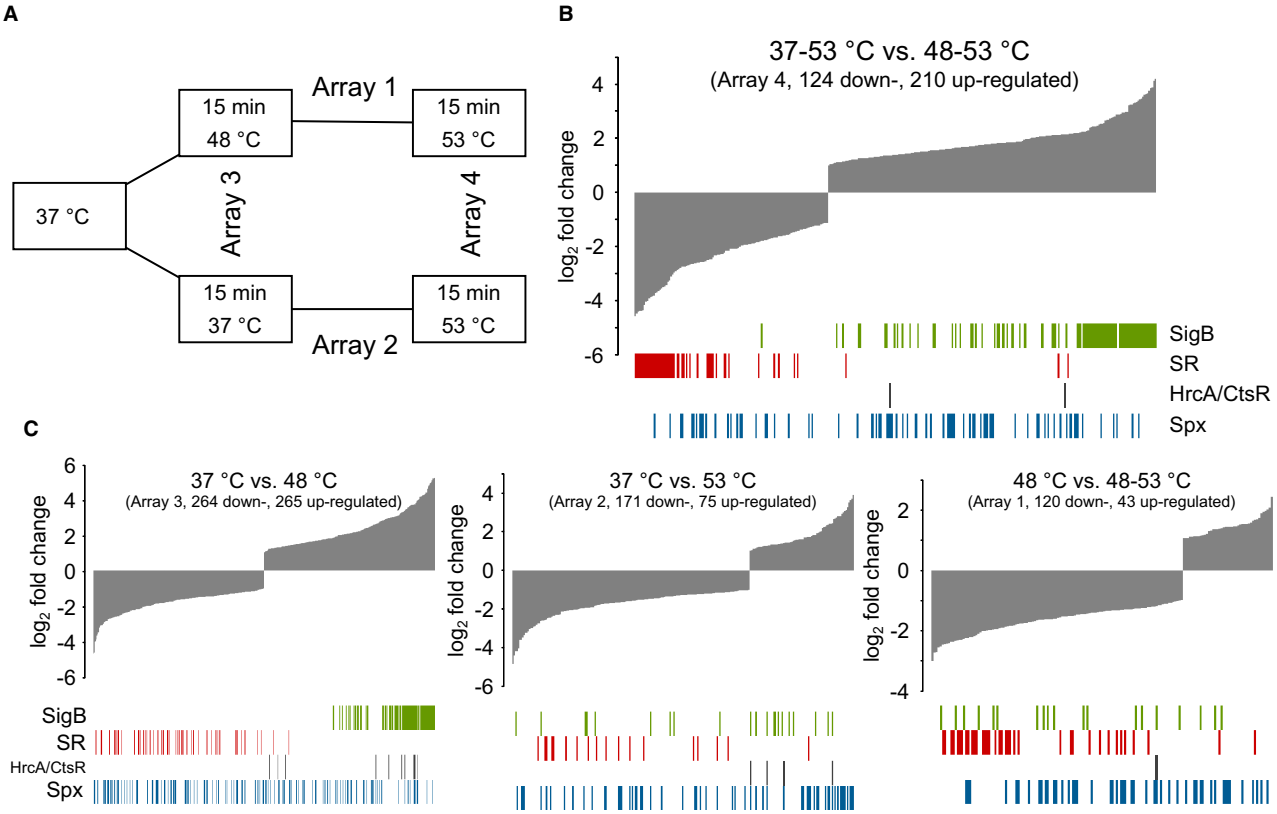

Fig. 1. Differentially regulated genes during thermotolerance.
A. Schematic representation of the thermotolerance protocol and the analysed microarrays.
B. The distribution of regulated genes in Array 4 (37–53°C vs. 48–53°C). Bar tracks indicate the number and distribution of genes of the σ^B regulon (SigB), regulated by the stringent response (SR), the HrcA and CtsR regulons (HrcA/CtsR) or the Spx regulon (Spx) as defined by (Nakano et al., 2003a; Rochat et al., 2012),
C. The distribution of arrays 1–3, representing the conditions 37°C vs. 48°C, 37°C vs. 53°C or 48°C vs. 48–53°C and bars representing the respective regulons with abbreviations as above. [Colour figure can be viewed at wileyonlinelibrary.com]

differentially regulated more than twofold. This set of genes partially overlapped with the set obtained from array 4 (Fig. 1C). Consistent with previous reports, we noticed an extensive up-regulation of genes controlled by the heat-shock regulators HrcA and CtsR as well as many genes of the general stress response regulon controlled by SigB (116 genes), (Helmann *et al.*, 2001) (array 3; Fig. 1C). However, we observed relatively little additional induction for most of these genes in the microarray comparing 48°C vs 48–53°C, suggesting that these regulons were already fully induced at 48°C with little potential for further adjustments upon more severe stress conditions (Array 1; Fig. 1C).

Moreover, array 2 (37–53°C) displayed a lower induction of heat shock genes controlled by HrcA, CtsR or SigB than array 2 (37–48°C). The applied lethal heat shock conditions (Völker *et al.*, 1999; Runde *et al.*, 2014) most likely also contributed to the diminished ability of the cells to efficiently change their gene expression. Nevertheless, a significant number of Spx-controlled genes were differentially transcribed under these conditions.

Spx had emerged from these experiments as an important heat shock regulator (Runde *et al.*, 2014), since a substantial fraction of previously identified Spx-regulated genes was observed in all investigated thermotolerance conditions (Fig. 1) (Nakano *et al.*, 2003a; Rochat *et al.*, 2012). While the regulons of SigB, HrcA and CtsR were almost exclusively up-regulated, the Spx regulon differed markedly from this pattern of transcriptional changes, since it exhibited both up- and down-regulation which appeared mostly equally distributed in all tested conditions (Fig. 1B and C, Tables S1 and S2). Interestingly, a Δ*spx* strain exhibited the most severe thermosensitivity phenotype assessed by growth on plates at 55°C compared to the also strongly impaired Δ*sigB* mutant strain, while both Δ*hrcA* or Δ*ctsR* were not strongly affected (Fig. S1A). In addition, Δ*sigB*, Δ*hrcA* or Δ*ctsR* strains were much less affected in thermotolerance development (Fig. S1B) compared to the previously investigated Δ*spx* (Runde *et al.*, 2014), corroborating an apparent difference in the role of these heat shock transcription factors.

Taken together with our previous results showing a strong impact of Spx on the development of thermotolerance (Runde *et al.*, 2014), these findings (Figs. 1 and S1, Table S1) further establish Spx as an important stress response regulator intricately involved in heat stress response.

Transcriptional changes of ΔclpX vs ΔclpX Δspx mutant that mediate thermotolerance controlled by Spx

To understand the role of Spx in thermotolerance development and the previously observed heat-resistant phenotype of the Δ*clpX* mutant strain in more detail (Runde *et al.*, 2014), we carried out microarray experiments comparing Δ*clpX* vs Δ*clpX* Δ*spx* mutant cells in the absence of stress at 37°C (Fig. 2). Since Spx is stabilized in cells lacking ClpX, we compare in this experiment the transcriptome of cells containing raised Spx levels, with cells lacking Spx, allowing to track the contributions of Spx on changes in the transcriptome (Nakano *et al.*, 2002b; Runde *et al.*, 2014).

In total, we observed 378 differentially transcribed genes (>2-fold change, 201 up-regulated, 177 down-regulated, Fig. 2A, Table S4) in this experiment. Besides up-regulated genes encoding proteins with unknown functions, there were many up-regulated genes encoding proteins of the general and oxidative stress response, in accordance with previous observations (Nakano *et al.*, 2003a; Rochat *et al.*, 2012) (Fig. 2A). Notably, this included the thioredoxin-system (*trxAB*), superoxide dismutase (*sodF*) and genes required for the synthesis of bacillithiol (*bsaA, bshB2*) but also genes of the heat-shock response (*clpC*, *htpG*, *ytvA*, *lonA*). Furthermore, we noticed the up-regulation of genes that mediate resistance against heavy metals (*cadA*, *copA*) or antibiotics (*ycbJ*, *ybxI*). Some of these genes were previously described to be regulated by Spx, but other transcripts were not yet known to be Spx-regulated (Table S4) (Gaidenko *et al.*, 2006; Rochat *et al.*, 2012; Gaballa *et al.*, 2013). Down-regulated transcription units were enriched in genes required for motility, translation or stringent response (see below) and genes of prophages (PBSX- and SPβ-prophages) (Rochat *et al.*, 2012; Molière *et al.*, 2016).

We observed a large overlap when comparing the regulated genes of the Δ*clpX* vs Δ*clpX* Δ*spx* microarray with Spx-regulated genes identified in (Nakano *et al.*, 2003a; Rochat *et al.*, 2012) (Fig. 2A, blue bars denoted 'Spx', 114 out of 378 genes). As expected, we also observed a strong overlap with the regulated genes of the heat-shock arrays (overlap with array 3: 100 genes; array 4: 93 genes, Fig. 2A) and observed a good correlation of up- and down-regulated transcripts between these datasets.

We confirmed the Spx-dependent regulation of selected genes in Δ*clpX* and Δ*clpX*Δ*spx* mutants by northern blotting (Fig. S2). However, since a Δ*clpX* deletion strain displays a considerable growth defect and a pleiotropic phenotype with both Spx dependent and independent regulated traits (Elsholz *et al.*, 2017), we aimed to confirm the Spx-dependent regulation of selected genes in independent experiments by RT-qPCR (Fig. 2B–D). Therefore, we utilized the conditional induction of the stabilized Spx^{DD} variant at 37°C in the absence of stress (Fig. 2B). In a second approach, we treated wild-type or Δ*spx* mutant cells with a mild heat-shock at 50°C for 15 min, which is not yet lethal to the heat-sensitive Δ*spx* mutant strain (Fig. 2C–E). Thereby, we could confirm the Spx-dependent regulation of *trxB*, *hslO* and *ytvA*, as described

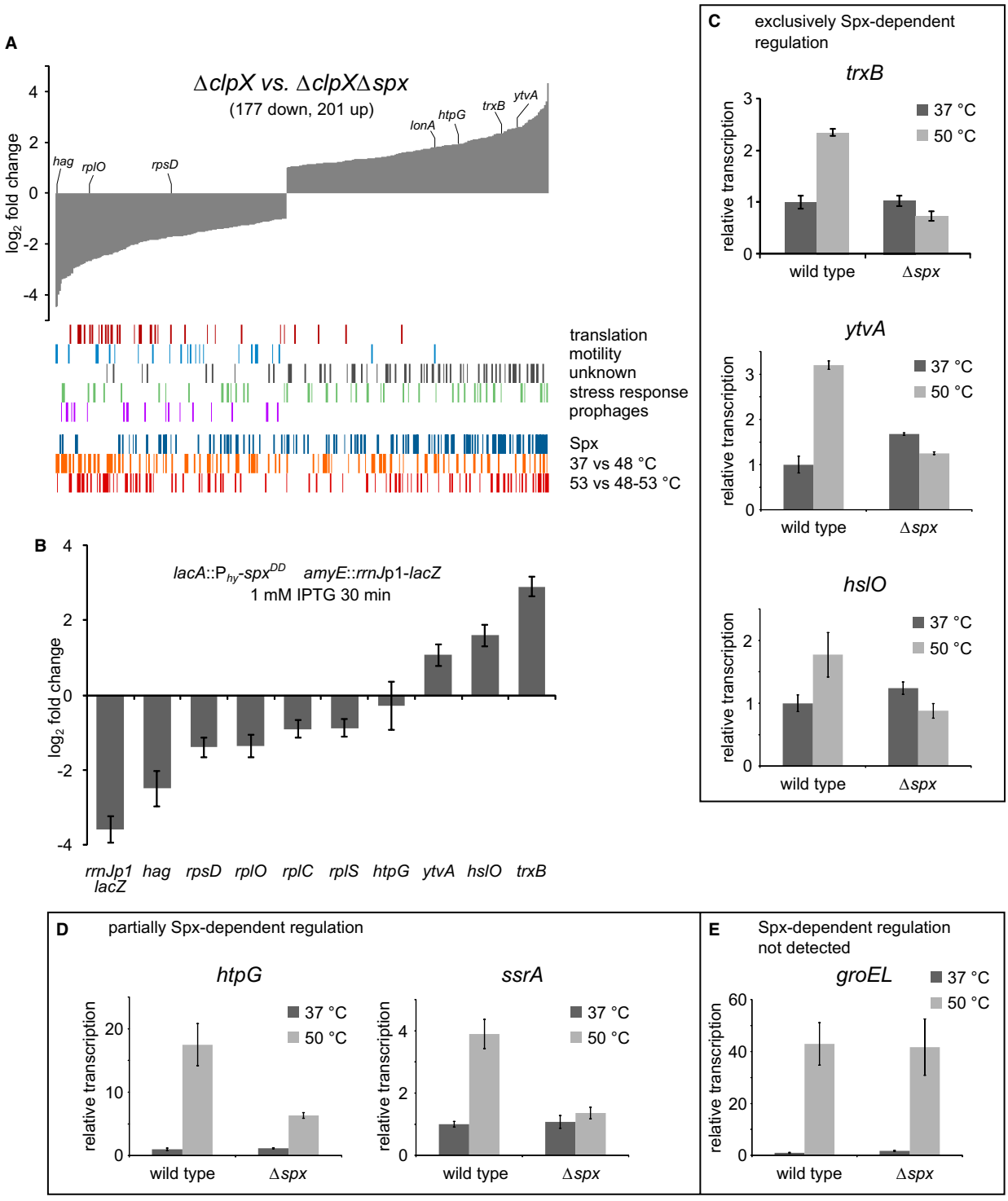

Fig. 2. Transcriptional changes mediated by Spx-accumulation under stress and non-stress conditions.
A. Differentially regulated genes of the Δ*clpX* vs. Δ*clpX*Δ*spx* microarray. Bar tracks indicate the number and distribution of regulated genes of the respective functional group (translation, motility, unknown, stress response, prophages), the overlap with the Spx-regulon (Spx) as defined by (Nakano *et al*., 2003a; Rochat *et al*., 2012) and the overlap with the arrays 3 and 4.
B. Changes in relative transcription of selected targets after treatment of a culture of P_{hy}-spx^{DD} cells (BHS225) with or without 1 mM IPTG for 30 min (to induce Spx^{DD}) as determined by RT-qPCR. Means and standard errors of three biological replicates are shown.
C–E. Relative expression changes after application of heat stress as determined by RT-qPCR. Means and standard errors of three biological replicates are shown. All strains carried *rrnJ* P1 -*lacZ* in the *amyE* site. Log-phase cultures of wild-type *rrnJ* P1-*lacZ* (BHS220) or Δ*spx rrnJ* P1-*lacZ* (BHS222) were divided and incubated at 37°C or 50°C for 15 min, then harvested. [Colour figure can be viewed at wileyonlinelibrary.com]

earlier (Nakano *et al.*, 2003a; Rochat *et al.*, 2012) (Fig. 2B) and observed that their heat-induced expression is completely dependent on Spx (Fig. 2C). Interestingly, *htpG*, encoding a HSP90 homolog, was up-regulated by Spx in the Δ*clpX* vs Δ*clpX* Δ*spx* microarray experiment (Table S4). It was previously observed that Spx in complex with RNAP binds to the promoter region of *htpG*, but no Spx-dependent regulation was detected (Rochat *et al.*, 2012). While an up-regulation of this locus upon expression of Spx^{DD} was not observed (Fig. 2B), we noticed a clear reduction in its heat-induced expression in the Δ*spx B. subtilis* strain (Fig. 2D). These observations suggest a partial, but not exclusive Spx-dependent heat regulation of *htpG* transcription (Schulz and Schumann, 1996; Versteeg *et al.*, 2003). Similar Spx-binding sites were also reported for other heat shock loci, such as *smpB-ssrA* and *groES-groEL* transcription units (Rochat *et al.*, 2012). We could confirm a Spx-dependent induction of the *ssrA* gene transcription at elevated temperature but observed no Spx-dependent heat regulation of the *groEL* locus (Fig. 2D and E). Overall, we observed a strong up-regulation of genes required for stress response and down-regulation of genes active during vegetative growth.

Spx down-regulates ribosomal promoters in vivo

Interestingly, we found the majority of r-proteins to be strongly down-regulated by Spx in the Δ*clpX* vs Δ*clpX* Δ*spx* dataset (26 of 55 CDS down-regulated, with the exception of *rpmEB* being up-regulated, Fig. 3A). In addition, genes encoding subunits of RNAP (*rpoA, rpoC*) or with functions in translation elongation (*lepA*) or secretion (*secY*) were also found to be down-regulated, while genes required for rRNA and tRNA maturation (*trmB, mrnC, cspR*) were up-regulated. The same pattern of strongly down-regulated genes for ribosomal proteins and other translation related genes associated with stringent response could be clearly observed for the thermotolerance array (Array 4 Fig. 1B, Tables S1–S3).

A similar down-regulation of r-proteins and other genes associated with the RelA-dependent stringent response (Eymann *et al.*, 2002) was observed for *B. subtilis* cells exposed to various stress conditions where Spx could be activated and involved in the response (Leichert *et al.*, 2003; Mostertz *et al.*, 2004; Rochat *et al.*, 2012). Interestingly, the additional ChIP–chip experiments by

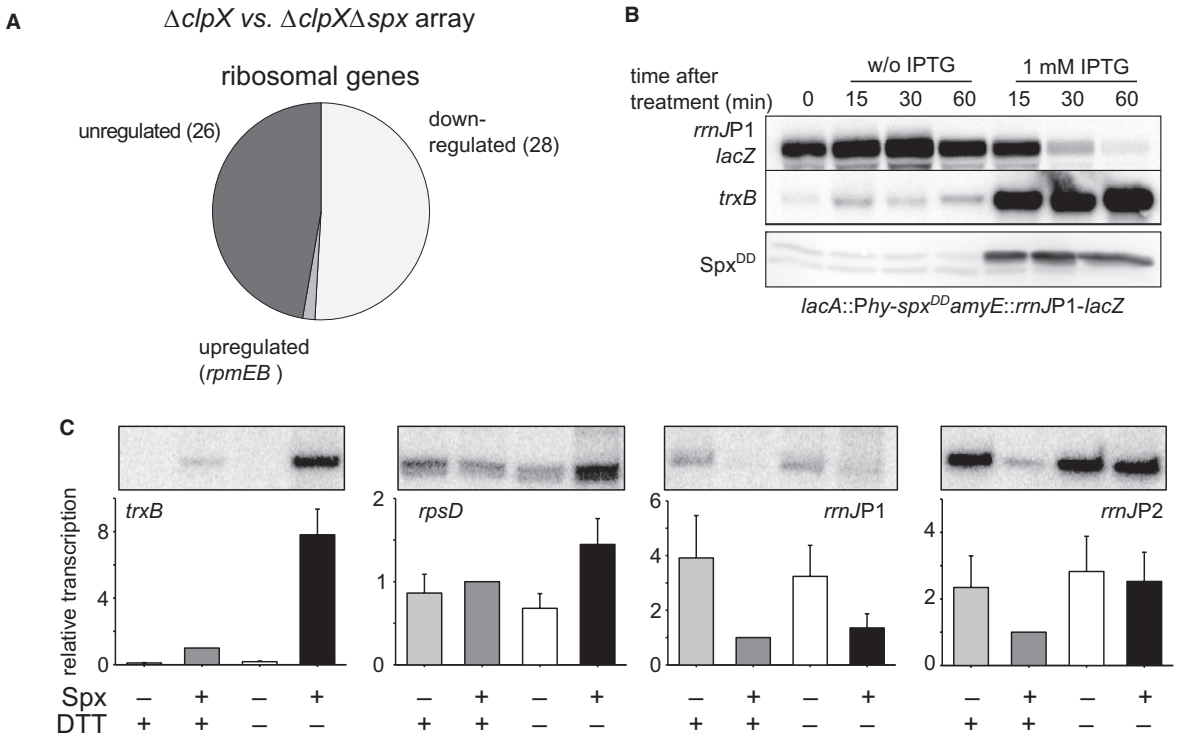

Fig. 3. Spx down-regulates transcription of ribosomal promoters *in vivo* and *in vitro*.
A. The fraction of ribosomal genes differentially regulated by Spx in the Δ*clpX* vs. Δ*clpX*Δ*spx* microarray.
B. Northern blots (*rrnJ* P1 -*lacZ*, *trxB*) and western blot (α-Spx). A mid-log culture (OD_{600} of 0.3–0.35) of BHS225 cells was divided and treated with or without 1 mM IPTG. Samples were withdrawn at the time points indicated and 2 μg total RNA or 10 μg total protein per lane were subjected to northern or western blotting.
C. *In vitro* transcription assays with *B. subtilis* RNA polymerase with or without Spx and/or dithiothreitol (DTT). The transcription rate with Spx and DTT was set to 1. Means and standard deviations of four replicates and a representative experiment are shown.

Rochat *et al.* suggest that the complex of Spx and RNAP specifically interacts with binding sites in front of the promotor regions of the *rrn* operons and other genes important for translation (such as, e.g. *rplC*, *secY*, *ssrA*, *ffh*) (Rochat *et al.*, 2012).

These observations strongly suggested that Spx may contribute to regulation of these genes. Therefore, we went on to investigate in more detail the possible role of Spx in the repression of rRNA and other translation-related genes.

In order to assess the effect of Spx on rRNA expression, we combined an array of strains carrying transcriptional *rrn-gfp* fusions in the *amyE* site (Rosenberg *et al.*, 2012) with an IPTG-inducible copy of spx^{DD} in the *lacA* site (Fig. S3A). Northern blot experiments revealed that the transcription from all tested promoters was repressed by the induction of spx^{DD} with 1 mM IPTG for 30 min (Fig. S4). Subsequently, we selected the *rrnJ-rrnW* operon as our model system. We constructed a transcriptional fusion of a 132 bp fragment carrying *rrnJ* P1, the first of the two promoters of the *rrnJ-rrnW* transcription unit (Koga *et al.*, 2006; Natori *et al.*, 2009), to the *lacZ* reporter gene (Fig. S3B, see Materials and Methods). Northern blot experiments revealed that the activity of the *rrnJ* P1 promoter was strongly decreased shortly after the induction of Spx^{DD} by the addition of IPTG. As expected, the addition of IPTG also resulted in a strong increase in the *trxB* control mRNA and an accumulation of the Spx protein, as revealed by western blotting (Fig. 3B). RT-qPCR experiments using the same strain confirmed that, after 30 min of treatment with IPTG, the transcription of the *lacZ* gene from the *rrnJ* P1 promoter and the selected transcripts of rRNA genes were strongly down-regulated. (Fig. 2B). Taken together, these results suggest that the observed down-regulation of ribosomal genes can be directly or indirectly caused by the activity of Spx.

Spx down-regulates rrnJ P1 in vitro

In order to confirm the observed *in vivo* down-regulation of the *rrnJ* P1 promoter and to assess whether this inhibition can be directly caused by Spx or requires another factor, *in vitro* transcription experiments using *B. subtilis* RNAP were carried out with either reduced (+DTT) or oxidized (–DTT) Spx (Fig. 3C). The results demonstrated that transcription from *rrnJ* P1 was inhibited upon Spx addition regardless of its oxidation state. Interestingly, transcription of *rrnJ* P2 was inhibited only by the reduced (+DTT) but not by oxidized Spx (–DTT). Transcription from the *rpsD* promoter was not strongly affected by Spx while transcription of the *trxB* promoter was significantly stimulated by oxidized Spx, as described previously (Nakano *et al.*, 2005; Rochat *et al.*, 2012). From these experiments, we conclude that Spx can directly act on the ribosomal promoters *rrnJ* P1 and *rrnJ* P2 and inhibit their transcription without the need for additional factors.

Spx-RpoA interaction and the influence of specific upstream sequences (UP-elements) are crucial for Spx-dependent regulation of the RNAP

The promoters of the *rrn* operons are well-conserved but do not contain the Spx binding motif (-45-AGCA-42) (Helmann, 1995; Reyes and Zuber, 2008; Nakano *et al.*, 2010; Rochat *et al.*, 2012). However, transcription from the *rrn*-promoters in *B. subtilis* is stimulated by contacts of α-CTD with AT-rich upstream sequences (UP-elements) (Ross *et al.*, 1993; Rao *et al.*, 1994; Krásný and Gourse, 2004; Murayama *et al.*, 2015). As the transcriptional regulation by Spx depends on its interaction with α-CTD, it was suggested that the Spx/α-CTD complex could recognize promoters by interaction with sequence motifs associated with the UP-element upstream of the core promoter (Reyes and Zuber, 2008; Nakano *et al.*, 2010). Therefore, we hypothesized that regulatory upstream sequence motifs associated with the UP-elements of *rrn*-promoters could also be required for the observed Spx-dependent transcriptional down-regulation.

To test whether there are any sequence-specific determinants for Spx-dependent repression within the upstream regulatory elements, we constructed a series of transcriptional promoter-*lacZ* fusions, each comprising the 41 bp *rrnJ* P1 core promoter and upstream sequences of variable-length. In addition, a 22 nt GC-rich sequence that is not recognized by α-CTD (denoted 'SUB' sequence, (Rao *et al.*, 1994)) and a 22 nt sequence upstream of the P_{trxB} promoter were fused to the same core promoter sequence as well (Fig. 4A). The influence of Spx^{DD} synthesis, induced *in trans*, on the *in vivo* transcription from these promoters was assessed by northern blotting and RT-qPCR (Fig. 4B and C).

In the absence of the inducer IPTG, the shorter fragments (61 nt or 41 nt) with truncated upstream elements showed a decreased promoter activity compared to the 110 nt fragment with the longest upstream region and the fusion of the SUB sequence further diminished the promoter activity (Fig. 4B and C), consistent with previous reports (Rao *et al.*, 1994; Krásný and Gourse, 2004). Upon induction of Spx^{DD}, the activity of all promoter fragments was decreased, but the magnitude of repression differed between the tested fragments. For the 110 nt fragment, the promoter activity decreased about 6–7 times, whereas for the 61 nt- or 41 nt fragments, the reduction of promoter activity was about 4–6 and 2–3 times respectively. This deletion analysis suggested a direct influence of the upstream elements on the Spx-dependent down-regulation.

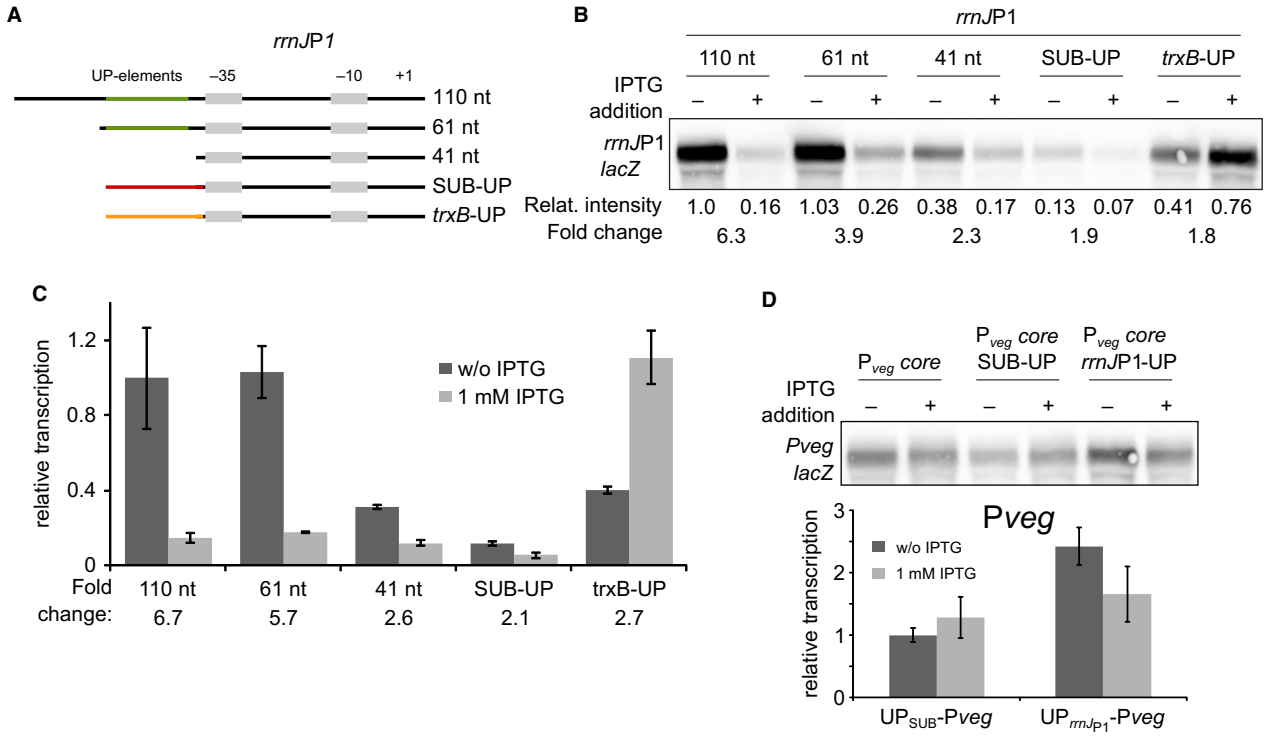

Fig. 4. UP elements are important for Spx-mediated up- and down-regulation.
A. Schematic drawing of the different *rrnJ* P1 variants with truncated or replaced upstream sequences.
B. Northern blot of BHS807 (110 nt), BHS516 (61 nt), BHS517 (41 nt), BHS601 (SUB-UP) or BHS602 (*trxB*-UP) cells treated with or without 1 mM IPTG for 30 min to induce Spx^{DD}. Relative band intensities were calculated using ImageJ, Ratios indicate the absolute value of fold change.
C. Relative transcription of *lacZ* in a similar but independent experiment as panel B. Means and standard errors of two biological replicates are shown. Ratios indicate the absolute value of fold change.
D. Northern blot of BHS573 (P*veg-lacZ*) BHS668 (SUB-P*veg-lacZ*) and BHS669 (*rrnJ* P1 UP-P*veg-lacZ*) cells treated with or without 1 mM IPTG for 30 min and relative transcription of *lacZ* in a similar but independent experiment as determined by RT-qPCR. Means and standard errors of two biological replicates are shown. [Colour figure can be viewed at wileyonlinelibrary.com]

Replacement of the upstream elements with the SUB sequence, which is not acting as an UP-element (Rao *et al.*, 1994), significantly decreased the promoter activity. However, the promoter was still repressed about two times when Spx^{DD} was synthesized *in trans* (Fig. 4B and C). This suggested that features of the core promoter might also influence the Spx-mediated down-regulation. Another possibility could be that Spx can recognize sequences within the GC-rich SUB element. However, when we fused the upstream sequences of P_{trxB} that carries known binding sites for Spx (Reyes and Zuber, 2008; Nakano *et al.*, 2010), the basal activity of the *rrnJ* P1 core promoter was significantly increased about three times and the transcript level was further raised about 2–3 times upon induction of Spx^{DD} synthesis (Fig. 4B and C), emphasizing the role of the upstream sequences for alpha-subunit-dependent activation together with Spx.

To confirm our findings, we fused the upstream elements of *rrnJ* P1 as well as the SUB sequence to the P_{veg} promoter. This promoter was previously shown to be constitutively active (Fukushima *et al.*, 2003; Sojka *et al.*, 2011; Nicolas *et al.*, 2012; Radeck *et al.*, 2013). The activity of the P_{veg} core promoter alone and the promoter with the SUB element was unchanged upon induction of Spx^{DD} synthesis (Fig. 4D). However, when the upstream sequence of the *rrnJ* P1 promoter was fused to the P_{veg} core promoter, a higher transcript level was observed, consistent with the finding that these upstream elements, containing the UP-elements, can stimulate promoter activity. Importantly, this promoter construct was sensitive to Spx and was down-regulated upon induction of Spx^{DD} synthesis, although to a lesser extent than observed for the *rrnJ* P1 promoter (Fig. 4B and C). We also confirmed by RT-qPCR measurements that the transcription of P_{veg} fused to the SUB element was not regulated by Spx^{DD} induction while P_{veg} fused to the UP-element of *rrnJ* P1 was down-regulated by Spx^{DD} (Fig. 4D).

Taken together, these experiments indicate that sequence elements upstream of the core promoter are a key factor for both positive and negative modulation of promoter activity by Spx in conjunction with the alpha

subunit of RNAP. Nevertheless, the properties of the *rrnJ* P1 core promoter also appear to influence the Spx-dependent down-regulation.

The positive and negative influence of Spx on transcription can be separated by a mutation in the alpha subunit of the RNAP

To gain insights into the mechanism of the Spx^{DD}-dependent down-regulation of *rrnJ* P1 we introduced the previously described point mutants *rpoA*Y263C or *rpoA*V260A (*cxs*-1 or *cxs*-2) into the *rpoA* gene of the *rrnJ* P1-*lacZ* reporter strain. Both mutations change the interaction surface of α-CTD with Spx and suppress the detrimental effects of Spx accumulation on growth by disturbing the interaction α-CTD-Spx required for the activity of Spx (Nakano *et al.*, 2000; Nakano *et al.*, 2003b). Northern blot experiments revealed that the negative regulatory impact of Spx^{DD} induction is completely suppressed in the *cxs*-1 background and almost completely suppressed in the *cxs*-2 background (Fig. 5A). This finding is in agreement with the previous reports that both mutations suppress any activity of Spx on gene expression (Nakano *et al.*, 2000; Nakano *et al.*, 2003b). Interestingly, only the *rpoA*Y263C (*cxs*-1) variant also abolished transcription of *trxB*. In contrast, *trxB* transcription was still activated by Spx^{DD} induction similarly to the wild-type in cells carrying the *rpoA*V260A (*cxs*-2) variant (Fig. 5A).

These experiments support the hypothesis that the tested ribosomal promoter is directly down-regulated by a repressor activity of Spx while interacting with the α-subunit. This activity is distinct from its previously described functions for (1) transcriptional repression by interfering with transcriptional activators (Nakano *et al.*, 2003b) or its second observed function as (2) direct activator of the thiol stress response (Nakano *et al.*, 2003a). Since both mutations (*cxs*-1 and 2) also restore growth (Fig. S6B) and viability of cells carrying P_{hy}-spx^{DD} on agar plates supplemented with IPTG (Fig. 5B), we assume that the inhibitory effects of Spx accumulation on growth could be caused rather by the depletion of rRNA than by Spx-dependent induction of the synthesis of a toxic factor, such as a toxin-antitoxin system.

Down-regulation of rrnJ P1 and r-proteins can occur in vivo in the absence of Spx

Next, we wished to determine to what extent Spx contributes to downregulation of rRNA genes during heat stress. Therefore, we analysed the transcriptional response of *rrnJ* P1 to heat stress in wild-type and Δ*spx* cells (Fig. 6A). Upon a temperature upshift from 37 to 50°C we noticed a pronounced down-regulation of *rrnJ* P1. Surprisingly, a comparable downshift was observed also in the Δ*spx* mutant. We observed this Spx-independent down-regulation of rRNA genes also during oxidative and cell wall stress (Fig. S5A and C).

Hence, we asked whether another protein might function similarly to, and substitute for, Spx in the cell. Indeed, *B. subtilis* possesses a paralog of Spx, MgsR, which modulates the expression of a sub-regulon of the general stress response controlled by σ^B. Given the high sequence similarity between MgsR and Spx, MgsR may have a similar negative regulatory activity on ribosomal promoters as observed for Spx and might be able to complement a deletion of *spx* in this regard. To test this hypothesis, we constructed and assessed the influence of Δ*mgsR* and Δ*spx* Δ*mgsR* deletion strains on heat-mediated *rrnJ* P1 transcriptional down regulation. However, despite a slightly increased transcription rate during non-stress conditions in strains with the Δ*mgsR* background, we still observed the down-regulation of *rrnJ* P1 and r-protein genes in the double deletion strain during heat stress (Fig. S5B).

Thus, we confirmed the transcriptional down-regulation of rRNA and r-proteins during severe stress conditions. However, a deletion of *spx* and/or its paralog *mgsR* did not significantly affect this down-regulation, suggesting that the Spx-dependent downregulation of rRNA expression may play a role under different conditions.

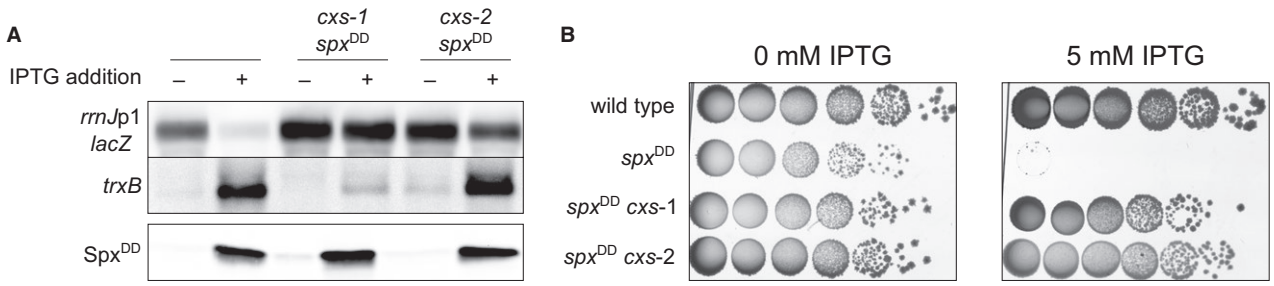

Fig. 5. The impact of *cxs* mutations in *rpoA* on Spx-mediated transcriptional regulation.
A. Northern blot (*rrnJ* P1-*lacZ, trxB*) and western blot (α-Spx) of BHS225, BHS729 and BHS730 cells. Mid-log cultures were divided and treated with or without 1 mM IPTG for 30 min. 2 µg total RNA or 10 µg protein per lane were analysed.
B. Serial dilutions of *B. subtilis* wild type, BHS225, BHS729 or BHS730 cells spotted on agar plates without (left) or with 5 mM IPTG (right).

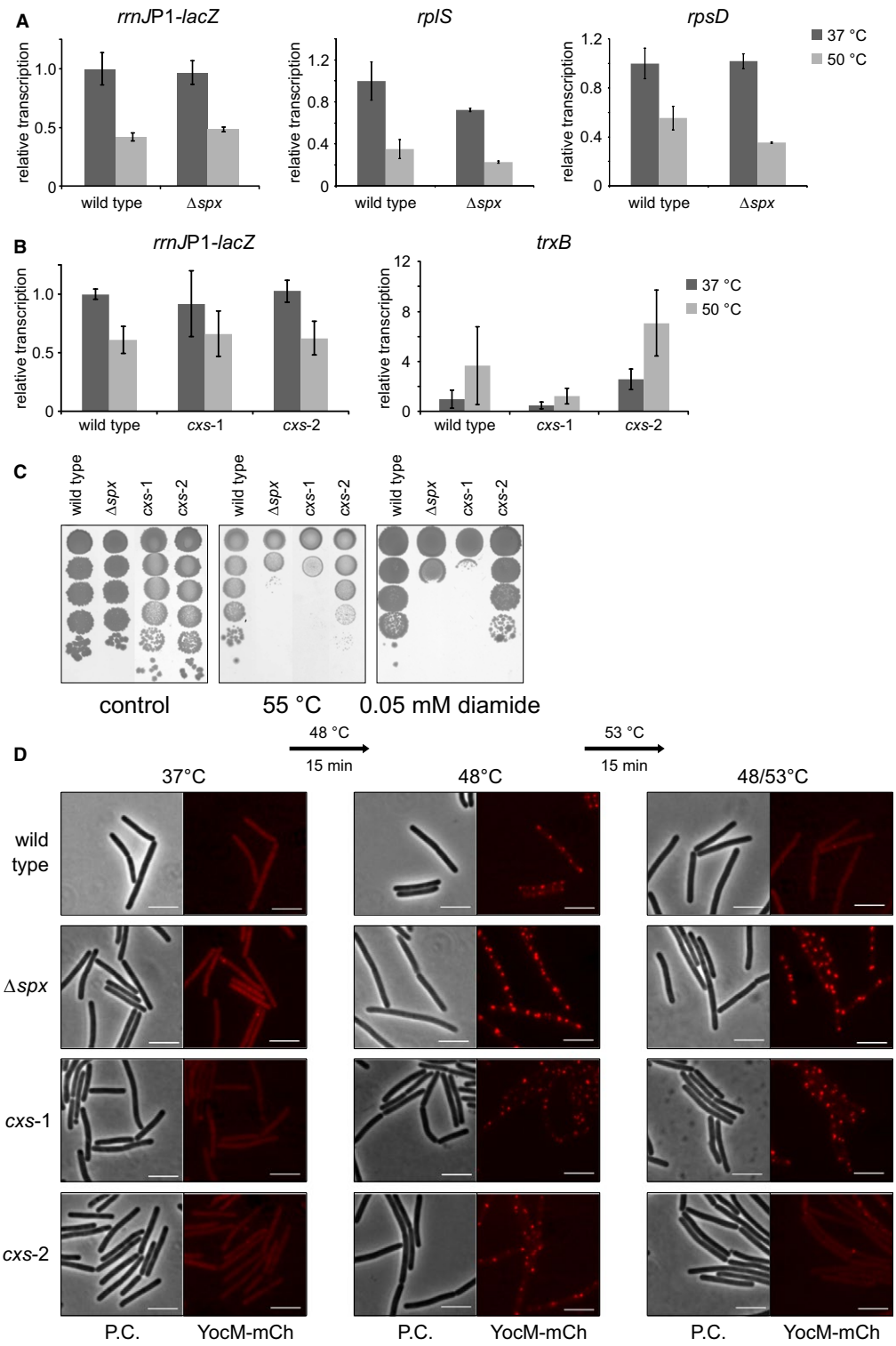

Fig. 6. Up-regulation of stress response-genes but not down-regulation of rRNA contributes to survival of stress.
A, B. Relative expression changes after application of heat stress as determined by RT-qPCR. Means and standard errors of three biological replicates are shown. All strains carried *rrnJ* P1-*lacZ* in the *amyE* site. Log-phase cultures of BHS220 (wild-type *rrnJ* P1-*lacZ*), BHS222 (Δ*spx*), BHS549 (*cxs*-1) and BHS550 (*cxs*-2) were divided and incubated at 37°C or 50°C for 15 min, then harvested.
C. Growth of wild type, Δ*spx* (BNM111), *cxs*-1 (BHS475) or *cxs*-2 cells (BHS476) on agar plates incubated ON at 37°C, 55°C or supplemented with 0.05 mM diamide and incubated at 37°C.
D. Subcellular protein aggregation of wild type, Δ*spx*, *cxs*-1 or *cxs*-2 cells carrying a YocM-mCherry fusion after heat shock. Scale bars are 5 μm. Phase contrast images (P.C.) and fluorescence images with RFP-filters (YocM-mCherry) are shown. [Colour figure can be viewed at wileyonlinelibrary.com]

Up-regulation of stress-response genes and not down-regulation of rRNA is the important activity for Spx-mediated thermoresistance in vivo

As Spx appeared to be dispensable for the stress-mediated down-regulation of rRNA genes, we explored the roles of the *cxs*-1 (Y263C) and *cxs*-2 (V260A) mutants in stress resistance and survival. We observed the strong down-regulation of *rrnJ* P1 upon heat shock, independent of Spx and regardless of the *rpoA* point mutations in the strain background (Fig. 6A and B). However, in line with the observation from Fig. 5A, the heat-induced transcription of *trxB* was abolished in the *cxs*-1 mutant (Y263C), but not in the *cxs*-2 mutant (V260A). We assayed the growth of wild-type and *rpoA* mutant cells on agar plates incubated at high temperatures or supplemented with diamide, a strong oxidizing agent (Fig. 6C). Both the Δ*spx* and the *cxs*-1 mutant exhibited a high sensitivity to both stress conditions (Nakano *et al*., 2003a). However, the strain carrying the *cxs*-2 mutation, which still allows the upregulation of the thiol stress response, displayed only slightly reduced growth compared to the wild type under both stress conditions.

Previously, we reported that Spx protects the cells from heat-induced protein aggregates that can be visualized by fluorescence microscopy (Runde *et al*., 2014). We developed a tool utilizing *yocM*, a member of the small heat shock proteins in *B. subtilis*, fused to the fluorescent mCherry protein, that localizes to and thus visualizes protein aggregates *in vivo* as similarly demonstrated with the sHsp-GFP fusion from *E. coli* (Lindner *et al*., 2008; Runde *et al*., 2014; Hantke *et al*., 2018). Upon the non-lethal preshock, some protein aggregates were visible as fluorescent foci at the cell poles in all strains, that disappeared during prolonged heat exposure in primed wild-type cells, but not in the Δ*spx* mutant (Fig. 6C) (Runde *et al*., 2014). Cells carrying the *cxs*-1 mutation displayed a severe protein aggregation phenotype, similar to the Δ*spx* mutant in accordance with the other observations, while cells with the *cxs*-2 mutation accumulated only slightly more protein aggregates than the wild type (Fig. 6C).

From these observations, we infer that up-regulation of stress response-genes is a crucial activity for the protective role of Spx during stress conditions and that a Spx-mediated down-regulation of rRNA is either dispensable under the tested conditions or can be complemented by other redundant different stress response mechanisms active under these conditions.

Spx-levels exhibit heterogeneity during outgrowth from the stationary phase

Finally, the possible different conditions, mentioned in the preceding paragraphs, must entail a high cellular level of Spx as the Spx-dependent inhibition of rRNA promoters was observed either upon overexpression of stabilized Spx^{DD} or in a strain where Spx was stabilized (Δ*clpX*). To identify and search for such conditions where Spx might accumulate either in all cells or in a subpopulation of cells, we investigated the cellular Spx levels during growth and stress response in a population of cells on a single-cell level. For these experiments, we utilized a recently constructed *B. subtilis* strain carrying a translational GFP-Spx fusion that retains the native transcriptional and post-translational regulation (Riley *et al*., 2018).

We observed that upon heat or cell wall stress all the cells of an exponentially growing cell population synthesized the GFP-Spx fusion in high amounts with a broad but unimodal distribution. We also examined the different growth phases and observed for late stationary phase cells from an over-night culture that a significant number of the population displayed bright fluorescence (Fig. 7). The establishment of the Spx positive subpopulation took place late in stationary phase as Spx positive cells started to appear after 540 min of incubation (Fig. S7). This sub-population of cells with elevated Spx concentration diminished when exponential growth was resumed (time points 90, 120, 180 min in Fig. S7) after dilution into fresh medium (Figs 7 and S7). The increased fluorescence of the subpopulation was not caused by differential autofluorescence of the stationary phase cells (Fig. S7 wild-type cells lacking GFP (PY97)). Furthermore, when we correlated the cell size with the fluorescence signal we observed a strong correlation between small cell sizes, which are typical for non-growing stationary phase cells, and high levels of Spx (Fig. S7). These results suggest that Spx may contribute to the growth arrest of this distinct subpopulation, possibly also by downregulating rRNA expression. This observed heterogeneity of Spx levels in stationary phase cells could represent an additional role of Spx during outgrowth from stationary phase, where the inhibition of growth activity by Spx in these cells might play a role establishing a persister-like phenotype and support the survival of the cell population when confronted with antibiotics or environmental stress (Dubnau and Losick, 2006; Veening *et al*., 2008; Fridman *et al*., 2014).

Discussion

We analysed the transcriptome of *B. subtilis* cells during thermotolerance development and observed that Spx, a transcription factor interacting with the alpha-subunit of the RNA polymerase, is a central player orchestrating heat shock response. We characterized a distinct activity of Spx to inhibit transcription from specific promoters of genes related to translation. The concurrent

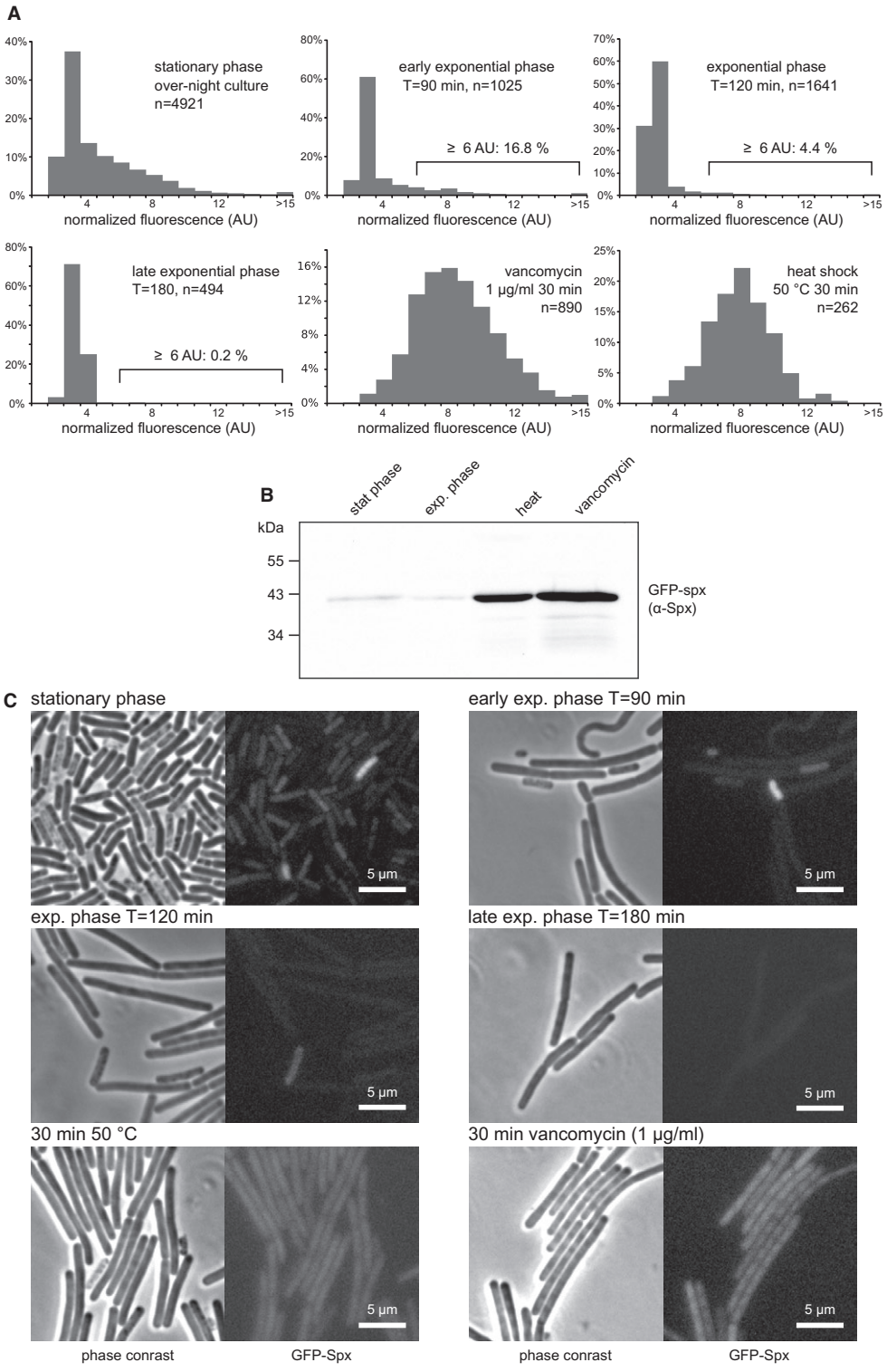

Fig. 7. Spx-activity displays heterogeneity during outgrowth from the stationary phase.
A. Histograms showing the distribution of GFP-Spx fluorescence among N single cells from different growth phases and indicated treatments.
B. Relative levels of GFP-Spx as determined by western blotting.
C. Representative images showing heterogeneous levels of GFP-Spx
in different growth phases or treatments. Scale bars are 5 µm. Phase contrast images (P.C.) and fluorescence images with GFP-filters (GFP-spx) are shown.

downregulation of translation as part of a protein folding stress response would result in a reduction in the metabolic burden accompanying protein repair mediated by the upregulated chaperone systems. However, only the concurrent up-regulation activity of stress genes by Spx is essential for mediating stress response and in a *spx* deletion mutant strain the inhibitory activity could be compensated *in vivo* by other differently acting cellular stress response processes. Nevertheless, the impact of Spx on limiting translation might become important under different conditions.

Thermotolerance development as a concerted process involving multiple regulators

The heat shock response in *B. subtilis* has been divided into different classes, which depend on the activity of different transcription factors such as SigB, and the repressors CtsR and HrcA (Hecker *et al.*, 1996). Recently, Spx the transcription factor controlling thiol stress response was also identified as a heat stress sensing transcription factor (Runde *et al.*, 2014). Our results (Figs 1 and 2, Tables S1, S2 and S4) and other studies suggest that Spx controls or influences the expression of heat shock proteins such as ClpC, LonA and HtpG as well as tmRNA, general stress proteins (YtkL, YraA, GabD, YfhF, YvgN) or oxidative stress response (TrxA, TrxB) (Nakano *et al.*, 2003a; Rochat *et al.*, 2012; Runde *et al.*, 2014). It further indicates that there exists a considerable overlap between the Spx regulon and the heat shock regulons controlled by CtsR and SigB (Figs 1 and 2). However, although bindings sites for Spx near the promoters of the HrcA-controlled chaperone systems *dnaKJE* and *groESL* were reported, neither we nor others could detect an influence of Spx on the expression of these transcription units in response to heat- (Fig. 2) or oxidative stress (Rochat *et al.*, 2012).

Spx-mediated down-regulation of ribosomal promoters

We showed that Spx down-regulates promoters that initiate transcription of rRNA and, to a lesser extent, promoters of ribosomal proteins (Figs 2A and B and 3A and B). This activity could be reconstituted *in vitro* and appears to be partly dependent on the state of the CXXC redox-switch of Spx in a promoter-specific manner (Fig. 3C). The inhibitory effect of Spx on *rrnJ* P1 could be reduced but not completely abolished by substitution of the UP element with a mock sequence (Fig. 4) and reconstituted on an unregulated promoter P_{veg} by fusing the UP element of a regulated promoter (Fig. 4D).

Spx was first described as an 'anti-alpha' factor as it could displace certain transcription factors and thereby interfere with their activity without sequence-specific requirements to the promoter (Nakano *et al.*, 2003b). Later it was shown that Spx also has a stimulatory activity on certain promoters, which requires interactions with an upstream sequence motif and is thought to re-position the RNAP for better promoter-recognition (Nakano *et al.*, 2003a; Reyes and Zuber, 2008; Nakano *et al.*, 2010).

As Spx binds close to the surface of α-CTD, which contacts the upstream UP elements (Gaal *et al.*, 1996; Zhang *et al.*, 2006; Birch *et al.*, 2017), it could act by influencing the recognition and productive interactions of α-CTD with the specific upstream sequences. The strong transcription of rRNA promoters is also dependent on these UP-elements (Fig. 4B and C) (Ross *et al.*, 1993; Rao *et al.*, 1994). Therefore, accumulation of Spx could lead to a down-regulation of the respective transcription units in a sequence-independent manner similar to the previously observed 'anti-alpha' activity. A specific interaction of Spx with the alpha subunit appears to be very important for the observed repressor activity of Spx, since both *rpoA* mutations *cxs*-1 and *cxs*-2 result in the alleviation of down-regulation of *rrnJ* P1 while cxs-2 can still upregulate the transcription of e.g. *trxB*.

Interestingly, SoxS, the activator of the superoxide stress response in *E. coli*, which shares no homology to Spx, can modulate RNAP holoenzyme activity with a 'pre-recruitment' mechanism by binding the DNA-recognition surface of α-CTD. SoxS then redirects RNAP to promoters that feature a degenerate 'soxbox' upstream of or within the – 35 promoter element (Griffith *et al.*, 2002; Martin *et al.*, 2002). Concurrently, binding of SoxS to α-CTD renders the complex unable to interact with UP elements, thereby decreasing the strong transcription of the *rrnB* P1 promoter (Shah and Wolf, 2004).

Nevertheless, the interaction and architecture of Spx/α-CTD is different from SoxS, which reprograms the up-element recognition and is therefore not directly applicable for Spx-mediated promoter regulation (Browning and Busby, 2016). A simple model in which Spx interferes like SoxS with the recognition of UP elements by α-CTD might not explain different promoter-specific *in vitro* down-regulation of oxidized or reduced form of Spx on *rrnJ* P1 and *rrnJ* P2 (Fig. 3C) or residual regulation of UP_{SUB}-*rrnJ* P1 *in vivo* (Fig. 4B and C). However, it was demonstrated that the Spx-mediated up-regulation and the sequence specific recognition and binding to promoters for different thiol-stress or redox chaperone genes is controlled by the redox state of the CXXC switch (Nakano *et al.*, 2003a; Reyes and Zuber, 2008; Nakano *et al.*, 2010; Lin *et al.*, 2013). Therefore, it appears that Spx regulates these promoters by a not yet understood mechanism, which might require sequence-specific contact of the Spx-RNAP complex with sequences upstream of or within the core promoter as observed for the up-regulated

promoters. This hypothesis is supported by the observation of Spx-RNAP binding sites in close proximity of *rrn* promoters (Rochat *et al.*, 2012).

In addition, it was recently observed that Spx interacts not only with α but also β or β′ subunits of RNAP (Birch *et al.*, 2017). Such a more complex interaction or different binding mode of Spx and RNAP might also contribute to the inhibition of transcription at the *rrnJ* P1 promoter. However, mechanistic details are not yet known, and more experiments are required to fully understand the different impacts which Spx can have on RNAP activity at these different promoters. Our results reveal that the ability of the unusual transcription factor Spx to influence the RNAP activity, especially via the alpha subunit is much more versatile than expected.

It should be emphasized that the experiments employing induction of Spx^{DD} synthesis were carried out in the absence of stress. Therefore, we assume that the CxxC redox switch of Spx remains in its reduced state (Nakano *et al.*, 2005; Rojas-Tapias and Helmann, 2018a). Interestingly, we observed that both the reduced and oxidized form of Spx were able to down-regulate transcription of *rrnJ* P1, while only the reduced state of Spx could down-regulate transcription of *rrnJ* P2 (Fig. 3C). These experiments suggest that the regulatory activity of Spx on *rrn* promoters might be particularly required during adverse conditions that do not involve oxidative stress.

Significance of down-regulation of ribosomal promoters for stress resistance and survival

During heat and oxidative stress, we and others observed a pronounced down-regulation of genes involved in transcription, translation and protein secretion (Figs 1B and C and 4) (Helmann *et al.*, 2001; Price *et al.*, 2001; Leichert *et al.*, 2003; Mostertz *et al.*, 2004; Chi *et al.*, 2011; Rochat *et al.*, 2012). We showed that Spx down-regulates the respective promoters during non-stress conditions. However, we also observed that this down-regulation occured independently of Spx during heat stress (Fig. 6A and B). Therefore, we conclude that other redundant regulatory stress responsive mechanisms must exist, that mediate the strong repression of these genes during heat or oxidative stress.

Moreover, a point mutation in *rpoA* conferring loss of Spx-mediated repression of *rrn* transcription did not affect survival or protein aggregation during heat stress (Fig. 6C and D). Thus, the up-regulation of genes of the heat and oxidative stress response (Fig. 2) appears to be the crucial activity of Spx for survival and stress resistance, while the negative impact on *rrn* transcription is dispensable for survival (Fig. 6C and D).

We believe that the herein described additional activity of Spx may impose benefits for the *B. subtilis* cell population under stress. Since the majority of total RNA synthesized during fast growth is rRNA, Spx might actively withdraw RNAP holoenzyme from the transcription of rRNA and concurrently re-deploy the complex to promoters of stress-related genes. This strategy could ensure reallocation of RNAP for a fast and efficient transcription of stress response genes during emerging stress. Furthermore, regulation of *rrn* transcription by Spx may become essential under environmental conditions not tested in this work. Finally, down-regulation of rRNA ultimately leads to a depletion of active ribosomes and the total translation capacity which diminishes the burden for protein quality control and reduces the cell growth. This would also provide tolerance to antibiotics and proteotoxic stress conditions.

An important role of Spx in antibiotic resistance especially against antibiotics interfering with cell-wall biosynthesis is already well-established (Luo and Helmann, 2012; Rojas-Tapias and Helmann, 2018a; 2018b) and we could clearly confirm the induction of Spx by vancomycin (Figs 7 and S5C). *B. subtilis* strains where Spx is stabilized due to mutations in *clpX*, *clpP* or *yjbH* are impaired in growth and a longer lag phase after inoculation can be observed. This growth impairment can be relieved by suppressor mutants appearing in *rpoA* (Fig. S6) (Nakano *et al.*, 2000; 2003a; Molière *et al.*, 2016). The high Spx concentration observed in the heterogenous population of stationary phase cells could also contribute to the growth inhibition of this subpopulation, most likely resulting in a longer lag phase for this sub-population. This is reminiscent of the antibiotic tolerance in type 1 persister cell formation, which could also be influenced by a variation of the lag-phase during outgrowth from the stationary phase (Balaban *et al.*, 2004; Fridman *et al.*, 2014). It is possible that in *B. subtilis* cell populations Spx is involved in two different processes conferring antibiotic tolerance. First through the Spx mediated stress response signalled by cell-wall stress through application of antibiotics like vancomycin, which affect cell-wall synthesis. Thereby the Spx-mediated upregulation of, e.g., redox-stress response genes might also enable a generally improved stress resistance. In a second process, a higher Spx level of a sub-population of stationary phase cells might facilitate possible persister-cell-like behaviour, which might also contribute to a raised antibiotic resistance of *B. subtilis* cells.

Redundancy in stress signal sensing, transduction and subsequent gene regulation would allow a much more robust cellular stress response. Therefore, we hypothesize, that the negative regulatory activity of Spx on *rrn* promoters may be complemented or superseded by additional stress response systems under these

conditions (Fig. 4A). In previous studies, the observed down-regulation of ribosomal RNA and proteins was frequently attributed to the (p)ppGpp-mediated stringent response. However, despite a considerable number of reports on this topic for *B. subtilis* and other organisms (Hecker *et al.*, 1989; Yang and Ishiguro, 2003; Abranches *et al.*, 2009; Fitzsimmons *et al.*, 2018), persuasive evidence for these hypotheses is scarce and molecular details on the regulation of stress-mediated (p)ppGpp-synthesis is lacking. However, the second messenger-based stringent response could act relatively fast on the protein level by shutting down translation and protecting ribosomes (Beckert *et al.*, 2017). This fast response could very well be complemented by down-regulation of the transcription of translation-related genes through (p)ppGpp induced changes in the GTP concentration (Krásný and Gourse, 2004; Kriel *et al.*, 2012) together with the here described Spx activity. A possible role of (p)ppGpp as a messenger during heat and other stress conditions in *B. subtilis* will have to be addressed in future studies.

Experimental procedures

Growth media and thermotolerance

B. subtilis strains were grown in a water bath at 37°C with orbital shaking at 200 rpm in Lysogeny Broth LB medium (5 g l^{-1} yeast extract, 10 g l^{-1} tryptone-peptone, 10 g l^{-1} NaCl). Belitzky minimal medium (Stülke *et al.*, 1993) supplemented with 0.05% yeast extract was used for experiments where diamide was added to the medium. Thermotolerance development and survival assays were performed as described previously (Runde *et al.*, 2014).

Cloning and strain construction

PCR-amplification using Phusion® high-fidelity polymerase (NEB), cloning using *E. coli* DH5α and transformation of *B. subtilis* 168 was carried out according to standard methods (Spizizen, 1958; Inoue *et al.*, 1990; Sambrook and Russell, 2001). All utilized primers are listed in Table S7. Transformants were selected on agar plates supplemented with 100 µg ml^{-1} ampicillin, 5 µg ml^{-1} chloramphenicol, 1 µg ml^{-1} erythromycin and 25 µg ml^{-1} lincomycin, 10 µg ml^{-1} kanamycin or 100 µg ml^{-1} spectinomycin when appropriate. Correct insertion of integrative plasmids into the *amyE* or *lacA* site was facilitated by digestion with *Sca*I or *Bsa*I and screening for loss of α-amylase activity.

To construct pMAD*hrcA,* flanking regions of *hrcA* were amplified using primers pMADhrcAp1-4, fused by overlap-extension PCR and cloned into the *Bam*HI/*Sal*I sites of pMAD. Transformation of this plasmid into *B. subtilis* 168 and successive recombination yielded BAH42 (Arnaud *et al.*, 2004). pBSIIE-spxDD was generated by amplification of a fragment containing Phy-*spx*^{DD} and *lacI* from pSN56 (Nakano *et al.*, 2003a) using primers p222/p223 and ligation into the *Eco*RI/*Spe*I sites of pBSIIE (Radeck *et al.*, 2013). To construct transcriptional fusions to the *lacZ* reporter gene, the respective promoter fragments were amplified from *B. subtilis* 168 genomic DNA and cloned into pDG268 (Antoniewski *et al.*, 1990). Substituted upstream elements are encoded on the 5' region of the forward-primer. To construct pDG268-Pveg, primer p445 and p446 were annealed in a 1:1 ratio and directly ligated into pDG268 digested with *Hind*III/*Eco*RI. The insert for pDG268-SUB-Pveg was created by annealing and extension of p493 and p494 in a standard PCR reaction without additional template. The insert for pDG268-rrnJ-Pveg was created by annealing and extension of p493 and p495 without additional template and a subsequent PCR reaction with p491 and p493 using the product of the first reaction as template.

The generated and used strains and plasmids are listed in Supplementary Tables S5 and S6.

In vitro transcription

His-tagged Spx was expressed from the plasmid pQE60-*spx* in *E. coli* FI1202 and purified by nickel affinity chromatography as described previously (Runde *et al.*, 2014). Purification of His-tagged *B. subtilis* RNA polymerase and *in vitro* transcription was carried out as described previously (Rochat *et al.*, 2012). Briefly, control promoters P_{trxB}, P_{rpsD} and *rrnJ* P1 P2 were PCR amplified, cloned via EcoRI and HindIII into the p770 vector (Ross *et al.*, 1990). For *in vitro* transcription reactions, plasmid DNA was linearized with EcoRI, and the restriction enzyme was inactivated at 65°C for 15 min. RNAP from the *spx*-null strain was reconstituted with saturating concentration of σ^A (ratio 1:5) in storage buffer (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 50% (v/v) glycerol) for 15 min at 37°C. Spx either was or was not pre-incubated with 5 mM DTT for 30 min at 37°C before addition to the transcription reaction. Multiple round transcription reactions were carried out in 10 µL reaction volumes with 30 nM RNAP holoenzyme and 50 ng of linearized plasmid DNA with tested promoters. The transcription buffer contained 40 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg mL^{-1} BSA and 150 mM KCl and NTPs (ATP, CTP were at 200 µM; GTP 1300 µM; UTP was 10 µM plus 30 nM radiolabeled [α-^{32}P] UTP). All transcription reactions were allowed to proceed for 10 min at 37°C and were stopped with equal volumes of formamide stop solution (95% formamide, 20 mM EDTA, pH 8.0). Samples were loaded onto 7 M urea-7% polyacrylamide gels and electrophoresed. The dried gels were scanned with a Molecular Imager FX (Bio-Rad) and visualized and analysed using the Quantity One software (Bio-Rad). The lengths of the transcribed fragments were 233 nt (*rrnJ* P1), 148 nt (*rrnJ* P2), 216 nt (p*rpsD*) or 228 nt (p*trxB*) respectively.

Preparation of total RNA

Total RNA was prepared from cells from 15 to 25 mL cell culture using the illustra RNAspin Mini Kit (GE Healthcare). Cells were resuspended in 350 µl buffer Lysis Buffer,

supplemented with 0.2 mL zircomium/glass beads (0.1 mm dia.) and lysed by vigorously shaking the suspension on a Vortex-Genie 2 (Scientific Industries) for 2 minutes. Further steps were carried out as recommended by the manufacturer. Additionally, RNA was treated with RNase-free DNase I (NEB) for 15 min at 37°C. Integrity of the RNA was checked by native agarose gel electrophoresis or methylene blue stain of blotted samples.

Northern blotting

About 2 µg total RNA per sample was denatured for 10 min at 65°C in sample buffer (50% formamide, 20 mM MOPS pH 7.0, 50 mM sodium acetate, 19 mM EDTA, 2.2% formaldehyde, 1.5% Ficoll 70) and run on a 1.3% agarose, 6.6% formaldehyde gel in 1x MOPS buffer (20 mM MOPS pH 7.0, 50 mM sodium acetate, 10 mM EDTA) for 1.5 h at 100 V. The RNA was transferred to a positively charged nylon membrane by upwards capillary transfer overnight (~16 h) using 10x standard saline citrate buffer (10x SSC; 150 mM sodium citrate, 1.5 M sodium chloride, pH 7.0) and crosslinked to the membrane by irradiation with 120 mJ cm^{-2} in a Stratalinker UV cross-linker apparatus. The membrane was stained with methylene blue dye (0.02% methylene blue, 300 mM sodium acetate pH 5.5) to verify integrity and equal loading of the RNA, scanned and subsequently destained with 1% SDS in 0.2x SSC. Digoxygenin-labelled RNA probes were generated by *in vitro*-transcription with T7 RNA-polymerase (NEB) and labelled DIG RNA Labelling Mix (Roche) in a 20 µl reaction for 3 h at 37°C. The templates were generated by PCR with primers listed in Table S7 that carry the sequence of the T7-promoter.

Membranes were hybridized with labelled probes in hybridization buffer (5x SSC, 0.02% sodium dodecyl sulphate, 0.1% N-laurylsarcosine, 2% blocking reagent (Roche), 20 mM sodium maleate, 4 M urea, pH 7) at 68°C over night as described in (Simard *et al.*, 2001). The membrane was blocked by incubation in 100 mM maleic acid pH 7.5, 150 mM NaCl, 1% w/v Blocking reagent (Roche Applied Sciences) for 1 h. Anti-digoxigenin antibodies conjugated to alkaline phosphatase (Roche Applied Sciences) were diluted 1:5000 in the same buffer and applied to the blot for 2 h with mild shaking. The membrane was washed twice for 15 min in 100 mM maleic acid pH 7.5, 150 mM NaCl and equilibrated in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$. CDP-Star solution (Tropix Inc.) was used as the substrate and signals were detected in a ChemiBIS 4.2 imaging system (DNR).

RT-qPCR

Total RNA was prepared as described above. cDNA from was synthesized from 500 ng total RNA using Protoscript® II reverse transcriptase (NEB) in a 20 µl reaction with 3.5 µM random hexamer primers for 1 h at 42°C and diluted in TE-Buffer (10 mM TRIS, 1 mM EDTA pH 8). qPCR was performed using Luna® Universal qPCR Master Mix (NEB) in a 20 µl reaction with 0.25 µM primers and cDNA equivalent to 5 ng RNA (or 0.5 ng RNA for rRNA targets). Cycling conditions were: 95°C for 60 s followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. Primer efficiency was calculated using a standard curve with serial 10-fold dilutions of cDNA. 23S rRNA was used as a reference and the $2^{\Delta\Delta CT}$ Method (Livak and Schmittgen, 2001) was used to calculate relative gene expression. The primers used for RT-qPCR are listed in Table S8.

Western blotting

Samples of 10 ml were collected by centrifugation for 5 min, 4°C, washed once in buffer TE (10 mM TRIS-HCl pH 8.0, 1 mM EDTA) and resuspended in the same buffer supplemented with 0.1 mM PMSF. Lysates were prepared by sonication and cleared by centrifugation for 5 min at 11.000 × g. Western blotting was carried out with antibody sera as described previously (Molière *et al.*, 2016). Signals were detected either using alkaline phosphatase (AP)-conjugated antibody or the ECF-reagent (GE healthcare) (Molière *et al.*, 2016) or HRP-conjugated antibody (Mruk and Cheng, 2011). Images were acquired using the MF-ChemiBIS 4.2 imaging system (DNR Bio-Imaging Systems) or ChemoStar imaging system (Intas, Göttingen, Germany).

Growth on agar plates

Stationary-phase cultures were adjusted to an OD_{600} of 1.0 and diluted in 0.9% NaCl. 5 µl cell suspension was spotted on LB agar plates with or without IPTG or diamide as indicated. Plates were incubated overnight (18 h) at 37°C.

Microarray experiments

For thermotolerance experiments, *B. subtilis* wild-type cells were grown in LB medium at 37°C and shaking at 300 rpm to the mid-exponential phase ($OD_{600\ nm}$ 0.6) and divided in four 50 ml cultures. Two cultures were incubated for 15 min at 37 or 48°C and harvested. The other cultures were incubated for 15 min at 37 or 48°C, then incubated at 53°C for additional 15 min, harvested by centrifugation at 3860 × g for 5 min and flash-frozen in liquid nitrogen. Δ*clpX* (BNM107) cells or Δ*clpX*Δ*spx* (BNM112) cells were grown in LB medium to the mid-exponential phase at 37°C and 300 rpm, harvested by centrifugation and frozen in liquid nitrogen.

Total RNA was prepared from frozen pellets using the ® FastRNA Pro Blue Kit (Qbiogene, Inc., CA), resuspended in 100 µl DEPC treated water, treated with DNase I at 37°C for 20 min and purified by phenol/chloroform extraction and ethanol precipitation. The quality of the RNA was checked by agarose gel electrophoresis. cDNA was synthesized from 15 µg total RNA and 5 µg random hexamer primers in a 30 µl scale using SuperScript® Plus Indirect cDNA Labelling Kit (Invitrogen), purified using the Low Elution cDNA Purification Module (Invitrogen) and labelled using the Alexa Fluor® 555 and 647 Reactive Dye modules (Invitrogen) according to the manufacturer's instructions.

The labelled cDNAs were concentrated to 6 µl, mixed with 35 µl prewarmed SlideHyb Glass Array Hybridization Buffer #1 (Ambion) and applied to an oligonucleotide microarray

prepared by the Center of Applied Genomics (ICPH, UMDNJ, Newark) in a Micro-Array Hybrid Chamber (Amlab) and incubated in a water bath over night at 55°C. The array was then washed in buffer 1 (2 × SSC, 0.5% SDS), buffer 2 (0.5 × SSC, 0.5% SDS) and buffer 3 (0.5 × SSC, 0.03% SDS) for 5 min each at 55°C and then washed with buffer 4 (0.2 × SSC), buffer 5 (0.1 × SSC) and buffer 6 (0.01 × SSC) for 5 min each at room temperature. The array was read in a Genepix 4100 Laserscanner (Molecular Devices) using auto PMT and the GenePix Pro 6.1 software. The dye swap and further analysis was performed using the Acuity 4.0 software (Axon) and Microsoft Excel 2010. Functional groups and regulon annotations were inferred from subtiwiki (Michna *et al*., 2016).The data have been deposited NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible through GEO Series accession numbers GSE45972 and GSE50102.

Fluorescence microscopy

Cells carrying a xylose-inducible copy of a translational fusion of *yocM* with mCherry were grown in LB medium supplemented with 0.5% (w/v) xylose. Upon an OD600 of 0.4, cells were treated with a 15 min pre-shock at 48°C followed by a shift to 53°C. Strain BER550, carrying a translational *in cis* GFP-*spx* fusion was inoculated in LB medium from a stationary phase overnight culture. Cells were briefly concentrated by centrifugation at 5000 × g for 2 min. Samples of 3 µl were subjected to phase contrast or fluorescence microscopy on agarose-coated slides with a Axio Imager.Z2 (Zeiss) using the GFP or RFP filter set with a fixed exposure time of 3000 ms (Runde *et al*., 2014). Images were obtained with an AxioCam MRm (Zeiss). Fluorescence intensity of individual cells was integrated on background-substracted images using the Fiji distribution of ImageJ (Schindelin *et al*., 2012) and normalized to the cell area.

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Author contributions

The authors HS (i, ii, iii), AH (ii), PS (ii), MV (ii), IH (ii), LK (ii, iii), KT (i, ii, iii) have made major contributions to (i) the conception or design of the study, (ii) the acquisition, analysis, or interpretation of the data; and (iii) writing of the manuscript.

References

Abranches, J., Martinez, A.R., Kajfasz, J.K., Chávez, V., Garsin, D.A. and Lemos, J.A. (2009) The molecular alarmone (p)ppGpp mediates stress responses, vancomycin tolerance, and virulence in *Enterococcus faecalis*. *Journal of Bacteriology*, **191**, 2248–2256.

Antelmann, H., Scharf, C. and Hecker, M. (2000) Phosphate starvation-inducible proteins of *Bacillus subtilis*: proteomics and transcriptional analysis. *Journal of Bacteriology*, **182**, 4478–4490.

Antoniewski, C., Savelli, B. and Stragier, P. (1990) The spoIIJ gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. *Journal of Bacteriology*, **172**, 86–93.

Arnaud, M., Chastanet, A. and Débarbouillé, M. (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Applied and Environment Microbiology*, **70**, 6887–6891.

Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L. and Leibler, S. (2004) Bacterial persistence as a phenotypic switch. *Science*, **305**, 1622–1625.

Balchin, D., Hayer-Hartl, M. and Hartl, F.U. (2016) In vivo aspects of protein folding and quality control. *Science*, **353**, aac4354.

Beckert, B., Abdelshahid, M., Schäfer, H., Steinchen, W., Arenz, S., Berninghausen, O., *et al.* (2017) Structure of the *Bacillus subtilis* hibernating 100S ribosome reveals the basis for 70S dimerization. *EMBO Journal*, **36**, 2061–2072.

Birch, C.A., Davis, M.J., Mbengi, L. and Zuber, P. (2017) Exploring the amino acid residue requirements of the RNA polymerase (RNAP) α subunit C-terminal domain for productive interaction between Spx and RNAP of *Bacillus subtilis*. *Journal of Bacteriology*, **199**, e00124.

Browning, D.F. and Busby, S.J.W. (2016) Local and global regulation of transcription initiation in bacteria. *Nature Reviews Microbiology*, **14**, 638–650.

Chi, B.K., Gronau, K., Mäder, U., Hessling, B., Becher, D. and Antelmann, H. (2011) S-bacillithiolation protects against hypochlorite stress in *Bacillus subtilis* as revealed by transcriptomics and redox proteomics. *Mol Cell Proteomics MCP*, **10**(M111), 009506.

Darmon, E., Noone, D., Masson, A., Bron, S., Kuipers, O.P., Devine, K.M., et al (2002) A novel class of heat and secretion stress-responsive genes is controlled by the autoregulated CssRS two-component system of *Bacillus subtilis*. *Journal of Bacteriology*, **184**, 5661–5671.

Delumeau, O., Lecointe, F., Muntel, J., Guillot, A., Guédon, E., Monnet, V., et al. (2011) The dynamic protein partnership of RNA polymerase in *Bacillus subtilis*. *Proteomics*, **11**, 2992–3001.

Dubnau, D. and Losick, R. (2006) Bistability in bacteria. *Molecular Microbiology*, **61**, 564–572.

Edgar, R., Domrachev, M. and Lash, A.E. (2002) Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Research*, **30**, 207–210.

Elsholz, A.K.W., Michalik, S., Zühlke, D., Hecker, M. and Gerth, U. (2010) CtsR, the gram-positive master regulator of protein quality control, feels the heat. *EMBO Journal*, **29**, 3621–3629.

Elsholz, A.K.W., Birk, M.S., Charpentier, E. and Turgay, K. (2017) Functional diversity of AAA+ protease complexes in *Bacillus subtilis*. *Front Mol Biosci*, **4**, 44.

Engman, J. and von Wachenfeldt, C. (2015) Regulated protein aggregation: a mechanism to control the activity of the ClpXP adaptor protein YjbH. *Molecular Microbiology*, **95**, 51–63.

Eymann, C., Homuth, G., Scharf, C. and Hecker, M. (2002) *Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. *Journal of Bacteriology*, **184**, 2500–2520.

Fitzsimmons, L.F., Liu, L., Kim, J.-S., Jones-Carson, J. and Vázquez-Torres, A. (2018) *Salmonella* reprograms nucleotide metabolism in its adaptation to nitrosative stress. *MBio*, **9**, e00211–e00218.

Fridman, O., Goldberg, A., Ronin, I., Shoresh, N. and Balaban, N.Q. (2014) Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature*, **513**, 418–421.

Fukushima, T., Ishikawa, S., Yamamoto, H., Ogasawara, N. and Sekiguchi, J. (2003) Transcriptional, functional and cytochemical analyses of the veg gene in *Bacillus subtilis*. *Journal of Biochemistry*, **133**, 475–483.

Gaal, T., Ross, W., Blatter, E.E., Tang, H., Jia, X., Krishnan, V.V., et al. (1996) DNA-binding determinants of the alpha subunit of RNA polymerase: novel DNA-binding domain architecture. *Genes & Development*, **10**, 16–26.

Gaballa, A., Antelmann, H., Hamilton, C.J. and Helmann, J.D. (2013) Regulation of *Bacillus subtilis* bacillithiol biosynthesis operons by Spx. *Microbiology*, **159**, 2025–2035.

Gaidenko, T.A., Kim, T.-J., Weigel, A.L., Brody, M.S. and Price, C.W. (2006) The blue-light receptor YtvA acts in the environmental stress signaling pathway of *Bacillus subtilis*. *Journal of Bacteriology*, **188**, 6387–6395.

Garg, S.K., Kommineni, S., Henslee, L., Zhang, Y. and Zuber, P. (2009) The YjbH protein of *Bacillus subtilis* enhances ClpXP-catalyzed proteolysis of Spx. *Journal of Bacteriology*, **191**, 1268–1277.

