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The general stress response of *Bacillus subtilis*

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Chapter 2: Differentiation of function among the RsbR paralogs in the general stress response of *Bacillus subtilis* with regard to light perception

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2.1. Abstract

The general stress response of *Bacillus subtilis* can be activated by a wide range of signals, including low intensities of visible light. It is regulated by a dedicated σ factor via a complex signal transduction pathway that makes use of stressosomes: hetero-oligomeric complexes that include one or more of the RsbR proteins (RsbRA, RsbRB, RsbRC, and RsbRD). The response to blue light is mediated by the photoreceptor YtvA. We show here which of the four RsbR proteins are necessary for the activation of the σ^B response by blue light. Experiments performed with single-, double- and triple-deletion strains in the *rsbR* genes show that RsbRB and RsbRA function antagonistically, with the former being a negative regulator and the latter a positive regulator of the YtvA-dependent light activation of the stress response. A strain with RsbRB as the only RsbR protein is unable to respond to light activation of σ^B . Furthermore, RsbRC and RsbRD can replace RsbRA's function only in the absence of RsbRB. This differentiation of function is confined to light stress, since strains with RsbRA or RsbRB as the only RsbR protein behave similarly in our experimental conditions in response to physicochemical stresses. Interestingly, RsbRB's absence is sufficient to result in light activation of the general stress response at wild-type expression levels of *ytvA*, while it was previously reported that YtvA could only activate σ^B when overproduced, or when cells are supplemented with an additional environmental stress.

2.2. Introduction

The general stress response (GSR) of *Bacillus subtilis*, mediated by the alternative sigma factor B (σ^B), is activated when cells encounter 'energy' (or nutritional) stresses, such as carbon, phosphorus, or oxygen limitation, or 'environmental' (or physicochemical) stresses, such as acid, ethanol, heat, or NaCl shock (32, 47, 492). In addition, it was recently discovered that illumination with blue light can activate the GSR (16). In *B. subtilis*, σ^B regulates the expression of approximately 150 genes that are involved in processes such as carbon metabolism, macromolecular turnover, and envelope function and in counteracting stresses, either directly or indirectly (179, 372, 378). Several important pathogens, such as *Staphylococcus aureus* and *Listeria monocytogenes*, have a GSR-like regulatory network that is involved in regulation of virulence (221).

The activity of σ^B is regulated by a partner switch mechanism, which in turn is regulated by two convergent signal transduction pathways. In unstressed cells, σ^B is held inactive by the anti-sigma factor RsbW. Upon exposure to stress, the phosphorylated form of the anti-anti-sigma factor RsbV (RsbV-P) is dephosphorylated, whereupon RsbV binds to RsbW and σ^B is released, leaving it free to associate with RNA polymerase and activate the GSR. When the stress signal has decayed, RsbV-P is no longer actively dephosphorylated, and the balance shifts back towards rephosphorylation of RsbV by the kinase activity of RsbW. This results in the liberation of RsbW from RsbV, which switches off the GSR (for a review, see reference (178)).

Dephosphorylation of RsbV-P is catalyzed by two phosphatases. RsbP, one of the two phosphatases that contains an N-terminal PAS (Per-ARNT-Sim) domain, is required for the response to energy stress (485) and, as shown recently, to red light (18). Although the activation mechanism of RsbP remains unclear, a correlation with a drop in ATP levels has been reported (521), and the activity of an α/β -hydrolase (RsbQ) is required for this response (54).

More is known about RsbU, the other phosphatase, which is activated by environmental stresses (513). RsbU activation is based on interaction between its N-terminal domain and the kinase RsbT (105, 513). RsbT is normally held inactive by a high-molecular-weight complex called the stressosome, which is speculated to function as a signal integration hub (106, 292). The stressosome contains at least three classes of proteins: one or more of the RsbR proteins (see below), RsbS, and the aforementioned kinase RsbT (78, 106, 231). Both RsbS and the RsbR proteins contain a conserved STAS (sulfate transporter and anti-sigma-factor antagonist) domain, which can be phosphorylated by RsbT upon activation by environmental stress (5, 79, 143, 230). This results in release of RsbT, which activates RsbU. When the stress signal is extinguished, the prestress condition is restored by the phosphatase RsbX, which dephosphorylates RsbS and RsbRA, resulting in a stressosome capable of recapturing RsbT (79, 513). Genome sequence analyses have shown that the stressosome is not unique to *B. subtilis*, and that it can likely be coupled to a variety of output functions (359).

