The general stress response of Bacillus subtilis

van der Steen, J.B.

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Chapter 1: General introduction

Jeroen B. van der Steen
1.1. Abstract

This chapter contains a comprehensive introduction to *Bacillus subtilis*, the subject of this thesis. Its general properties and relevance are reviewed, with special emphasis on the different strategies this bacterium uses to cope with environmental, often severely limiting, conditions. Furthermore, the general stress response and its regulatory network will be introduced, mostly describing the state of the research field prior to the start of the work described in this thesis.

1.2. *Bacillus subtilis*

1.2.1. Introduction and history

*Bacillus subtilis* is a Gram-positive, rod-shaped bacterium. It is readily isolated from diverse natural environments such as soil and water, and from the proximity of plants (347). It was first identified in 1835 by the German scientist Christian Ehrenberg, who called it *Vibrio subtilis*, or ‘thin, bent rod’ (119). Ferdinand Cohn later gave this bacterium its current name (90). Since then, *B. subtilis* has become the primary model organism for all Gram-positive bacteria. Its genome was sequenced early on, and has been updated several times since (22, 254, 302, 322). The most recent revision contains 4,215,606 nucleotides, coding for 4244 proteins (22, 259). *B. subtilis* was traditionally regarded as an obligate aerobe, but was later shown to be able to grow anaerobically by nitrate respiration and by fermentation (332).

1.2.2. Spores

One of the most distinguishing properties of *B. subtilis* is its ability to form endospores, or simply ‘spores’. Spores are specialized survival-capsules designed to protect the DNA of the bacterium under unfavorable conditions. The core of the spore, which contains its DNA, is surrounded by a series of membrane, peptidoglycan, and protein layers (417, 418). In the core, the DNA is protected by, amongst other things, a high concentration of dipicolinic acid, a low water-content, and protective small, acid-soluble, proteins (SASPs) that saturate the DNA (417, 418). These aspects result in an extreme tolerance of spores to, for example, heat, cold, radiation, pressure, desiccation and various noxious chemicals (346). In fact, spores have been described as the ‘hardest known form of life on Earth’ (346).

The existence of spores of *B. subtilis* was already noted by Ehrenberg in 1838, but they were first studied by Cohn and by Robert Koch in 1876 (91, 160, 239). Koch was also the first to observe a complete life cycle of spores in 1888 (160, 240). When a cell enters the sporulation process, usually upon nutrient limitation, it asymmetrically divides into a larger mother cell and a smaller daughter cell that is called the ‘forespore’ or ‘prespore’. When this division is complete, the forespore is internalized, followed by lysis of the mother cell and release of the mature spore (reviewed in e.g. (185, 186, 374)). When the spore encounters favorable conditions it can convert back into a vegetative cell through
processes called germination and outgrowth. The core is rehydrated and several layers of the spore are degraded, which is accompanied by re-initiation of metabolism and escape of the vegetative cell from the outer layers of the spore (reviewed in e.g. (314, 315, 364, 416)).

Spores are so resistant to extreme conditions that they can survive in outer space under certain conditions (196, 346, 349). Thus, spores have the potential to be transported between planets and possibly even between planetary systems, which makes them a prime candidate for theories such as exogenesis (i.e., that life on Earth originated elsewhere) and panspermia (i.e., that the universe is full of life, which is transported between astronomical bodies) (196, 348, 349). Those theories are evidently controversial, but research done on the viability of spores in space has made it clear that contamination of other astronomical bodies with bacteria from human spacecraft is a serious concern (349).

Apart from providing increased resistance, spores have another function: to serve as time capsules (346, 347). Because they are metabolically inactive they can, in principle, survive indefinitely, though at some point the accumulated damage will prevent germination and outgrowth. It is generally assumed that spores can remain viable for at least thousands of years under favorable conditions (224). Claims of isolation of revived cells from spores of up to 650 million year old have been made, but these claims have generally been regarded with skepticism (224). More recently, there have been reports of the isolation of a Bacillus species from the stomach of an extinct bee encapsulated in 25-to-50-million-year-old amber (63) and of a bacterium related to Bacillus from a 250-million-year-old brine inclusion (500). Because stringent controls were employed, the more recent results have been received with less skepticism, though they remain controversial (365, 404).

1.2.3. Habitat

Although B. subtilis has been studied extensively in the laboratory, it is not yet clear what natural habitat it occupies (117, 347). B. subtilis can readily be isolated from soil, water, and the rhizosphere, and is generally assumed to be a soil-bacterium for that reason (117, 347). Indeed, growth, sporulation, and exchange of genetic material by B. subtilis have been demonstrated in soil (161, 162, 430, 486). It has also been argued that growth of multicellular communities of B. subtilis on plant roots may be the most important natural habitat, and indeed B. subtilis can easily be isolated from such environments (117, 132, 490). Interestingly, the genome sequence of B. subtilis illustrates its capability of growing on many different carbon sources, including many plant-derived molecules (22, 254). However, the remarkable properties of spores mean that there is no guarantee that B. subtilis is isolated from an environment where it can actually grow (117, 346).

More recently, evidence has been mounting that a significant part of the natural life cycle of B. subtilis may take place in the gut of animals. B. subtilis can be isolated from animal and human feces in frequencies that seem too high to be explained by the
presence of spores in the diet (23, 97, 98, 117, 192, 193, 450). In addition, *B. subtilis* spores can survive the gastro-intestinal tract (GIT), and even appear to be able to germinate and re-sporulate inside it (66, 97, 117, 131, 263, 450). Following this, some have gone as far as to refer to *B. subtilis* as a ‘gut commensal’ (98).

Although ‘the’ natural habitat of *B. subtilis* has not yet been established with certainty, it should be noted that *B. subtilis* may well be opportunistic enough to grow under a multitude of circumstances. Thus, growth in soil, close association with plant roots and the GIT may all occur, and perhaps there are many other environments that also allow (subspecies of) *B. subtilis* to thrive.

### 1.3. Responses to fluctuating environmental conditions

#### 1.3.1. Stationary-phase responses

Regardless of the natural environment in which *B. subtilis* grows, it will be exposed to constantly fluctuating conditions (177). Here, such fluctuating conditions are referred to as ‘stresses’. It is not surprising that the bacterium has evolved a wide variety of ways to survive or counter these fluctuations. The most dramatic responses are to conditions that severely limit growth and cause a transition from exponential growth to the stationary phase of growth, where the number of cells no longer increases. The most prominent of these responses is the formation of endospores through sporulation, as discussed above. Other responses, which will be briefly discussed below, include changes in motility, the production of extracellular degradative enzymes, the uptake of extracellular DNA, the formation of complex multicellular communities, and predation upon sister cells.

Intriguingly, these responses are usually activated in a subpopulation of cells, and tend to be mutually exclusive (273). The regulatory networks that control these processes are highly intertwined and mostly revolve around a few central regulators. The most prominent of these is the master regulator of sporulation, Spo0A (273).

#### 1.3.2. Spo0A and initiation of sporulation

The transcriptional regulator Spo0A is active in the phosphorylated form (Spo0A-P), which is generated via a phosphorelay in which several kinases of the Kin family phosphorylate Spo0F in response to various stimuli (185, 210). The phosphoryl group is subsequently transferred to Spo0A via Spo0B. The details of this phosphorelay and the subsequent sporulation process will not be considered here, but have been extensively reviewed elsewhere (116, 125, 185, 186, 273, 373, 374, 515).

Interestingly, only a part of any given culture will reach the threshold of Spo0A-P required to initiate sporulation (88, 140, 185, 273). This heterogeneity is thought to be caused by a wide range of feedback loops that are present in, or act upon, the Spo phosphorelay (474, 479), with additional (indirect) feedback loops still being discovered (64). However, recent evidence suggests that fluctuations in the components of the phosphorelay may be the primary cause of heterogeneity (73, 126, 127).
1.3.3. Biofilm formation and matrix production

*B. subtilis* is not confined to growth in liquid culture, but can also form complex multicellular communities on solid surfaces and at liquid-air interfaces, called biofilms and pellicles, respectively (48, 490). In biofilms, cells are held together by an extracellular matrix consisting of exopolysaccharides and of several proteins (49), including TasA and BslA (formerly YuaB) (49, 187, 237, 238, 247, 483).

Like sporulation, the production of the extracellular matrix is regulated by Spo0A-P (48, 149, 222, 316, 422). However, matrix production requires lower levels of Spo0A-P than sporulation, and can therefore be initiated earlier (67, 140). At low or intermediate levels of phosphorylation, Spo0A-P results in the production of the regulator SinI, which in turn deactivates the master repressor of matrix production (SinR) (67, 87, 222). If Spo0A-P levels continue to rise they start to inhibit production of SinI, turning matrix production off and promoting sporulation instead (69).

Given that sporulation exhibits heterogeneity and matrix production is regulated via the same master regulator, Spo0A-P, it is perhaps not surprising that only a part of a culture or biofilm turns into matrix producers (67, 490). In fact, biofilms are highly structured and dynamic. For example, colonies form elevated bundles of cells that have been referred to as fruiting bodies, veins or wrinkles, which serve as preferential sites for sporulation (48, 475, 489).

1.3.4. Motility, cell chaining and extracellular degradative enzymes

Throughout growth, *B. subtilis* switches between a motile and a sessile state, the latter of which has cells growing in long chains (68, 223). The switch between these two states is in part controlled by the phosphorylation state of the regulator DegU, which needs to be in the non-phosphorylated form to activate the genes required for motility as well as the autolysins that are required for cell separation (8, 82, 237, 375, 482).