Griffith, K.L., Shah, I.M., Myers, T.E., O'Neill, M.C. and Wolf, R.E. (2002) Evidence for "pre-recruitment" as a new mechanism of transcription activation in *Escherichia coli*: the large excess of SoxS binding sites per cell relative to the number of SoxS molecules per cell. *Biochemical and Biophysical Research Communications*, **291**, 979–986.

Hantke, I., Schäfer, H., Janczikowski, A. and Turgay, K. (2018) YocM a small heat shock protein can protect *Bacillus subtilis* cells during salt stress. *Molecular Microbiology*. https://doi.org/10.1111/mmi.14164

Hauryliuk, V., Atkinson, G.C., Murakami, K.S., Tenson, T. and Gerdes, K. (2015) Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nature Reviews Microbiology*, **13**, 298–309.

Hecker, M., Völker, U. and Heim, C. (1989) RelA-independent (p)ppGpp accumulation and heat shock protein induction after salt stress in *Bacillus subtilis*. *FEMS Microbiology Letters*, **58**, 125–128.

Hecker, M., Schumann, W. and Völker, U. (1996) Heat-shock and general stress response in *Bacillus subtilis*. *Molecular Microbiology*, **19**, 417–428.

Hecker, M., Pane-Farre, J. and Völker, U. (2007) SigB-dependent general stress response in *Bacillus subtilis* and related gram-positive bacteria. *Annual Review of Microbiology*, **61**, 215–236.

Helmann, J.D. (1995) Compilation and analysus of *Bacillus Subtilis* σ^A -dependent promoter sequences: evidence for extended contact between RNA polymerse and upstream promoter DNA. *Nucleic Acids Research*, **23**, 2351–2360.

Helmann, J.D., Wu, M.F., Kobel, P.A., Gamo, F.J., Wilson, M., Morshedi, M.M., et al. (2001) Global transcriptional response of *Bacillus subtilis* to heat shock. *Journal of Bacteriology*, **183**, 7318–7328.

Höper, D., Völker, U. and Hecker, M. (2005) Comprehensive characterization of the contribution of individual SigB-dependent general stress genes to stress resistance of *Bacillus subtilis*. *Journal of Bacteriology*, **187**, 2810–2826.

Inoue, H., Nojima, H. and Okayama, H. (1990) High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, **96**, 23–28.

Jervis, A.J., Thackray, P.D., Houston, C.W., Horsburgh, M.J. and Moir, A. (2007) SigM-responsive genes of *Bacillus subtilis* and their promoters. *Journal of Bacteriology*, **189**, 4534–4538.

Kim, Y.E., Hipp, M.S., Bracher, A., Hayer-Hartl, M. and Hartl, F.U. (2013) Molecular chaperone functions in protein folding and proteostasis. *Annual Review of Biochemistry*, **82**, 323–355.

Kirstein, J., Molière, N., Dougan, D.A. and Turgay, K. (2009) Adapting the machine: adaptor proteins for Hsp100/Clp and AAA+ proteases. *Nature Reviews Microbiology*, **7**, 589–599.

Koga, K., Ikegami, A., Nakasone, K., Murayama, R., Akanuma, G., Natori, Y., et al. (2006) Construction of *Bacillus subtilis* strains carrying the transcriptional bgaB fusion with the promoter region of each rrn operon and their differential transcription during spore development. *Journal of General and Applied Microbiology*, **52**, 119–124.

Krásný, L. and Gourse, R.L. (2004) An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. *EMBO Journal*, **23**, 4473–4483.

Kriel, A., Bittner, A.N., Kim, S.H., Liu, K., Tehranchi, A.K., Zou, W.Y., et al. (2012) Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. *Molecular Cell*, **48**, 231–241.

Krüger, E. and Hecker, M. (1998) The first gene of the *Bacillus subtilis* clpC operon, ctsR, encodes a negative regulator of its own operon and other class III heat shock genes. *Journal of Bacteriology*, **180**, 6681–6688.

Lamour, V., Westblade, L.F., Campbell, E.A. and Darst, S.A. (2009) Crystal structure of the in vivo-assembled *Bacillus subtilis* Spx/RNA polymerase alpha subunit C-terminal domain complex. *Journal of Structural Biology*, **168**, 352–356.

Larsson, J.T., Rogstam, A. and von Wachenfeldt, C. (2007) YjbH is a novel negative effector of the disulphide stress regulator, Spx, in *Bacillus subtilis*. *Molecular Microbiology*, **66**, 669–684.

Leelakriangsak, M., Kobayashi, K. and Zuber, P. (2007) Dual negative control of spx transcription initiation from the P3 promoter by repressors PerR and YodB in *Bacillus subtilis*. *Journal of Bacteriology*, **189**, 1736–1744.

Leichert, L.I.O., Scharf, C. and Hecker, M. (2003) Global characterization of disulfide stress in *Bacillus subtilis*. *Journal of Bacteriology*, **185**, 1967–1975.

Lin, A.A., Walthers, D. and Zuber, P. (2013) 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. *Journal of Bacteriology*, **195**, 3967–3978.

Lindner, A.B., Madden, R., Demarez, A., Stewart, E.J. and Taddei, F. (2008) Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. *Proceedings of the National Academy of Sciences*, **105**, 3076–3081.

Lindquist, S. (1986) The heat-shock response. *Annual Review of Biochemistry*, **55**, 1151–1191.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*, **25**, 402–408.

Luo, Y. and Helmann, J.D. (2012) Analysis of the role of *Bacillus subtilis* σ(M) in β-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Molecular Microbiology*, **83**, 623–639.

Martin, R.G., Gillette, W.K., Martin, N.I. and Rosner, J.L. (2002) Complex formation between activator and RNA polymerase as the basis for transcriptional activation by MarA and SoxS in *Escherichia coli*: transcriptional activator-RNP complexes. *Molecular Microbiology*, **43**, 355–370.

Michna, R.H., Zhu, B., Mäder, U. and Stülke, J. (2016) SubtiWiki 2.0–an integrated database for the model organism *Bacillus subtilis*. *Nucleic Acids Research*, **44**, D654–D662.

Mogk, A., Homuth, G., Scholz, C., Kim, L., Schmid, F.X. and Schumann, W. (1997) The GroE chaperonin machine is a major modulator of the CIRCE heat shock regulon of *Bacillus subtilis*. *EMBO Journal*, **16**, 4579–4590.

Mogk, A., Huber, D. and Bukau, B. (2011) Integrating protein homeostasis strategies in prokaryotes. *Cold Spring Harbor Perspectives in Biology*, **3**(4), a004366–a004366.

Molière, N., Hoßmann, J., Schäfer, H. and Turgay, K. (2016) Role of Hsp100/Clp protease complexes in controlling the regulation of motility in *Bacillus subtilis*. *Frontiers in Microbiology*, **7**, 315.

Mostertz, J., Scharf, C., Hecker, M. and Homuth, G. (2004) Transcriptome and proteome analysis of *Bacillus subtilis* gene expression in response to superoxide and peroxide stress. *Microbiology*, **150**, 497–512.

Mruk, D.D. and Cheng, C.Y. (2011) Enhanced chemiluminescence (ECL) for routine immunoblotting: an inexpensive alternative to commercially available kits. *Spermatogenesis*, **1**, 121–122.

Murayama, S., Ishikawa, S., Chumsakul, O., Ogasawara, N. and Oshima, T. (2015) The role of α-CTD in the genome-wide transcriptional regulation of the *Bacillus subtilis* cells. *PLoS ONE*, **10**, e0131588.

Nakano, M.M., Zhu, Y., Liu, J., Reyes, D.Y., Yoshikawa, H. and Zuber, P. (2000) Mutations conferring amino acid residue substitutions in the carboxy-terminal domain of RNA polymerase alpha can suppress clpX and clpP with respect to developmentally regulated transcription in *Bacillus subtilis*. *Molecular Microbiology*, **37**, 869–884.

Nakano, M.M., Hajarizadeh, F., Zhu, Y. and Zuber, P. (2001) Loss-of-function mutations in yjbD result in ClpX- and ClpP-independent competence development of *Bacillus subtilis*. *Molecular Microbiology*, **42**, 383–394.

Nakano, M.M., Nakano, S. and Zuber, P. (2002a) Spx (YjbD), a negative effector of competence in *Bacillus subtilis*, enhances ClpC-MecA-ComK interaction. *Molecular Microbiology*, **44**, 1341–1349.

Nakano, S., Zheng, G., Nakano, M.M. and Zuber, P. (2002b) Multiple pathways of Spx (YjbD) proteolysis in *Bacillus subtilis*. *Journal of Bacteriology*, **184**, 3664–3670.

Nakano, S., Küster-Schöck, E., Grossman, A.D. and Zuber, P. (2003a) Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences*, **100**, 13603–13608.

Nakano, S., Nakano, M.M., Zhang, Y., Leelakriangsak, M. and Zuber, P. (2003b) A regulatory protein that interferes with activator-stimulated transcription in bacteria. *Proceedings of the National Academy of Sciences*, **100**, 4233–4238.

Nakano, S., Erwin, K.N., Ralle, M. and Zuber, P. (2005) Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx. *Molecular Microbiology*, **55**, 498–510.

Nakano, M.M., Lin, A., Zuber, C.S., Newberry, K.J., Brennan, R.G. and Zuber, P. (2010) Promoter recognition by a complex of Spx and the C-terminal domain of the RNA polymerase α subunit. *PLoS ONE*, **5**, e8664.

Natori, Y., Tagami, K., Murakami, K., Yoshida, S., Tanigawa, O., Moh, Y., et al. (2009) Transcription activity of individual rrn operons in *Bacillus subtilis* mutants deficient in (p)ppGpp synthetase genes, relA, yjbM, and ywaC. *Journal of Bacteriology*, **191**, 4555–4561.

Newberry, K.J., Nakano, S., Zuber, P. and Brennan, R.G. (2005) Crystal structure of the *Bacillus subtilis* anti-alpha, global transcriptional regulator, Spx, in complex with the alpha C-terminal domain of RNA polymerase. *Proceedings of the National Academy of Sciences*, **102**, 15839–15844.

Nicolas, P., Mäder, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., et al. (2012) Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science*, **335**, 1103–1106.

Petersohn, A., Antelmann, H., Gerth, U. and Hecker, M. (1999) Identification and transcriptional analysis of new members of the sigmaB regulon in *Bacillus subtilis*. *Microbiol Read Engl*, **145**(Pt 4), 869–880.

Petersohn, A., Brigulla, M., Haas, S., Hoheisel, J.D., Volker, U. and Hecker, M. (2001) Global analysis of the general stress response of *Bacillus subtilis*. *Journal of Bacteriology*, **183**, 5617–5631.

Price, C.W., Fawcett, P., Cérémonie, H., Su, N., Murphy, C.K. and Youngman, P. (2001) Genome-wide analysis of the general stress response in *Bacillus subtilis*. *Molecular Microbiology*, **41**, 757–774.

Radeck, J., Kraft, K., Bartels, J., Cikovic, T., Dürr, F., Emenegger, J., et al. (2013) The Bacillus BioBrick Box: generation and evaluation of essential genetic building blocks for standardized work with *Bacillus subtilis*. *Journal of Biological Engineering*, **7**, 29.

Rao, L., Ross, W., Appleman, J.A., Gaal, T., Leirmo, S., Schlax, P.J., et al. (1994) Factor independent activation of rrnB P1. An "extended" promoter with an upstream element that dramatically increases promoter strength. *Journal of Molecular Biology*, **235**, 1421–1435.

Reder, A., Hoper, D., Gerth, U. and Hecker, M. (2012) Contributions of individual B-dependent general stress genes to oxidative stress resistance of *Bacillus subtilis*. *Journal of Bacteriology*, **194**, 3601–3610.

Reyes, D.Y. and Zuber, P. (2008) Activation of transcription initiation by Spx: formation of transcription complex and identification of a Cis-acting element required for transcriptional activation. *Molecular Microbiology*, **69**, 765–779.

Riley, E.P., Trinquier, A., Reilly, M.L., Durchon, M., Perera, V.R., Pogliano, K. and Lopez-Garrido, J. (2018) Spatiotemporally regulated proteolysis to dissect the role of vegetative proteins during *Bacillus subtilis* sporulation: cell-specific requirement of σ^{H} and σ^{A}: Spatiotemporally regulated proteolysis in *Bacillus*. *Molecular Microbiology*, **108**, 45–62.

Rochat, T., Nicolas, P., Delumeau, O., Rabatinová, A., Korelusová, J., Leduc, A., et al. (2012) Genome-wide identification of genes directly regulated by the pleiotropic transcription factor Spx in *Bacillus subtilis*. *Nucleic Acids Research*, **40**, 9571–9583.

Rojas-Tapias, D.F. and Helmann, J.D. (2018a) Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in *Bacillus subtilis*. *Molecular Microbiology*, **107**, 659–674.

Rojas-Tapias, D.F. and Helmann, J.D. (2018b) Stabilization of *Bacillus subtilis* Spx under cell wall stress requires the anti-adaptor protein YirB. *PLoS Genetics*, **14**, e1007531.

Rosenberg, A., Sinai, L., Smith, Y. and Ben-Yehuda, S. (2012) Dynamic expression of the translational machinery during *Bacillus subtilis* life cycle at a single cell level. *PLoS ONE*, **7**, e41921.

Ross, W., Thompson, J.F., Newlands, J.T. and Gourse, R.L. (1990) *E. coli* Fis protein activates ribosomal RNA transcription in vitro and in vivo. *EMBO Journal*, **9**, 3733–3742.

Ross, W., Gosink, K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., et al. (1993) A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. *Science*, **262**, 1407–1413.

Runde, S., Molière, N., Heinz, A., Maisonneuve, E., Janczikowski, A., Elsholz, A.K.W., et al. (2014) The role of thiol oxidative stress response in heat-induced protein aggregate formation during thermotolerance in *B acillus subtilis*: Thiol oxidation in protein aggregate formation. *Molecular Microbiology*, **91**, 1036–1052.

Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012) Fiji: an open-source platform for biological-image analysis. *Nature Methods*, **9**, 676–682.

Schulz, A. and Schumann, W. (1996) hrcA, the first gene of the *Bacillus subtilis* dnaK operon encodes a negative regulator of class I heat shock genes. *Journal of Bacteriology*, **178**, 1088–1093.

Shah, I.M. and Wolf, R.E. (2004) Novel protein-protein interaction between *Escherichia coli* SoxS and the DNA binding determinant of the RNA polymerase α subunit: SoxS functions as a co-sigma factor and redeploys RNA polymerase from UP-element-containing promoters to SoxS-dependent promoters during oxidative stress. *Journal of Molecular Biology*, **343**, 513–532.

Simard, C., Lemieux, R. and Côté, S. (2001) Urea substitutes toxic formamide as destabilizing agent in nucleic acid hybridizations with RNA probes. *Electrophoresis*, **22**, 2679–2683.

Sojka, L., Kouba, T., Barvík, I., Sanderová, H., Maderová, Z., Jonák, J. and Krásny, L. (2011) Rapid changes in gene expression: DNA determinants of promoter regulation by the concentration of the transcription initiating NTP in *Bacillus subtilis*. *Nucleic Acids Research*, **39**, 4598–4611.

Spizizen, J. (1958) Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proceedings of the National Academy of Sciences*, **44**, 1072–1078.

Stülke, J., Hanschke, R. and Hecker, M. (1993) Temporal activation of beta-glucanase synthesis in *Bacillus subtilis* is mediated by the GTP pool. *Journal of General Microbiology*, **139**, 2041–2045.

Veening, J.W., Smits, W.K. and Kuipers, O.P. (2008) Bistability, epigenetics, and bet-hedging in bacteria. *Annual Review of Microbiology*, **62**, 193–210.

Versteeg, S., Escher, A., Wende, A., Wiegert, T. and Schumann, W. (2003) Regulation of the *Bacillus subtilis* heat shock gene htpG is under positive control. *Journal of Bacteriology*, **185**, 466–474.

Völker, U., Maul, B. and Hecker, M. (1999) Expression of the sigmaB-dependent general stress regulon confers multiple stress resistance in *Bacillus subtilis*. *Journal of Bacteriology*, **181**, 3942–3948.

Wickner, S., Maurizi, M.R. and Gottesman, S. (1999) Posttranslational quality control: folding, refolding, and degrading proteins. *Science*, **286**, 1888–1893.

Wiegert, T. and Schumann, W. (2001) SsrA-mediated tagging in *Bacillus subtilis*. *Journal of Bacteriology*, **183**, 3885–3889.

Yang, X. and Ishiguro, E.E. (2003) Temperature-sensitive growth and decreased thermotolerance associated with relA mutations in *Escherichia coli*. *Journal of Bacteriology*, **185**, 5765–5771.

Zhang, Y. and Zuber, P. (2007) Requirement of the zinc-binding domain of ClpX for Spx proteolysis in *Bacillus subtilis* and effects of disulfide stress on ClpXP activity. *Journal of Bacteriology*, **189**, 7669–7680.

Zhang, Y., Nakano, S., Choi, S.-Y. and Zuber, P. (2006) Mutational analysis of the *Bacillus subtilis* RNA polymerase α C-terminal domain supports the interference model of Spx-dependent repression. *Journal of Bacteriology*, **188**, 4300–4311.

Zuber, P. (2004) Spx-RNA polymerase interaction and global transcriptional control during oxidative stress. *Journal of Bacteriology*, **186**, 1911–1918.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

SUPPLEMENTARY INFORMATION

Spx, the central regulator of the heat- and oxidative stress response in *B. subtilis*, can repress transcription of translation-related genes

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Supplementary tables

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See supplementary excel data file.

Table S2 Regulated genes of the thermotolerance arrays

See supplementary excel data file.

Table S3 the most up- and down-regulated genes of array 4 (37/53 °C vs 48/53 °C)

See supplementary excel data file.

Table S4 Regulated genes in the Δc*lpX* vs Δc*lpX*Δ*spx* array

See supplementary excel data file.

Table S5 List of strains

Strain	genotype	Source/construction
B. subtilis 168	*trpC2*	(Spizizen, 1958)
B. subtilis PY79		(Youngman *et al.*, 1984)
BNM107	*trpC2* Δ*clpX::kan*	(Runde *et al.*, 2014)
BNM111	*trpC2* Δ*spx::kan*	(Runde *et al.*, 2014)
BNM112	*trpC2* Δ*spx::kan* Δ*clpX::spec*	(Runde *et al.*, 2014)
BNM810	*trpC2 amyE:: P_{hy}-spx^{DD} lacI spec*	(Runde *et al.*, 2014)
BAH34	*trpC2* ΔctsR*::kan*	This work, BHL-5 → *B. subtilis 168* (Krüger *et al.*, 2001)
BAH35	*trpC2* Δ*sigB::cat*	This work, ML-6 → *B. subtilis 168* (Igo *et al.*, 1987)
BAH42	*trpC2* Δ*hrcA*	This work, pMADΔhrcA → *B. subtilis 168*
BNM855	Δ*yjbH::spec*	(Molière *et al.*, 2016)
BHS201	*trpC2 lacA::P_{hy}-spx^{DD} lacI erm*	This work, pBSIIE-spxDD → *B. subtilis 168*
BHS220	*trpC2 amyE::RrnJP1(-107/+25)-lacZ Cm*	This work, pDG268-RrnJP1-132 → *B. subtilis 168*
BHS222	*trpC2 amyE::RrnJP1-lacZ Cm* Δ*spx::kan*	This work, BNM111 → BHS220
BHS225	*trpC2 amyE::RrnJP1-lacZ Cm lacA::P_{hy}-spx^{DD} lacI erm*	This work, BHS201 → BHS220
BHS475	*trpC2 $rpoA^{Y263C}$*	This work, pYZ37 → → *B. subtilis 168* (Nakano *et al.*, 2000)
BHS476	*trpC2 $rpoA^{V260A}$*	This work, pYZ38 → → *B. subtilis 168* (Nakano *et al.*,

		2000)
BHS516	*trpC2 amyE::RrnJP1(-59/+3)-lacZ cat*	This work, pDG268-RrnJP1-62 → *B. subtilis 168*
BHS517	*trpC2 amyE::RrnJP1(-38/+3)-lacZ cat*	This work, pDG268-RrnJP1-41 → *B. subtilis 168*
BHS549	*trpC2 rpoA^{Y263C} amyE::RrnJP1(-107/+25)-lacZ cat*	This work, BHS220 →BHS475
BHS550	*trpC2 rpoA^{V260A} amyE::RrnJP1(-107/+25)-lacZ cat*	This work, BHS220 →BHS476
BHS569	*trpC2 amyE::Pveg (-38/+1) -lacZ cat*	This work, pDG268-Pveg → *B. subtilis 168*
BHS573	*trpC2 amyE::Pveg (-38/+1) -lacZ cat lacA::P$_{hy}$-spx^{DD} lacI erm*	This work, BHS201 → BHS569
BHS591	*trpC2 amyE::RrnJP1(SUB -38/+3)-lacZ cat*	This work, pDG268-SUB-RrnJP1 → *B. subtilis 168*
BHS592	*trpC2 amyE::RrnJP1(trxB_UP -38/+3)-lacZ cat*	This work, pDG268-TRXB-RrnJP1 → *B. subtilis 168*
BHS601	*trpC2 amyE::RrnJP1(SUB -38/+3)-lacZ cat lacA::P$_{hy}$-spx^{DD} lacI erm*	This work, BHS201 → BHS591
BHS602	*trpC2 amyE::RrnJP1(trxB_UP -38/+3)-lacZ cat lacA::P$_{hy}$-spx^{DD} lacI erm*	This work, BHS201 → BHS592
BHS652	*trpC2 amyE::Pveg (SUB -38/+1) -lacZ cat*	This work, pDG268-SUB-Pveg → *B. subtilis 168*
BHS653	*trpC2 amyE::Pveg (rrnJ_UP -38/+1) -lacZ cat*	This work, pDG268-rrnJ-Pveg → *B. subtilis 168*
BHS668	*trpC2 amyE::Pveg (SUB -38/+1) -lacZ cat lacA::P$_{hy}$-spx^{DD} lacI erm*	This work, BHS201 → BHS652
BHS669	*trpC2 amyE::Pveg (rrnJ_UP -38/+1) -lacZ cat lacA::P$_{hy}$-spx^{DD} lacI erm*	This work, BHS201 → BHS653
BHS729	*trpC2 rpoA^{Y263C} amyE::RrnJP1(-107/+25)-lacZ cat lacA::P$_{hy}$-spx^{DD} lacI erm*	This work, BHS201 → BH549
BHS730	*trpC2 rpoA^{V260A} amyE::RrnJP1(-107/+25)-lacZ cat lacA::P$_{hy}$-spx^{DD} lacI erm*	This work, BHS201 → BHS550
BHS800	*trpC2 amyE::RrnJP1(-98/+3)-lacZ cat*	This work, pDG268-RrnJP1-101 → *B. subtilis 168*
BHS807	*trpC2 amyE::RrnJP1(-98/+3)-lacZ cat lacA::P$_{hy}$-spx^{DD} lacI erm*	This work, BHS201 → BHS800
BHS882	*trpC2 amyE::RrnJP1-lacZ Cm ΔmgsR::erm*	This work, BAR1 → BHS220 (Reder *et al.*, 2008)
BHS883	*trpC2 amyE::RrnJP1-lacZ Cm Δspx::kan ΔmgsR::erm*	This work, BAR1→ BHS222 (Reder *et al.*, 2008)
BHS932	*PY79 amyE::PrrnA-gfpmut2 spc lacA::P$_{hy}$-spx^{DD} lacI erm*	This work, pBSIIE-spxDD → AR13 (Rosenberg *et*

		al., 2012)
BHS933	*PY79 amyE::PrrnB-gfpmut2 spc lacA::P_{hy}-spx^{DD} lacI erm*	This work, pBSIIE-spxDD → AR14 (Rosenberg *et al.*, 2012)
BHS934	*PY79 amyE::PrrnD-gfpmut2 spc lacA::P_{hy}-spx^{DD} lacI erm*	This work, pBSIIE-spxDD → AR15 (Rosenberg *et al.*, 2012)
BHS935	*PY79 amyE::PrrnE-gfpmut2 spc lacA::P_{hy}-spx^{DD} lacI erm*	This work, pBSIIE-spxDD → AR16 (Rosenberg *et al.*, 2012)
BHS936	*PY79 amyE::PrrnO-gfpmut2 spc lacA::P_{hy}-spx^{DD} lacI erm*	This work, pBSIIE-spxDD → AR17 (Rosenberg *et al.*, 2012)
BHS937	*PY79 amyE::PrrnI-gfpmut2 spc lacA::P_{hy}-spx^{DD} lacI erm*	This work, pBSIIE-spxDD → AR18 (Rosenberg *et al.*, 2012)
BHS938	*PY79 amyE::PrrnJ-gfpmut2 spc lacA::P_{hy}-spx^{DD} lacI erm*	This work, pBSIIE-spxDD → AR19 (Rosenberg *et al.*, 2012)
BIH369	*trpC2 lacA::Pxyl yocM-mCherry erm*	(Hantke *et al.*, 2018)
BIH632	*trpC2 rpoA^{Y263C} lacA::Pxyl yocM-mCherry erm*	BIH369 →BHS475
BIH633	*trpC2 rpoA^{Y263C} lacA::Pxyl yocM-mCherry erm*	BIH369 →BHS476
BER550	PY79 *gfp-spx cat*	(Riley *et al.*, 2018)
LK1119	*rpoC-His10, spx::aphA-3*	(Rochat *et al.*, 2012)

Table S6 List of plasmids

Plasmid	**Relevant features**	**Source or cloning primers**
pYZ37	*rpoA^{Y263C}*	(Nakano *et al.*, 2000)
pYZ38	*rpoA^{V260A}*	(Nakano *et al.*, 2000)
pMADhrcA	Δ*hrcA*	pMADhrcAp1-4
pDG268-RrnJP1-132	*amyE3' cat* RrnJP1(-107/+25)-*lacZ amyE5'*	p249/p250
pDG268-RrnJP1-102	*amyE3' cat* RrnJP1(-98/+3)-*lacZ amyE5'*	p377/p616
pDG268-RrnJP1-62	*amyE3' cat* RrnJP1(-59/+3)-*lacZ amyE5'*	p377/379
pDG268-RrnJP1-41	*amyE3' cat* RrnJP1(-38/+3)-*lacZ amyE5'*	p377/378
pDG268-SUB-RrnJP1	*amyE3' cat* RrnJP1(SUB -38/+3)-*lacZ amyE5'*	p377/p463
pDG268-TRXB-RrnJP1	*amyE3' cat* RrnJP1(trxB_UP -38/+3)-*lacZ amyE5'*	p377/p464
pDG268-Pveg	*amyE3' cat* P*veg* (-38/+1)-*lacZ amyE5'*	p455/p456

pDG268-SUB-Pveg	*amyE3' cat* P*veg* (SUB-38/+1)-*lacZ amyE5'*	p493/p494
pDG268-rrnJ-Pveg	*amyE3' cat* P*veg* (trxB_UP-38/+1)-*lacZ amyE5'*	p493/p495, p491/p493
pBSIIE-spxDD	*lacA5' erm Phy-spx^{DD} lacI lacA3'*	p222/p223
P770-rrnJ P1 P2	*amp* rrnJ P1 P2 (-108/+88)	pLK2037/pLK2039
pEDJ160	*amp* p770-PtrxB	(Rochat *et al.*, 2012)
pEDJ163	*amp* p770-PrpsD	(Rochat *et al.*, 2012)
pCD2	*B. subtilis sigA*	(Chang and Doi, 1990)

Table S7 List of oligonucleotides

primer	**Sequence (5'-3')**
pMADhrcAp1	CCCCGGATCCGGATATGATCAACCGCGTGC
pMADhrcAp2	CCCCGTCGACCATCACCTCTGTTAGCACTC
pMADhrcAp3	CCCCGTCGACGCTTCAGCATGTGACTTCGG
pMADhrcAp4	CTTTGACCATGGAAGGGCG
p249_EcoRI_PrrnJ_P1_for	CCGGAATTCAAGAGCGGTATCCTCCATAG
p250_BamHI_PrrnJ_P1_rev	CGCGGATCCCGTTATCGCCTTGTTTAGCG
p377_RrnJP1_+3_rev	CGCGGATCCAACGAATAATAATATACCACC
p378_RrnJP1_-39_for	CCGGAATTCTATTGCACTATTATTTACTAGG
p379_RrnJP1_-60_for	CCGGAATTCTTAGTATTTCTTCAAAAAAACTATTGC
p616_RrnJP1_102	ATAGAATTCATCCTCCATAGGGAAAGG
p463_PrrnJ_SUB_up_for	CCGGAATTCTCGACTGCAGTGGTACCTAGGCTATTGCACTATTATTTACTAGG
p464_trxB_UP_rrnJ_core	CCGGAATTCGAATACATTTAATCGTGTTGAGCAAAAATATTGCACTATTATTTACTA
222_pDR111_for	CAGGAATTCGACTCTCTAGC
223_pDR111_rev	taACTAGTATAATGGATTTCCTTACGCG
p455_Pveg_core_for	AATTCTATTTGACAAAAATGGGCTCGTGTTGTACAATAAATGTAA
p456_Pveg_core_rev	AGCTTTACATTTATTGTACAACACGAGCCCATTTTTGTCAAATAG
491_RrnJP1_up	ataGAATTCGATGCCGCTCTTTTTAAATCCCTTAGTATTTCTTCAAAAAAA
493_Pveg_core_rev	tatAAGCTTTACATTTATTGTACAACACGAGCCCATTTTTGTCAAATA
494_SUB_UP_Pveg	ataGAATTCTCGACTGCAGTGGTACCTAGGTATTTGACAAAAATGG
495_RrnJP1_UP_Pveg	CTTAGTATTTCTTCAAAAAAATATTTGACAAAAATGGGC
LK2037/rrnJ_F	GCGAATTCAAGAGCGGTATCCTCCATAG
LK2039/rrnJP1+P2_R	GCAAGCTTGACTTTATTATTATAACTCG

Table S8 List of oligonucleotides for qPCR experiments

primer	**Sequence (5'-3')**

585_qPCR_lacZ_rev	CGTTTCACCCTGCCATAAAG
586_qPCR_lacZ_for	GGAAGATCAGGATATGTGGC
595_qPCR_rplC_for	TCCGGTAACTGTTATCGAGG
596_qPCR_rplC_rev	GACCAACTTCATACGCATCC
599_qPCR_trxB_for	CCGTGCTGTCATCATTGCTG
600_qPCR_trxB_rev	TATACGCCTTCTTCAACCGC
605_qPCR_23S_for	CTTTGATCCGGAGATTTCCG
606_qPCR_23S_rev	GTACAGAGTGTCCTACAACC
638_qPCR_rplS_for	GGTGGAATCAGCGAAACGTT
639_qPCR_rplS_rev	TAATACGAGCCGCTTTTCCG
640_qPCR_rpsD_for	GGCTCGCTATACAGGTCCAT
641_qPCR_rpsD_rev	TGCGGAATTGACGTTCGTTT
648_qPCR_ssrA_for	CGAGCTCTTCCTGACATTGC
649_qPCR_ssrA_rev	AACCCACGTCCAGAAACATC
650_qPCR_rplO_for	GTCGTGGTATTGGTTCTGGC
651_qPCR_rplO_rev	GTGACTTCCGTTCCTTCTGC
722_qPCR_ytvA_for	ATTGGCCCAAGTGAACGAAC
723_qPCR_ytvA_rev	ATCGGAAGCACTTTAACGGC
726_qPCR_hag_for	CATGCGATCCTTCAACGTGT
727_qPCR_hag_rev	TGCAGGAGTAGCTGTGTCAA
758_qPCR_groEL_for	GGTGATCGCCGTAAAGCAAT
759_qPCR_groEL_rev	TGTTTCTTCCACTTGAGCGC
762_qPCR_htpG_for	GGCATAGACACGGATGAGGA
763_qPCR_htpG_rev	GCTGTCAGGCATCGCATTTA
765_qPCR_hslO_for	ACGATGCCTGTCAGATTCCA
766_qPCR_hslO_rev	TAGTTTGGTCACGAAGCCCT

References

Chang, B.Y., and Doi, R.H. (1990) Overproduction, purification, and characterization of Bacillus subtilis RNA polymerase sigma A factor. *J Bacteriol* **172**: 3257–3263.

Hantke, I., Schäfer, H., Janczikowski, A., and Turgay, K. (2018) YocM a small heat shock protein can protect Bacillus subtilis cells during salt stress. *Mol Microbiol* .

Igo, M., Lampe, M., Ray, C., Schafer, W., Moran, C.P., and Losick, R. (1987) Genetic studies of a secondary RNA polymerase sigma factor in Bacillus subtilis. *J Bacteriol* **169**: 3464–3469.

Krüger, E., Zühlke, D., Witt, E., Ludwig, H., and Hecker, M. (2001) Clp-mediated proteolysis in Gram-positive bacteria is autoregulated by the stability of a repressor. *EMBO J* **20**: 852–863.

Molière, N., Hoßmann, J., Schäfer, H., and Turgay, K. (2016) Role of Hsp100/Clp Protease Complexes in Controlling the Regulation of Motility in Bacillus subtilis. *Front*

Microbiol **7** http://journal.frontiersin.org/Article/10.3389/fmicb.2016.00315/abstract. Accessed February 1, 2018.

Nakano, M.M., Zhu, Y., Liu, J., Reyes, D.Y., Yoshikawa, H., and Zuber, P. (2000) Mutations conferring amino acid residue substitutions in the carboxy-terminal domain of RNA polymerase alpha can suppress clpX and clpP with respect to developmentally regulated transcription in Bacillus subtilis. *Mol Microbiol* **37**: 869–884.

Reder, A., Höper, D., Weinberg, C., Gerth, U., Fraunholz, M., and Hecker, M. (2008) The Spx paralogue MgsR (YqgZ) controls a subregulon within the general stress response of Bacillus subtilis. *Mol Microbiol* **69**: 1104–1120.

Riley, E.P., Trinquier, A., Reilly, M.L., Durchon, M., Perera, V.R., Pogliano, K., and Lopez-Garrido, J. (2018) Spatiotemporally regulated proteolysis to dissect the role of vegetative proteins during *Bacillus subtilis* sporulation: cell-specific requirement of σ^{H} and σ^{A}: Spatiotemporally regulated proteolysis in *Bacillus*. *Mol Microbiol* **108**: 45–62.

Rochat, T., Nicolas, P., Delumeau, O., Rabatinová, A., Korelusová, J., Leduc, A., *et al.* (2012) Genome-wide identification of genes directly regulated by the pleiotropic transcription factor Spx in Bacillus subtilis. *Nucleic Acids Res* **40**: 9571–9583.

Rosenberg, A., Sinai, L., Smith, Y., and Ben-Yehuda, S. (2012) Dynamic Expression of the Translational Machinery during Bacillus subtilis Life Cycle at a Single Cell Level. *PLoS ONE* **7**: e41921.

Runde, S., Molière, N., Heinz, A., Maisonneuve, E., Janczikowski, A., Elsholz, A.K.W., *et al.* (2014) The role of thiol oxidative stress response in heat-induced protein aggregate formation during thermotolerance in *B acillus subtilis*: Thiol oxidation in protein aggregate formation. *Mol Microbiol* **91**: 1036–1052.

Spizizen, J. (1958) TRANSFORMATION OF BIOCHEMICALLY DEFICIENT STRAINS OF BACILLUS SUBTILIS BY DEOXYRIBONUCLEATE. *Proc Natl Acad Sci U S A* **44**: 1072–1078.

Youngman, P., Perkins, J.B., and Losick, R. (1984) A novel method for the rapid cloning in Escherichia coli of Bacillus subtilis chromosomal DNA adjacent to Tn917 insertions. *Mol Gen Genet* **195**: 424–433.

Supplementary Figures

Figure S1: Growth and survival of strains with deletions of individual heat shock regulators.

A: Growth of *B. subtilis* wild type, Δ*sigB*, Δ*ctsR*, Δ*hrcA* or Δ*spx* cells spotted on agar plates at 37 °C (left) or 55 °C (right) over night. **B:** Survival of wild type (black lines) and mutant strains Δ*sigB*, Δ*ctsR*, or Δ*hrcA* (red lines) during thermotolerance. Solid lines: 15 min pre-shock at 48 °C, dashed lines: no pre-shock. Means of normalized

log10 colony forming units and standard errors of 3 (mutants) or 48 (wild type) biological replicates are shown.

Figure S2: Relative levels of selected transcripts in mutant strains with increased or decreased Spx levels.

Samples of exponentially growing Δ*clpX or* Δ*spx*Δ*clpX* cells were subjected to western or northern blotting.

Figure S3: Genetic organisation of the transcriptional promoter fusions.

Transcriptional *rrn-gfp* fusions **(A)** or *rrnJ-lacZ* fusions **(B)** were integrated into the *amyE* locus. The Digoxigenin-11-UTP labelled RNA probe binds within the *gfp* or *lacZ* mRNA.

Figure S4: Spx acts similarly on all *rrn*-promoters

Northern blots of *rrn-gfp* transcripts. Mid-log cultures (OD_{600} of 0.3-0.35) of BHS923 - BHS938 cells, carrying transcriptional fusions of 7 *rrn* promoters to *gfp*, were divided and treated with or without 1 mM IPTG. Samples were withdrawn after 30 min and 2 µg total RNA per lane were subjected to northern blotting. The relative position of the 16 S and 23 S band is indicated.

Figure S5: Relative transcription of selected targets during oxidative stress and in Δ*spx*Δ*mgsR* mutant cells

Relative expression changes as determined by RT-qPCR. Means and standard errors of three biological replicates are shown. All strains carried *rrnJ* P1 -*lacZ* in the *amyE* site. **A:** Log-phase cultures of BHS220 (wild type *rrnJ* P1-*lacZ*) and BHS222 (Δ*spx rrnJ* P1-*lacZ*) were grown in minimal medium, divided and supplemented with or without 1 mM diamide for 15 min, then harvested. **B:** Log-phase cultures of BHS220 (wild type *rrnJ* P1-*lacZ*), BHS222 (Δ*spx rrnJ* P1-*lacZ*), BHS882 (Δ*mgsR rrnJ*

P1-*lacZ*) and BHS883 (Δ*spx* Δ*mgsR rrnJ* P1-*lacZ*) were divided and incubated at 37 °C or 50 °C for 15 min, then harvested. C: Northern blot showing the *rrnJ* P1-*lacZ* and *trxB* transcript. Log-phase cultures of BHS220 (wild type *rrnJ* P1-*lacZ*), BHS549 (*cxs*-1) and BHS550 (*cxs*-2) were divided and treated with or without 1 µg/mL vancomycin for 15 min and then harvested.

Figure S6: Impaired growth of strains synthesizing Spx^{DD} *in trans*

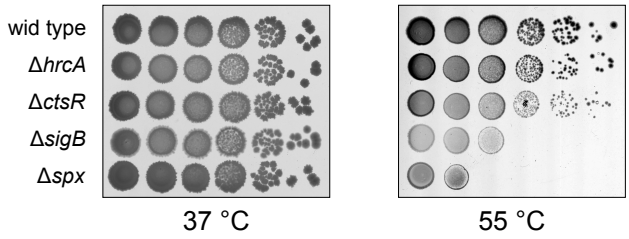
A: Growth of BHS148 (*amyE::Phy-spx^{DD}*) cells in LB medium. The mid-log culture was divided and supplemented with (red bar) or without (black bar) 1 mM IPTG. **B:** Growth of BHS148 (*amyE::Phy-spx^{DD}*), BHS535 (*amyE::Phy-spx^{DD} rpoA^{Y263C}*) and BHS536 (*amyE::Phy-spx^{DD} rpoA^{V260A}*) cells with (squares and dashed lines) or without (diamonds and solid lines) 1 mM IPTG added from the start.

Figure S7: Distribution of GFP-Spx fluorescence and cell size

Scatter plots and histograms showing the distribution and the relationship of GFP-Spx fluorescence and cell area among single cells of the experiment shown in Fig. 7.

Figure S1

A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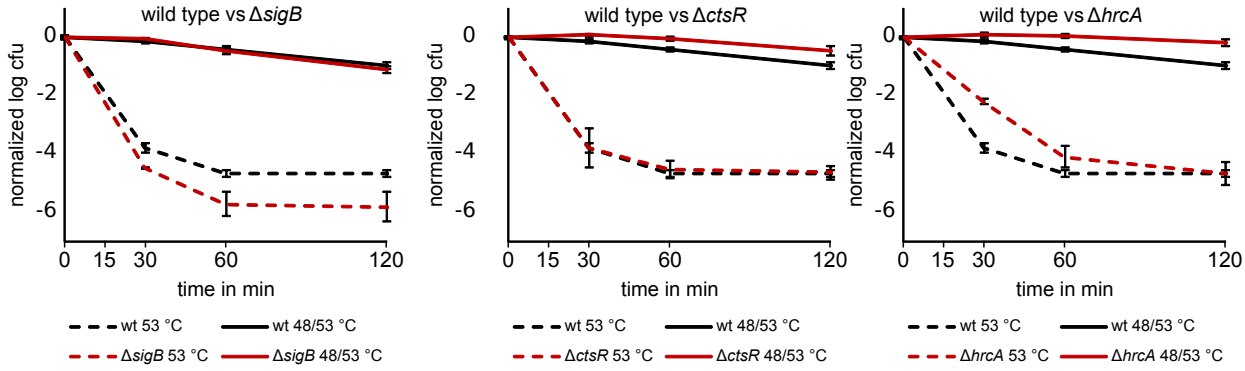
ΔctsR
ΔsigB
Δspx
37 °C
55 °C

B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wild type vs ΔctsR
wild type vs ΔhrcA
normalized log cfu
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-2
-4
-6
0 15 30 60 120
time in min
wt 53 °C
wt 48/53 °C
ΔsigB 53 °C
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ΔctsR 53 °C
ΔctsR 48/53 °C
ΔhrcA 53 °C
ΔhrcA 48/53 °C

Figure S2

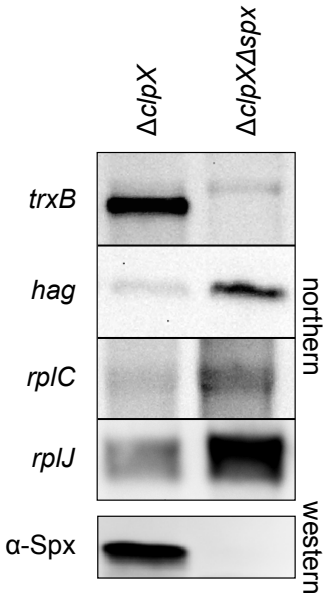

Figure S3

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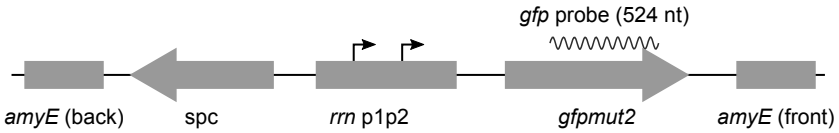
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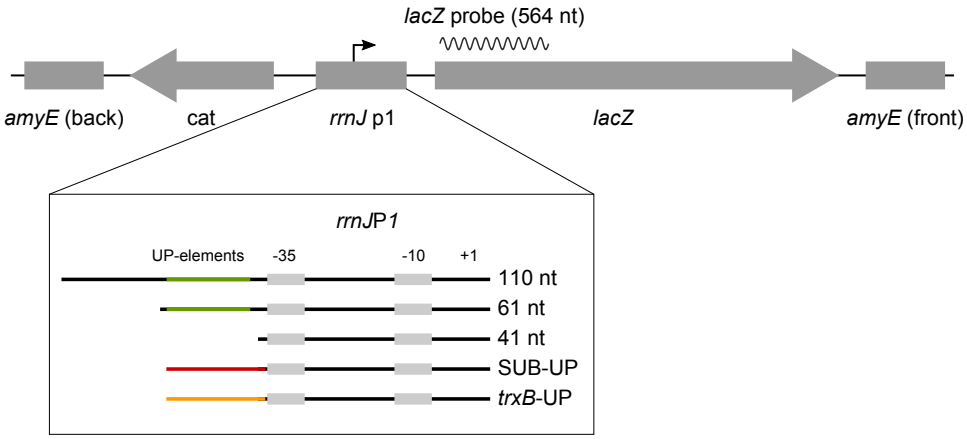
spc
rrn p1p2
gfpmut2
amyE (front)

B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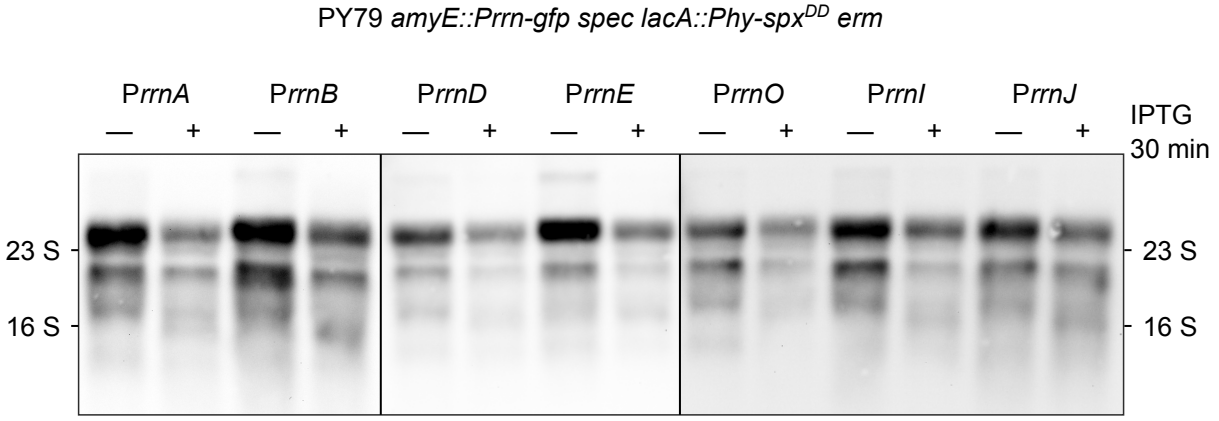
61 nt
41 nt
SUB-UP
trxB-UP

Figure S4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PrrnB
PrrnD
PrrnE
PrrnO
PrrnI
PrrnJ
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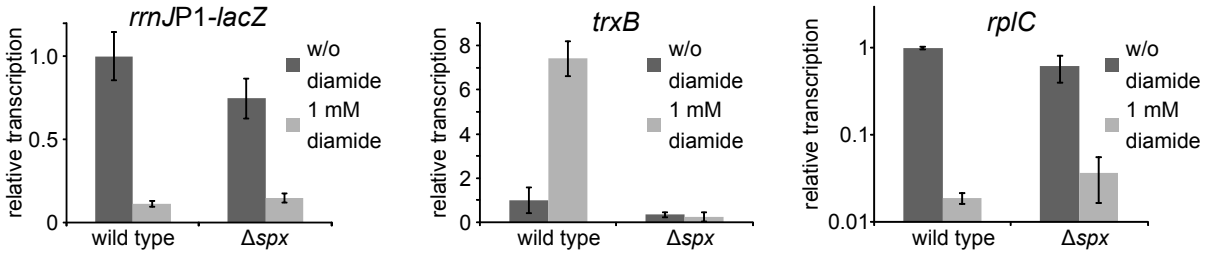
IPTG
30 min
23 S
16 S
23 S
16 S

Figure S5

A



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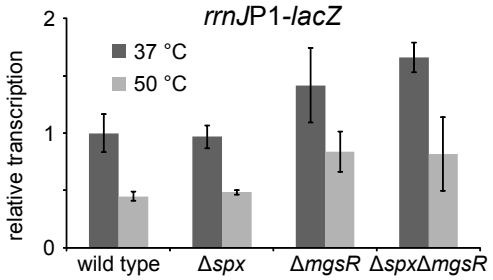

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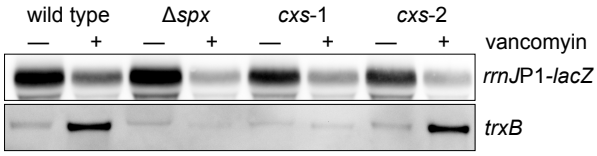
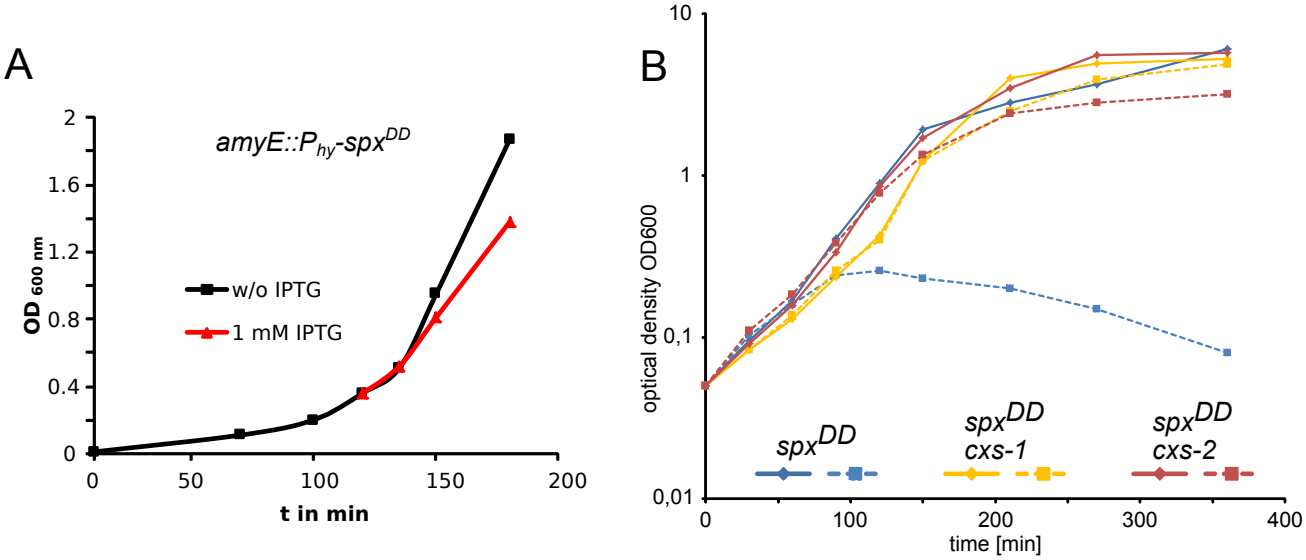


Figure S6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OD 600 nm
w/o IPTG
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t in min
B
optical density OD600
spx^DD
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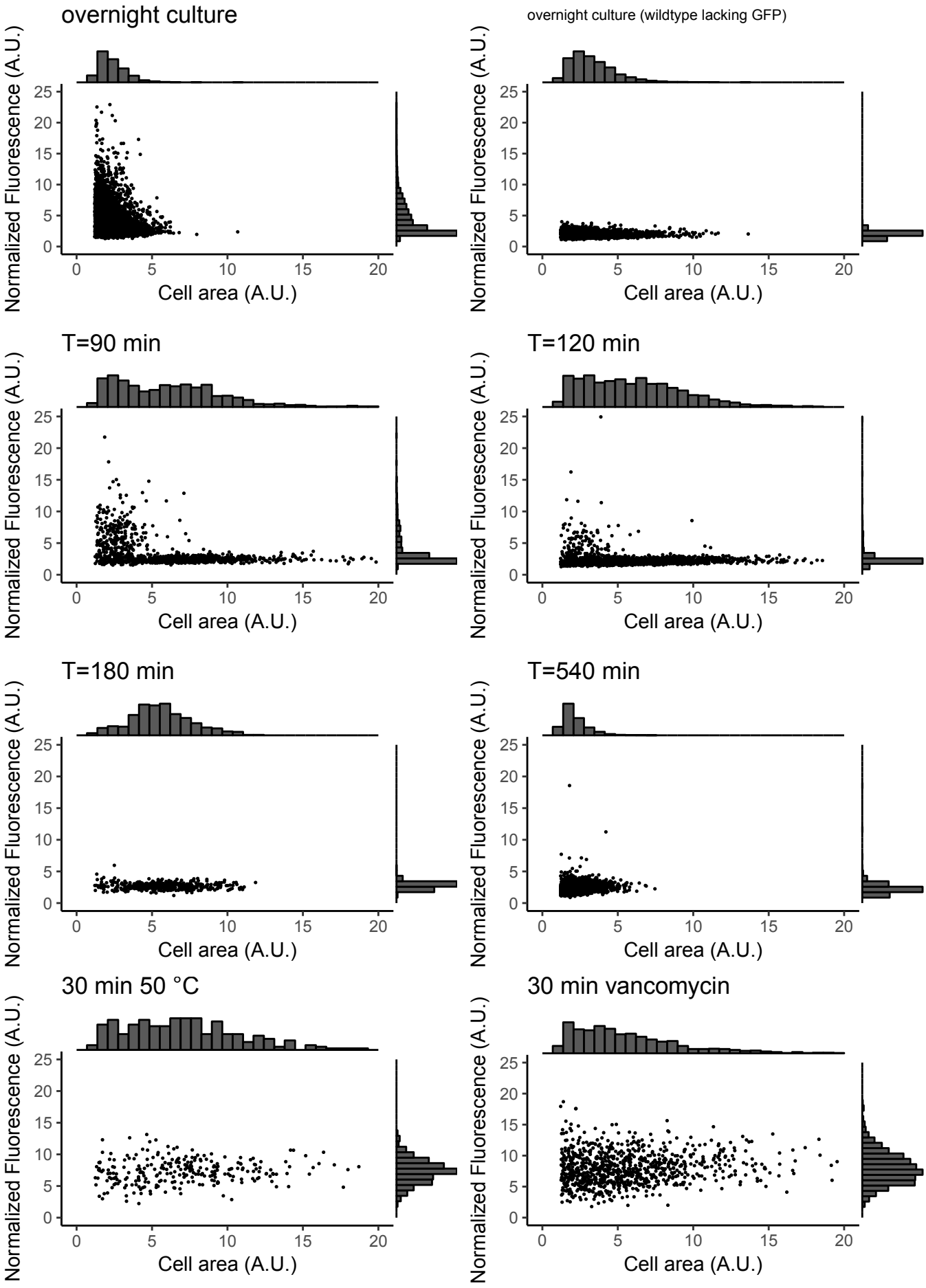
spx^DD cxs-2
time [min]

Figure S7


overnight culture
overnight culture (wildtype lacking GFP)
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T=120 min
T=180 min
T=540 min
30 min 50 °C
30 min vancomycin
Normalized Fluorescence (A.U.)
Cell area (A.U.)
0
5
10
15
20
25

2.2 Structure of the *Bacillus subtilis* hibernating 100S ribosome reveals the basis for 70S dimerization

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Authorship: Co- author

Share of work: 20 %

Contributions: DNW, GB, and KT designed and supervised the study. MA prepared the Bs100S sample for cryo-EM and performed all sucrose gradient analyses. OB collected the cryo-EM data. BB, MA, and SA processed the cryo-EM data. BB, MA, RB, and DNW interpreted the cryo-EM data. WS cloned and purified the BsHPF protein variants and performed the SEC and SLS. HS generated all BsHPF expression strains and performed Western blotting, growth curves, and ribosome pelleting assays. DNW, GB, and KT wrote the manuscript with comments from all authors.

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Structure of the *Bacillus subtilis* hibernating 100S ribosome reveals the basis for 70S dimerization

Bertrand Beckert[1,†], Maha Abdelshahid[1,†], Heinrich Schäfer[2], Wieland Steinchen[3], Stefan Arenz[1], Otto Berninghausen[1], Roland Beckmann[1], Gert Bange[3,*], Kürşad Turgay[2,**] & Daniel N Wilson[1,4,***]

Abstract

Under stress conditions, such as nutrient deprivation, bacteria enter into a hibernation stage, which is characterized by the appearance of 100S ribosomal particles. In *Escherichia coli*, dimerization of 70S ribosomes into 100S requires the action of the ribosome modulation factor (RMF) and the hibernation-promoting factor (HPF). Most other bacteria lack RMF and instead contain a long form HPF (LHPF), which is necessary and sufficient for 100S formation. While some structural information exists as to how RMF and HPF mediate formation of *E. coli* 100S (*Ec*100S), structural insight into 100S formation by LHPF has so far been lacking. Here we present a cryo-EM structure of the *Bacillus subtilis* hibernating 100S (*Bs*100S), revealing that the C-terminal domain (CTD) of the LHPF occupies a site on the 30S platform distinct from RMF. Moreover, unlike RMF, the *Bs*HPF-CTD is directly involved in forming the dimer interface, thereby illustrating the divergent mechanisms by which 100S formation is mediated in the majority of bacteria that contain LHPF, compared to some γ-proteobacteria, such as *E. coli*.

Keywords cryo-EM; hibernation; HPF; RMF; translation
Subject Categories Microbiology, Virology & Host Pathogen Interaction; Protein Biosynthesis & Quality Control; Structural Biology
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The EMBO Journal (2017) 36: 2061–2072

See also: **I Khusainov *et al*** (July 2017) *and* **RL Gonzalez Jr** (July 2017)

Introduction

The translational activity of the bacterial cell is able to respond rapidly to a variety of environmental cues. This is exemplified by the decrease in translational activity observed in bacteria entering into stationary growth phase due to stress conditions, such as nutrient deprivation. Under such circumstances, the decrease in translational activity is correlated with the appearance of 100S particles, which arise due to the dimerization of 70S ribosomes (Wada *et al*, 1990), reviewed by Yoshida and Wada (2014). In *E. coli*, 100S formation requires the presence of the ribosome modulation factor (RMF) and the hibernation-promoting factor (HPF, previously referred to as YhbH; Yamagishi *et al*, 1993; Wada *et al*, 1995; Maki *et al*, 2000; Ueta *et al*, 2005, 2008). Stationary phase *E. coli* cells also express a homolog of HPF (Fig 1A), termed YfiA (also referred to as pY or RaiA), which binds and inactivates 70S ribosomes (Agafonov & Spirin, 2004; Vila-Sanjurjo *et al*, 2004), and is antagonistic to RMF and HPF action by preventing 100S formation (Maki *et al*, 2000; Ueta *et al*, 2005). The hibernation state (Yoshida *et al*, 2002) appears to be important for bacterial survival since inactivation of the *rmf* gene leads to loss of viability in stationary phase cells (Yamagishi *et al*, 1993; Wada *et al*, 2000; Shcherbakova *et al*, 2015) as well as increased sensitivity to osmotic (Garay-Arroyo *et al*, 2000), heat (Niven, 2004), and acid stress (El-Sharoud & Niven, 2007).

Phylogenetic analyses have revealed that the presence of RMF and HPF is restricted to a subset of γ-proteobacteria, including *E. coli*, whereas the majority of other bacteria lack both RMF and YfiA, and instead contain a long form of HPF (LHPF; Fig 1A; Ueta *et al*, 2008, 2013; Yoshida & Wada, 2014). LHPFs comprise an N-terminal domain (NTD) homologous to the short form HPF (SHPF) and a unique C-terminal domain (CTD; Fig 1A), which was proposed to have weak homology with RMF (Ueta *et al*, 2010). LHPFs have been shown to be necessary and sufficient for 100S formation in a variety of different bacteria, including *Staphylococcus aureus* (Ueta *et al*, 2010, 2013; Basu & Yap, 2016), *Lactobacillus paracasei*, *Thermus thermophilus* (Ueta *et al*, 2010, 2013), *Lactococcus lactis* (Puri *et al*, 2014), and *B. subtilis* (Tagami *et al*, 2012; Akanuma *et al*, 2016). Unlike *E. coli* SHPF-100S (*Ec*100S), low levels of LHPF-containing 100S are also observed in exponentially growing cells (Ueta *et al*, 2010, 2013; Akanuma *et al*, 2016). Proteomics studies indicate that expression levels of *Bs*LHPF increase under conditions of nutrient deprivation, but also in response to antibiotics, heat, salt, and ethanol stress (Drzewiecki *et al*, 1998;

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†These authors contributed equally to this work

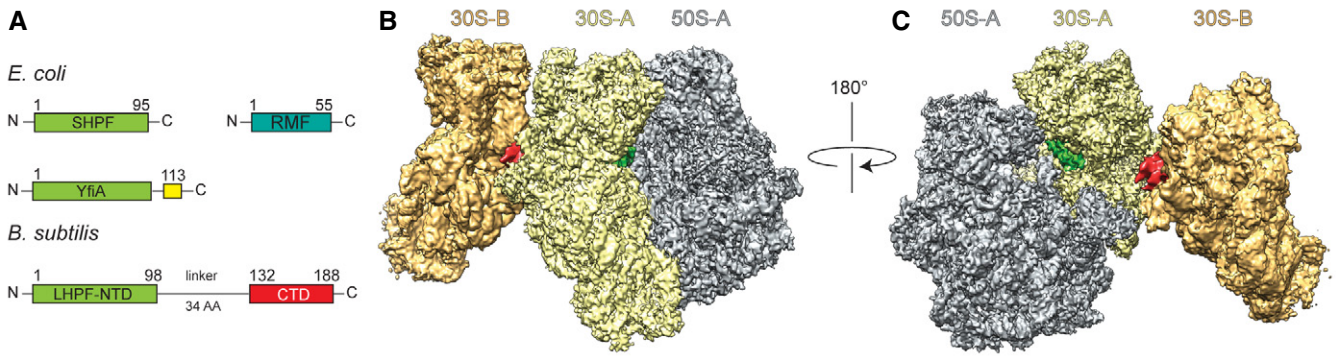

Figure 1. Cryo-EM reconstruction of the *Bs*70S-30S subcomplex.

A Schematic representation of the domain structure of *Escherichia coli* short form HPF (SHPF), RMF, and YfiA (C-terminal extension in yellow) compared to *Bacillus subtilis* long form HPF (LHPF) harboring an N-terminal (NTD, green) and C-terminal domains (CTD, red).

B, C Two views of the cryo-EM map of the *Bs*70S-30S subcomplex, with separated densities for the 30S-A (yellow), 50S-A (gray), 30S-B (orange), and additional densities in green and red.

Reiss *et al*, 2012; Tagami *et al*, 2012). In *Listeria monocytogenes*, LHPF is necessary for tolerance of bacteria to aminoglycoside antibiotics during stationary phase (McKay & Portnoy, 2015) and for optimal fitness and pathogenesis (Kline *et al*, 2015).

Cryo-EM and cryo-electron tomography (cryo-ET) structures of the *Ec*100S have revealed that the 70S monomers interact with each other via the back of the 30S subunits (Kato *et al*, 2010; Ortiz *et al*, 2010), consistent with earlier negative stain images (Wada, 1998; Yoshida *et al*, 2002). Unfortunately, the low resolution (18–38 Å) of these structures was insufficient to resolve the binding positions of the RMF and SHPF proteins within the *Ec*100S (Kato *et al*, 2010; Ortiz *et al*, 2010). However, structures of *E. coli* SHPF and RMF were subsequently determined on the *T. thermophilus* 70S ribosome by X-ray crystallography (Polikanov *et al*, 2012), providing insight into how SHPF and RMF dimerize 70S ribosomes and inactivate translation in γ-proteobacteria. To date, there is, however, little structural information available as to how LHPFs interact with 70S ribosomes to mediate 100S formation in the majority of bacteria other than *E. coli* and its close relatives.

Here we present a cryo-EM structure of the *B. subtilis* 100S particle (*Bs*100S) revealing the binding site for the *Bs*HPF (also referred to as YvyD). The *Bs*HPF-NTD binds in a position overlapping the mRNA, A- and P-tRNAs, analogous to YfiA, SHPF, and the NTD of the LHPF from spinach chloroplasts (Vila-Sanjurjo *et al*, 2004; Sharma *et al*, 2007, 2010; Polikanov *et al*, 2012; Graf *et al*, 2016; Bieri *et al*, 2017), indicating how LHPFs inhibit translation (Ueta *et al*, 2013; Basu & Yap, 2016). Unexpectedly, we observe that the *Bs*HPF-CTD forms a homodimer with the CTD of the *Bs*HPF from the second 70S ribosome, thus providing a structural basis for LHPF-mediated 100S formation. Our findings reveal that 100S formation mediated by RMF and HPF in γ-proteobacteria, such as *E. coli*, is mechanistically unrelated to 100S formation mediated by LHPF in the majority of other bacteria.

Results

Cryo-EM structure of *Bs*100S

*Bs*100S ribosomal particles were isolated from lysates of late exponential phase cells using sucrose density gradient centrifugation (Fig EV1A, see Materials and Methods). Negative stain electron microscopy images of the isolated *Bs*100S revealed the characteristic dimer arrangement of 70S monomers interacting via their 30S subunits (Fig EV1B), as observed previously for *B. subtilis* (Tagami *et al*, 2012), *Lactococcus lactis* (Puri *et al*, 2014), but distinct from *Ec*100S (Wada, 1998; Yoshida *et al*, 2002; Kato *et al*, 2010). The presence of the *Bs*HPF (YvyD) in the *Bs*100S was further confirmed using mass spectrometry. The LHPF-containing 100S particles were then subjected to single particle cryo-EM analysis (see Materials and Methods). Processing of the *Bs*100S was performed by aligning the 70S ribosomes within each 100S to a vacant 70S reference. The box size was maintained large enough so that the majority of the small 30S subunit of the second 70S ribosome in the dimer would also be represented during the reconstruction. The initial reconstructions revealed significant flexibility in the 100S, which was indicated by a stable aligned ribosome (70S-A) with a blurred density for the second 70S ribosome (70S-B). By implementing *in silico* sorting procedures, we were able to obtain a subpopulation of 100S particles with better-defined density for the 70S-B ribosome (Fig EV2). Subsequent refinement yielded a cryo-EM reconstruction of the *Bs*70S-30S subcomplex (Fig 1B and C) with an average resolution of 3.8 Å (Fig EV3A–D and Table EV1). Local resolution calculations indicate that the resolution for the 70S-A monomer ranges in the core between 3.5 and 5.0 Å, whereas, as expected, the resolution for 70S-B is worse, ranging between 5.0 and 10 Å (Fig EV3B and C). The cryo-EM map was fitted with the molecular model of the *B. subtilis* 70S ribosome (Sohmen *et al*, 2015), revealing that the 70S-A monomer adopts a classic non-rotated state, as observed previously (Sohmen *et al*, 2015). Moreover, the swivel of head observed when *E. coli* SHPF and RMF bind to *T. thermophilus* 70S ribosomes (Polikanov *et al*, 2012) is not observed in the *Bs*100S, indicating that dimerization of *B. subtilis* 70S ribosomes, unlike *E. coli*, does not require head movement. After fitting of the 70S models, two unassigned densities remained, one located within the intersubunit space of the 70S-A ribosome and a second located on the back of the 30S platform at the interface of the 70S-A and 70S-B ribosomes (Fig 1B and C).

Binding site of the *Bs*HPF-NTD on the small 30S subunit

The additional map density within the intersubunit space located between the head and body of the 30S subunit was assigned to the N-terminal domain of *Bs*HPF (*Bs*HPF-NTD; Fig 2A). This was based on the high sequence similarity of the *Bs*HPF-NTD with *E. coli* YfiA and HPF (Fig EV1C), both of which were shown to bind to this region of the ribosome (Vila-Sanjurjo *et al*, 2004; Polikanov *et al*, 2012). The local resolution of the *Bs*HPF-NTD ranged between 3.5 and 5.0 Å (Fig EV3F–G), enabling an unambiguous fit of the homology model to the density (Fig 2B). Aligning the *E. coli* SHPF-70S structure (Polikanov *et al*, 2012) to the 70S-A ribosome in the *Bs*100S based on the 16S rRNA revealed the expected similarity in their binding positions (Fig 2C). As noted previously for *E. coli* YfiA and HPF (Vila-Sanjurjo *et al*, 2004; Polikanov *et al*, 2012) and for the NTD of the LHPF from Spinach chloroplast (Sharma *et al*, 2007, 2010; Graf *et al*, 2016; Bieri *et al*, 2017), the binding position of *Bs*HPF-NTD overlaps with the mRNA and anticodon-stem loop regions of tRNAs bound in the ribosomal A- and P-sites (Fig 2D), thus explaining the observed inhibitory effect by LHPFs when added to *in vitro* translation assays (Ueta *et al*, 2013; Basu & Yap, 2016). The *Bs*HPF-NTD is connected by a 34 aa linker to the CTD (Fig 1A). Map density for the linker region was not observed in the cryo-EM map of the *Bs*100S, indicating that it is highly flexible. An exception is the 5–6 aa stretch of the linker region that directly follows the terminal α-helix of the *Bs*HPF-NTD (Fig 2B). Map density for this N-terminal part of the linker passes, analogous to mRNA, through the opening created by the β-hairpin of ribosomal protein S7 and helix h23 of the 16S rRNA, and extends in the general direction of the platform cavity at the back of the 30S subunit (Fig EV4).

*Bs*HPF-CTD is present as a dimer on the small 30S subunit

Given the general direction of the linker, we assigned the additional density located on the back of the 30S platform to the *Bs*HPF-CTD (Fig 3A and B). It was possible to generate a homology model for the *Bs*HPF-CTD based on the deposited crystal structure of the LHPF-CTD from a closely related Firmicute, *Clostridium acetobutylicum* (PDB ID 3KA5; Fig EV1D). Curiously, the *C. acetobutylicum* LHPF-CTD is present as a dimer in the crystal, and it was possible to make an unambiguous rigid body fit of the homology model of the *Bs*HPF-CTD dimer into the unassigned map density of the cryo-EM map (Fig 3C). We note that while the structurally conserved *L. monocytogenes* HPF-CTD (PDB ID 3K2T) appears as a monomer in the asymmetric unit, the homodimer forms across the crystallographic twofold symmetry. This suggests that the LHPF-CTDs are not only dimeric on the ribosome, but are likely to be dimeric in solution. To investigate this further, we performed size-exclusion chromatography (SEC) on the recombinantly expressed and purified wild-type *Bs*HPF and *Bs*HPF variants (see Materials and Methods). Analysis of the full-length *Bs*HPF and the *Bs*HPF-CTD revealed that they have apparent molecular masses of 56 and 14 kDa, respectively, rather than the expected 23 kDa and 8 kDa (Fig 3D–G), indeed suggesting that both proteins are dimeric in solution as well as on the ribosome. The apparent migration behavior of *Bs*HPF on SEC reflects the elongated shape of the dimer as also seen in our cryo-EM structure of the *Bs*100S. Based on the structures of the dimeric *C. acetobutylicum* and *L. monocytogenes*

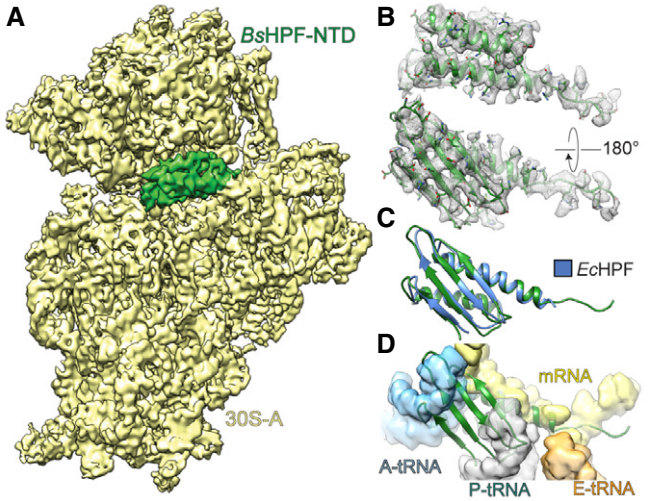

Figure 2. Interaction of the *Bs*HPF-NTD with the ribosome.

A Interface view of cryo-EM map of the 30S-A (yellow) from the *Bs*70S-30S subcomplex with separated *Bs*HPF-NTD density (green).

B Map density (gray mesh) with model of *Bs*HPF-NTD (green).

C, D Comparison of *Bs*HPF-NTD (green) with (C) *Escherichia coli* SHPF (*Ec*HPF, blue; Polikanov *et al*, 2012), and (D) mRNA (yellow surface), A- (cyan), P- (gray), and E-tRNAs (orange; Jenner *et al*, 2011).

LHPF-CTD, we rationalized that the highly conserved Phe160 in the *Bs*HPF-CTD is critical for dimerization (Fig 3H). Phe160 is present within the very hydrophobic dimer interface where it forms stacking interactions with Phe160 of the second monomer (Fig 3H). We predicted that a mutation of Phe160 to Glu (F160E) would disrupt the dimer interface via introduction of a negative charge into the hydrophobic environment. To test this, we also subjected the full-length *Bs*HPF-F160E protein to SEC (Fig 3D and E), revealing that the protein eluted with an apparent molecular mass of 40 kDa, smaller than the 56 kDa observed for the wild-type *Bs*HPF (Fig 3G). Although 40 kDa is larger than the expected size of 22.8 kDa, we believe this is due to retardation of the NTD and subsequent linker. Indeed, a *Bs*HPF variant lacking the CTD (*Bs*HPF-NTD) eluted with an apparent molecular mass of 28 kDa (rather than the expected 13.1 kDa; Fig 3G). This observation is in good agreement with structural information on the NTDs of other hibernation factors showing a non-globular shape (Polikanov *et al*, 2012). Our conclusions based on SEC were also confirmed using static light scattering (SLS), revealing the full-length *Bs*HPF had an absolute molecular mass of 42.8 ± 0.9 kDa, corresponding with a dimer (46 kDa), whereas the mass of the *Bs*HPF-F160E variant (28 ± 2.1 kDa) was more consistent with a monomer (22.8 kDa; Fig 3G). Taken together, our biochemical data clearly show that *Bs*HPF forms a homodimer in solution that is mediated via its CTD.

Dimerization of 70S ribosomes via the *Bs*HPF-CTD

While the limited resolution of the *Bs*HPF-CTD (Fig EV3H and I) does not allow a detailed analysis of the contacts with the ribosomal components to be made, the fitted model nevertheless enables a general description of the interaction mode (Fig 4A). The *Bs*HPF-CTD appears to interact exclusively with ribosomal proteins S2 and

S18 and does not establish contact with the 16S rRNA. Importantly, each *Bs*HPF-CTD monomer contacts S2 from the 70S to which the corresponding *Bs*HPF-NTD is bound, whereas the interaction with the N-terminal extension of S18 is from the second 70S ribosome (Fig 4A). The 100S dimer is also stabilized by direct interactions between the 70S-A and 70S-B monomers (Fig 4A and B). In addition to the contacts established between the N-terminal helix of S2 and the N-terminal extension of S18, the N-terminal β-hairpin and proximal region of the α2-helix of S2 establish a large interaction surface with the stem-loop of helix h26 of the 16S rRNA of the second 70S (Fig 4B). Thus, the dimerization of the HPF-CTDs stabilizes and facilitates direct interaction between the 70S-A and 70S-B monomers in the *Bs*100S. Our findings highlight the importance of the *Bs*HPF-CTD for 70S dimerization, and therefore 100S formation, which is in complete agreement with biochemical studies demonstrating that truncation of the CTD from LHPF leads to loss of 100S formation (Puri *et al*, 2014; Basu & Yap, 2016). Moreover, it was reported that the CTD of the LHPF from *Lactococcus lactis* can dimerize *E. coli* 70S ribosomes, but only when acting in concert with the SHPF from *E. coli* (Puri *et al*, 2014). This observation supports to some extent the previous assertion that the HPF-CTD functions analogously to RMF; an assertion that was partly based on proposed sequence homology between HPF-CTD and RMF (Ueta *et al*, 2010). However, comparison of the structures of *Bs*HPF-CTD with that of RMF on the ribosome (Polikanov *et al*, 2012) reveals that there is no structural similarity in terms of the protein fold and, despite both binding at the platform region at the back of the 30S subunit, there is no overlap in their binding sites on the ribosome (Fig 4C). The binding position of RMF was suggested to inhibit translation by sterically preventing formation of the Shine-Dalgarno-helix (SD-helix) between the 5′ end of the mRNA and the 3′ end of the 16S rRNA (Polikanov *et al*, 2012). In contrast, the HPF-CTD does not overlap with the SD-helix (Fig 4D), although we cannot exclude the possibility that the flexible linker of *Bs*HPF traverses the RMF binding site since it was not visualized in the cryo-EM map.

Importance of the linker–CTD for 100S formation

To assess the importance of the linker and CTD of *Bs*HPF for 100S formation *in vivo*, we generated a *B. subtilis* 168 strain where the *yvyD* gene was inactivated (Δ*Bs*HPF), as confirmed by Western blotting using antibodies specific to *Bs*HPF (Fig 5A). We then re-introduced the wild-type *yvyD* gene, as well as *yvyD* variants, into the *amyE* locus and monitored the IPTG-induced expression of the *Bs*HPFs (Fig 5A). To investigate the importance of the linker between the NTD and CTD of *Bs*HPF, we generated Δ*Bs*HPF strains expressing *Bs*HPF deletion variants lacking 10 aa (*Bs*HPF-LΔ10AA, lacking residues 110–119) or 20 aa (*Bs*HPF-LΔ20AA, lacking residues 105–124) from the central region of the linker (Fig 5A). In addition, we generated a *Bs*HPF variant bearing the F160E mutation in the CTD (*Bs*HPF-F160E), which interferes with homodimerization (Fig 3G). Western blotting of cell extracts from stationary phase bacteria indicated that all *Bs*HPF variants inserted into the *amyE* locus were expressed in the presence of IPTG at similar levels to wild-type *Bs*HPF observed in the parental *Bs*168 strain (Fig 5A). Pelleting experiments indicated that full-length *Bs*HPF co-migrated with the ribosome fraction as expected, as did the *Bs*HPF-LΔ10AA variant (Fig 5B). In contrast, the *Bs*HPF-LΔ20AA

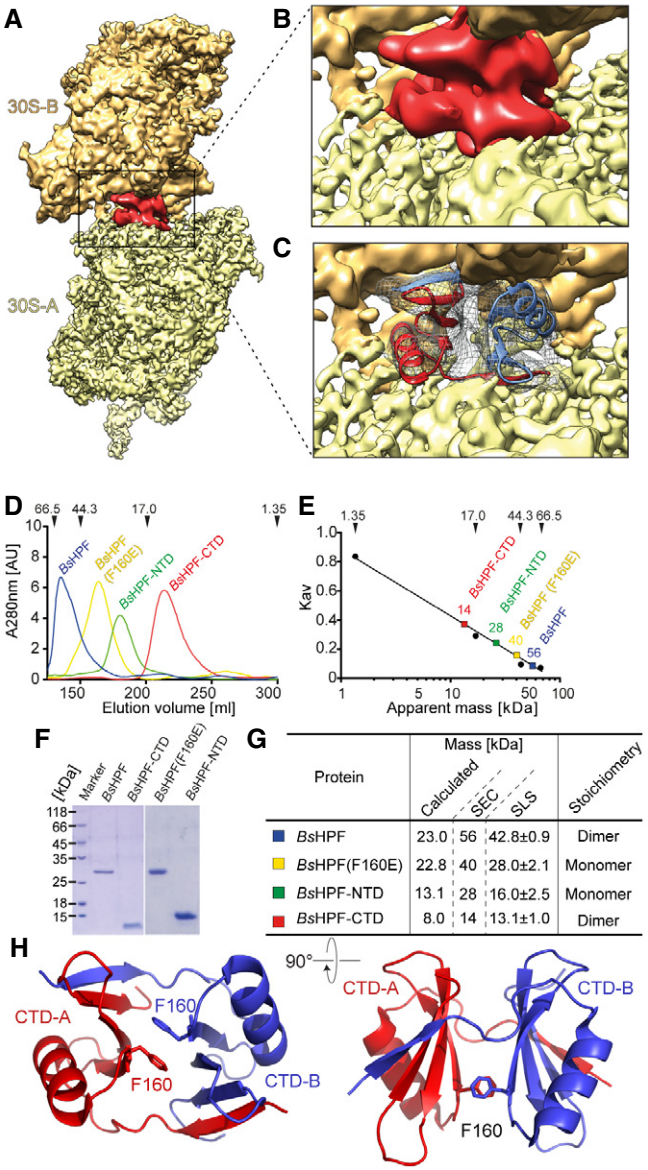

Figure 3. Binding site of dimeric LHPF-CTD on the *Bs*70S-30S subcomplex.

A Cryo-EM map of the 30S-A (yellow) from the *Bs*70S-30S subcomplex with separated LHPF-CTD density (red).

B, C Density (gray mesh) with fitted model of dimeric LHPF-CTD with monomers from 70S-A and 70S-B colored red and blue, respectively.

D Gel-filtration profiles of full-length *Bs*HPF (blue), *Bs*HPF-F160E (yellow), *Bs*HPF-NTD (green), and *Bs*HPF-CTD (red). Arrows indicate the molecular mass in kDa of the size standard.

E Standard curve with estimated molecular masses for full-length *Bs*HPF (blue), *Bs*HPF-F160E (yellow), *Bs*HPF-NTD (green), and *Bs*HPF-CTD (red). Arrows indicate the molecular mass in kDa of the size standard.

F Coomassie-stained SDS–PAGE of the peak fractions containing *Bs*HPF or its variants.

G Table summarizing the actual and apparent molecular mass of proteins in (D-F). Size-exclusion chromatography (SEC) and static light scattering (SLS) determined the apparent and absolute MWs, respectively. "Stoichiometry" indicates whether *Bs*HPF and its variants exist as mono- or homodimer.

H Homology model of the *Bs*HPF-CTD homodimer illustrating the position of Phe160 (F160) at the dimer interface.