Initially only one RsbR protein was known, but the availability of the genome sequence of *B. subtilis* led to the identification of three paralogs. These four proteins have been renamed RsbRA (previously RsbR), RsbRB (YkoB), RsbRC (YojH), and RsbRD (YqhA) (5, 231). All have a highly conserved C-terminal STAS domain, which forms the core of the stressosome, together with the STAS domain of RsbS (291). Their N-terminal domains, in contrast, are diverse (5). For clarity, these four proteins are referred to here as 'RsbR proteins'. Two additional paralogs, each having a C-terminal STAS domain, were identified: the split paralog YetI/YezB, which does not appear to be involved in the regulation of the GSR and will not be considered here, and YtvA (5). YtvA was demonstrated to sense blue light with its N-terminal LOV (light oxygen voltage) domain *in vitro* (277). The loss or overproduction of YtvA abolishes or enhances, respectively, the environmental σ^B response induced by moderate intensities of blue light (16, 18, 448). However, the light enhancement of the GSR by YtvA only becomes apparent when YtvA is overproduced or when light is combined with an additional environmental stress, such as NaCl shock (16, 448). YtvA has been shown to act upstream of RsbU and RsbT (144), suggesting that it interacts with the stressosome. However, its role in or on the stressosome is not yet resolved. YtvA, RsbRB, RsbRC and RsbRD can all be co-purified with RsbRA from *B. subtilis* cell extracts (79, 106, 144, 231), strongly suggesting that YtvA is associated with the stressosome. However, RsbT is able to phosphorylate all four RsbR proteins, but not YtvA (5), and stressosomes can be made *in vitro* by mixing RsbS with any of the four RsbR

proteins, but this has not yet been reported for YtvA (78, 106). In addition, a quadruple knockout of the RsbR proteins leads to a constitutive and high level of activation of the GSR, presumably due to the inability to form (sufficient) stressosomes to capture RsbT, even though YtvA is still present in such strains (5, 231).

The specific role of each individual RsbR protein in the stressosome has remained unclear. The most intuitive explanation would be that each paralog senses a different (set of) stress(es) via its N-terminal domain. However, this appears unlikely, since mutants with any three of the four RsbR proteins deleted respond in a very similar way to NaCl and ethanol shock (5, 231). An alternative explanation is that all four RsbR proteins respond to a common intracellular signal(s), presumably created by all environmental stresses, but with a different sensitivity or threshold. This would allow the cell to fine-tune the sensitivity of the stressosome as required. This hypothesis is supported by the fact that each *rsbR* gene is contained in a different transcription unit, implying that differential regulation of expression may be important. Indeed, transcriptional analyses have shown that *rsbRD* is upregulated by σ^B (372), whereas *rsbRA* and *rsbRB* are upregulated by the antimicrobials 2-methylhydroquinone and 6-brom-2-vinyl-chroman-4-on, respectively (344). Additional studies have shown that transcription of *ytvA* increases in strains that overproduce Spx and σ^E : important regulators involved in disulfide stress and sporulation, respectively (121, 144). Intriguingly, many other bacteria that contain the genes for a stressosome also contain more than one RsbR paralog (359).

Because neither the separate input signals for each of the four RsbR proteins nor the upstream components of the environmental stress pathway are known, it is difficult to determine their individual functions *in vivo*. Therefore, we studied the roles of the RsbR proteins with respect to YtvA-mediated light stress. To this end, we performed a systematic study of the activation of the GSR in strains with all possible combinations of null mutations in the four *rsbR* genes. The photoreceptor YtvA was overproduced in these strains, and the GSR activation was measured in the presence or absence of light. Our results show a differentiation of function, most notably between RsbRB and the other RsbR proteins. In addition, we show that a single deletion of *rsbRB* is sufficient for a light-sensitive GSR, without the need for overproduction of YtvA or an additional environmental stress.