As biofilms contain a high proportion of cells growing in chains, it is not surprising that regulation of matrix production and cell chaining is linked. The level of phosphorylated DegU (DegU-P) needs to be low for complex colony architecture to be formed (482). In addition, activation of matrix production indirectly leads to repression of the flagella, and to repression of the expression of autolysin and motility genes (44, 68, 169), ensuring that matrix-producing cells do not move. Nevertheless, motile cells do have a place within biofilms (489).

*B. subtilis* cells are also capable of producing extracellular degradative enzymes, such as proteases, which presumably provide extra nutrients to starving cells (324). In contrast to motility processes, the production of these enzymes requires a high level of DegU-P (8, 99, 482). As a result, cells are not motile when they express the extracellular degradative enzymes. This makes sense, given that close proximity is required to benefit from the nutrients released by the degradative enzymes (273). Intriguingly, only a subpopulation produces these enzymes, while the entire population presumably benefits (477).
1.3.5. Competence

*B. subtilis* is naturally able to take up extracellular DNA through a process called genetic or natural competence (9, 439). In fact, this property was one of the important reasons that *B. subtilis* became a model organism, as it results in a relatively straightforward genetic accessibility (517). Competence is regulated by a complex regulatory pathway with many links to other stationary-phase responses (175, 273). One of these links is DegU, which is required for initiation of competence (99, 174).

Intriguingly, only about 10 to 20% of a population becomes transiently competent during the transition from exponential to stationary phase (175). This heterogeneity has been shown to involve noisy gene expression and feedback loops surrounding the regulator ComK (15, 283, 284, 437, 446, 447, 476, 478). Spo0A is also involved, through direct and indirect mechanisms (273), and Spo0A-P has been proposed to define a time window of increased chance to become competent (307). Conversely, competent cells temporarily block initiation of sporulation by transiently keeping Spo0A-P levels low (273).

1.3.6. Cannibalism

Cells that initiate sporulation exhibit an interesting phenomenon, called cannibalism, in which they kill sister cells that have not yet initiated sporulation (89, 156, 157). Killing is the result of the production of Skf (sporulation killing factor) and Sdp (sporulation delay protein), which are both peptides with post-translational modifications (123, 156, 268). Their production is regulated by low levels of Spo0A-P, which is also responsible for resistance against these factors, and thus sister cells that have not (yet) reached sufficient levels of Spo0A-P are left unprotected (123, 140, 156).

Why *B. subtilis* cannibalizes its siblings is not yet completely clear. Speculations include that it may be favorable to delay the costly process of sporulation as long as possible, or that sporulation may not be able to proceed to completion if the non-sporulating population exhausts all nutrients, but available data do not specifically support these hypotheses (89, 157). Interestingly, it has been shown that the cannibalistic subpopulation also produces the extracellular matrix in biofilms (274). Thus, cannibalism may enhance biofilm formation by enriching the relative abundance of matrix producing cells (274). It has also been reported that the three-dimensional wrinkles in complex colony structures are associated with cell death (14), although they may also be formed via other mechanisms (456). Finally, it is interesting to note that the toxins also show antimicrobial activity against related bacteria (157, 258, 268) and that *B. subtilis* prefers to kill *Escherichia coli* cells in co-culture prior to cannibalism (339), suggesting that cannibalism may also be a defense mechanism.

1.3.7. Timing of stationary-phase responses

The heterogeneity in the abovementioned responses probably serves to increase the fitness of the population as a whole, by preparing the population for a range of future circumstances (273). In addition, it is clear that induction of all responses in all cells would
lead to a massive energetic burden (273). However, the mutually exclusive nature of the various responses does require an impressive regulatory network with many intricate feedback systems and cross-connections (273).

As discussed, one of the primary regulatory determinants of cell fate is the level of Spo0A-P (140). Responses such as competence, matrix production and cannibalism are induced at low levels of Spo0A-P, whereas sporulation requires high levels (67, 140, 307). Therefore, many cells that sporulate were matrix producers (489), and matrix producers are the cannibals of a culture (274). The level of Spo0A-P also needs to increase gradually to induce efficient sporulation, presumably due to sequential expression of genes induced at low and at high levels of Spo0A-P (141).

The level of Spo0A-P depends on noise to generate heterogeneity (185, 273, 474, 479). However, the activation of Spo0A is also strictly regulated, with more and more direct activating signals of the phosphorelay kinases being discovered (185, 275, 301). Interesting examples are that KinB appears to be activated by a block in respiration (244), while KinC appears to be activated by potassium leakage and a drop in membrane potential (272, 426). KinD is especially interesting, given that it appears to first keep Spo0A-P levels low until it is activated by the presence of extracellular matrix, and causes Spo0A-P levels to rise (3, 401). Information about the growth phase of the culture (306) and the phase in the cell-cycle (480) are also fed into the network, to ensure the optimal decision at all times.

### 1.3.8. Effect of domestication on complex behavior

Not all of the abovementioned responses are present or prominent in strain 168, the type strain of *B. subtilis*. Strain 168 was originally isolated as a tryptophan auxotroph after various rounds of X-ray mutagenesis and selection (60). It became the type strain when it turned out to be much more competent than wild-type isolates (9, 439, 517), which already underlines a clear difference between wild-type isolates and the domesticated lab strain.

There are more differences in competence between strains. The ‘less-domesticated’ strain NCIB3610 excretes fragments of genomic DNA, dependent on early regulators of the competence pathway, which can then be used for transformations (516). Domesticated strains also release some DNA, but appear to do this without much regulation and possibly by different mechanisms (276, 428, 516).

Other differences exist as well. For example, as shown in Figure 1, lab strains generally form poorer complex colony structures than wild-type isolates, although the colony structures of individual strains also tend to vary massively (2, 48, 475, 490). Interestingly, the biofilm robustness of strain 168 can be restored to the level of NCIB3610 by restoration of the function of four genes, and the introduction of one gene that is present on a plasmid that NCIB3610 carries but 168 does not (300), suggesting that complex traits are relatively easily lost under laboratory conditions. In support of this, it has been shown...
Figure 1: Colony morphology of cells spotted on MOPS plates with 1.5% agar and incubated at 30°C, basically as described in (475). A. Time series of a colony of *B. subtilis* strain 168 1A700 over four consecutive days. B. Comparison between *B. subtilis* PB198 (46), a 168 strain that is commonly used in our lab, and strain 1A700, after two days of incubation under identical conditions. The 1A700 strain used to produce this figure also carried a $P_{\text{spolAA}}$-gfp reporter from IIA-gfp (474) (not shown).

That propagation of a strain for several thousands of generations in rich media can lead to loss of several complex traits (57).

Such differences are important to keep in mind when comparing results obtained in different strains, and especially when attempting to interpret complex behavior of laboratory strains in relation to their natural environment.

1.4. General stress response

1.4.1. Introduction

The responses to severe stationary-phase inducing stresses outlined in the previous section all result in major alterations of the lifestyle of *B. subtilis*. However, there are less severe ways for the cell to protect itself against non-lethal stress. The most prominent of these is called the general stress response (GSR). It is induced by a wide range of conditions, which are generally subdivided into two main groups: energy (or nutritional) stresses and environmental (or physical) stresses. Energy stresses include starvation of glucose (35, 492), phosphate (492, 521), and oxygen (492, 498). The energy stress pathway is also induced by other compounds, such as the RNA-polymerase-inhibitor rifampin (21), and by chemicals which deplete the ATP pool, such as azide (521), CCCP (6, 492, 521), NO (318), and mycophenolic acid (297, 521). Environmental stresses include salt shock (47, 492, 498), ethanol shock (47, 492), heat shock (32, 47, 492, 498), acid shock (492), sodium nitroprusside (318), and blue light (16, 18, 448). The GSR is also induced by growth at high or low temperature (52, 59, 189, 303), exposure to $\text{Mn}^{2+}$ (165, 494), and by several antibiotics that cause cell-wall stress, such as vancomycin and bacitracin (295).

1.4.2. Alternative sigma factor $\sigma^B$

Bacterial RNA polymerase requires a sigma factor subunit for promoter recognition (173). Generally, one sigma factor drives transcription of housekeeping genes, while alternative sigma factors regulate expression of large sets of genes involved in processes such as
development, differentiation and stress response (173). The GSR is one such process, regulated by the alternative sigma factor $\sigma^B$.

$\sigma^B$ was isolated from RNA polymerase complexes as the first alternative sigma factor identified in non-phage-infected bacteria (38, 115, 172). It was initially thought to be associated with sporulation, but a null mutation of sigB, the gene coding for $\sigma^B$, sporulated normally (38, 115). At the time, this caused the interest in $\sigma^B$ to decline somewhat, especially since the null mutation did not even negatively affect normal growth (38, 115, 177). Interest was rapidly restored, however, when it turned out that the activity of $\sigma^B$ went up in stationary phase and in response to many different stress factors (177). This could be correlated with the induction of production of what had been known as ‘general stress proteins’: proteins that appeared on 2D gels after application of various stresses. Most of these turned out to be $\sigma^B$-dependent, thus linking $\sigma^B$ and the general stress response (177, 498).

1.4.3. General stress response regulon

Importance of the regulon

The regulon controlled by $\sigma^B$ and the GSR contains approximately 200 genes (179, 340, 372, 378). Some of these genes are involved in direct stress protection, but many are either of unknown function or involved in general processes such as regulation, transport, metabolism, and cell-envelope function (178, 378).