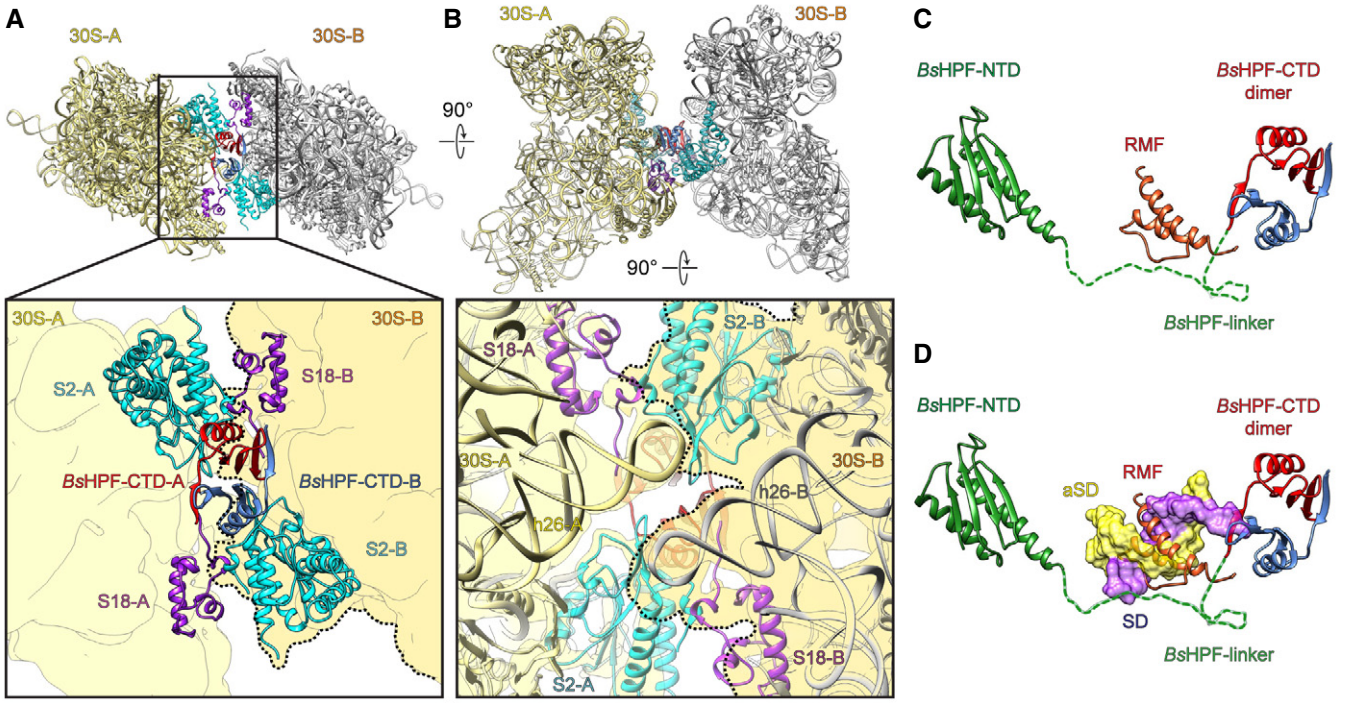

Figure 4. Dimerization interface of the *Bs*70S-30S subcomplex.

A, B Distinct views of the dimer interface between 30S-A (yellow) with *Bs*HPF-CTD-A (red) and 30S-B (gray, darker yellow with dashed line in zoomed panel) with *Bs*HPF-CTD-B (blue). Ribosomal proteins S2 (cyan), S18 (purple), and 16S rRNA are shown only, and the surface outline of the 30S subunit is included schematically for reference.

C, D Binding site of *Bs*HPF-NTD (green) and dimeric *Bs*HPF-CTD (red, blue) relative to (C) RMF (orange; Polikanov *et al*, 2012) and (D) SD–anti-SD helix (yellow-purple surface; Sohmen *et al*, 2015). The dashed line indicates the linker and is shown only to illustrate that the 34 amino acids are more than sufficient to connect the NTD and CTD; however, no density for the linker was observed, suggesting it does not adopt a defined conformation on the ribosome.

and *Bs*HPF-F160E variants had significantly reduced association with the ribosomal pellets (Fig 5B), suggesting that the deletion of 20 aa within the linker or preventing homodimerization via the CTD disrupts the interaction of *Bs*HPF with the ribosome. This is consistent with previous studies using *S. aureus* LHPF where C-terminal truncations of 42 aa (ΔCTD) and 90 aa (Δlinker–CTD) led to progressive loss in ribosome binding (Basu & Yap, 2016).

We next employed sucrose density gradient centrifugation to monitor the formation of 100S ribosomes using the different *Bs*168 strains (Fig 5C–G). As controls, the wild-type *Bs*168 strain was harvested during exponential phase, where a large 70S peak and lots of polysomes were observed, but little or no 100S were evident (Fig 5C). In contrast, a short heat treatment of the wild-type cells led to a complete loss of polysomes and the appearance of a prominent 100S peak (Fig 5C), as observed previously for *B. subtilis* (Akanuma *et al*, 2016). Formation of 100S was never observed in the Δ*Bs*HPF strain (Fig 5C) regardless of the stress conditions tested, in agreement with the strict dependence on *Bs*HPF for 70S dimerization (Akanuma *et al*, 2016). However, when the *yvyD* gene was reintroduced into the *amyE* locus of the Δ*Bs*HPF strain, 100S formation (and loss of polysomes) was observed, but only when *Bs*HPF expression was induced by the presence of IPTG (Fig 5D). No significant increase in the 100S peak, nor reduction in polysomes, was observed when expression of the *Bs*HPF-LΔ20AA variant was induced (Fig 5E), consistent with the lack of ribosome binding (Fig 5B). Surprisingly, similar results were obtained for *Bs*HPF-LΔ10AA (Fig 5F), suggesting that although the *Bs*HPF-LΔ10AA can still bind to the ribosome (Fig 5B), it is impaired in 100S formation. *Bs*HPF variants where the 10 aa or 20 aa were substituted (rather than deleted) by glycine-serine (GS) repeats, creating *Bs*HPF-$(GS)_5$ or *Bs*HPF-$(GS)_{10}$, respectively, also led to both a reduction in ribosome binding and 100S formation (Fig EV4C–E), suggesting that the sequence and not just the length of the linker is critical for *Bs*HPF activity. Lastly, we also monitored 100S formation in the *Bs*168 strain expressing the *Bs*HPF-F160E variant. As expected, no increase in the 100S peak or decrease in the polysome peaks was observed upon *Bs*HPF-F160E induction (Fig 5G), indicating that *Bs*HPF-CTD homodimerization is necessary for 100S formation.

Further support for the loss of activity of the *Bs*HPF-LΔ20AA and *Bs*HPF-F160E variants comes from growth assays. Compared to the wild-type *Bs*168 strain, the Δ*Bs*HPF strain exhibits a lag phase when stationary phase cells are diluted into fresh media (Fig 5H), as reported previously (Akanuma *et al*, 2016). The lag phenotype can be restored by expression of wild-type *Bs*HPF, but not by *Bs*HPF-LΔ20AA and *Bs*HPF-F160E variants (Fig 5H). Curiously, the *Bs*HPF-LΔ10AA variant also rescued the growth phenotype (Fig 5H), suggesting that ribosome binding rather than

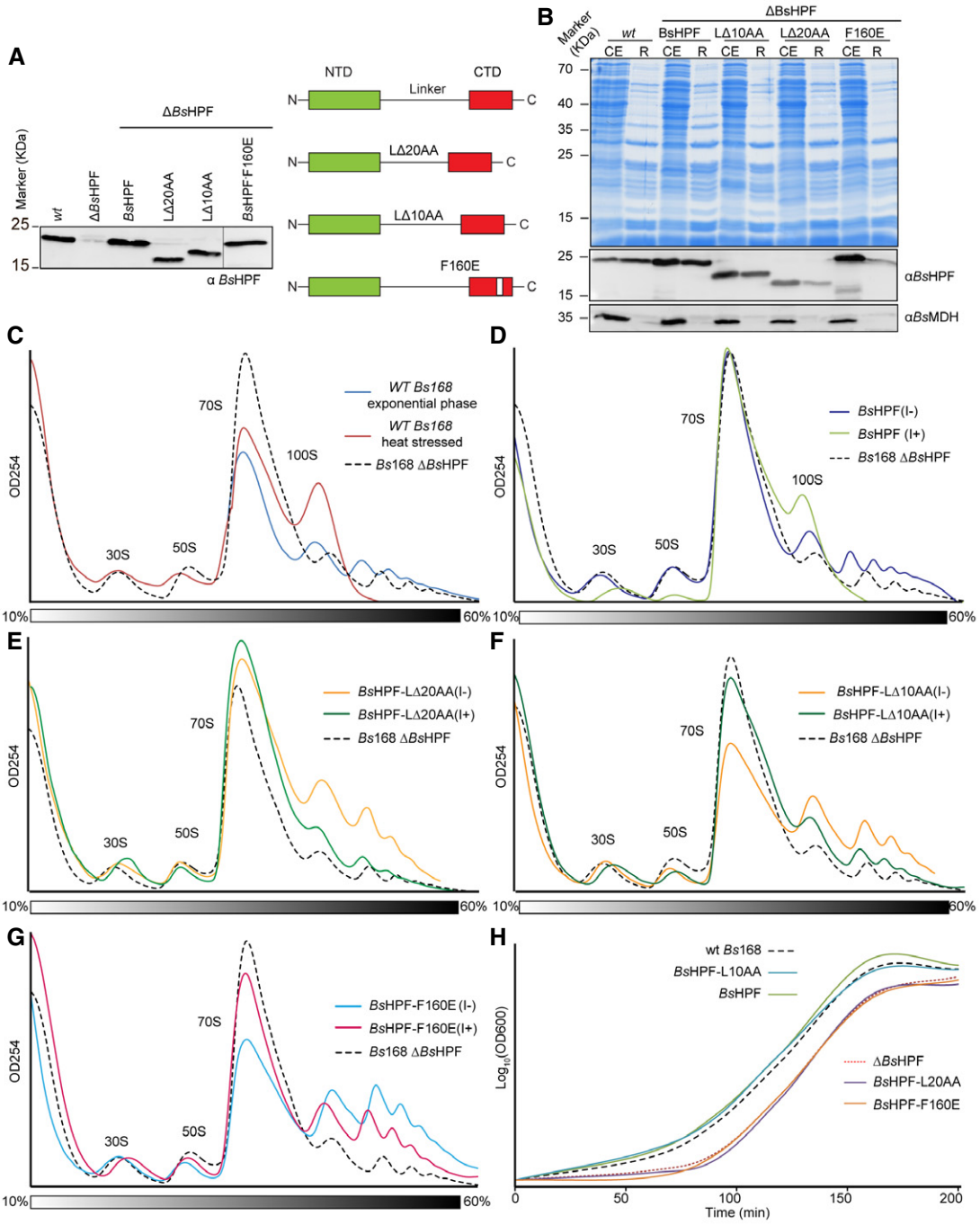

Figure 5. Monitoring 100S formation *in vivo* for *Bs*HPF variants.

A Western blot using antibodies raised against *Bs*HPF to assess the levels of *Bs*HPF in cell extracts of wild-type *Bs*168 (wt), Δ*Bs*HPF, and Δ*Bs*HPF strains expressing either wild-type *Bs*HPF or *Bs*HPF-LΔ20AA, *Bs*HPF-LΔ10AA, *Bs*HPF-F160E variants.

B Coomassie (above) and Western blot of cell extracts (CE) and ribosome pelleted fractions (R) of the wild-type *Bs*168 (wt) strain or the Δ*Bs*HPF strains expressing either wild-type *Bs*HPF, *Bs*HPF-LΔ10AA, *Bs*HPF-LΔ20AA, and *Bs*HPF-F160E.

C Sucrose gradient profiles of cell extracts from the wild-type *Bs*168 (wt) strain in exponential phase (blue) or heat stressed (red), compared with the extract from the *Bs*168 Δ*Bs*HPF strain (dashed line).

D–G Sucrose gradient profiles of cell extracts from the (D) *Bs*168 Δ*Bs*HPF amyE::*Bs*HPF strain, (E) *Bs*168 Δ*Bs*HPF amyE::*Bs*HPF-LΔ20AA strain, (F) *Bs*168 Δ*Bs*HPF amyE::*Bs*HPF-LΔ10AA strain, and (G) *Bs*168 Δ*Bs*HPF amyE::*Bs*HPF-F160E strain in the absence (I−) or presence (I+) of IPTG. The dashed line of the *Bs*168 Δ*Bs*HPF strain from (C) is shown for reference.

H Growth curves illustrating the recovery from stationary phase of the wild-type *Bs*168 (wt), Δ*Bs*HPF, and Δ*Bs*HPF strains expressing either wild-type *Bs*HPF or *Bs*HPF-LΔ20AA, *Bs*HPF-LΔ10AA, *Bs*HPF-F160E variants.

Source data are available online for this figure.

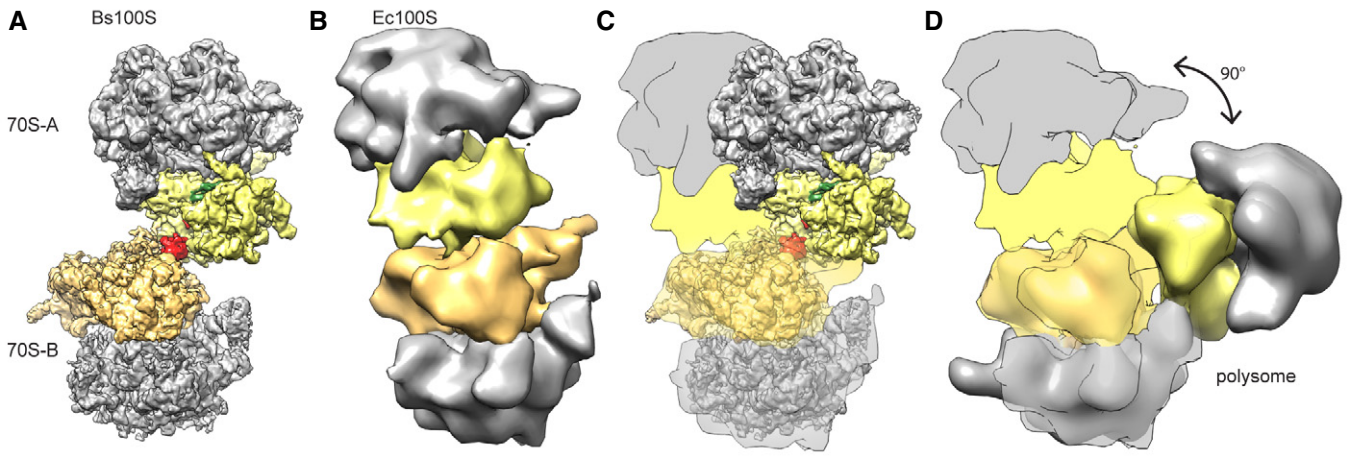

Figure 6. Spatial organization of *Bs*100S, *Ec*100S, and polysomes.

A–D Comparison of the 70S-A and 70S-B monomer arrangement in (A) *Bs*100S, compared with (B, C) *Ec*100S (Ortiz *et al*, 2010) and (D) *Escherichia coli* polysomes (Brandt *et al*, 2009). The 30S-A (yellow), 30S-B (orange), 50S (gray), *Bs*HPF-NTD (green), and *Bs*HPF-CTD (red) are colored for reference, and schematics of the *Ec*100S are presented in (C) and (D) for ease of comparison.

100S formation may be important for efficient stationary phase recovery.

Distinct arrangement of 70S monomers in the *Bs*100S

In order to obtain a reconstruction of the complete *Bs*100S particle to compare with previous *Ec*100S reconstructions, we also reprocessed the cryo-EM data using a larger box size that completely encompassed both 70S monomers (Fig EV2). Despite the inherent flexibility between the 70S monomers, we were able to obtain a reconstruction of the *Bs*100S (Fig 6A) with an average resolution of 6.2 Å (Fig EV5A–C). The relative orientation of the 70S-A and 70S-B monomers within the *Bs*100S was related by a 180° rotational symmetry with the axis of rotation centered on the dimeric *Bs*HPF-CTD (Fig EV5D). As expected, we observed additional density for the HPF-NTD within the intersubunit space and for the HPF-CTD at the back of the 30S subunit (Fig EV5E and F). Comparison of the *Bs*100S with the previous cryo-EM and cryo-ET reconstructions of the *Ec*100S (Fig 6B) revealed a dramatically different monomer arrangement (Fig 6C). While *Ec*100S dimerization involves a "back-to-back" interaction of the 30S subunits of each 70S monomers, *Bs*100S dimerization involves a more "side-to-side" (platform-to-platform) interaction of the 30S subunits. In the *Ec*100S, dimerization is proposed to be stabilized by contacts between S2 on one 70S with the cavity formed by S3/S4/S5 on the other (Kato *et al*, 2010), which may be facilitated by a swivel movement of the head of the 30S subunit that was observed upon RMF binding (Polikanov *et al*, 2012). In contrast, the head position of the *Bs*100S is identical to that observed in the classic post-translocational state ribosome (Sohmen *et al*, 2015) and, unlike RMF, the *Bs*HPF-CTD directly comprises part of the dimerization interface. The spatial orientation of the 70S monomers in the *Bs*100S (Fig 6A) could be considered intermediate between that observed in the *Ec*100S (Fig 6B; Kato *et al*, 2010) and the orientation observed in the cryo-ET reconstructions of *E. coli* polysomes (Fig 6D; Brandt *et al*, 2009).

Discussion

The appearance of hibernating 100S ribosomes is a near universal response of bacteria to adapt to a variety of stress conditions, in particular nutrient limitation (Ueta *et al*, 2013; Yoshida & Wada, 2014). Under these circumstances, bacteria employ second messenger signaling molecules, such as (p)ppGpp and cyclic AMP (cAMP), to reprogram the cellular activity network, down-regulating genes associated with translation and up-regulating stress response and amino acid biogenesis pathways (Hauryliuk *et al*, 2015; Steinchen & Bange, 2016). In *E. coli*, transcription of *rmf*, the gene encoding RMF, is up-regulated by (p)ppGpp when amino acids become limiting (Izutsu *et al*, 2001) and by cAMP upon carbon starvation (Shimada *et al*, 2013). Transcription of *yvyD*, the gene encoding *Bs*LHPF, is under the control of the sigma factors σ^H and σ^B (Drzewiecki *et al*, 1998; Tam le *et al*, 2006; Akanuma *et al*, 2016), and up-regulated by the presence of the alarmone (p)ppGpp (Eymann *et al*, 2001; Tagami *et al*, 2012; Shimada *et al*, 2013; Fig 7A). Similarly, in the cyanobacterium *Synechococcus elongatus*, LHPF is also up-regulated by (p)ppGpp to enable dark adaptation (Hood *et al*, 2016).

The up-regulation of LHPF leads to increased 100S formation, indicating that LHPF competes effectively with translation factors, as evidenced by LHPF inhibition of *in vitro* translation systems (Ueta *et al*, 2013; Basu & Yap, 2016). Since we observed that *Bs*HPF is dimeric in solution, we favor a model whereby dimeric *Bs*HPF interacts independently with two 70S ribosomes (Fig 7B). In this model, we propose that *Bs*HPF utilizes the free NTDs and long linker to initially bring 70S ribosomes into close proximity, and then further stabilizes the 70S dimer using the *Bs*HPF-CTD ribosome interface (Fig 7B). However, we cannot exclude that at a fraction of *Bs*HPF resides as a monomer *in vivo*, and these *Bs*HPF monomers bind separately to the 70S ribosome, such that 100S formation could then occur concomitantly with *Bs*HPF-CTD homodimerization. Moreover, it remains unclear how hibernating 100S ribosomes exactly provide protection against stress. What is

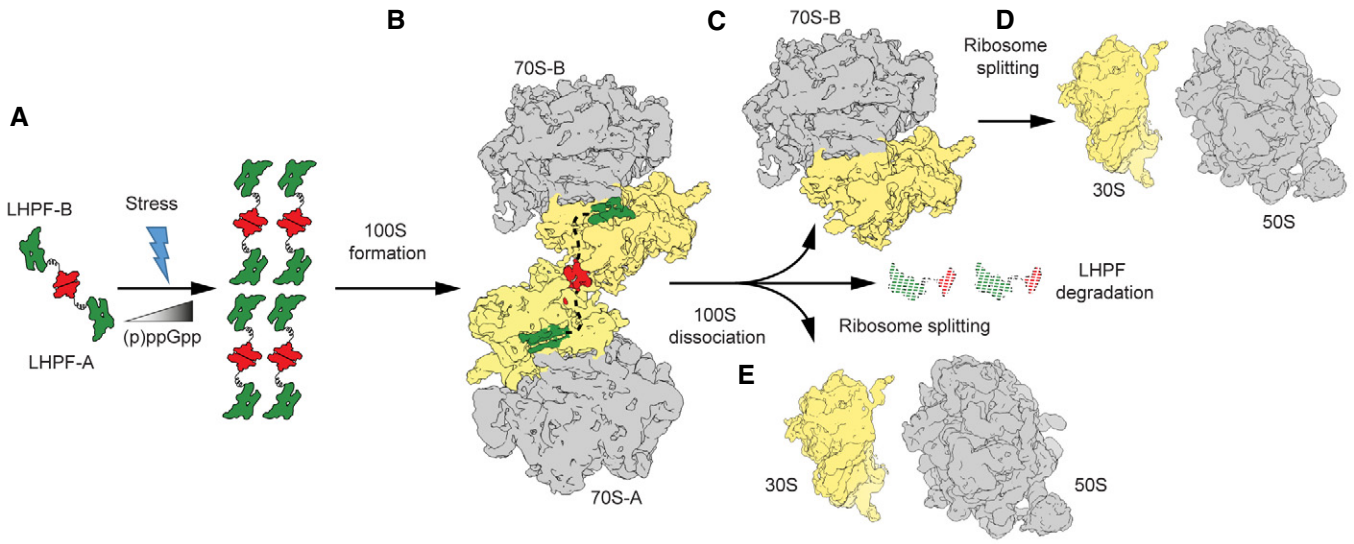

Figure 7. Model for *Bs*HPF-induced 100S formation.

A Stress conditions, such as nutrient deprivation, lead to elevated levels of (p)ppGpp, which up-regulates expression of the LHPF (NTD, green; CTD, red). The LHPF-CTD can interact to form homodimers in solution and therefore may also be present as dimers in the cell.

B The long linker of the dimeric LHPF enables the LHPF-NTD to interact with two independent 70S ribosomes and by bringing them in to close proximity stabilizes the 70S dimers, forming 100S.

C–E Following removal of the stress conditions, *Bs*HPF levels decline leading to (C) dissociation of 100S into 70S ribosomes and (D) eventually ribosome splitting into 30S and 50S subunits, or (E) alternatively directly in 30S and 50S subunits.

clear however is that in the absence of 100S, 70S ribosomes are slowly degraded leading to early cell death, suggesting that hibernating 100S are less susceptible to degradation by RNases (Fukuchi *et al*, 1995; Wada, 1998; Niven, 2004; Shcherbakova *et al*, 2015; Akanuma *et al*, 2016). Because 100S formation does not significantly alter the large rRNA surface exposed to RNases, we believe LHPF binding and 100S formation may interfere with a specific ribosome degradation pathway, rather than preventing non-specific RNase action on ribosomes. The identification of *Bs*HPF variant, such as the *Bs*HPF-LΔ10AA, which binds to the ribosome but does not promote 100S formation, may allow the contribution of these activities to ribosome protection to be dissected further.

In *E. coli*, disassembly of 100S is rapid and occurs within 1 min upon transfer to fresh medium, suggesting that an active mechanism exists to remove *Ec*HPF and RMF from the ribosome (Wada, 1998; Aiso *et al*, 2005). In contrast, *Bs*100S are more stable than *Ec*100S (Ueta *et al*, 2013) and upon transfer to fresh media significant dissociation of *Bs*100S was only observed after 120 min, where LHPF protein levels were also significantly decreased (Akanuma *et al*, 2016). Nevertheless, recycling factors, such as IF3, RRF, and EF-G, which have been reported to remove LHPF (PSRP-1) from Spinach chloroplast ribosomes (Sharma *et al*, 2010), might also be involved in *Bs*HPF release and 100S dissociation (Fig 7C–E).

In conclusion, the high conservation of the LHPF proteins suggests that most, if not all, LHPF proteins are present as dimers in the cell, with the implication that the majority of bacteria are likely to utilize an identical mechanism to induce 100S formation as we have described here for *B. subtilis* (Fig 7).

Materials and Methods

Cloning of *Bs*HPF and *Bs*HPF variants for protein purification

The *yvyD* gene encoding *Bs*HPF was amplified from *B. subtilis* PY79 genomic DNA by polymerase chain reaction using Phusion High-Fidelity DNA Polymerase (NEB) according to the manufacturer's manual. The *forward* primer encoded a hexa-histidine tag in-frame with the DNA sequence of *yvyD*. The fragment was cloned via *Nco*I/*Xho*I restriction sites into a modified pGAT2-vector incorporating a GST-tag N-terminal of His_6-*Bs*HPF. *Bs*HPF-CTD (amino acids 130–189 of *Bs*HPF), *Bs*HPF-NTD (amino acids 1–104 of *Bs*HPF), and *Bs*HPF(F160E) containing an N-terminal hexa-histidine tag were amplified by PCR as described above and cloned via *Nco*I/*Xho*I restriction sites into pET24d(+) vector (Novagen). Mutations within *Bs*HPF were introduced by overlapping PCR.

Protein production and purification for SEC and SLS

Escherichia coli BL21(DE3) cells (NEB) carrying the expression plasmid were grown in lysogeny broth (LB) medium supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) and D(+)-lactose-monohydrate (12.5 g/l) for 16 h at 30°C under rigorous shaking (180 rpm). The cells were harvested (3,500 × *g*, 20 min, 4°C), resuspended in lysis buffer (20 mM HEPES-KOH, pH 8.0, 20 mM KCl, 20 mM $MgCl_2$, 500 mM NaCl, 40 mM imidazole) and lysed using a M-110L Microfluidizer (Microfluidics). After centrifugation (47,850 × *g*, 20 min, 4°C), the clear supernatant was loaded on a HisTrap HP 1 ml column (GE Healthcare) equilibrated with 15 column volumes (CV) of lysis buffer. After

washing with 15 CV of lysis buffer, the protein was eluted with 5 CV of elution buffer (lysis buffer containing 500 mM imidazole). The GST-tag was removed from *Bs*HPF variants by incubation with 100 U of bovine thrombin (Merck Millipore) for 2 h at 20°C. After dilution with 12 volume parts of lysis buffer without imidazole, *Bs*HPF variants were resubjected to Ni-NTA affinity chromatography as described above and the elution fraction containing *Bs*HPF were collected. *Bs*HPF and *Bs*HPF variants were then concentrated using an Amicon Ultracel-10K or 3K, respectively (Merck Millipore), and applied to size-exclusion chromatography (HiLoad 26/600 Superdex 75 pg, GE Healthcare) equilibrated in SEC buffer (20 mM HEPES-KOH, pH 8.0, 20 mM KCl, 20 mM $MgCl_2$, 500 mM NH_4Cl). Protein-containing fractions were pooled and concentrated to ~500 μM as determined by a spectrophotometer (NanoDrop Lite, Thermo Scientific).

Analysis of oligomerization states of *Bs*HPF variants by SEC and SLS

The apparent molecular weight was analyzed by size-exclusion chromatography using a HiLoad 26/600 Superdex 75 pg column (GE Healthcare) equilibrated in SEC buffer. A standard curve for molecular mass determination was obtained using BSA (66.5 kDa), ovalbumin (chicken, 44.3 kDa), myoglobin (horse, 17 kDa), and vitamin B12 (1.35 kDa). The absolute molecular weight was determined by static light scattering (SLS) with a DelsaMax CORE (Beckmann Coulter) according to the manufacturer's instructions.

Cloning of *Bs*HPF and *Bs*HPF variants for *in vivo* studies

Full-length *yvyD* was amplified from genomic DNA by PCR as described above with the forward primer encoding the strong ribosomal binding site of the *gsiB* gene (AGGAGGAATTCAAA) and cloned via *Sal*I/*Sph*I restriction sites into the pDR111 plasmid (Ben-Yehuda *et al*, 2003). The *Bs*HPF-LΔ10AA, *Bs*HPF-LΔ20AA, and *Bs*HPF-F160E mutation were introduced by overlap extension PCR and cloned via SalI/SphI restriction sites as described above. The resulting plasmids were linearized by digestion with *Sca*I and transformed into naturally competent *B. subtilis* cells. Proper integration into the *amyE* locus was checked by growing selected transformants on LB-Agar containing 1% starch overnight and staining the plates with a solution of 0.5% (w/v) iodine, 1% (w/v) potassium iodine. Strains and oligonucleotides used in this study are presented in Tables EV2 and EV3.

Western blotting of *Bs*HPF variants

Strains expressing HPF variants *in trans* were grown in LB medium supplemented with 1 mM IPTG with rigorous shaking to until the mid-exponential phase (OD_{600} of ~0.8), harvested by centrifugation at 11,000 × g, 4°C for 5 min, washed once in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and disrupted by sonication three times for 30 s on ice in TE buffer supplemented with 1 mM PMSF. The soluble protein was cleared from cell debris by centrifugation at 11,000 × g, 4°C for 5 min. 10 μg of the protein extract (as determined by the Bradford assay) was analyzed by SDS–PAGE and Western Blotting onto a nitrocellulose membrane. As controls, equally treated samples from a stationary phase overnight culture of *B. subtilis* wild-type or *Δhpf* cells were loaded. The *Bs*HPF protein was detected using a polyclonal antibody raised against *Bs*HPF (Pineda Antibody Service) and a polyclonal Goat anti-Rabbit IgG alkaline Phosphatase conjugated antibody (Antikörper Online). Western blotting using an antibody against the malate dehydrogenase (MDH) was used as a loading control. The ECF reagent (GE Healthcare) was used as a substrate according to the manufacturer's manual, and chemifluorescent signals were detected using a cooled CCD camera in a ChemiBIS 4.2 Bioimaging system (DNR).

Binding assay for *Bs*HPF variants with pelleted ribosomes

Bacillus subtilis cells were grown in 200-ml LB medium supplemented with 1 mM IPTG with rigorous shaking (200 rpm) to the mid-exponential phase (OD_{600} ~0.8) and harvested by centrifugation at 15,300 × *g*, 10 min, 4°C. Ribosomes were pelleted as described in (Schmalisch *et al*, 2002). Briefly, cells were washed once in buffer A (20 mM Tris–HCl, 100 mM NH_4Cl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, pH 7.5), resuspended in 3 ml of the same buffer with 1 mM PMSF and disrupted in a French Pressure Cell three times at 1,000 psi. The lysate was cleared from cell debris by centrifugation for 30 min at 29,953 × g, 4°C (SW55-Ti, Beckman Coulter), layered on top of a 8 ml 1.1 M sucrose cushion in buffer A, and centrifuged for 16 h at 119,307 × g, 4°C (SW40-Ti, Beckman Coulter). The cell pellet was washed three times in buffer A and resuspended in buffer B (20 mM Tris–HCl, 100 mM NH_4Cl, 6 mM $MgCl_2$, 2 mM DTT). The suspension was centrifuged at 10,000 × g, 10 min, 4°C, and the supernatant containing the ribosomes was collected. 10 μg of the total soluble protein ("CE", as determined by the Bradford assay) and an equal volume of the ribosome suspension ("R") was subjected to 15% SDS–PAGE and subsequent stained with Coomassie using standard procedures or Western blotting as described above.

Growth recovery from stationary phase

Precultures of *B. subtilis* cells were grown in 5-ml LB medium supplemented with 0.5 mM IPTG at 37°C for 18 h, to ensure the cells reached the stationary growth phase. The cultures were then diluted to an OD_{600} of 0.05 into 20-ml fresh LB medium and grown at 37°C with rigorous shaking. The cell growth was monitored by determining the optical density at 600 nm (OD_{600}) at regular intervals.

Sucrose density gradient centrifugation analysis

Analysis of 100S formation was performed as described previously for *B. subtilis* (Akanuma *et al*, 2016). Briefly, 50-ml LB medium was inoculated at a 1:100 dilution with an overnight culture. Expression was induced using 1 mM IPTG at an OD_{600} of 0.4. Cells were harvested at the stationary phase by centrifugation at 4,000 × *g* for 10 min at 4°C (Hettich Rotanta 46R) and the cell pellet re-suspended in buffer C (50 mM HEPES-KOH, pH7.4, 100 mM KOAc, 25 mM Mg $(OAc)_2$, 6 mM β-mercaptoethanol). Cells were lysed using the sonifier three times, with each cycle consisting of 30 s at 30% power followed by centrifugation at 16,000 × *g* for 15 min at 4°C to remove cellular debris. A total OD_{260} of 10 of the cleared lysate was loaded onto sucrose density gradients (10–60% sucrose in buffer C)

by centrifugation at 154,693 × *g* (SW-40 Ti, Beckman Coulter) for 3 h at 4°C and then analyzed using a Gradient Station (Biocomp) with an Econo UV Monitor (Bio-Rad) and a FC203B Fraction Collector (Gilson).

Preparation of *Bacillus subtilis* S12 extract

Bacillus subtilis S12 extract was prepared as described (Sohmen *et al*, 2015). Briefly, an "INFORCE HT minifors" bench top fermenter was used to grow *B. subtilis* strain 168 cells to an OD_{600} 4.5 in 2× YPTG medium (16 g/l peptone, 10 g/l yeast extract, 5 g/l NaCl, 22 mM NaH_2PO_4, 40 mM Na_2HPO_4, 19.8 g/l glucose (sterile filtered)), with extra glucose feeding at 37°C while maintaining a pH 7.0 and oxygen level (60%). After collecting cells at 5,000 × *g* at 4°C for 15 min, they were washed 3× in cold Buffer A (10 mM Tris–acetate (pH 8.2), 14 mM magnesium acetate, 60 mM potassium glutamate, 1 mM dithiothreitol, and 6 mM β-mercaptoethanol). Cells were then snap-frozen in liquid nitrogen and stored at −80°C. 15 g of cells was thawed on ice, resuspended in 10 ml of cold buffer B (buffer A missing β-mercaptoethanol), and lysed 3× at 15,000 psi in an "microfluidics model 110I lab homogenizer". The lysate was cleared at 12,000 × *g* and 4°C for 10 min and incubated in a water bath for 30 min at 37°C. The cell extract was aliquoted, snap-frozen, and stored at −80°C. Extracts were analyzed on sucrose density gradients (10–50% sucrose in buffer C), by centrifugation at 89,454 × *g* (SW-28, Beckman Coulter) for 4 h at 4°C. For 100S purification, 100S fractions were collected using a Gradient Station (Biocomp) with an Econo UV Monitor (Bio-Rad) and a FC203B Fraction Collector (Gilson). Purified 100S ribosomes were concentrated by centrifugation at 92,159 × *g* for 2.5 h at 4°C (TLA110 rotor, Beckman Coulter).

Negative stain electron microscopy

Ribosomal particles were diluted in buffer C to a final concentration of 0.2 OD_{260}/ml. A 3.5 μl sample was applied onto a carbon-coated grid. After 30 s, the grids were washed with distilled water and then stained with 2% aqueous uranyl acetate for 15 s. The remaining liquid was removed by touching the grid with filter paper. Micrographs were taken using a Morgagni transmission electron microscope (FEI).

Cryo-electron microscopy and single particle reconstruction

A total of 4 OD_{260}/ml *Bs*100S sample were applied to 2 nm precoated Quantifoil R3/3 holey carbon-supported grids and vitrified using Vitrobot Mark IV (FEI Company). Data collection was performed using EM-TOOLS (TVIPS GmbH) on a Titan Krios transmission electron microscope equipped with a Falcon II direct electron detector (FEI Company) at 300 kV at a pixel size of 1.084 Å and a defocus range of 0.7–2.2 μm. Ten frames (dose per frame of 2.5 e^-/Å) were aligned using Motion Correction Software (Li *et al*, 2013). Power-spectra, defocus values, and astigmatism were then determined using CTFFIND4 software (Rohou & Grigorieff, 2015). Micrographs showing Thon rings beyond 3.5 Å were manually inspected for a good areas and power-spectra quality. Automatic particle picking was then performed using SIGNATURE (Chen & Grigorieff, 2007), and single particles were windowed out in small box able to contain a 70S ribosome together with the majority of the small 30S subunit of the neighboring 70S ribosome. The particles were then further processed using FREALIGN (Grigorieff, 2007). The 253,905 particles were first subjected to an extensive 3D classification (Fig EV2A and B), and the selected 24,546 *Bs*100S particles of class 8 were then subjected to refinement using 30S-70S mask resulting in a final reconstruction of 3.8 Å (0.143 FSC) average resolution (Figs EV2C and EV3). Local resolution was finally calculated using ResMap (Kucukelbir *et al*, 2014). For the processing of the complete *Bs*100S, the coordinates of the selected 24,546 particles were carefully re-inspected in order to remove particles that were within close proximity of another particle, so as not to include particles twice in the final reconstruction; 5,511 particles were identified and removed from class 8, and the rest of particles were windowed out using a larger box size that encompassed two 70S ribosomes (Fig EV2D). The remaining 19,335 particles were then realigned and refined, resulting in a final reconstruction with an average resolution of 6.2 Å (0.143 FSC; Fig EV5A–C).

Molecular modeling, refinement, and validation

The molecular model for the ribosomal proteins and rRNA of the 70S ribosome of the *Bs*100S was based on the molecular model from the recent cryo-EM reconstruction of the *B. subtilis* 70S ribosome (PDB ID 3JW9; Sohmen *et al*, 2015). The molecular model was fitted as a rigid body into the cryo-EM density maps using UCSF Chimera (Pettersen *et al*, 2004). For *Bs*HPF-NTD domain, a homology model was generated using HHPred (Soding *et al*, 2005) based on the HPF protein template from *E. coli* (PDB ID 4V8H; Polikanov *et al*, 2012; Fig EV1C). Molecular models were fitted and adjusted by using COOT (Emsley & Cowtan, 2004) and refined in Phenix using *phenix.real_space_refine* (Adams *et al*, 2010). Model over-fitting was evaluated through its refinement against one cryo-EM half map as described previously (Brown *et al*, 2015). FSC curves were calculated between the resulting model and the half map used for refinement, as well as between the resulting model and the other half map for cross-validation (Fig EV3E). The final refinement statistics were determined using MolProbity (Chen *et al*, 2010) and are provided in Table EV1. For *Bs*HPF-CTD domain, a homology model was generated using HHPred based on the template from *C. acetobutylicum* (PDB ID 3KA5; Fig EV1D). The molecular model was rigid body fitted using UCSF Chimera (Pettersen *et al*, 2004).

Figure preparation

Figures showing map densities and atomic models were generated using UCSF Chimera (Pettersen *et al*, 2004).

Accession numbers

The cryo-EM map of the *Bs*70S-30S subcomplex and the complete *Bs*100S have been deposited in the EMDB with the accession codes EMD-3656 and EMD-3664, respectively. Atomic coordinates have been deposited in the Protein Data Bank with accession code PDB ID 5NJT.

Expanded View for this article is available online.

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Author contributions

DNW, GB, and KT designed and supervised the study. MA prepared the *Bs*100S sample for cryo-EM and performed all sucrose gradient analyses. OB collected the cryo-EM data. BB, MA, and SA processed the cryo-EM data. BB, MA, RB, and DNW interpreted the cryo-EM data. WS cloned and purified the *Bs*HPF protein variants and performed the SEC and SLS. HS generated all *Bs*HPF expression strains and performed Western blotting, growth curves, and ribosome pelleting assays. DNW, GB, and KT wrote the manuscript with comments from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

Note added in proof

The recent cryo-EM structure of the *Staphylococcus aureus* 100S determined by Khusainov *et al* (2017) reveals that the mechanism of 70S dimerization mediated by the *S. aureus* long-form HPF appears to be similar to that observed here for *Bacillus subtilis*.

References

Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH (2010) PHENIX: a comprehensive python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2): 213 – 221

Agafonov DE, Spirin AS (2004) The ribosome-associated inhibitor A reduces translation errors. *Biochem Biophys Res Commun* 320: 354 – 358

Aiso T, Yoshida H, Wada A, Ohki R (2005) Modulation of mRNA stability participates in stationary-phase-specific expression of ribosome modulation factor. *J Bacteriol* 187: 1951 – 1958

Akanuma G, Kazo Y, Tagami K, Hiraoka H, Yano K, Suzuki S, Hanai R, Nanamiya H, Kato-Yamada Y, Kawamura F (2016) Ribosome dimerization is essential for the efficient regrowth of *Bacillus subtilis*. *Microbiology* 162: 448 – 458

Basu A, Yap MN (2016) Ribosome hibernation factor promotes Staphylococcal survival and differentially represses translation. *Nucleic Acids Res* 44: 4881 – 4893

Ben-Yehuda S, Rudner DZ, Losick R (2003) RacA, a bacterial protein that anchors chromosomes to the cell poles. *Science* 299: 532 – 536

Bieri P, Leibundgut M, Saurer M, Boehringer D, Ban N (2017) The complete structure of the chloroplast 70S ribosome in complex with translation factor pY. *EMBO J* 36: 475 – 486

Brandt F, Etchells SA, Ortiz JO, Elcock AH, Hartl FU, Baumeister W (2009) The native 3D organization of bacterial polysomes. *Cell* 136: 261 – 271

Brown A, Long F, Nicholls RA, Toots J, Emsley P, Murshudov G (2015) Tools for macromolecular model building and refinement into electron cryo-microscopy reconstructions. *Acta Crystallogr D Biol Crystallogr* 71(Pt 1): 136 – 153

Chen JZ, Grigorieff N (2007) SIGNATURE: a single-particle selection system for molecular electron microscopy. *J Struct Biol* 157: 168 – 173

Chen VB, Arendall WB III, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, Richardson DC (2010) MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr* 66(Pt 1): 12 – 21

Drzewiecki K, Eymann C, Mittenhuber G, Hecker M (1998) The yvyD gene of *Bacillus subtilis* is under dual control of sigmaB and sigmaH. *J Bacteriol* 180: 6674 – 6680

El-Sharoud WM, Niven GW (2007) The influence of ribosome modulation factor on the survival of stationary-phase *Escherichia coli* during acid stress. *Microbiology* 153(Pt 1): 247 – 253

Emsley P, Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60: 2126 – 2132

Eymann C, Mittenhuber G, Hecker M (2001) The stringent response, sigmaH-dependent gene expression and sporulation in *Bacillus subtilis*. *Mol Gen Genet* 264: 913 – 923

Fukuchi JI, Kashiwagi K, Yamagishi M, Ishihama A, Igarashi K (1995) Decrease in cell viability due to the accumulation of spermidine in spermidine acetyltransferase-deficient mutant of *Escherichia coli*. *J Biol Chem* 270: 18831 – 18835

Garay-Arroyo A, Colmenero-Flores JM, Garciarrubio A, Covarrubias AA (2000) Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. *J Biol Chem* 275: 5668 – 5674

Graf M, Arenz S, Huter P, Donhofer A, Novacek J, Wilson DN (2016) Cryo-EM structure of the spinach chloroplast ribosome reveals the location of plastid-specific ribosomal proteins and extensions. *Nucleic Acids Res* 45: 2887 – 2896

Grigorieff N (2007) FREALIGN: high-resolution refinement of single particle structures. *J Struct Biol* 157: 117 – 125

Hauryliuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K (2015) Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nat Rev Microbiol* 13: 298 – 309

Hood RD, Higgins SA, Flamholz A, Nichols RJ, Savage DF (2016) The stringent response regulates adaptation to darkness in the cyanobacterium *Synechococcus elongatus*. *Proc Natl Acad Sci USA* 113: E4867 – E4876

Izutsu K, Wada A, Wada C (2001) Expression of ribosome modulation factor (RMF) in *Escherichia coli* requires ppGpp. *Genes Cells* 6: 665 – 676

Jenner L, Demeshkina N, Yusupova G, Yusupov M (2011) Structural rearrangements of the ribosome at the tRNA proofreading step. *Nat Struct Mol Biol* 17: 1072 – 1078

Kato T, Yoshida H, Miyata T, Maki Y, Wada A, Namba K (2010) Structure of the 100S ribosome in the hibernation stage revealed by electron cryomicroscopy. *Structure* 18: 719 – 724

Khusainov I, Vicens Q, Ayupov R, Usachev K, Myasnikov A, Simonetti A, Validov S, Kieffer B, Yusupova G, Yusupov M, Hashem Y (2017) Structures and dynamics of hibernating ribosomes from *Staphylococcus aureus* mediated by intermolecular interactions of HPF. *EMBO J* 36: 2073 – 2087

Kline BC, McKay SL, Tang WW, Portnoy DA (2015) The listeria monocytogenes hibernation-promoting factor is required for the formation of 100S ribosomes, optimal fitness, and pathogenesis. *J Bacteriol* 197: 581 – 591

Kucukelbir A, Sigworth FJ, Tagare HD (2014) Quantifying the local resolution of cryo-EM density maps. *Nat Methods* 11: 63 – 65

Li X, Mooney P, Zheng S, Booth CR, Braunfeld MB, Gubbens S, Agard DA, Cheng Y (2013) Electron counting and beam-induced motion correction

enable near-atomic-resolution single-particle cryo-EM. *Nat Methods* 10: 584 – 590

Maki Y, Yoshida H, Wada A (2000) Two proteins, YfiA and YhbH, associated with resting ribosomes in stationary phase *Escherichia coli*. *Genes Cells* 5: 965 – 974

McKay SL, Portnoy DA (2015) Ribosome hibernation facilitates tolerance of stationary-phase bacteria to aminoglycosides. *Antimicrob Agents Chemother* 59: 6992 – 6999

Niven GW (2004) Ribosome modulation factor protects *Escherichia coli* during heat stress, but this may not be dependent on ribosome dimerization. *Arch Microbiol* 182: 60 – 66

Ortiz JO, Brandt F, Matias VR, Sennels L, Rappsilber J, Scheres SH, Eibauer M, Hartl FU, Baumeister W (2010) Structure of hibernating ribosomes studied by cryoelectron tomography *in vitro* and *in situ*. *J Cell Biol* 190: 613 – 621

Pei J, Kim BH, Grishin NV (2008) PROMALS3D: a tool for multiple protein sequence and structure alignments. *Nucleic Acids Res* 36: 2295 – 2300

Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF chimera – a visualization system for exploratory research and analysis. *J Comput Chem* 25: 1605 – 1612

Polikanov YS, Blaha GM, Steitz TA (2012) How hibernation factors RMF, HPF, and YfiA turn off protein synthesis. *Science* 336: 915 – 918

Puri P, Eckhardt TH, Franken LE, Fusetti F, Stuart MC, Boekema EJ, Kuipers OP, Kok J, Poolman B (2014) *Lactococcus lactis* YfiA is necessary and sufficient for ribosome dimerization. *Mol Microbiol* 91: 394 – 407

Reiss S, Pane-Farre J, Fuchs S, Francois P, Liebeke M, Schrenzel J, Lindequist U, Lalk M, Wolz C, Hecker M, Engelmann S (2012) Global analysis of the *Staphylococcus aureus* response to mupirocin. *Antimicrob Agents Chemother* 56: 787 – 804

Rohou A, Grigorieff N (2015) CTFFIND4: fast and accurate defocus estimation from electron micrographs. *J Struct Biol* 192: 216 – 221

Schmalisch M, Langbein I, Stulke J (2002) The general stress protein Ctc of *Bacillus subtilis* is a ribosomal protein. *J Mol Microbiol Biotechnol* 4: 495 – 501

Sharma MR, Wilson DN, Datta PP, Barat C, Schluenzen F, Fucini P, Agrawal RK (2007) Cryo-EM study of the spinach chloroplast ribosome reveals the structural and functional roles of plastid-specific ribosomal proteins. *Proc Natl Acad Sci USA* 104: 19315 – 19320

Sharma MR, Donhofer A, Barat C, Marquez V, Datta PP, Fucini P, Wilson DN, Agrawal RK (2010) PSRP1 is not a ribosomal protein, but a ribosome-binding factor that is recycled by the ribosome-recycling factor (RRF) and elongation factor G (EF-G). *J Biol Chem* 285: 4006 – 4014

Shcherbakova K, Nakayama H, Shimamoto N (2015) Role of 100S ribosomes in bacterial decay period. *Genes Cells* 20: 789 – 801

Shimada T, Yoshida H, Ishihama A (2013) Involvement of cyclic AMP receptor protein in regulation of the rmf gene encoding the ribosome modulation factor in *Escherichia coli*. *J Bacteriol* 195: 2212 – 2219

Soding J, Biegert A, Lupas AN (2005) The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res* 33(Web Server issue): W244 – W248

Sohmen D, Chiba S, Shimokawa-Chiba N, Innis CA, Berninghausen O, Beckmann R, Ito K, Wilson DN (2015) Structure of the *Bacillus subtilis* 70S ribosome reveals the basis for species-specific stalling. *Nat Commun* 6: 6941

Steinchen W, Bange G (2016) The magic dance of the alarmones (p)ppGpp. *Mol Microbiol* 101: 531 – 544

Tagami K, Nanamiya H, Kazo Y, Maehashi M, Suzuki S, Namba E, Hoshiya M, Hanai R, Tozawa Y, Morimoto T, Ogasawara N, Kageyama Y, Ara K, Ozaki K, Yoshida M, Kuroiwa H, Kuroiwa T, Ohashi Y, Kawamura F (2012) Expression of a small (p)ppGpp synthetase, YwaC, in the (p)ppGpp(0) mutant of *Bacillus subtilis* triggers YvyD-dependent dimerization of ribosome. *Microbiologyopen* 1: 115 – 134

Tam le T, Antelmann H, Eymann C, Albrecht D, Bernhardt J, Hecker M (2006) Proteome signatures for stress and starvation in *Bacillus subtilis* as revealed by a 2-D gel image color coding approach. *Proteomics* 6: 4565 – 4585

Ueta M, Yoshida A, Wada C, Baba T, Mori H, Wada A (2005) Ribosome binding proteins YhbH and YfiA have opposite functions during 100S formation in the stationary phase of *Escherichia coli*. *Genes Cells* 10: 1103 – 1112

Ueta M, Ohniwa RL, Yoshida H, Maki Y, Wada C, Wada A (2008) Role of HPF (hibernation promoting factor) in translational activity in *Escherichia coli*. *J Biochem* 143: 425 – 433

Ueta M, Wada C, Wada A (2010) Formation of 100S ribosomes in *Staphylococcus aureus* by the hibernation promoting factor homolog SaHPF. *Genes Cells* 15: 43 – 58

Ueta M, Wada C, Daifuku T, Sako Y, Bessho Y, Kitamura A, Ohniwa RL, Morikawa K, Yoshida H, Kato T, Miyata T, Namba K, Wada A (2013) Conservation of two distinct types of 100S ribosome in bacteria. *Genes Cells* 18: 554 – 574

Vila-Sanjurjo A, Schuwirth BS, Hau CW, Cate JHD (2004) Structural basis for the control of translational initiation during stress. *Nature Struct Mol Biol* 11: 1054 – 1059

Wada A, Yamazaki Y, Fujita N, Ishihama A (1990) Structure and probable genetic location of a ribosome modulation factor associated with 100S ribosomes in stationary-phase *Escherichia coli* cells. *Proc Natl Acad Sci USA* 87: 2657 – 2661

Wada A, Igarashi K, Yoshimura S, Aimoto S, Ishihama A (1995) Ribosome modulation factor: stationary growth phase-specific inhibitor of ribosome functions from *Escherichia coli*. *Biochem Biophys Res Commun* 214: 410 – 417

Wada A (1998) Growth phase coupled modulation of *Escherichia coli* ribosomes. *Genes Cells* 3: 203 – 208

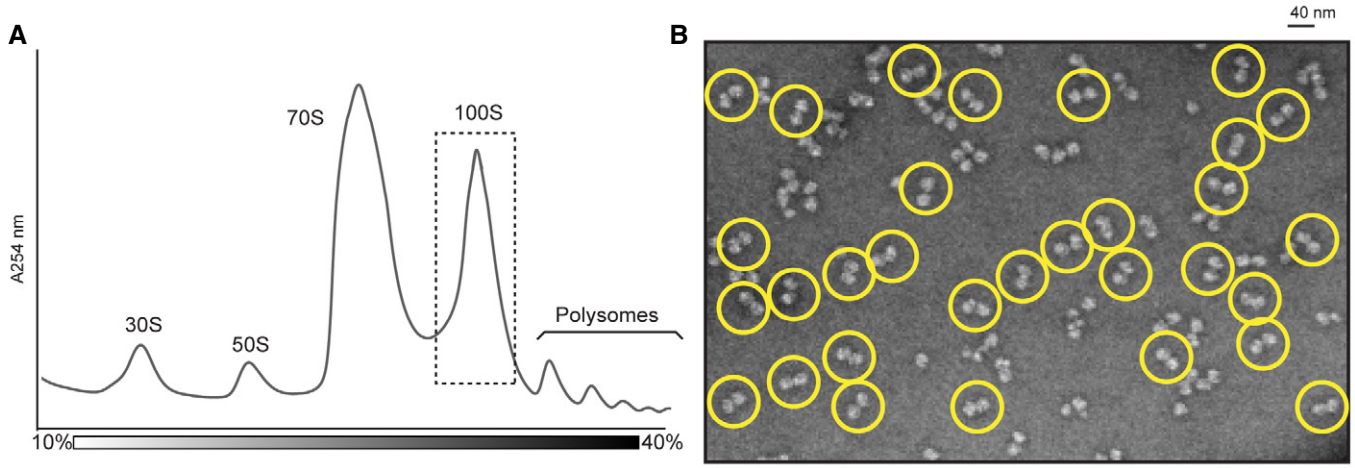
Wada A, Mikkola R, Kurland CG, Ishihama A (2000) Growth-phase coupled changes of the ribosome profile in natural isolates and laboratory strains of *Escherichia coli*. *J Bacteriol* 182: 2893 – 2899

Yamagishi M, Matsushima H, Wada A, Sakagami M, Fujita N, Ishihama A (1993) Regulation of the *Escherichia coli* rmf gene encoding the ribosome modulation factor - growth phase-dependent and growth rate-dependent control. *EMBO J* 12: 625 – 630

Yoshida H, Maki Y, Kato H, Fujisawa H, Izutsu K, Wada C, Wada A (2002) The ribosome modulation factor (RMF) binding site on the 100S ribosome of *Escherichia coli*. *J Biochem* 132: 983 – 989

Yoshida H, Wada A (2014) The 100S ribosome: ribosomal hibernation induced by stress. *Wiley Interdiscip Rev RNA* 5: 723 – 732

Expanded View Figures



C

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Conservation:    9  99 9 9 9 9 99   9  9  999 999      9                9
EcHPF          1  MQLNITGNNVEITEALREFVTAKFAKLEQYFDRINQVYVVLKVEKV---THTSDATLHVNGGEIHASAEG   67
4V8H.pdb       1  MQLNITGNNVEITEALREFVTAKFAKLEQYFDRINQVYVVLKVEKV---THTSDATLHVNGGEIHASAEG   67
BsNTD_HPF      1  MNYNIRGENIEVTPALKDHVERKIGKLERYFDHSVDADVNVNLKFYNDKESKVEVTIPMTDLALRSEVHN   70
Consensus_aa:     MphNIpGpNlElT.AL+-@Vp.KhtKLEpYFD+.sph.Vslplcbh...ppps-hTl.hss..l+tphcs
Consensus_ss:      eeeeeeeeee  hhhhhhhhhhhhhhhhh    eeeeeeeee       eeeeeeee    eeeeeee

Conservation:      999 999    99 99  999 9
EcHPF          68  QDMYAAIDGLIDKLARQLTKHKDKLKQH-----------   95
4V8H.pdb       68  QDMYAAIDGLIDKLARQLTKHKDKLKQH-----------   95
BsNTD_HPF      71  EDMYNAIDLATNKLERQIRKHKTKVNRKFREQGSPKYLL  109
Consensus_aa:      pDMYsAID.hhsKL.RQlpKHKsKlpp+..........
Consensus_ss:        hhhhhhhhhhhhhhhhhhhhhhhhhh
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D

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Conservation:                  99  999  999  999 9 9  99999999 9   9  9999 9 99 999999
3KA5 chainA  1    ----------EIVKTKRFAIKPMSEEEAVLEMELLGHNFFVFQNGDSNEVNVVYKRKDGNYGLIEPE-   57
BsCTD        121  VQDDIEEEESLDIVRQKRFNLKPMDSEEAILQMNMLGHNFFVFTNAETNLTNVVYRRNDGKYGLIEPTE  189
Consensus_aa:     ..........-IV+pKRFslKPMspEEAlLpMphLGHNFFVFpNt-oNbhNVVY+RpDGpYGLIEPp.
Consensus_ss:                eeeeeeee     hhhhhhhhh     eeeee      eeeeeee    eeeeee
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Figure EV1. Isolation of *Bacillus subtilis* 100S and sequence alignments of *Bs*HPF with *Ec*HPF-NTD and *Ca*CTD.

A Sucrose density gradient profile of *B. subtilis* extract from late log phase cells, with 30S, 50S, 70S, 100S, and polysome peaks indicated.

B Negative stain electron microscopy images of purified *Bs*100S from (A), with selected 70S dimers circled in yellow.

C PROMALS3D (Pei *et al*, 2008) sequence alignment of *Bs*HPF-NTD with *Escherichia coli* HPF (PDB 4V8H)(Polikanov *et al*, 2012) that was used to generate the homology model for *Bs*HPF-NTD.

D PROMALS3D (Pei *et al*, 2008) sequence alignment of *Bs*HPF-CTD with *Clostridium acetobutylicum* HPF-CTD (*Ca*CTD; PDB ID 3KA5) that was used to generate the homology model for *Bs*HPF-CTD.

Data information: In (C) and (D), fully conserved residues are indicated with "9" and are bold in the Consensus_aa, whereas similar residues are indicated with a "+". Consensus_ss indicates β-sheet (e) and helical (h) regions.

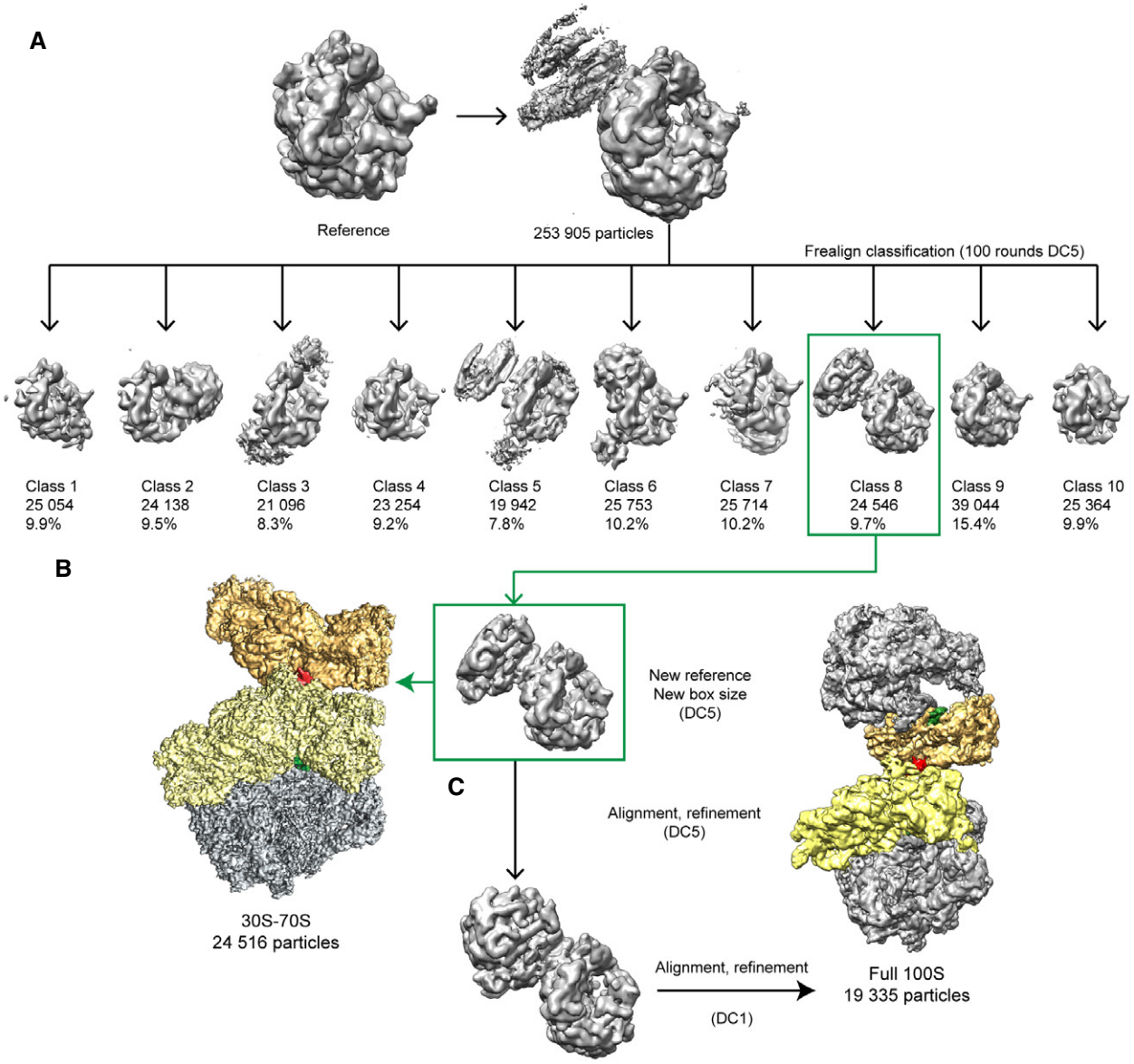

Figure EV2. *In silico* sorting and refinement scheme for the *Bs*70S-30S subcomplex and complete *Bs*100S.

A–C 253,905 particles were sorted into 10 classes. Class 8 had the most defined density for the 70S-B and was taken for further refinement using (B) a box size that includes the 70S-A ribosome and the 30S part of the 70S-B, and (C) a larger box size that encompasses both the 70S-A and 70S-B ribosomes.

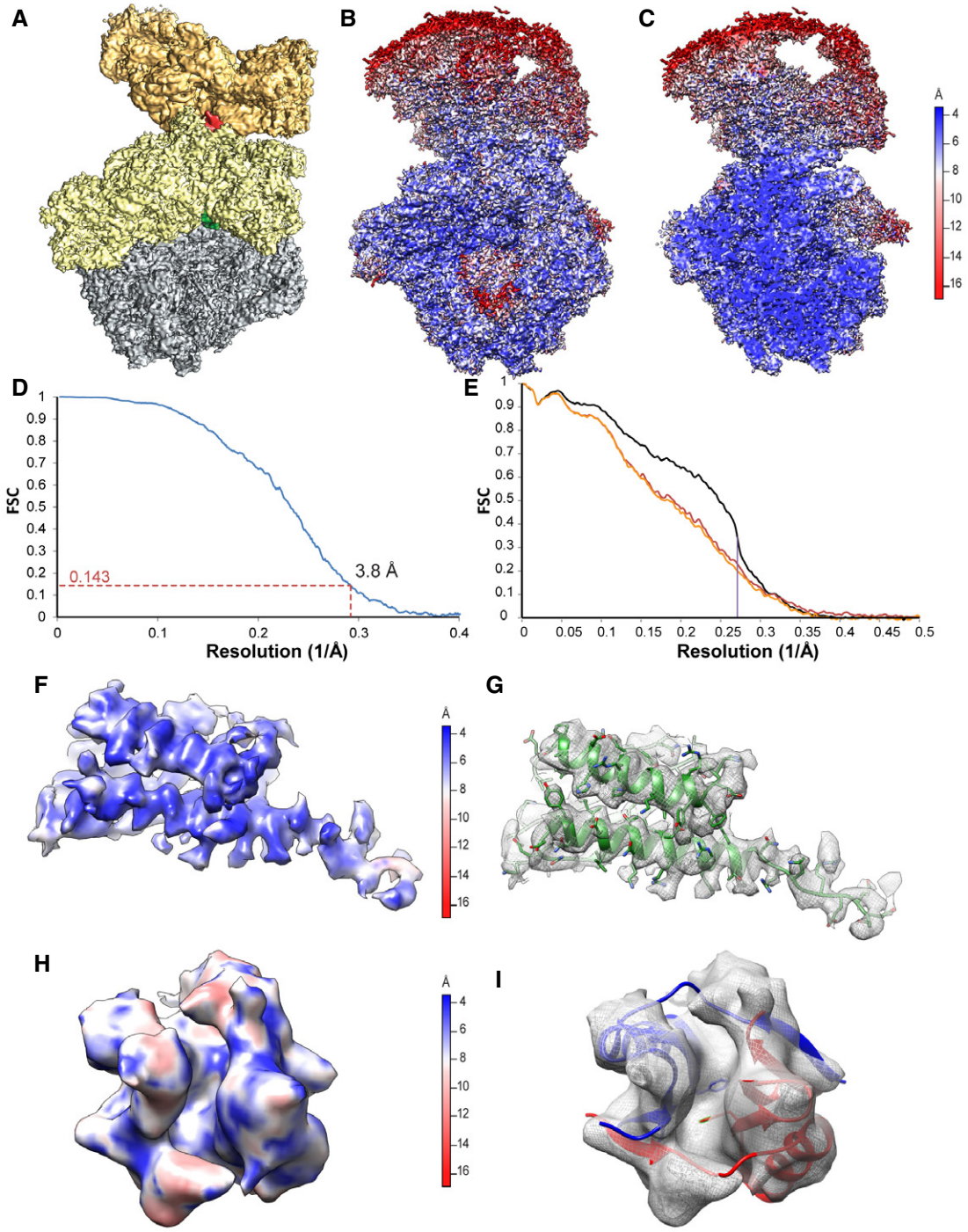

Figure EV3. Resolution of 70S-A in the *Bs*70S-30S subcomplex.

A Overview of the *Bs*70S-30S subcomplex with 30S-A (yellow), 50S-A (gray), and 30S-B (orange), as well as *Bs*HPF-NTD (green) and *Bs*HPF-CTD (red).

B, C Overview (B) and transverse section (C) of the *Bs*70S-30S subcomplex colored according to the local resolution, as calculated using ResMap (Kucukelbir *et al*, 2014).

D Fourier-shell correlation curve of the refined cryo-EM map, indicating the average resolution of 70S-A in the *Bs*70S-30S subcomplex is 3.8 Å.

E Fit of models to maps. FSC curves calculated between the refined model and the final map (black), with the self- and cross-validated correlations in orange and red, respectively. Information beyond 4 Å was not used during refinement and preserved for validation.

F–I Map density for the (F, G) *Bs*HPF-NTD and (H, I) *Bs*HPF-CTD, which are (F, H) colored according to the local resolution, as calculated using ResMap (see Materials and Methods), or (G, I) shown as a gray mesh with molecular models (G) for *Bs*HPF-NTD (green) or (I) *Bs*HPF-CTD for 70S-A (red) and 70S-B (blue), using the same respective view as in (F, H).

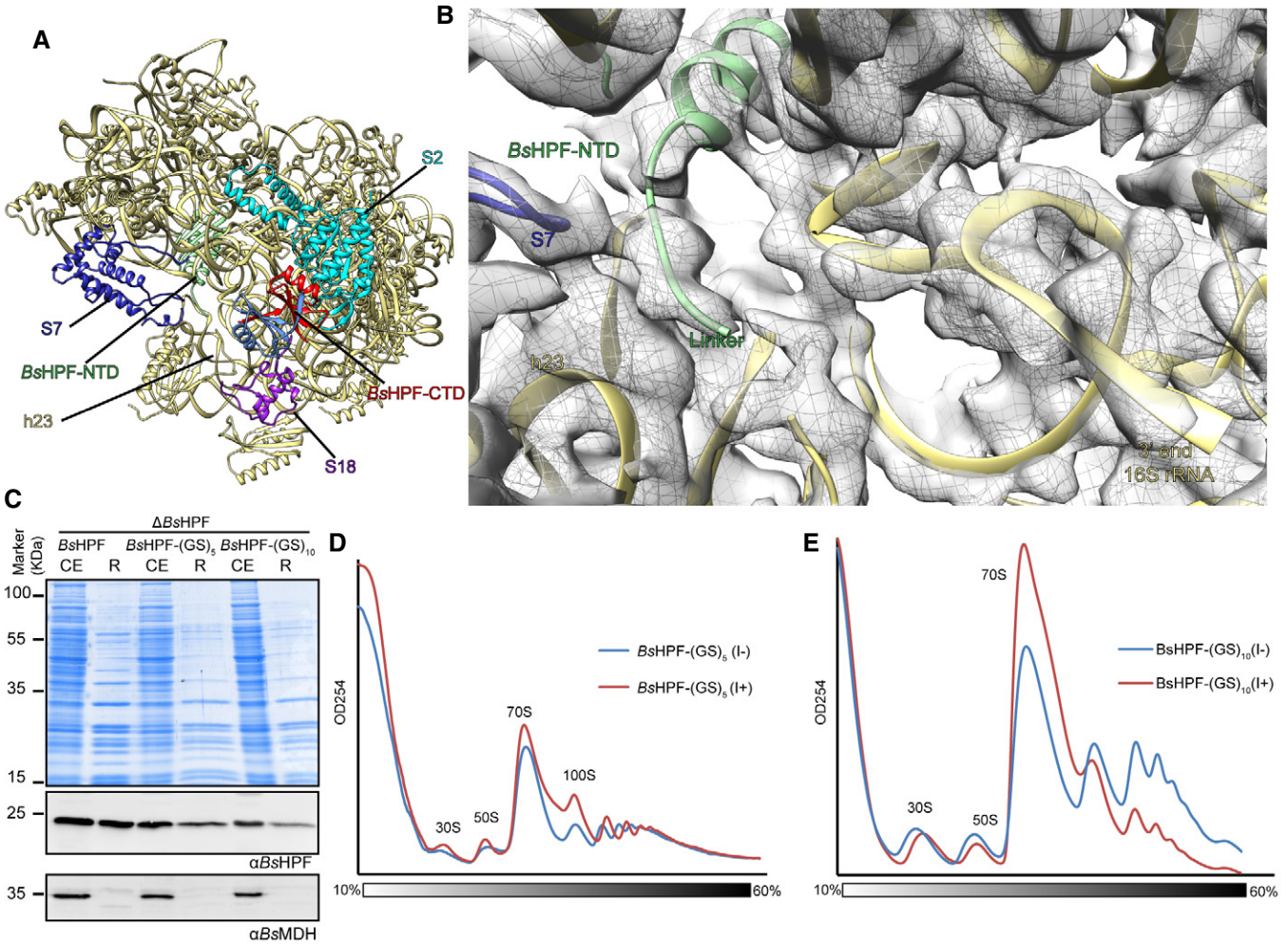

Figure EV4. *Bs*HPF linker region approaches the 30S platform cavity.

A Overview of the 30S cavity region showing *Bs*HPF-NTD (green) and *Bs*HPF-CTD (red) and 30S (yellow), except S2 (cyan), S7 (blue), and S18 (purple).

B Zoom of (A), showing map density (gray mesh) for the N-terminal part of the linker region of *Bs*HPF (green) as well as for the 3′ end of the 16S rRNA.

C Coomassie (upper panel) and Western blot of cell extracts (CE) and ribosome pelleted fractions (R) of the wild-type *Bs*168 (wt) strain or the Δ*Bs*HPF strains expressing either wild-type *Bs*HPF, *Bs*HPF-$(GS)_5$, or *Bs*HPF-$(GS)_{10}$.

D, E Sucrose gradient profiles of cell extracts from the (D) *Bs*168 Δ*Bs*HPF amyE::*Bs*HPF-$(GS)_5$ strain and (E) *Bs*168 Δ*Bs*HPF amyE::*Bs*HPF-$(GS)_{10}$ strain, in the absence (I−) or presence (I+) of IPTG.

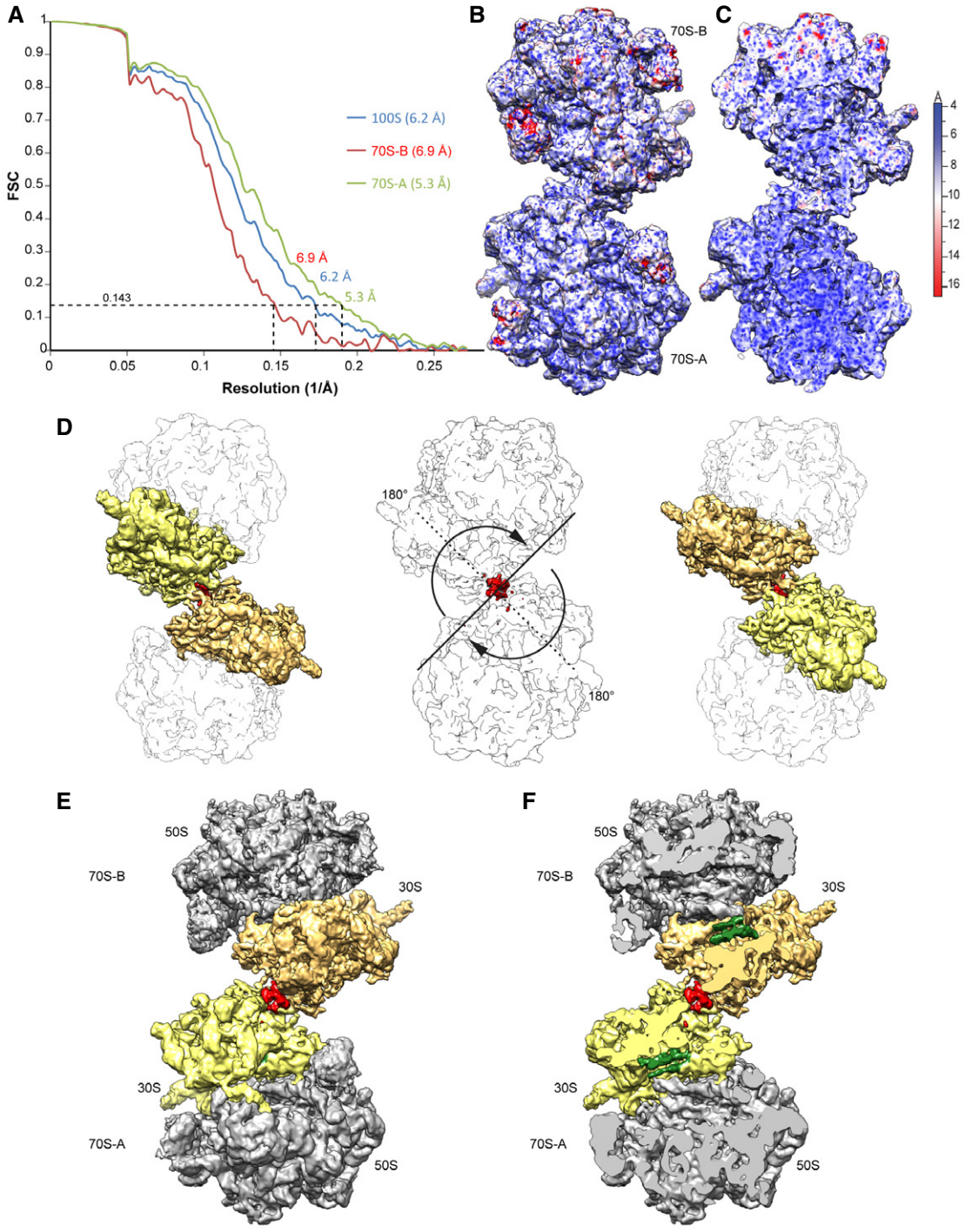

Figure EV5. Resolution of the complete dimeric *Bs*100S.

A Fourier-shell correlation curve of the refined cryo-EM map, indicating the average resolution of 70S-A, 70S-B, and the complete *Bs*100S is 5.3, 6.9 and 6.2 Å, respectively.

B, C Cryo-EM map of the dimeric *Bs*100S colored according to local resolution showing (B) overview and (C) transverse section of the complete 100S disome.

D The 70S-A and 70S-B monomers in the Bs100S are related by rotational symmetry of ~180°.

E, F Cryo-EM map of the (E) dimeric *Bs*100S with 30S-A (yellow), 30S-B (orange) and 50S (gray), and (F) transverse section of (E) highlighting the densities for the *Bs*HPF-NTD (green) and *Bs*HPF-CTD (red).

2.3 The alarmone (p)ppGpp is part of the heat shock response of *Bacillus subtilis*

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Authorship: First author

Share of work: 80 %

Contributions: The authors HS (i, ii, iii), BB (ii), WS (ii), AN (i, ii), MB (ii), IH (ii), PS (ii), LK (ii, iii), VK (ii, iii), PD (ii, iii), GB (ii, iii), DW (ii, iii), KT (i, ii, iii) have made major contributions to (i) the conception or design of the study, (ii) the acquisition, analysis, or interpretation of the data; and (iii) writing of the manuscript.

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The alarmone (p)ppGpp is part of the heat shock response of *Bacillus subtilis*

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Short title: The interplay of heat shock and stringent response

Abstract

Here, *B. subtilis* was used as a model organism to investigate how cells respond and adapt to proteotoxic stress conditions. Our experiments suggested that the stringent response, caused by raised levels of the (p)ppGpp alarmone, plays a role during thermotolerance development and the heat shock response. Accordingly, our experiments revealed a rapid increase of cellular (p)ppGpp levels upon heat shock as well as salt- and oxidative stress. Strains lacking (p)ppGpp exhibited increased stress sensitivity, while raised (p)ppGpp levels conferred increased stress tolerance to heat- and oxidative stress. During thermotolerance development, stress response genes were highly up-regulated together with a concurrent transcriptional down-regulation of the rRNA, which was influenced by the second messenger (p)ppGpp and the transcription factor Spx. Remarkably, we observed that (p)ppGpp appeared to control the cellular translational capacity and that during heat stress the raised cellular levels of the alarmone were able to curb the rate of protein synthesis. Furthermore, (p)ppGpp controls the heat-induced expression of Hpf and thus the formation of translationally inactive 100S disomes. These results indicate that *B. subtilis* cells respond to heat-mediated protein unfolding and aggregation, not only by raising the cellular repair capacity, but also by decreasing translation involving (p)ppGpp mediated stringent response to concurrently reduce the protein load for the cellular protein quality control system.

Author Summary

Here we demonstrate that the bacterial stringent response, which is known to slow down translation upon sensing nutrient starvation, is also intricately involved in the stress response of *B. subtilis* cells. The second messengers (p)ppGpp act as pleiotropic regulators during the adaptation to heat stress. (p)ppGpp slows down translation and is also involved in the transcriptional down-regulation of the translation machinery, together with the transcriptional stress regulator Spx. The stress-induced elevation of cellular (p)ppGpp levels confers increased stress tolerance and facilitates an improved protein homeostasis by reducing the load on the protein quality control system.

Introduction

Bacteria have evolved complex and diverse regulatory networks to sense and respond to changes in the environment, which can include physical stresses or nutrient limitations [1]. The protein quality control system (PQS) comprises a conserved set of chaperones and proteases that monitor and maintain protein homeostasis is present in all cells. Various physical stresses, such as heat stress, favor the unfolding and aggregation of cellular proteins, which can be sensed by heat shock response systems, allowing an appropriate cellular stress response. This response includes the induction of the expression of chaperones and proteases of the PQS, also known as heat shock proteins [2,3].

Interestingly, in all cells including *B. subtilis*, a short exposure to a raised but non-lethal temperature induces thermotolerance, an acquired resistance to otherwise lethal temperatures. Investigating the adaptation to such adverse conditions, also known as priming, allows the molecular mechanisms and interplay of the various cellular processes involved in the cellular stress and heat shock response to be studied [4,5]. In *B. subtilis*, the heat shock response is orchestrated by multiple transcriptional regulators, including the heat-sensitive repressors HrcA & CtsR, which control the expression of the PQS and other general stress genes [6,7]. HrcA regulates the expression of chaperones, while CtsR controls the expression of the AAA+ protease complexes [8–10]. The general stress response, activated by the alternative sigma factor σ^B, is controlled by a complex regulatory network that integrates diverse stress and starvation signals, including heat [11]. In addition, Spx is a central regulator of the heat and thiol stress response, which is important for the development of thermotolerance. Spx activates the expression of many genes of the heat shock response, including *clpX* and the oxidative stress response e.g. thioredoxin [5,12,13]. Interestingly, Spx can also mediate the inhibition of cell growth by the concurrent transcriptional down-regulation of many translation-related genes [14].

Another fast acting bacterial stress response system is the stringent response (SR), which is mediated by the second messenger alarmones (p)ppGpp [15]. The synthesis and hydrolysis of (p)ppGpp is catalyzed by RelA/SpoT homologs (RSH) which contain within the N-terminal part synthetase and hydrolase domains (bifunctional Rel or SpoT subgroup) or an active synthetase and an inactive hydrolase domain (RelA subgroup) together with additional regulatory domains at the C-terminus [16]. RSH can therefore direct both synthesis and, in the case of Rel, hydrolysis of (p)ppGpp.