2.3. Materials and methods

2.3.1. Bacterial strains and genetic manipulation

The bacterial strains and plasmids used in this investigation are listed in Table I. DNA manipulations and molecular genetic techniques were carried out according to standard procedures. All *B. subtilis* strains used in the present study are derivatives of the wild-type PB2 (46).

Table I: Plasmids and strains used in this study.

Plasmid or strain	Genotype ^a	Reference or construction ^b
pDG148-Stu	P _{spac} -MCS <i>bla kan ble</i> (cloning vector)	(212)
pYtvA	P _{spac} - <i>ytvA bla kan ble</i> (replicating plasmid)	(16)
PB2	<i>trpC2</i> (168 wild type)	(46)
PB198	<i>amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	(46)
PB491	<i>rsbRAD amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	(4)
PB495	<i>rsbUD amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	(485)
PB528	<i>rsbRBD::kan amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	(5)
PB565	<i>ytvAD::ery amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	(5)
PB574	<i>rsbRCD::ery amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	(5)
PB520	<i>rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	(5)
PB198/pYtvA	<i>amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	(16)
PB491/pYtvA	<i>rsbRAD amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → PB491
PB528/pYtvA	<i>rsbRBD::kan amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → PB528
PB574/pYtvA	<i>rsbRCD::ery amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → PB574
PB520/pYtvA	<i>rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → PB520
JBS10011	<i>rsbRBD::kan ytvAD::ery amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB565 → PB528
MA1	<i>rsbRAD rsbRCD::ery amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB574 → PB491
MA2	<i>rsbRAD rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB520 → PB491
MA3	<i>rsbRCD::ery rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB520 → PB574
MA4	<i>rsbRAD rsbRBD::kan amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB528 → PB491
MA5	<i>rsbRBD::kan rsbRCD::ery amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB528 → PB574
MA6	<i>rsbRBD::kan rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB528 → PB520
MA7	<i>rsbRBD::kan rsbRCD::ery rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB528 → MA3
MA8	<i>rsbRAD rsbRCD::ery rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB520 → MA1
MA9	<i>rsbRAD rsbRBD::kan rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB520 → MA4
MA10	<i>rsbRAD rsbRBD::kan rsbRCD::ery amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB528 → MA1
MA11	<i>rsbRAD rsbRBD::kan rsbRCD::ery rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i>	PB520 → MA10
MA1/pYtvA	<i>rsbRAD rsbRCD::ery amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → MA1
MA2/pYtvA	<i>rsbRAD rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → MA2
MA3/pYtvA	<i>rsbRCD::ery rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → MA3
MA4/pYtvA	<i>rsbRAD rsbRBD::kan amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → MA4
MA5/pYtvA	<i>rsbRBD::kan rsbRCD::ery amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → MA5
MA6/pYtvA	<i>rsbRBD::kan rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → MA6
MA7/pYtvA	<i>rsbRBD::kan rsbRCD::ery rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → MA7
MA8/pYtvA	<i>rsbRAD rsbRCD::ery rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → MA8
MA9/pYtvA	<i>rsbRAD rsbRBD::kan rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → MA9
MA10/pYtvA	<i>rsbRAD rsbRBD::kan rsbRCD::ery amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → MA10
MA11/pYtvA	<i>rsbRAD rsbRBD::kan rsbRCD::ery rsbRDA::spc amyE::</i> (P _{ctc} - <i>lacZ cat</i>) <i>trpC2</i> pYtvA	pYtvA → MA11

^a Genes for antibiotic selection: *bla* = ampicillin; *ble* = phleomycin; *cat* = chloramphenicol; *ery* = erythromycin; *kan* = kanamycin; *spc* = spectinomycin.

^b Arrows indicate the direction of transformation. DNA (left) was transformed into a strain (right).

2.3.2. Plasmid and strain constructions

The construction of pYtvA, a plasmid for overexpression of *ytvA* based on pDG148-Stu (212), has been reported elsewhere (16). This plasmid features the *ytvA* gene under the control of the IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible *spac* promoter.