Not surprisingly, the GSR regulon is important for resistance against the environmental insults that activate it. For example, mutants in sigB show a 50- to 100-fold decrease in survival when confronted with severe heat shock, ethanol shock, salt shock or acid shock, or when frozen or desiccated (499). Similarly, a sigB mutant loses viability in stationary phase at alkaline or acidic pH (142) and at low temperatures (303), and growth of such a mutant is impaired at 15°C (52).

Interestingly, the induction of the GSR also results in non-specific resistance to future stresses. For example, adaptation of wild-type cells to mild salt stress or nutrient-poor conditions increases survival of severe salt stress (499). This effect extends beyond the stresses that normally activate the GSR. In wild-type cells, entry into the stationary phase by glucose-starvation triggers a sigB-dependent resistance to oxidative stress (124, 389). This cross-resistance is striking because the GSR is normally not induced by oxidative stress, although there may be a minor induction upon severe oxidative stress, and there is some overlap of the GSR regulon and specific responses to oxidative stress (47, 124, 180, 321, 340).

Many individual genes in the $\sigma^B$ regulon are directly important for survival under harsh conditions, despite the fact that their function in stress resistance is often not (yet) understood. In a large screen of null mutations in 94 GSR-regulon genes it was found that 85% exhibited an increased sensitivity to at least one of the four severe GSR-inducing stresses tested (ethanol shock, salt shock, and survival and growth at low temperatures).
A follow-up screen, using the same mutant library, revealed that null mutations in 66% of these genes led to a decreased survival in response to stress generated by exposure to hydrogen peroxide or the superoxide-generating agent paraquat (389).

Because of the abovementioned characteristics, the GSR is said to confer multiple, non-specific and preventive stress resistance to *B. subtilis* (178).

**Cross-talk with specific stress responses**

Many stresses that activate the GSR also activate specific stress responses. A clear example of this is salt stress, which is known to induce the extra-cytoplasmic function (ECF) sigma factors $\sigma^W$, $\sigma^M$ and $\sigma^X$ (170, 195, 197, 372, 442), which have significantly overlapping regulons (296, 530). One possible explanation for these separate responses is that the GSR takes care of general resistance while the more specific responses strengthen the cell against osmotic stress. The temporal induction patterns of the various responses to salt stress support the idea that these regulons have different functions. After severe salt stress the GSR is immediately activated, which is followed by sequential induction of the $\sigma^W$ and $\sigma^M$ regulons (170, 195). In fact, it appears that the GSR is strongly activated after a rapid salt shock, while specific responses are more important during gradual increases of the same stress (514). Taken together, this suggests that the GSR functions to non-specifically protect the cells after a rapid change in conditions.

**Cross-talk with other stationary-phase responses**

Because the GSR is activated when cells transition to the stationary phase of growth, it can be viewed as a stationary-phase response. Thus, one may expect that regulation of the GSR is connected to regulation of other responses, such as sporulation and biofilm formation.

Indeed, there is evidence that the GSR can inhibit sporulation indirectly via a $\sigma^B$-dependent promoter in front of *spoOE*, which encodes a phosphatase of Spo0A-P (387, 388). Similarly, old data suggest that near-UV and blue light can delay sporulation in at least one strain of *B. subtilis* and in several other *Bacillus* species (379). Although a link has not been established, it is interesting to note that blue light is also capable of inducing the GSR (16, 18, 448).

Similarly, strain 168 1A700, a lab strain that can still form complex colony architecture ([475] and Figure 1), can increase the complexity of colonies in a GSR-dependent manner under certain conditions (unpublished results in Figure 2).
Figure 2: Colony morphology of derivatives of *B. subtilis* strain 168 1A700 after incubation at 30°C on NB plates with 1.5% agar (J. B. van der Steen *et al.*, unpublished results). **A.** Time series of a strain carrying plasmid pYtvA, which carries an IPTG-controlled copy of *ytvA* (16). Where indicated, overexpression was induced with 1 mM IPTG in the plate. As can be seen, colony morphology is markedly different in the light when *ytvA* is overexpressed. **B.** Colonies of strains carrying either pYtvA (16) or the negative-control plasmid pDG148-Stu (212) incubated for three days in different conditions. The knockouts of *sigB*, the gene coding for σ^B^, were created via transformation with genomic DNA of strain PB344 (47). Where indicated, overexpression was induced with 1 mM IPTG in the plate. As can be seen, the effect of YtvA in the light depends on the presence of σ^B^. No phenotype was apparent for a *ytvA* knockout (data not shown).
1.5. Signal transduction pathway that activates the general stress response

1.5.1. The general stress response is tightly controlled
The GSR is an extensive response, controlling almost 5% of the protein-coding genes of *B. subtilis*, which means that induction of the GSR is a significant energetic burden (177, 178, 408). It is, therefore, not surprising that a tight regulatory network limits unnecessary activation of the GSR (Figure 3). As described in more detail below, this pathway comprises of a central partner switch that is activated by two converging signal transduction pathways—one for energy stress and one for environmental stress. The proteins involved in this pathway are named Rsb, for regulator of sigmaB (46). The primary rsb genes form a single operon (Figure 4) (216, 507). The operon is transcribed from a σ^A^-dependent promoter (P_A) (507) and an internal σ^B^-dependent promoter (P_b) in front of the downstream half of the operon, thus creating a feedback loop (216).

1.5.2. A partner switch controls the activity of σ^B_
To activate transcription of the GSR regulon, σ^B_ needs to bind to RNA polymerase. The formation of a complex of σ^B_ with the anti-sigma-factor RsbW prevents activation of the regulon in unstressed cells (7, 31, 33, 46). Upon stress, RsbW is captured by the anti-anti-sigma-factor RsbV (7, 113, 493). Formation of the RsbV-RsbW and RsbW-σ^B_ complexes is mutually exclusive and the affinity of RsbW for RsbV is higher than for σ^B_, and thus σ^B_ is released to activate the GSR regulon as soon as RsbV is activated (7, 104). This mechanism is referred to as a ‘partner switch’ because the activity of σ^B_ is determined by a switch in binding partner of RsbW (6).

The affinity of RsbV for RsbW is modulated by its phosphorylation state (7, 113, 513). In unstressed cells, RsbV is kept phosphorylated (RsbV-P) by the kinase activity of RsbW (7, 113). When a stress signal is received, one or more phosphatases dephosphorylate RsbV-P (494). The dephosphorylated form of RsbV then displaces σ^B_ from the RsbW-σ^B_ complex, thus activating the response.

There are two known phosphatases that dephosphorylate RsbV-P: RsbU and RsbP. They each relay signals from a separate branch of the stress signaling pathway: RsbU is required for transmission of environmental stress (114, 492, 494), while RsbP is required for transmission of energy stress (485). Both proteins have a C-terminal PP2C domain (protein phosphatase 2C), but differ in their N-terminal domains.

1.5.3. RsbU activates the general stress response in response to environmental stress
RsbU was the first RsbV-P phosphatase identified (491, 492, 507). As stated above, it is essential for GSR activation in response to environmental stress, but not to energy stress (114, 492, 494). The phosphatase activity of RsbU is activated in stressed cells by association with the kinase RsbT (493, 513). To prevent unwanted activation of RsbU and the GSR in the absence of stress, the availability of RsbT is limited through binding to a
complex of RsbRA and RsbS (4, 218, 493, 513), which both contain a STAS (sulfate transporter and anti-sigma-factor antagonist) domain (13). When a stress signal is received, the kinase RsbT phosphorylates the STAS domains of both RsbRA and RsbS, leading to release of RsbT (4, 143, 218, 219, 513). The pre-stress condition can be restored by the phosphatase RsbX, which dephosphorylates RsbS and RsbRA, allowing them to recapture RsbT (436, 495, 513). Thus, RsbT is the central part of another partner switch, with a role much like RsbW in the downstream part of the GSR-activation pathway (218, 513).

1.5.4. RsbRA and RsbS form a large complex called the ‘stressosome’
RsbRA and RsbS, the negative regulators of RsbT in the environmental stress partner switch, were shown to interact (4, 493), but were purified as dimers (114). Thus, the interaction was assumed to be similar to the RsbV-RsbW-σ^B partner switch, in which RsbV and RsbW also function as dimers (104). However, gel filtration experiments consistently
revealed a significant portion of the upstream regulators of RsbU in the void volume (114, 411).

As it turns out, these aggregates found in the void volume are highly significant, as RsbRA and RsbS form a large protein complex (78, 291). The first hints to the structure of this complex, which was renamed the 'stressosome', were obtained from cryo-transmission electron microscopy (cryo-EM). This revealed that the proteins most likely formed a spherical complex (78, 106). Using averaging of the cryo-EM images of more than 10,000 of these particles, together with analysis of crystal structures and homology models of the protein domains involved, Marles-Wright and co-workers were able to construct a detailed model of the stressosome (Figure 5) (290-293).

The model has 10 dimers of RsbS and 20 dimers of RsbRA in a pseudo-icosahedral symmetry. Thus, there are 60 proteins in each stressosome, with a total mass of 1.5 MDa (291). The STAS domains of RsbS and RsbRA form the core structure of the complex, while the N-terminal domains of RsbRA protrude outwards (290, 291). RsbT can bind above RsbS in between the gaps left in between the N-terminal domains of RsbRA, increasing the size of the stressosome to approximately 1.8 MDa (291, 293).