The enzyme activity of RelA or Rel is stimulated by association with uncharged tRNAs with the ribosome, thereby mediating (p)ppGpp synthesis upon amino acid starvation [17–21]. In addition to this long multidomain RSH form, monofunctional small alarmone synthetases (SAS) or small alarmone hydrolases (SAH) with single synthetase or hydrolase domains are present in many bacteria [16]. In *B. subtilis*, alarmone levels are controlled by Rel (often referred to as RelA), a bifunctional, RSH-type synthetase/hydrolase as well as two SAS proteins [22,23].

The synthesis and hydrolysis of (p)ppGpp allows the activation or repression of different cellular pathways by modulating various enzyme activities involved in GTP homeostasis, replication, transcription and translation, not only in response to amino acid starvation, but also to various other signals or stresses. It was observed for different bacteria that additional and diverse starvation or stress signals can activate the SR via interacting proteins or metabolites that bind and modulate the activity of RSH-type enzymes, or by transcriptional or post-translational regulation of monofunctional SAS [24,25]. *B. subtilis* and related Firmicutes lack a DksA homolog and a direct binding site for (p)ppGpp on RNA polymerase (RNAP) which mediate positive and negative stringent regulation in *E. coli* and other proteobacteria. Instead, in *B. subtilis* (p)ppGpp can exert transcriptional regulation via a drop in GTP levels caused by the direct inhibition of multiple enzymes of the GTP synthesis pathway [26,27]. Thereby, transcription of ribosomal RNA (rRNA) and ribosomal protein (r-protein) genes from promoters that initiate transcription with GTP is strongly reduced, while in turn promoters that initiate with ATP are activated [28,29]. In addition, the global regulator CodY is regulated by GTP via an allosteric binding site and de-represses amino acid biosynthesis genes and other pathways during the SR [30]. Beyond regulation of transcription, (p)ppGpp can inhibit translation initiation and elongation by binding, for example, to the translation initiation factor IF-2 and other ribosome-associated GTPases [31–33]. With its ability to inhibit translation and growth, the SR was also implied in persister cell formation and development of antibiotic tolerance [34]. In addition, virulence as well as survival of pathogens during infection was strongly affected in *rel* and (p)ppGpp^0 mutant strains [15,35].

During exposure to heat and oxidative stress, we and others previously observed in *B. subtilis* a pronounced down-regulation of rRNA and r-protein genes that resembled the pattern of the SR [13,14,39,40]. Thus, we hypothesized that the alarmone (p)ppGpp and the SR-like response could be

part of the heat shock response of *B. subtilis*. Therefore, we investigated the role of the SR and its intricate and mutual involvement with the cellular stress response during various proteotoxic stress conditions, including heat shock conditions, such as growth at high temperatures (50 °C), or thermoresistance and thermotolerance development [5,41].

Consistent with our hypothesis, we show here that the cellular level of (p)ppGpp was increased upon heat shock and also upon salt and oxidative stress. In addition, artificially raised alarmone levels conferred increased stress tolerance and a (p)ppGpp^0 strain appeared more stress sensitive. The presence of the bifunctional Rel was necessary and sufficient for the observed stress induced increase of (p)ppGpp. We analyzed changes in the transcriptome with RNA-sequencing (RNA-seq) experiments of wildtype, *rel* and (p)ppGpp^0 *B. subtilis* strains at raised temperatures and observed pleiotropic adjustments of transcription typical for SR, which was heat-dependent but also partially influenced by (p)ppGpp levels. However, the presence or absence of this second messenger had a more significant and immediate impact on limiting the translation of heat shocked cells. Our results suggest a model in which (p)ppGpp and Spx appear to play a complementary and partially redundant role in stress-mediated readjusting of transcription. In addition, we observed a prominent and instantaneous effect of the cellular alarmone (p)ppGpp levels on limiting translation, allowing the fast reallocation of cellular resources by raising the cellular repair capacity and concurrently reducing the protein load on the PQS during stress.

Results

Regimes for monitoring of heat shock stress response in *B. subtilis*

In this study, we monitored the stress response of *B. subtilis* by application of different, but related, heat shock conditions: (i) growth and heat shock at 50°C, a temperature that is non-lethal in *B. subtilis* but already induces a significant heat shock response with a raised expression of chaperones and proteases, (ii) resistance to severe heat shock by measuring the survival of exponentially growing cells exposed to a severe, lethal heat shock at 53 °C, which can also be considered as thermoresistance (37/53°C) (Fig. 1A), and (iii) the development of thermotolerance by measuring the survival of expo-

nentially growing cells primed by a 15 min mild pre-shock at 48 °C before their exposure to the severe heat shock at 53 °C (48/53°C) (Fig. 1A). We experimentally established that 55°C was an appropriate temperature to examine the impact of severe heat on *B. subtilis* cells growing on agar plates. In addition to exposure to these various heat conditions, we also examined other potentially proteotoxic stresses, such as salt and oxidative stress [5,14,41].

Cellular (p)ppGpp levels increase during heat shock exposure

To investigate the impact of heat on the stringent response, we first assessed the intracellular levels of the alarmones pGpp, ppGpp and pppGpp during the heat shock response at 50 °C. To do so, cells were grown at 37 °C in minimal medium to an optical density at 600 nm (OD_{600nm}) of 0.4, and subsequently they were treated with a single, non-lethal temperature upshift to 50 °C in order to induce the heat shock response. After 2, 5 and 10 minutes of incubation at 50 °C, the intracellular levels of the three alarmones (i.e. pGpp, ppGpp and pppGpp) were examined by liquid chromatography (LC)-coupled mass spectrometry (LC-MS). Already after 2 minutes, the alarmone levels increased approx. seven-fold (from 13 to 88 pmol OD^{-1} ml^{-1}) (Fig. 1B). The observed alarmone accumulation after 2 minutes at 50 °C was in a similar range to that previously observed upon amino acid starvation induced by DL-norvaline, serine hydroxamate, salt stress induced by 6 % (w/v) NaCl or 0.5 mM diamide, a strong oxidant of thiol groups (Fig. 1 C) [39,42]. It should be noted that the (p)ppGpp levels increased only transiently during heat shock and reduced to almost basal levels after approximately 10 minutes (Fig. 1B). Thus, we conclude that exposure to a non-lethal heat shock at 50°C elicits a fast, but transient, increase of the alarmones pGpp, ppGpp and pppGpp.

Having shown that (p)ppGpp levels transiently increase during heat shock, we next assessed the levels of the alarmones under thermoresistance conditions (37/53°C), after priming (37/48°C), as well as under thermotolerance conditions (48/53°C) (Fig. 1A & D). When we examined (p)ppGpp levels upon those temperature shifts, we observed transiently increased (p)ppGpp levels (Fig. 1D). The alarmone levels were particularly high during the severe heat shock shift at 37/53 °C (about 25-fold increase) and the induction was lower both for a 37/ 48°C or 48/53°C shift (about 2-3 fold increase) (Fig. 1D). Thermotolerant cells that were exposed to 48/53 °C showed a comparable alarmone level to cells exposed to 48 °C or 50 °C after 5 min (2-3 fold), while cells only exposed to a higher lethal heat

shock of 37/53 °C display a relative much higher alarmone level (Fig. 1B,C). The primed thermotolerant cells appear to be able to somehow limit the alarmone synthesis, when exposed to the lethal heat shock. The synthesis of (p)ppGpp that occurs during activation of the SR is normally accompanied by a fast reduction of cellular GTP levels in cells treated with serine hydroxamate (SHX) or DL-norvaline (NV) [27] and also after exposure to salt or diamide (Fig. S1A). Therefore, we were also interested in monitoring changes in GTP levels under conditions of heat shock but interestingly we do not observe a reduction in GTP levels after exposure to 50 °C (Fig. S1A). Notably, the GTP levels were at a comparable high level (FigS1B) during temperature upshifts of 37/48 °C, 37/53 °C and 48/53 °C, however GTP levels appeared a little lower for all temperature upshifts after 15 min incubation (Fig. S1B).

Taken together, we show that exposure to heat shock elicits a fast, but transient, increase of the alarmones pGpp, ppGpp and pppGpp, while not immediately affecting the GTP levels. Therefore, it seems that alarmone levels exhibit a graded response to stress, which appears to correlate to the temperature levels and possibly the heat stress intensity the cells are exposed to.

Rel is the main source for (p)ppGpp synthesis during stress response

Next, we aimed to identify the major source of (p)ppGpp during the heat stress response. To this end, strains with mutations that disrupt the (p)ppGpp synthetase activity of the proteins encoded by either *sasA/ywaC* and *sasB/yjbM* (*sasA/B*$^{-}$ strain) or *rel* (*rel*^{E324V}; inactive synthetase) were assayed for (p)ppGpp accumulation and GTP levels upon heat shock at 50 °C for 2 min (Fig. 1E, S1C). As a control, (p)ppGpp accumulation was also measured in a (p)ppGpp^{0} strain bearing inactivating mutations in all three alarmone synthetase genes (*sasA*, *sasB* and *rel*) (Fig 1E). In addition to monitoring (p)ppGpp accumulation directly, the (p)ppGpp-dependent transcription of *hpf* was employed as an additional read-out for the activation of the stringent response (Fig. S1D) [43,44]. As expected, alarmone nucleotides were not detected in the (p)ppGpp^{0} mutant under any conditions, neither stress or non-stress, consistent with finding that Rel, SasA and SasB are the only sources of (p)ppGpp in *B. subtilis* [23] (Fig. 1E). We observed that the *sasA*/*B*$^{-}$ strain also exhibited accumulation of (p)ppGpp (Fig. 1E) and up-regulation of the *hpf* transcript similar to the wildtype *B. subtilis* cells upon heat exposure (Fig. S1D), indicating that the activity of SasA and SasB is dispensable for (p)ppGpp production during heat stress. By contrast, the *rel*^{E324V} strain accumulated negligible amounts of (p)ppGpp in

response to heat, with the levels even dropping after heat shock (Fig. 1E). Consistently, up-regulation of the *hpf* transcript and accumulation of the Hpf protein in response to stress was also strongly impaired in the *rel*^{E324V} strain (Fig. S1D, S12D). Together, these results strongly suggest that Rel is the main source of (p)ppGpp during heat stress.

Activation of Rel during amino acid starvation requires the presence of uncharged tRNA on the ribosome [17,18]. A first indication of such a connection between SR and Rel activation in conjunction with the ribosome was the initial observation that (p)ppGpp accumulation upon starvation for amino acids was almost completely suppressed in the presence of the translation-inhibitor chloramphenicol [45]. To probe, whether Rel activation during heat or oxidative stress could utilize a similar pathway, we measured alarmone levels in stressed cells in the presence or absence of chloramphenicol (Fig. 1F, S1E). Interestingly, the addition of chloramphenicol completely suppressed alarmone accumulation and resulted in increased GTP levels upon heat and diamide treatment. Notably, chloramphenicol treatment of unstressed cells did not induce a SR, but decreased the basal (p)ppGpp levels and slightly increased GTP (Fig. 1F, S1E). These observations indicate that heat and oxidative stress could activate Rel in a similar manner to each other and similar to the pathway suggested for amino acid starvation.

B. subtilis cells lacking the alarmone are more sensitive to stress

To assess the importance of alarmone production for cellular survival under heat stress, we monitored growth of the wildtype, (p)ppGpp^0, *sasA/B*$^-$ and *rel*^{E324V} strains at 37 °C and 55 °C

(Fig. 2A, B). As expected, no obvious growth defects were observed for any of the strains at 37 °C. While the cellular survival of the *sasA/B*$^-$ strain at 55 °C was identical to that of the wildtype strain, strong growth defects were evident for the (p)ppGpp^0 and *rel*^{E324V} strains at 55 °C. These findings suggested that production of (p)ppGpp by Rel, but not SasA/B, is critical for survival of *B. subtilis* cells under heat stress. This prompted us to also investigate whether production of (p)ppGpp by Rel is critical for survival of *B. subtilis* cells under other stress conditions, such as high salt or oxidative stress. Indeed, severe growth defects were observed for both the (p)ppGpp^0 and *rel*^{E324V} strains under oxidative heat and salt stress, whereas the growth behavior of the *sasA/B*$^-$ strain again resembled the wildtype strain under the same conditions (Fig. 2C, D). Collectively, these findings suggest that pro-

duction of (p)ppGpp by Rel is critical for survival of *B. subtilis* cells, not only under heat stress, but also conditions of oxidative and salt stress.

High cellular (p)ppGpp levels confer elevated heat stress resistance

Next, we asked whether (p)ppGpp alarmone levels influence thermotolerance development and survival. To do this, we utilized the (p)ppGpp^0 strain, which cannot synthesize (p)ppGpp (Fig. 1E) as well as a *rel* deletion strain that displays raised (p)ppGpp (Fig. 2F) and lowered GTP levels (Fig. S2A). The high (p)ppGpp levels in the *rel* deletion strain arise because Rel is the only alarmone hydrolase in *B. subtilis* and causes an overall decrease in growth rate (Fig. 2F, S2A, B), as reported previously [23,46]. For completeness, we also assayed *sasA* and *sasB* deletion strains. As expected, exposure of wildtype *B. subtilis* cells to heat shock at 37/53°C led to a dramatic reduction in survival, e.g. 1000-fold (3-log) reduction in viability with 60 min heat shock at 53 °C, whereas survival remained unaltered when cells received a pre-shock at 48 °C for 15 min before being exposed to the lethal heat shock at 53 °C (Fig. 2G). Similarly, *B. subtilis* strains with single deletions in *sasA* or *sasB* phenocopied the wildtype strain for thermotolerance development (Fig. S2C-D), as they did for heat shock resistance (Fig. 2A-B and Fig. S2E). By contrast, we observed that *rel* deletion resulted in strongly increased thermoresistance, which was apparent from the high number of cells still able to form colonies during the otherwise lethal heat shock (Fig 2G). Consistently, we also observed a strong reduction in protein aggregation during the 37/53 °C heat shock (Fig 2 I). While no significant effect on thermotolerance development was observed in the (p)ppGpp^0 strain (Fig. 2 H), the (p)ppGpp^0 strain exhibited more protein aggregation when exposed to 37/53 °C heat shock (Fig 2 I).

To confirm that the elevated heat resistance phenotype of the *rel* strain was caused by the elevated levels of the alarmone (p)ppGpp, rather than the absence of the Rel protein, we expressed a truncated form of the *E. coli* RelA (*RelA$_{hyper}$*) that exhibits constitutive and hyperactive alarmone synthetase activity *in trans* in wildtype *B. subtilis* cells [47,48]. As a control, we also expressed a truncated form of the *E. coli* RelA (*RelA$_{inactive}$*) that has no alarmone synthetase activity [47,48]. In a second approach, we examined the (p)ppGpp^0 strain expressing *in trans B. subtilis* Rel with mutations that inactivate either the synthetase (Rel^{E324V}) or hydrolase (Rel^{H77AD78A}) domains. Expression of RelA$_{hyper}$ or hydrolase-inactive Rel^{H77AD78A} resulted in increased alarmone levels (Fig. S3A) and conferred high

thermoresistance (Fig. S3B,C), as observed for the Δ*rel* strain (Fig. 2G). By contrast, strains expressing $RelA_{inactive}$ or the synthetase-inactive Rel^{E324V} did not display increased alarmone levels (Fig. 3E), nor increased survival to severe heat stress (Fig. S3D,E).

High (p)ppGpp levels during the SR lead to a decrease in cellular GTP levels and this decrease is known to be intricately involved in causing the transcriptional changes during SR [27,28] (Fig. S1, S2A, S3A). To examine, whether the resistance to heat stress observed in the Δ*rel* strain could be mediated simply by lowering cellular GTP levels, wildtype cells were treated with decoyinine, an inhibitor of GMP synthetase, which results in a significant drop of cellular GTP levels (> 3-fold) without increasing (p)ppGpp levels [49,50]. Treatment with 250 or 400 μg ml^{-1} decoyinine resulted only in moderately increased thermoresistance and moderately decreased thermotolerance (Fig. S4). However, we could not observe the strongly increased thermoresistance as we observed before in the presence of raised (p)ppGpp levels (Fig 2 F, G, Fig S3). In addition, higher decoyinine concentrations (1000 μg ml^{-1}) even abolished both thermoresistance and thermotolerance development (Fig S4). These experiments suggest that lowered cellular GTP levels, which turn the transcriptional stringent response on [27,28,51], is not sufficient to elicit heat resistance as observed in strains with elevated (p)ppGpp levels (Fig 2 F, G, Fig S3).

From these observations we infer that raised (p)ppGpp levels are sufficient to confer increased stress resistance and reduced levels of heat-induced protein aggregates. This phenotype is dependent on the levels of alarmones and not the presence or absence of the specific Rel protein *per se*, since it could be reconstituted by *in trans* expression of full-length or truncated Rel or RelA protein variants from *B. subtilis* or *E. coli* that actively synthesized (p)ppGpp (Fig. 2, S3). The SR mediated drop in cellular GTP levels was not observed during heat shock response (Fig. S1) and an artificial reduction of cellular GTP levels had only a moderate effect on thermoresistance and even abolished thermotolerance (Fig. S4). Taken together, these experiments suggest that the cellular level of the second messenger (p)ppGpp *per se* appears to be important for the modulation and enhancement of the heat shock response in *B. subtilis* cells, since a strain lacking the alarmone is more stress sensitive (Fig. 2 A-D, H, I) and strains with constitutively raised alarmone levels are much more stress resistant (Fig. 2 F, G, I).

Constitutive stringent response in Δ*rel* cells results in global transcriptional changes

To obtain further insights into the impact of *rel* deletion and (p)ppGpp accumulation on transcriptome changes, we performed RNA-seq analyses and annotated transcription start sites (TSS) of exponentially growing wildtype, (p)ppGpp^0 and Δ*rel* strains (Fig. S5, see S1 Text for a detailed analysis, Dataset S1, Dataset S2, Dataset S3). Since down-regulation of "stable" rRNA is a hallmark of the SR, we introduced a previously established chromosomal *rrnJ*p1-*lacZ* fusion into the assessed strains, thereby allowing us to follow the activity of this rRNA promoter using the *lacZ* reporter [14]. Only small changes between wildtype and (p)ppGpp^0 strains (45 genes significantly regulated, Fig. 3A, Fig. S6B) were observed during non-stressed growth, while Δ*rel* cells exhibit broad transcriptional changes compared to wildtype cells (494 genes regulated, Fig. 3A & Fig. S6C). However, the full extent of the impact of (p)ppGpp was revealed when we compared the transcriptome of the (p)ppGpp^0 with Δ*rel* strain (Fig. 3A, B). Here we observed a differential regulation of the expression of 682 genes with a broad down-regulation of translation-related genes known to be part of the SR regulon (Fig. 3B). We performed RT-qPCR experiments with independent samples of the (p)ppGpp^0 and Δ*rel* strains, measuring the transcripts of selected genes known to be under stringent control and observed a good correlation with the RNA-seq data (Fig. 3C). The *rrnJ*p1-*lacZ* transcript was down-regulated 1.7-fold in the RNA-seq experiment and confirmed by RT-qPCR (Fig. 3C). Furthermore, we noticed an extensive up-regulation of genes that function in amino acid synthesis, indicating a de-repression of the CodY regulon (e.g. *ilvB* 294-fold up-regulated, Fig. 3B,D,E, S8, Dataset S2) [27,52]. Interestingly, we detected a strong decrease in the transcription of CcpA-regulated genes required for the utilization of alternative carbon sources (e.g. *rbsC* 181-fold down-regulated) and a broad regulation of stress-related genes accompanied by an activation of the SigB regulon (e.g. *dps* 2.8-fold up-regulated, *ssrA* 4.5 fold up-regulated). We also observed a reduced transcription of genes regulated by the regulators HrcA and CtsR (e.g. *dnaK* 6.4-fold down-regulated, *clpE* 7.1-fold down-regulated) (Fig. 3C, D, E and Fig. S7, S8, Dataset S2), when comparing the (p)ppGpp^0 and Δ*rel B. subtilis* strains at 37 °C without heat exposure. Notably, the transcription of *hpf* (*yvyD*), encoding the hibernation promoting factor Hpf, was induced by raised (p)ppGpp levels (24-fold up-regulated, Fig. 3C,E, Fig. S8), confirming that the increased transcription of *hpf* can be considered as a reporter for the activation of the SR [43,44].

(p)ppGpp modulates transcription during heat stress response

To study the impact of the SR on the transcriptome during heat exposure, we examined the (p)ppGpp^0 and Δ*rel* strains not only at 37 °C (Fig. 3), but also at 48 °C, in the same RNA-seq experiment that was used to investigate the thermoresistance (37/53 °C) and thermotolerance (48°/53 °C) conditions (Fig.4 & Fig. 1A) [5,14]. Thermotolerant cells (48/53 °C) exhibited a pronounced up-regulation of the heat-specific stress response (median 3.4-fold up-regulated) or general stress response (median 3.0-fold up-regulated) as well as comprehensive down-regulation of translation-related genes (median 2.6-fold down-regulated, Fig. 4A, B, S7, Dataset S2) that was, to a lesser extent, also observed in the mild pre-shock (48 °C, median 1.3-fold down-regulated) and severe heat shock (37/53 °C, median 1.2-fold down-regulated) conditions (Fig. S6 A, S7) in agreement with previous observations [14]. The transcription of translation-related genes was generally lower than wildtype levels in Δ*rel* cells (median 1.6-fold down-regulated) and higher in (p)ppGpp^0 strains under non-stress conditions (37 °C) (Fig. 4C, S7). We could confirm by RT-qPCR that down-regulation of *rrnJ*p1-*lacZ* is partially (p)ppGpp-dependent during thermotolerance development (Fig. 4C,D), which became even more pronounced when the 50 °C heat shock condition was examined (Fig. S8) [14]. Nevertheless, the transcription of the genes encoding conserved chaperones and proteases of the heat shock response were strongly up-regulated upon all temperature up-shifts, independently of the presence or absence of (p)ppGpp (Fig. 4 C). Interestingly, additional qPCR experiments applying a 50 °C heat shock revealed that the heat-induced expression of some SigB regulated genes was impaired in the (p)ppGpp^0 background, e.g. of *ssrA* (approx. 2-fold lower expression in (p)ppGpp^0 cells at 50 °C) and *dps* (approx. 3-fold lower expression), indicating a functional connection between the SR and the general stress response (Fig. S8) [53,54]. However, the majority of genes of the SigB regulon were found to be induced in the (p)ppGpp^0 strain similarly to wildtype cells at 48 °C (Fig. S7). Likewise, while CcpA-regulated genes were repressed in wildtype and (p)ppGpp^0 cells under heat shock conditions (Fig. 3B,D, Fig. 4A-C, S7), some genes (e.g. *rbsD, ganP, licH*) were less down-regulated or even induced at 48 °C in the (p)ppGpp^0 strain (Fig. 4C). In contrast, motility-genes were particularly strongly down-regulated by heat in the (p)ppGpp^0 mutant (median 3.0-fold change, Fig. 4C, S6B, S7), while the down-regulation of these genes appeared to be not significant in wildtype cells at 48 °C (median 1.14-fold change, S7)

[55]. Notably, the expression of the *hpf* and *ilvB* transcripts, the transcription of which is positively regulated by the SR, was lower during heat stress in the (p)ppGpp^0 strain compared to wildtype cells (*hpf*: 3.7-fold lower in (p)ppGpp^0 cells relative to wildtype at 50 °C, *ilvB*: 1.6-fold lower, Fig. 4D, S8).

Spx and the stringent response act complementary during heat shock

Previously, we reported that Spx, a central regulator of the heat and oxidative stress response, can down-regulate the transcription of translation-related genes and rRNA (ref). However, an *spx* deletion was not impaired in the heat-mediated down-regulation of these genes [14]. Here, we noticed a complex, but clearly detectable, involvement of the SR in the down-regulation of specific genes during heat stress (Fig. 4C-D), suggesting an intricate regulation of these genes by different factors, including Spx and (p)ppGpp. To test for such a concurrent and complementary transcriptional regulation, a *B. subtilis* strain combining a *spx* deletion with the (p)ppGpp^0 mutations was constructed. Strikingly, down-regulation of *rrnJ*p1-*lacZ* upon heat shock was completely abolished in this (p)ppGpp^0 Δ*spx* strain, indicating a concurrent and complementary activity of both regulators on this promoter (Fig. 5A). However, the transcription of some r-protein genes was also down-regulated in the (p)ppGpp^0 Δ*spx* strain (Fig. S9A), suggesting additional factors beyond Spx and (p)ppGpp, that can also influence the promoter and/or the stability of these transcripts. Interestingly, this (p)ppGpp^0 Δ*spx* strain, lacking both regulators, also displayed a slow growth phenotype at 37 °C and a more severe growth defect at 50 °C compared to the strains with single deletions of (p)ppGpp^0 or Δ*spx* (Fig. 5B, Fig. S9B). This experiment suggests a genetic interaction of the SR and the *spx* regulon under heat stress conditions. In addition, the (p)ppGpp^0 Δ*spx* strain accumulated more heat-induced protein aggregates at 50 °C than cells lacking either (p)ppGpp or *spx* (Fig. S9 C).

When mutations in *rpoA* were introduced in the (p)ppGpp^0 strain that abolish Spx-mediated up- and down-regulation (*cxs*-1/*rpoA*^{Y263C}), or interfere only with Spx-mediated repression of rRNA while still allowing up-regulation of redox chaperones (*cxs*-2 / *rpoA*^{V260A}) [14], only the (p)ppGpp^0 *cxs*-1 strain displayed a severe growth defect as observed for the (p)ppGpp^0 Δ*spx* strain (Fig. S9B). This experiment suggests that the Spx-mediated up-regulation of stress response genes, and not the ability to down-regulate translation related genes, is required for efficient growth in the (p)ppGpp^0 background. Notably, (p)ppGpp is sufficient for the down-regulation of translation-related genes dur-

ing norvaline-induced amino acid limitation, while Spx is not required for this process (Fig. S10A). Conversely, Spx can act on rRNA promoters independently of (p)ppGpp *in vivo* (Fig. S10B) [14]. In addition, *in vitro* transcription experiments with purified Spx and RNAP gave no indications that ppGpp could directly influence Spx transcriptional activation or inhibition of RNAP (Fig. S10C).

Together, these observations suggest that both regulatory systems act concurrently but independently on rRNA and r-protein promoters, allowing the inhibition of transcription of translation-related genes by Spx and to a minor extend also by the (p)ppGpp-mediated transcriptional response.

(p)ppGpp curbs translation during heat stress

We observed that the raised levels of (p)ppGpp, but not the transcriptional reprogramming during SR, appears to be necessary for the observed strong heat stress resistance (Fig 2, 3, 4, Fig S3 & S4). Therefore, we wanted to determine the impact of (p)ppGpp on translation during heat stress. To this end, a method for pulse-labeling newly synthesized nascent peptide chains using low amounts of puromycin was utilized to estimate protein synthesis rates (see S1 Text, Fig. S11). When we examined growing cells at 50° C, or cells exposed to thermotolerance conditions (48/53 °C), we observed that the Δ*rel* strain always exhibited a lower translation rate compared to the wildtype cells (Fig. 6A,B), consistent with its raised (p)ppGpp levels and the observed "stringent" phenotype of this strain. By contrast, the "relaxed" (p)ppGpp^0 strain always exhibited higher translation rates (Fig. 6A,B), indicating a more deregulated translation. During the non-lethal 50 °C heat shock, translation rates transiently increased in all strains (Fig. 6A), corresponding with the high growth rate at this temperature (Fig. S9B). Nevertheless, the (p)ppGpp^0 strain still displayed significantly higher translation rates compared to wildtype and the Δ*rel* strains (Fig. 6A and 6B).

Treatment with a lethal temperature shift (37/53 °C) without pre-shock resulted in a strong decrease in translation efficiency in wildtype and (p)ppGpp^0 strains, whereas translation in Δ*rel* cells was transiently increased (Fig. S12A), in agreement with the observed high heat-resistance of this strain (Fig. 2G). Interestingly, translation was strongly decreased in (p)ppGpp^0 cells at 37/53 °C, while wildtype cells still maintained active translation under this condition (Fig. S 12A). The lowered translation activity in (p)ppGpp^0 cells appears to be accompanied by a strong reduction of the levels of

cellular 16S rRNA (Fig. S12C), which could indicate a defect in 16S rRNA maturation and the assembly and/or activity of the small ribosomal subunit.

The (p)ppGpp^0 strain also failed to induce expression of the *hpf* gene during heat stress and did not accumulate the Hpf protein (Fig. 4C,D, S12D). Thus, the formation of 100 S disomes upon heat stress, which was clearly visible in the ribosome profiles of wt and Δ*rel* cells, especially under thermotolerance conditions, was strongly reduced in the (p)ppGpp^0 strain (Fig. 6C). However, the observed apparent degradation of the 16S rRNA under severe stress conditions was not prevented by *in trans* expression of Hpf (Fig. S12 E) and overexpression of Hpf could not rescue the heat-sensitive phenotype of (p)ppGpp^0 strains (Fig. S12F). Also, the addition of translation-inhibiting antibiotics could not rescue this phenotype, indicating that inhibition of translation *per se* is not sufficient to protect ribosomes during severe heat stress (Fig. S12G).

The observed influence of (p)ppGpp on translation suggests that the major impact of (p)ppGpp appears not to be its effect on transcription (Fig. 4, 5), but the direct modulation of translation (Fig. 6, S12), possibly by directly interfering with the activity of different translational GTPases [31,33]. Conversely, Spx appears to act on transcriptional regulation of stress-response and translation-related genes [14]. To assess the relative impact of Spx on translation, we examined the translation rate in a *B. subtilis* strain encoding an inducible gene for the synthesis of a stable Spx^{DD} variant and observed only a 20 % reduction of translation by Spx^{DD} induction (Fig. S9D). This reduction may be an indirect result of the Spx^{DD}-mediated repression of rRNA synthesis with the ensuing decreased synthesis of new ribosomes [14].

In summary, these observations indicate that the intracellular (p)ppGpp second messenger can immediately control translation during heat stress and is involved in the protection of ribosomes from damage upon severe heat stress (Fig. 7).

Discussion

In this study, we analyzed the role of the SR during heat shock in *B. subtilis*. We could demonstrate that upon heat shock the second messenger (p)ppGpp is rapidly synthesized mostly by Rel and can confer enhanced thermoresistance to these cells. Our data suggests that (p)ppGpp is a pleiotropic regu-

lator, affecting several transcriptional processes, but mostly modulates and protects translation during heat stress. The SR- and Spx-mediated heat shock responses can act concurrently and might be able to complement one another in the down-regulation of rRNA transcription during stress. Overall, our results suggest that limiting translation is an integral part of the *B. subtilis* stress response (Fig. 7).

The activation of the stringent response during heat stress

The presented results clearly demonstrate a rapid accumulation of (p)ppGpp during heat and other environmental stresses (Fig. 1). In addition, strains unable to synthesize (p)ppGpp are rendered sensitive to high temperatures and accumulate more heat-induced protein aggregates (Fig. 2A-D,H,I). Interestingly, (p)ppGpp synthesis and heat tolerance are solely dependent on the synthetase activity of Rel, indicating that this enzyme is the major contributor of (p)ppGpp under these conditions (Fig. 1E, 2A-D). Activation of the SR by Rel-mediated (p)ppGpp synthesis during heat and oxidative stress has been reported for diverse taxa, suggesting that the underlying mechanisms could be conserved [56–58]. However, little is known about the mechanism of Rel activation upon environmental stress.

RSH-type enzymes have been implicated in sensing and integrating many environmental cues beyond amino acid starvation. These additional signals may be transmitted by direct interaction of RSH-type with additional regulatory proteins, which expand and adapt SR-signalling to the respective requirements of the environmental niche [24,25]. For example, growth inhibition in competent *B. subtilis* cells has recently been shown to be mediated by a specific interaction between Rel and ComGA, a membrane associated ATPase which is involved in uptake of DNA in competent cells [59]. It was suggested that the interaction of ComGA with Rel inhibits its hydrolase activity, resulting in the accumulation of (p)ppGpp and the inhibition of rRNA synthesis and growth. Furthermore, the growth-inhibition by the SR promotes increased antibiotic tolerance of competent cells and therefore contributes to bet-hedging and improves fitness of the population [59].

Our experiments demonstrate that Rel activation during heat- or oxidative stress can be inhibited by chloramphenicol similarly as during amino acid starvation (Fig. 1F, S1E). Therefore, the underlying activation mechanisms during environmental stress likely shares some similarities to the well-studied SR-activation upon amino acid deprivation and may also involve the sensing of uncharged tRNA on the ribosome [17,18,56,58].

Proteotoxic and oxidative stress results in the inactivation of labile enzymes and may thereby impair uptake or biosynthesis of certain amino acids and/or modulate the activity of aminoacyl-tRNA synthetases, resulting in an accumulation of uncharged tRNA which can serve as a signal to activate Rel [58,60]. In addition, tRNAs and proteins of the translational machinery are prone to oxidation or modification upon stress, leading to translation stalling, which can also elicit the SR [61].

The role of (p)ppGpp and the transcription factor Spx in global transcriptional regulation upon heat stress

Using RNA-seq, we observed large transcriptomic alterations mediated by (p)ppGpp in the Δ*rel* mutant (Fig. 3). Since *B. subtilis* lacks a DksA homolog, regulation of transcription by (p)ppGpp is achieved indirectly by lowering GTP levels, which reduces transcription of promoters that initiate with GTP. Accordingly, transcription of rRNA and r-protein genes was strongly reduced in the Δ*rel* strain as a consequence of the lower GTP level [28,62]. In addition, the CodY activity is under allosteric control by cellular GTP levels [52]. While the effect of both mechanisms is prominently visible in Δ*rel* cells where the GTP level is strongly reduced, their impact is less noticeable in heat-stressed cells where a transient increase of alarmones, but no decrease of the GTP concentration, was observed (Fig. 1, S1). It is possible that the transient pulse, its kinetic and the generated total amount of (p)ppGpp induced by the raised temperature might not be sufficient to promote the strong reduction of cellular GTP that is observed during amino acid starvation (Fig. S1) [27]. It should be noted that a strong reduction of cellular GTP levels would most likely also interfere with the ability of *B. subtilis* cells to grow at 50 °C with a growth rate comparable to that at 37 °C (Fig. S9 B). When designing the RNA-seq experiment, we choose 48 °C as a simple heat shock condition for the mutant strains since it resembled the thermotolerance protocol (Fig. 1A) and the condition of previously published microarrays [14]. However, many phenotypes of Spx and (p)ppGpp could be observed best upon a stronger, but non-lethal, heat shock at 50 °C [14]. In contrast, while wildtype cells treated with 37/53 °C exhibit a strong increase of (p)ppGpp within the first minutes of stress (Fig 1D), the examination of cellular physiology is confounded by the rapid reduction of viability at this lethal condition (Fig. 2G) [5,14].

We reported previously that transcription of rRNA can be down-regulated by the global regulator Spx. During heat stress however, down-regulation of rRNA was independent of Spx, suggesting

that the loss of Spx was compensated by additional regulators [14]. Strikingly, we now observed that (p)ppGpp also engages in this down-regulation of rRNA during heat stress and that the concurrent activity of both Spx and (p)ppGpp is required to reach the full strength of this effect (Fig. 5A). This functional relationship of Spx and the SR is corroborated by the observation that the (p)ppGpp^0 Δ*spx* mutant strain displays strongly impaired growth at both 37 °C and 50 °C (Fig. 5B). In addition, the observation that a (p)ppGpp^0 *cxs*-2 strain, in which Spx can still up-regulate the stress response, exhibits a much less impaired growth than a (p)ppGpp^0 Δ*spx* or (p)ppGpp^0 *cxs*-1 strain in which Spx activity is fully disrupted. This suggests that at least either (p)ppGpp or Spx is required for the transcription of unknown factors necessary for efficient growth under adverse conditions. The observation that transcription of *spx* is also activated by (p)ppGpp via CodY in *Enterococcus faecalis* and *rel* transcription is activated by the disulfide-stress regulator σ^R in *Streptomyces coelicolor* points toward possible functional connection of these two regulators [63,64].

Our RNA-seq dataset also indicates a possible activation of the SigB-dependent general stress response by (p)ppGpp during stress- and non-stress conditions (Fig S7). SigB becomes activated by decreased GTP levels as elicited by decoyinine [53,54]. In addition, a requirement of L11, which is necessary for Rel synthetase activity, and Obg, a ribosome-associated GTPase that interacts with ppGpp, for the activation of SigB upon physical stress and an interaction of Obg with components of the SigB regulatory cascade was reported, suggesting an intricate connection between the ribosome, Rel and the general stress response [53,65,66].

Control and protection of translation by (p)ppGpp and the role of Hpf during heat stress

Our results suggest that (p)ppGpp acts as a negative regulator of translation during heat shock. (p)ppGpp was shown to bind and inhibit many ribosome-associated GTPases thus interfering with ribosome assembly and arresting translation [32,33]. The relative reduction of translation during stress would reduce the load on the protein quality control systems, thus alleviating the burden for cellular protein homeostasis upon protein folding stress [3,67]. This hypothesis is supported by the observation that the (p)ppGpp^0 mutant accumulated more protein aggregates during heat stress, whereas a Δ*rel* mutant strain exhibited significantly lower translation, but at the same time generated also significantly less protein aggregates (Fig. 2I). (p)ppGpp is also required for the efficient transcription and synthe-

sis of the Hpf protein that promotes the formation of translationally inactive 100S disomes, which supports the fast regrowth of cells after stress conditions have ceased [43,68,69]. Furthermore, we observed that the 16S rRNA was degraded and translation was diminished in the (p)ppGpp^0 mutant upon severe stress at 37/53 °C. This phenotype was neither rescued by *in trans* expression of *hpf* or the addition of antibiotics that inhibit translation, suggesting that a specific mechanism for the protective action of (p)ppGpp and not an inhibition of translation is required for this process. However, cells were reported to be able to fully recover from the heat-induced rRNA degradation [70]. It was recently observed *B. subtilis* that tRNA maturation defects could lead to an inhibition of rRNA processing and 30S assembly via the synthesis of (p)ppGpp [71]. These observations might be important to understand possible stress signaling pathways and also the protective effect of (p)ppGpp on translation under proteotoxic stress conditions.

The role of the SR during the heat stress response

Taken together, our data suggest a model in which cells respond in a concerted manner to heat-mediated protein unfolding and aggregation, not only by raising the repair capacity, but also by decreasing translation to concurrently reduce the load on the cellular protein quality control systems (Fig. 7A). Upon heat shock, Rel is activated and rapidly synthesizes alarmones. These alarmones mediate, in conjunction with Spx, a strong down-regulation of ribosomal promoters together with the up-regulation of stress response-genes such as *hpf* during heat shock (Fig. 7B), while chaperones and stress-response genes controlled by Spx and other regulators are concurrently up-regulated. In addition, the second messenger (p)ppGpp could directly control the activity of translation factors and may thereby mediate a fast and immediate response to slow down translation during stress. Together, the combined readjustments on transcription and translation allow an efficient reallocation of cellular resources to the synthesis of stress response proteins and concurrently minimize the load on the protein quality control systems, thus contributing to protein homeostasis [3,67,72]. The unfolded protein response to misbalances in protein homeostasis in the endoplasmic reticulum of eukaryotic cells is a well-studied and analogous stress response mechanism where the up-regulation of chaperones is also coupled to the concurrent down-regulation of translation, albeit by different mechanisms [3,73].

Interestingly, accumulation of (p)ppGpp upon heat or oxidative stress and its importance for stress resistance has also been reported in other *Firmicutes* and also *Proteobacteria* that differ widely in terms of (p)ppGpp signaling [56–58,74,75]. Accumulation of (p)ppGpp was shown to protect cells from salt or osmotic stress [76,77]. Conversely, the lack of (p)ppGpp is known to renders cells sensitive to heat or oxidative stress [58,78,79], suggesting that activation of the SR, allowing the fast down regulation of translation, is an important and conserved part of the response to environmental stress in bacteria. It is interesting to note that SR was also implicated in *B. subtilis* competence development, facilitating a cellular state (also referred to as the K-state) where cells cease to divide, and most transcription and translation is strongly down-regulated. In these cells only competence proteins, together with DNA repair and recombination genes, are expressed, allowing the uptake and possible utilization of homologous of DNA in this specific cellular state of a subpopulation of stationary phase cells [59]. Bacterial cells thus appear to utilize the (p)ppGpp second messenger, which can interfere directly with basic cellular processes such as translation, replication and growth, as an important part of different regulatory networks, facilitating and allowing the survival of bacterial cells in fast changing environments with limited nutrient availability and exposure to various stress conditions.

Methods

Construction of strains and plasmids

Strains, plasmids and primers are listed in S1 Table. PCR-amplification and molecular cloning using *E. coli* DH5α as host was carried out according to standard protocols [80]. Point mutations were introduced via overlap-extension PCR. To generate pBSII-spxDD-spec, a fragment carrying spx^{DD}, *lacI* and the spectinomycin resistance cassette was amplified from pSN56 [12] with primers p289/p223 and ligated using *Spe*I/*Nsi*I sites into the pBSIIE backbone amplified with primers p203/p288. Integrative plasmids were linearized by digestion with *Sca*I or *Bsa*I prior to transformation. Point mutations in the *rel* gene were first cloned in the pMAD vector and then re-amplified for cloning into pDR111.

Transformation of *B. subtilis* strains, the generation of scarless mutations using the pMAD system and the introduction of *cxs-1/2* mutations in *rpoA* was carried out as described previously [81–83]. Mu-

tants were selected on 100 µg ml^{-1} spectinomycin, 10 µg ml^{-1} kanamycin, 1 µg ml^{-1} erythromycin, 25 µg ml^{-1} lincomycin or 5 µg ml^{-1} chloramphenicol, respectively. To obtain the (p)ppGpp^0 strain (BHS214), markerless *sasA*^{E154V} and *sasB*^{E139V} mutations were introduced into *B. subtilis 168* cells by successive transformation and recombination of plasmids pMAD-sasA^{E154V} and pMAD-sasB^{E139V}, yielding strain BHS204. Next, a PCR amplified fragment carrying *rel::erm* [22] and flanking homologous regions was transformed to generate BHS214. Since the (p)ppGpp^0 strain fails to develop natural competence, additional mutations were introduced in BHS204 and transformed with a PCR-amplified *rel::erm* fragment or BHS214 genomic DNA in a second step.

Growth conditions

B. subtilis strains were grown in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone-peptone, 10 g L^{-1} NaCl) or minimal medium [84] supplemented with 0.5 % casamino acids in water baths with 200 rpm orbital shaking at the desired temperatures. 1 mM IPTG or 0.4 % xylose was supplemented if required.

Survival and viability assays

The assays for thermotolerance development, survival and preparation of protein aggregate are described previously [5]. 1 mM IPTG was added to induce expression of recombinant proteins 30 min before the division of the culture. The influence of decoyinine on thermotolerance was tested in 1.5 mL tubes in thermoshakers. Detection of aggregates by fluorescence microscopy was described previously in [41]. Spot colony formation assays were carried out as described previously and incubated at the indicated temperatures [14].

Transcription analysis

Strains were grown in LB and treated as indicated. Samples of 15-25 mL were harvested by centrifugation for 3 min at 3.860 xg at 4 °C and frozen in liquid nitrogen. Isolation of total RNA, treatment with DNase I (NEB) and quality control by native agarose gel electrophoresis, methylene blue staining and northern blotting was described previously [14]. Northern blotting, hybridization with DIG-labeled RNA probes and detection was carried out as described previously [14]. Primers for the synthesis of probes are listed in S1 Table. Reverse transcription and qPCR were carried out as described previously [14]. The primers are listed in S1 Table. 23S rRNA was used as a reference.

RNA sequencing

Cells of BHS220, BHS319 and BHS368 were grown in 150 mL LB medium in 500 mL flasks in water baths at 37 °C and 200 rpm. In the mid-exponential phase ($OD_{600\ nm}$ ~ 0.4), the culture was divided and shifted to 48 °C or left at 37 °C. After 15 min, samples were withdrawn and both cultures were shifted to 53 °C for another 15 min and harvested. Cells from 25 mL medium were pelleted by centrifugation for 3 min at 3.860 x g and 4 °C and flash-frozen in liquid nitrogen. RNA was prepared the using phenol/trizol method as described in [85] and treated with TURBO DNase (Invitrogen). RNA quality was assessed on a Bioanalyzer 2100 System (Agilent).

rRNA depletion from total RNA using MICROBExpress (Ambion), treatment with tobacco acid pyrophosphatase (TAP) for +TAP libraries, library preparation, Illumina sequencing and quality control of the sequencing output was carried out as described previously [86]. Reads were mapped to the *Bacillus subtils* 168 genome with insertion of *rrnJp1-lacZ* in the *amyE* site (strain BHS220, *amyE::rrnJp1-lacZ cat*) using Bowtie2 (version 2.1.0) reads [87] with default parameters and filtered for uniquely mapped reads using SAMtools [88]. The DEseq2 package with default parameters was used for the detection of differentially expressed genes from raw count data of triplicate experiments [89]. Expression changes were considered significant if differentially regulated by at least 4-fold (p-value ≤ 0.05). The data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE125467 [90]. Identification of transcription start sites (TSS) and gene set enrichment analysis (GSEA) is described in S1 Text.

***In vitro* transcription**

In vitro transcription assays using purified *B. subtilis* RNA polymerase and Spx protein was carried out as described previously [14].

Fluorescence microscopy

Strain BIH369 (*lacA::Pxyl-yocM-mCherry erm*) was grown in LB medium + 0.5 % xylose. The culture was divided in the mid-exponential phase, supplemented with puromycin for 15 min and subjected to fluorescence microscopy in a Axio Imager.Z2 (Zeiss) microscope using the RFP filter set [14].

SDS PAGE and Western blotting

Strains were grown in LB medium and treated as indicated, harvested by centrifugation for 5 min at 3.860 xg at 4 °C, washed in TE buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 8.0) and disrupted by sonication in TE supplemented with 1 mM PMSF. Equal amounts of protein were separated by SDS-PAGE and stained with coomassie or subjected to western blotting [91–93]. For signal detection, polyclonal α-Hpf antibody (1:5000) [69] or monoclonal anti-puromycin antibody (1:10.000, Merck) and HRP-conjugated anti-mouse or anti-rabbit antibodies (1:10.000, Roth) were used in conjunction with the ECL-system as described previously [14]. Images were acquired using a ChemoStar Imaging System (Intas, Göttingen, Germany)

Translation rate analysis

Strains were grown in LB medium and treated as indicated. For *in vivo* labeling, 10 mL medium were separated, supplemented with 1 µg mL^{-1} puromycin (Roth) and incubated for 15 min at the same conditions. Then, samples were supplemented with 25 µg mL^{-1} chloramphenicol, harvested by centrifugation for 5 min at 3.860 xg at 4 °C, washed in TE buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 8.0) and disrupted by sonication in TE supplemented with 1 mM PMSF. Equal amounts of protein were directly spotted on nitrocellulose membranes (5 µg) or subjected to SDS-PAGE and western blotting [80]. Puromycin-signals were detected using monoclonal anti-puromycin antibody (1:10.000, Merck), HRP-conjugated anti-mouse antibody (1:10.000, Roth) and the ECL-system in a ChemoStar imaging system (Intas, Göttingen, Germany). Signals were analyzed using Fiji distribution of ImageJ [94].

Sucrose density gradient centrifugation analysis

Early exponential phase cultures of *B. subtilis* strains grown in LB medium were treated with heat shock at 48 °C or 48 °C/53 °C for 15 min each. Samples of 50 mL were supplemented with 50 µg mL^{-1} chloramphenicol to stall translation and harvested by centrifugation at 4000 x g for 10 min at 4 °C. Cells were resuspended in 25 mM HEPES-KOH, pH 7.5, 150 mM KOAc, 25 mM $Mg(OAc)_2$, 1 mM dithiothreitol (DTT), n-Decyl−β−D-thiomaltopyranoside (DTM), 5 % (w/v) sucrose) and lysed by sonication. The lysate was cleared by centrifugation at 16,000 x g for 15 min at 4 °C. 10 OD_{260} units

were loaded on a 10 mL 5-45 % (w/v) sucrose gradient prepared in the same buffer, run in a SW-40 Ti rotor (Beckman Coulter) at 57471 x g for 16.5 h and analyzed using a Gradient Station (Biocomp) with an Econo UV Monitor (Bio-Rad).

Quantification of nucleotides

Cells were grown in minimal medium supplemented with 0.5 % casamino acids to support the growth of (p)ppGpp deficient strains [95] and treated as indicated. Samples of 2 mL were removed, supplemented with 75 µL 100 % formic acid and incubated on ice for 30 min. Extraction of nucleotides was carried out as described in [96] and detected by HPLC-ESI-MS/MS on a QTRAP 5500 instrument. Analytes were separated on a Hypercarb column (30 x 4.6 mm, 5 µm particle size) in a linear gradient of solvent A (10 mM ammonium acetate pH 10) and solvent B (acetonitrile) at a flow rate of 0.6 mL/min from 96 % A + 4 % B (0 min) to 40 % A + 60 % B (8 min) into the ESI ion source at 4.5 kV in positive ion mode. Tenofovir was used as internal standard. pGpp and pppGpp standards were synthesized *in vitro* from ATP and GTP or GMP as described previously [97]. ppGpp was purchased from Trilink Biotechnologies.

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References

1. Storz G, Hengge R, American Society for Microbiology, editors. Bacterial stress responses. 2nd ed. Washington, DC: ASM Press; 2011.
2. Mogk A, Huber D, Bukau B. Integrating protein homeostasis strategies in prokaryotes. Cold Spring Harb Perspect Biol. 2011;3. doi:10.1101/cshperspect.a004366
3. Hartl FU, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. Nature. 2011;475: 324–332. doi:10.1038/nature10317
4. Völker U, Mach H, Schmid R, Hecker M. Stress proteins and cross-protection by heat shock and salt stress in Bacillus subtilis. J Gen Microbiol. 1992;138: 2125–2135. doi:10.1099/00221287-138-10-2125
5. Runde S, Molière N, Heinz A, Maisonneuve E, Janczikowski A, Elsholz AKW, et al. The role of thiol oxidative stress response in heat-induced protein aggregate formation during thermotolerance in *B acillus subtilis*: Thiol oxidation in protein aggregate formation. Molecular Microbiology. 2014;91: 1036–1052. doi:10.1111/mmi.12521
6. Hecker M, Schumann W, Völker U. Heat-shock and general stress response in *Bacillus subtilis*. Molecular microbiology. 1996;19: 417–428.
7. Schumann W. The *Bacillus subtilis* heat shock stimulon. Cell Stress Chaperones. 2003;8: 207–217.
8. Mogk A, Homuth G, Scholz C, Kim L, Schmid FX, Schumann W. The GroE chaperonin machine is a major modulator of the CIRCE heat shock regulon of *Bacillus subtilis*. EMBO J. 1997;16: 4579–4590. doi:10.1093/emboj/16.15.4579
9. Krüger E, Hecker M. The first gene of the *Bacillus subtilis* clpC operon, ctsR, encodes a negative regulator of its own operon and other class III heat shock genes. J Bacteriol. 1998;180: 6681–6688.
10. Elsholz AKW, Michalik S, Zühlke D, Hecker M, Gerth U. CtsR, the Gram-positive master regulator of protein quality control, feels the heat. The EMBO Journal. 2010;29: 3621–3629. doi:10.1038/emboj.2010.228
11. Hecker M, Pané-Farré J, Uwe V. SigB-Dependent General Stress Response in *Bacillus subtilis* and Related Gram-Positive Bacteria. Annual Review of Microbiology. 2007;61: 215–236. doi:10.1146/annurev.micro.61.080706.093445
12. Nakano S, Küster-Schöck E, Grossman AD, Zuber P. Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. Proc Natl Acad Sci USA. 2003;100: 13603–13608. doi:10.1073/pnas.2235180100
13. Rochat T, Nicolas P, Delumeau O, Rabatinová A, Korelusová J, Leduc A, et al. Genome-wide identification of genes directly regulated by the pleiotropic transcription factor Spx in *Bacillus subtilis*. Nucleic Acids Res. 2012;40: 9571–9583. doi:10.1093/nar/gks755
14. Schäfer H, Heinz A, Sudzinová P, Voß M, Hantke I, Krásný L, 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: 514–533. doi:10.1111/mmi.14171
15. Potrykus K, Cashel M. (p)ppGpp: still magical? Annu Rev Microbiol. 2008;62: 35–51. doi:10.1146/annurev.micro.62.081307.162903
16. Atkinson GC, Tenson T, Hauryliuk V. The RelA/SpoT Homolog (RSH) Superfamily: Distribution and Functional Evolution of ppGpp Synthetases and Hydrolases across the Tree of Life. Stiller JW, editor. PLoS ONE. 2011;6: e23479. doi:10.1371/journal.pone.0023479
17. Haseltine WA, Block R. Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes. Proc Natl Acad Sci USA. 1973;70: 1564–1568.
18. Wendrich TM, Blaha G, Wilson DN, Marahiel MA, Nierhaus KH. Dissection of the Mechanism for the Stringent Factor RelA. Molecular Cell. 2002;10: 779–788. doi:10.1016/S1097-2765(02)00656-1
19. Arenz S, Abdelshahid M, Sohmen D, Payoe R, Starosta AL, Berninghausen O, et al. The stringent factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis. Nucleic Acids Res. 2016;44: 6471–6481. doi:10.1093/nar/gkw470

20. Brown A, Fernández IS, Gordiyenko Y, Ramakrishnan V. Ribosome-dependent activation of stringent control. Nature. 2016;534: 277–280. doi:10.1038/nature17675
21. Loveland AB, Bah E, Madireddy R, Zhang Y, Brilot AF, Grigorieff N, et al. Ribosome•RelA structures reveal the mechanism of stringent response activation. Elife. 2016;5. doi:10.7554/eLife.17029
22. Wendrich TM, Marahiel MA. Cloning and characterization of a relA/spoT homologue from *Bacillus subtilis*. Mol Microbiol. 1997;26: 65–79.
23. Nanamiya H, Kasai K, Nozawa A, Yun C-S, Narisawa T, Murakami K, et al. Identification and functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*. Mol Microbiol. 2008;67: 291–304. doi:10.1111/j.1365-2958.2007.06018.x
24. Boutte CC, Crosson S. Bacterial lifestyle shapes stringent response activation. Trends Microbiol. 2013;21: 174–180. doi:10.1016/j.tim.2013.01.002
25. Irving SE, Corrigan RM. Triggering the stringent response: signals responsible for activating (p)ppGpp synthesis in bacteria. Microbiology. 2018;164: 268–276. doi:10.1099/mic.0.000621
26. Lopez JM, Dromerick A, Freese E. Response of guanosine 5'-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation. J Bacteriol. 1981;146: 605–613.
27. Kriel A, Bittner AN, Kim SH, Liu K, Tehranchi AK, Zou WY, et al. Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. Mol Cell. 2012;48: 231–241. doi:10.1016/j.molcel.2012.08.009
28. Krásný L, Gourse RL. An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. EMBO J. 2004;23: 4473–4483. doi:10.1038/sj.emboj.7600423
29. Krásný L, Tišerová H, Jonák J, Rejman D, Šanderová H. The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in *Bacillus subtilis*. Molecular Microbiology. 2008;69: 42–54. doi:10.1111/j.1365-2958.2008.06256.x
30. Geiger T, Wolz C. Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria. Int J Med Microbiol. 2014;304: 150–155. doi:10.1016/j.ijmm.2013.11.013
31. Svitil AL, Cashel M, Zyskind JW. Guanosine tetraphosphate inhibits protein synthesis *in vivo*. A possible protective mechanism for starvation stress in *Escherichia coli*. J Biol Chem. 1993;268: 2307–2311.
32. Milon P, Tischenko E, Tomsic J, Caserta E, Folkers G, La Teana A, et al. The nucleotide-binding site of bacterial translation initiation factor 2 (IF2) as a metabolic sensor. Proc Natl Acad Sci USA. 2006;103: 13962–13967. doi:10.1073/pnas.0606384103
33. Corrigan RM, Bellows LE, Wood A, Gründling A. ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. Proc Natl Acad Sci USA. 2016;113: E1710-1719. doi:10.1073/pnas.1522179113
34. Bokinsky G, Baidoo EEK, Akella S, Burd H, Weaver D, Alonso-Gutierrez J, et al. HipA-triggered growth arrest and β-lactam tolerance in Escherichia coli are mediated by RelA-dependent ppGpp synthesis. J Bacteriol. 2013;195: 3173–3182. doi:10.1128/JB.02210-12
35. Dalebroux ZD, Swanson MS. ppGpp: magic beyond RNA polymerase. Nat Rev Microbiol. 2012;10: 203–212. doi:10.1038/nrmicro2720
36. Kanjee U, Ogata K, Houry WA. Direct binding targets of the stringent response alarmone (p)ppGpp: Protein targets of ppGpp. Molecular Microbiology. 2012;85: 1029–1043. doi:10.1111/j.1365-2958.2012.08177.x
37. Zhang Y, Zborníková E, Rejman D, Gerdes K. Novel (p)ppGpp Binding and Metabolizing Proteins of *Escherichia coli*. MBio. 2018;9. doi:10.1128/mBio.02188-17
38. Wang B, Dai P, Ding D, Del Rosario A, Grant RA, Pentelute BL, et al. Affinity-based capture and identification of protein effectors of the growth regulator ppGpp. Nat Chem Biol. 2019;15: 141–150. doi:10.1038/s41589-018-0183-4
39. Leichert LIO, Scharf C, Hecker M. Global characterization of disulfide stress in *Bacillus subtilis*. J Bacteriol. 2003;185: 1967–1975.
40. Mostertz J, Scharf C, Hecker M, Homuth G. Transcriptome and proteome analysis of *Bacillus subtilis* gene expression in response to superoxide and peroxide stress. Microbiology (Reading, Engl). 2004;150: 497–512. doi:10.1099/mic.0.26665-0

41. Hantke I, Schäfer H, Janczikowski A, Turgay K. YocM a small heat shock protein can protect *Bacillus subtilis* cells during salt stress. Mol Microbiol. 2019;111: 423–440. doi:10.1111/mmi.14164
42. Hecker M, Richter A, Schroeter A, Wölfel L, Mach F. [Synthesis of heat shock proteins following amino acid or oxygen limitation in *Bacillus subtilis* relA+ and relA strains]. Z Naturforsch, C, J Biosci. 1987;42: 941–947.
43. Drzewiecki K, Eymann C, Mittenhuber G, Hecker M. The yvyD gene of *Bacillus subtilis* is under dual control of sigmaB and sigmaH. J Bacteriol. 1998;180: 6674–6680.
44. Tagami K, Nanamiya H, Kazo Y, Maehashi M, Suzuki S, Namba E, et al. Expression of a small (p)ppGpp synthetase, YwaC, in the (p)ppGpp(0) mutant of *Bacillus subtilis* triggers YvyD-dependent dimerization of ribosome. Microbiologyopen. 2012;1: 115–134. doi:10.1002/mbo3.16
45. Cashel M. The Control of Ribonucleic Acid Synthesis in Escherichia coli : IV. RELEVANCE OF UNUSUAL PHOSPHORYLATED COMPOUNDS FROM AMINO ACID-STARVED STRINGENT STRAINS. Journal of Biological Chemistry. 1969;244: 3133–3141.
46. Srivatsan A, Han Y, Peng J, Tehranchi AK, Gibbs R, Wang JD, et al. High-precision, whole-genome sequencing of laboratory strains facilitates genetic studies. PLoS Genet. 2008;4: e1000139. doi:10.1371/journal.pgen.1000139
47. Schreiber G, Metzger S, Aizenman E, Roza S, Cashel M, Glaser G. Overexpression of the relA gene in *Escherichia coli*. J Biol Chem. 1991;266: 3760–3767.
48. Nouri H, Monnier A-F, Fossum-Raunehaug S, Maciag-Dorszynska M, Cabin-Flaman A, Képès F, et al. Multiple links connect central carbon metabolism to DNA replication initiation and elongation in *Bacillus subtilis*. DNA Res. 2018; doi:10.1093/dnares/dsy031
49. Lopez JM, Marks CL, Freese E. The decrease of guanine nucleotides initiates sporulation of *Bacillus subtilis*. Biochim Biophys Acta. 1979;587: 238–252.
50. Tojo S, Satomura T, Kumamoto K, Hirooka K, Fujita Y. Molecular Mechanisms Underlying the Positive Stringent Response of the *Bacillus subtilis* ilv-leu Operon, Involved in the Biosynthesis of Branched-Chain Amino Acids. J Bacteriol. 2008;190: 6134–6147. doi:10.1128/JB.00606-08
51. Tojo S, Kumamoto K, Hirooka K, Fujita Y. Heavy involvement of stringent transcription control depending on the adenine or guanine species of the transcription initiation site in glucose and pyruvate metabolism in *Bacillus subtilis*. J Bacteriol. 2010;192: 1573–1585. doi:10.1128/JB.01394-09
52. Ratnayake-Lecamwasam M, Serror P, Wong KW, Sonenshein AL. Bacillus subtilis CodY represses early-stationary-phase genes by sensing GTP levels. Genes Dev. 2001;15: 1093–1103. doi:10.1101/gad.874201
53. Zhang S, Haldenwang WG. RelA is a component of the nutritional stress activation pathway of the *Bacillus subtilis* transcription factor sigma B. J Bacteriol. 2003;185: 5714–5721.
54. Zhang S, Haldenwang WG. Contributions of ATP, GTP, and redox state to nutritional stress activation of the *Bacillus subtilis* sigmaB transcription factor. J Bacteriol. 2005;187: 7554–7560. doi:10.1128/JB.187.22.7554-7560.2005
55. Molière N, Hoßmann J, Schäfer H, Turgay K. Role of Hsp100/Clp Protease Complexes in Controlling the Regulation of Motility in *Bacillus subtilis*. Front Microbiol. 2016;7: 315. doi:10.3389/fmicb.2016.00315
56. Gallant J, Palmer L, Pao CC. Anomalous synthesis of ppGpp in growing cells. Cell. 1977;11: 181–185.
57. VanBogelen RA, Kelley PM, Neidhardt FC. Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli*. J Bacteriol. 1987;169: 26–32.
58. Fitzsimmons LF, Liu L, Kim J-S, Jones-Carson J, Vázquez-Torres A. *Salmonella* Reprograms Nucleotide Metabolism in Its Adaptation to Nitrosative Stress. Aballay A, editor. mBio. 2018;9: e00211-18. doi:10.1128/mBio.00211-18
59. Hahn J, Tanner AW, Carabetta VJ, Cristea IM, Dubnau D. ComGA-RelA interaction and persistence in the *Bacillus subtilis* K-state. Mol Microbiol. 2015;97: 454–471. doi:10.1111/mmi.13040

60. Pöther D-C, Liebeke M, Hochgräfe F, Antelmann H, Becher D, Lalk M, et al. Diamide triggers mainly S Thiolations in the cytoplasmic proteomes of *Bacillus subtilis* and *Staphylococcus aureus*. J Bacteriol. 2009;191: 7520–7530. doi:10.1128/JB.00937-09
61. Katz A, Orellana O. Protein Synthesis and the Stress Response. In: Biyani M, editor. Cell-Free Protein Synthesis. InTech; 2012. doi:10.5772/50311
62. Natori Y, Tagami K, Murakami K, Yoshida S, Tanigawa O, Moh Y, et al. Transcription Activity of Individual rrn Operons in *Bacillus subtilis* Mutants Deficient in (p)ppGpp Synthetase Genes, relA, yjbM, and ywaC. Journal of Bacteriology. 2009;191: 4555–4561. doi:10.1128/JB.00263-09
63. Paget MSB, Molle V, Cohen G, Aharonowitz Y, Buttner MJ. Defining the disulphide stress response in Streptomyces coelicolor A3(2): identification of the sigmaR regulon. Molecular Microbiology. 2001;42: 1007–1020. doi:10.1046/j.1365-2958.2001.02675.x
64. Gaca AO, Abranches J, Kajfasz JK, Lemos JA. Global transcriptional analysis of the stringent response in *Enterococcus faecalis*. Microbiology (Reading, Engl). 2012;158: 1994–2004. doi:10.1099/mic.0.060236-0
65. Scott JM, Haldenwang WG. Obg, an essential GTP binding protein of *Bacillus subtilis*, is necessary for stress activation of transcription factor sigma(B). J Bacteriol. 1999;181: 4653–4660.
66. Zhang S, Scott JM, Haldenwang WG. Loss of ribosomal protein L11 blocks stress activation of the *Bacillus subtilis* transcription factor sigma(B). J Bacteriol. 2001;183: 2316–2321. doi:10.1128/JB.183.7.2316-2321.2001
67. Bruel N, Castanié-Cornet M-P, Cirinesi A-M, Koningstein G, Georgopoulos C, Luirink J, 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: 44435–44446. doi:10.1074/jbc.M112.418525
68. Akanuma G, Kazo Y, Tagami K, Hiraoka H, Yano K, Suzuki S, et al. Ribosome dimerization is essential for the efficient regrowth of *Bacillus subtilis*. Microbiology. 2016;162: 448–458. doi:10.1099/mic.0.000234
69. Beckert B, Abdelshahid M, Schäfer H, Steinchen W, Arenz S, Berninghausen O, et al. Structure of the *Bacillus subtilis* hibernating 100S ribosome reveals the basis for 70S dimerization. EMBO J. 2017;36: 2061–2072. doi:10.15252/embj.201696189
70. Miller LL, Ordal ZJ. Thermal injury and recovery of *Bacillus subtilis*. Appl Microbiol. 1972;24: 878–884.
71. Trinquier A, Ulmer JE, Gilet L, Figaro S, Hammann P, Kuhn L, et al. tRNA Maturation Defects Lead to Inhibition of rRNA Processing via Synthesis of pppGpp. Molecular Cell. 2019;0. doi:10.1016/j.molcel.2019.03.030
72. Maaβ S, Wachlin G, Bernhardt J, Eymann C, Fromion V, Riedel K, et al. Highly precise quantification of protein molecules per cell during stress and starvation responses in *Bacillus subtilis*. Mol Cell Proteomics. 2014;13: 2260–2276. doi:10.1074/mcp.M113.035741
73. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. Science. 2011;334: 1081–1086. doi:10.1126/science.1209038
74. Abranches J, Martinez AR, Kajfasz JK, Chávez V, Garsin DA, Lemos JA. The molecular alarmone (p)ppGpp mediates stress responses, vancomycin tolerance, and virulence in *Enterococcus faecalis*. J Bacteriol. 2009;191: 2248–2256. doi:10.1128/JB.01726-08
75. Gaca AO, Kudrin P, Colomer-Winter C, Beljantseva J, Liu K, Anderson B, et al. From (p)ppGpp to (pp)pGpp: Characterization of Regulatory Effects of pGpp Synthesized by the Small Alarmone Synthetase of *Enterococcus faecalis*. J Bacteriol. 2015;197: 2908–2919. doi:10.1128/JB.00324-15
76. Rallu F, Gruss A, Ehrlich SD, Maguin E. Acid- and multistress-resistant mutants of *Lactococcus lactis* : identification of intracellular stress signals. Molecular Microbiology. 2000;35: 517–528. doi:10.1046/j.1365-2958.2000.01711.x
77. Okada Y, Makino S, Tobe T, Okada N, Yamazaki S. Cloning of rel from Listeria monocytogenes as an osmotolerance involvement gene. Appl Environ Microbiol. 2002;68: 1541–1547.
78. Yang X, Ishiguro EE. Temperature-Sensitive Growth and Decreased Thermotolerance Associated with relA Mutations in *Escherichia coli*. J Bacteriol. 2003;185: 5765–5771. doi:10.1128/JB.185.19.5765-5771.2003

79. Khakimova M, Ahlgren HG, Harrison JJ, English AM, Nguyen D. The stringent response controls catalases in Pseudomonas aeruginosa and is required for hydrogen peroxide and antibiotic tolerance. J Bacteriol. 2013;195: 2011–2020. doi:10.1128/JB.02061-12
80. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press; 2001.
81. Spizizen J. TRANSFORMATION OF BIOCHEMICALLY DEFICIENT STRAINS OF BACILLUS SUBTILIS BY DEOXYRIBONUCLEATE. Proc Natl Acad Sci USA. 1958;44: 1072–1078.
82. Nakano MM, Zhu Y, Liu J, Reyes DY, Yoshikawa H, Zuber P. Mutations conferring amino acid residue substitutions in the carboxy-terminal domain of RNA polymerase alpha can suppress clpX and clpP with respect to developmentally regulated transcription in *Bacillus subtilis*. Mol Microbiol. 2000;37: 869–884.
83. Arnaud M, Chastanet A, Débarbouillé M. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl Environ Microbiol. 2004;70: 6887–6891. doi:10.1128/AEM.70.11.6887-6891.2004
84. Stülke J, Hanschke R, Hecker M. Temporal activation of beta-glucanase synthesis in *Bacillus subtilis* is mediated by the GTP pool. J Gen Microbiol. 1993;139: 2041–2045. doi:10.1099/00221287-139-9-2041
85. Lamy M-C, Zouine M, Fert J, Vergassola M, Couve E, Pellegrini E, et al. CovS/CovR of group B streptococcus: a two-component global regulatory system involved in virulence: The CovS/CovR regulatory system of Streptococcus agalactiae. Molecular Microbiology. 2004;54: 1250–1268. doi:10.1111/j.1365-2958.2004.04365.x
86. Nuss AM, Heroven AK, Waldmann B, Reinkensmeier J, Jarek M, Beckstette M, et al. Transcriptomic Profiling of Yersinia pseudotuberculosis Reveals Reprogramming of the Crp Regulon by Temperature and Uncovers Crp as a Master Regulator of Small RNAs. Sharma CM, editor. PLoS Genet. 2015;11: e1005087. doi:10.1371/journal.pgen.1005087
87. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9: 357–359. doi:10.1038/nmeth.1923
88. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25: 2078–2079. doi:10.1093/bioinformatics/btp352
89. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11: R106. doi:10.1186/gb-2010-11-10-r106
90. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002;30: 207–210.
91. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA. 1979;76: 4350–4354.
92. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227: 680–685.
93. Neuhoff V, Arold N, Taube D, Ehrhardt W. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis. 1988;9: 255–262. doi:10.1002/elps.1150090603
94. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9: 676–682. doi:10.1038/nmeth.2019
95. Kriel A, Brinsmade SR, Tse JL, Tehranchi AK, Bittner AN, Sonenshein AL, et al. GTP Dysregulation in *Bacillus subtilis* Cells Lacking (p)ppGpp Results in Phenotypic Amino Acid Auxotrophy and Failure To Adapt to Nutrient Downshift and Regulate Biosynthesis Genes. Journal of Bacteriology. 2014;196: 189–201. doi:10.1128/JB.00918-13
96. Ihara Y, Ohta H, Masuda S. A highly sensitive quantification method for the accumulation of alarmone ppGpp in Arabidopsis thaliana using UPLC-ESI-qMS/MS. J Plant Res. 2015;128: 511–518. doi:10.1007/s10265-015-0711-1

97. Steinchen W, Schuhmacher JS, Altegoer F, Fage CD, Srinivasan V, Linne U, et al. Catalytic mechanism and allosteric regulation of an oligomeric (p)ppGpp synthetase by an alarmone. Proc Natl Acad Sci USA. 2015;112: 13348–13353. doi:10.1073/pnas.1505271112
98. Nicolas P, Mäder U, Dervyn E, Rochat T, Leduc A, Pigeonneau N, et al. Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. Science. 2012;335: 1103–1106. doi:10.1126/science.1206848

Supporting information captions

S1 Dataset: List of identified transcription start sites.

In this dataset, all identified transcriptional start sites and their classification is shown.

S2 Dataset: Results of the gene set enrichment analysis.

This dataset lists all enriched functional categories and regulons for each for each condition in separate sheets.

S3 Dataset: List of differentially expressed genes

Global gene expression changes for all conditions are listed in separate sheets.

Figures

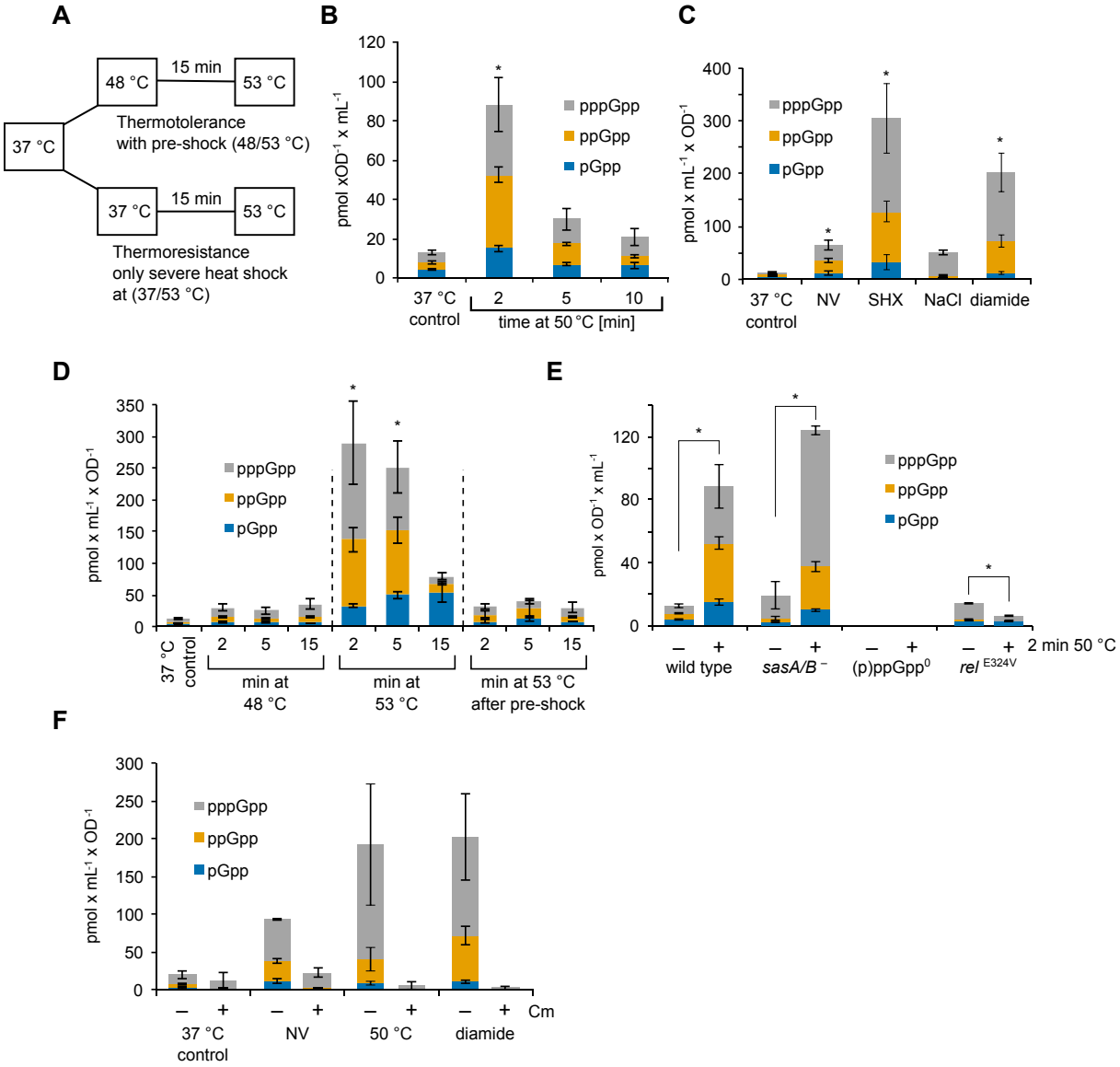

Figure 1: (p)ppGpp levels are increased by heat shock and stress.

(A) Outline of the thermotolerance protocol. A culture of cells growing exponentially at 37 °C is divided and incubated at 48 °C or left at 37 °C. After 15 min, both cultures are shifted to 53 °C. **(B-F)** Levels of pGpp, ppGpp and pppGpp under different conditions. **(B)** Cells were grown in minimal medium to OD_{600} of 0.4 and transferred to 50 °C or treated with 0.5 mg ml^{-1} DL-norvaline for 10 min. Means and and standard error of mean (SEM) of four independent experiments are shown. Asterisks (*) indicate significance ($p_{adj.} \leq 0.05$) of combined pGpp, ppGpp and pppGpp levels according to the Kruskal-Wallis and Dunn-Bonferrroni test**.** **(C)** Cells were grown in minimal medium to the mid-exponential phase (OD_{600} ~ 0.4) and treated with DL-norvaline (NV; 0.5 mg ml^{-1}), serine hydroxamate (SHX; 5 µg ml^{-1}), NaCl (6 %) or diamide (0.5 mM) for 10 min. Means and SEM of three to four independent experiments are shown. Asterisks (*) indicate significance ($p_{adj.} \leq 0.05$) of combined pGpp, ppGpp and pppGpp levels according to the Kruskal-Wallis and Dunn-Bonferrroni test. **(D)** Wildtype cells were grown at 37 °C and shifted to 48 °C for 15 min (pre-shock), then to 53 °C or directly to 53 °C. Samples were taken at 2, 5 and 15 min. Means and SEM of four independent experiments are shown. Asterisks (*) indicate significance ($p_{adj.} \leq 0.05$) of combined pGpp, ppGpp and pppGpp levels according to the Kruskal-Wallis and Dunn-Bonferrroni test. **(E)** Wildtype cells or strains with mutations in (p)ppGpp synthetases (*sasA/B*$^{-}$: BHS204, *rel*^{E324V}: BHS709; (p)ppGpp^{0}: BHS214) were treated with or without heat shock at 50 °C for 2 min. Means and SEM of three to six independent experiments are shown. No alarmone peaks were detected in the (p)ppGpp^{0} mutant (lower limit of quantification: 0.26 pmol x mL^{-1} x OD^{-1}).. Asterisks (*) indicate significant changes ($p \leq 0.05$) of combined pGpp, ppGpp and pppGpp levels according to Welch's *t*-test. **(F)** The influence of chloramphenicol on alarmone accumulation during stress. Cells were grown in minimal medium and treated with DL-norvaline (0.5 mg ml^{-1}) for 10 min, heat shock at 50 °C for 2 min or diamide (1 mM) for 10 min. Chloramphenicol (Cm, 25 µg ml^{-1}) was added at the same time to one part. Means and SEM of two independent experiments are shown.

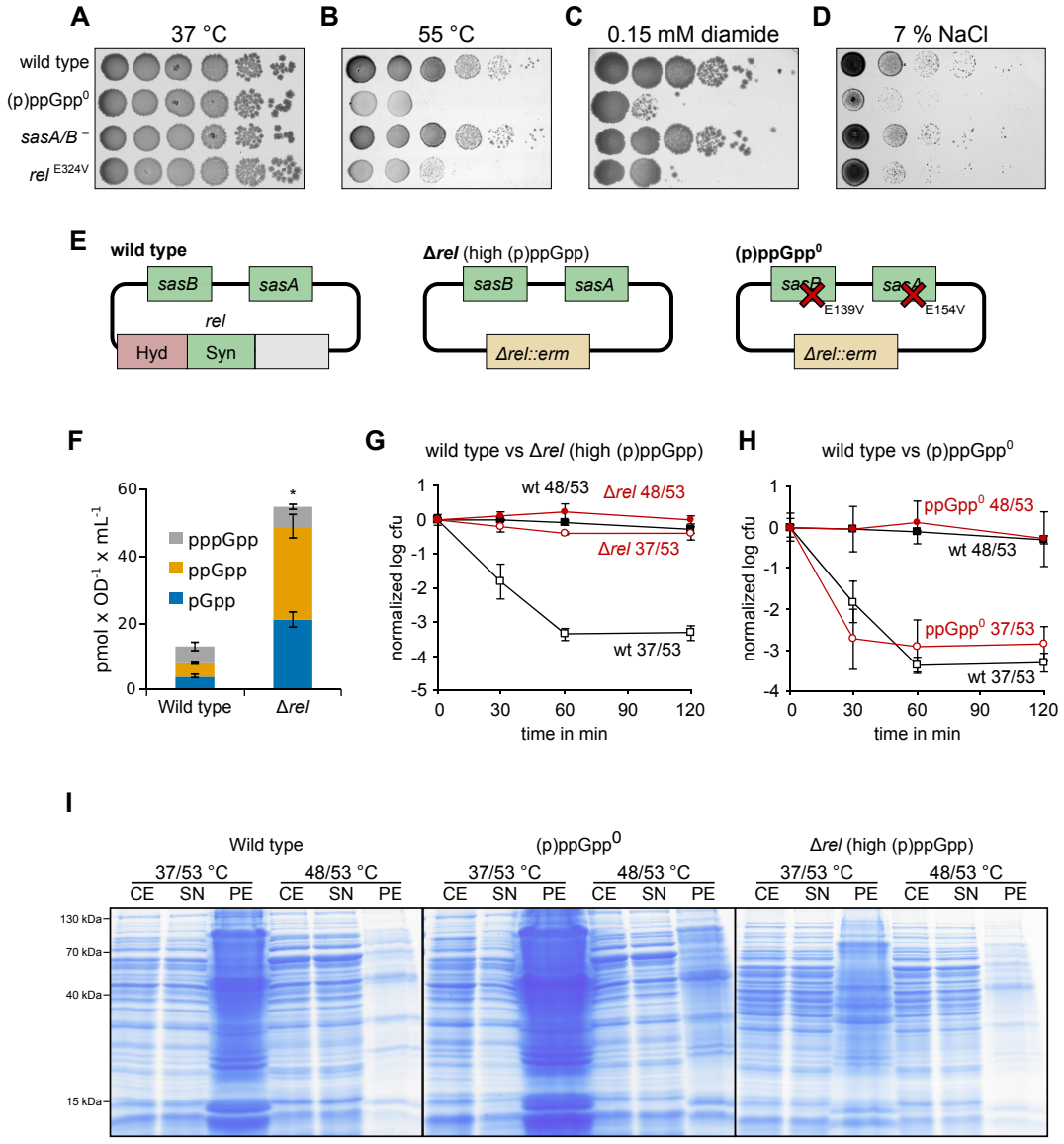

Figure 2 Increased (p)ppGpp levels confer high heat stress resistance.