For strain constructions, transformants were selected on Bacto tryptic soy broth (TSB, 30 g/l) agar plates, containing, when appropriate, 10 μ g/ml kanamycin, 5 μ g/ml chloramphenicol, 0.5 μ g/ml erythromycin, 100 μ g/ml spectinomycin or 10 μ g/ml phleomycin, after overnight incubation at 37°C or 30°C. Transformations of *B. subtilis* were carried out according to a previously described protocol (253). The double, triple and quadruple null mutant strains MA1 to MA11, as well as JBS10011, were obtained by transforming the parent strain with chromosomal DNA of the strain bearing the desired mutation (see Table I for details). The pYtvA plasmid was also introduced into a number of strains by standard transformation. The only exception to this was strain MA11, which we could not transform using natural competence. For this strain, electrotransformation was used instead, according to a previously described protocol (510).

The identities of all transformants were checked by the presence of the appropriate antibiotic resistance(s) and by various PCRs to check for the presence of the correct knockout(s) and/or plasmid(s).

2.3.3. Growth conditions and β -galactosidase assays

The medium used for the β -galactosidase assays was TSB supplemented with 0.5% glucose, 5 μ g/ml chloramphenicol, and 10 μ g/ml kanamycin or 10 μ g/ml phleomycin, where applicable, to keep selective pressure on the replicating plasmids. The antibiotics did not result in a reduction in growth rate in our conditions. TSB medium was inoculated with a single colony from a fresh plate of the particular strain and incubated overnight in a shaking water bath at 37 °C and approximately 250 rpm. The overnight cultures were diluted and allowed to grow until they had reached the exponential growth phase, re-diluted, and distributed over different Erlenmeyer flasks to initiate an experiment. No volume larger than 10 ml in a 100-ml Erlenmeyer flask was used to assure sufficient aeration. The Erlenmeyer flasks were placed in the shaking water bath at 37 °C and approximately 250 rpm in the light or in the dark. Dark controls were wrapped tightly in aluminum foil. When the cultures had reached an optical density at 600 nm (OD_{600}) of 0.3 to 0.6 (early exponential growth phase) sampling was started. Depending on the specific assay conditions, described in the text and figure legends, a stress was usually induced at this point. YtvA overproduction was induced by adding IPTG to a final concentration of 1 mM (from a 1 M stock solution). Environmental stresses were induced by adding the stress factor (NaCl or ethanol; for amounts see the text) or light (by turning on a lamp). Whenever a stress factor was added an equal amount of water was added to controls without stress induction to correct for any dilution effects. Samples of the cultures were taken at various time points in complete darkness or with a minimal red background light when necessary (see the illumination conditions below). The samples were immediately

transferred to an ice/water mixture, flash-frozen with liquid nitrogen and stored at -80°C . In all of the experiments reported here, sampling was stopped before the cells reached stationary phase. β -galactosidase activities were measured and expressed in Miller units to correct for cell growth according to a previously described protocol (225). To compare the many different strains in the experiments with YtvA overproduction, each strain was assayed together with the “wild-type” strain (PB198/pYtvA, *i.e.*, the strain with intact copies of all four *rsbR* genes) under the same conditions (light and dark). The relative GSR activation was then calculated by dividing the actual activation by the activation in the presence of light of the “wild-type” strain at the same time point. In all graphs, error bars represent the variation of two biological replicates in the same experiment. Error bars are only shown when they exceed the size of the symbols. The data shown are from representative experiments, which were repeated at least two times with two replicates each.

