The general stress response of Bacillus subtilis

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Chapter 6: General discussion

Jeroen B. van der Steen
6.1. Abstract
The stressosome is undoubtedly one of the most interesting and prominent features of the signal transduction pathway that regulates the general stress response in Bacillus subtilis. Shortly after work on this thesis was started, Marles-Wright and co-workers published a landmark paper in Science about the structure of in vitro reconstituted stressosomes (291). Although much has been learned since then, many important questions still remain unanswered. For example, it is not yet clear what intracellular signal(s) the stressosome responds to and how, what the exact role of the individual RsbR proteins is, and how the signal(s) are propagated inside the stressosome to ultimately result in the release of RsbT. There are additional unanswered questions regarding the function and role of YtvA.

Similarly, many questions regarding the energy stress response pathway remain. Do further upstream components of this pathway remain to be identified, how do RsbP and RsbQ interact to sense and propagate a signal, and what is the biological role of light in this response?

Here, the progress made by us and others in the past years towards answering these questions will be discussed.

6.2. Stressosome function
6.2.1. Input signal(s)
The discovery of the input signal(s) of the stressosome is crucial for a full understanding of the general stress response (GSR) in Bacillus subtilis. Uncovering the input of the stressosome will increase our understanding of the effects of environmental stress on the cell, what the different stresses have in common, and what the cell does to counter them. On the protein level, knowledge of the input signals of the stressosome is essential for an understanding of the mechanisms in—and properties of—the stressosome itself.

With the accumulating evidence that the photoreceptor YtvA is part of the stressosome (see Chapter 4 and below), it has become clear that blue light is sensed directly by the stressosome. However, there is no clear evidence that other environmental stresses are also sensed directly. Thus, there may be unknown upstream components of the pathway, or general effects of environmental stresses on the cell that may in turn be sensed directly or indirectly. One candidate for such a general effect is a secondary oxidative stress, which many environmental stresses have in common (see Chapter 1 for a more detailed review). However, it remains to be seen if oxidative stress has anything to do with the activation of the stressosome.

Interestingly, experiments in which the primary components of the pathway were expressed in Escherichia coli yielded a pathway which could be induced by deletion of rsbX, but not by environmental stress (410). This suggests that a Bacillus-specific factor may be required—and that direct sensing of the stress factor is unlikely (410). In contrast,
an RsbR homolog from *Listeria monocytogenes* (LmRsbR) was shown to be functional as the only RsbR protein in *B. subtilis*, which suggests a conserved or common signal (294). It should be noted, however, that interpretation of both these studies is difficult, and that neither directly proves anything related to the input signal(s). In addition, the GSR activation network of *L. monocytogenes* is very similar to that of *B. subtilis* (see Chapter 1), which makes conservation of a specific input signal more likely.

The increasing availability of genome sequences has made it clear that the genes for stressosome-like modules occur in many organisms, some of which are evolutionarily very far removed from *B. subtilis* (see Chapter 1). These stressosome-like modules can be involved in the regulation of diverse responses. Thus, it will be very interesting to exchange stressosome components between these organisms, as they are expected to respond to very different stimuli. Placing the well-studied stressosome from *B. subtilis* in another organism, much as was done for *E. coli*, is another promising way to see if input signals are conserved across organisms with and without stressosome-like complexes. These experiments will become much more powerful when it becomes possible to measure the output of the stressosome directly. Towards that end, visualization of the release of RsbT will be highly useful (see below).

### 6.2.2. Signal propagation

Regardless of the input signal(s), the result of activation of the stressosome is clear: RsbT is released to activate the downstream pathway. Phosphorylation of RsbS and the RsbR proteins is critical for this release, although little is known about the mechanisms that facilitate this. It is plausible that some structural rearrangement is required to allow RsbT access to the phosphorylation sites in the STAS (sulfate transporter and anti-sigma-factor antagonist) domains of the other stressosome proteins. The nature of this putative rearrangement and the trigger that starts it are still unclear. Blue-light-dependent activation of the GSR through YtvA is the one exception, which will be further discussed below.