(A-D) Growth of strains with mutations in (p)ppGpp synthetases (*sasA/B*$^{-}$: BHS204, *rel*^{E324V}: BHS709; (p)ppGpp^{0}: BHS214) on agar plates at 37 °C, during heat stress (55 °C), oxidative stress (0.1 mM diamide) or salt stress (total concentration of 7 % (w/v) NaCl) over night. **(E)**
Outline of the genotypes and the (p)ppGpp synthesis capabilities of the assessed wildtype, Δ*rel* (BHS126 and BHS368) and (p)ppGpp^{0} (BHS214 and BHS319) strains. **(F)** Cellular alarmone levels of wildtype and Δ*rel* strains. Asterisks indicate significant changes ($p \leq 0.05$) of combined pGpp, ppGpp and pppGpp levels according to Welch's *t*-test. Means and SEM of three independent experiments are shown. **(G/H)** Thermotolerance and survival of wildtype (black lines) and mutant strains (red lines) at 53 °C. Means and SEM of at least three independent experiments are shown. Open symbols: no pre-shock, closed symbols: 15 min pre-shock at 48 °C. **(I)** Accumulation of protein aggregates during heat stress at 53 °C without (37/53 °C) or with (48/53 °C) pre-shock. CE: cell extract, SN: supernatant, PE: pellet (aggregated protein fraction).

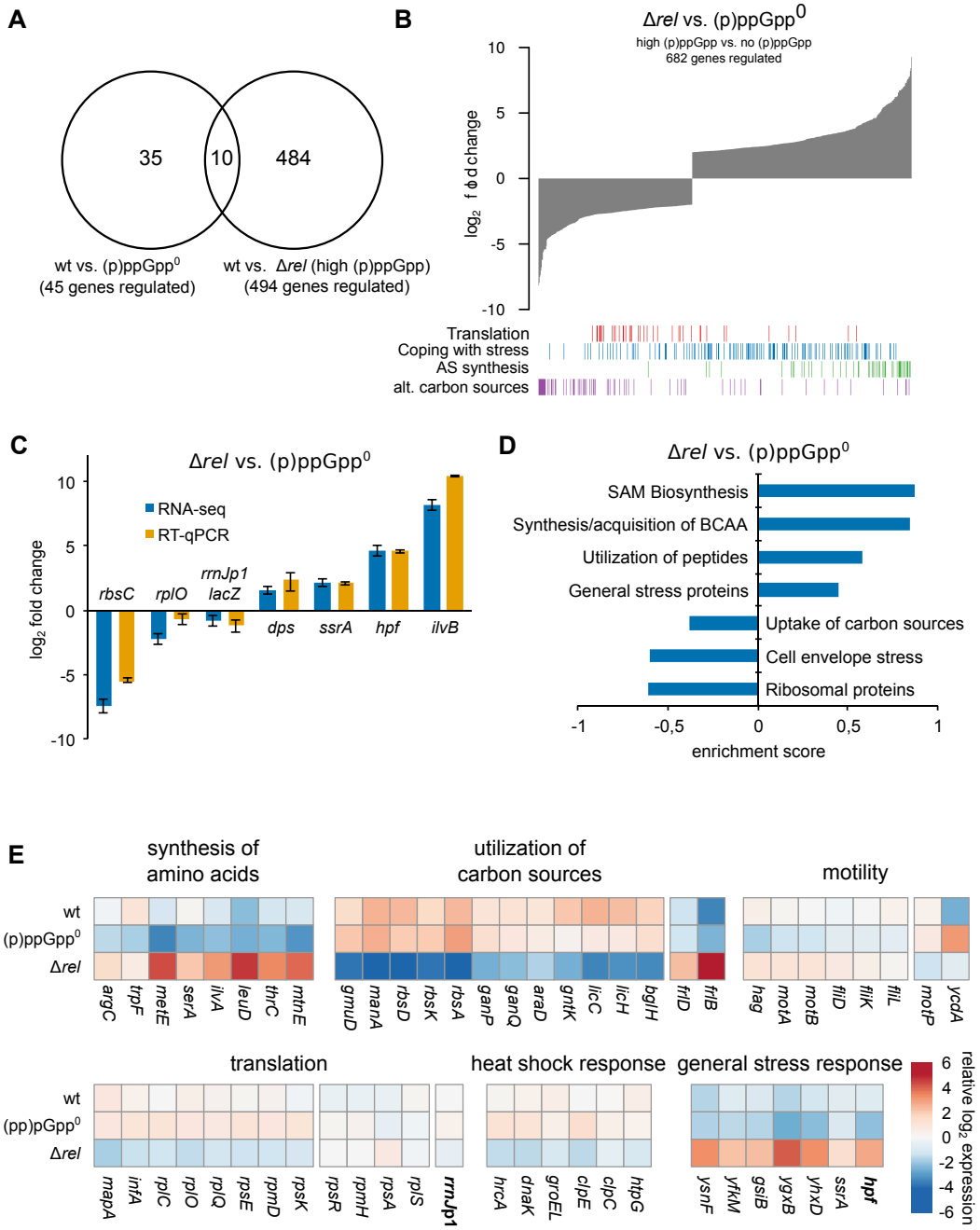

Figure 3: (p)ppGpp- mediated global changes in the transcriptome.

(A) Venn diagram showing the number of significantly regulated genes in Δ*rel* or (p)ppGpp^0 strains vs. wildtype. **(B)** Global differences in gene expression in Δ*rel* versus (p)ppGpp^0 strains. Bar tracks indicate the distribution of genes in the respective functional groups. **(C)** Comparison of the relative transcription changes of selected genes in, Δ*rel* and (p)ppGpp^0 strains during exponential growth at 37 °C as determined by RNA-seq or RT-qPCR from independent experiments. Means and SEM of three replicates are shown. **(D)** Selected category results of the gene set enrichment analysis from regulated transcripts in Δ*rel* vs. (p)ppGpp^0 cells. Positive/negative enrichment scores represent enrichment in the up- or down-regulated genes. **(E)** Heatmap showing the expression changes of selected transcripts in wildtype, (p)ppGpp^0 or Δ*rel* strains. Values represent normalized $\log_2$ scaled read counts centered on the mean expression level of each transcript.

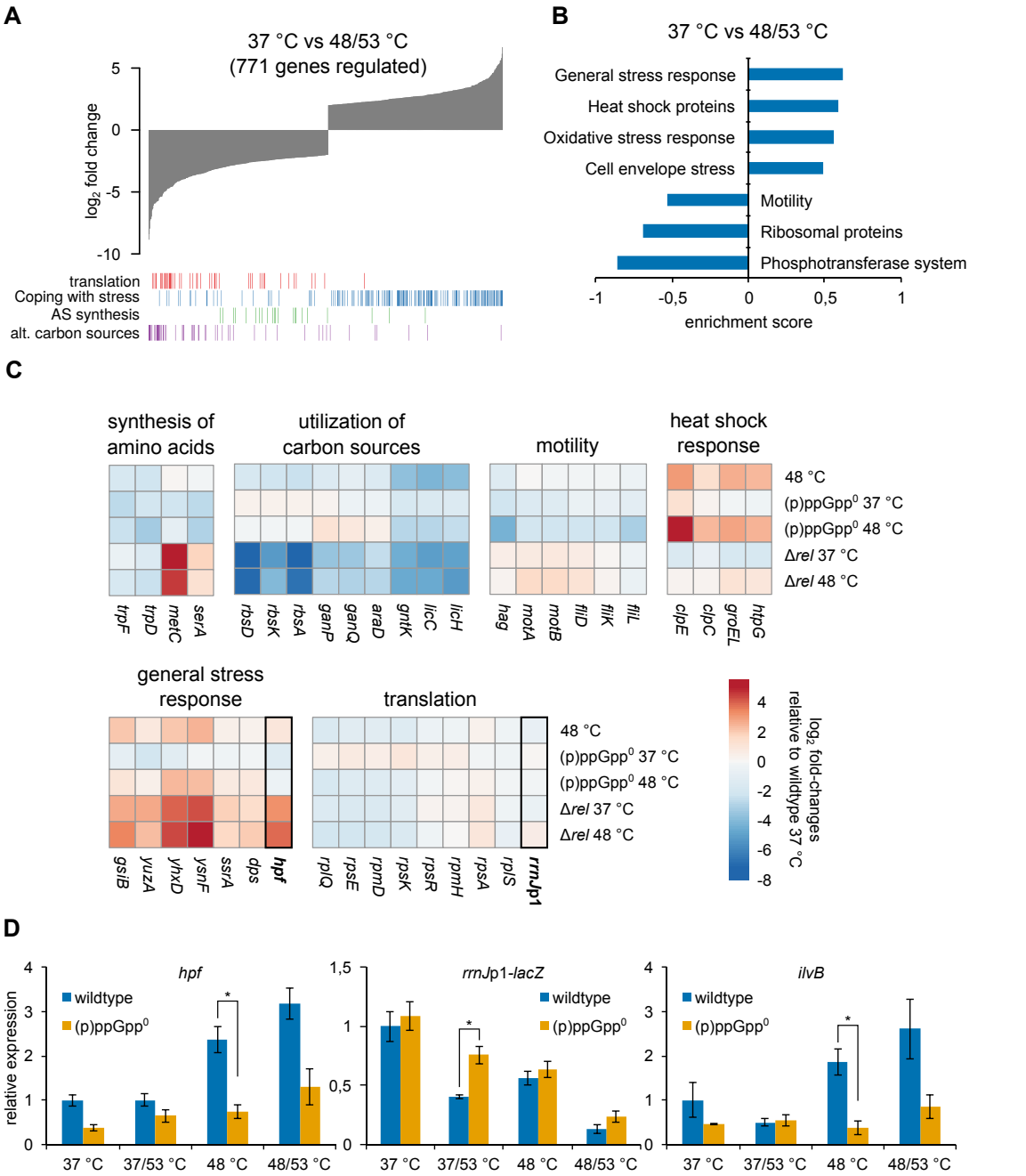

Figure 4: (p)ppGpp mediated transcriptional changes during heat stress.
(A) Global differences in gene expression of heat shocked, thermotolerant cells (48/53 °C) versus untreated cells (37 °C). Bar tracks indicate the distribution of genes in the respective functional groups. **(B)** Selected category results of the gene set enrichment analysis from regulated transcripts in heat shocked (48/53 °C) cells. Positive/negative enrichment scores represent enrichment in the up- or down-regulated genes. **(C)** Heatmap showing expression changes of selected transcripts during mild heat stress in wildtype, (p)ppGpp^0 or Δ*rel* cells. Values represent $\log_2$ fold changes of transcript levels relative to wildtype cells at 37 °C. **(D)** Relative changes in the transcription of selected genes during heat shock in wildtype and (p)ppGpp^0 strains determined by RT-qPCR. Means and SEM of three replicates are shown. Asterisks indicate significance ($p \leq 0.05$) according to Welch's *t*-test.

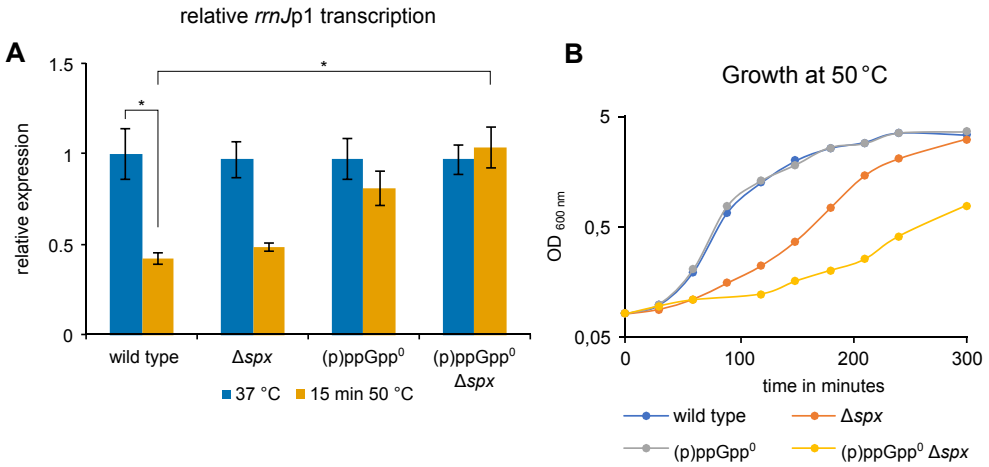

Figure 5: (p)ppGpp and Spx act complementary during heat shock.
(A) Heat mediated down-regulation *of rrnJ*p1-*lacZ* transcription in wildtype (BHS220), Δ*spx* (BHS222), (p)ppGpp^0 (BHS319) and Δ*spx* (p)ppGpp^0 (BHS766) strains as determined by RT-qPCR. Means and SEM of three independent experiments are shown. Asterisks indicate significant changes ($p \leq 0.05$) of combined pGpp, ppGpp and pppGpp levels according to Welch's *t*-test. **(B)** Growth of the same strains in LB medium at 50 °C.

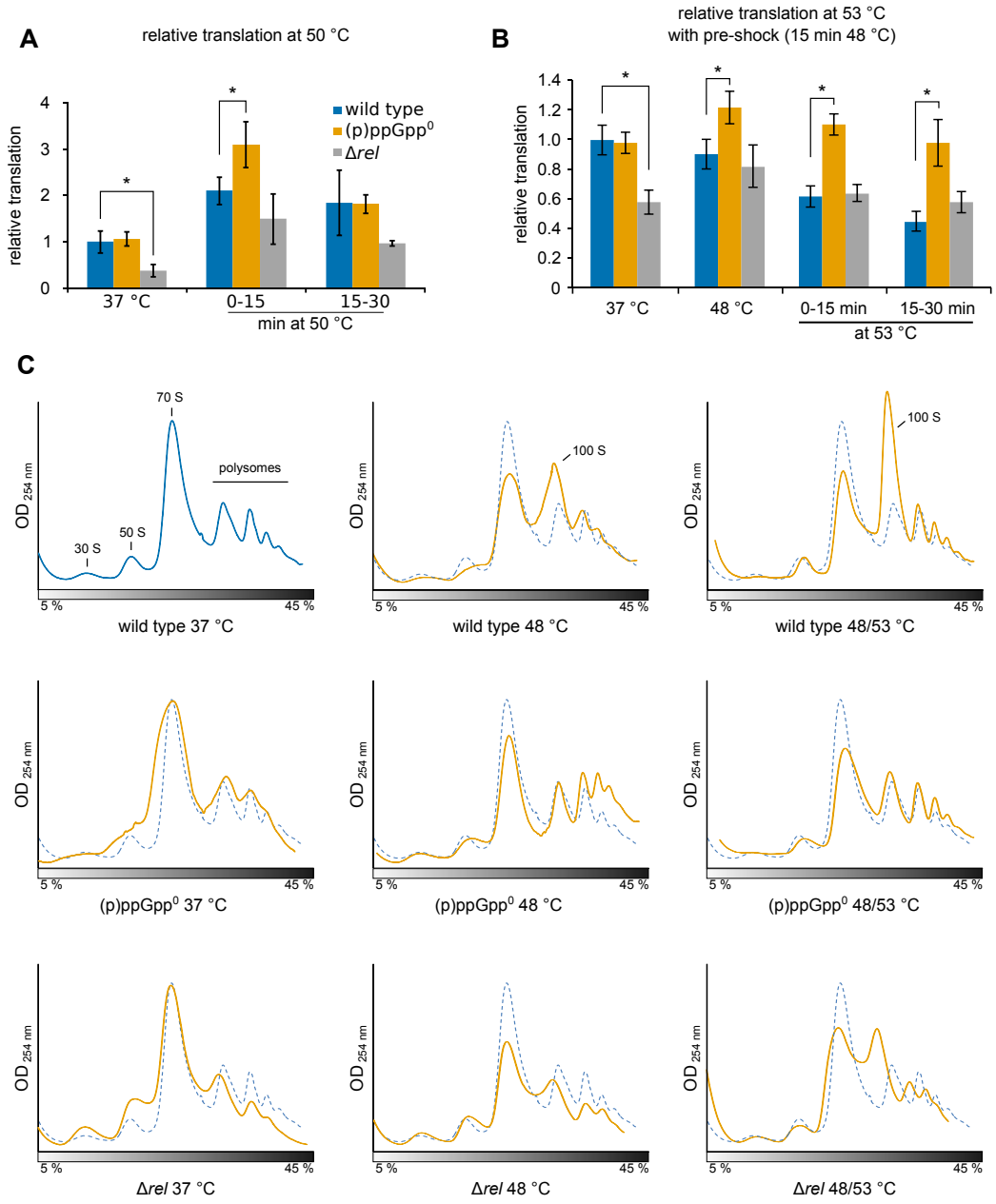

Figure 6: (p)ppGpp modulates translation during stress response.
(A/ B) Relative translation (estimated from puromycin incorporation) of wildtype, (p)ppGpp^0 (BHS214) and Δ*rel* (BHS126) strains during heat stress **(A)** at 50 °C or **(B)** at 48 °C, 53 °C or 48/53 °C. 1 µg ml^{-1} puromycin was added for 15 min to the medium directly after (0-15 min) or 15 min after shifting the sample to the indicated temperatures. Means and SEM of four independent experiments are shown. Asterisks indicate significance ($p \leq 0.05$) relative to wildtype according to Welch's *t*-test. **(C)** Sucrose gradient profiles of extracts from wildtype,(p)ppGpp^0 (BHS214) or Δ*rel* cells (BHS126) with or without heat shock at 48 °C or 48/53 °C for 15 min each**.**

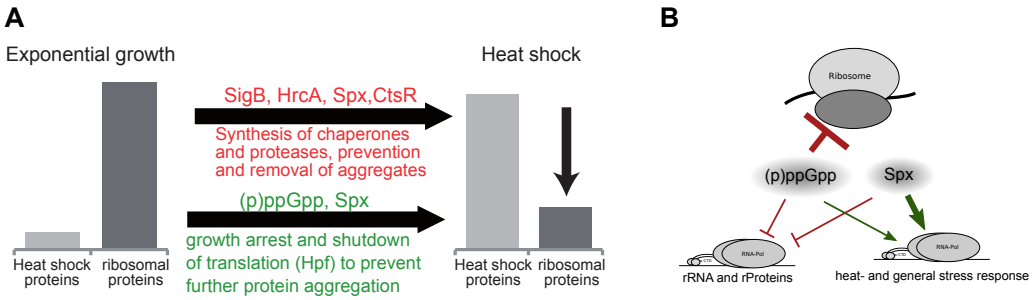

Figure 7: The interplay of the stringent response and the heat shock response.
(A) Model of the role of the stringent response in the regulatory network of the heat shock response. **(B)** The interplay of Spx activity and the stringent response in the regulation of transcription and translation during the heat shock response.

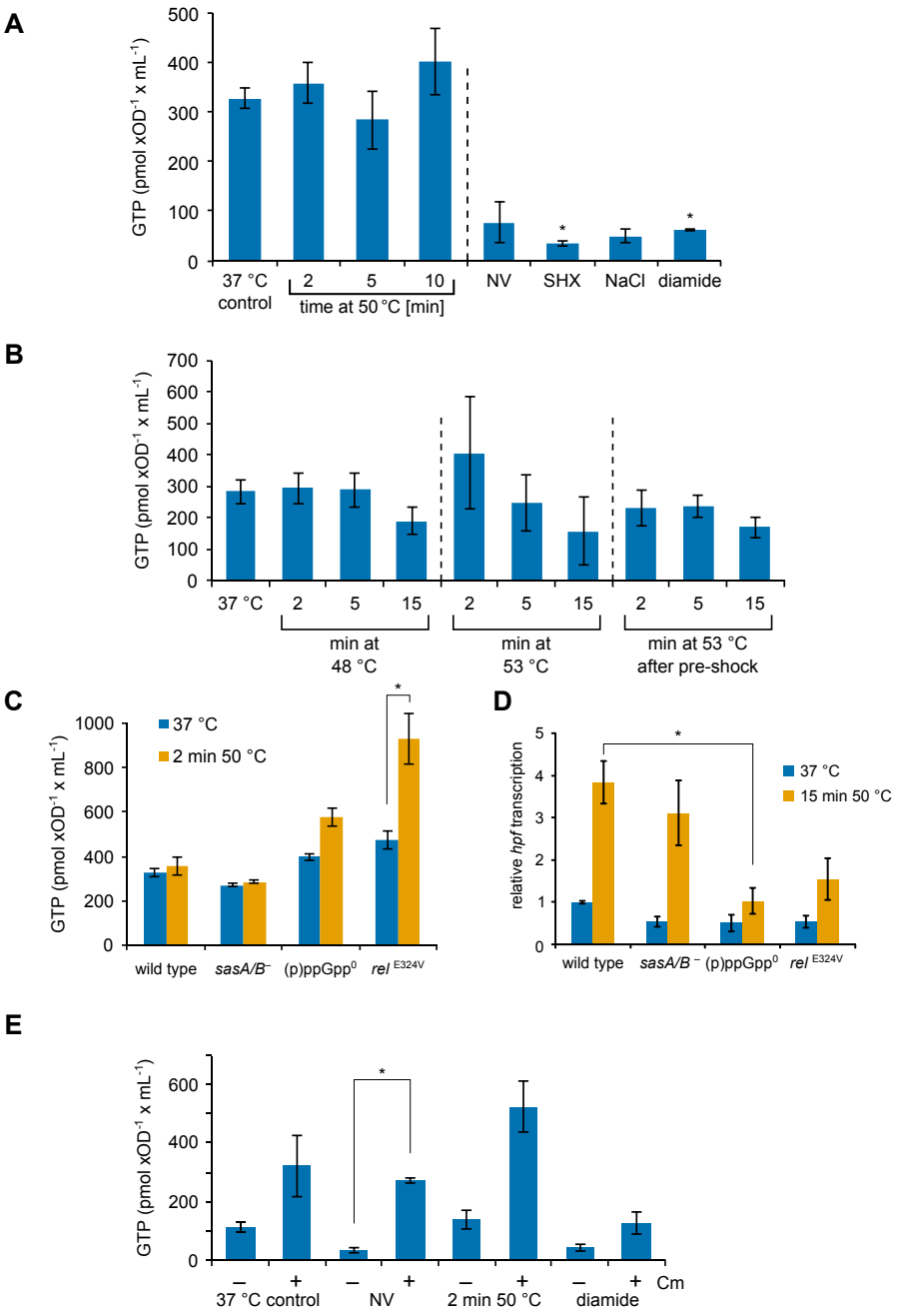

Figure S 1 Alarmone and GTP levels during stress and starvation.
(A) Means and SEM of GTP after the application of different stress conditions. Sample sizes and treatments are the same as in Fig. 1 A, B. NV: DL-norvaline, SHX: serine hydroxamate. Asterisks (*) indicate significance ($p_{adj.} \leq 0.05$) of combined pGpp, ppGpp and pppGpp levels according to the Kruskal-Wallis and Dunn-Bonferrroni test. **(B)** Levels of GTP during thermotolerance development. Wildtype cells were grown at 37 °C and shifted to 48 °C for 15 min (pre-shock), then to 53 °C or directly to 53 °C. Samples were taken at 2, 5 and 15 min. Means and SEM of four independent experiments are shown. All changes are not significant ($p \leq 0.05$) according to the Kruskal-Wallis test. **(C)** Means and SEM of GTP levels during heat shock in of wildtype cells or strains with mutations in (p)ppGpp synthetases (*sasA/B*⁻: BHS204, *rel*^{E324V}: BHS709; (p)ppGpp^0: BHS214). Sample sizes are the same as in Fig. 1E. Asterisks indicate significant changes ($p \leq 0.05$) according to Welch's *t*-test. **(D)** Relative changes in the transcription of *hpf* during heat shock in the same strains (15 min 50 °C). Means and SEM of three independent experiments are shown. Asterisks (*) indicate significance ($p_{adj.} \leq 0.05$) according to the Kruskal-Wallis and Dunn-Bonferrroni test. **(E)** The influence of chloramphenicol on GTP levels during stress. Sample sizes and treatments are the same as in Fig. 1F. Asterisks indicate significant changes ($p \leq 0.05$) according to Welch's *t*-test.

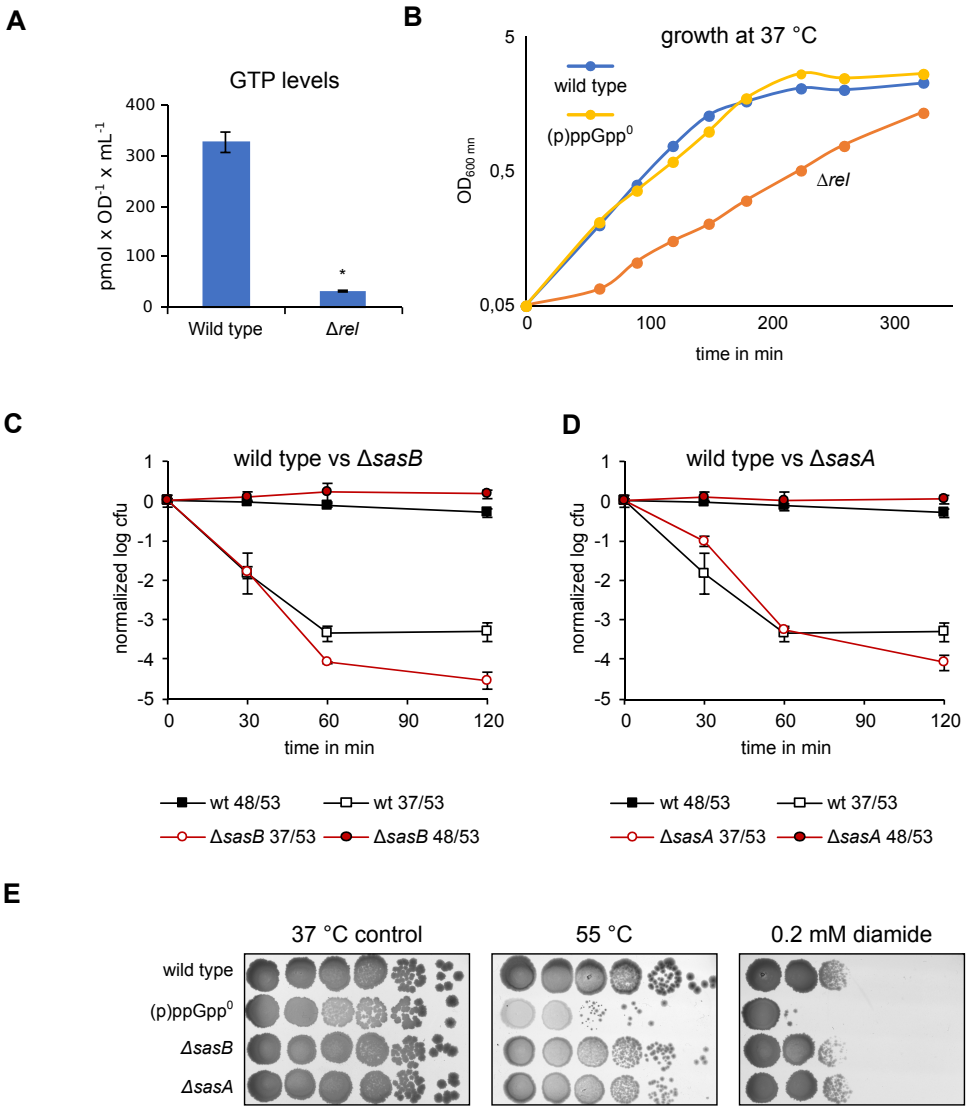

Figure S 2 Phenotype of single deletions of (p)ppGpp synthetase genes.
(A) Cellular GTP levels in wildtype or Δ*rel*: (BHS126) strains. **(B)** Growth of strains with mutations or deletions in (pp)pGpp metabolizing enzymes in rich LB medium. Δ*rel*: BHS126, (p)ppGpp^0: BHS214. **(C/D)** Survival of wildtype (black lines) and mutant strains (Δ*sasB*: BHS127 or Δ*sasA*: BHS128) red lines at 53 °C with (48/53 °C) or without (37/53 °C) pre-shock. Means and SEM of at least three independent experiments are shown. Open symbols: no pre-shock, closed symbols: 15 min pre-shock at 48 °C. **(E)** Growth of wildtype cells or strains with deletions in *sasB* (BHS127) or *sasA* (BHS128) on agar plates at 37 °C, during heat stress (55 °C) or oxidative stress (0.2 mM diamide).

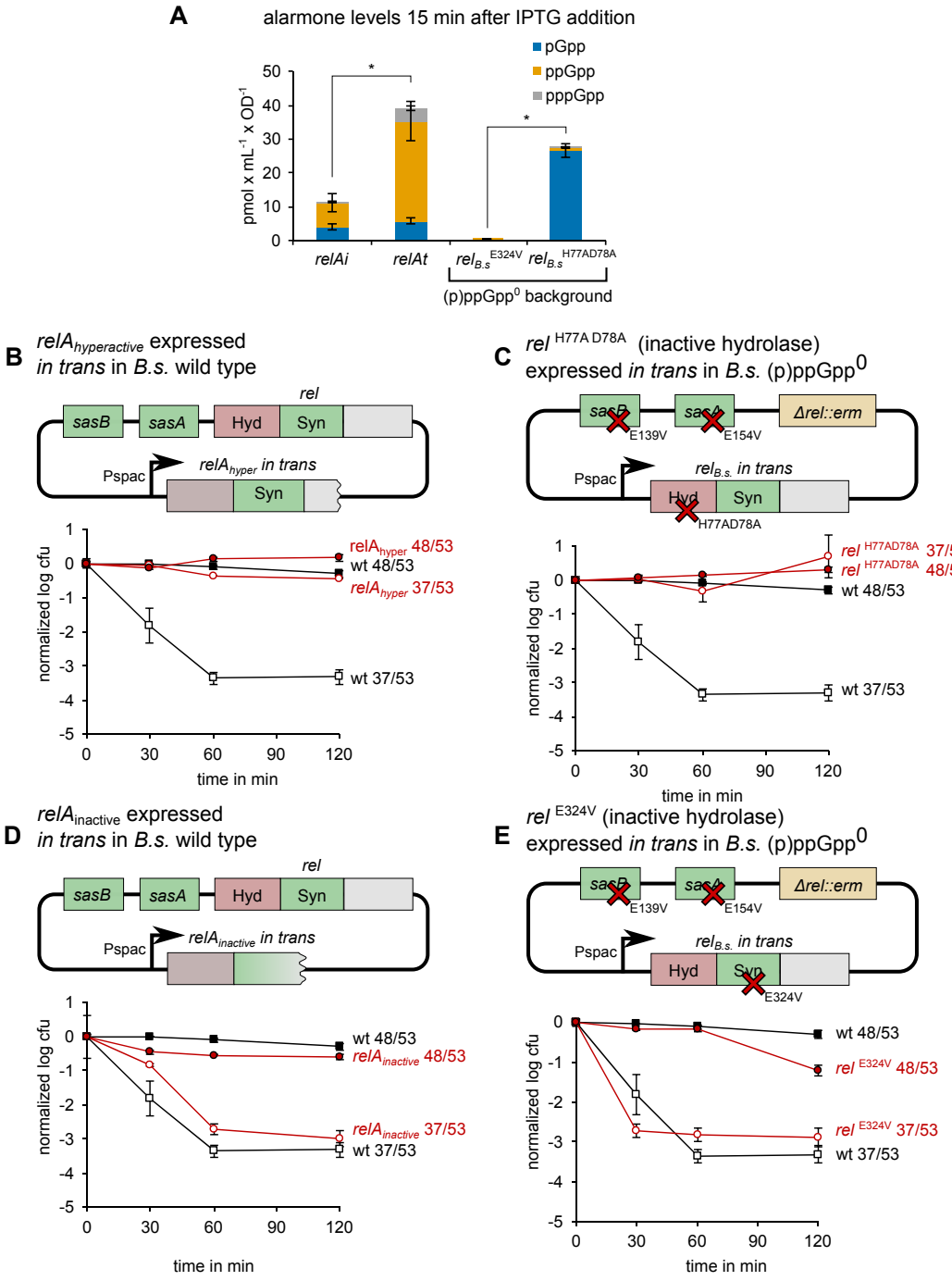

Figure S 3: Thermotolerance and survival of strains expressing *rel* variants *in trans*.
(A) Levels of alarmones in these strains after the application of 1 mM IPTG for 15 min. Asterisks indicate significant changes ($p \leq 0.05$) of combined alarmone levels according to Welch's *t*-test. **(B-E)** Survival of wildtype (black lines) and mutant strains (red lines) at 53 °C without pre-shock (37/53 °C; open symbols) or with pre-shock (15 min 48 °C/53 °C; closed symbols). Means and SEM of at least three independent experiments are shown. Strains were supplemented with 1 mM IPTG 15 min prior to temperature shift. **(B)** Expression of a truncated, hyperactive *rel* variant from *E. coli* (designated $relA_{hyper}$). **(C)** Expression of $rel_{B.s.}$ with inactive hydrolase domain (E77A D78A) in the (pp)pGpp^0 strain. **(D)** Expression of a truncated, inactive *relA* variant from *E. coli* ($relA_{inactive}$). **(E)** Expression of $rel_{B.s.}$ with inactive synthetase domain (E324V) in the (pp)pGpp^0 strain.

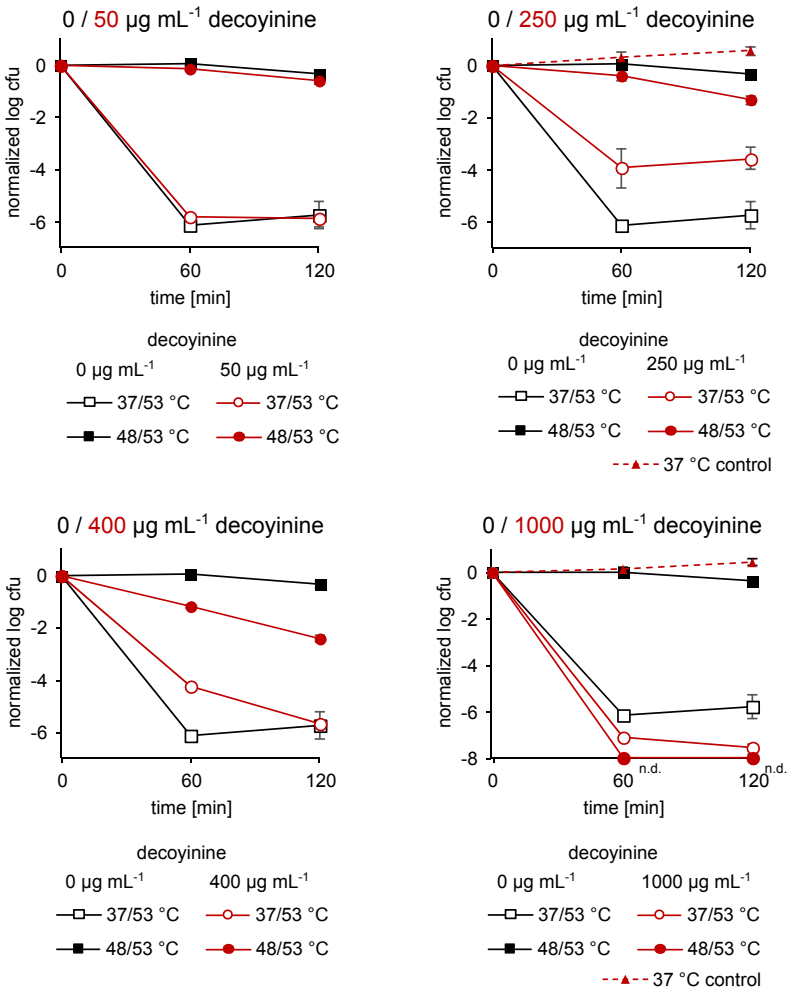

Figure S 4 thermotolerance and survival of strains expressing treated with decoyinine
Thermotolerance development and survival of wildtype cells treated with decoyinine (red lines) or left untreated (black lines). Means and SEM of at least three independent experiments are shown. Strains were supplemented with 50, 250, 400 or 1000 µg ml^{-1} decoyinine 15 min before heat treatment. Open symbols: no pre-shock, closed symbols: 15 min pre-shock at 48 °C. n.d.: not determined, no cfu could be detected from 100 µl cell culture.

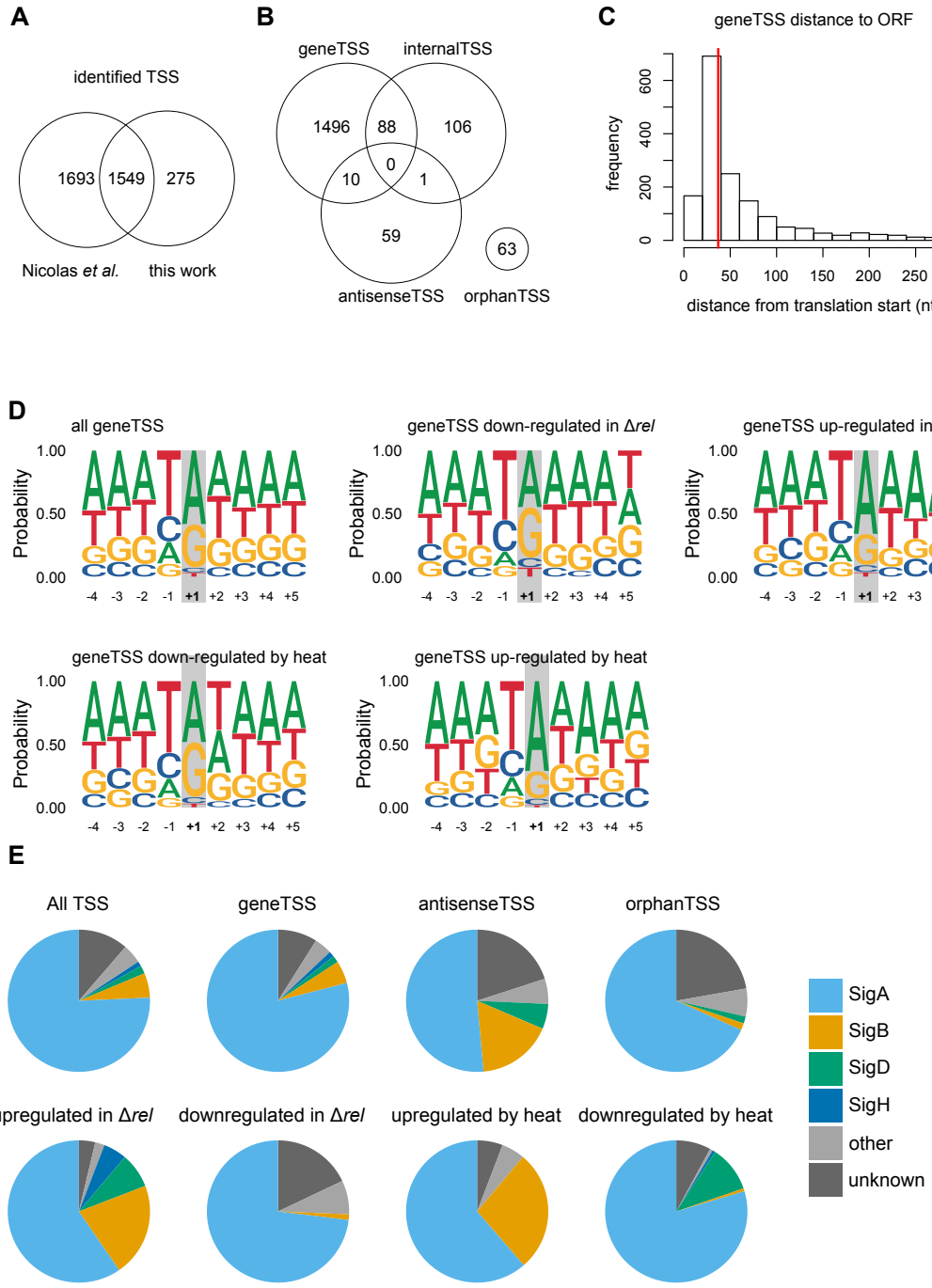

Figure S 5 Analysis of transcription start sites
(A) Venn Diagram showing the overlap of TSS identified in this study with transcription upshifts identified in [98]. **(B)** Venn Diagram depicting the classification of the identified TSS. **(C)** Length distribution of the distance from the TSS to the translation initiation site. **(D)** Sequence logos of the region around the TSS of genes up- or down-regulated during different conditions. **(E)** Predicted sigma factors in the different TSS classes.

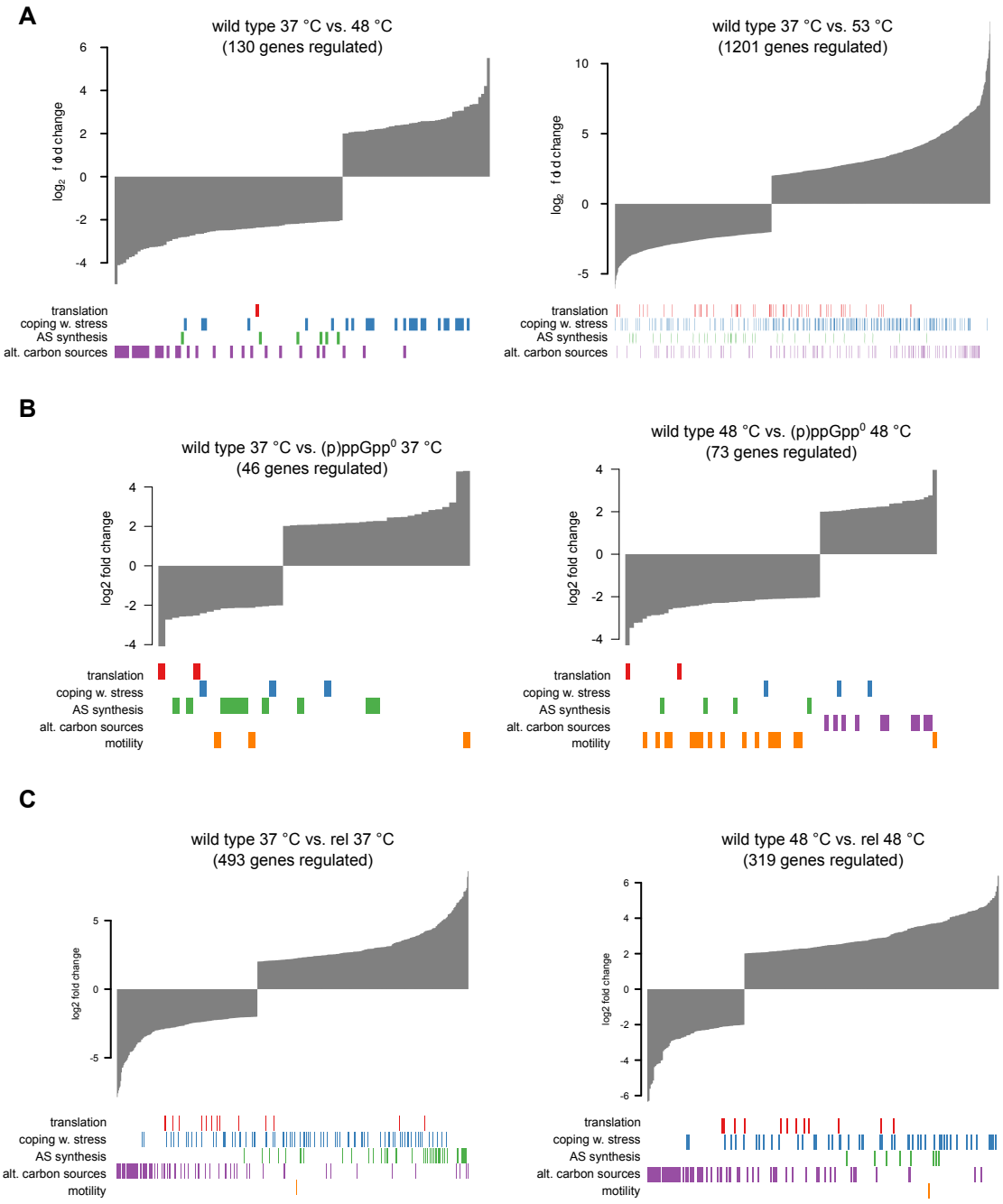

Figure S 6.: Global differences in gene expression of heat shocked
The distributions of all up- and down-regulated genes for the indicated conditions are shown. Bar tracks indicate the distribution of the respective functional groups. **(A)** Wildtype cells (BHS220) heat shocked at 48 °C or 53 °C versus unstressed cells. **(B)** Wildtype (BHS220) versus (p)ppGpp^0 cells (BHS319) at37 or 48 °C. **(C)** wildtype (BHS220) versus Δ*rel* cells (BHS368) at 37 °C or 48 °C.

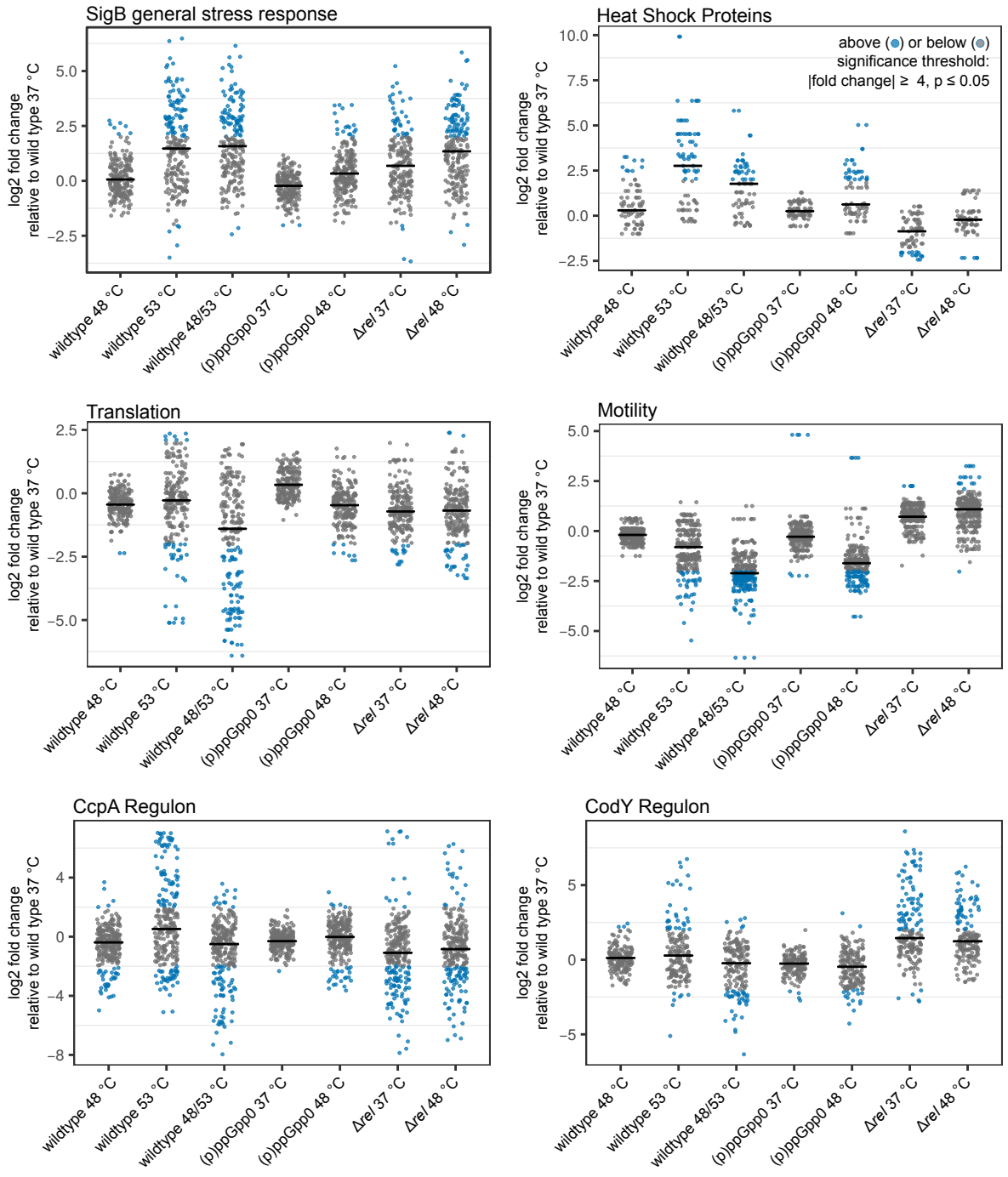

Figure S 7 : Up- or down-regulation of regulons or gene categories.
Points in the scatterplot represent log2-transformed up- or down-regulation of individual genes of the respective regulons relative to wildtype cells at 37 °C. Blue/gray color indicates transcriptional changes above/below the significance threshold (see Materials and Methods). Horizontal bars represent the median expression changes of the whole gene set.

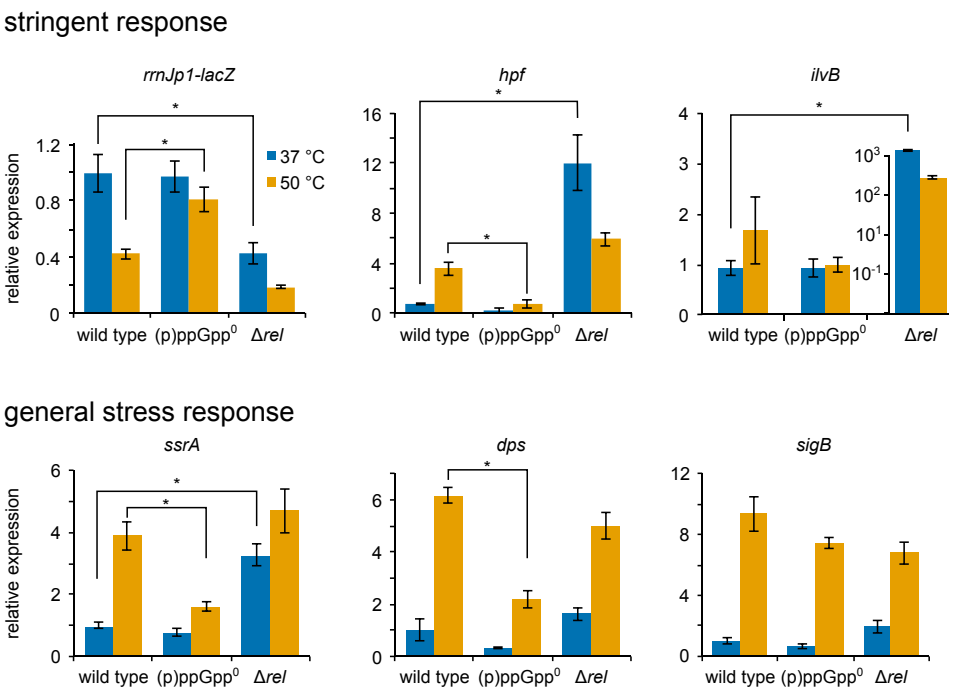

Figure S 8 : (p)ppGpp mediated transcriptional changes during heat stress
Relative changes in the transcription of selected genes during heat shock at 50 °C in wildtype (BHS220), (p)ppGpp^0 (BHS319) and Δ*rel* (BHS368) strains determined by RT-qPCR. Means and SEM of three replicates are shown. Asterisks indicate significance ($p \leq 0.05$) according to Welch's *t*-test, *n.s.*: not significant.

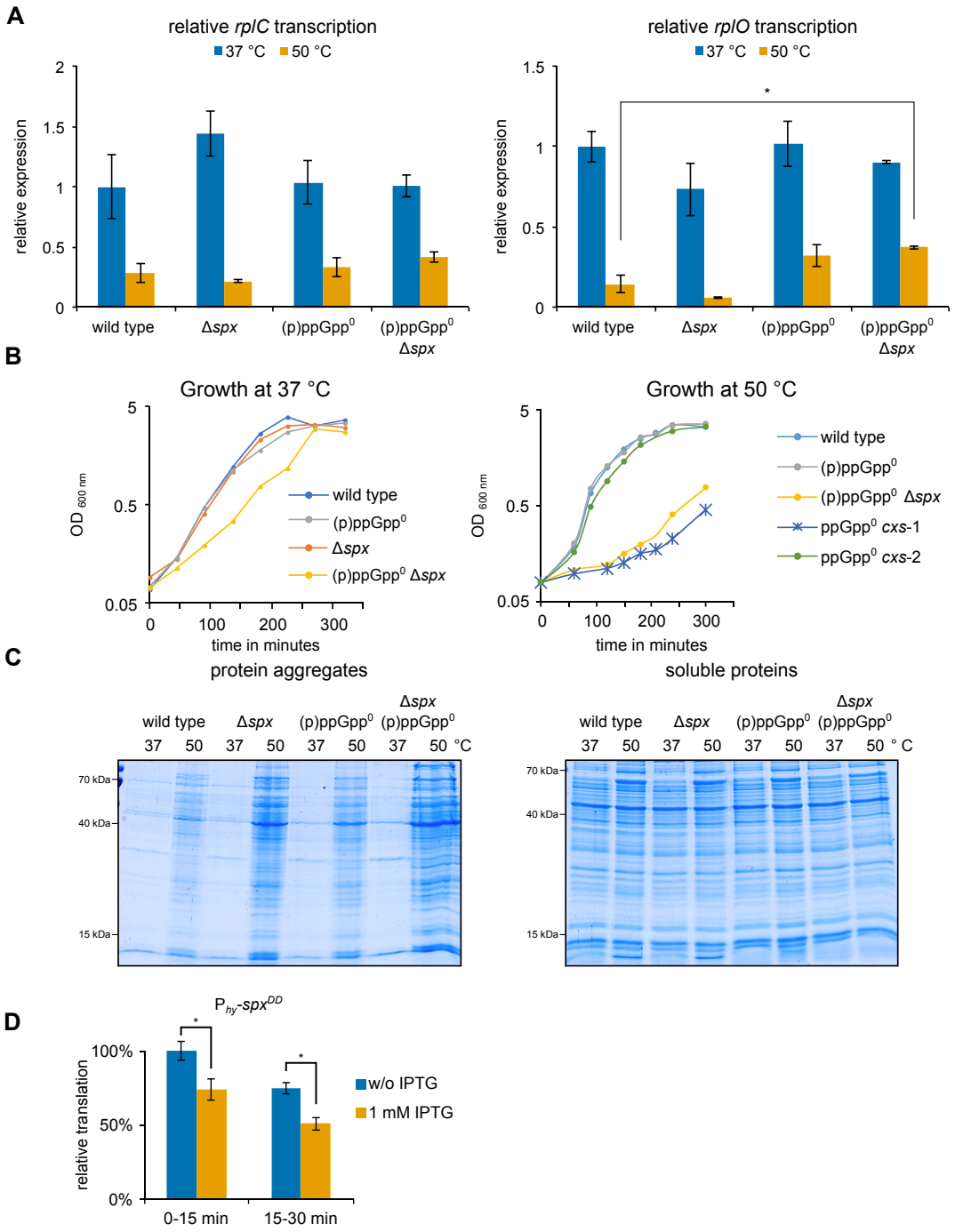

Figure S 9.: (pp)pGpp and Spx act complementary.
(A) RT-qPCR experiment showing the relative transcription of *rplC* and *rplO* in wildtype (BHS220), Δ*spx* (BHS222), (pp)pGpp^0 (BHS319) or (pp)pGpp^0 Δ*spx* (BHS766) cells treated with or heat stress at 50 °C for 15 min. Means and SEM of three replicates are shown. Asterisks indicate significant changes ($p \leq 0.05$) of transcript levels according to Welch's *t*-test, **(B)** Growth of wildtype (BHS220), Δ*spx* (BHS222), (pp)pGpp^0 (BHS319), (pp)pGpp^0 Δ*spx* (BHS766), (pp)pGpp^0 cx*s*-1 (BHS954) or (pp)pGpp^0 cx*s*-2 (BHS949) cell in LB medium at 37 °C or 50 °C. **(C)** The fraction of aggregated proteins (left) or soluble proteins (right) in wildtype, Δ*spx* (BHS014), (pp)pGpp^0 (BHS214) or (pp)pGpp^0 Δ*spx* (BHS766) cells treated with or heat stress at 50 °C for 15 min. **(D)** Relative translation of a strain carrying an inducible copy of Spx^{DD} (BHS201) with and without the addition of IPTG. Means and SEM of seven independent experiments are shown. Asterisks indicate significance ($p \leq 0.05$) according to Welch's *t*-test.

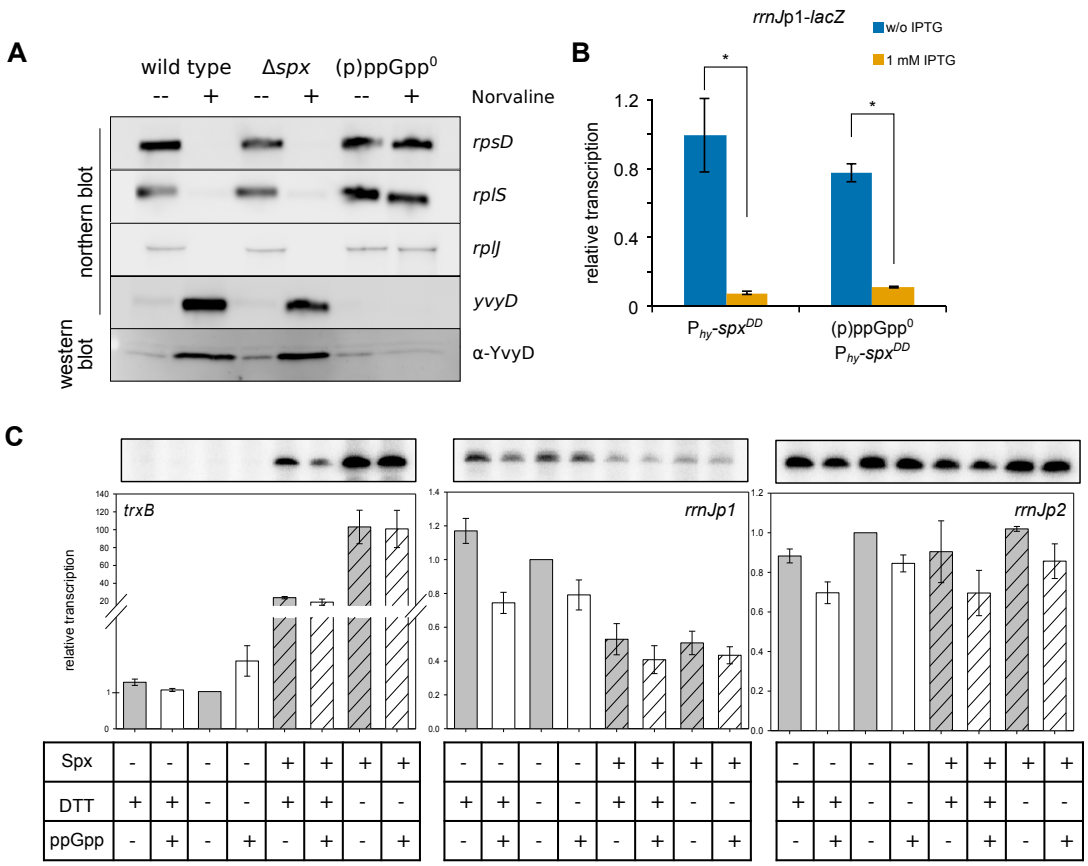

Figure S 10.: (pp)pGpp and Spx act independently.
(A) Northern and western blot of wildtype, Δ*spx* (BHS014) or (pp)pGpp^0 (BHS214) strains treated with or without DL-norvaline. Cells were grown in minimal medium supplemented with 0.5 % casamino acids to OD_{600} 0.4. The medium was removed by centrifugation and the cells were resuspended in fresh medium with casamino acids (--) or 0.5 mg/ml DL-norvaline (+) and grown for 30 min. **(B)** Relative transcription of *rrnJp1-lacZ* with or without expression of *spx*^{DD} with 1 mM IPTG for 30 min in the wildtype or (pp)pGpp^0 background. Means and SEM of three replicates are shown. **(C)** *in vitro* transcription from selected promoters with or without Spx and ppGpp under reducing (+ DTT) or oxidizing (- DTT) conditions. Means and SEM of three replicates and a representative autoradiogram are shown.

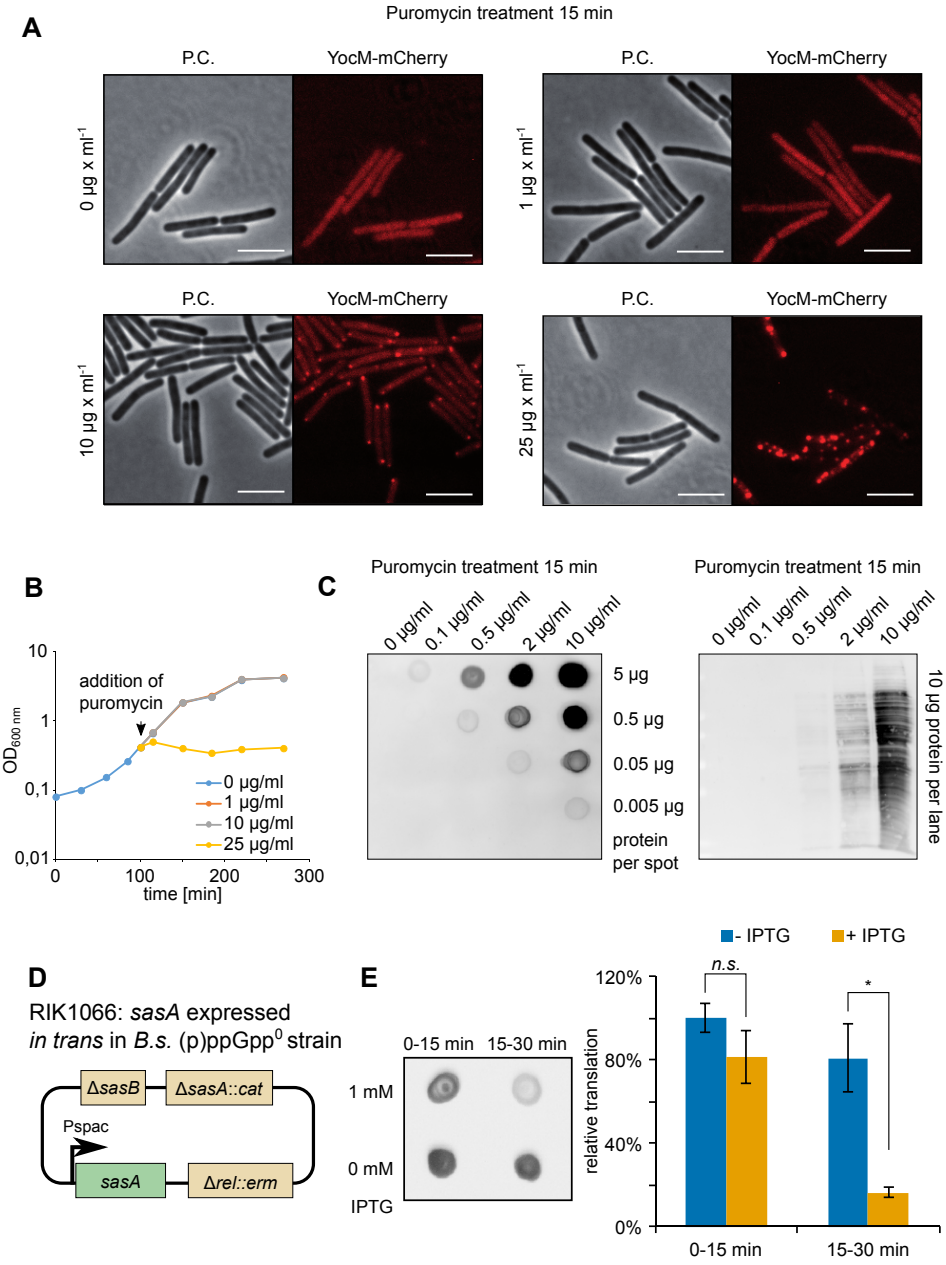

Figure S 11 Puromyin labels nascent proteins and does not disturb protein homeostasis at low concentration.

(A) Accumulation of subcellular protein aggregates (fluorecent spots) after the addition of puromycin visualized by YocM-mCherry. BIH369 cells were grown in LB + 0.5 % xylose and treated with 1, 10 or 25 µg ml^{-1} puromycin or left untreated for 15 min. Phase contrast images (P.C.) and fluorescence images with RFP-filters (YocM-mCherry) are shown. **(B)** The effect of puromycin on growth. Wildtype cells were grown in LB to the mid-exponential phase (OD_{600} 0.4) and supplemented with puromycin at the indicated concentrations. **(C)** Dot blot or western blot of puromycin-labeled proteins. Exponentially growing cells grown in LB were treated with the indicated concentrations of puromycin for 15 min. **(D)** Outline of the genotypes of the RIK1066 strain, carrying an inducible copy of *sasA* in the (p)ppGpp^0 background. **(E)** Relative puromycin incorporation in RIK1066 cells treated with or without 1 mM IPTG. Cells were incubated with 1 mg ml^{-1} puromycin for 15 min, added directly to the medium after the addition of IPTG (0-15 min) or after 15 min (15-30 min), then harvested. One representative experiment and means and SEM from the quantification of three independent experiments are shown. Asterisks indicate significance ($p \leq 0.05$) according to Welch's *t*-test.

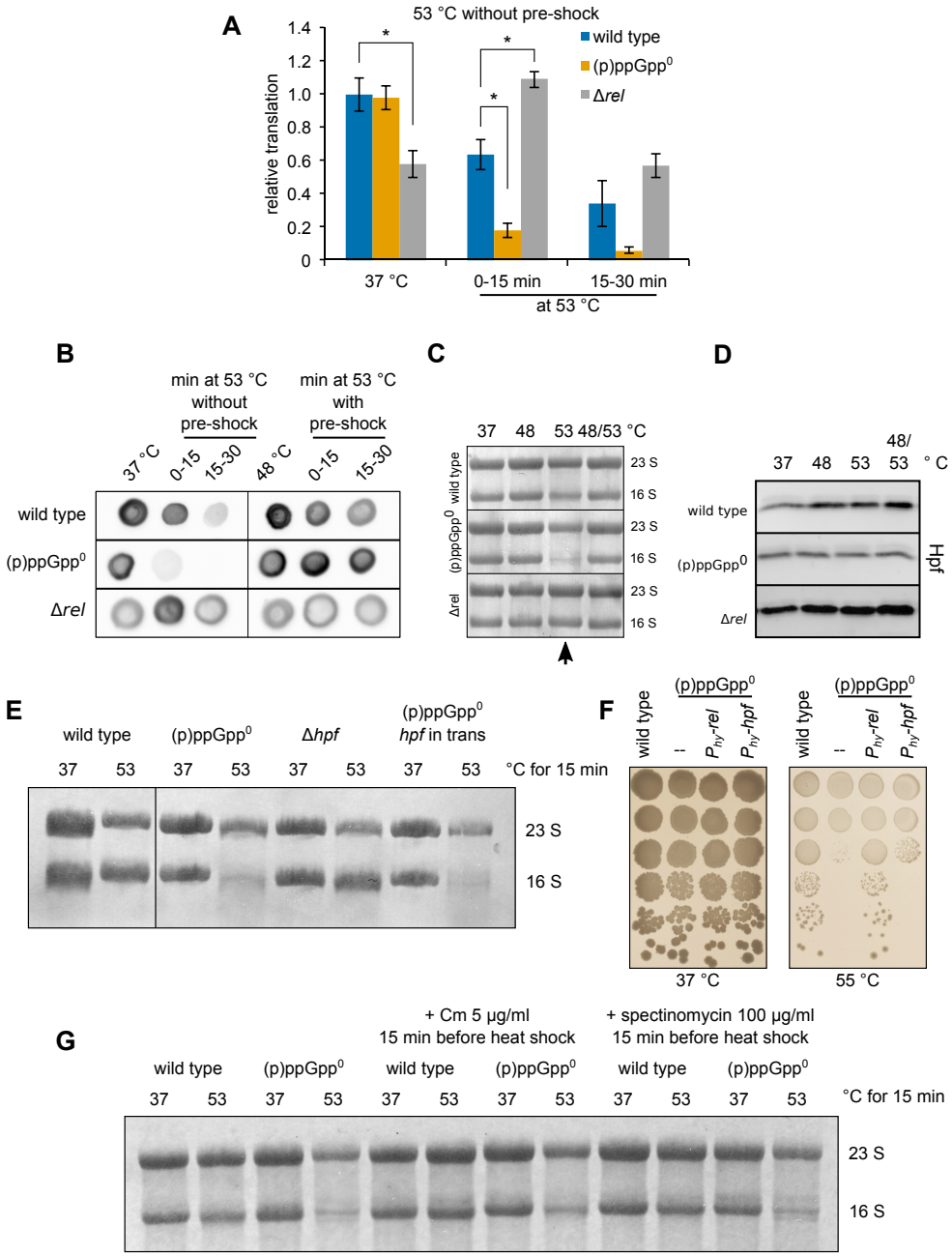

Figure S 12.: Relative translation of heat shocked cells.

(A) Relative translation (puromycin incporpration) of wildtype, (p)ppGpp^0 (BHS214) and Δ*rel* (BHS126) strains during heat stress at 53 °C. 1 µg ml^{-1} puromycin was added for 15 min to the medium directly after (0-15 min) or 15 min after the temperature upshift. Means and SEM of three independent experiments are shown. Asterisks indicate significance ($p \leq 0.05$) according to Welch's *t*-test. **(B)** Representative experiment from Fig. 6 B & S9 A. **(C)** Methylene blue stained membranes showing the integrity or degradation of rRNA after severe heat stress (53 °C). Wildtype, (p)ppGpp^0 (BHS214) or Δ*rel* (BHS126) cells were heat-shocked at 48 °C, 53 °C or 48/53 °C for 15 min each. 2 µg total RNA was separated on denaturing agarose gels and blotted on nylon membranes. **(D)** Western blot showing Hpf levels during thermotolerance development in wildtype, (p)ppGpp^0 (BHS214) or Δ*rel* (BHS126) strains. Cells were heat shocked for 15 min each at the indicated temperature(s). **(E)** Methylene blue stained membranes showing the integrity or degradation of rRNA. Wildtype, (p)ppGpp^0 (BHS214) Δ*hpf* (BHS008) or (p)ppGpp^0 P_{spac}-*hpf* (BHS626) cells were treated with or without heat shock at 53 °C for 15 min. 1 mM IPTG was added to the strains to induce the expression of *hpf* 15 min prior to heat shock. 2 µg total RNA was separated on denaturing agarose gels and blotted on nylon membranes. **(F)** Wildtype, (p)ppGpp^0 (BHS214) (p)ppGpp^0 P_{spac}-*rel* (BHS622) or (p)ppGpp^0 P_{spac}-*hpf* (BHS626) were spotted on agar plates supplemented with 1 mM IPTG and incubated over night at 37 °C or 55 °C. **(G)** rRNA degradation after severe heat stress (53 °C) in wildtype or (p)ppGpp^0 (BHS214) cells left untreated or treated with 5 µg ml^{-1} chloramphenicol or 100 µg ml^{-1} spectinomycin 15 min prior to the application of stress. 2 µg total RNA was separated on denaturing agarose gels and blotted on nylon membranes.

Text S1 Supporting Results

Supporting Materials and Methods

Identification of transcription start sites

Transcription start sites were annotated from the comparison of rRNA-depleted, tobacco acid pyrophosphatase (TAP) treated libraries that allow adaptor-ligation to 5' primary transcripts and libraries, where TAP treatment was omitted using the TSSpredator v1.06 software [1] in the "more sensitive" parameter preset and manually reviewed. TSS were classified as geneTSS (located ≤ 300 nt upstream of a gene), internal TSS (within an annotated gene), antisense TSS (in antisense direction within or in a 100 nt window downstream of a gene) or orphan TSS if not classified otherwise [1]. The results are presented in DataSet S1. Sequences around geneTSS directly upstream of significantly regulated genes were extracted and GNU R version 3.5.1 with the ggseqlogo package version 0.1 was used for the generation of sequence logos [2,3].

Gene set enrichment analysis

A gene set enrichment analysis (GSEA) of the significantly regulated genes was carried out on the Category (SW1 to SW4) and regulon datasets provided by SubtiWiki [4]. The GNU R software v. 3.5.1 [3] and the clusterProfiler library v. 3.10.1 [5] was used. P values were adjusted according to the Benjamini-Hochberg (BH) method and $P_{adjust} \leq 0.05$ was set as significance threshold.

Supporting Results

Analysis of transcription start sites

1547 TSS were selected by manual review and 276 additional sites were manually added that TSSpredator failed to detect, resulting in a total number of 1823 annotated TSS that are in good agreement to data published earlier by [6] (Fig. S5A, DataSet S1). About 75 % of TSS are located upstream of known genes (geneTSS) with a median distance of 39 nt to the start codon (Fig. S5 B, C).

Analysis of the sequence surrounding the TSS showed that ATP was the most frequent initiating nucleotide, followed by GTP (Fig. S5 D). Interestingly, the TSS of genes down-regulated in the Δ*rel* strain

exhibited an overrepresentation of GTP as the initiating nucleotide, while adenine was overrepresented for genes up-regulated in Δ*rel* cells. These observations are in accordance with the observed decreased GTP levels during the SR (Fig. S1) which are known to negatively influence the transcription of genes possessing guanine at the TSS, while genes possessing adenine at the +1 position are positively affected [7–10]. Conversely, the same over-representation of guanine or adenine was observed for the TSS of genes down- or up-regulated by heat (Fig. S5).

By comparing the TSS dataset with the transcription upshifts reported earlier by Nicolas et al [6], a cognate sigma factor could be assigned to more than 80 % of the identified TSS, with SigA-dependent promoters representing the most abundant group (Fig. S5 E, DataSet S1). Interestingly, promoters recognized by the alternative sigma factors SigB, SigD and SigH appear to be enriched upstream of genes up-regulated in Δ*rel* cells, while promoters of genes down-regulated in Δ*rel* cells are mostly transcribed by SigA (Fig. S5 E). In accordance with the our observations (Fig. 3), a large share of SigB-dependent promoters was up-regulated by heat (48/53 °C), while SigA- and SigD promoters were down-regulated (Fig. S5 E). Interestingly, SigB- and SigD promoters were also found enriched in the set of antisense TSS, indicating a possible indirect regulation by interference by these alternative sigma factors.

Gene set enrichment analysis

A gene set enrichment analysis (GSEA) from significantly regulated genes was conducted using the functional categories and regulons provided by Subtiwiki [4]. The results are summarized in DataSet S2. Among others, heat shock- and general stress genes controlled by SigB were enriched upon all temperature upshifts in wildtype and mutant strains. Down-regulated genes during heat stress (48/53 °C) were enriched in ribosomal- and flagellar protein genes as well as genes of the phosphotransferase system (PTS). For example, the CodY- AbrB-, SigD-, stringent response- and CcpA-regulons were enriched as down-regulated during heat.

Up-regulated genes of Δ*rel* cells were enriched in sporulation-specific genes, genes for the acquisition or biosynthesis of S-adenosylmethionine and branched-chain amino acids and general stress proteins, while PTS-specific genes required for the uptake of alternative carbon sources were enriched among the down-regulated genes. The regulons of CodY, SigB and ScoC (up-regulated) as well as stringent

response, AbrB and CcpA (down-regulated), were found to be enriched, in the regulated genes of Δ*rel* cells (Dataset S2).

Estimating translation rates by labelling of nascent peptides with puromyin

The antibiotic puromycin mimics charged tRNA and binds to the ribosomal A site. It becomes covalently linked to the nascent chain, resulting in premature termination of the translation and the release of a puromycyl-peptide [11,12]. High puromycin concentrations inhibit protein synthesis and induce the accumulation of misfolded proteins, as visualized by fluorescence microscopy using the YocM-mCherrry protein which localizes to cellular protein aggregates (Fig. S11) [13,14].

However, treatment with low concentrations (1 μg mL^{-1}) does not perturb protein homeostasis and growth (Fig. S11 A, B). This dose-dependent, unspecific incorporation of puromycin into nascent polypeptides can be detected using monoclonal anti-puromycin antibodies and utilized as readout for the rate of translation (Fig. S11 C) [15,16]. To verify this approach, we analyzed the translation rates of a strain expressing the small alarmone synthetase *ywaC in trans* which results in accumulation of high (p)ppGpp levels [17] As expected, activation of *ywaC* expression resulted in a strong decrease of puromycin incorporation corresponding to the inhibited translation in these cells (Fig. S11 D, E) [17–19].