2.3.4. Western blots

Samples for Western blots were taken from cultures grown and treated exactly as the cultures for the β -galactosidase assays (see above). Instead of inducing a stress at an OD_{600} of 0.3 to 0.6, samples were taken at an OD_{600} of 0.8 to 1.0—*i.e.*, in the middle of the growth phase where the reporter-enzyme experiments were performed. Cells were harvested by centrifugation and lysed with lysozyme and boiling prior to loading an amount corrected for small variations in OD_{600} on a SDS-15% PAGE gel. Proteins were subsequently transferred to Immobilon-P transfer membrane (Millipore). Complete transfer was ensured by monitoring the transfer efficiency of a prestained protein ladder (Fermentas). Immunoblotting was performed with polyclonal anti-N-terminal-RsbRA antibodies (78), which were kindly provided by Jon Marles-Wright (Newcastle University), in 5% (w/v) milk powder in phosphate-buffered saline (PBS; 80 mM Na_2PO_4 , 20 mM NaH_2PO_4 , 100 mM NaCl, pH 7.5) for 2 h. After thorough washing in PBS, the blots were incubated with goat anti-rabbit peroxidase antibodies (Thermo Fisher Scientific) in 5% (w/v) milk powder in PBS for 1 h, after which they were thoroughly washed again prior to visualization using Amersham Hyperfilm ECL and an Amersham ECL Plus Western blot detection system (GE Healthcare). The blot shown is from a representative experiment, repeated three times. Minimal processing was done in Adobe Photoshop CS5: the image was cropped, an irrelevant lane was removed, and the contrast was enhanced equally on the entire image for printability.

2.3.5. Illumination conditions

Without further specification, ‘light’ refers to white light from a compact fluorescent lamp. Moderate white light intensities were set in the range of 25 to 35 $\mu\text{Einstein}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ —more than enough to saturate the YtvA-dependent light response (18). Illumination with this intensity of visible light does not significantly affect growth rate or growth yield of *B. subtilis*. Dark controls were wrapped tightly in aluminum foil. Occasionally, a low-intensity

red LED that emits wavelengths unable to activate YtvA (λ_{\max} 632 nm with a spectral width at half maximum of ~ 25 nm) was used as a background light for sampling in the dark.

2.4. Results

Previous studies have shown that YtvA is a photoreceptor that acts in the environmental signaling pathway of the GSR, on (or upstream of) the main structural hub in this pathway: the stressosome (16, 144, 291, 448). The stressosome can contain one or more proteins of the RsbR family—RsbRA, RsbRB, RsbRC, and RsbRD—which appear to be redundant (5, 231). As a first step towards elucidation of the role of the individual RsbR proteins, the requirement of each RsbR protein in the YtvA-dependent light activation of the GSR was systematically analyzed. All possible single, double, triple, and quadruple null mutant combinations of the four RsbR proteins were constructed and the plasmid pYtvA, which has the *ytvA* gene under the control of the IPTG-inducible *spac* promoter, was introduced in all of these strains. In addition, all strains contained a fusion between a σ^B -dependent promoter (P_{ctc}) and a promoter-less gene encoding a reporter-enzyme (*lacZ*) in their genome, of which the β -galactosidase activity (expressed in Miller units) was determined as a measure for the level of σ^B (and thus GSR) activation. The ability of YtvA to activate the GSR was assayed by comparing light- and dark-grown cultures at different time points after the induction of overexpression of YtvA by the addition of IPTG (for details, see Materials and methods).

Due to the large number of strains that this procedure required, not all could be assayed at the same time. To allow for a comparison of the results of different experiments, the “wild-type” strain (PB198/pYtvA, with all four genes of the *rsbR* family present) was always included as a control. The GSR activation was then normalized on the level of activation in the “wild-type” strain in the light. Thus, a value of 1 corresponds to the activity of the control strain in the light. To create the bar graphs in Figure 1 and Figure 2, the time point at 120 min after IPTG induction was chosen because it was previously determined to be most suitable (17).

2.4.1. A mutant that lacks RsbRA has no YtvA-dependent light activation of the GSR

Figure 1 shows the relative σ^B activation of each strain that contains a single null mutation in one of the *rsbR* genes (PB491/pYtvA, PB528/pYtvA, PB574/pYtvA and PB520/pYtvA), compared to the control strain (PB198/pYtvA), grown both in the absence or presence of light. The absence of RsbRA abolishes the YtvA-dependent light activation of the GSR, while the absence of any of the other three RsbR proteins increases the level of the activation of the GSR by light by ca. 75%. In the case of the *rsbRC*-null mutation the GSR activation in the dark is also increased, which could imply a small suppressive role for RsbRC even in unstressed cells.