The core of the stressosome is made up of the STAS domains of RsbS and the RsbR proteins, while the N-terminal domains of the RsbR proteins protrude out from this core as ‘turrets’ (291). Based on this, the N-terminal domains of the RsbR proteins were proposed to act as the signal input domains (291). The signal may subsequently be transmitted through a linker region to the STAS domains in the core, resulting in rearrangements that lead to phosphorylation of the stressosome and release of RsbT.

Although this hypothesis is plausible, there is no direct evidence to support it. Mutations in the globin-like N-terminal domain of RsbRA (N-RsbRA) can affect the basal output level of the GSR in unstressed cells, but none have been identified with significant effects on stress induction of the GSR (145, 327). Similarly, effects of mutations in the linker between N-RsbRA and the C-terminal STAS domain are mostly confined to the basal level of GSR activation in unstressed cells (146). It is possible that the correct mutations to disrupt signal sensing in N-RsbRA simply remain to be identified. However, available data
are also consistent with the stress signal entering the stressosome elsewhere, such as directly in the C-terminal STAS domains of the RsbR proteins (146). In such a model, the N-terminal domains of the RsbR proteins could function as modulators of the basal activity of the stressosome (146). This modulation of the basal activity could still happen in response to a signal, allowing the stressosome to integrate multiple simultaneous signals into a single response. Alternatively, the N-terminal domains may be involved in binding of RsbT, which would also be consistent with their proposed role in the modulation of the basal activity of the stressosome.

Exchanging stressosome components between organisms, as mentioned above, should also aid in answering which component of the stressosome is sensing the signal. Intriguingly, the previously mentioned RsbR homolog from *L. monocytogenes* (LmRsbR) also allowed the stressosome to show some response to energy stress (294). This apparent transfer of stress-specificity from one organism to another suggests that the RsbR proteins function in signal sensing. However, a response to energy stress was also found for two *B. subtilis* RsbR proteins, which may have been a secondary effect of changes in the basal activation of the GSR in unstressed cells (294). Thus, the response to energy stress may not be a feature of LmRsbR itself. Also, these experiments reveal nothing about the domain involved in signal sensing, and do not exclude the possibility that the RsbR proteins are involved in modulation of the basal level of GSR activation.

One way to uncover the domain responsible for specificity is to create fusion proteins between the N-terminal domain of one RsbR protein and the C-terminal domain of another. This requires a clearly observable difference in function, such as the observed different basal level of GSR activation in mutants with different sole RsbR proteins (see Chapter 2). Alternatively, a fusion protein of the domains of RsbRA and RsbRB may be created, as they have an easily observable difference in function towards YtvA-mediated light stress (see Chapter 2).

To visualize the rearrangements needed to release RsbT, structures of active and inactive stressosomes would be ideal. The recent advances in creating stressosomes containing YtvA (215) have opened up the exciting possibility to compare the structure of light- and dark-adapted stressosome particles, which would literally ‘shine light’ on the activation of the stressosome.

**6.2.3. Role of the RsbR proteins**

Experiments *in vivo* and *in vitro* have made it clear that the stressosome can be composed of just RsbS and one of the RsbR proteins, with the optional attachment of RsbT in unstressed cells (see Chapter 1). However, there are four RsbR proteins *in vivo*: RsbRA, RsbRB, RsbRC, and RsbRD. At the start of the work described in this thesis, they were regarded as ‘mostly redundant’: any one of the four is enough for a functional stress response, with only relatively minor differences in, e.g., the basal level of activation of the GSR in unstressed cells (see Chapter 1). However, no systematic study of the effects of all knockout combinations of RsbR proteins had been performed.
We performed such a systematic study using blue-light stress transmitted by YtvA as the input signal (Chapter 2). The results clearly show that the RsbR proteins have opposing functions when it comes to the activity of YtvA: RsbRA and RsbRC enable the YtvA light-effect, while RsbRB inhibits it. The role of RsbRD was less clear, possibly due to relatively low protein levels in vivo (392). Interestingly, the localization of a fusion protein between a fluorescent reporter and YtvA appears to be different in strains with either RsbRA or RsbRB as the sole RsbR protein. An apparent loss of localization of YtvA in a strain with only RsbRB suggests that YtvA may not be able to incorporate into stressosomes formed by RsbRB (see Chapter 4). To confirm this, it will be very interesting to see if in vitro reconstitution of the stressosome (such as in reference (215)) leads to different results if YtvA is mixed with RsbRB instead of RsbRA.