References

1. Dugar G, Herbig A, Förstner KU, Heidrich N, Reinhardt R, Nieselt K, et al. High-Resolution Transcriptome Maps Reveal Strain-Specific Regulatory Features of Multiple Campylobacter jejuni Isolates. Hughes D, editor. PLoS Genetics. 2013;9: e1003495. doi:10.1371/journal.pgen.1003495
2. Wagih O. ggseqlogo: A "ggplot2" Extension for Drawing Publication-Ready Sequence Logos [Internet]. 2017. Available: https://CRAN.R-project.org/package=ggseqlogo
3. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2018. Available: https://www.R-project.org/
4. Zhu B, Stülke J. SubtiWiki in 2018: from genes and proteins to functional network annotation of the model organism *Bacillus subtilis*. Nucleic Acids Research. 2018;46: D743–D748. doi:10.1093/nar/gkx908
5. Yu G, Wang L-G, Han Y, He Q-Y. clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. OMICS: A Journal of Integrative Biology. 2012;16: 284–287. doi:10.1089/omi.2011.0118
6. Nicolas P, Mäder U, Dervyn E, Rochat T, Leduc A, Pigeonneau N, et al. Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. Science. 2012;335: 1103–1106. doi:10.1126/science.1206848
7. Krásný L, Gourse RL. An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. EMBO J. 2004;23: 4473–4483. doi:10.1038/sj.emboj.7600423

8. Krásný L, Tišerová H, Jonák J, Rejman D, Šanderová H. The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in *Bacillus subtilis*. Molecular Microbiology. 2008;69: 42–54. doi:10.1111/j.1365-2958.2008.06256.x
9. Tojo S, Kumamoto K, Hirooka K, Fujita Y. Heavy involvement of stringent transcription control depending on the adenine or guanine species of the transcription initiation site in glucose and pyruvate metabolism in *Bacillus subtilis*. J Bacteriol. 2010;192: 1573–1585. doi:10.1128/JB.01394-09
10. Kriel A, Bittner AN, Kim SH, Liu K, Tehranchi AK, Zou WY, et al. Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. Mol Cell. 2012;48: 231–241. doi:10.1016/j.molcel.2012.08.009
11. Yarmolinsky MB, Haba GL. INHIBITION BY PUROMYCIN OF AMINO ACID INCORPORATION INTO PROTEIN. Proc Natl Acad Sci USA. 1959;45: 1721–1729.
12. Nathans D. PUROMYCIN INHIBITION OF PROTEIN SYNTHESIS: INCORPORATION OF PUROMYCIN INTO PEPTIDE CHAINS. Proc Natl Acad Sci USA. 1964;51: 585–592.
13. Krüger E, Witt E, Ohlmeier S, Hanschke R, Hecker M. The clp proteases of *Bacillus subtilis* are directly involved in degradation of misfolded proteins. J Bacteriol. 2000;182: 3259–3265.
14. Hantke I, Schäfer H, Janczikowski A, Turgay K. YocM a small heat shock protein can protect *Bacillus subtilis* cells during salt stress. Mol Microbiol. 2019;111: 423–440. doi:10.1111/mmi.14164
15. Starck SR, Green HM, Alberola-Ila J, Roberts RW. A General Approach to Detect Protein Expression In Vivo Using Fluorescent Puromycin Conjugates. Chemistry & Biology. 2004;11: 999–1008. doi:10.1016/j.chembiol.2004.05.011
16. Schmidt EK, Clavarino G, Ceppi M, Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. Nat Methods. 2009;6: 275–277. doi:10.1038/nmeth.1314
17. Tagami K, Nanamiya H, Kazo Y, Maehashi M, Suzuki S, Namba E, et al. Expression of a small (p)ppGpp synthetase, YwaC, in the (p)ppGpp(0) mutant of *Bacillus subtilis* triggers YvyD-dependent dimerization of ribosome. Microbiologyopen. 2012;1: 115–134. doi:10.1002/mbo3.16
18. Svitil AL, Cashel M, Zyskind JW. Guanosine tetraphosphate inhibits protein synthesis *in vivo*. A possible protective mechanism for starvation stress in *Escherichia coli*. J Biol Chem. 1993;268: 2307–2311.
19. Corrigan RM, Bellows LE, Wood A, Gründling A. ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. Proc Natl Acad Sci USA. 2016;113: E1710-1719. doi:10.1073/pnas.1522179113

Table S1: List of plasmids, strains and oligonucleotides

List of *B. subtilis* strains

strain	genotype	Source/construction
wild type	trpC2	[1]
BNM111	*trpC2 spx::kan*	[2]
BIH369	*trpC2 lacA::P_{xyl}-yocM-mCherry erm*	[3]
RIK900	*trpC2 rel::erm*	[4]
RIK908	*trpC2 ywaC::spec*	[4]
RIK909	*trpC2 yjbM::cat*	[4]
RIK1066	*trpC2 ΔyjbM ywaC::cat rel::erm aprE::Pspac-ywaC spc*	[5]
BHS008	*hpf::kan*	[6]
BHS014	*spx::kan*	[7]
BHS126	*trpC2 rel::erm*	this work, Δ*rel* from RIK900 in wild type
BHS127	*trpC2 yjbM::cat*	this work, Δ*yjbM* from RIK909 in wild type
BHS128	*trpC2 ywaC::spec*	this work, Δ*ywaC* from RIK908 in wild type
BHS157	*trpC2 $yjbM^{E139V}$*	this work, pMAD-yjbME139V in wild type
BHS158	*trpC2 $ywaC^{E154V}$*	this work, pMAD-ywaCE154V in wild type
BHS204	*trpC2 $ywaC^{E154V}$ $yjbM^{E139V}$*	this work, pMAD-yjbME139V in BHS158
BHS214	*trpC2 $ywaC^{E154V}$ $yjbM^{E139V}$ rel::erm*	this work, Δ*rel* in BHS204
BHS220	*trpC2 amyE::rrnJp1-lacZ cm*	[7]
BHS222	*trpC2 spx::kan amyE::rrnJp1-lacZ cm*	[7]
BHS225	*trpC2 amyE::rrnJp1-lacZ cm lacA::P_{hy}-spx^{DD} erm*	[7]
BHS313	*trpC2 $ywaC^{E154V}$ $yjbM^{E139V}$ amyE::rrnJp1-lacZ cm*	this work, pDG268-rrnJp1 [7] in BHS204
BHS319	*trpC2 $ywaC^{E154V}$ $yjbM^{E139V}$ amyE::rrnJp1-lacZ cm rel::erm*	this work, Δ*rel* in BHS313
BHS368	*trpC2 rel::erm amyE::rrnJp1-lacZ cm*	this work, Δ*rel* in BHS220
BHS610	*trpC2 amyE::Phyperspank-relAT spec*	this work, DGRM415 [8] in wild type
BHS611	*trpC2 amyE::Phyperspank-relAi spec*	this work, DGRM416 [8] in wild type
BHS618	*trpC2 $ywaC^{E154V}$ $yjbM^{E139V}$ amyE::Phy-rel spec*	this work, pDR111-rel in BHS204

BHS619	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *amyE::Phy-rel*^{E324V} *spec*	this work, pDR111-relE324V in BHS204
BHS620	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *amyE::Phy-rel*$^{H77A\ D78A}$ *spec*	this work, pDR111-relHDAA in BHS204
BHS621	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *amyE::Phy-hpf spec*	this work, pDR111-hpf in BHS204
BHS622	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *amyE::Phy-rel spec rel::erm*	this work, Δ*rel* in BHS618
BHS624	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *amyE::Phy-rel*^{E324V} *spec rel::erm*	this work, Δ*rel* in BHS619
BHS625	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *amyE::Phy-rel*$^{H77A\ D78A}$ *spec rel::erm*	this work, Δ*rel* in BHS620
BHS626	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *amyE::Phy-hpf spec rel::erm*	this work, Δ*rel* in BHS621
BHS709	*trpC2 rel* ^{E324V}	this work, pMAD-rel E324V in wild type
BHS755	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *amyE::rrnJp1-lacZ cm spx::kan*	this work, Δ*spx* in BHS313
BHS766	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *amyE::rrnJp1-lacZ cm spx::kan rel::erm*	this work, Δ*rel* in BHS755
BHS889	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *amyE::rrnJp1-lacZ cm lacA::*P_{hy}*-spx*^{DD} *spec*	this work, pBSII-spxDD-spec in BHS313
BHS890	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *amyE::rrnJp1-lacZ cm lacA::*P_{hy}*-spx*^{DD} *spec*	this work, Δ*rel* in BHS890
BHS942	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *rpoA*^{V260A}	this work, PYZ38 [9] in BHS204
BHS948	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *rpoA*^{V260A} *amyE::rrnJp1-lacZ cm*	this work, pDG268-rrnJp1 [7] in BHS942
BHS949	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *rpoA*^{V260A} *amyE::rrnJp1-lacZ cm rel::erm*	this work, Δ*rel* in BHS948
BHS952	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *rpoA*^{Y263C}	this work, PYZ37 [9] in BHS204
BHS953	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *rpoA*^{Y263C} *amyE::rrnJp1-lacZ cm*	this work, pDG268-rrnJp1 [7] in BHS952
BHS954	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *rpoA*^{Y263C} *amyE::rrnJp1-lacZ cm rel::erm*	this work, Δ*rel* in BHS953

List of plasmids

strain	**Source or cloning primers**
pBSIIE	[10]
pDG268-rrnJp1	[7]
pDR111	[11]
pMAD	[12]
pDR111-hpf	[6]
pSN56 (pDR111-spxDD)	[13]
pBSII-spxDD-spec	p203, p223, p288, p289
pDR111-rel	p174, p274

pDR111-rel E324V	p174, p274
pDR111-rel H77A D78A	p174, p274
pMAD-yjbME139V	WS3-WS6
pMAD-ywaCE154V	WS7-WS10
pMAD-rel E324V	WS11, WS12, Ws13, WS14
pMAD-rel H77A D78A	WS11, WS12, Ws15, WS16

List of oligonucleotides used for cloning/sequencing

ID	name	Sequence
p68	ywaC_seq	GAACCTTGCAGCAGACAGGG
p69	ywaC_do_rev	CTATGACGCCAAACCTGTCG
p70	ywaC_up_for	TTGCCTATGGATCCAGATCGC
p71	yjbM_up_for	CTGATACCTCTGAAAGCTGC
p72	yjbM_do_rev	CCTTATTGTAGGCTGTGCTG
p73	yjbM_seq	GCAAACTATGGAGAAGAAATGG
p74	rel_up_for	GTGTGCTGTCTGTTGTGAGC
p75	rel_do_rev	CAAAACGGCAAAACTGCTCG
p76	rel_seq_for	TCTGCTCTTTACATCTTTCG
p77	rel_seq_rev	CTGTATCATCGTGAGTGATG
p174	SphI_rel_rev	ACATGCATGCTTAGTTCATGACGCGGCGCAC
p203	pBS2E_for	TATACTAGTAGCGGCCGCTG
p219	rel_intra_seq	TACGATTTGTTGGCTGTCCG
p223	pDR111_rev	TAACTAGT ATAATGGATTTCCTTACGCG
p274	SalI_SD_rel_for	ACGCGTCGACTTGGGGGATGTATGATGGCGAACGAACAAGTATTG
p288	NsiI_pBSIIE_rev	GATCATGCAT CCCTAGACTCTAGGACTCTC
p289	NsiI_pdr111_for	GATCATGCAT CCCTATGCAAGGGTTTATTG
WS01	Bs*rel*-H420E-for	CTTACCGGATTGAATCTGAAATCGGC
WS02	Bs*rel*-H420E-rev	GCCGATTTCAGATTCAATCCGGTAAG
WS03	Bs*yjbM*-flk1-EcoRI-F	TTAAGAATTCCCGCCCTGTAAATCTTATTT
WS04	Bs*yjbM* -flk2-NcoI-R	AATTCCATGGGTGCTGCCTGATGGAGTTGA
WS05	Bs*yjbM* -E139V-F	GAAAAGCATGTTCTCGTAGTAATACAGATCCGTACAC
WS06	Bs*yjbM* -E139V-R	GTGTACGGATCTGTATTACTACGAGAACATGCTTTTC
WS07	Bs*ywaC* -EcoRI-F	TTAAGAATTCATGGATTTATCTGTAACAC
WS08	Bs*ywaC* +fla-NcoI-R	TTAACCATGGAATCCAGCCGTACGGCTGC
WS09	Bs*ywaC* -E154V-F	GTCAAAGCAGTAATTC
WS10	Bs*ywaC* -E154V-R	GAATTACTGCTTTGAC
WS11	BsRelA-EcoRI-F	TTAAGAATTCATGGCGAACGAACAAG
WS12	BsRelA-Nco-R	TTAACCATGGTTAGTTCATGACGCGGCG
WS13	Bs*rel*_E324V_for	GCGATCCGCTTGTAGTGCAGATCCG
WS14	Bs*rel*_E324V_rev	CGGATCTGCACTACAAGCGGATCGC
WS15	Bs*rel*_H77AD78A_for	GATTTTTGGCCGCTGTCGTGGAAGATAC
WS16	Bs*rel*_H77AD78A_rev	GTATCTTCCACGACAGCGGCCAAAAATC

Oligonucleotides used for synthesis of RNA probes

ID	name	Sequence (T7 promoter underlined)
p162	hpf_probe_for	CGTTAAAGGATCATGTCGAG
p163	hpf_T7_rev	CTAATACGACTCACTATAGGGAGACGTCATTTCTGCGGTACACG
p297	rpsD_probe_for	GGAATCTCTCTTAGCGGTAC
p299	rpsD_T7_rev	CTAATACGACTCACTATAGGGAGAGCAAGTTCAGAACGCTCAGG
p300	rplS_probe_for	CTTCGTACTGATCTTCCTGC
p302	rplS_T7_rev	CTAATACGACTCACTATAGGGAGACTGATCTCTTTAATACGAGCC
p422	rplJ_probe_for	ATGAGCAGCGCAATTGAAAC
p423	rplJ_T7_rev	CTAATACGACTCACTATAGGGAGAGCGCCTTGTTCTTCCTTTTG

oligonucleotides used in RT-qPCR experiments

ID	name	Sequence
p585	qPCR_lacZ_rev	CGTTTCACCCTGCCATAAAG
p586	qPCR_lacZ_for	GGAAGATCAGGATATGTGGC
p595	qPCR_rplC_for	TCCGGTAACTGTTATCGAGG
p596	qPCR_rplC_rev	GACCAACTTCATACGCATCC
p601	qPCR_hpf_for	AGGATCATGTCGAGAGGAAG
p602	qPCR_hpf_rev	GCTTACGGATTTGACGTTCC
p605	qPCR_23S_for	CTTTGATCCGGAGATTTCCG
p606	qPCR_23S_rev	GTACAGAGTGTCCTACAACC
p642	qPCR_sigB_for	AGCCTTATCCGTTGACCACA
p643	qPCR_sigB_rev	GCGAGACGTGCATTTGAGAT
p648	qPCR_ssrA_for	CGAGCTCTTCCTGACATTGC
p649	qPCR_ssrA_rev	AACCCACGTCCAGAAACATC
p650	qPCR_rplO_for	GTCGTGGTATTGGTTCTGGC
p651	qPCR_rplO_rev	GTGACTTCCGTTCCTTCTGC
p664	qPCR_dps_for	ACCGATTGCAACAATGAAGGA
p665	qPCR_dps_rev	GTCCCCTGTTGTTTCGTCAC
p797	qPCR_rbsC_for	GATGATTGTGTCCGGTGTCG
p798	qPCR_rbsC_rev	GTAGTTTGTGCCAAGTCCGG
p799	qPCR_ilvB_for	CATATCCTTCCCCGTCACGA
p800	qPCR_ilvB_rev	GCTTCCTGAAATGCATCGCT

References

[1] J. Spizizen, “TRANSFORMATION OF BIOCHEMICALLY DEFICIENT STRAINS OF BACILLUS SUBTILIS BY DEOXYRIBONUCLEATE,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 44, no. 10, pp. 1072–1078, Oct. 1958.

[2] S. Runde *et al.*, “The role of thiol oxidative stress response in heat-induced protein aggregate formation during thermotolerance in *B acillus subtilis*: Thiol oxidation in protein aggregate formation,” *Mol. Microbiol.*, vol. 91, no. 5, pp. 1036–1052, Mar. 2014.

[3] I. Hantke, H. Schäfer, A. Janczikowski, and K. Turgay, "YocM a small heat shock protein can protect *Bacillus subtilis* cells during salt stress," *Mol. Microbiol.*, vol. 111, no. 2, pp. 423–440, Feb. 2019.
[4] H. Nanamiya *et al.*, "Identification and functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*," *Mol. Microbiol.*, vol. 67, no. 2, pp. 291–304, Jan. 2008.
[5] K. Tagami *et al.*, "Expression of a small (p)ppGpp synthetase, YwaC, in the (p)ppGpp(0) mutant of *Bacillus subtilis* triggers YvyD-dependent dimerization of ribosome," *MicrobiologyOpen*, vol. 1, no. 2, pp. 115–134, Jun. 2012.
[6] B. Beckert *et al.*, "Structure of the *Bacillus subtilis* hibernating 100S ribosome reveals the basis for 70S dimerization," *EMBO J.*, vol. 36, no. 14, pp. 2061–2072, 14 2017.
[7] H. Schäfer *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.*, vol. 111, no. 2, pp. 514–533, Feb. 2019.
[8] H. Nouri *et al.*, "Multiple links connect central carbon metabolism to DNA replication initiation and elongation in *Bacillus subtilis*," *DNA Res. Int. J. Rapid Publ. Rep. Genes Genomes*, Sep. 2018.
[9] M. M. Nakano, Y. Zhu, J. Liu, D. Y. Reyes, H. Yoshikawa, and P. Zuber, "Mutations conferring amino acid residue substitutions in the carboxy-terminal domain of RNA polymerase alpha can suppress clpX and clpP with respect to developmentally regulated transcription in *Bacillus subtilis*," *Mol. Microbiol.*, vol. 37, no. 4, pp. 869–884, Aug. 2000.
[10] J. Radeck *et al.*, "The Bacillus BioBrick Box: generation and evaluation of essential genetic building blocks for standardized work with *Bacillus subtilis*," *J. Biol. Eng.*, vol. 7, no. 1, p. 29, Dec. 2013.
[11] S. Ben-Yehuda, D. Z. Rudner, and R. Losick, "RacA, a bacterial protein that anchors chromosomes to the cell poles," *Science*, vol. 299, no. 5606, pp. 532–536, Jan. 2003.
[12] M. Arnaud, A. Chastanet, and M. Débarbouillé, "New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria," *Appl. Environ. Microbiol.*, vol. 70, no. 11, pp. 6887–6891, Nov. 2004.
[13] S. Nakano, E. Küster-Schöck, A. D. Grossman, and P. Zuber, "Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 100, no. 23, pp. 13603–13608, Nov. 2003.

2.4 Additional studies on the stringent response

A number of additional experiments and observations are described in this section, which address previously unresolved questions from the preceding chapters.

2.4.1 The role of tRNA and L11 in Rel activation

In section 2.3, it could be demonstrated that Rel is the main source of (p)ppGpp during heat stress. In addition, the observation that the accumulation of alarmones during heat- and oxidative stress could be suppressed by chloramphenicol suggests that these stresses may activate Rel by a mechanism similar as during amino acid limitation which probably also involves sensing of uncharged tRNA at the ribosome (section 2.3 Figure 1).

To further explore the mechanism of Rel activation, mutations were analyzed which block the activation of Rel during amino acid starvation. In addition to direct measurements of alarmone levels, the accumulation of the *hpf* transcript or Hpf protein was used as readout for the SR, as established in section

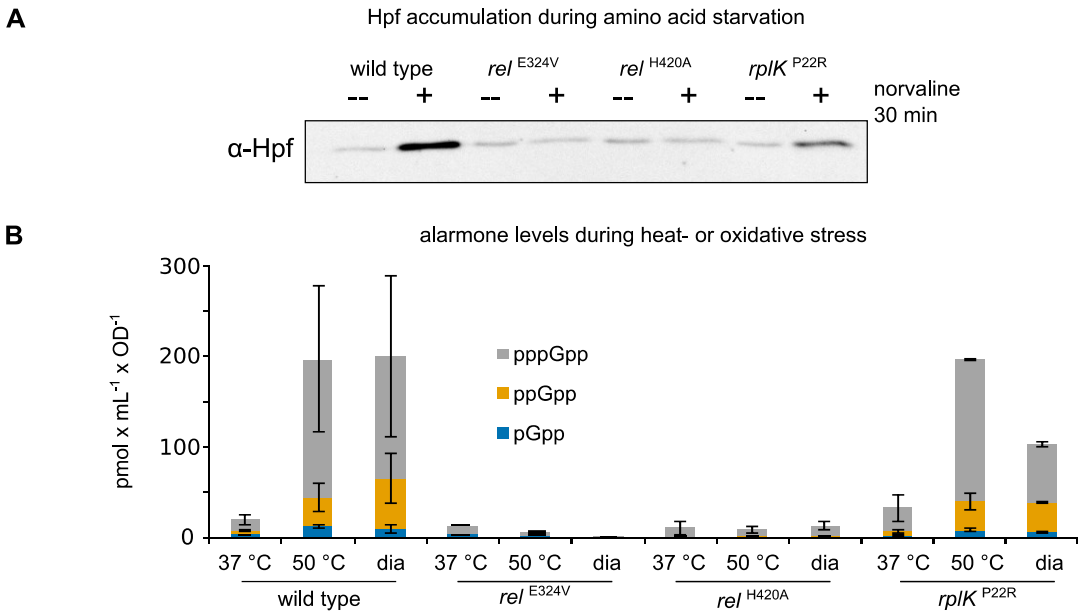

Figure 7: Genetic requirements for the activation of Rel during environmental stress

(A) Western Blot of Hpf as readout for the activation of the SR during amino acid starvation elicited by DL-norvaline in wild type or mutant strains. Cells were grown in minimal medium with 0.5 % CAA, pelleted by centrifugation, resuspended in fresh medium containing 0.5 % CAA or 0.05 % DL-norvaline and incubated for 30 min before harvesting. (B) Levels of pGpp, ppGpp and pppGpp in the same strains during unstressed growth (37 °C), heat stress (2 min 50 °C) or diamide treatment (dia; 1 mM 10 min). Mean and SEM of at least two replicates are shown.

2.3 (Figure 7). The E324V mutation in Rel inactivates the (p)ppGpp synthetase by mutation of its catalytic center while the H420E mutation interferes with tRNA binding (equivalent to H432E in *E. coli*) [191]. A P22R mutation in the conserved proline-loop of L11 (*rplK*) was reported to confer thiostrepton resistance but also blocks activation of the SR [179], [289]. As expected, all mutant strains exhibit a strongly impaired SR upon DL-norvaline treatment, as indicated by the abolished Hpf accumulation (Figure 7 A).

Interestingly, the *rel* ^{H420E} strain also failed to accumulate (p)ppGpp in response to heat- and oxidative stress similar to the *rel* ^{E324} strain (Figure 7 B). This observation further corroborates the hypothesis that SR activation upon heat stress is dependent on the sensing of uncharged tRNA. In contrast, the *rplK* ^{P22R} mutant strain exhibits (p)ppGpp accumulation during heat stress similar to wild type and alarmone accumulation was only moderately reduced during diamide-induced oxidative stress (Figure 7 B). These results indicate, that either the *rplK* ^{P22R} mutation is not sufficient to fully abolish activation of Rel or

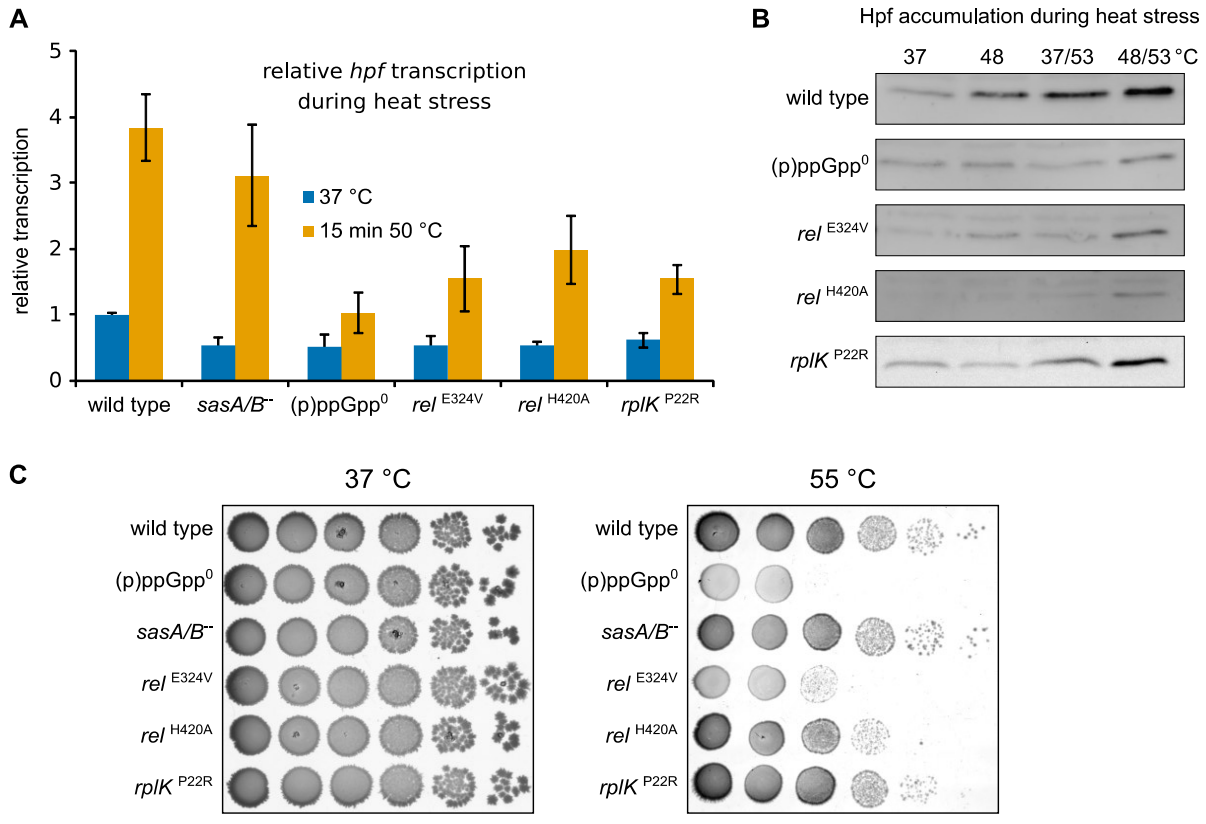

Figure 8: Analysis of mutations, which block SR activation.

(A) Regulation of the *hpf* transcript during heat shock in wild type or mutant strains with impaired SR. For details, see text. (B) Levels of the Hpf protein during thermotolerance development in the same strains. (C) Growth and s urvival of the same strains on agar plates during heat stress (55 °C).

that the SR during environmental stress could be independent of L11. In contrast to the results obtained by direct alarmone-measurements, both the *rel* ^{H420E} and *rplK* ^{P22R} mutants displayed reduced *hpf* expression during heat shock at 50 °C similar to the *rel* ^{E324V} strain. Likewise, accumulation of the Hpf protein was similarly impaired in the same strains during thermotolerance development (Figure 8 A, B), indicating, that both tRNA and L11 may be involved and required for the full activation of the SR upon environmental stress.

As an additional readout, the same strains were also tested for survival on agar plates during severe heat stress (Figure 9 C). The ability to form colonies during stress is dependent on (p)ppGpp and was strongly impaired in (p)ppGpp^0 and *rel* ^{E324V} strains as described in section 2.3. The *rel* ^{H420E} and *rplK* ^{P22R} strains also reproducibly displayed a somewhat impaired growth during severe heat stress. However, both strains reproducibly exhibited a less pronounced phenotype than the *rel* ^{E324V} strain.

Taken together, these observations strongly suggest that tRNA binding by the H420 residue of Rel is strictly required for its activation upon heat- or oxidative stress. The slightly less-strong stress sensitivity of the *rel* ^{H420E} strain (Figure 8C) may be conferred via basal (p)ppGpp synthesis by Rel, which is only fully abolished in the *rel* ^{E324V} strain (Figure 7 B). However, the phenotypes observed for the *rplK* ^{P22R} strain do not allow for an unambiguous interpretation. The observation that both (p)ppGpp accumulation during heat- and oxidative stress and stress resistance on agar plates was only marginally impaired in the *rplK* ^{P22R} strain (Figure 7 B, Figure 8 C), suggested that in contrast to amino acid starvation, the activation of Rel does not require L11 during heat stress.

In contrast, the impaired accumulation of *hpf* and Hpf during heat shock indicates that L11 is involved in SR regulation during heat stress in some way or the other (Figure 8 A, B). The *rplK* ^{P22R} mutation may for example lead to a delayed activation of the SR or otherwise affect its kinetics. Furthermore, the point mutation may have other unexpected effects on the cellular physiology, which may alter the expression of *hpf*.

2.4.2 Subcellular localization of Rel during stress and starvation

Interestingly, an N-terminal GFP-Rel fusion displayed an unusual localization during stress and starvation, which may be used as additional readout (Figure 9). The GFP-Rel fusion protein was inactive

and could not complement Rel with respect to SR-activation upon amino acid starvation. However, the GFP-Rel fusion formed subcellular foci during DL-norvaline treatment visible by fluorescence microscopy. The foci may represent aggregated fusion protein, which becomes misfolded due to the bulky N-

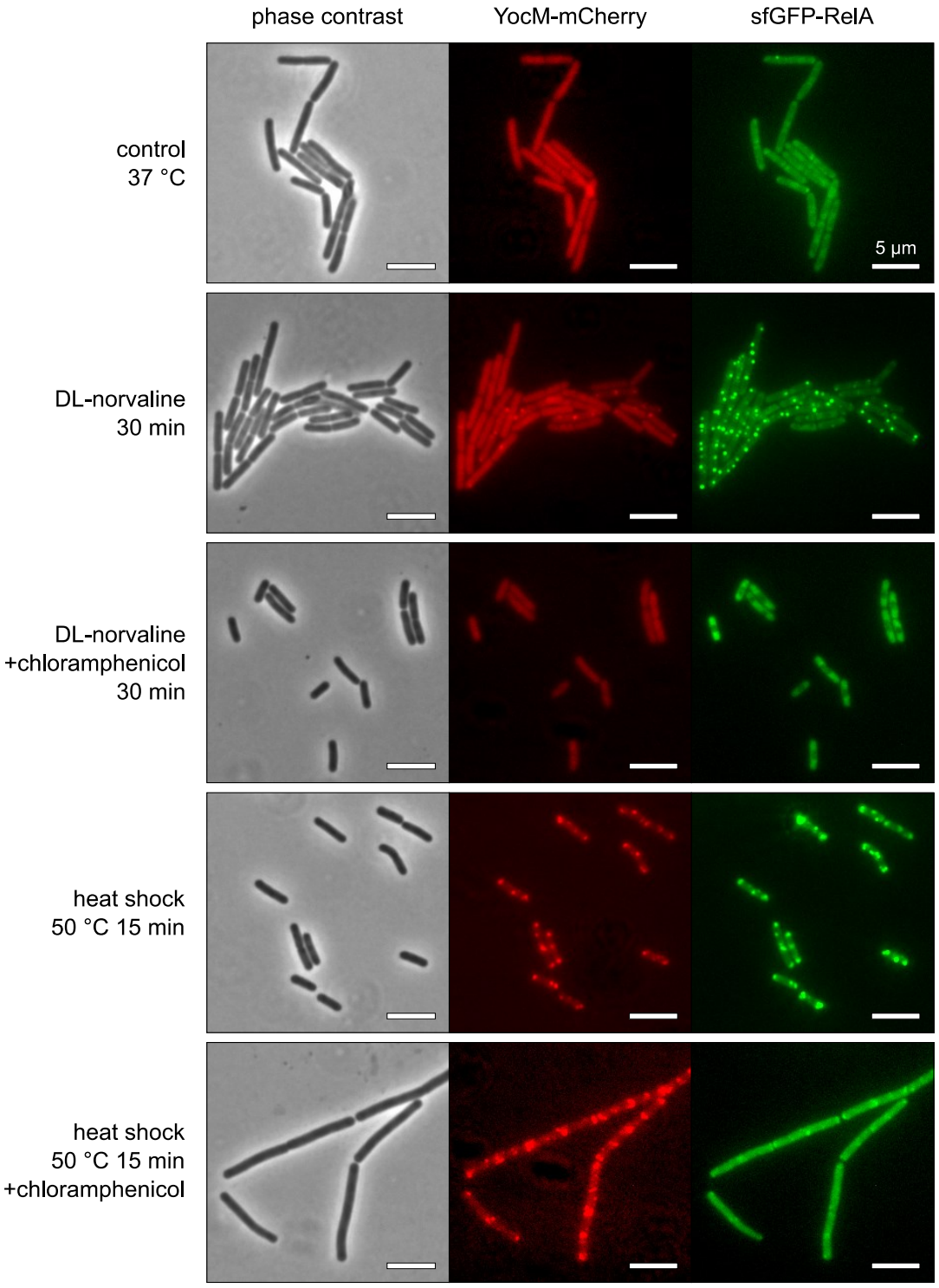

Figure 9: Subcellular localization of YocM-mCherry and GFP-RelA during starvation and heat stress.

Cells were grow in BMM supplemented with 0.5 % CAA and 0.4 % xylose and treated with DL-norvaline (0.5 mg mL^{-1}) for 30 min or heat shock at 50 °C for 15 min with or without chloramphenicol addition (25 µg mL^{-1}). Phase contrast and fluorescent images using the GFP or RFP filter set are shown.

terminal GFP domain during the starvation-induced transition from the closed, auto-inhibited conformation to the ribosome-associated open complex (see section 1.3.2).

Consistent with this hypothesis, the formation of foci was completely inhibited by the addition of chloramphenicol, which suppresses the SR (Figure 9). The YocM-mCherry fusion protein, which localizes to protein aggregates [290], was distributed mostly homogeneous, indicating that treatment with DL-norvaline does not generally provoke the formation of protein aggregates and that the localization of GFP-Rel is a specific response to starvation and not a general perturbation of protein homeostasis.

Heat shock resulted in the formation of large subcellular protein aggregates visualized as fluorescent foci by the YocM-mCherry fusion protein, as observed previously [290]. The GFP-Rel fusion formed similar foci which partially co-localized with the YocM-mCherry foci. However, foci formation of GFP-Rel during heat shock could be suppressed almost completely by chloramphenicol, while the formation of protein aggregates stained by YocM-mCherry was unaffected (Figure 9).

The reversibility of the GFP-Rel foci formation by chloramphenicol addition during heat stress indicates that the foci formation is not the consequence of general heat-induced misfolding and aggregation of the protein but rather of specific conformation changes similar to those observed during DL-norvaline treatment. These observations support the hypothesis, that heat stress elicits a partial amino acid deprivation and stimulates (p)ppGpp synthesis activity of Rel by uncharged tRNA (see discussion).

2.4.3 Reduced translation can both enhance and abolish thermotolerance

During the analysis of the SR and its role in the HSR, it became apparent that mutant strains with elevated (p)ppGpp levels exhibit decreased sensitivity to an otherwise lethal heat shock, while cells with lowered alarmone levels are more sensitive to stress (see section 2.3). This stress resistance phenotype consistentently correlated with (p)ppGpp levels across different strains which lack a functional *rel* gene or express mutated *rel* or *relA* genes, suggesting that the phenotype was solely conferred by the increased level of alarmones and not the presence or absence of certain proteins (see section 2.3).

Interestingly, a similar thermoresistant phenotype was observed upon examination of a *B. subtilis* Δ*rplK* strain. Similar to the *rplK* ^{P22R} mutation described above, the SR cannot be activated by Rel or RelA upon amino acid limitation in Δ*rplK* cells [179]. However, while the *rplK* ^{P22R} mutation confers a relaxed phenotype but permits otherwise normal growth, Δ*rplK* cells exhibit severe translation defects and strongly decreased growth rate (Figure 10 A) [95], [179], [291]. Interestingly, *B. subtilis* Δ*rplK* cells displayed strongly decreased sensitivity to an otherwise lethal temperature shift to 53 °C when assayed for thermotolerance (Figure 10 B). This strain still accumulated the HSP GroEL during thermotolerance development, albeit at a decreased rate which presumably reflects the translation defect (Figure 10 C). This observation suggests that reduced translation rates *per se*, which are also observed in strains with constitutive SR (see section 2.3), may be a critical determinant for the increased stress resistance.

To further investigate the relationship between translation rates and thermoresistance, cells were analyzed with inhibited translation by treatment with either chloramphenicol (5 µg mL^{-1}), which halts translation but does not induce (p)ppGpp synthesis [181] or DL-norvaline (0.5 mg mL^{-1}), an inhibitor of leucyl- and isoleucyl-tRNA synthetases, which causes the accumulation of uncharged tRNA and thereby reduces translation while (p)ppGpp synthesis is turned on [214], [276].

However, the addition of antibiotics or inhibitors may interfere with the ability of cells to form colonies on agar plates and thereby confound the interpretation of the results. Therefore, the amount of cellular protein aggregates was taken as alternative readout for protein homeostasis and heat stress

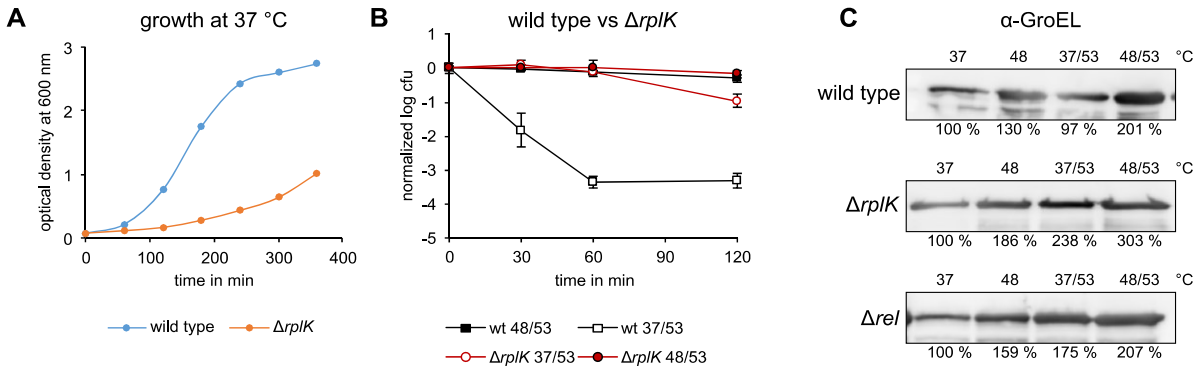

Figure 10: Thermotolerance and HSP accumulation of a Δ*rplK* mutant strain.

(A) Growth of wild type (blue) and Δ*rplK* mutant (orange) strains in rich medium. (B) Survival of *B. subtilis* wild type (black) and Δ*rplK* mutant (red) strains during thermotolerance with adaptation at 48 c (closed symols) or without (open symbols). (C) Western Blot showing the accumulation of GroEL during thermotolerance development in wild type, Δ*rplK* and Δ*rel* cells. The band intensity is given relative unstressed cells at 37 °C.

resistance. The results are depicted in Figure 11. Interestingly, treatment with either chloramphenicol or DL-norvaline abolished the protective influence of the priming step at 48 °C and resulted in accumulation of large amounts of protein aggregates in both primed and unprimed cells (Figure 11). In addition, both inhibitors also stopped GroEL accumulation, indicating that protein synthesis was completely inhibited.

Taken together, the data suggests that slowing down translation can be beneficial for the survival of heat stress. The high-resistance phenotype can be achieved by increased accumulation of (p)ppGpp, e.g. in *rel* mutants (see section 2.3), but also independently of (p)ppGpp accumulation by a deletion of *rplK*. However, inhibition of translation *per se* does not lead to increased thermoresistance in all instances since treatment with inhibitors of protein synthesis or tRNA-synthetases resulted in the opposite effect (Figure 11). The opposing phenotypes may be the consequence of different mechanisms by which protein synthesis rates are reduced, the different extents to which translation is either slowed down or completely inhibited or additional side effects elicited by (p)ppGpp or treatments with protein synthesis inhibitors. Clearly, more experiments are required to dissect the relationship between translation rates and stress resistance.

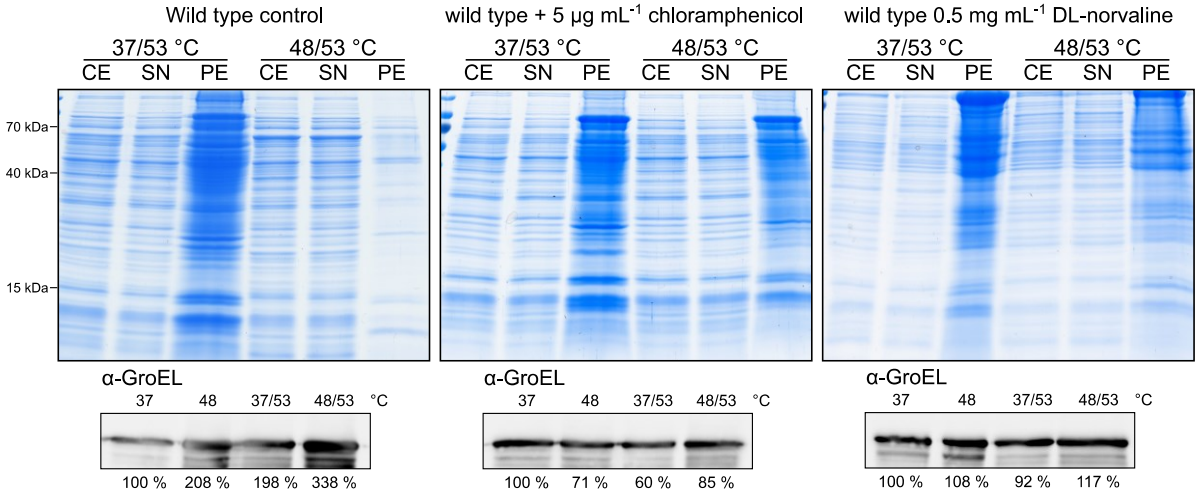

Figure 11: Accumulation of protein aggregates an HSP in cells with inhibited translation.

Wild type cells grown in BMM and treated with 5 µg mL-1 chloramphenicol 0.5 mg mL-1 DL-norvaline 15 min before the application of the temperature shift or left untreated. Top: Coomassie stained gels showing total proteins (CE, cell extract), soluble proteins (SN, supernatant) or protein aggregates (PE, pellet). Bottom: Western Blot of the total protein fraction depicting the GroEL levels. The band intensity is given relative to untreated cells at 37 °C.

2.4.4 CodY has only a limited influence on stress resistance

Next, the significance of CodY in the (p)ppGpp-dependent stress resistance phenotype was examined by asessing Δ*codY* mutant strains in wild type and *rel* ^{E324V} backgrounds, in which the synthetase activity of Rel is abolished (Figure 12). The rationale for this experiment was two-fold: First, the results described in section 2.3 suggest, that the SR mediates only modest transcriptional changes during heat stress, suggesting that transcriptional adjustments are probably not a critical determinant for stress resistance conferred by (p)ppGpp. In contrast, de-repression of the CodY regulon was found to mediate large transcriptional alterations in highly thermoresistant Δ*rel* strains (section 2.3). In addition, it was recently reported that the SR confers tolerance to nitrosative stress in *Salmonella* by reestablishing the pools of amino acids, which becomes limited during stress (see also section 3.1) [292]. In particular, the up-regulation of biosynthesis pathways for BCAA during stress was of importance in *Salmonella*, which are controlled by CodY in *B. subtilis*.

Therefore, the Δ*codY* mutant strain was tested for resistance to heat- salt- and oxidative stress. The Δ*codY* strain exhibited a slightly increased sensitivity to heat- and salt stress, but no phenotype was observed upon oxidative stress. It was also tested, whether a *codY* deletion could rescue the diminished stress resistance of (p)ppGpp^0 mutants. Since a (p)ppGpp^0 Δ*codY* strain could not be created due to incompatible resistance cassettes, a Δ*codY rel* ^{E324V} strain was constructed and assessed. Interestingly, a

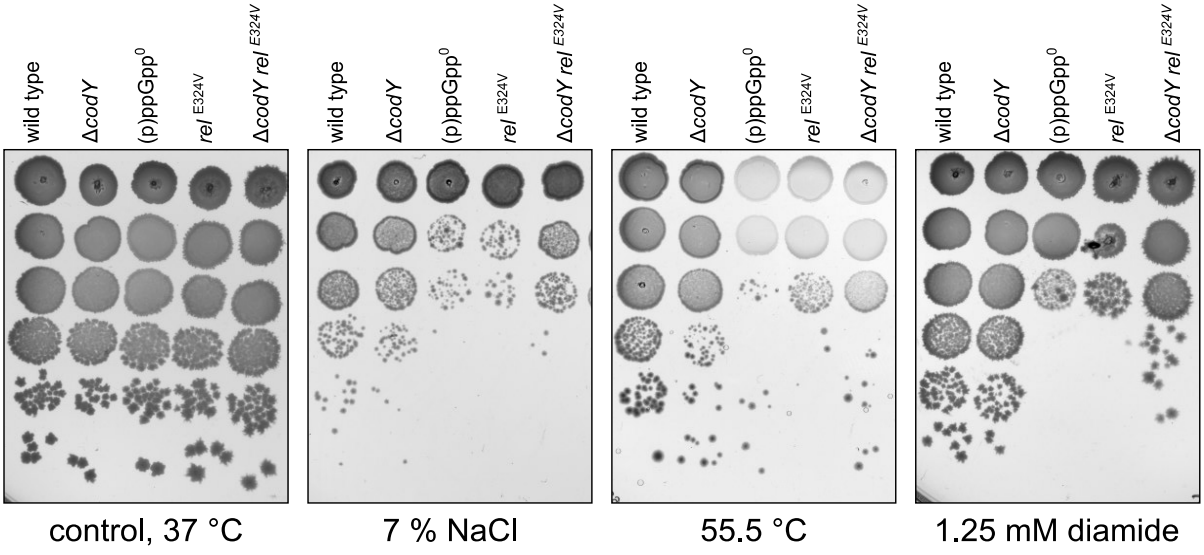

Figure 12: The influence of *codY* deletions on stress tolerance

Wild type, Δ*codY*, (p)ppGpp^0, rel ^{E324V} or Δ*codY rel* ^{E324V} strains were spotted on agar plates supplemented with NaCl or diamide and grown over night at 37 °C or 55.5 °C.

codY deletion slightly increased stress resistance, but was unable to fully rescue the stress-sensitive phenotype of a *rel* ^{E324V} strain. Together, these results suggest, that CodY is not critical for stress resistance and that (p)ppGpp confers stress resistance mostly independently of CodY.

2.4.5 *In silico* identification of non-coding RNA candidates

The data obtained in the RNA-sequencing experiment (see section 2.3) allows a strand specific identification of transcripts with high sensitivity. Particularly by the analysis of transcription start sites (TSS), which are enriched in the libraries, new candidates for RNA based regulation can be identified with high sensitivity [293]. To identify new candidates for non-coding, regulatory RNAs, TSS were classified and analyzed. In total, 1823 TSS were identified (section 2.3). Based on their distance and orientation to known open reading frames, 229 TSS were selected which are probably not involved in the transcription of protein coding genes (see section 2.3, Figure S5).

TSS within or down-stream of a gene with opposing direction are likely to represent *cis*-acting RNAs and were classified as 'antisense' (asRNA) and 70 of such TSS were identified. However, a manual review of these TSS revealed that only a small number of the RNA candidates significantly overlap sense RNA reads. On the other hand, other events could also lead to the transcription of anti-sense RNAs. For example, a 3' extended UTR of a sense transcript could extend into a down-stream gene in antisense direction without this being detected by the classification of TSS. Therefore, for a more comprehensive screening of antisense RNA candidates, all uniquely mapped reads with antisense orientation to annotated genes were filtered and counted. Using an expression threshold of 30 reads per kilobase per million reads (RPKM) in at least one condition, 171 genes with antisense transcript candidates were identified. The genes with identified putative anti-sense RNAs are listed in Table 1. A large overlap was noted between the candidates detected here and the results of Nicolas *et al*. (117 out of 171 putative RNAs), indicating that the applied approach yields reliable results and may be better suited for the identification of *cis*-acting regulatory RNAs than the TSS-based approach. Since this dataset was fairly large, it was filtered prior to further analysis to select for RNA candidates, which might be involved in transcriptional regulation during heat shock or the SR. By filtering for differential (above 4 fold) expression in any of the examined heat shock conditions and (p)ppGpp mutant strains, 41 genes with putative antisense RNAs were selected (Figure 13). About 60 % these asRNA candidates could be matched with

non-coding RNAs identified previously by Nicolas *et al.* (2012) [9]. Notably, most asRNA candidates were up-regulated during heat stress (48 °C, 37/53 °C or 48/53 °C) and in the Δ*rel* strain, while the expression of only few asRNAs candidates was reduced under these conditions (Figure 13).

Out of these candidate asRNAs, two examples were selected which might be of elevated interest for the scope of this thesis (bold gene names in Figure 13). The first asRNA example overlaps the coding region of the *sigA* (*rpoD*) gene, which encodes the housekeeping sigma factor σ^A (Figure 14). The *sigA* gene is transcribed from multiple promoters recognized by σ^A, σ^D and σ^H located directly upstream of the *sigA* gene or upstream of the *yqxD-dnaG-sigA* operon [294], [295]. The antisense transcript is transcribed from at least one promoter downstream of the *sigA* reading frame. Notably, this RNA was already annotated as S951 in the dataset of Nicolas *et al.* (2012), but no function has been assigned. During unstressed growth, the RNA is transcribed at a basal level and the intensity of the antisense transcript

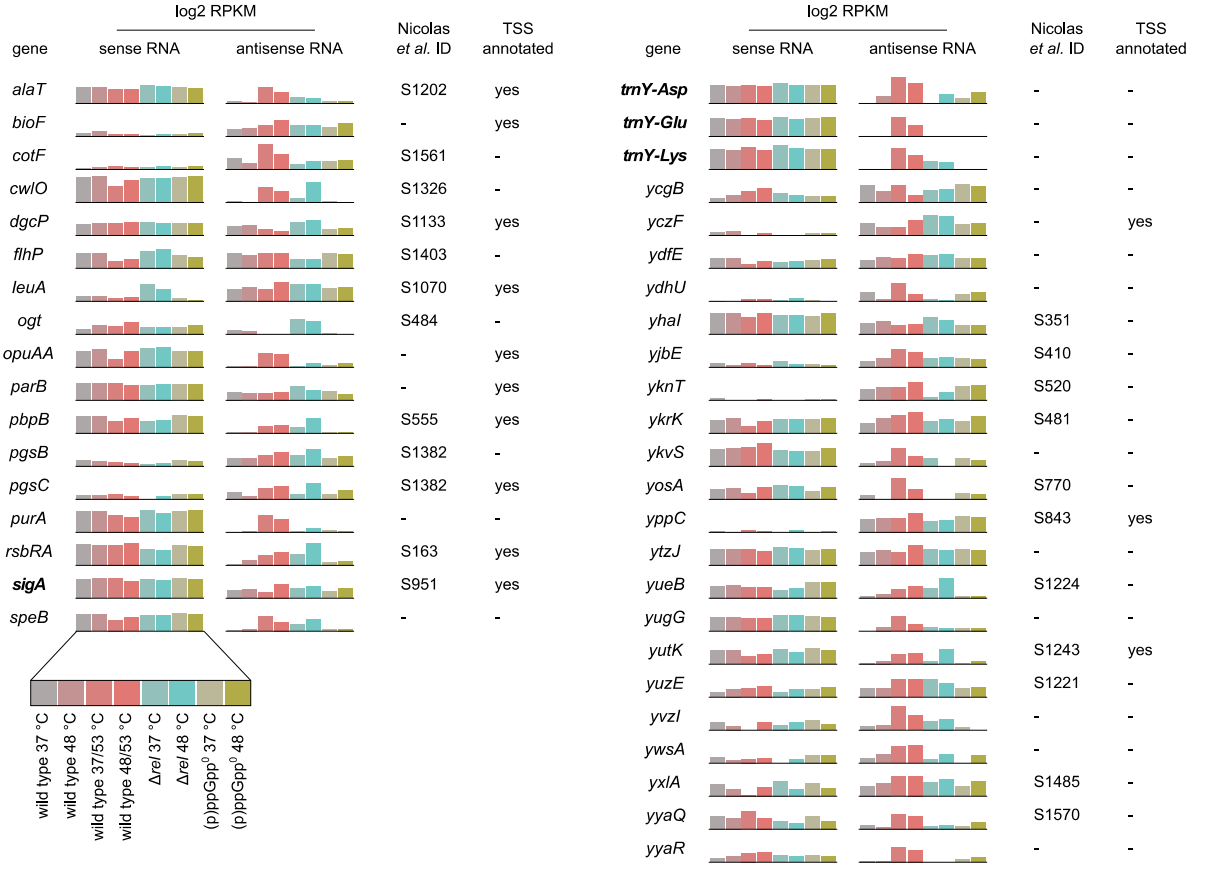

Figure 13 Expression of identified antisense RNA candidates

Expression changes of sense- and antisense-RNAs for selected genes. Bars represent expression (log_2 RPKM) of sense- and anti-sense transcripts in wild type and mutant cells treated with different heat shock conditions. Additional columns indicate the ID of the anti-sense RNA if it was also described in Nicolas et al (2012) and whether a transcription start site (TSS) could be annotated for the anti-sense RNA.

was increased about five-fold during thermotolerance (48/53 °C), indicating that the RNA is implicated in the response to stress (Figure 14). Concurrently, the abundance of the sense transcript was reduced about two-fold (Figure 14). However, the abundance of the asRNA was not regulated in the Δ*rel* or (p)ppGpp^0 strain (not shown).

A second stress induced asRNA candidate overlaps an operon encoding four tRNA genes as well as the *purA* gene, which encodes an adenyl-succinate synthetase of the purine biosynthesis pathway (Figure 15). Transcription of the asRNA is initiated from at least one promoter downstream of the tRNA operon, but a consensus sequence for any sigma factor could not be identified. Interestingly, considerable read coverage close to the promoter region of the asRNA candidate was already detected under un-stressed growth. However, the full antisense transcript was only detected during thermotolerance (48/53 °C, Figure 15). This unusual profile may indicate a regulation of the RNA based transcript termination/anti-termination. Severe heat stress, but not high or low (p)ppGpp levels resulted in strongly increased levels of the asRNA candidate, while the transcription of the sense transcripts was reduced five to ten-fold. Notably, this RNA was not described in Nicolas et al. (2012) [9].

In addition of the identification of putative *cis*-acting regulatory RNAs, it was also aimed to identify *trans*-acting RNAs. An automatic bioinformatics identification of such RNAs was not feasible, the set of TSS from section 2.3 was manually reviewed. This process resulted in the annotation of 34 transcripts, which are listed in Table 2. Notably, 26 of these 34 RNAs have been identified previously in the studies

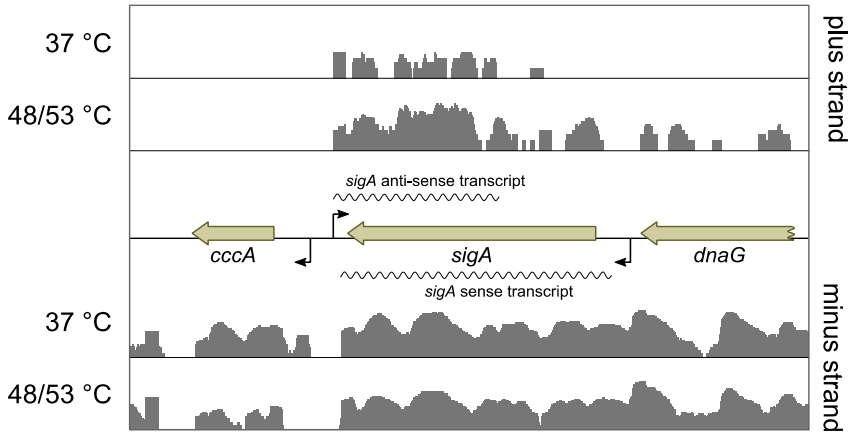

Figure 14: Transcription profile of a putative anti-*sigA* antisense RNA

Gray bars depict the log-scaled read coverage on the plus (top) or minus strand (bottom) during control (37 °C) and thermotolerance condition (48/53 °C). A schematic representation of the reading frames (bold arrows), promoters (small arrows) and transcripts (wobbly lines) is depicted in the center.

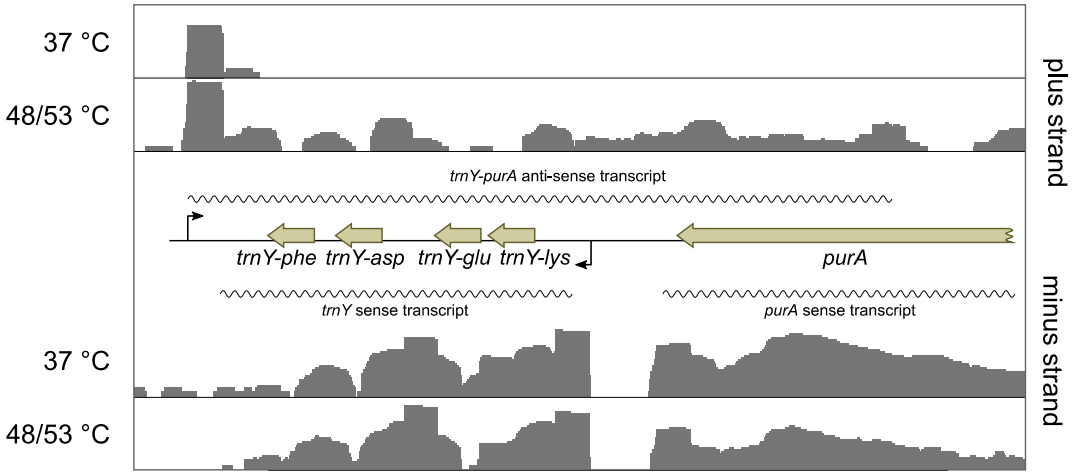

Figure 15: Transcription profile of a putative antisense RNA to the *trnY* operon.

Gray bars depict the log-scaled read coverage on the plus (top) or minus strand (bottom) during control (37 °C) and thermotolerance condition (48/53 °C). A schematic representation of the reading frames (bold arrows), promoters (small arrows) and transcripts (wobbly lines) is depicted in the center.

of Nicolas *et al.* (2012) or Irnov *et al.* (2010) [9], [296]. The vast majority of the *trans*-acting RNA candidates could be detected in all analyzed condition with no apparent regulation and only a small number was regulated during the different heat shock conditions or in the a Δ*rel* or (p)ppGpp^0 strains. Since most identified *trans*-acting RNA candidates are unregulated under the conditions tested and no apparent function could be deduced for these RNAs, no detailed analysis was performed.

Table 1: List of genes with identified anti-sense RNA candidates

The gene names, for which an anti-sense RNA candidate was predicted, are given. The identifiers of corresponding RNAs identified in Nicolas *et al.* (2012) are given in parentheses. [9]

trnY-Phe (na)	*lytE (S327)*	*yqhO (na)*	*yuxN (S1272)*
trnY-Asp (na)	*yheN (na)*	*yqgS (S931)*	*gerAC (S1275)*
trnY-Glu (na)	*yhaI (S351)*	*yqgB (S938)*	*opuBD (S1290)*
trnY-Lys (na)	*hpr (S352)*	*zur (S941)*	*opuBC (S1290)*
yaaC (S8)	*yhzF (S353)*	*sigA (S951)*	*yvbG (S1299)*
dck (S15)	*comK (S365)*	*antE (na)*	*sigL (S1310)*
yaaI (S16)	*yisI (S381)*	*holA (na)*	*yvdQ (S1315)*
yabE (S25)	*yisQ (S389)*	*sda (S965)*	*cwlO (S1326)*
gerD (S61)	*yisX (na)*	*yqeF (S969)*	*cypX (S1335)*
ybaN (S63)	*yjzB (S401)*	*yqdB (S976)*	*yvmA (S1338)*
rtpA (na)	*yjbE (S410)*	*yqaI (na)*	*yvlD (S1339)*
opuAA (na)	*yjbH (S416)*	*oatA (na)*	*yvkN (S1340)*
ycgA (na)	*yjdG (na)*	*yrhK (na)*	*yvzB (na)*
ycgB (na)	*yjdH (na)*	*yrzK (S1044)*	*ggaA (S1367)*
nasF (S120)	*yjfC (na)*	*leuA (S1070)*	*yvzI (na)*
nasE (S120)	*yjzI (na)*	*ytvI (S1102)*	*pgsC (S1382)*
tlpC (S125)	*guaD (S468)*	*ytzJ (na)*	*pgsB (S1382)*
yczF (na)	*ykzO (na)*	*nrnA (S1110)*	*ywsA (na)*
yczH (S133)	*ykzB (S475)*	*ytpI (S1113)*	*flhP (S1403)*
yczI (S135)	*sspD (S481)*	*braB (S1123)*	*ywnJ (na)*
yczJ (S136)	*ykrK (S481)*	*hisJ (S1127)*	*spoIIQ (S1406)*
ydzA (na)	*ogt (S484)*	*yttP (S1130)*	*ywnG (S1408)*
ydbN (na)	*mtnU (S486)*	*dgcP (S1133)*	*ywjG (S1440)*
ydbO (S154)	*ykvS (na)*	*ytzH (S1146)*	*speB (na)*
ydbP (S155)	*yknT (S520)*	*pbuO (S1149)*	*ywhB (S1454)*
ydcA (S161)	*ylaK (S539)*	*bioF (na)*	*bacA (S1459)*
ndoA (S163)	*ylzH (S553)*	*ytqB (na)*	*ywdH (na)*
rsbRA (S163)	*pbpB (S555)*	*ytoA (S1163)*	*sacT (S1466)*
ydfE (na)	*pelB (S696)*	*ytkA (na)*	*yxlH (S1484)*
ydhU (na)	*yoaM (S696)*	*ytjA (S1173)*	*yxlA (S1485)*
ydzT (na)	*yosA (S770)*	*cdoA (S1187)*	*yxzE (S1488)*
yefB (S240)	*yopS (S782)*	*alaT (S1202)*	*yxeE (S1521)*
yezA (S244)	*yoyJ (na)*	*yugG (na)*	*yxaH (S1535)*
yflL (S263)	*yonT (na)*	*yuzC (S1217)*	*yxnA (na)*
yfkT (S272)	*degR (S829)*	*yuzE (S1221)*	*yyzO (na)*
yfkS (S272)	*ypzG (S838)*	*yuzF (na)*	*purA (na)*
yfkR (S272)	*yppC (S843)*	*yueB (S1224)*	*dnaC (na)*
yfkF (S276)	*ypjB (na)*	*yutK (S1243)*	*cotF (S1561)*
sspK (S290)	*ypuF (na)*	*yuzD (na)*	*yyaR (na)*
yfhS (S298)	*ypzC (S884)*	*yutC (S1248)*	*yyaQ (S1570)*
spo0M (na)	*ansR (S895)*	*frlR (S1254)*	*yyaC (na)*
ygaJ (na)	*yqjU (S899)*	*sspG (S1255)*	*parB (na*
yhzB (S311)	*yqjB (S908)*	*yurS (S1255)*	

Table 2: Identified non-coding, *trans*-acting RNA candidates

a: The left and right boundary and the orientation is given relative to the BHS220 genome (*amyE::rrnJ*p1-*lacZ*) is given. **b:** the nearest annotated gene. **c:** Differential expression across the tested examined conditions in any heat-shock condition (heat) or by high/low (p)ppGpp levels in the mutant strains ((p)ppGpp). **d:** Corresponding IDs from Nicolas *et al.* (2012) (SXXX) or Irnov *et al*. (2010) (ncrXXX) [9], [296].

Start-end (strand)[a]	Near gene[b]	regulation[c]	Found in other publications?[d]	Comments
1,813-1,897 (-)	*dnaA*	none	-	
557,710-557,857 (-)	*yddR*	none	-	
563,671-563,821 (-)	*cspC*	none	ncr1566	
656,183-656,420 (-)	*groEL*	none	-	
824,878-825,051 (+)	*yfmG*	(p)ppGpp	ncr1566, S254	
977,136-977,217 (-)	*yhbF*	none	S313	
1,060,601-1,060,826 (+)	*yhaX*	none	S354	
1,075,938-1,076,180 (-)	*yhaJ*	none	S348	
1,154,686-1,155,483 (+)	*yisI*	none	S381	
1,208,137-1,208,395 (+)	*argF*	none	-	
1,237,641-1,237,757 (+)	*yizD*	(p)ppGpp	ncr629	
1,267,112-1,267,388 (-)	*yjcM*	Heat	S429	
1,283,247-1,283,663 (+)	*yjdJ*	none	S444	
1,361,662-1,362,044 (-)	*htrA*	heat	S481	
1,451,017-1,451,317 (+)	*ykzR*	none	S499, ncr721	
1,451,036-1,451,319 (-)	*ykzR*	none	S498	
1,457,580-1,457,766 (-)	*zosA*	none	ncr1755	
1,525,472-1,525,522 (-)	*ktrC*	none	-	
1,904,726-1,905,069 (-)	*yncF*	none	S641	
1,921,711-1,921,851 (+)	*lexA*	none	S659, ncr982	
1,942,126-1,942,663 (-)	*ynfC*	none	U1466	
2,001,960-2,002,267 (-)	*ppsA*	none	-	
2,058,203-2,058,811 (+)	*xynA*	none	S708, ncr1015	
2,212,785-2,213,024 (-)	*yopM*	none	S786	Prophage(SPß)
2,303,505-2,303,633 (+)	*ypiP*	none	-	
2,381,628-2,381,830 (-)	*trpE*	none	S857	
2,493,034-2,493,746 (+)	*yqjB*	heat	S908	Prophage (skin element)
2,521,802-2,521,953 (-)	*spo0A*	none	-	Probably encodes a small protein [297]
2,701,258-2,701,325 (+)	*yqaI*	none	Ncr1160	
2,751,836-2,751,980 (+)	*yraM*	(p)ppGpp	-	
2,777,979-2,778,101 (+)	*cypB*	none	Ncr1175	
3,229,922-3,230,043 (+)	*alaT*	heat	Ncr1241, S1202	
3,277,920-3,278,909 (+)	*yukB*	heat	S1225	
3,646,464-3,646,711 (+)	*xvzI*	none	S1369	

2.4.6 Methods

The methods described here refer only to the results of this section presented above.

Growth conditions. Strains are listed in Table 3. Cells were grown in baffled flasks in LB medium or Belitzkie minimal medium (BMM) [298] supplemented with 0.5 % (w/v) casamino acids (CAA) at 37 °C and 200. Exponential phase cells were treated to a one-step heat shock (15 min 50 °C) or the thermotolerance protocol (15 min 37 °C or 48 °C followed by 15 min 53 °C) in pre-heated water bathes or treated with diamide (1 mM) as described in section 2.1). Measurements of thermotolerance and thermoresistance have been described previously [50]. For amino acid starvation, cells grown in minimal medium supplemented with casamio acids were harvested by centrifugation for 5 min at 3,860 xg and resuspended in fresh minimal medium supplemented with 0.5 mg mL^{-1} DL-norvaline, but without casamino acids. The colony formation assay is described in section 2.1. Agar plates were supplemented with NaCl or diamide as indicated and incubated over night at different temperatures.

Cloning and strain construction. Molecular cloning using *E. coli* DH5α as host and transformation of *B. subtilis* was carried out according to standard procedures [6], [299]. Point mutations were introduced by overlap-extension PCR. Cells were selected on 100 µg mL^{-1} ampicillin, 1 µg mL^{-1} erythromycin, 25 µg mL^{-1} lincomycin or 100 µg mL^{-1} spectinomycin, when appropriate. Markerless point mutations were introduced using the pMAD system as established previously [300]. Plasmids and primers used for cloning are listed in Table 4 and Table 5.

Biochemical methods. Extraction and quantification of nucleotides was carried out as described in section 2.3. Preparation of subcellular protein aggregates is described in Runde *et al.* (2014) [50]. SDS-PAGE and western blotting was carried out according to standard procedures.

RT-qPCR. Extraction of total RNA and reverse transcription is described in section 2.1. Oligonucleotides used for the amplification of *hpf* are listed in section 2.3.

Bioinformatic analysis. The aligned reads of the RNA-seq dataset described in section 2.3 were counted using Python 3.6 and pysam. GNU R 3.5.1 was used for filtering and normalization of read counts and the generation of heat maps. Putative regulatory RNAs were also searched and reviewed manually using Integrated Genome Viewer (version 2.4.14) and the TSS of section 2.3.

Table 3: List of strains used in section 2.4

Name	Genotype	Contruction or reference
Wild type	*trpC2*	[6]
BHS126	*trpC2 rel::erm*	See section 2.3
BHS204	*trpC2 ywaC*^{E154V} *yjbM*^{E139V}	See section 2.3
BHS214	*trpC2 ywaC*^{E154V} *yjbM*^{E139V} *rel::erm*	See section 2.3
BHS709	*trpC2 rel* ^{E324V}	See section 2.3
BHS794	*trpC2 rel* ^{H420E}	Using pMAD-rel-H420E
BIH369	*lacA::Pxyl-yocM-mcherry erm*	[290]
BHS805	*lacA::Pxyl-yocM-mcherry erm amyE::Pxyl-sfGFP-rel spec*	pSG1729-rel in BIH369
BHS856	*trpC2 rplK* ^{P22R}	Using pMAD-rplK-P22R
BHS859	*trpC2 rplK::erm*	[301]
BNM143	*codY::erm*	[33]
BHS1046	*trpC2 rel* ^{E324V} *codY::erm*	*codY* in BHS709

Table 4: List of oligonucleotides used in section 2.4

Name	Sequence	purpose
286_codY_up_for	CGTTCATTCTTTCAACCAAAGC	Transformation of *codY::erm*
287_codY_do_rev	ATGACAGTCAGAGAAGCCAG	Transformation of *codY::erm*
rplK_P22R_for	GAAAAGCTAACCCAGCACGACCAGTTGGACCTGCAC	pMAD-rplK-P22R
rplK_P22R_rev	GTGCAGGTCCAACTGGTCGTGCTGGGTTAGCTTTTC	pMAD-rplK-P22R
rplK_up_for	ataGGATCC TAGACGGACCTTTTGCTAAC	pMAD-rplK-P22R
rplK_do_rev	ataGAATTC GTTAAAACTCGCAACCCGAC	pMAD-rplK-P22R
BsRelA-EcoRI-F	TTAAGAATTCATGGCGAACGAACAAG	pMAD-rel-H420E
BsRelA-Nco-R	TTAACCATGGTTAGTTCATGACGCGGCG	pMAD-rel-H420E
Bsrel-H420E-for	CTTACCGGATTGAATCTGAAATCGGC	pMAD-rel-H420E

Bsrel-H420E-rev	GCCGATTTCAGATTCAATCCGGTAAG	MAD-rel-H420E
rel_pSG1729_rev	atatGAATTC TTAGTTCATGACGCGGCGC	pSG1729-rel
rel_pSG1729_for	atatGGATCC AC GCGAACGAACAAGTATTGAC	pSG1729-rel

Table 5: List of Plasmids used in section 2.4

Name	Relevant genotype
pSG1729-rel	5'*amyE sfGFP-rel spec amyE* 3'
pMAD-rel H420E	*rel* ^{H420E} *erm*
pMAD-rplK P22R	*rplK* ^{P22R} *erm*

3. Concluding discussion

In this thesis, the role of the SR in the HSR of *B. subtilis* was examined. It could be demonstrated that the alarmone (p)ppGpp is rapidly synthesized in response to heat stress and other stress conditions. It was observed that Δ*rel* cells, which lack the only bifunctional alarmone hydrolase while the small synthetases SasA and SasB are still active, exhibit increased alarmone levels which conferred enhanced thermoresistance. In contrast, the (p)ppGpp^0 mutant strain displayed increased sensitivity to stress. The alarmones (p)ppGpp mediated pleiotropic transcriptional alterations and a reduction of the translation capacity during heat stress. Furthermore, the SR was also required for the formation of 100S disomes by controlling the synthesis of the required factor Hpf. Taken together, the data presented in the preceding sections clearly demonstrate that the SR is an important regulator of the HSR. In addition, it could be observed that Spx, a central transcription factor of the heat- and oxidative stress response, is also a negative regulator of rRNA transcription. In the following sections, these findings and their significance for the HSR will be discussed in more detail and a model of the role and interplay of Spx and (p)ppGpp during the heat shock response is being presented.

3.1 Transcriptional changes during the heat shock and stringent response

An initial microarray-based characterization of thermotolerance development in *B. subtilis* was carried out by Anja Heinz (presented in section 2.1) [288], [302]. The experiment revealed a broad up-regulation of stress-response genes together with a strong down-regulation of translation-related genes during thermotolerance conditions (48/53 °C), which was reminiscent of the typical regulatory pattern of the SR (section 2.1) [302]. To further characterize transcriptional changes during thermotolerance development and to assess the role of the SR in this process, RNA sequencing (RNA-seq) was employed in collaboration with the group of Prof. Petra Dersch (Helmholtz Centre for Infection Research, Braunschweig & Institute of Infectiology, University of Münster, Germany). Since the regulation of rRNA, which is very abundant and transcribed from multiple identical *rrn* operons, cannot be assessed by RNA-seq, a transcriptional reporter fusion of the *rrnJp1*promoter fused to *lacZ* was introduced into the *amyE* locus of all strains, as established in section 2.1.

3.1.1 Transcriptional regulations during the heat shock response

At all temperature upshifts, an extensive transcriptional up-regulation of the heat shock- and general stress response regulated by HrcA, CtsR, SigB and Spx (see also section 1.1) could be observed (section 2.1 Figure 1; 2.2, Figure 4, S8). The results of the RNA-seq experiment of the heat shock conditions mostly resemble the microarray data with regard to its key characteristics. The induction of heat shock gene expression was generally the highest in thermotolerant cells at 48/53 °C but could also be observed to a lesser extent at 48 °C. In contrast, the induction of heat shock gene expression was less strong at 37/53 °C and it should be noted that the sudden exposure to the harsh heat stress condition without prior priming may already be lethal for *B. subtilis* cells [50]. The rationale for using the different heat shock conditions (48 °C, 48/53 °C and 37/53 °C) was to characterize the transcriptional changes and molecular mechanisms underlying thermotolerance development, which was established using these temperatures [50], [288]. Furthermore, in the course of this thesis, it was found that many effects and phenotypes of Spx and (p)ppGpp (see below) can be best observed during a single temperature upshift to 50 °C. This temperature is slightly higher than the priming step at 48 °C and led to a more pronounced response in many assays, while still being non-lethal. Therefore, many experimental setups and readouts were assessed at both temperature regimes.

At 48 °C and 48/53 °C, an extensive up-regulation of the σ^B dependent general stress response could be observed (section 2.3 Figure S7) [113], [303]. A strain with a deletion of *sigB* displayed reduced growth at high temperatures; however a deletion of *sigB* has no impact on the development of thermotolerance under the tested conditions (section 2.1 Figure 1 & S1). In contrast, Runde *et al.* (2014) could demonstrate that Δ*spx* cells are severely impaired in thermotolerance development, suggesting that Spx is an important transcription factor of the HSR in *B. subtilis* [50].

To better understand the role and impact of Spx in the HSR, the transcriptional changes observed in the different heat shock conditions were compared to a microarray which aimed to define the Spx regulon by comparing Δ*clpX* cells (high levels of Spx, see section 1.2.4) with Δ*clpX*Δ*spx* cells. The analysis suggests that many heat-regulated genes are under Spx dependent transcriptional control (section 2.1 Figure 1,2) [62], [103]. Furthermore, the analysis also revealed that Spx is implicated in the transcriptional control of additional heat induced genes such as *htpG* or *ssrA*, for which the transcriptional

regulation was previously unknown (designated class IV; section 2.1 Figure 2). Taken together, Spx appears to be a central and critical transcriptional stress response transcription factor which controls a large sub-regulon of the HSR and is critical for thermotolerance and survival [50], [62], [103].

3.1.2 Regulation of translation related genes during stress

The strong up-regulation of stress response genes during heat stress is accompanied by a broad down-regulation of translation-related genes, which was initially observed in the microarrays (section 2.1 Figure 1). The comprehensive down-regulation of both rRNA and ribosomal protein (RP) genes during heat shock could then be confirmed by RNA-seq, northern blotting and RT-qPCR experiments (section 2.1 and 2.3) in various experimental setups and heat shock conditions. Similarly, different stress conditions such as oxidative stress or antibiotic treatment resulted in a similar decrease of rRNA expression (see section 2.1) [286], [287]. Down-regulation of rRNA and RP genes was particularly strong during thermotolerance (48/53 °C) (section 2.1 Figure 1 & section 2.3 Figure 4).

The transcriptional down-regulation of translation-related genes is likely to result in reduced ribosome biogenesis during stress. Since both rRNA and RP synthesis is costly and accounts for a large share of the total RNA and protein synthesis during exponential growth [304], [305], a main objective for the down-regulation of ribosome biogenesis could be the re-allocation of cellular resources and synthesis capacity to facilitate the rapid production and accumulation of heat shock proteins during stress. Furthermore, the down-regulation of ribosome biosynthesis could contribute to long-term adaptation to stress conditions and the establishment of dormant-like states by decreasing the cellular ribosome content [306], [307]. However, since transcriptional regulation of rRNA and RP genes is expected to result only in the reduction of newly synthesized ribosomes and does not affect the activity of existing ribosomes, this transcriptional response is probably not involved in the direct control of translation rates as a short-term response to proteotoxic stress, which will be discussed in detail in section 3.2. Nevertheless, the down-regulation of rRNA and RP genes may act complementary to such a translational response (section 3.2).

Furthermore, ribosome assembly is an intricate process and sensitive to perturbations by exposure to heat- and other proteotoxic stresses [308]. The maturation of ribosomal subunits was demonstrated to be impaired upon heat stress in *E. coli* and required the activity of the DnaKJE chaperone system [309].

In addition, ribosomal proteins can accumulate in large quantities and are prone to aggregation when not bound to rRNA scaffolds during perturbations of ribosome assembly. Therefore, a shutdown of ribosome synthesis could also serve to prevent the toxic accumulation damaged ribosomal subunits and -proteins.

3.1.3 (p)ppGpp dependent and independent heat-induced transcriptional alterations

The comprehensive down-regulation of rRNA and RP genes observed during heat stress was highly reminiscent of the transcriptional pattern of the SR elicited by amino acid starvation [214] and similar transcriptional alterations could be observed by RNA-seq in Δ*rel* cells, which exhibit increased (p)ppGpp levels, while the cellular concentration of GTP is decreased (section 2.3 Figure 3).

To assess the influence of (p)ppGpp on the heat-induced down-regulation of translation related genes, the transcriptional response of a (p)ppGpp^0 strain to heat stress was examined and compared to wild type cells using RNA-seq and RT-qPCR. The different experiments consistently confirm that the *rrnJp1-lacZ* promoter fusion was significantly less down-regulated in the (p)ppGpp^0 strain during heat stress, thus clearly demonstrating the participation of the SR in the heat-induced down-regulation of ribosomal genes (section 2.3 Figure 4, 5). However, the experiments also revealed a broad down-regulation of many RP genes which was similar in both wild type and (p)ppGpp^0 cells, suggesting that the SR is not essential for the transcriptional down-regulation of most translation-related genes during heat shock (section 2.3 Figure 4, S8).

In *B. subtilis*, stringent control of transcription is mediated indirectly via decreased GTP levels. Ribosomal promoters, which are regulated by the SR feature conserved guanosine residues at the +1 position of the TSS and the rate of transcription initiation is thought to be highly sensitive to the GTP concentration [215], [228]. Increasing GTP concentrations stabilize the open transcription initiation complex of GTP-sensitive promoters and thereby facilitate transcription initiation, while decreased GTP levels have the opposite effect [215], [228].

In contrast, GTP levels were only slightly decreased upon heat shock and during thermotolerance development compared to other stress- and starvation conditions examined (section 2.3 Figure S1). It appears likely that the short, transient (p)ppGpp accumulation observed during heat shock may be insufficient to mediate a strong decrease of GTP. These relatively small changes in the GTP level may

likely affect only certain rRNA promoters which are particularly sensitive to changes in the GTP level and could explain why the SR has only a limited role in the regulation of rRNA and RP genes during heat stress. In agreement with this notion no significant de-repression of the CodY-regulon, which is controlled by GTP-levels as well [310], was visible in the RNA-seq data upon heat stress (section 2.3 Figure 4 & S8). It is however tempting to speculate that the SR may have a more pronounced impact on transcriptional regulation during other stress condition which resulted in a more pronounced drop of the cellular GTP level, such as salt- or oxidative stress (section 2.3 Figure S1). Nevertheless, the strong down-regulation of RP genes observed in both wild type and (p)ppGpp^0 cells upon heat shock strongly suggests the existence of additional, (p)ppGpp-independent pathways and mechanisms for the transcriptional control of ribosomal genes during stress.

3.1.4 Spx-dependent, direct down-regulation of ribosomal promoters

Interestingly, a new activity of Spx characterized in this work may represent such an additional mechanism for the stress-dependent repression of translation related genes. During heat- and other stress conditions during which the reduction of rRNA and RP gene expression was observed, Spx levels are strongly induced and it was already demonstrated to play a central role in the HSR (see section 3.1.1) [50]. Spx has already been reported to interfere with various processes on the transcriptional level and inhibit for example motility, biofilm formation or sporulation [62], [122], [132]. A first hint that Spx could be also implicated in the regulation of translation related genes came from the observation, that many RP genes were generally down-regulated in the microarray comparing Δ*clpX* (Spx high) with Δ*clpX*Δ*spx* (no Spx) strains (section 2.1, Figure 2). More experiments using Spx^{DD}, an inducible and stable variant, revealed that the accumulation of Spx leads to a strongly decreased expression of rRNA and RP genes as well as a reduced growth rate (section 2.1, Figures 2, 3 & S6).

Spx belongs to a family of unusual transcriptional regulators which do not bind and recognize DNA sequences on its own. Spx was identified as an ‘anti-alpha’ factor since it can bind to αCTD and interfere with transcriptional activators. Furthermore, it was demonstrated to stimulate the transcription of certain genes by appropriation of RNAP on the promoter, which required interaction of the Spx·αCTD complex with sequence elements upstream of the promoter (see section 1.2.1) [103], [106], [109], [111], [122].

The results presented in section 2.1 suggest, that the down-regulation of rRNA and RP genes follows a direct mechanism which is different from the previously reported indirect down-regulation by interference [106], [122]. First, negative regulation of rRNA promoters could also be observed *in vitro* using purified Spx protein and RNAP, suggesting that the Spx dependent down-regulation is independent of additional factors. Furthermore, the promoters of rRNA operons are very conserved and do not require stimulation by transcriptional activators. However, these promoters are stimulated by conserved AT-rich upstream sequences (UP-elements), which are recognized by the αCTD subunit of RNAP [311]–[314]. Truncation or replacement of the UP elements resulted in decreased promoter activity but concurrently alleviated Spx-dependent repression (section 2.1, Figures 4). The available crystal structures of the Spx·αCTD complex suggest that Spx binds αCTD close to the residues which also interact with UP elements [107], [312], [315]. However, the down-regulation of rRNA promoters by Spx is probably not exerted by simple disruption of the interaction between αCTD and UP elements similar to the interference model, since this model cannot account for the observed promoter-specific dependence on Spx' redox state (section 2.1, Figures 3). Furthermore, a ChIP-Chip experiment by Rochat *et al.* (2012) suggests that the Spx·RNAP complex binds and recognizes certain sequence motifs in close proximity to rRNA promoters [62].

Notably, a V260A mutation in αCTD abolished down-regulation of rRNA promoters while still allowing up-regulation of stress response genes, indicating that different residues in the interaction surface of Spx and αCTD are required for Spx-dependent stimulation and repression of promoters (section 2.1, Figures 5). Thus, it appears that Spx can down-regulate ribosomal promoters by modulating RNAP via a novel, not yet understood mechanism, which requires sequence-specific contact of the Spx·αCTD complex with regions within or upstream of the promoter region. Notably, Spx may also interact with β or β' subunits of RNAP, suggesting a complex and intricate interaction between Spx and RNAP [110], [316].

Together, the experiments carried out *in vitro* and *in vivo* demonstrate that Spx is a *bona fide* negative regulator of rRNA promoters (section 2.1, Figures 3). Microarray and RT-qPCR experiments also indicate that the transcription of many RP genes can be negatively influenced by Spx. However, the regulation of RP genes was less strong *in vivo* compared to rRNA promoters (section 2.1, Figures 3) and the

tested *rpsD* transcript was not down-regulated by Spx *in vitro* (section 2.1, Figures 3) [62]. These observations indicate that Spx-dependent down-regulation concerns mainly rRNA promoters, while the promoters of RP genes are regulated to a lesser extent. Since the transcription of many RP genes is governed by auto-repression by their gene products, it is also tempting to speculate that some RP genes are regulated indirectly by lowering the availability of transcribed rRNA [317].

It is interesting to note that the concurrent activation of stress-response genes and down-regulation of translation related genes by Spx is reminiscent of the regulator SoxS in *E. coli*. Similar to Spx, SoxS binds αCTD, recognizes degenerate recognition sites in upstream regions of its target promoters and activates gene expression by appropriation of RNAP [318]. Concurrently, SoxS binding to αCTD mediates the repression of rRNA transcription by interfering with the recognition of UP elements, which appear to be of greater importance for efficient rRNA transcription in *E. coli* than in *B. subtilis* [306], [318].