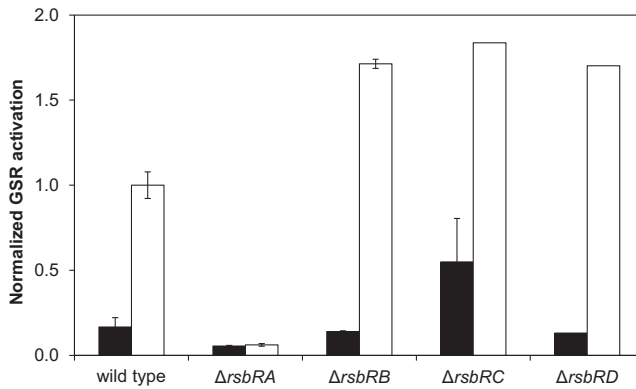


Figure 1: Effects of single null mutations of *rsbR* genes on the YtvA-dependent light activation of the GSR. All strains contained a copy of *lacZ*, controlled by the σ^B -dependent *ctc* promoter, and pYtvA, a plasmid for overexpression of *ytvA*. The β -galactosidase activity was determined 2 h after IPTG induction of YtvA overexpression. Absolute activation expressed in Miller units was normalized on the activation of the “wild-type” strain PB198/pYtvA in the light, which was set to 1. Filled bars correspond to dark conditions; open bars correspond to light conditions. The other strains used were PB491/pYtvA ($\Delta rsbRA$), PB528/pYtvA ($\Delta rsbRB$), PB574/pYtvA ($\Delta rsbRC$) and PB520/pYtvA ($\Delta rsbRD$).

2.4.2. RsbRB is a strong negative regulator of the YtvA-dependent light activation of the GSR

When comparing the GSR activation in strains that contain null mutations in two of the four *rsbR* genes (Figure 2), it is clear that the presence of *rsbRB* is of key importance. When an *rsbRA*-null mutation is combined with the *rsbRB* mutation (MA4/pYtvA) a small light effect is restored. Combining an *rsbRB* deletion with an *rsbRD* (MA6/pYtvA) or *rsbRC* (MA5/pYtvA) deletion increases the response to light, as compared to the single mutants (Figure 1). Similarly, the presence of RsbRB is the common factor of the other three strains (MA1/pYtvA, MA2/pYtvA and MA3/pYtvA), in all of which the light effect is significantly decreased or completely abolished as compared to the single mutants (Figure 1).

RsbRA, in contrast, appears to have a positive effect on the light activation of the GSR. This is especially clear when comparing null mutants in both *rsbRA* and either *rsbRC* (MA1/pYtvA) or *rsbRD* (MA2/pYtvA) with the single null mutants of *rsbRC* and *rsbRD* (Figure 1): all of the light enhancement of the GSR in those single null mutants is lost in the double-deletion strains. In strains in which RsbRA is present but RsbRB is absent (MA5/pYtvA and MA6/pYtvA) the activity of the GSR is further enhanced, but in the strain with RsbRA and RsbRB as the only RsbR proteins (MA3/pYtvA) the light activation is lost, indicating that RsbRB is able to suppress the activating effect of RsbRA.

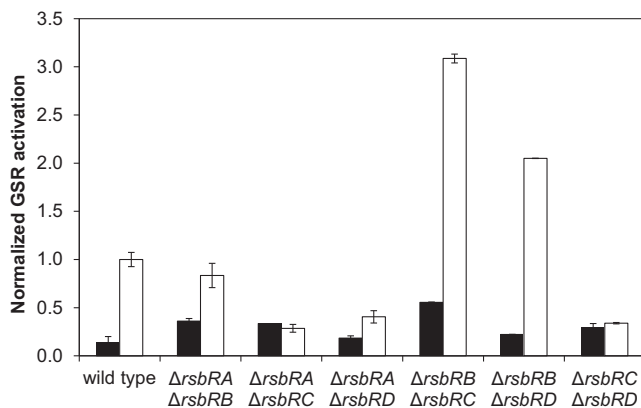


Figure 2: Effects of double null mutations of *rsbR* genes on the YtvA-dependent light activation of the GSR. The GSR activation was determined and normalized as described for Figure 1. Filled bars correspond to dark conditions; open bars correspond to light conditions. The strains used were MA4/pYtvA ($\Delta rsbRA \Delta rsbRB$), MA1/pYtvA ($\Delta rsbRA \Delta rsbRC$), MA2/pYtvA ($\Delta rsbRA \Delta rsbRD$), MA5/pYtvA ($\Delta rsbRB \Delta rsbRC$), MA6/pYtvA ($\Delta rsbRB \Delta rsbRD$) and MA3/pYtvA ($\Delta rsbRC \Delta rsbRD$).