Taken together, these results suggest that regulation of YtvA may be one of the primary functions of RsbRB. Thus, the regulation of protein levels of RsbRB may reveal under which conditions light is deemed important by B. subtilis.

It is not yet clear if the differentiation in function towards light stress extends to some other environmental stresses as well, as most have not yet been (systematically) studied in relation to the different RsbR proteins. Thus, it is possible that other environmental stresses are similarly regulated by changing the ratio of the various RsbR proteins in the stressosome. In addition, some combinations of RsbR proteins may allow activation by stresses that have not yet been identified because they have not yet been tried as inducers of the GSR under the right conditions. The tentative observation that stressosomes with RsbRC or RsbRD as the sole RsbR proteins respond to energy stress could be one such example (294).

### 6.3. YtvA and blue light

#### 6.3.1. Relevance of light for B. subtilis

It has been convincingly shown that YtvA activates the stressosome in response to blue light (16, 18, 448). Thus, low-intensity blue light is an environmental stress (464). This raises the interesting question of why light is perceived as stressful by a non-phototrophic bacterium like B. subtilis.

One possibility is that the presence of light, and especially of blue light, serves as an indicator for harmful UV-radiation, which can lead to all sorts of cellular damage (462). Similarly, very high light intensities of blue light could lead to damage. Thus, cells growing in the light may benefit from the general protection offered by the GSR.

It is interesting to note that, at least under laboratory conditions, moderate light intensities do not cause a detectable activation of the GSR without one of the following: 1) overproduction of YtvA (16), 2) the addition of another environmental stress such as salt (16, 448), or 3) removal of the inhibitor RsbRB (Chapter 2). The last observation in particular suggests that there may be conditions in which light is perceived as a stress by itself. Interestingly, the presence of salt stress in the light is apparently more harmful than
in the dark. Thus, light may also serve as an indicator for salt stress, for example because sunlight may cause osmotic stress through desiccation of the upper layers of soil. Similarly, increased light intensities can correlate with increased temperatures, which are also sensed by the stressosome. Thus, light input into the GSR may help the cell prepare for some environmental stresses before they occur.

Light may also signal the position of *B. subtilis* relative to its preferred habitat. Whether *B. subtilis* prefers living in the top layers of soil, in association with plant roots, or in animal gastrointestinal tracts, light may signal a deviation from these habitats, and thus an increased potential for stress.

Another alternative worth mentioning is that light-sensing may function as a very primitive version of a circadian clock (18). Daylight could signal an increased possibility for environmental stresses such as desiccation and heat, but could also predict changes in other organisms in the proximity of *B. subtilis* (including microorganisms and plants). This information can be used to prepare the GSR, but may also be used to indirectly influence important lifestyle decisions. This is not implausible, since all stationary-phase responses are highly intertwined, and links between the GSR and sporulation and biofilm formation have also been demonstrated (see Chapter 1 and Chapter 5).

The examples of possible roles of light-sensing mentioned above are, of course, not mutually exclusive. What the dominant reason to sense light is, and how the light effect on energy stress ties in (see Chapter 5 and below), remains to be discovered.

### 6.3.2. Relevance of light for experiments on *B. subtilis*

It has become increasingly clear that light matters, even for organisms that were not traditionally viewed as light-sensitive. The light effects in the regulatory network of the GSR are clear examples of this. However, even the laboratory-favorite *Escherichia coli* was recently shown to have a blue-light photoreceptor involved in biofilm regulation (385, 457), and domains for photoreceptors are wide-spread throughout the bacterial kingdom (122, 462).

These observations underline the importance of a controlled light environment for experiments on bacteria—especially on bacteria with known photoreceptors. In the case of *B. subtilis*, the results presented in Chapter 3 clearly demonstrate that typical laboratory light intensities can be enough to cause significant differences in results. Thus, every experiment performed on the GSR should be done under controlled light conditions. Since working in the dark is often impractical, it is advisable to keep light intensities for all samples within a controlled range, and to avoid any light-intensity bias between samples and controls. As the GSR influences many processes directly or indirectly, so will light. If this is not taken into consideration, experimental results can become difficult to interpret.