Despite the availability of a detailed model, it is not yet clear why the cell requires the stressosome to regulate the environmental stress response. Speculations include that it may integrate various signals or fine-tune the stress response (78, 106). A function in tuning is supported by the observation that the environmental stress response exhibits a form of cooperativity in vivo, while the energy stress response does not (291).

1.5.5. Sequential phosphorylation of stressosome components

As mentioned previously, phosphorylation of the stressosome is critical for release of RsbT and the initiation of the GSR (4, 143, 218, 508, 513). RsbS has one residue that is phosphorylated by RsbT: Ser59 (218, 513). RsbRA can be phosphorylated on both Thr171 and Thr205, though in vitro studies suggest that RsbT has a higher affinity for Thr171 (79, 143). The phosphorylation of RsbRA on Thr171 appears to be necessary for the release of RsbT, but by itself it is not sufficient (231). Instead, phosphorylation of RsbRA on Thr171 is thought to enhance the ability of RsbT to phosphorylate RsbS, and only after RsbS is

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**Figure 4:** Genomic organization of the genes involved in the primary regulatory network of the general stress response. The arrows representing genes and their relative spacing are drawn to scale.
phosphorylated can RsbT be released (78, 230, 231). In support of this, RsbRA is always partially phosphorylated—even in unstressed cells (129, 230, 231).

1.5.6. RsbP activates the general stress response in response to energy stress

Activation of the GSR by energy stress does not rely on RsbU, and no other phosphatase was initially identified (492). However, it was quickly noted that many signals that induce energy stress also decrease ATP levels (6, 7, 297, 492, 521). Therefore, it was postulated that low ATP levels may directly decrease the kinase activity of RsbW, which would lead to activation of the GSR (6, 7, 297, 492, 521).

This view of the energy stress response pathway changed when the phosphatase RsbP was identified based on sequence homology with RsbU. RsbP is essential for activation of the GSR by energy stress (485). Its gene lies in an operon with the gene for RsbQ, a small protein with an α/β-hydrolase fold (54). The proposed catalytic triad of RsbQ, consisting of Ser96, His247 and Asp219, is essential for energy stress transduction (54, 217). This suggests that enzymatic activity of RsbQ is required, and that RsbQ modifies either RsbP or a co-factor for RsbP (54, 217). Based on the crystal structure of RsbQ, which shows that the putative active site is buried in a small, hydrophobic cavity that is poorly solvent-accessible, the latter scenario seems more likely (217).

Interestingly, yeast-two-hybrid assays suggest that RsbP and RsbQ directly interact (54, 329). The crystal structure of RsbQ somewhat supports the idea of a direct protein-protein interaction because RsbQ has a loop region that is distinct from, and unique

Figure 5: Model of the stressosome. Images were generated from coordinates provided by Dr. Jon Marles-Wright (291) using YASARA View v13.9.8 (250). A. Model of RsbRA and RsbS. The STAS domains of RsbRA and RsbS are shown in blue and red, respectively. The N-terminal domains of RsbRA are shown in yellow. B. Like panel A, but also including RsbT in purple.
amongst, other proteins with a similar fold (217). This loop region has been speculated to be involved in association with RsbP, which may open up the active site of RsbQ (217).

Next to its C-terminal PP2C phosphatase domain, RsbP has an N-terminal PAS (Per-ARNT-Sim) domain (485). Both domains are linked by a region with a predicted coiled-coil structure (55). PAS domains can function as signal input domains, and are known to be able to associate with small molecules (183). Thus, an attractive hypothesis is that the PAS domain of RsbP activates the phosphatase domain when a signal is received (485). In support of this, a mutant of RsbP lacking the PAS domain is unable to activate the GSR (55).

This idea is further supported by the isolation of randomly mutated versions of RsbP that are able to restore phosphatase activity in strains lacking RsbQ (55). A number of mutations could be identified in the coiled-coil region and in the first predicted helices of the PP2C domain but, notably, none could be identified in the PAS domain (55). Interestingly, a deletion of both the PAS domain and the coiled-coil region is constitutively active, independent of RsbQ (55). These results are consistent with the observation that the PAS domain of RsbP is sufficient for RsbQ to bind to in a yeast-two-hybrid assay (329).

Taking everything together, the simplest model is that RsbQ modifies or creates a small molecule that is bound by the PAS domain of RsbP. This then causes a conformational change propagating through the coiled-coil to activate the phosphatase domain in response to energy stress (55). However, until the hypothesized small molecule is identified, this model remains speculative. The crystallization of the PAS domain of RsbP has been reported, but no structure or co-factor has yet been published (286).

1.5.7. RsbV-independent activation of the general stress response

Most stresses characterized to date activate the GSR through either the energy stress (RsbP-dependent) or the environmental stress (RsbU-dependent) pathway. These all depend on the presence and activity of RsbV, which is needed to capture RsbW and release σ^8. However, there are indications that there may also be RsbV-independent ways to induce the GSR.

One such induction is observed when *B. subtilis* is grown at 51°C—close to its maximal growth temperature. This induces the GSR continuously, which is dependent on RsbU and not on RsbP, as would be expected given that heat shock is also an environmental stress (189). However, the dependence of the GSR activation on RsbU is overcome in a mutant lacking RsbV, which is contrary to expectations and which may suggest that RsbW itself is somehow sensitive to high temperatures (189). Similarly, growth close to the minimal growth temperature of *B. subtilis* causes a continuous expression of the GSR regulon that is (mostly) independent of RsbV, RsbU and RsbP (52, 59).

Both observations may suggest the presence of one or more additional pathways that feed into the signal transduction network at RsbW. However, it should be noted that it is also possible that extreme temperatures may lead to uncharacterized cell-wide or local
side-effects, such as alteration of the binding affinity of the RsbW-σ8 complex or the stability of one of the Rsb proteins.

1.6. Paralogs of RsbRA

1.6.1. RsbR protein family

In the paragraphs above, the stressosome has been described as a complex consisting of two core proteins, RsbRA and RsbS. However, the completion of the genome sequence of *B. subtilis* revealed this to be an oversimplification. Homology searches returned a number of proteins with C-terminal STAS domains similar to RsbRA, amongst which were: YkoB, YojH, YqhA, and the split paralog YezB/YetI (Figure 6) (5). Of these, all but the split paralog were shown to be involved in the environmental stress branch of the GSR (5). The split paralog, therefore, will not be further considered here.

YkoB, YojH and YqhA all behave very similar to RsbRA. For example, they are all phosphorylated by RsbT in vitro and in vivo (5, 130), and they all co-purify with RsbRA from cell-extracts (106, 144, 231). Furthermore, all are able to form stressosome-like complexes with RsbS when mixed in vitro (106). Together, this suggests that these three proteins occupy a place in the stressosome similar to the place of RsbRA. Their function also appears to overlap: only when RsbRA, YkoB, YojH and YqhA are all lost is the cell unable to form (functional) stressosomes, with a more than 400-fold increase in GSR activation as result (5, 231). Therefore, these proteins are generally called redundant, although there are some minor differences in, for example, the absolute level of GSR activation following stress when there is only one of the four proteins present (231). To

![Figure 6: Paralogs of RsbRA involved in the regulation of the general stress response. A. Clustal Omega (159, 432) alignment of the STAS domain envelopes predicted by Pfam 27.0 (380). The residues that can be phosphorylated by RsbT are depicted in bold and indicated with arrows. The predicted STAS domain envelope of RsbRB extends 18 amino acids further to the N-terminus than any other RsbR protein; these amino acids are not shown here for clarity. B. Domain organization of the paralogs. Domains as predicted by Pfam 27.0 (380) are the STAS domain, LOV domain, RsbR N-terminal domain (R_N) and the RsbRD N-terminal domain (RD_N). Proteins and domains are drawn to scale and aligned on the equivalent of Ser59 of RsbS.](image-url)
reflect their similar roles, they have been renamed to RsbRB (YkoB), RsbRC (YojH) and RsbRD (YqhA) (231). Here, RsbRA, RsbRB, RsbRC and RsbRD will collectively be referred to as the ‘RsbR proteins’.

It is not at all clear why *B. subtilis* requires four different paralogs that appear to be largely redundant. The simplest hypothesis would be that each paralog senses or transmits a different stress signal. However, this does not appear to be the case, given that any of the RsbR proteins is sufficient for a response to e.g. salt and ethanol stress (5, 231).

Alternatively, the RsbR proteins may be involved in fine-tuning the magnitude of the response in varying conditions by responding differently to either a single signal or a set of signals. This hypothesis is supported by the fact that all four genes lie in different transcriptional units (Figure 4), which suggests that differential regulation of their expression is important. Further support comes from *in vitro* experiments that show that the RsbR paralogs can displace each other in pre-formed stressosomes, suggesting that the stressosome is a dynamic complex (106).

1.6.2. Blue-light photoreceptor YtvA

**Photoreceptors and LOV domains**

The ability to sense or use light is wide-spread in all kingdoms of life. Interestingly, light-regulated gene expression is not just confined to phototrophic organisms that use light as an energy source (122, 462). Many chemotrophic organisms, including bacteria, also have one or more proteins involved in light sensing (122, 462). These proteins, called photoreceptors, typically bind a small molecule called a chromophore to be able to absorb light in the UV/visible range of the spectrum (461). Different classes of photoreceptors are distinguished, one of which is the family containing the blue-light-absorbing LOV (light, oxygen, or voltage) domain.