3.1.5 Overlapping activities of Spx and (p)ppGpp

Different stress conditions which lead to the stabilization of Spx (see section 1.2.4) such as heat stress, oxidative stress or exposure to vancomycin, correlated with a strong down-regulation of the *rrnJ*p1-*lacZ* reporter and RP genes, while the transcription of *trxB* was increased. However, while the transcription of *trxB* was clearly Spx-dependent, the stress-induced down-regulation of *rrnJ*p1 and several tested RP genes was unchanged by the either presence or absence of Spx and the same both in wild type and Δ*spx* mutant cells (section 2.1 Figure 6 & S5).

The observation that the heat-mediated down-regulation of ribosomal promoters was for the most part unaffected by either (p)ppGpp^0 mutations (see above) or a Δ*spx* deletion gave rise to the hypothesis that both regulators may act concurrently on the promoters of translation related genes and that both regulators could compensate their mutual loss to a certain extent. To test this hypothesis, a strain in which both the (p)ppGpp^0 and the Δ*spx* mutations were combined, was constructed and examined. Strikingly, down-regulation of *rrnJ*p1 during heat stress, which was unchanged in a Δ*spx* deletion strain (section 2.1 Figure 6) and only partly impaired in (p)ppGpp^0 cells (section 2.3 Figure 4 & S8), was completely abolished in the (p)ppGpp^0 Δ*spx* deletion strain, in which both regulators were inactivated

(section 2.3 Figure 5). These results suggest, that rRNA promoters are controlled by the concurrent activity of both Spx and (p)ppGpp during stress (Figure 16 A).

It is interesting to note the parallels between Spx and DksA proteins, which are found in proteobacteria and regulate RNAP activity synergistically with (p)ppGpp, with respect to their activity as pleiotropic regulators of both ribosomal and stress response genes [218], [307]. However, unlike DksA, the function and regulation of Spx and (p)ppGpp appear to be independent of each other (section 2.3 Figure S10).

It should be noted that, while the regulation of *rrnJ*p1 appears to be solely dependent on (p)ppGpp and Spx during heat stress, the expression of most RP genes was still significantly down-regulated in the (p)ppGpp^0 Δ*spx* strain by heat stress (section 2.3 Figure S9), hinting towards the activity of even more regulatory mechanisms (Figure 16 B). It is conceivable that additional, so far unidentified transcriptional regulators could modulate the activity of the respective promoters to down-regulate the expression of RP genes during stress at the stage of transcription initiation. For example, the transcription activator Ifh1, which is essential for the activation of RP genes in *Saccharomyces cerevisiae*, was very recently demonstrated to be sensitive to protein aggregates in the nucleus and becomes rapidly inactivated by co-aggregation during proteotoxic stress, thus down-regulating RP gene expression. [319]. However, such additional transcriptional regulators are unknown in *B. subtilis*. Yet, the expression of RP genes is frequently auto-regulated by transcriptional anti-termination switches in many prokaryotes including *B. subtilis*. By binding to the leader regions of their own mRNA and triggering the termination of transcription, certain RPs can moonlight as transcriptional regulators and thus prevent excess production of RPs, when assembly of ribosomes is impaired during stress [317], [320], [321] (Figure 16 B). Furthermore, it is possible, that the transcription of RP genes could be modulated by the activity of *cis*- or *trans*-acting non-coding RNAs. For example, it was recently demonstrated that a σ^B controlled antisense-RNA repressed the transcription of the *rpsD* gene, encoding the ribosomal protein S4, during environmental stress [322] (Figure 16 C). However, additional RNA-based regulation of other RP genes is unknown and yet to be described (see also below). Additional experiments using reporter fusions to promoter fragments of different length could be employed to separate regulation of transcription initiation, and may aid the identification of additional *cis*-regulatory elements responsive to heat stress.

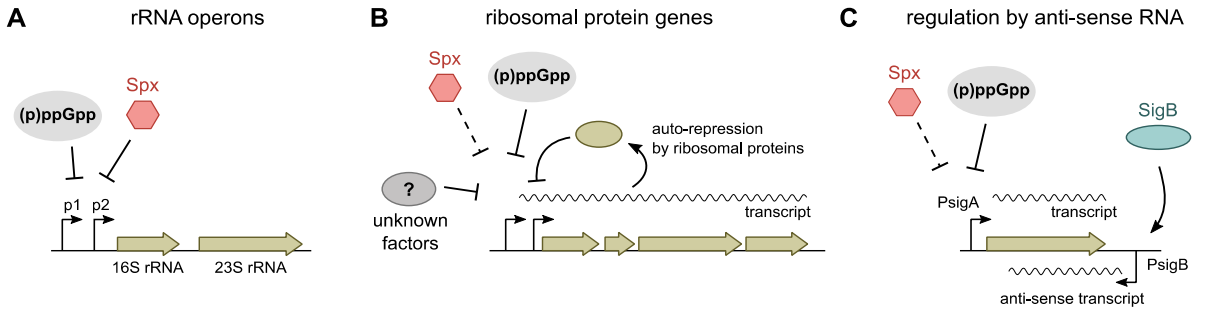

Figure 16: Down-regulation of rRNA and RP genes by Spx, (p)ppGpp and additional factors during heat stress.
(A) Transcription of the rRNA operon *rrnJ* is down-regulated by the concurrent action of (p)ppGpp and Spx during heat stress. (B) Many ribosomal protein (RP) genes are subject to down-regulation by (p)ppGpp and, to a lesser extent by Spx (see sections 2.1 &2.3). However, these genes continue to be down-regulated upon heat stress in the absence of both (p)ppGpp and Spx, indicating the activity of other, unknown transcriptional regulators. Furthermore, many RP operons are subject to transcriptional regulation via auto-repression mechanisms. For details, see text. (C) An interesting additional layer of regulation was described for the *rpsD* gene. During environmental stress, an antisense RNA is transcribed from a SigB dependent promoter and down-regulates transcription of the *rpsD* sense mRNA. In addition, the data presented in sections 2.1 &2.3 indicate, that *rpsD* is also subject to regulation via Spx and (p)ppGpp. For details, see text.

Interestingly, the (p)ppGpp^0 Δ*spx* mutant strain exhibits strongly impaired growth at 50 °C and accumulates large amounts of protein aggregates upon heat shock (section 2.3 Figures 5 & S9). These results further corroborate the functional interplay of Spx and (p)ppGpp during the heat shock response. However, a (p)ppGpp^0 *cxs*-2 strain, in which Spx-dependent down-regulation of rRNA is strongly impaired while still allowing the Spx-dependent up-regulation of stress-response genes (see sections 2.1 & 3.1.4), displayed much less impaired growth compared to a (p)ppGpp^0 *cxs*-1 strain in which the activity of Spx is fully abolished (section 2.3 Figure S9).

Thus, it appears that the up-regulation of unknown factors likely involved in proteostasis and stress response by either (p)ppGpp or Spx is important and required for efficient growth of *B. subtilis* during heat stress, while the lack of down-regulation of rRNA transcription, which is also fully abolished in a (p)ppGpp^0 *cxs*-2 strain, does not perturb growth at high temperature.

However, the (p)ppGpp^0 Δ*spx* strain also exhibits growth impairment at 37 °C in the absence of stress, while the detectable amount of cellular protein aggregates is not increased (section 2.3 Figure S9). While this growth defect in the absence of stress could also be attributed to impaired protein homeostasis, it is also tempting to speculate that the observed growth impairment during unstressed growth could be caused by the dys-regulation of additional cellular functions other than protein homeostasis. Further experiments, such as a global transcriptomic or proteomic approach using (p)ppGpp^0 *cxs*-1 and

(p)ppGpp^0 *cxs*-2 strains may give more insights into the functional and regulatory overlap of Spx and (p)ppGpp during stressed or unstressed growth.

3.1.6 Intersection of the stringent- and general stress response

Interestingly, the RNA-seq data revealed a correlation between (p)ppGpp levels and the general stress response. During unstressed growth at 37 °C, the expression of σ^B controlled general stress genes was found to be higher in Δ*rel* cells compared to wild type. In addition, the induction of the σ^B regulon in Δ*rel* cells was considerably higher upon heat shock at 48 °C than in wild type cells (section 2.3 Figure 4, S7, S8). Additional qPCR experiments from strains stressed at 50 °C confirmed this observation. Conversely, both basal and induced transcript levels of general stress genes were found to be lower than wild type in (p)ppGpp^0 cells (section 2.3 Figure 4, S7, S8). These experiments suggest a regulatory cross-talk between both stress response systems. It appears that activation of the SR also stimulates the general stress response to a certain extent. Nevertheless, the σ^B regulon was still stimulated by heat stress in the (p)ppGpp^0 strain, suggesting that (p)ppGpp is not essential for σ^B activity (see also section 1.1.3).

It can only be speculated about the interaction of both stress response systems. Exposure to decoyinine or mycophenolic acid, which inhibit GTP synthesis and elicit a drop of GTP levels similar to the SR *in vivo*, were reported to induce the σ^B-dependent general stress response in *B. subtilis*, suggesting that decreased GTP levels during the SR could mediate the activation σ^B [43], [90]. However, thorough investigations by Zhang and Haldenwang (2005) suggested, that the drop in the cellular GTP level *per se* is likely not a σ^B activating signal and that the activation of σ^B by decoyinine may be caused by secondary effects [90]. Furthermore, amino acid starvation stimulates (p)ppGpp synthesis and activates the SR but not the σ^B dependent general stress response [85], [92]. However, Rel-dependent activation of the general stress response was observed during amino acid starvation in *spo0A* or *spo0H* (encoding σ^H) mutants, suggesting that σ^H could compete with σ^B during stringent conditions [85], [92], [323].

A number of additional observations also suggest a genetic interaction between σ^B and the SR (see section 1.1.3). The presence of Rel has been reported to be essential for the activation of σ^B during phosphate or glucose starvation but not upon physical stress [92]. Interestingly, the requirement for Rel appears to be independent of its (p)ppGpp synthesis activity since missense mutations in *rel* or L11, which block the SR, could still support σ^B activation during nutritional stress [92]. Furthermore, L11

and Obg, which are both implicated in the SR, are also required in the activation of σ^B upon environmental stress [93]–[95]. Taken together, these observations strongly suggest a regulatory connection between the SR and the general stress response and emphasize the high degree of integration and interconnection between different stress response networks. However, more experiments are required to deduce the molecular details underlying this regulation.

The transcriptional regulation of the *hpf* gene represents another example for the close relationship between the stringent and general stress response. Transcription of *hpf* is stimulated by high (p)ppGpp levels during the SR, however it is also up-regulated during environmental stress as part of the general stress response [232], [249], [250]. Accordingly, the heat-induced expression of *hpf* was observed to be strongly dependent on the SR during heat stress (section 2.3 Figure 4).

It is interesting to note that the SR also positively regulates the general stress response in *E. coli* and related organisms by interfering with the intricate regulatory network which controls σ^S (RpoS) activity. First, (p)ppGpp and DksA are required for the efficient transcription of *rpoS* [324], [325]. Second, the long 5'-UTR of the *rpoS* mRNA forms an inhibitory secondary structure which blocks translation initiation. The UTR is targeted by several post-transcriptional regulators which promote or inhibit translation initiation and influence mRNA stability [326], [327]. One of them is the small regulatory RNA DsrA, which is transcriptionally up-regulated by (p)ppGpp/DksA. By binding to the 5'-UTR of the *rpoS* transcript, DsrA increases transcript stability and stimulates translation by inducing conformational changes that reveal the ribosome binding site [328]–[330]. In addition, the stability of the σ^S protein, which is subject to degradation by the ClpXP protease via the RssB adaptor protein, is increased by (p)ppGpp-dependent expression of the anti-adaptor proteins IraP and IraD [330]–[334]. Furthermore, (p)ppGpp influences sigma factor competition and contributes to the expression of the σ^{32} (RpoH) and σ^S regulons during stress in *E. coli* [275], [335].

3.1.7 The role of CodY in the stringent- and heat Shock reponse

Many transcriptional changes during the SR are mediated by CodY as a consequence of the decrease in cellular GTP levels [229], [310]. Accordingly, the CodY regulon was found to be strongly regulated in Δ*rel* cells, which display very low intracellular GTP levels (section 2.3 Figure 3, S7). In contrast, the CodY regulon was unregulated for the most part in the different heat shock conditions, where a transient

increase of (p)ppGpp but no pronounced decrease of GTP was observed (section 2.3 Figure S1). However, a weak de-repression of the CodY regulon could be detected upon a harsh heat shock (53 °C) together with an approximate two-fold decrease of cellular GTP levels after 15 min (section 2.3 Figure S1).

To further investigate the role of CodY during the heat shock response, *codY* mutant strains were tested for growth and survival during heat stress (section 2.4 Figure 12). The de-repression of the CodY regulon by deletion of *codY* was reported to rescue many phenotypes of a (p)ppGpp^0 strain, e.g. the ability to grow under nutrient-poor conditions [217]. Since a (p)ppGpp^0 Δ*codY* strain could not be created due to incompatible resistance cassettes, Δ*codY* was instead introduced in the *rel*^{E324V} strain, which displays heat sensitive phenotypes similar to the (p)ppGpp^0 strain due to the inactivated synthetase activity of Rel (section 2.3 Figure 2). However, a Δ*codY* deletion was unable to rescue the increased heat sensitivity of the *rel*^{E324V} strain and a deletion of *codY* in wild type cells did not increase heat stress resistance (section 2.4 Figure 12).

Together, these results suggest that CodY has only a minor role in the in transcriptional regulation and survival during the heat shock response, at least under the tested conditions. Furthermore, while the discussion in section 3.3 (see below) suggest, that *B. subtilis* cells may exhibit partial amino acid starvation when exposed to heat- or other environmental stress, the finding that Δ*codY* was unable to rescue *rel*^{E324V} indicates that the expression of CodY controlled amino acid synthesis pathways is not limiting for growth during heat stress [217].

The availability of certain amino acids and the expression of the CodY regulon may have a more pronounced impact during other stress conditions, in which stronger (p)ppGpp synthesis accompanied by a reduction of the GTP pool could be observed (section 2.3 Figure 1). For example, a transient reduction of certain amino acid pools was reported during diamide treatment in *B. subtilis* (see also section 3.3) [284], which may raise the requirement for the expression of the CodY regulon.

Interestingly, CodY regulates the expression of stress-response genes such as *spx*, *trxAB* or the *ctsR-clpC* operon in *Enterococcus faecalis*. which were found to be under positive stringent control, supporting the notion that the SR is interconnected with other key regulators and processes [336], [337].

3.1.8 Towards the identification of new non-coding RNAs

The high resolution and sensitivity of the RNA-seq experiment allows the identification of non-coding *trans*-acting RNAs and antisense RNAs. As a model organism, *B. subtilis* has been subject to extensive investigation of this matter [9], [296]. Consequently, the majority of identified features could be matched with previously published datasets (section 2.3 Figure S5 and section 2.4 Figure 13, Table 1 & Table 2).

Different approaches to identify new RNA-based regulatory systems were employed. By analyzing TAP-treated libraries in which primary and secondary 5' ends of RNA molecules are enriched, TSS can be identified with high accuracy and sensitivity. Therefore, the analysis of TSS is a very sensitive approach for the identification of new regulatory RNAs [293]. In total, 1823 TSS could be identified in at least one of the examined conditions (section 2.3 Figure S5). By classification of TSS according to their position and orientation relative to near genes, the majority of TSS could be associated with the transcription of mRNA or rRNA operons, while the remaining TSS are indicative for non-coding, *cis*- or *trans*-acting regulatory RNAs (section 2.3 Figure S5, section 2.4.5). In a second approach, all sequencing reads were filtered for reads which mapped to the reverse complement of coding regions, which allows a relative quantification of putative antisense RNAs (section 2.4.5, Figure 13).

Interestingly, many putative antisense RNAs appear to be up-regulated upon heat shock, especially at 48/53 °C, indicating that the heat shock response interferes with different processes via regulatory RNAs (section 2.4.5, Figure 13). In agreement with this finding, the general stress sigma factor σ^B was over-represented in the TSS of putative antisense RNAs (section 2.3 Figure S5). Two examples of particular interest for the subject of this work are putative antisense RNAs overlapping an operon of four tRNA genes or the *sigA* coding region (section 2.4.5 Figure 14, Figure 15). However, while the latter RNA was also identified in the work of Nicolas *et al*. (2012), a functional characterization of these RNAs is not available [9]. Nevertheless, it is tempting to speculate that both RNAs may contribute to the global transcriptional down-regulation of the translation apparatus and other processes during heat stress discussed above. Clearly, more experiments are required to characterize of the newly identified antisense RNA candidates and their role in the heat shock response.

A very similar and interesting example for RNA-based down-regulation of translation-related genes is the recent identification of an antisense RNA to the *rpsD* gene encoding the ribosomal protein S4. The σ^B dependent antisense RNA S1136-S1134 overlaps with the *rpsD* sense mRNA and cause its transcriptional down-regulation during stress, e.g. exposure to ethanol, which results in the decreased formation of 30S ribosomal particles [9], [322].

A manual review of TSS revealed 35 additional non-coding RNA candidates with no apparent *cis*-regulatory target (section 2.4.5 Table 2). These RNAs may therefore represent *trans*-acting regulatory RNAs. However, a recently published proteomic study suggests that at least some of the RNAs identified here encode previously unidentified small peptides [297]. Additional experiments are required to validate the putative regulatory RNAs and to investigate their function in the heat shock response.

3.2 (p)ppGpp dependent control of translation

3.2.1 Curbing protein synthesis as a part of protein quality control

The experiments presented in this thesis suggest that the (p)ppGpp attenuates translation rates during heat stress. Translation rates were generally higher that wild type in (p)ppGpp^0 cells during heat stress, while Δ*rel* cells exhibited reduced translation under stress- and non-stress conditions (section 2.3, Figure 6). (p)ppGpp is well-known as a potent inhibitor of translation by directly modulating many GTP-dependent steps during translation initiation, elongation and termination/ribosome recycling (see section 1.3.5) [239], [240]. Nascent, unfolded proteins emerging from the ribosome are particularly susceptible to misfolding and aggregation during proteotoxic stress [22]. Therefore curbing the rate of protein synthesis may support the maintenance of cellular protein homeostasis under stress by reducing the burden of the protein quality control system [21], [338]. In addition, the alarmone-mediated reduced translation rate may increase the translation accuracy and thus contribute to protein homeostasis by reducing mistranslation and the accumulation erroneous peptides [339]. Since (p)ppGpp can act immediately on protein synthesis without requiring the synthesis of additional factors, the transient (p)ppGpp-dependent decrease of translation may represent a fast acting primary response to stress which could be activated within a few seconds [181], [270]. In accordance, reduced accumulation of protein aggregates was

observed in Δ*rel* strains upon heat shock, while (p)ppGpp^0 cells exhibited increased aggregate accumulation (section 2.3, Figure 2).

Notably, it is also conceivable that the positive influence of (p)ppGpp on protein homeostasis (section 2.3, Figure 2) could be e.g. the result of increased chaperone synthesis. However, the transcriptomic experiments (section 2.3 Figure S7) and western blots (section 2.4 Figure 10) suggest that the up-regulation of chaperones during the heat shock response is mostly independent of the SR.

Interestingly, a similar, stress-dependent signaling system which mediates global reduction of translation during stress known as the "unfolded protein response" is well-conserved in eukaryotic organisms. Protein folding stress in the lumen of the endoplasmic reticulum activates three independent signaling branches which mediate a transcriptional response to increase the protein folding capacity [340]. Concurrently, the protein folding load is reduced by phosphorylation of a conserved serine residue of the eukaryotic translation initiation factor 2 alpha subunit (eIF2α) via the protein kinase PERK. The modification of eIF2α causes a global attenuation of CAP-dependent initiation of protein synthesis, while selected genes which rely alternative initiation mechanisms such as the global transcriptional regulator of stress response ATF4, are preferentially translated [338], [340]. In mammals, three additional protein kinases integrate many different stress- and starvation conditions and activate a common adaptive response pathway by eIF2α phosphorylation, the "integrated stress response" [338], [341].

Transient attenuation of translation during stress was also observed in other bacteria, although the molecular basis for these responses are not well-studied [273], [305]. In the light of the results obtained here, it is tempting to speculate that the SR could be a conserved regulator of translation rates in prokaryotes during stress.

Interestingly, a Δ*rplK* strain, in which translation rates are decreased due to the lack of the non-essential ribosomal protein L11, exhibited increased thermoresistance similar to Δ*rel* cells (Figure 10). Notably, the level of chaperones such as GroEL were still increased upon heat stress in both Δ*rplK* and Δ*rel* cells, indicating that the reduced translation rates in these strains are still sufficient to promote a HSR (Figure 10). The deletion of non-essential ribosomal proteins was also demonstrated to enhance the folding of heterologous proteins by decreasing translation rates in yeast [342] and an activation of the SR by serine hydroxamate or inhibition of translation by antibiotics conferred increased salt

tolerance in *E. coli* [343], [344] or *B. subtilis* [290]. Together, these observations indicate that both (p)ppGpp-dependent and –independent reduction of translation rates *per se* could be sufficient to facilitate protein homeostasis during stress conditions. Therefore, reducing translation rates during stress may represent an important aspect of the SR during proteotoxic stress, which appears to be crucial for adaptation and survival (section 2.3, Figure 6).

However, a complete inhibition of protein synthesis by chloramphenicol or high concentrations of DL-norvaline abolished accumulation of chaperones such as GroEL and had a detrimental effect on protein homeostasis during heat stress (Figure 10, Figure 11). These observations indicate that residual translation capacity, which is probably required for the synthesis of molecular chaperones, is important for the survival of heat stress. Interestingly, in a similar experiment, complete inhibition of translation resulted in a reduction of protein aggregates in salt-stressed *B. subtilis* cells [290]. Thus, heat- and salt stress may lead to different perturbations of protein homeostasis and cellular physiology with distinct requirements for chaperones levels and translation [290].

Given the requirement for active translation of heat shock proteins during heat stress, it is tempting to speculate that a preferential translation of stress-related mRNAs could take place to ensure the timely accumulation of chaperones and other stress-related proteins under adverse conditions that limit translation rates, for example by specialized ribosomes which translate only a subset of stress-related mRNAs. However, a conserved mechanism for selective translation of certain mRNAs similar to eIF2α phosphorylation known in eukaryotes is unknown in bacteria. It was reported that the toxin-antitoxin system MazEF could establish a pool of modified ribosomes by cleaving the 3' end of the 16S rRNA which comprises the anti-Shine-Dalgarno sequence. These modified ribosomes were proposed to selectively translate leaderless mRNAs during stress, which are also thought to be generated by MazF [345]. However, a global transcriptomic analysis suggests that the endonuclease MazF cleaves most transcripts at multiple sites and does not generate a pool of specialized ribosomes [346], [347], but this topic is still under active debate [348]–[351]. Sequence variations encoded in different copies of the rDNA operons may also establish heterogeneous ribosome populations with modulated functions under certain conditions [352]. Differential expression of rDNA operons during stress was suggested to modulate RpoS-mediated stress response, motility and biofilm formation in *E. coli* [353]. In *B. subtilis*, deletion of

multiple rDNA operons resulted in decreased sporulation frequency [354]. Clearly, more experiments are required to investigate the structural and functional heterogeneity of bacterial ribosomes and the implications for development and stress response [352], [355].

3.2.2 The stringent response protects ribosomes from stress-induced damage

Second to the control of translation and its implications for protein homeostasis discussed above, (p)ppGpp appears to have a protective role on the translational apparatus itself during heat stress. In (p)ppGpp^0 cells severely heat shocked at 53 °C, a strong reduction of the 16S rRNA could be observed (section 2.3, Figure S12), which may indicate a severe damage and disintegration of the small ribosomal subunit. Accordingly, translation in (p)ppGpp^0 cells was strongly reduced during severe heat stress and virtually abolished after 15 min, while translation was still observed in similarly treated wild type cells (section 2.3, Figure S12). Furthermore, only a small reduction in the intensity of the 16S rRNA was observed in wild type cell, while the rRNA was completely stabilized in Δ*rel* cells (section 2.3, Figure S12). Notably, the large ribosomal subunit appeared to be much more stable under the conditions tested. Together, these observations indicate, that (p)ppGpp is required for the stability of the small ribosomal subunit.

The exact mechanism how heat stress might impose such damage to the small ribosomal subunit is unknown. However, lethal heat stress was reported to cause a similar lesions in the 16S rRNA in *Staphylococcus aureus* and *Salmonella enterica*, indicating that these heat-induced alterations are a more general phenomenon not limited to a specific species [356]–[358]. Interestingly, supplementation of Mg^{2+}, which is known to stabilize the association of ribosomal subunits *in vitro*, strongly increased heat resistance of *Salmonella* cells and protected 16S rRNA from heat induced damage *in vivo* [358], [359]. Experiments with chloramphenicol- treated *S. aureus* cells during recovery from severe heat stress indicate that the *de novo* synthesis of 16S rRNA is sufficient for the assembly of new 30S particles after stress, while protein synthesis was not required, indicating that only the rRNA is damaged during stress while the RPs can be recycled [356], [360].

It can only be speculated how the SR protects the ribosome from damage during stress. Apparently, the alarmones stabilizes the 30S subunit in a concentration dependent manner during heat stress. Since a negative impact of the SR on translation rates during heat stress was already established (see preceding

section 3.2.1), it appears to be reasonable that unrestrained translation in cells lacking the SR could be the cause of the observed damage and the target for the protective effect of (p)ppGpp. However, the increased sensitivity of the 16S rRNA to severe heat stress in (p)ppGpp^0 cells could not be rescued by inhibition of translation via chloramphenicol or spectinomycin (section 2.3, Figure S12), indicating that a simple reduction of translation rates is not sufficient for ribosome protection. Thus, it is likely that the foundation for the protective phenotype reported here could be a specific modulation of the ribosome or a ribosome-associated enzyme activity by (p)ppGpp, which could for example control the assembly of the ribosomal subunits or regulate other processes [244], [361].

3.2.3 The role of Hpf in the heat shock response

Interestingly, (p)ppGpp is also implicated in the formation of 100S disomes, which may constitute an additional mechanism for ribosome protection and translation control during stress and starvation [252]. The translationally inactive ribosome-dimers are formed by the Hpf protein which features on its C-terminus a dimerization domain, while each N-terminus binds to the 30S subunit of a different 70S particle, yielding a 100S particle (see section 2.2). The binding site of Hpf on the ribosome overlaps with the A-site and P-site and occludes the binding of tRNA and mRNA (section 2.2), thereby interfering with protein synthesis [362], [363].

In the course this thesis, the levels of the Hpf protein and its cognate *hpf* mRNA were found to be versatile reporters for the SR (see sections 2.3 & 2.4.1). During heat stress, Hpf rapidly accumulated in wild type cells, but not in the (p)ppGpp^0 strain. In contrast, large amounts of Hpf could be detected in Δ*rel* cells even in the absence of stress (Figure 8).

During thermotolerance development, a pronounced peak depicting the formation of 100S particles could be observed in wild type- but not in Δ*hpf* cells. In accordance with the (p)ppGpp dependent regulation of Hpf, the formation of 100S disomes was also strongly reduced in (p)ppGpp^0 mutant while Δ*rel* cells exhibites considerable 100S formation already during non-stress conditions (section 2.3, Figure 6). The formation of inactive 100S particles may support the attenuation of translation and thereby contribute to protein homeostasis (see section 3.2.1). However, the contribution of Hpf to the arrest of translation and protein folding is yet to be determined.

In addition, Hpf-mediated formation of disomes could also protect the translation apparatus from stress-induced damage and the loss of 100S formation in (p)ppGpp^0 cells may be accountable for the increased damage of the small ribosomal subunit in this mutant strain (see section 2.3 and 3.2.2). To test this hypothesis, a (p)ppGpp^0 strain expressing *hpf in trans* was examined with regard to stress induced damage of the 16S rRNA. However, neither the absence of Hpf nor its overexpression had any effect on the damage of the small ribosomal subunit during severe heat stress (section 2.3, Figure S12). In addition, overexpression of *hpf* could not complement the heat-sensitive growth phenotype and conferred only very little growth improvement at 55 °C (section 2.3, Figure S12). Accordingly, no growth phenotype was observed for Δ*hpf* cells in the same essay (not shown).

In summary, a clearly (p)ppGpp dependent formation of 100S formation mediated by Hpf could be observed during thermotolerance development, but no phenotype was associated with the Δ*hpf* mutant and only limited increase of viability was observed upon *hpf* overexpression. However, it was frequently observed that multiple, partially redundant stress response systems may complement each other to a limited extent such that only strains with knockouts of multiple systems exhibit severe phenotypes [364], [365]. Thus, it is conceivable that the loss of Hpf could be buffered or complemented by other protective mechanisms.

While no phenotype for *hpf* overexpression or deletion could be observed in this work, Höper *et al.* (2005) and Reder *et al.* (2012) reported a stress sensitive phenotype of the Δ*hpf* (*yvyD*) mutant towards low temperatures, heat stress and oxidative stress during a screen of general stress genes. In addition, impaired thermotolerance development of Δ*hpf* cells was observed (Noël Molière, unpublished). Hpf has been associated with long-term survival and the adaptation to starvation. Δ*hpf* cells exhibit severely impaired viability during starvation accompanied by degradation of intact ribosomes. In addition, Δ*hpf* cells exhibit a long lag-phase during outgrowth after starvation [251], [362]. Thus, it is tempting to speculate that the significance of Hpf for the heat shock response may become more apparent during prolonged stress exposition or even during recovery from heat stress.

Interestingly, Hpf mediates 100S formation and translational arrest *in vitro*, while overexpression of *hpf in vivo* does not lead to growth arrest and significant inhibition of translation, although 100S formation can be observed (section 2.3) [250], [254], [258]. It is possible that the high number of ribosomes

during exponential growth provide excess translational capacity, which does not become growth-limiting when a subpopulation of ribosome is titrated into 100S disomes by Hpf [362]. However, this observation could also indicate the presence of additional regulatory factors, which post-translationally limit or regulate 100S formation *in vivo*. One such factor may be (p)ppGpp, which is already known to modulate translation initiation and ribosome assembly. Furthermore, Hpf was found to interact with ClpC·MecA in a pull-down experiment, suggesting that ClpC could also influence 100S formation (Janine Kirstein, unpublished). A co-sedimentation experiment indicated, that binding of Hpf to ribosomes is independent of heat stress or high (p)ppGpp levels during the SR *in vivo* (section 2.2). In addition, co-sedimentation of Hpf with ribosomes was not altered in a *clpC* deletion strain (not shown). Additional experiments, such as the analysis of 100S formation by sucrose gradient centrifugation, may contribute to the understanding of the role of these factors in the post-translational regulation of disome formation. For examples, the GTPase HflX has recently been identified to be involved in the disassembly of hibernating 100S ribosomes in *S. aureus* [258]. HflX belongs to the conserved family of small ribosome-associated GTPases and was previously implicated in the disassembly of 70S ribosomes [245]. Disassembly of 100S particles *in vitro* by HflX required the cleavage of GTP and could be inhibited by (p)ppGpp, thereby supporting the idea that (p)ppGpp implicated in the post-translational regulation of Hpf-dependent ribosome dimerization [244], [258].

3.3 Activation of the Stringent Response

The initial observation of increased (p)ppGpp levels upon heat shock, salt- or oxidative stress (section 2.3 Figure 1) raised many questions regarding the stress sensing, signal-transduction and SR activation during stress, some of which could not be answered conclusively. In particular:

(i) Is there a common signal which activates the SR upon heat, salt and oxidative stress?

(ii) How are these stress signal(s) sensed and what are their sensor(s)?

(iii) What is the pathway which triggers the synthesis of (p)ppGpp?

Interestingly, activation of the SR upon a plethora of stress stimuli such as heat stress, oxidative stress and treatment with alcohols, cytostatic quionones or heavy metal salts appears to be common in both Gram-positive and –negative bacteria such as *E. coli*, *S. enterica* or *B. subtilis* (see also section

1.3.7) [270], [271], [273], [274]. Given the multitude of adverse conditions which activate the SR, it appears likely that different stress stimuli are integrated by one or multiple stress sensors which then elicit a shared signal that triggers the activation of the SR. Under the assumption that the regulatory networks which activate the SR in response to these stresses have not evolved independently in the different organisms, these observations also argue for a common, conserved activation mechanism.

3.3.1 Potential mechanisms for the regulation of Rel during stress

Several pathways are conceivable by which environmental stress signals could lead to the activation of Rel. First, it is a tempting hypothesis that Rel is not only the effector which mediates the SR during stress but also the direct sensor of environmental stress conditions. Homologs of Rel and RelA were reported to have a high propensity to form aggregates *in vitro* [178], [366]. In addition, untagged Rel was found to be enriched in the aggregated protein fraction upon heat stress by western blotting (Ingo Hantke, personal communication) and the co-localization of the GFP-Rel fusion protein with YocM-mCherry labelled protein aggregates was observed by fluorescence microscopy (Figure 9).

Based on these observations, an initial model was developed by which Rel is an unstable and aggregation-prone protein and could thus a direct sensor of perturbed protein homeostasis, which may be a common consequence of many environmental stress conditions. During such stress, Rel could become inactivated by aggregation, thereby creating a phenotype that partially resembles Δ*rel* cells, which exhibit increased alarmone levels synthesized by the SAS enzymes and high thermoresistance (see section 2.3).

However, it could be demonstrated that Rel is the main source of (p)ppGpp during heat stress (section 2.3 Figure 1). Furthermore, GFP-Rel does not localize to large subcellular protein aggregates formed upon the addition of puromycin, which could be visualized by YocM-mCherry (Ingo Hantke, personal communication), suggesting that perturbed protein homeostasis *per se* does not provoke the aggregation of Rel. Together, these results were inconsistent with the model of Rel being an aggregation prone stress sensor. Instead, the observation that (p)ppGpp accumulation was abolished in *rel* ^{E324V} mutant cells clearly demonstrates that the alarmone synthetase activity of Rel is critical for (p)ppGpp accumulation upon stress (section 2.3 Figure 1). The transcription of the small alarmone synthetase *sasA* was found to be slightly up-regulated by heat stress. Nevertheless, the two SAS proteins appear to be dispensable

for both (p)ppGpp synthesis and survival during heat stress, indicating that their role in the HSR is negligible (section 2.3 Figure 1 & S1).

It may also be possible that different stress stimuli are intrinsically sensed by Rel and elicit moderate conformational changes which do not fully unfold and inactivate the enzyme but rather relieve the auto-inhibition of the synthetase activity and thereby stimulate (p)ppGpp synthesis. In line with this hypothesis it was reported that *E. coli* RelA can be activated *in vitro* to some extent by 20 % methanol in the absence of ribosomes [366]. However, additional data, such as the observation that the activation of Rel by heat stress can be modulated by inhibiting translation with chloramphenicol (section 2.3 Figure 1 and 2.4.2 Figure 9) suggests a different model (see below).

In *B. subtilis*, it was recently reported that the late competence protein ComGA modulates the SR by interaction with Rel and inhibition of its alarmone-hydrolase activity during the K-state [205]. In addition, an increasing number other protein factors or small molecules have been reported to modulate (p)ppGpp synthesis and hydrolysis by interaction with RSH or SAS proteins in response to different stress- and starvation signals in various species (see section 1.3.3). In the light of these observations, it is also an intriguing hypothesis that certain protein factors other than Rel may act as stress sensors and stimulate alarmone synthesis by direct interaction with Rel. Interestingly, an interaction between DksA, an RNAP-binding protein essential for direct, stringent transcriptional changes by (p)ppGpp in Proteobacteria [330], with the chaperone DnaJ was recently described in *Salmonella enterica* [367]. While it is tempting to speculate that Rel could be stimulated or otherwise modulated by interaction with chaperones or other proteins during stress, such interactions are yet unknown.

Instead, a number of observations suggest that during environmental stress, Rel may be activated by a mechanism similar as during amino acid deprivation (see section 2.3).

First, it could be demonstrated that inhibition of translation by chloramphenicol abolished accumulation of (p)ppGpp both during amino acid starvation as well as heat- and oxidative stress, suggesting that Rel activation during heat stress involves the ribosome and takes place in a pathway similar to that of amino acid starvation (section 2.3, Figure 1). Correspondingly, GFP-Rel was observed to form similar chloramphenicol-sensitive foci during both amino acid deprivation and heat stress (Figure 9),

A secondary limitation for certain amino acids may actually be an important signal for SR activation under such stress conditions. In the literature, it was first noted by Gallant *et al.* (1977), Lemaux *et al.* (1978) and others that the accumulation of (p)ppGpp in heat-treated *E. coli* strains could be eliminated by the supplementation of amino acids, particularly valine, leucine, isoleucine, serine and lysine [271], [272], [368]. Similarly, when tracking the *in vivo* diffusion behavior of single RelA molecules, English *et al.* (2011) noted the similar RelA diffusion patterns during amino acid starvation and heat shock, which correspond to its free or ribosome-bound states, suggesting that both stimuli provoke similar changes in the association of RelA to ribosomes [186].

3.3.2 Uncharged tRNA as a signal for the SR during environmental stress

To investigate the activation of Rel in more detail, mutant strains were constructed and analyzed which interfere with the activation of the SR at different steps. A mutation in histidine 420 of Rel (*rel* ^{H420E}) blocks (p)ppGpp synthesis upon amino acid starvation by abrogating the interaction of Rel with tRNA, while a mutation in the N-terminus of L11 (*rplK* ^{P22R}) impairs with the activation of Rel on the ribosome. Both mutations are sufficient to abolish a SR in response to amino acid starvation (Figure 7 A). Interestingly, (p)ppGpp accumulation was completely suppressed in the *rel* ^{H420E} strain during heat- or oxidative stress. (Figure 7 B). Likewise, this mutant strain exhibited impaired up-regulation of *hpf* and displayed a temperature-sensitive growth phenotype (Figure 8). Together, these results strongly support the notion that heat stress activates alarmone synthesis by Rel via the same "starvation-pathway" as during amino acid limitation. In contrast, the *rplK* ^{P22R} strain exhibited a strongly impaired response to amino acid starvation but was unimpaired in alarmone synthesis during stress (Figure 7 A, B) and was less sensitive to high temperatures compared to *rel* ^{E324V} or *rel* ^{H420E} strains (Figure 8 C). These observations contradict the hypothesis that both amino acids and heat stress activate Rel via a shared pathway. However, the *rplK* ^{P22R} mutation may still allow limited activation of the SR or influence Hpf levels independently of the SR. Together, these observations strongly suggest that Rel requires interaction with tRNA and possibly the ribosome for its activation by heat- or oxidative stress.

How could heat- and other environmental stress lead to amino acid shortage or otherwise activate the starvation-pathway of the SR? First, such stresses may provoke a drop in the cellular concentrations of certain amino acids by interfering with their uptake [271], [368] or biosynthesis (Figure 17 A, B). For

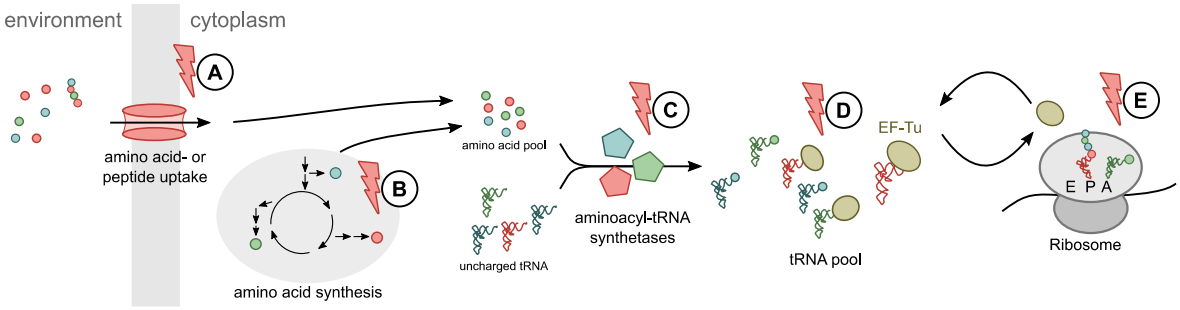

Figure 17: Possibly stress-sensitive processes which may provoke a stringent response.

Amino acids and short peptides are taken up by transporters or synthesized from intermediates of the central carbon metabolism and then coupled to their cognate tRNA by specific synthetases. At each step, these processes may be inhibited by heat stress or other stress conditions and thereby create a shortage of charged tRNA which may trigger a SR (A, B, C). Similarly, certain stress stimuli may lead to the modification or fragmentation of tRNA (D) or influence the activity of a ribosome-associated factor which could also provide a signal for the activation of Rel (E). For details, see text.

example, diamide was reported to provoke a transient decrease in the cellular concentrations of certain amino acids such as methionine, glycine, valine or isoleucine as well as other metabolites in *B. subtilis*, presumably by inactivating key metabolic enzymes [284]. Likewise, nitrosative stress induces a transient, functional auxotrophy for branched-chain and aromatic amino acids by reacting with the 4Fe-4S clusters in the active sites of CitB (aconitase) and IlvD in *Salmonella* and *E. coli* [292], [369], [370]. Furthermore, oxidative stress results in a depletion of the cellular cysteine pool in *B. subtilis* [284].

In addition, there are several indications that oxidative or proteotoxic stress could directly affect aminoacyl-tRNA synthetases (Figure 17 C) [339], [371]. For example, treatment of *E. coli* cells with the cytostatic quinone ACDQ (6-amino-7-chloro-5,8-dioxoquinoline) results in growth inhibition and activation of the SR, which can be suppressed by the addition of chloramphenicol or amino acids [372], [373]. It was demonstrated that ACDQ specifically inactivates Leucyl-tRNA synthetase by the oxidation of essential –SH groups while the actual leucine levels are unchanged [374], [375]. The hypothesis that aminoacyl-tRNA synthetases could be sensors of stress is further corroborated by the observation that heat- or oxidative stress and many other stress conditions, which provoke a SR also result in the accumulation of adenylated dinucleotides such as AppppA or ApppGpp [274], [278], [376], which are synthesized as a side reaction of tRNA synthetases from an aminoacyl-adenylate intermediate [377], [378].

Furthermore, tRNAs itself may be sensors of stress conditions and activate the SR (Figure 17 D). Like other macromolecules, tRNA can be damaged by stress and may become oxidized, fragmented or degraded, especially at the conserved, hypermodified bases, thereby directly affecting translation and

possibly also the SR [339], [379], [380]. It was recently demonstrated that tRNA maturation defects caused by the depletion of essential RNases trigger a Rel-dependent synthesis of (p)ppGpp in *B. subtilis* [361]. Likewise, the stringent response is constitutively activated in strains which cannot synthesize the threonyl-carbamoyl-adenosine modification at position 37 of certain tRNAs, suggesting that structural alterations or modifications of tRNA may also elicit a SR [381].

Interestingly, the conserved 4-thiouridine (s4U) modification at position 8 of tRNAs has been associated with the response to near-UV radiation. UV-exposure triggers a rapid RelA-dependent synthesis of (p)ppGpp and growth arrest in *Salmonella* and related organisms [382]. The basis for this response was found to be the quantitative photochemical oxidation of the s4U base which in some cases results in an intra-molecular crosslink with the cytidine at position 13 [383]. The oxidized tRNAs exhibit reduced affinity for their cognate aminoacyl-tRNA synthetase and a decreased incorporation into nascent polypeptide chains [384]. Concurrently, RelA dependent (p)ppGpp production as well as a rapid accumulation of adenylated dinucleotides is observed [382]. Mutant strains lacking the s4U modification (*nuv*) or relaxed (*relA*$^-$) strains do not exhibit a SR nor growth arrest upon short UV-exposure and initially continue growth, but display increased sensitivity and are rapidly killed upon continuous UV-radiation [382], [385].

The stress-induced modification or inactivation of other factors involved in translation could also be a trigger for the Rel-dependent SR (Figure 17 E). Patterson and Gillespie (1971) first hypothesized, that the heat-induced inactivation of an initiation factor for protein synthesis could provoke a SR in heat-shocked *E. coli* cells [270], [386]. Many ribosomal proteins or elongation factors are susceptible to modification by cysteine oxidation, disulfide formation or carbonylation during oxidative conditions [339], [387]. Interestingly, oxidation or *S*-bacillithiolation of a conserved cysteine of EF-Tu was observed in *B. subtilis* and other species [388]–[390]. This residue is critical for tRNA binding and may thereby represent a redox sensor which controls translation in response to stress and could possibly also promote a Rel-dependent SR [391], [392]. Likewise, oxidation of conserved cysteines in EF-G was also demonstrated to be a main cause of translational arrest during oxidative stress in the cyanobacterium *Synechocystis sp*. And *E. coli* [393], [394]. Furthermore, heat-induced lesions of the small ribosomal subunits could be observed, which could also contribute the activation of the SR (section 3.2.2).

In summary, the results presented here demonstrates that Rel is the main source of (p)ppGpp during heat- and oxidative stress. Furthermore, the experiments indicate that secondary amino acid limitation or other perturbations of the protein synthesis apparatus during stress appear to be a signal for the Rel-dependent SR. However, more experiments are required to support or reject the above hypotheses. First, *in vitro* and *in vivo* experiments could be devised to probe the mechanism of Rel activation during amino acid deprivation and to reveal similarities and differences to the heat-stress dependent activation. In particular, the requirement for a productive interaction with ribosomes for the activation of Rel could be investigated by devising additional mutations from the available RelA·ribosome structures [188]–[190]. Since there are good indications that heat- or oxidative stress may interfere with amino acid synthesis or activation of tRNAs (see above), it would also be a promising experiment to survey the cellular pool of amino acids and the tRNA charge-ratio during different stress conditions.

3.4 An integrated view on the heat shock- and stringent response

The results presented here support and extend the notion of the SR mediated by the second messenger (p)ppGpp as a global and pleiotropic adaptation to stress- and starvation conditions. It could be demonstrated that the SR is also part of the regulatory network which governs the HSR.

3.4.1 Transcriptional and translational adaptations during the HSR

The data supports a model by which the HSR of *B. subtilis* is a concerted and integrated adaptation program orchestrated by different heat shock transcription factors as well as the second messenger (p)ppGpp. Upon heat stress, cells respond with a strongly increased synthesis of chaperones and proteases, which together form the cellular protein quality control network. Together with the proteins of the general stress response, which provide the cell with a broad and preemptive resistance to many adverse conditions, the proteins synthesized during the HSR can account for more than half of the cellular protein synthesis capacity upon stress and accumulate in large quantities [43], [395]. This aspect of the heat shock response is well studied and regulated by the transcriptional regulators HrcA, CtsR and σ^B as well as other less well-characterized regulators (Figure 18, see also section 1.1.3) [58].

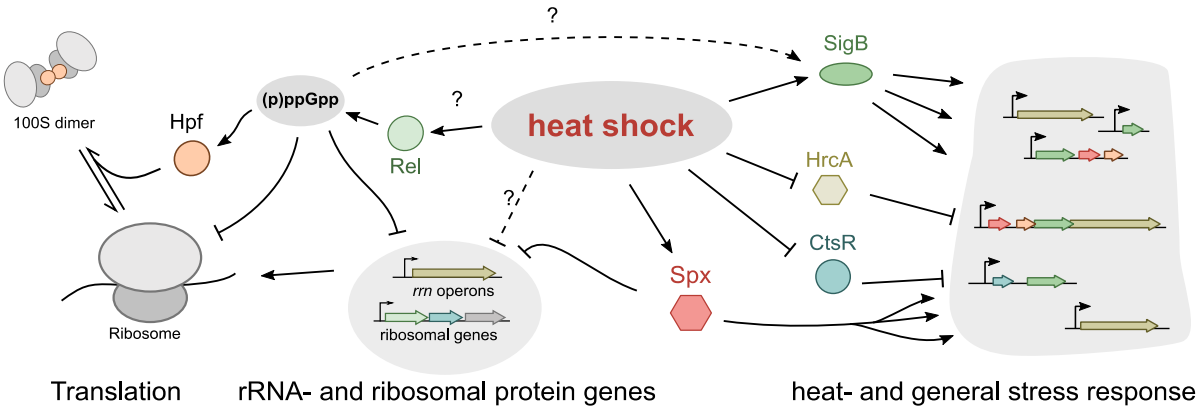

Figure 18: Model of the function and interplay of transcriptional and translational regulators during the heat shock response.

Heat stress leads to a broad and strong transcriptional up-regulation of heat- and general stress response genes (right side) controlled by the transcriptional regulators HrcA, CtsR, SigB and Spx. Concurrently, heat stress activates (p)ppGpp synthesis, which is implicated in the control of the translation apparatus and mediates, together with Spx and possibly additional, unknown factors, a comprehensive down-regulation of translation-related genes. For details see text.

Furthermore, the results of Runde *et al.* (2014) demonstrate that Spx is an additional regulator of the HSR (see section 1.2.2) [50]. The microarrays presented in this work (section 2.1) and previous publications suggest that Spx controls a large regulon of stress response genes [62], [103]. In particular, Spx appears to be a regulator of many genes of the HSR of which the transcriptional regulation was previously unknown (class IV heat shock genes), e.g. *clpX*, *hslO* or *htpG* (section 2.1 Figure 2) [62]. Furthermore, there appears to be an intricate connection and a mutual influence between Spx and other heat shock regulons. For example, Spx was demonstrated to co-regulate the CtsR-controlled *ctsR-mcsA-mcsB-clpC* operon [62]. On the other hand, Spx activity appears to be influenced by McsB, ClpC and YwlE *in vivo*, suggesting an intricate relationship between Spx and the CtsR regulon [139]. It was also demonstrated that Spx regulates *ytvA*, which encodes an activator of the σ^B dependent general stress response in response to blue light, suggesting that Spx could also influence σ^B activity as well [396]. Together, the findings that Spx controls a large sub-regulon of the HSR and that Δ*spx* deletion strains are associated with severe heat sensitive phenotypes support the notion that Spx is a central regulator of the HSR. In contrast, the regulation and synthesis of heat shock proteins was mostly independent of the alarmone (p)ppGpp (section 2.3 Figure 4, S7). However, the results of the RNA-seq experiment obtained in section 2.3 suggest that the SR can intricately influence and modulate the expression of the general

stress response controlled by σ^B. Furthermore, the heat-induced expression of *hpf* is strongly dependent on the SR (Figure 18).

The results presented in this thesis also suggests that the up-regulation of heat shock genes coincides with the concurrent down-shift of ribosome biogenesis by down-regulating the transcription of rRNA and RP genes during the HSR (Figure 18 and section 2.3 Figure 4). Although the transcriptional response of *B. subtilis* cells during the HSR was highly reminiscent of the SR, the experimental results indicate that (p)ppGpp is only partially involved in mediating this transcriptional adaptation. The results of section 2.3 support a model by which transcription of rRNA operons is down-regulated by the concurrent but independent activities of Spx and (p)ppGpp. In addition, the regulation of RP gene transcription during the HSR appears to be more complex and mainly influenced by unknown mechanisms other than (p)ppGpp and Spx (Figure 18).

The comprehensive transcriptional down-regulation of rRNA and RP genes during heat stress is accompanied by direct adjustments and attenuation of translation rates (section 2.3 Figure 6). The results suggests that the second messenger (p)ppGpp, which is known to be a regulator of many GTP-dependent steps of protein synthesis, is involved in the control and attenuation of cellular translational capacity during heat stress. Furthermore, (p)ppGpp is required and sufficient for the stress-induced synthesis of Hpf, which promotes the synthesis of translationally inactive 100S ribosome dimers (Figure 18).

Together, the HSR of *B. subtilis* appears to be a concerted program which includes (i) transcriptional up-regulation of chaperones and proteases and other heat shock proteins, (ii) the transcriptional repression of ribosomal genes and other processes which are not critical for the survival of the present stress conditions as well as (iii) the direct adjustment of translation to curb the rate of protein synthesis. This combined response allows a timely accumulation of the proteins of the cellular protein quality control system to prevent the stress-induced misfolding and aggregation of proteins and to remove aggregated and otherwise damaged proteins from the cell, while concurrently preventing further protein aggregation by restricting new protein synthesis and therefore reducing the load on the protein quality control system (Figure 19).

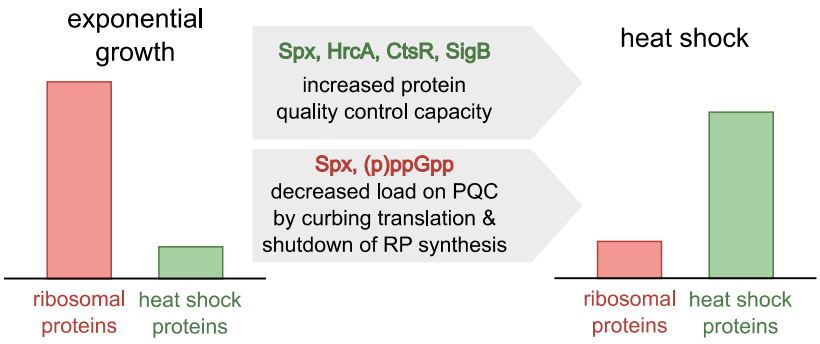

Figure 19: Model of the transcriptional and translational adaptations during the heat shock response.
During heat stress, the synthesis of chaperones and other heat shock proteins is strongy increased, regulated by the transcription factors HrcA, CtsR, Spx and SigB. Concurrently, the load on the protein quality control (PQC) system is reduced by transcriptional down-regulation of ribosomal protein (RP) genes as well as the direct restriction of protein synthesis rates. For details, see text.

The results indicate that the protective role of (p)ppGpp in the HSR may be primarily mediated by the direct modulation of translation rates, while (p)ppGpp mediated transcriptional adjustments appear to have a smaller role in the adaptation to heat stress. First, RNA-seq and RT-qPCR experiments revealed only little transcriptional differences between wild type and (p)ppGpp^0 cells during heat shock, which may be explained by the only modest changes of the GTP level during heat stress (section 2.3 Figure 4, S1, S7). Furthermore, even the complete dys-regulation of rRNA transcription in a (p)ppGpp^0 *cxs*-2 strain did not decrease viability at high temperatures (section 2.3 Figure S9). In contrast, the data clearly suggests that (p)ppGpp is implicated in the attenuation of global translation rates, the formation of 100S disomes and the stability of ribosomal subunits during stress. Interestingly, Δ*rplK* cells, in which translation rate are decreased independently of (p)ppGpp by deletion of the non-essential ribosomal protein L11, also exhibited strongly increased thermoresistance, implicating that the attenuation of translation during heat stress is supporting protein homeostasis and survival (section 2.4 Figure 10). Taken together, curbing translation rates appears to be an important aspect of the HSR and primarily mediated by (p)ppGpp during heat stress.

3.4.2 The role of (p)ppGpp in stress response and survival

It is tempting to speculate that the activation of the SR could represent a fast-acting first response to sudden proteotoxic stress. Upon stress, the accumulation of (p)ppGpp was found to be very rapid but transient and both the Rel-dependent synthesis of alarmones as well as its direct influence on protein

synthesis rates do not require the time-consuming *de novo* synthesis of macromolecules such as RNA or proteins. By transiently curbing the rate of protein synthesis, (p)ppGpp could possibly prevent unrestrained protein synthesis at the onset of stress, when the amount of accumulated HSPs is still low and could therefore counteract the formation of protein aggregate during the early stages of the HSR. However, to further investigate this hypothesis, additional experiments are required to determine the kinetics of protein synthesis rates with high sensitivity and temporal resolution. Furthermore, the determination of individual protein synthesis rates in a global proteomic approach would allow a better characterization of translational regulation during stress.

Strains with increased (p)ppGpp levels such as Δ*rel* cells exhibit decreased accumulation of protein aggregates together with higher thermoresistance to an otherwise lethal temperature upshift (37/53 °C). Many aspects of the SR in *B. subtilis* are mediated indirectly by the (p)ppGpp dependent reduction of GTP levels and can be reproduced by treatment with decoyinine [217]. However, the experiments using decoyinine suggest that lowered GTP levels are not sufficient to confer heat stress resistance as observed in strains with increased (p)ppGpp levels (section 2.3 Figure S4). These results suggest that increased (p)ppGpp levels, but not a drop in the cellular GTP level, is critical for the adaptation to stress and implicated in alterations of the cellular physiology during the HSR. In the light of the above discussion regarding the role of (p)ppGpp mediated transcriptional and translational adaptations in the HSR, it appears likely that the GTPases of the translation apparatus could be the major target subject to precise and direct modulation and inhibition during stress by (p)ppGpp, which cannot be mediated by decreased GTP concentration. In good agreement with this notion a strong increase of (p)ppGpp could be observed during heat shock, while GTP levels were for the most part unchanged. Previously, Kriel *et al.* (2014) used a strain in which the essential *guaB* gene was placed under an IPTG inducible promoter to study the impact of decreased GTP levels independently of (p)ppGpp [217]. Such a strain could be a useful tool to further explore the role of (p)ppGpp and GTP in stress response and survival.

In *B. subtilis*, the alarmone (p)ppGpp leads to decreased GTP levels by inhibition of HprT, Gmk and, to a lesser extent, GuaB [223]–[225] (see section 1.3.4). However, certain homologs of these enzymes from other species were reported to be insensitive to the competitive regulation by alarmones [225],

[397], [398]. The heterologous expression of these (p)ppGpp insensitive enzymes could possibly allow the construction of a strain in which the (p)ppGpp level can be adjusted without affecting GTP levels.

3.4.3 The stringent response as a conserved stress response system

Interestingly, rapid accumulation of (p)ppGpp, accompanied by stringent transcriptional regulation, could also be observed upon other environmental stress conditions, such as oxidative stress, salt stress or treatment with antibiotics in *B. subtilis* (section 2.1 Figure S5, section 2.3 Figure 1) [62], [284], [287], [390], indicating that the SR is commonly activated upon environmental stress and that the putative mechanisms of SR mediated stress tolerance discussed above could to also apply to other environmental stress conditions (see section 3.3). Furthermore, (p)ppGpp synthesis upon heat- and oxidative stress has been reported from diverse bacterial species such as *Escherichia, Salmonella* or *Enterococcus* [271], [278], [292], [372], [399]. Likewise, mutant strains of different bacterial families with impaired alarmone synthesis capabilities often exhibit impaired stress tolerance and survival [292], [399]–[401] and strains with increased (p)ppGpp levels display increased stress tolerance [343], [399], [402], [403]. Therefore, it is intriguing to speculate that the SR could represent a conserved stress response system in prokaryotes which not only responds to starvation for amino acids and other nutrients but has also mediates adaptation to the challenges imposed by different adverse environments by a concerted response involving both transcriptional and translational adjustments [159].

3.4.4 Concluding remarks

The discussion in the preceding sections suggest that the translation apparatus is an important target of (p)ppGpp mediated modulation in the adaptation to stress. However, alarmones may mediate crucial adaptations during the HSR by binding to other, yet unknown targets. In the past, the set of proteins known to bind (p)ppGpp could be considerably expanded in *E. coli* and *S. aureus*, suggesting that(p)ppGpp could be involved in the regulation of additional, as of yet unknown processes [173], [244], [267] (see section 1.3.7). Similarly, a genetic screen or a global proteomic approach to enrich and identify additional direct binding targets of (p)ppGpp could significantly enhance he understanding of the role of the SR in the control of cellular physiology and the adaptation to stress and starvation in *B. subtilis*. Clearly, regarding the role of nucleotide second messengers, much remains to be studied.

The SR has also been implicated in persister cell formation and antibiotic tolerance, where similar adaptive strategies, in particular a shut-down of translation, metabolism and growth, leads to the formation of dormant, non-growing cells which are highly tolerant to antibiotics and adverse environments [404]. Therefore, studying the impact of (p)ppGpp signaling, its mechanistic targets and its role in stress adaptation and survival may also lead to a deeper understanding of the phenomenon of antibiotic tolerance and persistence and the development of clinically relevant applications.

4. List of figures

5. List of tables

6. References

[1] G. Storz, R. Hengge, and American Society for Microbiology, Eds., *Bacterial stress responses*, 2nd ed. Washington, DC: ASM Press, 2011.

[2] F. Cohn, "Untersuchungen über Bacterien IV: Beiträge zur Biologie der Bacillen.," in *Beiträge zur Biologie der Pflanzen*, vol. 2, F. Cohn, Ed. Breslau, 1877, pp. 249–276.

[3] J. Errington, "Regulation of endospore formation in *Bacillus subtilis*," *Nat. Rev. Microbiol.*, vol. 1, no. 2, pp. 117–126, Nov. 2003.

[4] R. Losick, P. Youngman, and P. J. Piggot, "Genetics of endospore formation in *Bacillus subtilis*," *Annu. Rev. Genet.*, vol. 20, pp. 625–669, 1986.

[5] P. Stragier and R. Losick, "Molecular genetics of sporulation in *Bacillus subtilis*," *Annu. Rev. Genet.*, vol. 30, pp. 297–241, 1996.

[6] J. Spizizen, "TRANSFORMATION OF BIOCHEMICALLY DEFICIENT STRAINS OF BACILLUS SUBTILIS BY DEOXYRIBONUCLEATE," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 44, no. 10, pp. 1072–1078, Oct. 1958.

[7] F. Kunst *et al.*, "The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*," *Nature*, vol. 390, no. 6657, pp. 249–256, Nov. 1997.

[8] U. Völker and M. Hecker, "From genomics via proteomics to cellular physiology of the Gram-positive model organism *Bacillus subtilis*," *Cell. Microbiol.*, vol. 7, no. 8, pp. 1077–1085, Aug. 2005.

[9] P. Nicolas *et al.*, "Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*," *Science*, vol. 335, no. 6072, pp. 1103–1106, Mar. 2012.

[10] K. Kobayashi *et al.*, "Essential *Bacillus subtilis* genes," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 100, no. 8, pp. 4678–4683, Apr. 2003.

[11] N. Mirouze and D. Dubnau, "Chance and Necessity in *Bacillus subtilis* Development," *Microbiol. Spectr.*, vol. 1, no. 1, Oct. 2013.

[12] H. Vlamakis, Y. Chai, P. Beauregard, R. Losick, and R. Kolter, "Sticking together: building a biofilm the *Bacillus subtilis* way," *Nat. Rev. Microbiol.*, vol. 11, no. 3, pp. 157–168, Mar. 2013.

[13] D. Lopez, H. Vlamakis, and R. Kolter, "Generation of multiple cell types in *Bacillus subtilis*," *FEMS Microbiol. Rev.*, vol. 33, no. 1, pp. 152–163, Jan. 2009.

[14] P. Zuber, "Management of Oxidative Stress in *Bacillus*," *Annu. Rev. Microbiol.*, vol. 63, no. 1, pp. 575–597, Oct. 2009.

[15] M. Hecker, W. Schumann, and U. Völker, "Heat-shock and general stress response in *Bacillus subtilis*," *Mol. Microbiol.*, vol. 19, no. 3, pp. 417–428, 1996.

[16] A. K. W. Elsholz, M. S. Birk, E. Charpentier, and K. Turgay, "Functional Diversity of AAA+ Protease Complexes in *Bacillus subtilis*," *Front. Mol. Biosci.*, vol. 4, p. 44, 2017.

[17] C. B. Anfinsen, "Principles that govern the folding of protein chains," *Science*, vol. 181, no. 4096, pp. 223–230, Jul. 1973.

[18] C. B. Anfinsen, E. Haber, M. Sela, and F. H. White, "The kinetics of formation of native ribonuclease during oxidation of the reduced polypeptide chain," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 47, pp. 1309–1314, Sep. 1961.

[19] M.-J. Gething and J. Sambrook, "Protein folding in the cell," *Nature*, vol. 355, no. 6355, pp. 33–45, Jan. 1992.
[20] R. Jaenicke, "Protein folding: local structures, domains, subunits, and assemblies," *Biochemistry*, vol. 30, no. 13, pp. 3147–3161, Apr. 1991.
[21] F. U. Hartl, A. Bracher, and M. Hayer-Hartl, "Molecular chaperones in protein folding and proteostasis," *Nature*, vol. 475, no. 7356, pp. 324–332, Jul. 2011.
[22] D. Balchin, M. Hayer-Hartl, and F. U. Hartl, "*In vivo* aspects of protein folding and quality control," *Science*, vol. 353, no. 6294, pp. aac4354–aac4354, Jul. 2016.
[23] A. Mogk, D. Huber, and B. Bukau, "Integrating protein homeostasis strategies in prokaryotes," *Cold Spring Harb. Perspect. Biol.*, vol. 3, no. 4, Apr. 2011.
[24] S. Lindquist, "The Heat-Shock Response," *Annu. Rev. Biochem.*, vol. 55, no. 1, pp. 1151–1191, 1986.
[25] F. Ritossa, "A new puffing pattern induced by temperature shock and DNP in drosophila," *Experientia*, vol. 18, no. 12, pp. 571–573, Dec. 1962.
[26] A. L. Horwich, A. C. Apetri, and W. A. Fenton, "The GroEL/GroES cis cavity as a passive anti-aggregation device," *FEBS Lett.*, vol. 583, no. 16, pp. 2654–2662, Aug. 2009.
[27] O. Fayet, 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.*, vol. 171, no. 3, pp. 1379–1385, Mar. 1989.
[28] A. Mogk, E. Deuerling, S. Vorderwülbecke, E. Vierling, and B. Bukau, "Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation," *Mol. Microbiol.*, vol. 50, no. 2, pp. 585–595, Oct. 2003.
[29] D. Y. Reyes and H. Yoshikawa, "DnaK chaperone machine and trigger factor are only partially required for normal growth of *Bacillus subtilis*," *Biosci. Biotechnol. Biochem.*, vol. 66, no. 7, pp. 1583–1586, Jul. 2002.
[30] N. Molière and K. Turgay, "Chaperone-protease systems in regulation and protein quality control in *Bacillus subtilis*," *Res. Microbiol.*, vol. 160, no. 9, pp. 637–644, Nov. 2009.
[31] S. Versteeg, A. Mogk, and W. Schumann, "The Bacillus subtilis htpG gene is not involved in thermal stress management," *Mol. Gen. Genet. MGG*, vol. 261, no. 3, pp. 582–588, Apr. 1999.
[32] S. Wickner, M. R. Maurizi, and S. Gottesman, "Posttranslational quality control: folding, refolding, and degrading proteins," *Science*, vol. 286, no. 5446, pp. 1888–1893, Dec. 1999.
[33] N. Molière, J. Hoßmann, H. Schäfer, and K. Turgay, "Role of Hsp100/Clp Protease Complexes in Controlling the Regulation of Motility in *Bacillus subtilis*," *Front. Microbiol.*, vol. 7, p. 315, 2016.
[34] E. Krüger, U. Völker, and M. Hecker, "Stress induction of clpC in *Bacillus subtilis* and its involvement in stress tolerance," *J. Bacteriol.*, vol. 176, no. 11, pp. 3360–3367, Jun. 1994.
[35] E. Krüger, E. Witt, S. Ohlmeier, R. Hanschke, and M. Hecker, "The clp proteases of *Bacillus subtilis* are directly involved in degradation of misfolded proteins," *J. Bacteriol.*, vol. 182, no. 11, pp. 3259–3265, Jun. 2000.
[36] K. Turgay, L. W. Hamoen, G. Venema, and D. Dubnau, "Biochemical characterization of a molecular switch involving the heat shock protein ClpC, which controls the activity of ComK, the competence transcription factor of *Bacillus subtilis*.," *Genes Dev.*, vol. 11, no. 1, pp. 119–128, Jan. 1997.
[37] J. Kirstein, D. A. Dougan, U. Gerth, M. Hecker, and K. Turgay, "The tyrosine kinase McsB is a regulated adaptor protein for ClpCP," *EMBO J.*, vol. 26, no. 8, pp. 2061–2070, Apr. 2007.
[38] T. Schlothauer, A. Mogk, D. A. Dougan, B. Bukau, and K. Turgay, "MecA, an adaptor protein necessary for ClpC chaperone activity," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 100, no. 5, pp. 2306–2311, Mar. 2003.
[39] M. Persuh, I. Mandic-Mulec, and D. Dubnau, "A MecA paralog, YpbH, binds ClpC, affecting both competence and sporulation," *J. Bacteriol.*, vol. 184, no. 8, pp. 2310–2313, Apr. 2002.
[40] E. Deuerling, A. Mogk, C. Richter, M. Purucker, and W. Schumann, "The ftsH gene of *Bacillus subtilis* is involved in major cellular processes such as sporulation, stress adaptation and secretion," *Mol. Microbiol.*, vol. 23, no. 5, pp. 921–933, Mar. 1997.
[41] R. Schmidt, A. L. Decatur, P. N. Rather, C. P. Moran, and R. Losick, "*Bacillus subtilis* lon protease prevents inappropriate transcription of genes under the control of the sporulation transcription factor sigma G," *J. Bacteriol.*, vol. 176, no. 21, pp. 6528–6537, Nov. 1994.

[42] D. Höper, U. Völker, and M. Hecker, "Comprehensive characterization of the contribution of individual SigB-dependent general stress genes to stress resistance of *Bacillus subtilis*," *J. Bacteriol.*, vol. 187, no. 8, pp. 2810–2826, Apr. 2005.
[43] M. Hecker, J. Pané-Farré, and V. Uwe, "SigB-Dependent General Stress Response in *Bacillus subtilis* and Related Gram-Positive Bacteria," *Annu. Rev. Microbiol.*, vol. 61, no. 1, pp. 215–236, Oct. 2007.
[44] C. von Blohn, B. Kempf, R. M. Kappes, and E. Bremer, "Osmostress response in *Bacillus subtilis*: characterization of a proline uptake system (OpuE) regulated by high osmolarity and the alternative transcription factor sigma B," *Mol. Microbiol.*, vol. 25, no. 1, pp. 175–187, Jul. 1997.
[45] E. Krüger, T. Msadek, S. Ohlmeier, and M. Hecker, "The *Bacillus subtilis* clpC operon encodes DNA repair and competence proteins," *Microbiology*, vol. 143, no. 4, pp. 1309–1316, Apr. 1997.
[46] A. Reder, D. Hoper, U. Gerth, and M. Hecker, "Contributions of Individual B-Dependent General Stress Genes to Oxidative Stress Resistance of *Bacillus subtilis*," *J. Bacteriol.*, vol. 194, no. 14, pp. 3601–3610, Jul. 2012.
[47] M. Schmalisch, I. Langbein, and J. Stülke, "The general stress protein Ctc of *Bacillus subtilis* is a ribosomal protein," *J. Mol. Microbiol. Biotechnol.*, vol. 4, no. 5, pp. 495–501, Sep. 2002.
[48] H. Bügl *et al.*, "RNA Methylation under Heat Shock Control," *Mol. Cell*, vol. 6, no. 2, pp. 349–360, Aug. 2000.
[49] J.-H. Shin and C. W. Price, "The SsrA-SmpB ribosome rescue system is important for growth of *Bacillus subtilis* at low and high temperatures," *J. Bacteriol.*, vol. 189, no. 10, pp. 3729–3737, May 2007.
[50] S. Runde *et al.*, "The role of thiol oxidative stress response in heat-induced protein aggregate formation during thermotolerance in *B acillus subtilis*: Thiol oxidation in protein aggregate formation," *Mol. Microbiol.*, vol. 91, no. 5, pp. 1036–1052, Mar. 2014.
[51] U. Gerth, E. Krüger, I. Derré, T. Msadek, and M. Hecker, "Stress induction of the *Bacillus subtilis* clpP gene encoding a homologue of the proteolytic component of the Clp protease and the involvement of ClpP and ClpX in stress tolerance," *Mol. Microbiol.*, vol. 28, no. 4, pp. 787–802, May 1998.
[52] U. Völker, H. Mach, R. Schmid, and M. Hecker, "Stress proteins and cross-protection by heat shock and salt stress in *Bacillus subtilis*," *J. Gen. Microbiol.*, vol. 138, no. 10, pp. 2125–2135, Oct. 1992.
[53] M. Mols and T. Abee, "Primary and secondary oxidative stress in *Bacillus*: Oxidative stress in Bacilli," *Environ. Microbiol.*, vol. 13, no. 6, pp. 1387–1394, Jun. 2011.
[54] K. R. Messner and J. A. Imlay, "The Identification of Primary Sites of Superoxide and Hydrogen Peroxide Formation in the Aerobic Respiratory Chain and Sulfite Reductase Complex of *Escherichia coli*," *J. Biol. Chem.*, vol. 274, no. 15, pp. 10119–10128, Apr. 1999.
[55] J. A. Imlay, "Pathways of Oxidative Damage," *Annu. Rev. Microbiol.*, vol. 57, no. 1, pp. 395–418, Oct. 2003.
[56] C. Scharf, S. Riethdorf, H. Ernst, S. Engelmann, U. Völker, and M. Hecker, "Thioredoxin is an essential protein induced by multiple stresses in *Bacillus subtilis*," *J. Bacteriol.*, vol. 180, no. 7, pp. 1869–1877, Apr. 1998.
[57] A. Chastanet, J. Fert, and T. Msadek, "Comparative genomics reveal novel heat shock regulatory mechanisms in *Staphylococcus aureus* and other Gram-positive bacteria: Dual heat shock regulation by CtsR and HrcA in *S. aureus*," *Mol. Microbiol.*, vol. 47, no. 4, pp. 1061–1073, Feb. 2003.
[58] W. Schumann, "Regulation of bacterial heat shock stimulons," *Cell Stress Chaperones*, vol. 21, no. 6, pp. 959–968, Nov. 2016.
[59] A. K. W. Elsholz, U. Gerth, and M. Hecker, "Regulation of CtsR Activity in Low GC, Gram+ Bacteria," in *Advances in Microbial Physiology*, vol. 57, Elsevier, 2010, pp. 119–144.
[60] R. L. Gourse, "Classic Spotlight: the Heat Shock Response and the Discovery of Alternative Sigma Factors in *Escherichia coli*," *J. Bacteriol.*, vol. 198, no. 19, pp. 2550–2550, Oct. 2016.
[61] T. Yura, "Regulation and conservation of the heat-shock transcription factor sigma32," *Genes Cells Devoted Mol. Cell. Mech.*, vol. 1, no. 3, pp. 277–284, Mar. 1996.
[62] T. Rochat *et al.*, "Genome-wide identification of genes directly regulated by the pleiotropic transcription factor Spx in *Bacillus subtilis*," *Nucleic Acids Res.*, vol. 40, no. 19, pp. 9571–9583, Oct. 2012.

[63] G. Homuth, S. Masuda, A. Mogk, Y. Kobayashi, and W. Schumann, "The dnaK operon of *Bacillus subtilis* is heptacistronic," *J. Bacteriol.*, vol. 179, no. 4, pp. 1153–1164, Feb. 1997.
[64] A. Schmidt, M. Schiesswohl, U. Völker, M. Hecker, and W. Schumann, "Cloning, sequencing, mapping, and transcriptional analysis of the groESL operon from *Bacillus subtilis*.," *J. Bacteriol.*, vol. 174, no. 12, pp. 3993–3999, Jun. 1992.
[65] A. Schulz and W. Schumann, "hrcA, the first gene of the *Bacillus subtilis* dnaK operon encodes a negative regulator of class I heat shock genes.," *J. Bacteriol.*, vol. 178, no. 4, pp. 1088–1093, Feb. 1996.
[66] U. Zuber and W. Schumann, "CIRCE, a novel heat shock element involved in regulation of heat shock operon dnaK of *Bacillus subtilis*.," *J. Bacteriol.*, vol. 176, no. 5, pp. 1359–1363, Mar. 1994.
[67] A. Mogk, G. Homuth, C. Scholz, L. Kim, F. X. Schmid, and W. Schumann, "The GroE chaperonin machine is a major modulator of the CIRCE heat shock regulon of *Bacillus subtilis*," *EMBO J.*, vol. 16, no. 15, pp. 4579–4590, Aug. 1997.
[68] U. Völker *et al.*, "Analysis of the induction of general stress proteins of *Bacillus subtilis*," *Microbiol. Read. Engl.*, vol. 140 (Pt 4), pp. 741–752, Apr. 1994.
[69] I. Derré, G. Rapoport, and T. Msadek, "CtsR, a novel regulator of stress and heat shock response, controls clp and molecular chaperone gene expression in Gram-positive bacteria," *Mol. Microbiol.*, vol. 31, no. 1, pp. 117–131, Jan. 1999.
[70] E. Krüger and M. Hecker, "The first gene of the *Bacillus subtilis* clpC operon, ctsR, encodes a negative regulator of its own operon and other class III heat shock genes," *J. Bacteriol.*, vol. 180, no. 24, pp. 6681–6688, Dec. 1998.
[71] E. Krüger, T. Msadek, and M. Hecker, "Alternate promoters direct stress-induced transcription of the *Bacillus subtilis* clpC operon," *Mol. Microbiol.*, vol. 20, no. 4, pp. 713–723, May 1996.
[72] E. Krüger, D. Zühlke, E. Witt, H. Ludwig, and M. Hecker, "Clp-mediated proteolysis in Gram-positive bacteria is autoregulated by the stability of a repressor," *EMBO J.*, vol. 20, no. 4, pp. 852–863, Feb. 2001.
[73] A. K. W. Elsholz, S. Michalik, D. Zühlke, M. Hecker, and U. Gerth, "CtsR, the Gram-positive master regulator of protein quality control, feels the heat," *EMBO J.*, vol. 29, no. 21, pp. 3621–3629, Nov. 2010.
[74] J. Kirstein, D. Zühlke, U. Gerth, K. Turgay, and M. Hecker, "A tyrosine kinase and its activator control the activity of the CtsR heat shock repressor in *B. subtilis*," *EMBO J.*, vol. 24, no. 19, pp. 3435–3445, Oct. 2005.
[75] A. K. W. Elsholz *et al.*, "Activity control of the ClpC adaptor McsB in *Bacillus subtilis*," *J. Bacteriol.*, vol. 193, no. 15, pp. 3887–3893, Aug. 2011.
[76] A. K. W. Elsholz, K. Hempel, D.-C. Pöther, D. Becher, M. Hecker, and U. Gerth, "CtsR inactivation during thiol-specific stress in low GC, Gram+ bacteria: Dual activity control of CtsR," *Mol. Microbiol.*, vol. 79, no. 3, pp. 772–785, Feb. 2011.
[77] A. K. W. Elsholz *et al.*, "Global impact of protein arginine phosphorylation on the physiology of *Bacillus subtilis*," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 19, pp. 7451–7456, May 2012.
[78] J. Fuhrmann *et al.*, "McsB Is a Protein Arginine Kinase That Phosphorylates and Inhibits the Heat-Shock Regulator CtsR," *Science*, vol. 324, no. 5932, pp. 1323–1327, Jun. 2009.
[79] J. Fuhrmann, V. Subramanian, D. J. Kojetin, and P. R. Thompson, "Activity-Based Profiling Reveals a Regulatory Link between Oxidative Stress and Protein Arginine Phosphorylation," *Cell Chem. Biol.*, vol. 23, no. 8, pp. 967–977, 18 2016.
[80] A. K. Benson and W. G. Haldenwang, "*Bacillus subtilis* sigma B is regulated by a binding protein (RsbW) that blocks its association with core RNA polymerase," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 90, no. 6, pp. 2330–2334, Mar. 1993.
[81] A. Dufour and W. G. Haldenwang, "Interactions between a *Bacillus subtilis* anti-sigma factor (RsbW) and its antagonist (RsbV).," *J. Bacteriol.*, vol. 176, no. 7, pp. 1813–1820, Apr. 1994.
[82] S. Alper, A. Dufour, D. A. Garsin, L. Duncan, and R. Losick, "Role of Adenosine Nucleotides in the Regulation of a Stress-response Transcription Factor in*Bacillus subtilis*," *J. Mol. Biol.*, vol. 260, no. 2, pp. 165–177, Jul. 1996.
[83] X. Yang, C. M. Kang, M. S. Brody, and C. W. Price, "Opposing pairs of serine protein kinases and phosphatases transmit signals of environmental stress to activate a bacterial transcription factor," *Genes Dev.*, vol. 10, no. 18, pp. 2265–2275, Sep. 1996.

[84] U. Völker, A. Völker, and W. G. Haldenwang, "Reactivation of the *Bacillus subtilis* anti-sigma B antagonist, RsbV, by stress- or starvation-induced phosphatase activities," *J. Bacteriol.*, vol. 178, no. 18, pp. 5456–5463, Sep. 1996.

[85] U. Völker, A. Völker, B. Maul, M. Hecker, A. Dufour, and W. G. Haldenwang, "Separate mechanisms activate sigma B of *Bacillus subtilis* in response to environmental and metabolic stresses.," *J. Bacteriol.*, vol. 177, no. 13, pp. 3771–3780, Jul. 1995.

[86] A. K. Benson and W. G. Haldenwang, "The sigma B-dependent promoter of the *Bacillus subtilis* sigB operon is induced by heat shock," *J. Bacteriol.*, vol. 175, no. 7, pp. 1929–1935, Apr. 1993.

[87] C. M. Kang, M. S. Brody, S. Akbar, X. Yang, and C. W. Price, "Homologous pairs of regulatory proteins control activity of *Bacillus subtilis* transcription factor sigma(b) in response to environmental stress," *J. Bacteriol.*, vol. 178, no. 13, pp. 3846–3853, Jul. 1996.

[88] U. Völker, A. Dufour, and W. G. Haldenwang, "The *Bacillus subtilis* rsbU gene product is necessary for RsbX-dependent regulation of sigma B," *J. Bacteriol.*, vol. 177, no. 1, pp. 114–122, Jan. 1995.