### 6.3.3. Structure and place of YtvA in the stressosome

Despite some initial ambiguity, it is now firmly shown that YtvA forms a dimer in solution (213). This dimer is most likely oriented head-to-head, with LOV-LOV and STAS-STAS
interaction surfaces (214). The α helix (α) that connects the LOV (light oxygen voltage) domain and the STAS domain is predicted to form a coiled-coil, and to assist in inter-domain transmission of the light signal (17, 312).

Before the start of this work it was already known that YtvA could co-purify with components of the stressosome (144). We and others have further extended the evidence that YtvA associates with stressosomes (Chapter 4 and (215)). Based on the domain organization of YtvA it seems likely that it would occupy a place in the stressosome similar to the RsbR proteins. Indeed, YtvA was shown to displace RsbRA from \textit{in vitro} reconstituted stressosomes (215). Together, these results strongly suggest that YtvA occupies a place in the stressosome similar to the RsbR proteins, with its STAS domain in the core and its LOV domain protruding outwards. YtvA may do this as a homodimer, but given the requirement for at least one of the RsbR proteins to be present before it can form functional stressosomes (Chapter 2 and (5, 215, 231)), it may also do this as a heterodimer with an RsbR protein. More detailed analysis of stressosomes containing YtvA \textit{in vitro} should be able to clarify this.

Some experiments have suggested that YtvA binds GTP, and that the presumed GTP-binding motif is important for its biological function (17, 61, 62, 451). Based on this, it was proposed that YtvA may be a GTP sensor (61) or recruit GTP for the RsbT kinase (17). However, GTP binding has since been shown to be an artifact (107, 338). Thus, there is currently no known role for YtvA other than modulating the activity of the stressosome in response to blue light, suggesting that blue light is an important signal for the GSR.

6.3.4. YtvA as a tool

As a photoreceptor, YtvA has a unique position in the signal transduction network controlling the GSR because it is the only protein with a clearly defined input signal: blue light. In addition, this input signal is easy to control, both in intensity and duration. These properties make it the ideal tool to study the GSR, which we have exploited in the work reported in this thesis (Chapter 2 and Chapter 3). The photocycle of YtvA can be modeled and its molecular properties can be modified. For example, the lifetime of the activated state of YtvA \textit{in vivo} can be changed by mutations (Chapter 3). This makes light and YtvA the ideal quantitative input for a systems biology model of the entire σ^8 network. Similarly, YtvA is currently the best candidate to activate stressosomes \textit{in vitro}, and thus the most promising way to uncover the changes the stressosome undergoes upon activation by stress.

These properties of YtvA are valid for many other photoreceptors as well, including synthetic ones. Our increasing knowledge on the molecular mechanisms of photoreceptors like YtvA is enabling our ability to construct synthetic photoreceptors that are used to control cellular processes, like transcription. A growing number of examples of such optogenetic approaches already exist in literature (for reviews, see \textit{e.g.} (86, 110, 133, 313)).
Interestingly, the natural fluorescence of YtvA can be greatly enhanced by mutating the adduct-forming Cys62 to alanine. Using this mutant, a class of FMN-based fluorescent proteins (FbFPs) has recently been developed (108). This is particularly interesting because LOV domains do not need oxygen to mature, unlike the better-known derivatives of green fluorescent protein (GFP). Thus, FbFPs are a good alternative for imaging under anaerobic conditions (109). The recently developed near-infrared phytochrome-based fluorescent reporters (137, 138, 429) also do not require oxygen for maturation, but they do require a bilin co-factor which may have to be added or require oxygen for synthesis.

Because YtvA possesses a branching reaction that converts a small portion of the molecules that are in the light-induced signaling state back to the dark state (Chapter 3 and (281)), it even has a limited use in super-resolution microscopy (281).