LOV domains were originally identified in plant proteins called phototropins, which are named this way because they are involved in, amongst other things, phototropism—the moving of plant organs towards or away from light (85, 198). LOV domains bind a flavin mononucleotide (FMN) as chromophore through a highly conserved GXNCRFLQ motif (51, 84, 85). Absorption of light leads to the formation of a triplet excited state that rapidly decays to form a covalent adduct between the C4a atom of the FMN and the cysteine from the conserved sequence motif (51, 403). The resulting adduct state then decays on a relatively long timescale of seconds or minutes to the dark (ground) state (51, 403).

**YtvA has a LOV domain**

YtvA from *B. subtilis* was the first prokaryotic protein with a LOV domain to be identified (277). Its sequence bears the conserved FMN-binding motif with the cysteine that is essential for adduct formation (GKNCRFLQ in the case of YtvA). Indeed, YtvA was shown to bind FMN and to undergo a photocycle like the LOV domains of the phototropins (Figure
In the dark state, YtvA has an absorption maximum at 448 nm. The absorption of blue light results in formation of an excited triplet state within nanoseconds, which leads to adduct formation between Cys62 and the FMN on a microsecond timescale (277, 278). The resulting adduct converts back to the dark state with a recovery lifetime of approximately 43 minutes at 25°C, which is notably slower than the recovery of the LOV domains of the phototropins (277, 278).

**Involvement of YtvA in the general stress response**

YtvA is a protein with two distinct domains. Apart from the N-terminal LOV domain, YtvA also has a C-terminal STAS domain that shares significant homology with the STAS domains of the RsbR proteins (5). Based on this, its involvement in the activation of the GSR was tested, and YtvA was found to be a positive regulator of the GSR (5). However, these experiments were conducted before the photoreceptor function of YtvA was known, and light conditions were not controlled.

Evidence that YtvA regulates the GSR in a blue-light-dependent manner has since been provided (16, 448). YtvA activates the GSR in response to blue light when overproduced, but at wild-type expression levels this effect was not apparent (16). However, at wild-type expression levels there is a light-enhancement of the activation of the GSR in response to salt stress (16, 448). These effects of YtvA depend on the presence of the critical Cys62 residue that forms a covalent bond with the FMN co-factor of YtvA during its photocycle (144, 448). Thus, YtvA clearly sensitizes the GSR to blue light, even though *B. subtilis* is a non-phototrophic bacterium, for which it is not immediately apparent why the integration of blue light into a stress response would be important.
The presence of a C-terminal STAS domain in YtvA raises the question of whether YtvA can be considered as a fifth RsbR protein. In some ways YtvA appears to behave much as the RsbR proteins. For example, it co-purifies with RsbRA from cell-extracts, suggesting that it may be associated with the stressosome (144). In addition, YtvA was shown to require the presence of RsbT for its function, suggesting that it works on, or upstream of, the stressosome (144). However, YtvA is also markedly different in many aspects. For example, the phosphorylatable threonine residues of the RsbR proteins are absent in YtvA, where they appear to be replaced by glutamic acid residues (Figure 6). In agreement with this, YtvA cannot be phosphorylated by RsbT (5). In addition, any one of the four RsbR proteins is sufficient for a functional stressosome, but when none of the four RsbR proteins are present there is no functional stressosome, in spite of the presence of YtvA (5, 231). This suggests that YtvA is unable to form (stable and functional) stressosomes by itself, which makes it different from the RsbR proteins. Therefore, YtvA will not be considered to be an RsbR protein here.

Signal transduction within YtvA and between YtvA and the stressosome

The observation that YtvA most likely activates the stressosome in a light-dependent manner raises the question of how it transmits this light signal. A crystal structure of the LOV domain is available (Figure 8), but unfortunately the full-length protein has not been crystallized (311). The LOV domain forms a dimer in the crystal lattice with the flanking C-terminal ß-helix pointing away from the domain’s core (311). This helix appears to form a coiled-coil in the dimer of the full-length protein. Illumination results in formation of the adduct and in structural changes which are propagated throughout the LOV domain (311).

In an elegant study, a fusion protein was created between the LOV domain of YtvA and the histidine kinase domain of FixL from *Bradyrhizobium japonicum*, rendering the kinase domain sensitive to light (312). By systematically varying the properties of the coiled-coil linker, this linker was shown to be of critical importance for signal transduction in the fusion protein (312). Based on this, it was speculated that a rotation within the coiled-coil upon light absorption may be the way the signal is propagated (312). Whether this is true in YtvA as well remains to be uncovered, but the idea of signal propagation in a head-to-head dimer through a coiled-coil structure connecting the LOV and STAS domains certainly appears to fit with the stressosome model discussed above.

An intriguing possible role of YtvA emerged when it was noted that STAS domains include a conserved sequence motif that can be involved in GTP-binding. This led to the proposal that STAS domains, including those of the RsbR proteins and YtvA, may have a general GTP-binding role (13). Indeed, some STAS domains have been shown to bind GTP (330, 423-425). YtvA was shown to bind the fluorescent GTP-derivative BODIPY-GTP, which could be displaced by GTP and ATP (61, 62). Illumination of the LOV domain resulted in small but reproducible changes in the BODIPY-GTP-derived signal (61, 62). In addition, disruption of the proposed GTP-binding motif was shown to lower the affinity for BODIPY-GTP and to be deleterious for the function of YtvA in vivo (17, 451). This led to the
hypotheses that YtvA may be a sensor of GTP or ATP (61), or that YtvA may recruit nucleotides for the kinase activity of RsbT (17). However, subsequent work showed that BODIPY-GTP binds YtvA rather non-specifically, probably through hydrophobic interactions, including in the N-terminal part of the protein and in the coiled-coil linker between the LOV and STAS domains (107, 338). Also, GTP could not displace BODIPY-GTP (338), and was in fact shown not to bind to YtvA at all (107).

1.6.3. Transcriptional regulation of the rsbRA paralogs

The gene for *rsbRA* is located within the *sigB* operon (507), but the genes for *rsbRB* and *rsbRD* form single-gene operons elsewhere on the genome, and *rsbRC* lies in an apparent two-gene operon (5) (Figure 4). The first gene in the operon together with *rsbRC* is *norM*, a gene with significant homology to multidrug efflux proteins (254, 351, 386). Though the function of this gene in *B. subtilis* is not known, its annotation suggests that it may also be involved in stress protection.

**Figure 8:** Cartoon representation of the dimer formed in the crystal structure of the LOV domain of YtvA in the dark state, generated from PDB ID 2PR5 (311) using PyMOL v0.99 (Schrödinger, LLC). The two LOV domains are differently colored. FMN is shown, and the cysteine that forms a covalent adduct with FMN after illumination is colored yellow.
Not much is known of the regulation of transcription of the rsbR genes. Because rsbRA is part of the sigB operon, its transcription is regulated by the housekeeping sigma factor $\sigma^A$ (507). There may be a slight upregulation of rsbRA in response to the antimicrobial 2-methylhydroquinone, and similarly rsbRB may be slightly upregulated by the antimicrobial 6-brom-2-vinyl-chroman-4-on (344), though the underlying regulatory mechanisms remain unknown. Based on a bioinformatics analysis, the norM-rsbRC operon may be controlled by $\sigma^D$, an alternative sigma factor involved in motility and chemotaxis, but these genes have so far not been identified as members of this regulon (386, 415). The exception among the rsbR genes is rsbRD, which has been clearly shown to be transcribed in a $\sigma^B$-dependent manner (179, 340, 371, 378).

The transcriptional regulation of ytvA, which also lies in a single-gene operon, has been characterized relatively well. Promoter analysis and genome-wide transcriptome studies have identified Spx as a positive regulator of ytvA expression (144, 335). Interestingly, Spx is involved in protection against disulfide stress. Spx interacts directly with the $\alpha$-subunit of RNA polymerase to change its specificity towards certain promoters, and thereby both activates and represses transcription of several genes (335). Its activity is controlled by both a disulfide bond between two cysteine residues in a CxxC motif, and by proteolytic degradation via the ClpP protease (333, 334, 336, 337, 341).

1.7. Input signals and feedback in the general stress response

1.7.1. Input signals for the energy stress response pathway

Though the stresses that activate the GSR are well-known, it is not clear what the input signals for the signal transduction pathway are. As discussed above, many stresses that activate the energy stress response pathway have a decrease in ATP and GTP levels in common, which was thought to influence the kinase activity of RsbW (6, 7, 297, 492, 521) until RsbP was identified as primary regulator of energy stress (54, 485). Nevertheless, ATP levels may still be a direct or indirect signal for the activation of RsbP, and thereby the GSR. It is, however, also possible that the ATP drop is a consequence of the changes in the cell upon nutrient limitation, and that the relation with the energy stress response is not causal.

Arguing for a direct role of ATP levels in GSR activation is the observation that the decline in ATP levels, which follows phosphate limitation, precedes the induction of the GSR (521). The protonophoric uncoupler CCCP, a known inducer of the energy stress response (492), induces the GSR when cells are grown on the non-fermentable carbon source succinate but not when cells are grown on glucose (521). This suggests that it is ATP rather than a difference in proton motive force that leads to the activation of the GSR (521). Similarly, experiments have been done with a mutant in the $F_0F_1$ ATPase, which is responsible for the link between the electron transport chain and ATP synthesis (521). These experiments suggest that azide activates the GSR through its effect on the ATP pool rather than its effect on the electron transport chain (521). Finally, the relative effects of
inhibitors of ATP synthesis (azide) and GTP synthesis (mycophenolic acid) suggest that it is ATP rather than GTP that is more important for GSR activation (521).