[89] C.-C. Chen, R. J. Lewis, R. Harris, M. D. Yudkin, and O. Delumeau, "A supramolecular complex in the environmental stress signalling pathway of *Bacillus subtilis*: Large protein complex regulates stress response," *Mol. Microbiol.*, vol. 49, no. 6, pp. 1657–1669, Aug. 2003.

[90] S. Zhang and W. G. Haldenwang, "Contributions of ATP, GTP, and redox state to nutritional stress activation of the *Bacillus subtilis* sigmaB transcription factor," *J. Bacteriol.*, vol. 187, no. 22, pp. 7554–7560, Nov. 2005.

[91] K. Vijay, M. S. Brody, E. Fredlund, and C. W. Price, "A PP2C phosphatase containing a PAS domain is required to convey signals of energy stress to the sigmaB transcription factor of *Bacillus subtilis*," *Mol. Microbiol.*, vol. 35, no. 1, pp. 180–188, Jan. 2000.

[92] S. Zhang and W. G. Haldenwang, "RelA is a component of the nutritional stress activation pathway of the *Bacillus subtilis* transcription factor sigma B," *J. Bacteriol.*, vol. 185, no. 19, pp. 5714–5721, Oct. 2003.

[93] J. M. Scott, J. Ju, T. Mitchell, and W. G. Haldenwang, "The *Bacillus subtilis* GTP binding protein obg and regulators of the sigma(B) stress response transcription factor cofractionate with ribosomes," *J. Bacteriol.*, vol. 182, no. 10, pp. 2771–2777, May 2000.

[94] J. M. Scott and W. G. Haldenwang, "Obg, an essential GTP binding protein of *Bacillus subtilis*, is necessary for stress activation of transcription factor sigma(B)," *J. Bacteriol.*, vol. 181, no. 15, pp. 4653–4660, Aug. 1999.

[95] S. Zhang, J. M. Scott, and W. G. Haldenwang, "Loss of ribosomal protein L11 blocks stress activation of the *Bacillus subtilis* transcription factor sigma(B)," *J. Bacteriol.*, vol. 183, no. 7, pp. 2316–2321, Apr. 2001.

[96] E. Darmon *et al.*, "A Novel Class of Heat and Secretion Stress-Responsive Genes Is Controlled by the Autoregulated CssRS Two-Component System of *Bacillus subtilis*," *J. Bacteriol.*, vol. 184, no. 20, pp. 5661–5671, Oct. 2002.

[97] U. Zuber, K. Drzewiecki, and M. Hecker, "Putative sigma factor SigI (YkoZ) of *Bacillus subtilis* is induced by heat shock," *J. Bacteriol.*, vol. 183, no. 4, pp. 1472–1475, Feb. 2001.

[98] T.-Y. Liu, S.-H. Chu, Y.-N. Hu, J.-J. Wang, and G.-C. Shaw, "Genetic evidence that multiple proteases are involved in modulation of heat-induced activation of the sigma factor SigI in *Bacillus subtilis*," *FEMS Microbiol. Lett.*, vol. 364, no. 7, 01 2017.

[99] O. Ramaniuk *et al.*, "σI from *Bacillus subtilis*: Impact on Gene Expression and Characterization of σI-Dependent Transcription That Requires New Types of Promoters with Extended -35 and -10 Elements," *J. Bacteriol.*, vol. 200, no. 17, Sep. 2018.

[100] P. D. Thackray and A. Moir, "SigM, an extracytoplasmic function sigma factor of *Bacillus subtilis*, is activated in response to cell wall antibiotics, ethanol, heat, acid, and superoxide stress," *J. Bacteriol.*, vol. 185, no. 12, pp. 3491–3498, Jun. 2003.

[101] A. Schulz, S. Schwab, G. Homuth, S. Versteeg, and W. Schumann, "The htpG gene of *Bacillus subtilis* belongs to class III heat shock genes and is under negative control," *J. Bacteriol.*, vol. 179, no. 10, pp. 3103–3109, May 1997.

[102] S. Versteeg, A. Escher, A. Wende, T. Wiegert, and W. Schumann, "Regulation of the *Bacillus subtilis* heat shock gene htpG is under positive control," *J. Bacteriol.*, vol. 185, no. 2, pp. 466–474, Jan. 2003.

[103] S. Nakano, E. Küster-Schöck, A. D. Grossman, and P. Zuber, "Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 100, no. 23, pp. 13603–13608, Nov. 2003.
[104] P. A. Zuber, *Function and control of the Spx-family of proteins within the bacterial stress response*. New York: Springer, 2013.
[105] A. Reder, D. Höper, C. Weinberg, U. Gerth, M. Fraunholz, and M. Hecker, "The Spx paralogue MgsR (YqgZ) controls a subregulon within the general stress response of *Bacillus subtilis*," *Mol. Microbiol.*, vol. 69, no. 5, pp. 1104–1120, Sep. 2008.
[106] S. Nakano, M. M. Nakano, Y. Zhang, M. Leelakriangsak, and P. Zuber, "A regulatory protein that interferes with activator-stimulated transcription in bacteria," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 100, no. 7, pp. 4233–4238, Apr. 2003.
[107] K. J. Newberry, S. Nakano, P. Zuber, and R. G. Brennan, "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.*, vol. 102, no. 44, pp. 15839–15844, Nov. 2005.
[108] Y. Zhang, S. Nakano, S.-Y. Choi, and P. Zuber, "Mutational Analysis of the *Bacillus subtilis* RNA Polymerase α C-Terminal Domain Supports the Interference Model of Spx-Dependent Repression," *J. Bacteriol.*, vol. 188, no. 12, pp. 4300–4311, Jun. 2006.
[109] S. Nakano, K. N. Erwin, M. Ralle, and P. Zuber, "Redox-sensitive transcriptional control by a thiol/disulphide switch in the global regulator, Spx," *Mol. Microbiol.*, vol. 55, no. 2, pp. 498–510, Jan. 2005.
[110] A. A. Lin, 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.*, vol. 195, no. 17, pp. 3967–3978, Sep. 2013.
[111] D. Y. Reyes 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.*, vol. 69, no. 3, pp. 765–779, Aug. 2008.
[112] M. M. Nakano, A. Lin, C. S. Zuber, K. J. Newberry, R. G. Brennan, and P. Zuber, "Promoter Recognition by a Complex of Spx and the C-Terminal Domain of the RNA Polymerase α Subunit," *PLoS ONE*, vol. 5, no. 1, p. e8664, Jan. 2010.
[113] A. Petersohn, M. Brigulla, S. Haas, J. D. Hoheisel, U. Völker, and M. Hecker, "Global analysis of the general stress response of *Bacillus subtilis*," *J. Bacteriol.*, vol. 183, no. 19, pp. 5617–5631, Oct. 2001.
[114] A. Petersohn *et al.*, "Identification of sigma(B)-dependent genes in *Bacillus subtilis* using a promoter consensus-directed search and oligonucleotide hybridization," *J. Bacteriol.*, vol. 181, no. 18, pp. 5718–5724, Sep. 1999.
[115] P. Zuber *et al.*, "Phenotype enhancement screen of a regulatory spx mutant unveils a role for the ytpQ gene in the control of iron homeostasis," *PloS One*, vol. 6, no. 9, p. e25066, 2011.
[116] T. Zeller and G. Klug, "Thioredoxins in bacteria: functions in oxidative stress response and regulation of thioredoxin genes," *Naturwissenschaften*, vol. 93, no. 6, pp. 259–266, Jun. 2006.
[117] P. Gonzalez Porqué, A. Baldesten, and P. Reichard, "The involvement of the thioredoxin system in the reduction of methionine sulfoxide and sulfate," *J. Biol. Chem.*, vol. 245, no. 9, pp. 2371–2374, May 1970.
[118] K. C. Das and C. K. Das, "Thioredoxin, a Singlet Oxygen Quencher and Hydroxyl Radical Scavenger: Redox Independent Functions," *Biochem. Biophys. Res. Commun.*, vol. 277, no. 2, pp. 443–447, Oct. 2000.
[119] C. Berndt, C. H. Lillig, and A. Holmgren, "Thioredoxins and glutaredoxins as facilitators of protein folding," *Biochim. Biophys. Acta BBA - Mol. Cell Res.*, vol. 1783, no. 4, pp. 641–650, Apr. 2008.
[120] A. Gaballa *et al.*, "Biosynthesis and functions of bacillithiol, a major low-molecular-weight thiol in Bacilli," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 14, pp. 6482–6486, Apr. 2010.
[121] A. Gaballa, H. Antelmann, C. J. Hamilton, and J. D. Helmann, "Regulation of *Bacillus subtilis* bacillithiol biosynthesis operons by Spx," *Microbiol. Read. Engl.*, vol. 159, no. Pt 10, pp. 2025–2035, Oct. 2013.
[122] M. M. Nakano, F. Hajarizadeh, Y. Zhu, and P. Zuber, "Loss-of-function mutations in yjbD result in ClpX- and ClpP-independent competence development of *Bacillus subtilis*," *Mol. Microbiol.*, vol. 42, no. 2, pp. 383–394, Oct. 2001.

[123] L. W. Hamoen, "Controlling competence in *Bacillus subtilis*: shared use of regulators," *Microbiology*, vol. 149, no. 1, pp. 9–17, Jan. 2003.
[124] K. Turgay, J. Hahn, J. Burghoorn, and D. Dubnau, "Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor," *EMBO J.*, vol. 17, no. 22, pp. 6730–6738, Nov. 1998.
[125] Y. Weinrauch, R. Penchev, E. Dubnau, I. Smith, and D. Dubnau, "A *Bacillus subtilis* regulatory gene product for genetic competence and sporulation resembles sensor protein members of the bacterial two-component signal-transduction systems.," *Genes Dev.*, vol. 4, no. 5, pp. 860–872, May 1990.
[126] J. M. Solomon, R. Magnuson, A. Srivastava, and A. D. Grossman, "Convergent sensing pathways mediate response to two extracellular competence factors in *Bacillus subtilis*.," *Genes Dev.*, vol. 9, no. 5, pp. 547–558, Mar. 1995.
[127] L. W. Hamoen, H. Eshuis, J. Jongbloed, G. Venema, and D. Sinderen, "A small gene, designated comS, located within the coding region of the fourth amino acid-activation domain of srfA, is required for competence development in *Bacillus subtilis*," *Mol. Microbiol.*, vol. 15, no. 1, pp. 55–63, Jan. 1995.
[128] C. D'Souza, M. M. Nakano, and P. Zuber, "Identification of comS, a gene of the srfA operon that regulates the establishment of genetic competence in *Bacillus subtilis*," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 91, no. 20, pp. 9397–9401, Sep. 1994.
[129] M. M. Nakano, L. A. Xia, and P. Zuber, "Transcription initiation region of the srfA operon, which is controlled by the comP-comA signal transduction system in *Bacillus subtilis*," *J. Bacteriol.*, vol. 173, no. 17, pp. 5487–5493, Sep. 1991.
[130] M. M. Nakano, S. Nakano, and P. Zuber, "Spx (YjbD), a negative effector of competence in *Bacillus subtilis*, enhances ClpC-MecA-ComK interaction," *Mol. Microbiol.*, vol. 44, no. 5, pp. 1341–1349, Jun. 2002.
[131] S. J. Pamp, D. Frees, S. Engelmann, M. Hecker, and H. Ingmer, "Spx is a global effector impacting stress tolerance and biofilm formation in *Staphylococcus aureus*," *J. Bacteriol.*, vol. 188, no. 13, pp. 4861–4870, Jul. 2006.
[132] K. Kobayashi, "Inactivation of cysL inhibits biofilm formation by activating the disulfide stress regulator Spx in *Bacillus subtilis*," *J. Bacteriol.*, Feb. 2019.
[133] J. D. Helmann *et al.*, "Global transcriptional response of *Bacillus subtilis* to heat shock," *J. Bacteriol.*, vol. 183, no. 24, pp. 7318–7328, Dec. 2001.
[134] D. F. Rojas-Tapias and J. D. Helmann, "Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in *Bacillus subtilis*," *Mol. Microbiol.*, vol. 107, no. 5, pp. 659–674, Mar. 2018.
[135] M. Leelakriangsak, K. Kobayashi, and P. Zuber, "Dual Negative Control of spx Transcription Initiation from the P3 Promoter by Repressors PerR and YodB in *Bacillus subtilis*," *J. Bacteriol.*, vol. 189, no. 5, pp. 1736–1744, Mar. 2007.
[136] S. Nakano, G. Zheng, M. M. Nakano, and P. Zuber, "Multiple pathways of Spx (YjbD) proteolysis in *Bacillus subtilis*," *J. Bacteriol.*, vol. 184, no. 13, pp. 3664–3670, Jul. 2002.
[137] J. T. Larsson, A. Rogstam, and C. von Wachenfeldt, "YjbH is a novel negative effector of the disulphide stress regulator, Spx, in *Bacillus subtilis*," *Mol. Microbiol.*, vol. 66, no. 3, pp. 669–684, Nov. 2007.
[138] S. K. Garg, S. Kommineni, L. Henslee, Y. Zhang, and P. Zuber, "The YjbH protein of *Bacillus subtilis* enhances ClpXP-catalyzed proteolysis of Spx," *J. Bacteriol.*, vol. 191, no. 4, pp. 1268–1277, Feb. 2009.
[139] D. F. Rojas-Tapias 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.*, vol. 201, no. 13, Jul. 2019.
[140] P. Zuber, "Spx-RNA polymerase interaction and global transcriptional control during oxidative stress," *J. Bacteriol.*, vol. 186, no. 7, pp. 1911–1918, Apr. 2004.
[141] Y. Zhang and P. Zuber, "Requirement of the zinc-binding domain of ClpX for Spx proteolysis in *Bacillus subtilis* and effects of disulfide stress on ClpXP activity," *J. Bacteriol.*, vol. 189, no. 21, pp. 7669–7680, Nov. 2007.
[142] J. Engman and C. von Wachenfeldt, "Regulated protein aggregation: a mechanism to control the activity of the ClpXP adaptor protein YjbH," *Mol. Microbiol.*, vol. 95, no. 1, pp. 51–63, Jan. 2015.

[143] D. F. Rojas-Tapias and J. D. Helmann, "Stabilization of *Bacillus subtilis* Spx under cell wall stress requires the anti-adaptor protein YirB," *PLoS Genet.*, vol. 14, no. 7, p. e1007531, Jul. 2018.
[144] S. Kommineni, S. K. Garg, C. M. Chan, and P. Zuber, "YjbH-enhanced proteolysis of Spx by ClpXP in *Bacillus subtilis* is inhibited by the small protein YirB (YuzO)," *J. Bacteriol.*, vol. 193, no. 9, pp. 2133–2140, May 2011.
[145] R. L. Gourse and S. Crosson, "Classic Spotlight: Studies of the Stringent Response," *J. Bacteriol.*, vol. 198, no. 12, pp. 1710–1710, Jun. 2016.
[146] E. Borek, A. Ryan, and J. Rockenbach, "Nucleic acid metabolism in relation to the lysogenic phenomenon," *J. Bacteriol.*, vol. 69, no. 4, pp. 460–467, Apr. 1955.
[147] E. Borek, J. Rockenbach, and A. Ryan, "Studies on a mutant of Escherichia coli with unbalanced ribonucleic acid synthesis," *J. Bacteriol.*, vol. 71, no. 3, pp. 318–323, Mar. 1956.
[148] L. Alföldi, G. S. Stent, and R. C. Clowes, "The chromosomal site of the RNA control (RC) locus in Escherichia coli," *J. Mol. Biol.*, vol. 5, no. 3, pp. 348–355, Sep. 1962.
[149] G. S. Stent and S. Brenner, "A GENETIC LOCUS FOR THE REGULATION OF RIBONUCLEIC ACID SYNTHESIS," *Proc. Natl. Acad. Sci.*, vol. 47, no. 12, pp. 2005–2014, Dec. 1961.
[150] M. Swanton and G. Edlin, "Isolation and characterization of an RNA relaxed mutant of B. subtilis," *Biochem. Biophys. Res. Commun.*, vol. 46, no. 2, pp. 583–588, Jan. 1972.
[151] M. Cashel and J. Gallant, "Two compounds implicated in the function of the RC gene of *Escherichia coli*," *Nature*, vol. 221, no. 5183, pp. 838–841, Mar. 1969.
[152] M. Cashel and B. Kalbacher, "The control of ribonucleic acid synthesis in Escherichia coli. V. Characterization of a nucleotide associated with the stringent response," *J. Biol. Chem.*, vol. 245, no. 9, pp. 2309–2318, May 1970.
[153] W. A. Haseltine, R. Block, W. Gilbert, and K. Weber, "MSI and MSII made on Ribosome in Idling Step of Protein Synthesis," *Nature*, vol. 238, no. 5364, pp. 381–384, Aug. 1972.
[154] W. A. Haseltine and R. Block, "Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 70, no. 5, pp. 1564–1568, May 1973.
[155] E. Bouveret and A. Battesti, "The Stringent Response," in *Bacterial Stress Responses, Second Edition*, R. Hengge and G. Storz, Eds. American Society of Microbiology, 2011, pp. 231–250.
[156] K. Potrykus and M. Cashel, "(p)ppGpp: still magical?," *Annu. Rev. Microbiol.*, vol. 62, pp. 35–51, 2008.
[157] G. C. Atkinson, T. Tenson, and V. Hauryliuk, "The RelA/SpoT Homolog (RSH) Superfamily: Distribution and Functional Evolution of ppGpp Synthetases and Hydrolases across the Tree of Life," *PLoS ONE*, vol. 6, no. 8, p. e23479, Aug. 2011.
[158] E. A. van der Biezen, J. Sun, M. J. Coleman, M. J. Bibb, and J. D. Jones, "Arabidopsis RelA/SpoT homologs implicate (p)ppGpp in plant signaling," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 97, no. 7, pp. 3747–3752, Mar. 2000.
[159] C. C. Boutte and S. Crosson, "Bacterial lifestyle shapes stringent response activation," *Trends Microbiol.*, vol. 21, no. 4, pp. 174–180, Apr. 2013.
[160] S. E. Irving and R. M. Corrigan, "Triggering the stringent response: signals responsible for activating (p)ppGpp synthesis in bacteria," *Microbiology*, vol. 164, no. 3, pp. 268–276, Mar. 2018.
[161] J. Sy and F. Lipmann, "Identification of the synthesis of guanosine tetraphosphate (MS I) as insertion of a pyrophosphoryl group into the 3'-position in guanosine 5'-diphosphate," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 70, no. 2, pp. 306–309, Feb. 1973.
[162] J. Sy, "*In vitro* degradation of guanosine 5'-diphosphate, 3'-diphosphate," *Proc. Natl. Acad. Sci.*, vol. 74, no. 12, pp. 5529–5533, 1977.
[163] G. Schreiber, S. Metzger, E. Aizenman, S. Roza, M. Cashel, and G. Glaser, "Overexpression of the relA gene in *Escherichia coli*," *J. Biol. Chem.*, vol. 266, no. 6, pp. 3760–3767, Feb. 1991.
[164] W. Steinchen and G. Bange, "The magic dance of the alarmones (p)ppGpp: The structural biology of the alarmones (p)ppGpp," *Mol. Microbiol.*, vol. 101, no. 4, pp. 531–544, Aug. 2016.
[165] G. Mittenhuber, "Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins)," *J. Mol. Microbiol. Biotechnol.*, vol. 3, no. 4, pp. 585–600, Oct. 2001.
[166] H. Nanamiya *et al.*, "Identification and functional analysis of novel (p)ppGpp synthetase genes in *Bacillus subtilis*," *Mol. Microbiol.*, vol. 67, no. 2, pp. 291–304, Jan. 2008.

[167] T. M. Wendrich and M. A. Marahiel, "Cloning and characterization of a relA/spoT homologue from *Bacillus subtilis*," *Mol. Microbiol.*, vol. 26, no. 1, pp. 65–79, Oct. 1997.
[168] H. Xiao, M. Kalman, K. Ikehara, S. Zemel, G. Glaser, and M. Cashel, "Residual guanosine 3',5'-bispyrophosphate synthetic activity of relA null mutants can be eliminated by spoT null mutations," *J. Biol. Chem.*, vol. 266, no. 9, pp. 5980–5990, Mar. 1991.
[169] K. D. Murray and H. Bremer, "Control of spoT-dependent ppGpp synthesis and degradation in Escherichia coli," *J. Mol. Biol.*, vol. 259, no. 1, pp. 41–57, May 1996.
[170] J. D. Keasling, L. Bertsch, and A. Kornberg, "Guanosine pentaphosphate phosphohydrolase of Escherichia coli is a long-chain exopolyphosphatase," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 90, no. 15, pp. 7029–7033, Aug. 1993.
[171] U. Mechold, K. Potrykus, H. Murphy, K. S. Murakami, and M. Cashel, "Differential regulation by ppGpp versus pppGpp in Escherichia coli," *Nucleic Acids Res.*, vol. 41, no. 12, pp. 6175–6189, Jul. 2013.
[172] J. D. Wang, G. M. Sanders, and A. D. Grossman, "Nutritional control of elongation of DNA replication by (p)ppGpp," *Cell*, vol. 128, no. 5, pp. 865–875, Mar. 2007.
[173] Y. Zhang, E. Zborníková, D. Rejman, and K. Gerdes, "Novel (p)ppGpp Binding and Metabolizing Proteins of *Escherichia coli*," *mBio*, vol. 9, no. 2, 06 2018.
[174] T. Ooga *et al.*, "Degradation of ppGpp by nudix pyrophosphatase modulates the transition of growth phase in the bacterium Thermus thermophilus," *J. Biol. Chem.*, vol. 284, no. 23, pp. 15549–15556, Jun. 2009.
[175] V. Hauryliuk, G. C. Atkinson, K. S. Murakami, T. Tenson, and K. Gerdes, "Recent functional insights into the role of (p)ppGpp in bacterial physiology," *Nat. Rev. Microbiol.*, vol. 13, no. 5, pp. 298–309, May 2015.
[176] A. O. Gaca, C. Colomer-Winter, and J. A. Lemos, "Many means to a common end: the intricacies of (p)ppGpp metabolism and its control of bacterial homeostasis," *J. Bacteriol.*, vol. 197, no. 7, pp. 1146–1156, Apr. 2015.
[177] S. Ronneau and R. Hallez, "Make and break the alarmone: regulation of (p)ppGpp synthetase/hydrolase enzymes in bacteria," *FEMS Microbiol. Rev.*, Mar. 2019.
[178] T. M. Wendrich, G. Blaha, D. N. Wilson, M. A. Marahiel, and K. H. Nierhaus, "Dissection of the Mechanism for the Stringent Factor RelA," *Mol. Cell*, vol. 10, no. 4, pp. 779–788, Oct. 2002.
[179] I. Smith, P. Paress, and S. Pestka, "Thiostrepton-resistant mutants exhibit relaxed synthesis of RNA," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 75, no. 12, pp. 5993–5997, Dec. 1978.
[180] I. Smith, P. Paress, K. Cabane, and E. Dubnau, "Genetics and physiology of the rel system of Bacillus subtilis," *MGG Mol. Gen. Genet.*, vol. 178, no. 2, pp. 271–279, May 1980.
[181] M. Cashel, "The Control of Ribonucleic Acid Synthesis in Escherichia coli : IV. RELEVANCE OF UNUSUAL PHOSPHORYLATED COMPOUNDS FROM AMINO ACID-STARVED STRINGENT STRAINS," *J. Biol. Chem.*, vol. 244, no. 12, pp. 3133–3141, Jun. 1969.
[182] M. Gropp, Y. Strausz, M. Gross, and G. Glaser, "Regulation of Escherichia coli RelA requires oligomerization of the C-terminal domain," *J. Bacteriol.*, vol. 183, no. 2, pp. 570–579, Jan. 2001.
[183] X. Yang and E. E. Ishiguro, "Dimerization of the RelA protein of Escherichia coli," *Biochem. Cell Biol. Biochim. Biol. Cell.*, vol. 79, no. 6, pp. 729–736, 2001.
[184] U. Mechold, H. Murphy, L. Brown, and M. Cashel, "Intramolecular regulation of the opposing (p)ppGpp catalytic activities of Rel(Seq), the Rel/Spo enzyme from Streptococcus equisimilis," *J. Bacteriol.*, vol. 184, no. 11, pp. 2878–2888, Jun. 2002.
[185] T. Hogg, U. Mechold, H. Malke, M. Cashel, and R. Hilgenfeld, "Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp metabolism during the stringent response [corrected]," *Cell*, vol. 117, no. 1, pp. 57–68, Apr. 2004.
[186] B. P. English, V. Hauryliuk, A. Sanamrad, S. Tankov, N. H. Dekker, and J. Elf, "Single-molecule investigations of the stringent response machinery in living bacterial cells," *Proc. Natl. Acad. Sci.*, vol. 108, no. 31, pp. E365–E373, Aug. 2011.
[187] W. Li, E. Bouveret, Y. Zhang, K. Liu, J. D. Wang, and J. C. Weisshaar, "Effects of amino acid starvation on RelA diffusive behavior in live Escherichia coli," *Mol. Microbiol.*, vol. 99, no. 3, pp. 571–585, Feb. 2016.
[188] S. Arenz *et al.*, "The stringent factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis," *Nucleic Acids Res.*, vol. 44, no. 13, pp. 6471–6481, 27 2016.

[189] A. B. Loveland *et al.*, "Ribosome•RelA structures reveal the mechanism of stringent response activation," *eLife*, vol. 5, 19 2016.
[190] A. Brown, I. S. Fernández, Y. Gordiyenko, and V. Ramakrishnan, "Ribosome-dependent activation of stringent control," *Nature*, vol. 534, no. 7606, pp. 277–280, 09 2016.
[191] K. S. Winther, M. Roghanian, and K. Gerdes, "Activation of the Stringent Response by Loading of RelA-tRNA Complexes at the Ribosomal A-Site," *Mol. Cell*, vol. 70, no. 1, pp. 95-105.e4, 05 2018.
[192] G. S. Kushwaha, G. Bange, and N. S. Bhavesh, "Interaction studies on bacterial stringent response protein RelA with uncharged tRNA provide evidence for its prerequisite complex for ribosome binding," *Curr. Genet.*, Apr. 2019.
[193] P. Kudrin *et al.*, "The ribosomal A-site finger is crucial for binding and activation of the stringent factor RelA," *Nucleic Acids Res.*, vol. 46, no. 4, pp. 1973–1983, Feb. 2018.
[194] E. A. Heinemeyer and D. Richter, "Characterization of the guanosine 5'-triphosphate 3'-diphosphate and guanosine 5'-diphosphate 3'-diphosphate degradation reaction catalyzed by a specific pyrophosphorylase from Escherichia coli," *Biochemistry*, vol. 17, no. 25, pp. 5368–5372, Dec. 1978.
[195] P. Wout *et al.*, "The Escherichia coli GTPase CgtAE cofractionates with the 50S ribosomal subunit and interacts with SpoT, a ppGpp synthetase/hydrolase," *J. Bacteriol.*, vol. 186, no. 16, pp. 5249–5257, Aug. 2004.
[196] D. Richter, "Uncharged tRNA inhibits guanosine 3',5'-bis (diphosphate) 3'-pyrophosphohydrolase [ppGppase], the spoT gene product, from Escherichia coli," *Mol. Gen. Genet. MGG*, vol. 178, no. 2, pp. 325–327, 1980.
[197] A. Battesti and E. Bouveret, "Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism," *Mol. Microbiol.*, vol. 62, no. 4, pp. 1048–1063, Nov. 2006.
[198] M. Seyfzadeh, J. Keener, and M. Nomura, "spoT-dependent accumulation of guanosine tetraphosphate in response to fatty acid starvation in Escherichia coli," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 90, no. 23, pp. 11004–11008, Dec. 1993.
[199] A. Battesti and E. Bouveret, "Bacteria possessing two RelA/SpoT-like proteins have evolved a specific stringent response involving the acyl carrier protein-SpoT interaction," *J. Bacteriol.*, vol. 191, no. 2, pp. 616–624, Jan. 2009.
[200] A. A. Pulschen *et al.*, "The stringent response plays a key role in *Bacillus subtilis* survival of fatty acid starvation," *Mol. Microbiol.*, vol. 103, no. 4, pp. 698–712, Feb. 2017.
[201] G. Chaloner-Larsson and H. Yamazaki, "Effects of the *spoT* and *relA* Mutation on the Synthesis and Accumulation of ppGpp and RNA during Glucose Starvation," *Can. J. Biochem.*, vol. 56, no. 4, pp. 264–272, Apr. 1978.
[202] T. Nishino, J. Gallant, P. Shalit, L. Palmer, and T. Wehr, "Regulatory nucleotides involved in the Rel function of Bacillus subtilis," *J. Bacteriol.*, vol. 140, no. 2, pp. 671–679, Nov. 1979.
[203] D. R. Gentry and M. Cashel, "Mutational analysis of the Escherichia coli spoT gene identifies distinct but overlapping regions involved in ppGpp synthesis and degradation," *Mol. Microbiol.*, vol. 19, no. 6, pp. 1373–1384, Mar. 1996.
[204] J.-W. Lee, Y.-H. Park, and Y.-J. Seok, "Rsd balances (p)ppGpp level by stimulating the hydrolase activity of SpoT during carbon source downshift in *Escherichia coli*," *Proc. Natl. Acad. Sci.*, vol. 115, no. 29, pp. E6845–E6854, Jul. 2018.
[205] J. Hahn, A. W. Tanner, V. J. Carabetta, I. M. Cristea, and D. Dubnau, "ComGA-RelA interaction and persistence in the *Bacillus subtilis* K-state," *Mol. Microbiol.*, vol. 97, no. 3, pp. 454–471, Aug. 2015.
[206] M. Fang and C. E. Bauer, "Regulation of stringent factor by branched-chain amino acids," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 115, no. 25, pp. 6446–6451, 19 2018.
[207] V. Shyp *et al.*, "Positive allosteric feedback regulation of the stringent response enzyme RelA by its product," *EMBO Rep.*, vol. 13, no. 9, pp. 835–839, Sep. 2012.
[208] W. Steinchen *et al.*, "Catalytic mechanism and allosteric regulation of an oligomeric (p)ppGpp synthetase by an alarmone," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 112, no. 43, pp. 13348–13353, Oct. 2015.

[209] J. Beljantseva *et al.*, "Negative allosteric regulation of Enterococcus faecalis small alarmone synthetase RelQ by single-stranded RNA," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 114, no. 14, pp. 3726–3731, 04 2017.
[210] T. L. Czarny, A. L. Perri, S. French, and E. D. Brown, "Discovery of novel cell wall-active compounds using P ywaC, a sensitive reporter of cell wall stress, in the model gram-positive bacterium Bacillus subtilis," *Antimicrob. Agents Chemother.*, vol. 58, no. 6, pp. 3261–3269, Jun. 2014.
[211] K. Liu, A. N. Bittner, and J. D. Wang, "Diversity in (p)ppGpp metabolism and effectors," *Curr. Opin. Microbiol.*, vol. 24, pp. 72–79, Apr. 2015.
[212] U. Kanjee, K. Ogata, and W. A. Houry, "Direct binding targets of the stringent response alarmone (p)ppGpp: Protein targets of ppGpp," *Mol. Microbiol.*, vol. 85, no. 6, pp. 1029–1043, Sep. 2012.
[213] M. F. Traxler *et al.*, "The global, ppGpp-mediated stringent response to amino acid starvation in Escherichia coli," *Mol. Microbiol.*, vol. 68, no. 5, pp. 1128–1148, Jun. 2008.
[214] C. Eymann, G. Homuth, C. Scharf, and M. Hecker, "*Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis," *J. Bacteriol.*, vol. 184, no. 9, pp. 2500–2520, Jan. 2002.
[215] L. Krásný and R. L. Gourse, "An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation," *EMBO J.*, vol. 23, no. 22, pp. 4473–4483, Nov. 2004.
[216] M. Zhu and X. Dai, "Growth suppression by altered (p)ppGpp levels results from non-optimal resource allocation in *Escherichia coli*," *Nucleic Acids Res.*, Mar. 2019.
[217] A. Kriel *et al.*, "GTP Dysregulation in *Bacillus subtilis* Cells Lacking (p)ppGpp Results in Phenotypic Amino Acid Auxotrophy and Failure To Adapt to Nutrient Downshift and Regulate Biosynthesis Genes," *J. Bacteriol.*, vol. 196, no. 1, pp. 189–201, Jan. 2014.
[218] R. L. Gourse, A. Y. Chen, S. Gopalkrishnan, P. Sanchez-Vazquez, A. Myers, and W. Ross, "Transcriptional Responses to ppGpp and DksA," *Annu. Rev. Microbiol.*, vol. 72, no. 1, pp. 163–184, Sep. 2018.
[219] B. J. Paul *et al.*, "DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP," *Cell*, vol. 118, no. 3, pp. 311–322, Aug. 2004.
[220] P. Sanchez-Vazquez, C. N. Dewey, N. Kitten, W. Ross, and R. L. Gourse, "Genome-wide effects on *Escherichia coli* transcription from ppGpp binding to its two sites on RNA polymerase," *Proc. Natl. Acad. Sci.*, vol. 116, no. 17, pp. 8310–8319, Apr. 2019.
[221] S. Österberg, T. del Peso-Santos, and V. Shingler, "Regulation of Alternative Sigma Factor Use," *Annu. Rev. Microbiol.*, vol. 65, no. 1, pp. 37–55, Oct. 2011.
[222] C. Wolz, T. Geiger, and C. Goerke, "The synthesis and function of the alarmone (p)ppGpp in firmicutes," *Int. J. Med. Microbiol.*, vol. 300, no. 2–3, pp. 142–147, Feb. 2010.
[223] J. M. Lopez, A. Dromerick, and E. Freese, "Response of guanosine 5'-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation," *J. Bacteriol.*, vol. 146, no. 2, pp. 605–613, May 1981.
[224] A. Kriel *et al.*, "Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance," *Mol. Cell*, vol. 48, no. 2, pp. 231–241, Oct. 2012.
[225] K. Liu *et al.*, "Molecular Mechanism and Evolution of Guanylate Kinase Regulation by (p)ppGpp," *Mol. Cell*, vol. 57, no. 4, pp. 735–749, Feb. 2015.
[226] S. Tojo, K. Kumamoto, K. Hirooka, and Y. Fujita, "Heavy involvement of stringent transcription control depending on the adenine or guanine species of the transcription initiation site in glucose and pyruvate metabolism in *Bacillus subtilis*," *J. Bacteriol.*, vol. 192, no. 6, pp. 1573–1585, Mar. 2010.
[227] J. M. Lopez, C. L. Marks, and E. Freese, "The decrease of guanine nucleotides initiates sporulation of *Bacillus subtilis*," *Biochim. Biophys. Acta*, vol. 587, no. 2, pp. 238–252, Oct. 1979.
[228] L. Krásný, H. Tišerová, J. Jonák, D. Rejman, and H. Šanderová, "The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in *Bacillus subtilis*," *Mol. Microbiol.*, vol. 69, no. 1, pp. 42–54, Jul. 2008.
[229] M. Ratnayake-Lecamwasam, P. Serror, K. W. Wong, and A. L. Sonenshein, "*Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels," *Genes Dev.*, vol. 15, no. 9, pp. 1093–1103, May 2001.

[230] R. P. Shivers and A. L. Sonenshein, "Activation of the Bacillus subtilis global regulator CodY by direct interaction with branched-chain amino acids," *Mol. Microbiol.*, vol. 53, no. 2, pp. 599–611, Jul. 2004.
[231] T. Geiger and C. Wolz, "Intersection of the stringent response and the CodY regulon in low GC Gram-positive bacteria," *Int. J. Med. Microbiol.*, vol. 304, no. 2, pp. 150–155, Mar. 2014.
[232] C. Eymann, G. Mittenhuber, and M. Hecker, "The stringent response, sigmaH-dependent gene expression and sporulation in Bacillus subtilis," *Mol. Gen. Genet. MGG*, vol. 264, no. 6, pp. 913–923, Feb. 2001.
[233] K. Ochi, J. C. Kandala, and E. Freese, "Initiation of Bacillus subtilis sporulation by the stringent response to partial amino acid deprivation," *J. Biol. Chem.*, vol. 256, no. 13, pp. 6866–6875, Jul. 1981.
[234] T. Inaoka and K. Ochi, "RelA protein is involved in induction of genetic competence in certain Bacillus subtilis strains by moderating the level of intracellular GTP," *J. Bacteriol.*, vol. 184, no. 14, pp. 3923–3930, Jul. 2002.
[235] S. Tojo, K. Hirooka, and Y. Fujita, "Expression of kinA and kinB of Bacillus subtilis, necessary for sporulation initiation, is under positive stringent transcription control," *J. Bacteriol.*, vol. 195, no. 8, pp. 1656–1665, Apr. 2013.
[236] Y. Fujita, M. Ogura, S. Nii, and K. Hirooka, "Dual Regulation of Bacillus subtilis kinB Gene Encoding a Sporulation Trigger by SinR through Transcription Repression and Positive Stringent Transcription Control," *Front. Microbiol.*, vol. 8, p. 2502, 2017.
[237] P. Serror and A. L. Sonenshein, "CodY is required for nutritional repression of Bacillus subtilis genetic competence," *J. Bacteriol.*, vol. 178, no. 20, pp. 5910–5915, Oct. 1996.
[238] Q. O. Ababneh and J. K. Herman, "CodY Regulates SigD Levels and Activity by Binding to Three Sites in the fla/che Operon," *J. Bacteriol.*, vol. 197, no. 18, pp. 2999–3006, Sep. 2015.
[239] A. L. Svitil, M. Cashel, and J. W. Zyskind, "Guanosine tetraphosphate inhibits protein synthesis *in vivo*. A possible protective mechanism for starvation stress in *Escherichia coli*," *J. Biol. Chem.*, vol. 268, no. 4, pp. 2307–2311, Feb. 1993.
[240] L. Legault, C. Jeantet, and F. Gros, "Inhibition of in vitro protein synthesis by ppGpp," *FEBS Lett.*, vol. 27, no. 1, pp. 71–75, Oct. 1972.
[241] E. Hamel and M. Cashel, "Guanine nucleotides in protein synthesis. Utilization of pppGpp and dGTP by initiation factor 2 and elongation factor Tu," *Arch. Biochem. Biophys.*, vol. 162, no. 1, pp. 293–300, May 1974.
[242] P. Milon *et al.*, "The nucleotide-binding site of bacterial translation initiation factor 2 (IF2) as a metabolic sensor," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 38, pp. 13962–13967, Sep. 2006.
[243] K. Kihira *et al.*, "Crystal structure analysis of the translation factor RF3 (release factor 3)," *FEBS Lett.*, vol. 586, no. 20, pp. 3705–3709, Oct. 2012.
[244] R. M. Corrigan, L. E. Bellows, A. Wood, and A. Gründling, "ppGpp negatively impacts ribosome assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 113, no. 12, pp. E1710-1719, Mar. 2016.
[245] N. Verstraeten, M. Fauvart, W. Versées, and J. Michiels, "The universally conserved prokaryotic GTPases," *Microbiol. Mol. Biol. Rev. MMBR*, vol. 75, no. 3, pp. 507–542, second and third pages of table of contents, Sep. 2011.
[246] P. Pausch *et al.*, "Structural basis for (p)ppGpp-mediated inhibition of the GTPase RbgA," *J. Biol. Chem.*, vol. 293, no. 51, pp. 19699–19709, 21 2018.
[247] S. Gkekas *et al.*, "Structural and biochemical analysis of Escherichia coli ObgE, a central regulator of bacterial persistence," *J. Biol. Chem.*, vol. 292, no. 14, pp. 5871–5883, 07 2017.
[248] C. Kint, N. Verstraeten, J. Hofkens, M. Fauvart, and J. Michiels, "Bacterial Obg proteins: GTPases at the nexus of protein and DNA synthesis," *Crit. Rev. Microbiol.*, vol. 40, no. 3, pp. 207–224, Aug. 2014.
[249] K. Drzewiecki, C. Eymann, G. Mittenhuber, and M. Hecker, "The yvyD gene of *Bacillus subtilis* is under dual control of sigmaB and sigmaH," *J. Bacteriol.*, vol. 180, no. 24, pp. 6674–6680, Dec. 1998.
[250] K. Tagami *et al.*, "Expression of a small (p)ppGpp synthetase, YwaC, in the (p)ppGpp(0) mutant of *Bacillus subtilis* triggers YvyD-dependent dimerization of ribosome," *MicrobiologyOpen*, vol. 1, no. 2, pp. 115–134, Jun. 2012.

[251] G. Akanuma *et al.*, "Ribosome dimerization is essential for the efficient regrowth of *Bacillus subtilis*," *Microbiology*, vol. 162, no. 3, pp. 448–458, 2016.
[252] D. W. Gohara and M.-N. F. Yap, "Survival of the drowsiest: the hibernating 100S ribosome in bacterial stress management," *Curr. Genet.*, vol. 64, no. 4, pp. 753–760, Aug. 2018.
[253] M. Ueta *et al.*, "Conservation of two distinct types of 100S ribosome in bacteria," *Genes Cells Devoted Mol. Cell. Mech.*, vol. 18, no. 7, pp. 554–574, Jul. 2013.
[254] B. Beckert *et al.*, "Structure of the *Bacillus subtilis* hibernating 100S ribosome reveals the basis for 70S dimerization," *EMBO J.*, vol. 36, no. 14, pp. 2061–2072, 14 2017.
[255] B. Beckert *et al.*, "Structure of a hibernating 100S ribosome reveals an inactive conformation of the ribosomal protein S1," *Nat. Microbiol.*, vol. 3, no. 10, pp. 1115–1121, Oct. 2018.
[256] A. Wada, Y. Yamazaki, N. Fujita, and A. Ishihama, "Structure and probable genetic location of a 'ribosome modulation factor' associated with 100S ribosomes in stationary-phase Escherichia coli cells," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 87, no. 7, pp. 2657–2661, Apr. 1990.
[257] M. Ueta, R. L. Ohniwa, H. Yoshida, Y. Maki, C. Wada, and A. Wada, "Role of HPF (hibernation promoting factor) in translational activity in Escherichia coli," *J. Biochem. (Tokyo)*, vol. 143, no. 3, pp. 425–433, Mar. 2008.
[258] A. Basu and M.-N. F. Yap, "Disassembly of the Staphylococcus aureus hibernating 100S ribosome by an evolutionarily conserved GTPase," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 114, no. 39, pp. E8165–E8173, 26 2017.
[259] S. Autret, A. Levine, F. Vannier, Y. Fujita, and S. J. Séror, "The replication checkpoint control in Bacillus subtilis: identification of a novel RTP-binding sequence essential for the replication fork arrest after induction of the stringent response," *Mol. Microbiol.*, vol. 31, no. 6, pp. 1665–1679, Mar. 1999.
[260] A. Levine, S. Autret, and S. J. Séror, "A checkpoint involving RTP, the replication terminator protein, arrests replication downstream of the origin during the Stringent Response in Bacillus subtilis," *Mol. Microbiol.*, vol. 15, no. 2, pp. 287–295, Jan. 1995.
[261] J. Denapoli, A. K. Tehranchi, and J. D. Wang, "Dose-dependent reduction of replication elongation rate by (p)ppGpp in *Escherichia coli* and *Bacillus subtilis*," *Mol. Microbiol.*, vol. 88, no. 1, pp. 93–104, Apr. 2013.
[262] J. P. Merlie and L. I. Pizer, "Regulation of phospholipid synthesis in Escherichia coli by guanosine tetraphosphate," *J. Bacteriol.*, vol. 116, no. 1, pp. 355–366, Oct. 1973.
[263] T. N. Huynh, S. Luo, D. Pensinger, J.-D. Sauer, L. Tong, and J. J. Woodward, "An HD-domain phosphodiesterase mediates cooperative hydrolysis of c-di-AMP to affect bacterial growth and virulence," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 112, no. 7, pp. E747-756, Feb. 2015.
[264] F. Rao, R. Y. See, D. Zhang, D. C. Toh, Q. Ji, and Z.-X. Liang, "YybT is a signaling protein that contains a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain with ATPase activity," *J. Biol. Chem.*, vol. 285, no. 1, pp. 473–482, Jan. 2010.
[265] R. M. Corrigan, L. Bowman, A. R. Willis, V. Kaever, and A. Gründling, "Cross-talk between two nucleotide-signaling pathways in Staphylococcus aureus," *J. Biol. Chem.*, vol. 290, no. 9, pp. 5826–5839, Feb. 2015.
[266] F. M. Commichau, A. Dickmanns, J. Gundlach, R. Ficner, and J. Stülke, "A jack of all trades: the multiple roles of the unique essential second messenger cyclic di-AMP," *Mol. Microbiol.*, vol. 97, no. 2, pp. 189–204, Jul. 2015.
[267] B. Wang *et al.*, "Affinity-based capture and identification of protein effectors of the growth regulator ppGpp," *Nat. Chem. Biol.*, vol. 15, no. 2, pp. 141–150, Feb. 2019.
[268] F. C. Neidhardt, R. A. VanBogelen, and V. Vaughn, "The genetics and regulation of heat-shock proteins," *Annu. Rev. Genet.*, vol. 18, pp. 295–329, 1984.
[269] M. Cashel, D. R. Gentry, V. J. Hernandez, and D. Vinella, "The Stringent Response," in *Escherichia Coli and Salmonella: Typhimurium Cellular and Molecular Biology*, 2nd ed., vol. 1, F. C. Neidhardt and R. Curtiss, Eds. Washington, D.C: ASM Press, 1996, pp. 1458–1496.
[270] E. Lund and N. O. Kjeldgaard, "Metabolism of guanosine tetraphosphate in Escherichia coli," *Eur. J. Biochem.*, vol. 28, no. 3, pp. 316–326, Jul. 1972.
[271] J. Gallant, L. Palmer, and C. C. Pao, "Anomalous synthesis of ppGpp in growing cells," *Cell*, vol. 11, no. 1, pp. 181–185, May 1977.
[272] G. Chaloner-Larsson and H. Yamazaki, "Adjustment of RNA content during temperature upshift in Escherichia coli," *Biochem. Biophys. Res. Commun.*, vol. 77, no. 2, pp. 503–508, Jul. 1977.

[273] P. C. Lee, B. R. Bochner, and B. N. Ames, "AppppA, heat-shock stress, and cell oxidation.," *Proc. Natl. Acad. Sci.*, vol. 80, no. 24, pp. 7496–7500, Dec. 1983.
[274] R. A. VanBogelen, P. M. Kelley, and F. C. Neidhardt, "Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli*," *J. Bacteriol.*, vol. 169, no. 1, pp. 26–32, Jan. 1987.
[275] A. D. Grossman, W. E. Taylor, Z. F. Burton, R. R. Burgess, and C. A. Gross, "Stringent response in Escherichia coli induces expression of heat shock proteins," *J. Mol. Biol.*, vol. 186, no. 2, pp. 357–365, Nov. 1985.
[276] M. Hecker, A. Richter, A. Schroeter, L. Wölfel, and F. Mach, "[Synthesis of heat shock proteins following amino acid or oxygen limitation in *Bacillus subtilis* relA+ and relA strains]," *Z. Für Naturforschung C J. Biosci.*, vol. 42, no. 7–8, pp. 941–947, Aug. 1987.
[277] R. A. VanBogelen and F. C. Neidhardt, "Ribosomes as sensors of heat and cold shock in *Escherichia coli*," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 87, no. 15, pp. 5589–5593, Aug. 1990.
[278] B. R. Bochner, P. C. Lee, S. W. Wilson, C. W. Cutler, and B. N. Ames, "AppppA and related adenylylated nucleotides are synthesized as a consequence of oxidation stress," *Cell*, vol. 37, no. 1, pp. 225–232, May 1984.
[279] M. Hecker and A. Richter, "Physiologischer Untersuchungen zur Bildung von Hitzeschockproteinen inBacillus subtilis," *J. Basic Microbiol.*, vol. 27, no. 5, pp. 253–261, 1987.
[280] A. Richter and M. Hecker, "Heat-shock proteins in *Bacillus subtilis* : a two-dimensional gel electrophoresis study," *FEMS Microbiol. Lett.*, vol. 36, no. 1, pp. 69–71, Aug. 1986.
[281] M. Hecker and U. Völker, "General stress proteins in *Bacillus subtilis*," *FEMS Microbiol. Lett.*, vol. 74, no. 2–3, pp. 197–213, Dec. 1990.
[282] M. Hecker, U. Völker, and C. Heim, "RelA-independent (p)ppGpp accumulation and heat shock protein induction after salt stress in *Bacillus subtilis*," *FEMS Microbiol. Lett.*, vol. 58, no. 2–3, pp. 125–128, Apr. 1989.
[283] J. M. Lopez and P. Fortnagel, "Nitrofurantoin prompts the stringent response in Bacillus subtilis," *J. Gen. Microbiol.*, vol. 126, no. 2, pp. 491–496, Oct. 1981.
[284] D.-C. Pöther *et al.*, "Diamide triggers mainly S Thiolations in the cytoplasmic proteomes of *Bacillus subtilis* and *Staphylococcus aureus*," *J. Bacteriol.*, vol. 191, no. 24, pp. 7520–7530, Dec. 2009.
[285] S. Luche, E. Eymard-Vernain, H. Diemer, A. Van Dorsselaer, T. Rabilloud, and C. Lelong, "Zinc oxide induces the stringent response and major reorientations in the central metabolism of Bacillus subtilis," *J. Proteomics*, vol. 135, pp. 170–180, Mar. 2016.
[286] L. I. O. Leichert, C. Scharf, and M. Hecker, "Global characterization of disulfide stress in *Bacillus subtilis*," *J. Bacteriol.*, vol. 185, no. 6, pp. 1967–1975, Mar. 2003.
[287] J. Mostertz, C. Scharf, M. Hecker, and G. Homuth, "Transcriptome and proteome analysis of *Bacillus subtilis* gene expression in response to superoxide and peroxide stress," *Microbiol. Read. Engl.*, vol. 150, no. Pt 2, pp. 497–512, Feb. 2004.
[288] A. Heinz, "Die molekulare Charakterisierung der Thermotoleranz in Bacillus subtilis," Master Thesis, Freie Universität Berlin, Berlin.
[289] D. M. Cameron, J. Thompson, S. T. Gregory, P. E. March, and A. E. Dahlberg, "Thiostrepton-resistant mutants of Thermus thermophilus," *Nucleic Acids Res.*, vol. 32, no. 10, pp. 3220–3227, 2004.
[290] I. Hantke, H. Schäfer, A. Janczikowski, and K. Turgay, "YocM a small heat shock protein can protect *Bacillus subtilis* cells during salt stress," *Mol. Microbiol.*, vol. 111, no. 2, pp. 423–440, Feb. 2019.
[291] N. Van Dyke and E. J. Murgola, "Site of Functional Interaction of Release Factor 1 with the Ribosome," *J. Mol. Biol.*, vol. 330, no. 1, pp. 9–13, Jun. 2003.
[292] L. F. Fitzsimmons, L. Liu, J.-S. Kim, J. Jones-Carson, and A. Vázquez-Torres, "*Salmonella* Reprograms Nucleotide Metabolism in Its Adaptation to Nitrosative Stress," *mBio*, vol. 9, no. 1, pp. e00211-18, Feb. 2018.
[293] I. Rosinski-Chupin, O. Soutourina, and I. Martin-Verstraete, "Riboswitch Discovery by Combining RNA-Seq and Genome-Wide Identification of Transcriptional Start Sites," in *Methods in Enzymology*, vol. 549, Elsevier, 2014, pp. 3–27.
[294] H. L. Carter, L. F. Wang, R. H. Doi, and C. P. Moran, "rpoD operon promoter used by sigma H-RNA polymerase in Bacillus subtilis," *J. Bacteriol.*, vol. 170, no. 4, pp. 1617–1621, Apr. 1988.

[295] L. F. Wang, C. W. Price, and R. H. Doi, "Bacillus subtilis dnaE encodes a protein homologous to DNA primase of Escherichia coli," *J. Biol. Chem.*, vol. 260, no. 6, pp. 3368–3372, Mar. 1985.
[296] I. Irnov, C. M. Sharma, J. Vogel, and W. C. Winkler, "Identification of regulatory RNAs in *Bacillus subtilis*," *Nucleic Acids Res.*, vol. 38, no. 19, pp. 6637–6651, Jan. 2010.
[297] V. Ravikumar *et al.*, "In-depth analysis of *Bacillus subtilis* proteome identifies new ORFs and traces the evolutionary history of modified proteins," *Sci. Rep.*, vol. 8, no. 1, p. 17246, Nov. 2018.
[298] J. Stülke, R. Hanschke, and M. Hecker, "Temporal activation of beta-glucanase synthesis in *Bacillus subtilis* is mediated by the GTP pool," *J. Gen. Microbiol.*, vol. 139, no. 9, pp. 2041–2045, Sep. 1993.
[299] J. Sambrook and D. W. Russell, *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press, 2001.
[300] M. Arnaud, A. Chastanet, and M. Débarbouillé, "New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria," *Appl. Environ. Microbiol.*, vol. 70, no. 11, pp. 6887–6891, Nov. 2004.
[301] B.-M. Koo *et al.*, "Construction and Analysis of Two Genome-Scale Deletion Libraries for Bacillus subtilis," *Cell Syst.*, vol. 4, no. 3, pp. 291-305.e7, 22 2017.
[302] H. Schäfer *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.*, vol. 111, no. 2, pp. 514–533, Feb. 2019.
[303] A. Petersohn, H. Antelmann, U. Gerth, and M. Hecker, "Identification and transcriptional analysis of new members of the sigmaB regulon in *Bacillus subtilis*," *Microbiol. Read. Engl.*, vol. 145 (Pt 4), pp. 869–880, Apr. 1999.
[304] H. Bremer and P. P. Dennis, "Modulation of Chemical Composition and Other Parameters of the Cell at Different Exponential Growth Rates," *EcoSal Plus*, vol. 3, no. 1, Sep. 2008.
[305] S. Maaβ *et al.*, "Highly precise quantification of protein molecules per cell during stress and starvation responses in *Bacillus subtilis*," *Mol. Cell. Proteomics MCP*, vol. 13, no. 9, pp. 2260–2276, Sep. 2014.
[306] D. Shah, Z. Zhang, A. Khodursky, N. Kaldalu, K. Kurg, and K. Lewis, "Persisters: a distinct physiological state of *E. coli*," *BMC Microbiol.*, vol. 6, p. 53, Jun. 2006.
[307] H. Schäfer and K. Turgay, "Spx, a versatile regulator of the *Bacillus subtilis* stress response," *Curr. Genet.*, Mar. 2019.
[308] M. Kaczanowska and M. Ryden-Aulin, "Ribosome Biogenesis and the Translation Process in Escherichia coli," *Microbiol. Mol. Biol. Rev.*, vol. 71, no. 3, pp. 477–494, Sep. 2007.
[309] O. René and J.-H. Alix, "Late steps of ribosome assembly in E. coli are sensitive to a severe heat stress but are assisted by the HSP70 chaperone machine," *Nucleic Acids Res.*, vol. 39, no. 5, pp. 1855–1867, Mar. 2011.
[310] L. D. Handke, R. P. Shivers, and A. L. Sonenshein, "Interaction of Bacillus subtilis CodY with GTP," *J. Bacteriol.*, vol. 190, no. 3, pp. 798–806, Feb. 2008.
[311] W. Ross *et al.*, "A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase," *Science*, vol. 262, no. 5138, pp. 1407–1413, Nov. 1993.
[312] R. L. Gourse, W. Ross, and T. Gaal, "UPs and downs in bacterial transcription initiation: the role of the alpha subunit of RNA polymerase in promoter recognition," *Mol. Microbiol.*, vol. 37, no. 4, pp. 687–695, Aug. 2000.
[313] J. D. Helmann, "Compilation and analysis of *Bacillus Subtilis* σ^{A} -dependent promoter sequences: evidence for extended contact between RNA polymerse and upstream promoter DNA," *Nucleic Acids Res.*, vol. 23, no. 13, pp. 2351–2360, 1995.
[314] L. Rao *et al.*, "Factor independent activation of rrnB P1. An 'extended' promoter with an upstream element that dramatically increases promoter strength," *J. Mol. Biol.*, vol. 235, no. 5, pp. 1421–1435, Feb. 1994.
[315] V. Lamour, L. F. Westblade, E. A. Campbell, and S. A. Darst, "Crystal structure of the *in vivo*-assembled *Bacillus subtilis* Spx/RNA polymerase alpha subunit C-terminal domain complex," *J. Struct. Biol.*, vol. 168, no. 2, pp. 352–356, Nov. 2009.
[316] C. A. Birch, M. J. Davis, L. Mbengi, and P. Zuber, "Exploring the Amino Acid Residue Requirements of the RNA Polymerase (RNAP) α Subunit C-Terminal Domain for Productive Interaction between Spx and RNAP of *Bacillus subtilis*," *J. Bacteriol.*, vol. 199, no. 14, pp. e00124-17, Jul. 2017.

[317] L. V. Aseev and I. V. Boni, "Extraribosomal functions of bacterial ribosomal proteins," *Mol. Biol.*, vol. 45, no. 5, pp. 739–750, Oct. 2011.
[318] D. F. Browning and S. J. W. Busby, "Local and global regulation of transcription initiation in bacteria," *Nat. Rev. Microbiol.*, vol. 14, no. 10, pp. 638–650, Oct. 2016.
[319] B. Albert *et al.*, "A ribosome assembly stress response regulates transcription to maintain proteome homeostasis," *eLife*, vol. 8, p. e45002, May 2019.
[320] N. Choonee, S. Even, L. Zig, and H. Putzer, "Ribosomal protein L20 controls expression of the *Bacillus subtilis* infC operon via a transcription attenuation mechanism," *Nucleic Acids Res.*, vol. 35, no. 5, pp. 1578–1588, Jan. 2007.
[321] D. Dar *et al.*, "Term-seq reveals abundant ribo-regulation of antibiotics resistance in bacteria," *Science*, vol. 352, no. 6282, p. aad9822, Apr. 2016.
[322] R. A. T. Mars, K. Mendonça, E. L. Denham, and J. M. van Dijl, "The reduction in small ribosomal subunit abundance in ethanol-stressed cells of *Bacillus subtilis* is mediated by a SigB-dependent antisense RNA," *Biochim. Biophys. Acta*, vol. 1853, no. 10 Pt A, pp. 2553–2559, Oct. 2015.
[323] C. Eymann and M. Hecker, "Induction of sigma(B)-dependent general stress genes by amino acid starvation in a spo0H mutant of *Bacillus subtilis*," *FEMS Microbiol. Lett.*, vol. 199, no. 2, pp. 221–227, May 2001.
[324] D. R. Gentry, V. J. Hernandez, L. H. Nguyen, D. B. Jensen, and M. Cashel, "Synthesis of the stationary-phase sigma factor sigma s is positively regulated by ppGpp," *J. Bacteriol.*, vol. 175, no. 24, pp. 7982–7989, Dec. 1993.
[325] R. Lange, D. Fischer, and R. Hengge-Aronis, "Identification of transcriptional start sites and the role of ppGpp in the expression of rpoS, the structural gene for the sigma S subunit of RNA polymerase in Escherichia coli," *J. Bacteriol.*, vol. 177, no. 16, pp. 4676–4680, Aug. 1995.
[326] C. Cunning, L. Brown, and T. Elliott, "Promoter substitution and deletion analysis of upstream region required for rpoS translational regulation," *J. Bacteriol.*, vol. 180, no. 17, pp. 4564–4570, Sep. 1998.
[327] R. Hengge-Aronis, "Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase," *Microbiol. Mol. Biol. Rev. MMBR*, vol. 66, no. 3, pp. 373–395, table of contents, Sep. 2002.
[328] N. Majdalani, C. Cunning, D. Sledjeski, T. Elliott, and S. Gottesman, "DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 95, no. 21, pp. 12462–12467, Oct. 1998.
[329] C. A. McCullen, J. N. Benhammou, N. Majdalani, and S. Gottesman, "Mechanism of positive regulation by DsrA and RprA small noncoding RNAs: pairing increases translation and protects rpoS mRNA from degradation," *J. Bacteriol.*, vol. 192, no. 21, pp. 5559–5571, Nov. 2010.
[330] M. E. Girard, S. Gopalkrishnan, E. D. Grace, J. A. Halliday, R. L. Gourse, and C. Herman, "DksA and ppGpp Regulate the σS Stress Response by Activating Promoters for the Small RNA DsrA and the Anti-Adapter Protein IraP," *J. Bacteriol.*, vol. 200, no. 2, Jan. 2018.
[331] A. Bougdour and S. Gottesman, "ppGpp regulation of RpoS degradation via anti-adaptor protein IraP," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 31, pp. 12896–12901, Jul. 2007.
[332] H. Merrikh, A. E. Ferrazzoli, and S. T. Lovett, "Growth phase and (p)ppGpp control of IraD, a regulator of RpoS stability, in Escherichia coli," *J. Bacteriol.*, vol. 191, no. 24, pp. 7436–7446, Dec. 2009.
[333] Y. Zhou, S. Gottesman, J. R. Hoskins, M. R. Maurizi, and S. Wickner, "The RssB response regulator directly targets sigma(S) for degradation by ClpXP," *Genes Dev.*, vol. 15, no. 5, pp. 627–637, Mar. 2001.
[334] T. Schweder, K. H. Lee, O. Lomovskaya, and A. Matin, "Regulation of Escherichia coli starvation sigma factor (sigma s) by ClpXP protease," *J. Bacteriol.*, vol. 178, no. 2, pp. 470–476, Jan. 1996.
[335] M. Jishage, K. Kvint, V. Shingler, and T. Nyström, "Regulation of sigma factor competition by the alarmone ppGpp," *Genes Dev.*, vol. 16, no. 10, pp. 1260–1270, May 2002.
[336] A. O. Gaca, J. Abranches, J. K. Kajfasz, and J. A. Lemos, "Global transcriptional analysis of the stringent response in *Enterococcus faecalis*," *Microbiol. Read. Engl.*, vol. 158, no. Pt 8, pp. 1994–2004, Aug. 2012.
[337] A. O. Gaca, "The Physiological Significance of (p)ppGpp in Enterococcus faecalis," University of Rochester, 2015.

[338] M. Holcik and N. Sonenberg, "Translational control in stress and apoptosis," *Nat. Rev. Mol. Cell Biol.*, vol. 6, no. 4, pp. 318–327, Apr. 2005.
[339] A. Katz and O. Orellana, "Protein Synthesis and the Stress Response," in *Cell-Free Protein Synthesis*, M. Biyani, Ed. InTech, 2012.
[340] P. Walter and D. Ron, "The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation," *Science*, vol. 334, no. 6059, pp. 1081–1086, Nov. 2011.
[341] K. Pakos-Zebrucka, I. Koryga, K. Mnich, M. Ljujic, A. Samali, and A. M. Gorman, "The integrated stress response," *EMBO Rep.*, vol. 17, no. 10, pp. 1374–1395, 2016.
[342] X. Liao *et al.*, "Enhancing co-translational folding of heterologous protein by deleting non-essential ribosomal proteins in Pichia pastoris," *Biotechnol. Biofuels*, vol. 12, no. 1, p. 38, Dec. 2019.
[343] T. Tarusawa, S. Ito, S. Goto, C. Ushida, A. Muto, and H. Himeno, "(p)ppGpp-dependent and -independent pathways for salt tolerance in *Escherichia coli*," *J. Biochem. (Tokyo)*, vol. 160, no. 1, pp. 19–26, Jul. 2016.
[344] Y. Hase, T. Tarusawa, A. Muto, and H. Himeno, "Impairment of Ribosome Maturation or Function Confers Salt Resistance on Escherichia coli Cells," *PLoS ONE*, vol. 8, no. 5, p. e65747, May 2013.
[345] O. Vesper *et al.*, "Selective Translation of Leaderless mRNAs by Specialized Ribosomes Generated by MazF in *Escherichia coli*," *Cell*, vol. 147, no. 1, pp. 147–157, Sep. 2011.
[346] P. H. Culviner and M. T. Laub, "Global Analysis of the E. coli Toxin MazF Reveals Widespread Cleavage of mRNA and the Inhibition of rRNA Maturation and Ribosome Biogenesis," *Mol. Cell*, vol. 70, no. 5, pp. 868-880.e10, 07 2018.
[347] T. Mets, S. Kasvandik, M. Saarma, Ü. Maiväli, T. Tenson, and N. Kaldalu, "Fragmentation of Escherichia coli mRNA by MazF and MqsR," *Biochimie*, vol. 156, pp. 79–91, Jan. 2019.
[348] J. T. Wade and M. T. Laub, "Concerns about 'Stress-Induced MazF-Mediated Proteins in *Escherichia coli* ,'" *mBio*, vol. 10, no. 3, pp. e00825-19, /mbio/10/3/mBio.00825-19.atom, Jun. 2019.
[349] A. Nigam, T. Ziv, A. Oron-Gottesman, and H. Engelberg-Kulka, "Reply to Wade and Laub, 'Concerns about "Stress-Induced MazF-Mediated Proteins in *Escherichia coli* ,"'" *mBio*, vol. 10, no. 3, pp. e01063-19, /mbio/10/3/mBio.01063-19.atom, Jun. 2019.
[350] N. Kaldalu, Ü. Maiväli, V. Hauryliuk, and T. Tenson, "Reanalysis of Proteomics Results Fails To Detect MazF-Mediated Stress Proteins," *mBio*, vol. 10, no. 3, pp. e00949-19, /mbio/10/3/mBio.00949-19.atom, Jun. 2019.
[351] A. Nigam, T. Ziv, A. Oron-Gottesman, and H. Engelberg-Kulka, "Reply to Kaldalu et al., 'Reanalysis of Proteomics Results Fails To Detect MazF-Mediated Stress Proteins,'" *mBio*, vol. 10, no. 3, pp. e01068-19, /mbio/10/3/mBio.01068-19.atom, Jun. 2019.
[352] M. Sauert, H. Temmel, and I. Moll, "Heterogeneity of the translational machinery: Variations on a common theme," *Biochimie*, vol. 114, pp. 39–47, Jul. 2015.
[353] C. M. Kurylo *et al.*, "Endogenous rRNA Sequence Variation Can Regulate Stress Response Gene Expression and Phenotype," *Cell Rep.*, vol. 25, no. 1, pp. 236-248.e6, Oct. 2018.
[354] K. Yano *et al.*, "Multiple rRNA operons are essential for efficient cell growth and sporulation as well as outgrowth in Bacillus subtilis," *Microbiology*, vol. 159, no. Pt_11, pp. 2225–2236, Nov. 2013.
[355] M. B. Ferretti and K. Karbstein, "Does functional specialization of ribosomes really exist?," *RNA*, vol. 25, no. 5, pp. 521–538, May 2019.
[356] L. J. Rosenthal, S. E. Martin, M. W. Pariza, and J. J. Iandolo, "Ribosome synthesis in thermally shocked cells of Staphylococcus aureus," *J. Bacteriol.*, vol. 109, no. 1, pp. 243–249, Jan. 1972.
[357] L. J. Rosenthal and J. J. Iandolo, "Thermally induced intracellular alteration of ribosomal ribonucleic acid," *J. Bacteriol.*, vol. 103, no. 3, pp. 833–835, Sep. 1970.
[358] T. Tolker-Nielsen and S. Molin, "Role of ribosome degradation in the death of heat-stressed *Salmonella typhimurium*," *FEMS Microbiol. Lett.*, vol. 142, no. 2–3, pp. 155–160, Sep. 1996.
[359] G. Akanuma *et al.*, "Magnesium Suppresses Defects in the Formation of 70S Ribosomes as Well as in Sporulation Caused by Lack of Several Individual Ribosomal Proteins," *J. Bacteriol.*, vol. 200, no. 18, pp. e00212-18, /jb/200/18/e00212-18.atom, Jul. 2018.
[360] L. L. Miller and Z. J. Ordal, "Thermal injury and recovery of *Bacillus subtilis*," *Appl. Microbiol.*, vol. 24, no. 6, pp. 878–884, Dec. 1972.
[361] A. Trinquier *et al.*, "tRNA Maturation Defects Lead to Inhibition of rRNA Processing via Synthesis of pppGpp," *Mol. Cell*, p. S1097276519302345, Apr. 2019.

[362] A. Basu and M.-N. F. Yap, "Ribosome hibernation factor promotes Staphylococcal survival and differentially represses translation," *Nucleic Acids Res.*, vol. 44, no. 10, pp. 4881–4893, 02 2016.
[363] T. Brodiazhenko, M. J. O. Johansson, H. Takada, T. Nissan, V. Hauryliuk, and V. Murina, "Elimination of Ribosome Inactivating Factors Improves the Efficiency of *Bacillus subtilis* and Saccharomyces cerevisiae Cell-Free Translation Systems," *Front. Microbiol.*, vol. 9, p. 3041, 2018.
[364] P. Genevaux, F. Keppel, F. Schwager, P. S. Langendijk-Genevaux, F. U. Hartl, and C. Georgopoulos, "In vivo analysis of the overlapping functions of DnaK and trigger factor," *EMBO Rep.*, vol. 5, no. 2, pp. 195–200, Feb. 2004.
[365] K. Klobucar and E. D. Brown, "Use of genetic and chemical synthetic lethality as probes of complexity in bacterial cell systems," *FEMS Microbiol. Rev.*, vol. 42, no. 1, 01 2018.
[366] F. S. Pedersen and N. O. Kjeldgaard, "Analysis of the relA gene product of Escherichia coli," *Eur. J. Biochem.*, vol. 76, no. 1, pp. 91–97, Jun. 1977.
[367] J.-S. Kim *et al.*, "DksA-DnaJ redox interactions provide a signal for the activation of bacterial RNA polymerase," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 115, no. 50, pp. E11780–E11789, 11 2018.
[368] P. G. Lemaux, S. L. Herendeen, P. L. Bloch, and F. C. Neidhardt, "Transient rates of synthesis of individual polypeptides in *E. coli* following temperature shifts," *Cell*, vol. 13, no. 3, pp. 427–434, Mar. 1978.
[369] A. R. Richardson *et al.*, "Multiple Targets of Nitric Oxide in the Tricarboxylic Acid Cycle of Salmonella enterica Serovar Typhimurium," *Cell Host Microbe*, vol. 10, no. 1, pp. 33–43, Jul. 2011.
[370] D. R. Hyduke, L. R. Jarboe, L. M. Tran, K. J. Y. Chou, and J. C. Liao, "Integrated network analysis identifies nitric oxide response networks and dihydroxyacid dehydratase as a crucial target in Escherichia coli," *Proc. Natl. Acad. Sci.*, vol. 104, no. 20, pp. 8484–8489, May 2007.
[371] A. Katz, R. Banerjee, M. de Armas, M. Ibba, and O. Orellana, "Redox status affects the catalytic activity of glutamyl-tRNA synthetase," *Biochem. Biophys. Res. Commun.*, vol. 398, no. 1, pp. 51–55, Jul. 2010.
[372] R. A. VanBogelen, M. A. Acton, and F. C. Neidhardt, "Induction of the heat shock regulon does not produce thermotolerance in *Escherichia coli*," *Genes Dev.*, vol. 1, no. 6, pp. 525–531, Aug. 1987.
[373] A. Ogilvie, M. Lämmerman, K. Wiebauer, and W. Kersten, "Quinone induced stringent control. Accumulation of ppGpp and inhibition of RNA synthesis in stringent *Escherichia coli* by 5,8-dioxo-6-amino-7-chloroquinoline," *Biochim. Biophys. Acta*, vol. 395, no. 2, pp. 136–145, Jun. 1975.
[374] K. Wiebauer, A. Ogilvie, and W. Kersten, "The molecular basis of leucine auxotrophy of quinone-treated Escherichia coli. Active site-directed modification of leucyl-tRNA synthetase by 6-amino-7-chloro-5,8-dioxoquinoline," *J. Biol. Chem.*, vol. 254, no. 2, pp. 327–332, Jan. 1979.
[375] A. Ogilvie, K. Wiebauer, P. Spitzbarth, and W. Kersten, "Inhibition of leucyl-tRNA synthetase in Escherichia coli by the cytostatic 5,8-dioxo-6-amino-7-chloroquinoline," *Biochim. Biophys. Acta*, vol. 407, no. 3, pp. 357–364, Oct. 1975.
[376] P. C. Lee, B. R. Bochner, and B. N. Ames, "Diadenosine 5',5"'-P1,P4-tetraphosphate and related adenylylated nucleotides in Salmonella typhimurium," *J. Biol. Chem.*, vol. 258, no. 11, pp. 6827–6834, Jun. 1983.
[377] K. Randerath, C. M. Janeway, M. L. Stephenson, and P. C. Zamecnik, "Isolation and characterization of dinucleoside tetra- and tri-phosphates formed in the presence of lysyl-sRNA synthetase," *Biochem. Biophys. Res. Commun.*, vol. 24, no. 1, pp. 98–105, Jul. 1966.
[378] E. Rapaport, S. K. Svihovec, and P. C. Zamecnik, "Relationship of the first step in protein synthesis to ppGpp: formation of A(5')ppp(5')Gpp," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 72, no. 7, pp. 2653–2657, Jul. 1975.
[379] B. Nawrot, E. Sochacka, and M. Düchler, "tRNA structural and functional changes induced by oxidative stress," *Cell. Mol. Life Sci. CMLS*, vol. 68, no. 24, pp. 4023–4032, Dec. 2011.
[380] J. Zhong *et al.*, "Transfer RNAs Mediate the Rapid Adaptation of *Escherichia coli* to Oxidative Stress," *PLoS Genet.*, vol. 11, no. 6, p. e1005302, Jun. 2015.
[381] C. Benoist, C. Guérin, P. Noirot, and E. Dervyn, "Constitutive Stringent Response Restores Viability of *Bacillus subtilis* Lacking Structural Maintenance of Chromosome Protein," *PLOS ONE*, vol. 10, no. 11, p. e0142308, Nov. 2015.

[382] G. F. Kramer, J. C. Baker, and B. N. Ames, "Near-UV stress in Salmonella typhimurium: 4-thiouridine in tRNA, ppGpp, and ApppGpp as components of an adaptive response," *J. Bacteriol.*, vol. 170, no. 5, pp. 2344–2351, May 1988.
[383] A. Favre, A. M. Michelson, and M. Yaniv, "Photochemistry of 4-thiouridine in Escherichia coli transfer RNA1Val," *J. Mol. Biol.*, vol. 58, no. 1, pp. 367–379, May 1971.
[384] M. Yaniv, A. Chestier, F. Gros, and A. Favre, "Biological activity of irradiated tRNA Val containing a 4-thiouridine-cytosine dimer," *J. Mol. Biol.*, vol. 58, no. 1, pp. 381–388, May 1971.
[385] G. Thomas and A. Favre, "4-Thiouridine Triggers Both Growth Delay Induced by Near-Ultraviolet Light and Photoprotection," *Eur. J. Biochem.*, vol. 113, no. 1, pp. 67–74, Mar. 2005.
[386] D. Patterson and D. Gillespie, "Stringent response of RNA synthesis in E. coli produced by a temperature shift-up," *Biochem. Biophys. Res. Commun.*, vol. 45, no. 2, pp. 476–482, Oct. 1971.
[387] J. Mostertz and M. Hecker, "Patterns of protein carbonylation following oxidative stress in wild-type and sigB Bacillus subtilis cells," *Mol. Genet. Genomics MGG*, vol. 269, no. 5, pp. 640–648, Aug. 2003.
[388] B. K. Chi *et al.*, "S-bacillithiolation protects conserved and essential proteins against hypochlorite stress in firmicutes bacteria," *Antioxid. Redox Signal.*, vol. 18, no. 11, pp. 1273–1295, Apr. 2013.
[389] N. Brandes, A. Rinck, L. I. Leichert, and U. Jakob, "Nitrosative stress treatment of E. coli targets distinct set of thiol-containing proteins," *Mol. Microbiol.*, vol. 66, no. 4, pp. 901–914, Nov. 2007.
[390] L. I. Leichert *et al.*, "Quantifying changes in the thiol redox proteome upon oxidative stress in vivo," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 24, pp. 8197–8202, Jun. 2008.
[391] R. Yutthanasirikul *et al.*, "Oxidation of a Cysteine Residue in Elongation Factor EF-Tu Reversibly Inhibits Translation in the Cyanobacterium *Synechocystis* sp. PCC 6803," *J. Biol. Chem.*, vol. 291, no. 11, pp. 5860–5870, Mar. 2016.
[392] P. H. Anborgh, A. Parmeggiani, and J. Jonák, "Site-directed mutagenesis of elongation factor Tu. The functional and structural role of residue Cys81," *Eur. J. Biochem.*, vol. 208, no. 2, pp. 251–257, Sep. 1992.
[393] K. Kojima *et al.*, "Regulation of translation by the redox state of elongation factor G in the cyanobacterium Synechocystis sp. PCC 6803," *J. Biol. Chem.*, vol. 284, no. 28, pp. 18685–18691, Jul. 2009.
[394] T. Nagano *et al.*, "Elongation factor G is a critical target during oxidative damage to the translation system of Escherichia coli," *J. Biol. Chem.*, vol. 287, no. 34, pp. 28697–28704, Aug. 2012.
[395] J. Bernhardt, K. Büttner, C. Scharf, and M. Hecker, "Dual channel imaging of two-dimensional electropherograms in*Bacillus subtilis*," *Electrophoresis*, vol. 20, no. 11, pp. 2225–2240, Aug. 1999.
[396] T. A. Gaidenko, T.-J. Kim, A. L. Weigel, M. S. Brody, and C. W. Price, "The Blue-Light Receptor YtvA Acts in the Environmental Stress Signaling Pathway of *Bacillus subtilis*," *J. Bacteriol.*, vol. 188, no. 17, pp. 6387–6395, Sep. 2006.
[397] A. O. Gaca *et al.*, "Basal levels of (p)ppGpp in *Enterococcus faecalis*: the magic beyond the stringent response," *mBio*, vol. 4, no. 5, pp. e00646-00613, Sep. 2013.
[398] B. W. Anderson *et al.*, "Evolution of (p)ppGpp-HPRT regulation through diversification of an allosteric oligomeric interaction," *bioRxiv*, p. 621474, Jan. 2019.
[399] J. Abranches, A. R. Martinez, J. K. Kajfasz, V. Chávez, D. A. Garsin, and J. A. Lemos, "The molecular alarmone (p)ppGpp mediates stress responses, vancomycin tolerance, and virulence in *Enterococcus faecalis*," *J. Bacteriol.*, vol. 191, no. 7, pp. 2248–2256, Apr. 2009.
[400] X. Yang and E. E. Ishiguro, "Temperature-Sensitive Growth and Decreased Thermotolerance Associated with relA Mutations in *Escherichia coli*," *J. Bacteriol.*, vol. 185, no. 19, pp. 5765–5771, Oct. 2003.
[401] J. Wang *et al.*, "Identification and Functional Analysis of RelA/SpoT Homolog (RSH) Genes in Deinococcus radiodurans," *J. Microbiol. Biotechnol.*, vol. 26, no. 12, pp. 2106–2115, Dec. 2016.
[402] F. Rallu, A. Gruss, S. D. Ehrlich, and E. Maguin, "Acid- and multistress-resistant mutants of *Lactococcus lactis* : identification of intracellular stress signals," *Mol. Microbiol.*, vol. 35, no. 3, pp. 517–528, Feb. 2000.
[403] H. Y. Kim, J. Go, K.-M. Lee, Y. T. Oh, and S. S. Yoon, "Guanosine tetra- and pentaphosphate increase antibiotic tolerance by reducing reactive oxygen species production in *Vibrio cholerae*," *J. Biol. Chem.*, vol. 293, no. 15, pp. 5679–5694, Apr. 2018.

[404] E. Maisonneuve and K. Gerdes, “Molecular Mechanisms Underlying Bacterial Persisters,” *Cell*, vol. 157, no. 3, pp. 539–548, Apr. 2014.

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Schäfer, H. & Turgay, K. Spx, a versatile regulator of the *Bacillus subtilis* stress response. *Current Genetics* (2019). doi:10.1007/s00294-019-00950-6

Schäfer, H., Heinz, A., Sudzinová, P., Voß, M., Hantke, I., Krásný, L. & Turgay, K., Spx, the central regulator of the heat and oxidative stress response in *B. subtilis*, can repress transcription of translation-related genes. *Mol. Microbiol.* **111**, 514–533 (2019). doi: 10.1111/mmi.14171

Hantke, I., Schäfer, H., Janczikowski, A. & Turgay, K., YocM a small heat shock protein can protect *Bacillus subtilis* cells during salt stress. *Mol. Microbiol.* **111**, 423–440 (2019).doi: 10.1111/mmi.14164

Beckert, B., Abdelshahid, M., Schäfer, H., Steinchen, W., Arenz, S., Berninghausen, O., Beckmann, R., Bange, G., Turgay, K. & Wilson, D.N., Structure of the *Bacillus subtilis* hibernating 100S ribosome reveals the basis for 70S dimerization. *EMBO J.* **36**, 2061–2072 (2017). doi: 10.15252/embj.201696189

Molière, N., Hoßmann, J., Schäfer, H. & Turgay, K. Role of Hsp100/Clp Protease Complexes in Controlling the Regulation of Motility in *Bacillus subtilis*. *Front Microbiol* **7**, 315 (2016). doi: 10.3389/fmicb.2016.00315