### 6.4. Fluorescence microscopy

#### 6.4.1. Single-cell analysis of the general stress response

Environmental stress is known to cause a transient activation of the GSR, peaking at approximately 20 to 30 minutes after addition of the stress factor (178). However, such measurements were always done on entire cell populations, and their results thus represent a population average. What happens on the single-cell level was mostly unclear, until Elowitz and co-workers studied a fluorescent reporter of GSR activation (514).

Their experiments show that the temporal pattern of single cells largely follows that of the entire population: upon addition of an environmental stress factor, cells display a single synchronized ‘pulse’ of activity (514). Interestingly, the amplitude seems to depend on the rate of increase of the environmental stress rather than the absolute magnitude (514). Thus, the GSR appears to be a general protection mechanism that is activated when conditions rapidly deteriorate, while slower stresses are dealt with by specific responses (514).

These results sharply contrast those obtained for energy stress. Cells with a functional energy stress response pathway (i.e., the presence of RsbP and RsbQ), sporadically exhibit unsynchronized but sustained pulses of GSR activation (270). Increasing levels of energy stress result in increasing pulse frequency, amplitude and duration, but the frequency increase to up to 0.1 to 0.2 pulses per hour is most prominent (270). Thus, the energy stress response pathway appears to be frequency-regulated, in contrast with the amplitude-regulation of the environmental stress response. Like for many other stationary-phase responses, stochastic noise plays a role in this frequency regulation (270).

A simple model is sufficient to qualitatively explain these observations (270). In this model, the phosphatase activity of RsbP and the kinase activity of RsbW balance each other, with RsbW initially having the upper hand. Stochastic increases in the activity of RsbP allow it to overcome the activity of RsbW, which results in activation of $\sigma^B$. This leads to a feedback loop in which $\sigma^B$, RsbV and RsbW are upregulated, resulting in an increase in
total $\sigma^B$ activity. This continues until the kinase activity of RsbW reaches a threshold and overcomes the phosphatase activity of RsbP, at which point the system is shut off.

The abovementioned results reveal that fluorescence microscopy and the study of systems on the level of a single cell can reveal surprising mechanisms as compared to studies on the population level, and lead to entirely new insights.

6.4.2. Localization and dynamics

Fluorescence microscopy is also a powerful technique to study the regulators of the GSR in vivo. In Chapter 4, we have made initial steps towards this end, by visualizing stressosomes and providing evidence that YtvA is associated with stressosomes in vivo. This work is in its initial stages, but should eventually assist in answering many interesting questions, such as how many stressosomes there are per cell, if stressosomes have a preferred localization, and if the number of RsbRA and YtvA proteins matches expectations from in vitro data.

We have shown that stressosomes can be visualized in live cells, which raises interesting possibilities for future work. For example, the dynamic exchange of RsbR proteins or the real-time release of RsbT from the stressosome may be visualized in the future (see Chapter 4). Using such an approach, it should be possible to look at the GSR activation pulses with a more direct readout, with the option of collecting quantitative parameters on the system. Such data should prove valuable for a detailed systems biology model as well as for detailed understanding of the reasons for the existence of the stressosome.

6.5. Energy stress

6.5.1. Interaction between RsbQ and RsbP

Despite recent progress, understanding of the energy stress response pathway is still limited. It is not known what the role of RsbQ is, if there are any upstream components of the network, and how the phosphatase activity of RsbP is regulated.

There are multiple possibilities regarding the role of RsbQ. Given the importance of its proposed catalytic triad (54, 217), it seems plausible that enzymatic activity of RsbQ is required. Thus, RsbQ has been proposed to modify either RsbP or a co-factor for RsbP (54, 217). A direct interaction between RsbQ and RsbP is probably important, e.g. for co-factor delivery (54, 329). However, whether this interaction is permanent or transient in vivo, remains to be elucidated. In the near future, this question may be answerable by co-localization studies with fluorescence microscopy.