Despite all these data it remains difficult to draw solid conclusions. For example, not all stresses that induce the energy stress response result in an equally sharp decrease in the ATP pool (297), and given the general importance of ATP for cellular physiology there may be many indirect effects. Thus, it is not certain if the state of the ATP pool is sensed directly, if a consequence of the decrease in ATP is sensed, or if both the GSR and the decrease in ATP are a consequence of the energy stress. The involvement of RsbP and its PAS domain, which, as discussed above, most likely binds a small molecule modified by RsbQ, argues against a direct sensing mechanism of ATP. However, it is formally possible that RsbP and RsbQ are more involved in regulating a background level of dephosphorylation of RsbV-P, and that the RsbW kinase is the input for the ATP level (55), although this seems unlikely given the requirement for RsbP and RsbQ. The identification of the molecule that probably binds to RsbP will prove an important step in the elucidation of the details of this signaling pathway.

1.7.2. Input signals for the environmental stress response pathway
The input signal(s) for the stressosome have also not yet been resolved. It is not yet clear whether there are additional upstream regulators, or whether a component of the stressosome is responsible for direct signal sensing. When the entire sigB operon, including the stressosome, is expressed in E. coli, the pathway cannot be induced by ethanol (410). When rsbX is deleted, however, transcription from a σ^B-dependent promoter increases 20-fold, which suggests that the proteins are active, and that a factor specific for B. subtilis is required for activation of the stressosome by stress (410). However, it is not known whether this is a specific signal or an additional (unknown) component of the regulatory pathway.

One interesting fact to consider is that many environmental stresses result in a secondary oxidative stress (317, 529). Induction of specific oxidative stress protection genes has been demonstrated during, for example, severe ethanol stress (194), heat stress (317), salt stress (195) and mild acid stress (506), which all also induce the GSR. In addition, as mentioned previously, many genes in the GSR regulon are involved in oxidative stress resistance, and sigB is important for non-specific resistance against oxidative stress (124, 194, 389). However, like for energy stress, it is hard to identify cause and effect, all the more because oxidative stress itself is not a strong inducer of the GSR (47, 124, 180, 321, 340, 529).

Blue light via the LOV domain of YtvA is the only well-defined input signal for environmental stress (see above). This demonstrates that it is at least possible for signals to directly enter the signal transduction pathway at the stressosome. Further characterization of the signal transduction mechanisms inside the stressosome should help in gaining more insight in the mechanisms involved and in the other input signals for the stressosome.
It is interesting to note that the reason why blue light is biologically perceived as stress is not yet understood. However, one possibility is that blue light (and UV light) can be harmful due to the formation of reactive oxygen species (280). In that respect it is interesting to note that Spx regulates transcription of ytvA, and is itself activated by thiol-specific oxidative stress (see above). This is yet another tentative link between the GSR and oxidative stress, although its function is not yet understood.

1.7.3. Other proteins with an unknown link to the general stress response

Although no more is known of the upstream regulatory pathway than discussed above, some other proteins appear to be directly or indirectly involved via unknown mechanisms.

One example is Obg, a GTP-binding protein essential for growth and initiation of sporulation (242, 455, 484). Obg was isolated in a screen for potential interaction partners of known components of the GSR signal transduction pathway, and was found to be essential for the environmental stress response but not for the energy stress response (409). Subsequent work showed that Obg binds L13, a protein in the 50S ribosomal subunit, in a GTP-dependent manner, and that it co-elutes with ribosomes during gel filtration (411, 520). Initially the components of the stressosome were also found in these fractions, but this was later shown to be a coincidence (78, 255). It is possible to isolate mutations in Obg that allow normal growth but do not allow GSR activation or sporulation, suggesting that its involvement is not purely due to growth inhibition (256). However, the role of Obg in the GSR pathway remains to be uncovered.

The ribosomal protein L11 (RplK) is also essential for environmental stress but not energy stress (518). Mutants in either Obg or L11 can still be activated by overproduction of RsbT, suggesting that the regulators of the GSR are present and functional, but that the ribosomes may somehow be involved in generation of a signal for the GSR (409, 411, 518).

Another protein involved in the GSR through an unknown mechanism is the (p)ppGpp synthetase RelA, the primary regulator of the stringent response to amino acid starvation (441). A null mutation in relA was found to block the activation of the GSR following energy stress (519). Specific mutations in relA or rplK (the gene coding for L11) that block the stringent response did not block the energy stress response, suggesting that the stringent response itself is not involved (519). In agreement with this, amino acid starvation does not normally induce the GSR (128, 325, 492). Interestingly, a null mutation in relA also blocks the drop in ATP/GTP levels that normally accompanies GSR activation (519), and the addition of inhibitors of ATP or GTP synthesis (azide and mycophenolic acid, respectively) did result in GSR activation, irrespective of RelA (521). Since the effect of ATP synthesis inhibition was much stronger than of GTP synthesis inhibition, it has been speculated that RelA may be necessary because it is somehow essential for the drop of ATP levels that appears to accompany GSR activation (521).

It is interesting to note that RelA is also ribosome-associated. The influence of these three proteins on the GSR suggests a role of ribosomes in GSR activation, but it is
important to note that this role may be indirect, especially given the impaired growth of mutants in the genes that encode these proteins.

1.7.4. Feedback in the general stress response

An interesting feature of the GSR signal transduction pathway is that feedback clearly plays an important role (Figure 9). As discussed above, the transcription of the downstream members of the regulatory pathway is induced by $\sigma^B$ itself (216, 507), creating a complex feedback system with more $\sigma^B$, but also more of the anti-sigma-factor RsbW and the anti-anti-sigma-factor RsbV. RsbX, the phosphatase responsible for the dephosphorylation of the stressosome, is also a part of this transcriptional unit. This creates an additional feedback loop, in which both energy stress and environmental stress lead to an increased capacity to dephosphorylate (and thereby inactivate) the stressosome. In line with these results, it was shown that the GSR can be activated without the RsbX feedback loop, but that this activation persists longer (495). Similarly, the transcriptional control of rsbRD by $\sigma^B$ (179, 340, 371, 378) creates a feedback loop, since RsbRD may displace other RsbR proteins in the stressosome, although it is not immediately clear what the function or effect of this loop is.

Another interesting potential feedback loop with unknown function is provided by the protease ClpP. Strains lacking ClpP display increased activation of the GSR in both stressed and unstressed conditions, which appears to be mostly due to the persistence of the response (241, 390). This suggests that ClpP is somehow involved in the turnover of one or more factors involved in stress induction (390) or in restoring the signal transduction network to pre-stress levels. Interestingly, this may be another feedback loop, as

![Figure 9: Simplified schematic overview of the feedback mechanisms discussed in paragraph 1.7.4. See the text for a comprehensive description. Different types of arrows indicate different mechanisms: regular arrows indicate stimulation of transcription of the gene coding for the indicated protein, and flat and round arrowheads indicate inhibition and activation of the protein, respectively. It should be noted that the arrows drawn here are not necessarily direct interactions.](image_url)
expression of ClpP is partly controlled by σ^8 (150, 340, 372). However, this effect may also be indirect, as ClpP is a major determinant of protein turnover in stressed cells (241), and mutants in clpP are impaired in diverse stationary phase processes such as sporulation, competence and motility (323).

A possible complicated feedback loop is also present in the regulation of expression of ytvA. As mentioned above, YtvA is known to be induced by the regulator Spx (144, 335), the activity of which is kept in check for a large part by the protease ClpP (333, 334, 336). Just like ClpP, transcription of spx is also partially regulated by σ^8 (179, 340, 371, 372). This means that, especially under conditions where multiple stresses are present, the regulatory functions of Spx and σ^8 may modulate each other.

All-in-all, it is clear that the signal transduction pathway of the general stress response is very complex, and that it ties in with many other cellular processes.

1.8. Relevance of Bacillus subtilis

*Bacillus subtilis* is a highly relevant organism to study. As mentioned previously, it is the model organism of choice for Gram-positive and spore-forming bacteria (117). The intrinsic resistance and extreme longevity of spores further increase the relevance of *Bacilli* in many different fields. Several of these fields will be briefly reviewed below.

1.8.1. Health hazards

There have been rare cases in which *B. subtilis* was found associated with disease, mostly in immune-compromised patients, but this may well be a consequence of misidentification or of how ubiquitous *B. subtilis* is (102). In fact, given the prevalence of spores of *B. subtilis* in diverse environments, including the human gut, it is surprising that this bacterium is not isolated from sick patients more often (98, 271). Thus, *B. subtilis* has generally been recognized as a safe organism without notable health hazards (98, 102, 191, 271, 354, 438, 454). Several of its close relatives, however, are known to cause disease. The most notable examples are *Bacillus cereus*, which produces toxins that can cause classical food poisoning symptoms, and *Bacillus anthracis*, the causative agent of anthrax (118, 199, 208, 239, 246, 487). Studying *B. subtilis* is safer and easier than studying these pathogenic species directly.