6.5.2. Input signal(s)

The input signal and where this enters the pathway is unknown for the energy stress response, much as for the environmental stress response. Although the PAS (Per-ARNT-Sim) domain of RsbP, a well-known sensor domain, seems the most likely input site, it is also possible that the signal enters through RsbQ (55). Nothing is known of the possible
signal, but one interesting possibility comes from a yeast hydrolase with relatively high structural similarity to RsbQ. In this yeast hydrolase, a motif containing a cysteine renders the protein sensitive to inactivation by oxidation (262). As this motif is conserved in RsbQ (262), it would be interesting to see if oxidative stress has an effect on the energy stress response.

Alternatively, the RsbQP module may be involved in setting a basal level of GSR activation (55), much as proposed for the N-terminal domains of the RsbR proteins in the stressosome (see above). The signal for energy stress could then enter elsewhere in the network. The kinase activity of RsbW is one such place, which would fit well with the earlier hypothesis that a drop in ATP levels may directly modulate the kinase activity of RsbW (6, 7, 104, 297, 492).

The response may also be regulated by transcriptional control of the rsbQP operon (270). This idea is supported by the work on the pulsed activation of the GSR in response to energy stress. A change in the level of RsbQ and RsbP is sufficient to explain the increased frequency of pulses upon energy stress (270). In addition, transcription of the rsbQP operon increases following addition of mycophenolic acid (270). However, no discrimination between a regulation of the activity of RsbW or of the activity of RsbP could be made, as this has similar effects in the proposed pulse-generating circuit (270).

Although these last two models are attractive in their simplicity, they fail to explain why the RsbQ-PAS module is conserved as a sensing module coupled to diverse outputs in other organisms (329), and why RsbP requires them in the first place if RsbP’s activity is purely regulated by transcriptional control. Thus, these models are likely an oversimplification. However, like for the N-terminal domains of the RsbR proteins, it is possible that the primary role of the RsbQP module is to regulate the basal activity of the GSR in response to a signal. This signal may be either some direct input into the module or a transcriptional regulation, or a combination of both. It is also possible that all of the abovementioned mechanisms are utilized in vivo by different energy stresses.

6.5.3. Light activation of RsbP

During our characterization of YtvA-dependent blue-light stress, a (chronologically) second light effect appeared in the GSR activation network (Chapter 5). In ytvA null mutants, the level of energy stress in stationary phase was significantly enhanced by light. This effect required the presence of RsbP and RsbQ, as would be expected for an energy stress (Chapter 5).

In an attempt to further characterize the light effect we compared its magnitude in response to red and blue light. Interestingly, red light was more efficient than blue light, which is in sharp contrast to the YtvA-mediated light effect (Chapter 5). However, we have not yet characterized the action spectrum of this effect in detail. A systematic comparison of many different wavelengths will aid in the identification of the molecule that absorbs the light. Although such experiments are laborious and technically challenging, they
should be relatively straightforward on a ~20 nm resolution using differently colored light-emitting diodes (LEDs).

Where light enters the pathway that transmits energy stress is currently unknown. Light may act directly on RsbQ or RsbP, which is the simplest explanation in the absence of more knowledge of the upstream pathway. In this scenario, the PAS domain of RsbP is the most likely candidate to function as light sensor, since PAS domains have a well-described role as signaling domains that can bind diverse co-factors (183). Thus, we have tentatively assigned the light-sensing role to this domain (Chapter 5). If this hypothesis is correct, there is the exciting chance that the PAS domain of RsbP represents a new family of photoreceptors.

Nevertheless, it cannot be excluded that light acts somewhere upstream of the RsbQP module. This may be on an unidentified protein, but also on something involved in generating the signal for the RsbQP module, or on something controlling its expression. Given that the light-enhancement is only observed when cells encounter energy stress, light must act on something that is only active or present during energy stress.

The physiological role of the RsbP-dependent light effect is not yet clear. Although it is less sensitive than the light effect in strains overproducing YtvA, it is still apparent at moderate light intensities (Chapter 5). Thus, it seems plausible that B. subtilis would encounter sufficiently high light intensities in its natural environment for this effect to play a significant role, which may be similar to the role of YtvA (see above).

6.5.4. Further characterization of light activation of RsbP
If light enters the energy stress response pathway in the PAS domain of RsbP, this domain must bind a light-sensitive co-factor. PAS domains are well-known for the ability of many members of this family to bind diverse ligands, including flavins (such as in LOV domains), hemes, carboxylic acids, and divalent metal ions (183).

Unfortunately, we were unable to identify a co-factor attached to RsbP, purified from exponentially growing or stationary phase E. coli or B. subtilis cells, despite numerous attempts (unpublished results). Nevertheless, a single purification experiment revealed a tiny absorption at a wavelength corresponding to a heme-like co-factor. Thus, we tested whether RsbP was able to bind hemin in vitro. Addition of RsbP to hemin resulted in a characteristic shift of the absorption spectrum, indicating binding (Figure 1). The association persisted after dialysis, or when RsbP and hemin were incubated in the cell extract prior to purification (unpublished results).

To further clarify the association between RsbP and hemin we used electron paramagnetic resonance (EPR) spectroscopy (Figure 2; unpublished results). The major contribution in the obtained spectrum shows strong resemblance to proteins that bind heme via a cysteine residue, such as the cytochrome P450 family (see (30) for an example).
RsbP contains three cysteine residues in its PAS domain (Cys10, Cys56 and Cys104) and three in its STAS domain (Cys279, Cys357 and Cys396). Because ligand binding would be expected to happen at the PAS domain, we generated alanine substitutions of the first three cysteine residues. These mutants were then analyzed for hemin binding in vitro, followed by analysis by EPR. However, all three mutant proteins still bound hemin, and the characteristic EPR signal was not lost (unpublished results). In addition, the energy stress response was not abolished in vivo in any of the three mutants (unpublished results), which is consistent with a similar observation for Cys10 and Cys56 in recent literature (329). Similarly, hemin binding in vitro was not abolished by cysteine-to-alanine mutations in the three cysteine residues in the STAS domain (unpublished results).

Although these results are not encouraging, a homology model of the PAS domain based on the heme-binding PAS domain of FixL from Bradyrhizobium japonicum (163) revealed that it is possible that Cys56 and Cys104 are both oriented towards the putative binding pocket. Therefore, a double mutant would have to be tested before the involvement of cysteine residues in hemin-binding can be ruled out.

6.5.5. Pressing questions regarding the light activation of RsbP
Although we observed binding of RsbP to hemin in vitro, there is no indication that RsbP actually binds hemin in vivo. In fact, the presence of RsbQ, and the requirement of its active site for the energy stress response to function (54), suggests that the putative co-factor of RsbP may require specific modifications. Therefore, it is critical that RsbP is isolated with bound co-factor. It is possible that the co-factor is only generated when RsbQ is bound to RsbP. In this case, the entire rsbQP operon should be overexpressed. Similarly, the co-factor may only be generated during energy stress, and purification from starved cells may, therefore, be advisable.

It should be noted that even if RsbP binds a co-factor, it is not necessarily involved in the RsbP-dependent light effect. In fact, the spectrum of the RsbP-hemin complex does
not match the observation that red light is more efficient than blue light (Chapter 5), which is one indication that hemin may not be involved, and that further research is required.

An alternative approach to identifying the co-factor may be via crystallization of the PAS domain of RsbP. Although a preliminary crystallization report has been published some time ago (286), no structure is yet available. In addition, no mention of a co-factor was made (286).

If the RsbP-dependent light effect represents a true energy stress, it would be expected to behave similar to other energy stresses with regard to the frequency-increase of the GSR-activation pulses. Thus, it will be interesting to see if illumination with red light changes the pulse frequency.
6.6. Concluding remarks
During the work on this thesis our understanding of the regulatory network of the GSR has increased significantly, partly due to work described here. This increased understanding will serve as a basis for future research, which should lead to many new and exciting results in the coming years.

It will be exciting to uncover the mechanisms behind the effect of light on the energy stress pathway that is described in Chapter 5, especially given the possibility that a new class of photoreceptors may be involved. Similarly, a continuation of the fluorescence microscopy work described in Chapter 4 should yield many results in the coming years. Chapter 2, Chapter 3, and Chapter 4 have contributed to an increased understanding of YtvA and how it interacts with the stressosome. This will enable a more detailed study of the stressosome, and provide a quantitative input for a systems biology description of the GSR.