Although *B. subtilis* is benign it does cause some problems for the food industry. Most notably, it is one of the *Bacilli* responsible for ‘ropiness’ in bread, which is the most important cause of bread spoilage after moldiness (271, 369, 400). In addition, the prevalence of spores in soil and in association with plants means that spores readily enter the food processing line. There, they are hard to inactivate with mild preservation methods. Thus, *Bacillus* species including *B. subtilis* are a major cause of food spoilage, especially given the trend to move to less processed fresh-like food (58).
1.8.2. Health benefits
As discussed above, *B. subtilis* spores are able to survive passage through the gastrointestinal tract, where they can also germinate and re-sporulate. This property can be exploited in various ways. For example, *B. subtilis* and related species have been used as food supplements in humans, and as growth promoters and competitive exclusion agents in animals (reviewed in *e.g.* (98, 366)). Spores have also been used in a novel vaccination strategy, where the expression of antigens fused to the outer layers of the spore was shown to effectively induce an immune response and confer resistance in challenge experiments in mice. Similarly, the ability of spores to germinate in the GIT has been exploited with vaccines made from spores of antigen-producing cells (reviewed in *e.g.* (97, 111, 233)). When developed further, spore-based vaccines will have benefits such as: cheap production, easy preservation without the need for refrigeration, and administration through the nose or mouth instead of via needles—all of which make them highly suitable for use in developing countries (111).

1.8.3. Traditional fermentation
The ability of *B. subtilis* to ferment certain foodstuffs is used in many traditional fermentation strategies in Asia and Africa (reviewed in (366)). The best known example of this is the traditional Japanese food natto, which is made by fermenting soybeans with *B. subtilis* natto. Consumption of natto has long been associated with various health benefits (405). Other examples include fermentations of the seeds of several plants, such as dawadawa, which is made from the seeds of the néré tree (26, 366).

1.8.4. Industrial production
*B. subtilis* and its close relatives are the most important industrial producers of enzymes, with a market share of 50 to 60% in 2004 (405, 504). They have been used for decades for the production of *e.g.* proteases and amylases (354). *Bacillus* species are particularly suited for this purpose because they can easily grow under industrial production conditions, do not produce any toxic by-products, produce protein to high yields, and secrete the produced protein efficiently into the medium (354, 405, 504). The increasing understanding at the systems level of *B. subtilis* has the potential to lead to what has been referred to as a ‘next-generation super-secretion cell factory’ for enzyme production (465).

Apart from the production of enzymes, *B. subtilis* shows promise for the industrial production of other compounds, such as riboflavin (362, 405). It also has the ability to synthesize more than two dozen compounds with antibiotic activity, which may be used in the future as demand for antibiotics continues to rise (254, 443). One example of this is surfactin, a biosurfactant with antimicrobial activity produced by *B. subtilis* (287). Surfactin has medical applications as well as applications as a detergent and bio-emulsifier (20, 287, 326, 434).
1.8.5. Fuel and biomolecule production

The depletion of fossil fuels combined with the threat of global warming has led to an increased demand for biological fuel production (11, 112). *B. subtilis* natively produces a number of interesting compounds, such as ethanol, lactate, and 2,3-butanediol, although not with yields that generate commercial interest (96, 331). However, its easily accessible genetics have led to a recent increase in interest in *B. subtilis* as a producer of biofuels and related biomolecules. Ethanol has been successfully produced and the production has been optimized to close to the maximal theoretical yield, albeit with a low overall yield (398). Similarly, a few genetic modifications resulted in homo-lactic fermentation with a close to maximal yield, and with optically pure L-lactate as end-product (399). Production of acetoin (394, 501, 523), 2,3-butanediol (43, 350), and N-acetylglucosamine (269) have also been characterized and enhanced through genetic engineering. Production of isoprene, which can be used as biofuel or as precursor for synthetic rubber, has also been studied and significantly enhanced (511). More recently, there have also been attempts to have *B. subtilis* synthesize non-native compounds, such as isobutanol (209, 265, 266).

A general disadvantage of the production of bio-commodities in a non-photosynthetic bacterial system is that most bacteria require the supply of costly nutrients. In this sense, *B. subtilis* is highly attractive: added to its secretory abilities, safety and ease of use, it is able to grow on a wide range of carbon sources such as glucose, xylose, starch, and cellulo- and maltodextrins (362, 525). This has been exploited to produce L-lactate from cellobiose (399). More recently, *B. subtilis* has been engineered to grow on cellulose as the sole carbon source, which was applied to the one-step production of L-lactate from pre-treated cellulosic biomass (524). A similar approach has even led to the creation of a recombinant strain with the ability to grow on untreated cellulosic biomass (10). Although direct conversion of CO$_2$ and light into biofuels by cyanobacteria can, in principle, be more efficient and convenient (112), conversion of biomass waste into useful commodities may nevertheless prove to be highly valuable. In that light, it is interesting to note that residue from *B. subtilis* fermentations may be used as a feedstock for other bacteria that in turn produce alcohols (200).

1.8.6. Other applications

Other applications of *B. subtilis* include the use of biofilms as bio-control agents to reduce infections or promote growth in plants (282, 320). Pre-calibrated biofilms with spores have also been employed as biological UV-dosimeters in space during several space missions (196, 395), and similar personal dosimeters are in development for regular personal use on Earth (459). Spores of *B. subtilis* have been used as convenient (and reproducible) storage and packaging methods for strains that can be used as biosensors against *e.g.* arsenic and zinc (233).

Another intriguing application is found in a process called microbial enhanced oil recovery (MEOR), in which crude oil recovery from ‘depleted’ reservoirs is enhanced by
injection of microbes. *Bacillus* species have primarily been used in MEOR for their ability to produce surfactin and exopolysaccharide (20, 164, 413).

The (potential) applications of *B. subtilis* discussed above are but a few of many. It is interesting to note that many of the abovementioned applications make use of one of the ways that *B. subtilis* deals with stresses, such as biofilm formation or sporulation.

### 1.9. Pathways with similarity to the general stress response

#### 1.9.1. Introduction

The increased availability of genome sequences has made it clear that various parts of the network that regulates the GSR of *B. subtilis* are conserved in a variety of other organisms. Here, networks which have been (partly) characterized will be briefly reviewed, and their importance will be highlighted. It is relevant to note that the characterization of these pathways has generally relied heavily on work carried out in *B. subtilis*, which is often easier (or safer) to work with than the organisms discussed below.

#### 1.9.2. Close relatives of *Bacillus subtilis*

*Bacillus licheniformis*  
One of the closest relatives of *B. subtilis* is the industrially relevant *Bacillus licheniformis*, which possesses the complete eight-gene *sigB* operon of which the regulators are presumed to function as in *B. subtilis* (53). Like in *B. subtilis*, this σ^B_8 regulon is induced when cells are exposed to environmental stresses such as salt, heat or ethanol shock (53, 497), and peroxide stress may result in a partial activation of the regulon as well (407). However, no response could be detected to phosphate or glucose starvation (188, 496), even though entry into the stationary phase can cause σ^B_8 levels to rise (53). The absence of a functional energy stress response is likely due to the absence of the *rsbQP* module that senses energy stress in *B. subtilis* (178, 496).

*Listeria monocytogenes*  
*Listeria monocytogenes* is a food-borne, invasive pathogenic relative of *B. subtilis* that also carries the complete eight-gene *sigB* operon (27, 136, 153, 505). Like for *B. subtilis* and *B. licheniformis*, σ^B_8 can be activated by environmental shocks, such as those from acid, salt, cold, heat and ethanol, as well as by several cell-wall antibiotics (27, 74-76, 427, 458). Surprisingly, however, energy stress and CCCP also activate σ^B_8 (74, 75, 427), even though the *rsbQP* module is absent (136). How energy stress activates σ^B_8 is not completely understood. Although it requires RsbU, energy stress may enter the pathway independently of the stressosome and RsbT (74, 75, 427).

Interestingly, *L. monocytogenes* also carries a homolog of YtvA (LMO0799) that exhibits a photocycle *in vitro* (72) and is involved in activation of σ^B_8 in response to blue light *in vivo* (357). As higher temperatures destabilize FMN binding *in vitro*, it has been
speculated that the protein may integrate light and temperature sensing (72), but whether this is relevant in vivo remains to be seen.

The regulon controlled by $\sigma^B$ in L. monocytogenes is involved in functions such as direct stress protection, envelope function and cell-wall remodeling (171), much like in B. subtilis. The presence of $\sigma^B$ and its regulators has also been shown to be essential for optimal survival in several stress conditions (28, 77, 135, 355, 402, 503, 505, 526), including the imposition of antibiotic stress (29, 527). However, $\sigma^B$ is also strongly implicated in virulence of L. monocytogenes. The expression of several well-known virulence factors is controlled by $\sigma^B$ (76, 77, 171, 220), and sigB null mutants fare poorly in certain virulence model systems (76, 148, 355, 526). Because of its contribution to virulence and general stress resistance, $\sigma^B$ and its activation network are attractive targets for the development of antibiotics against this organism.

1.9.3. Conservation of the downstream partner switch in Staphylococcus

The identification of a sigB gene in Staphylococcus aureus, another important human pathogen, has received much attention. Only the three most downstream genes of the regulatory pathway are conserved in S. aureus (252, 414, 509). The anti-sigma factor RsbW, the anti-anti-sigma factor RsbV, and the stress-inducing phosphatase RsbU all function in roughly the same way as in B. subtilis (310, 414). Like in B. subtilis, environmental stresses such as shocks of heat, salt and alkaline pH induce $\sigma^B$ (151, 152, 360, 414). Ethanol and CCCP do not (71, 151, 360), although there does appear to be an induction upon entry into the stationary phase of growth (40, 360, 414). All these effects depend on the presence of RsbU, although there may also be poorly understood RsbU-independent effects (358, 361, 414). Interestingly, overexpression of rsbU is sufficient to activate $\sigma^B$ (361, 414), and the S. aureus RsbU protein is also constitutively active when expressed in B. subtilis (361). How RsbU is regulated in S. aureus remains to be discovered (178, 361).

Like in B. subtilis, $\sigma^B$ of S. aureus is involved in the regulation of processes such as cell envelope modification, metabolism, signaling pathways and membrane transport (41, 360). Mutants that lack sigB are impaired in resistance against various stresses, including hydrogen peroxide (71, 152, 251), UV (152), and desiccation (70), and are known to produce less pigment (152, 251, 345). Importantly, several virulence factors are influenced by $\sigma^B$ (41, 251), and some (though not all) studies show a significant role in infection and virulence (178, 184, 211, 308, 356). Similarly, (stress-induced) biofilm formation may be influenced by $\sigma^B$, although also here not all studies agree (25, 178, 229, 384). Finally, and perhaps most importantly, $\sigma^B$ is clearly involved in resistance against cell-wall antibiotics, including methicillin and vancomycin (39, 80, 204, 319, 435, 509).

The opportunistic pathogen Staphylococcus epidermidis carries a similar $\sigma^B$ network with the same operon structure (227, 234, 383) and protein function (94, 234, 235). Interestingly, the essential role of $\sigma^B$ in (stress-induced) biofilm formation has been firmly
established in this bacterium (94, 206, 207, 234, 235, 285, 376), and $\sigma^B$ is similarly involved in resistance against antibiotics (155, 236, 502).

1.9.4. Bacillus cereus group

*B. cereus*, a frequent cause of food-poisoning, also contains a $\sigma^B$-regulated general stress response that is activated by stresses such as heat, osmotic and ethanol shock (467), although its regulon bears only limited similarity to the GSR regulon of *B. subtilis* (468, 471).

Like in *B. subtilis*, $\sigma^B$ is controlled by the anti-sigma factor RsbW and the anti-anti-sigma factor RsbV (101, 470). The phosphatase RsbY is responsible for the activation of RsbV (470). However, the signaling pathway that controls RsbY is very different, with resemblance to chemotaxis because a two-component system and methylation are involved (101). RsbY is activated via its N-terminal response regulator receiver domain by the hybrid histidine kinase RsbK (81, 100). The genetic region encoding these proteins also contains a methyltransferase, RsbM, which has been shown to methylate RsbK (81). Deletion of *rsbM* results in failure to keep $\sigma^B$ activity in check, suggesting that it is a negative regulator (81). Interestingly, no methylesterase homolog has been found (yet) in *B. cereus* (81, 101).

*B. cereus* is closely related to *B. anthracis*, the causative agent of anthrax, and *Bacillus thuringiensis*, an important insect pathogen and biological pesticide (487). In fact, the relationship between these bacteria is so close that they are sometimes considered the same species with specific plasmids that cause different phenotypes (487). Not surprisingly, the genomic organization of the regulatory module of $\sigma^B$ is conserved in these organisms as well (100, 101), and $\sigma^B$ may contribute to virulence in *B. anthracis* (139).

1.9.5. Streptomyces coelicolor

*Streptomyces coelicolor* is an interesting bacterium that possesses 65 sigma factors, 48 homologs of RsbW and 17 homologs of RsbV (34, 309). One of these sigma factors is $\sigma^B$, which is viewed as a master regulator of osmotic stress and differentiation (83, 261). Its activity is induced by stresses like osmotic and acid shock, as well as by starvation (83, 232, 260, 261).

Like in *B. subtilis*, $\sigma^B$ is controlled by a partner switch of the anti-sigma factor RsbA and the anti-anti-sigma factor RsbV (260). Surprisingly, the gene for RsbV is not contained in the *sigB* operon but in a gene cluster close to the gene for another sigma factor, $\sigma^M$, which is related to $\sigma^B$ in structure and regulation (260). The *sigB* operon does contain an anti-anti-sigma factor homolog, called *rsbB*, but this does not appear to be involved in regulation of $\sigma^B$ at all (83, 260). Of note, the operon containing *rsbV* also contains homologs of a stressosome module, although it is not known what this stressosome regulates (260).

The known upstream components of the activation pathway somewhat resembles the regulators in *B. cereus* (101). The *S. coelicolor* pathway involves OsaB, a response regulator
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critical for differentiation, antibiotic production and osmo-adaptation (42). OsaB is activated by the histidine kinase OsaA, which resembles RsbK of B. cereus (101). OsaC is an interesting multi-domain protein with a phosphatase domain, several sensor domains and an RsbW-like kinase domain (134). Its activity is required to restore σB and OsaB activation to pre-stress levels after an osmotic shock (134). How this works is not clear, but the RsbW-like anti-sigma factor domain has been shown to be able to bind σB, and thus likely functions as a second anti-sigma factor for σB (134).

Several other sigma factors in S. coelicolor have also been shown to be regulated by partner switches. For example, σF is regulated by the anti-sigma factor RsfA, which in turn is regulated by at least one anti-anti-sigma factor (228). The same is true for σI, where Prsl is the anti-sigma factor and Arsl the anti-anti-sigma factor (190). Finally, σH, another stress-response transcription factor involved in differentiation (232, 419), is regulated by the anti-sigma factor UshX (PrsH) (420, 488) and the anti-anti sigma factor BldG (37, 421). This system is especially interesting because BldG is transcribed together with another anti-sigma factor, ApgA, with which it also interacts but which is not involved in regulation of σH (363, 421). Even more surprising, neither ApgA nor UshX appears to be able to phosphorylate BldG, leaving its regulation largely unclear (421).

It should be noted that many of these mechanisms have not been fully characterized and are thus somewhat tentative. Given that the regulators tend to lie in different operons or indeed in different genomic regions, it is very possible that some of the observed interactions are artifacts or that there is significant cross-talk that remains to be discovered. However, it is clear that partner switches play a central role in regulation in S. coelicolor.

1.9.6. Conservation of modules

Partner switches that do not control σB homologs
The involvement of a partner switch is not confined to regulatory networks of σB homologs. In fact, B. subtilis has another well-characterized partner switch which controls the activity of σF, an alternative sigma factor involved in the early stages of spore-formation (reviewed extensively in e.g. (116, 125, 186, 515)). In fact, the name ‘partner switch’ was first proposed for this module (6). The basics of the module are the same as the partner switches in the GSR pathway: an anti-sigma factor SpoIIAB switches between σF and the anti-anti sigma factor SpoIYA. The activity of SpoIYA is controlled by the kinase activity of SpoIIAB, and the phosphatase activity of the membrane protein SpoIIE.

Conservation of the stressosome
Similar to the ubiquitous partner switch module, the genes for the stressosome module are also highly conserved in a wide range of organisms (359, 460). Interestingly, the stressosome genes are found in diverse phyla, such as the Firmicutes, Actinobacteria, Proteobacteria, Cyanobacteria and even in at least one Archaea (359, 460). Genomic
organization in these organisms suggests that they are likely coupled to a range of output modules.

So far, *Moorella thermoacetica*, previously known as *Clostridium thermoaceticum* (92), is the only organism apart from *B. subtilis* in which a stressosome has been characterized (381, 382). The system consists of homologs of RsbRA, RsbS, RsbT and RsbX, and was shown to behave in much the same way as its *Bacillus* counterpart (382). Interestingly, the RsbT homolog of *M. thermoacetica* was shown to regulate the biosynthesis of the second messenger cyclic di-GMP (382).

**Conservation of the rsbQP module**

Although the energy-stress-sensing *rsbQP* module is the least conserved part of the *B. subtilis* GSR regulatory network, similar modules can be found in other bacteria. Bioinformatics analysis found genetic organizations of RsbQ-like hydrolases followed by an RsbP-like PAS domain in 45 bacteria of various families (329). Interestingly, these PAS domains could be coupled to a phosphatase domain, as in RsbP, but also to histidine protein kinase or diguanylate cyclase domains (329). Thus, coupling of sensing modules to diverse outputs seems to be a general property of the different parts of the GSR regulatory network.

**1.10. Scope and outline of this thesis**

The main goal of the work presented here was to increase our understanding of the mechanisms by which the stressosome functions. Because light is its only well-characterized input signal, we focused on the interaction between YtvA and the stressosome.

In Chapter 2, we used YtvA to systematically investigate the function of the RsbR proteins. By determining the strength of the light-induced activation of the GSR in all possible knockout combinations of the RsbR proteins, we show that they have different functions with regard to light stress.

In Chapter 3, we describe the construction and validation of a mathematical model of the photocycle of YtvA. We then applied this model on *in vivo* activation of the stress response as a first step towards a systems biology analysis of the input signals of the GSR.

In Chapter 4, we present the results of localization of RsbRA and YtvA, and discuss preliminary results of the first super-resolution microscopy on stressosomes. Our results are consistent with localization of YtvA to stressosomes *in vivo*.

Chapter 5 describes the discovery of a second, YtvA-independent, effect of light on the activation of the GSR. In contrast to the YtvA-mediated blue-light effect, this second effect activates the GSR via the energy stress pathway, and can also be activated with red light.

Finally, Chapter 6 contains a general discussion, in which the results reported here are placed in the context of recent developments in the field. Future directions of stressosome research are also discussed.