2.4.3. RsbRA is sufficient for YtvA-dependent light activation of the GSR

Based on the results presented thus far, it is difficult to distinguish between direct effects caused by the activity of the RsbR protein itself and indirect effects caused by displacement of other RsbR proteins by YtvA. Therefore, the four strains with three of the four *rsbR* genes knocked out were assayed next. Figure 3A shows that the strain with RsbRA as the only remaining RsbR protein is capable of transmitting the light signal to activate the GSR, even when YtvA overproduction is not induced (time zero, open triangles). The strain with only RsbRB, in contrast, shows no detectable activation of the GSR by light (Figure 3B), which is consistent with the results discussed above.

The other two triple null mutants show less pronounced, but nevertheless clear, effects. The strain with only RsbRC is able to transmit the light signal and activate the GSR (Figure 3C) whereas the strain with only RsbRD shows a light effect that is very small (Figure 3D).

We observed an increase of the activity of the GSR after induction of YtvA overproduction in the dark in the strain with only RsbRA (Figure 3A). This observation may point towards an effect of overproduced YtvA, even in the dark. The strains with only RsbRC or RsbRD show a high background activation that remains approximately constant throughout the experiment (Figure 3C and D), as has been observed before (5, 231).

2.4.4. There is no light effect in the absence of all four RsbR proteins

It has previously been shown that a strain with null mutations in all four *rsbR* genes has a high, constitutive activation of the GSR in unstressed conditions (5, 231). This suggests that YtvA is unable to form stressosomes without the presence of at least one of the RsbR

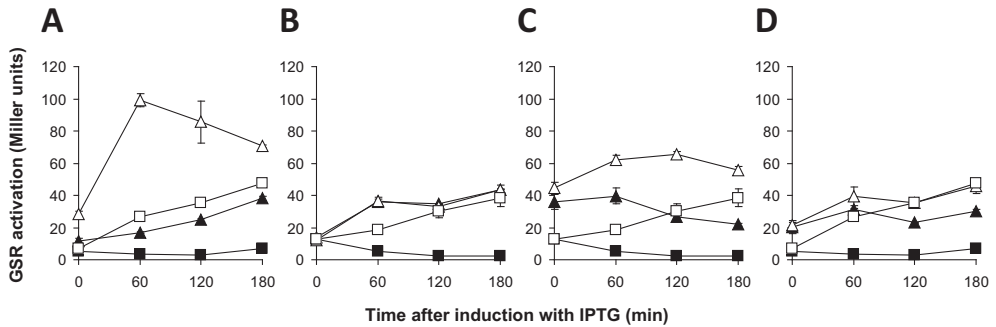


Figure 3: Effects of triple null mutations of *rsbR* genes on the YtvA-dependent light activation of the GSR. All strains contained a copy of *lacZ*, controlled by the σ^B -dependent *ctc* promoter, and pYtvA, a plasmid for overexpression of *ytvA*. The β -galactosidase activity was determined at various time points after IPTG induction of YtvA overexpression and expressed in Miller units. Open symbols represent cultures grown in the light; closed symbols represent cultures grown in the dark. The “wild-type”-like strain PB198/pYtvA is represented by squares in all panels, while each one of the triple null mutant strains is represented by triangles. **A.** The triple null mutant of *rsbRB*, *rsbRC* and *rsbRD* with pYtvA (MA7/pYtvA, with RsbRA as the only RsbR protein). **B.** The triple null mutant of *rsbRA*, *rsbRC* and *rsbRD* with pYtvA (MA8/pYtvA, with only RsbRB). Note that the lines for this mutant in the light and the dark overlay each other. **C.** The triple null mutant of *rsbRA*, *rsbRB* and *rsbRD* with pYtvA (MA9/pYtvA, with only RsbRC). **D.** The triple null mutant of *rsbRA*, *rsbRB* and *rsbRC* with pYtvA (MA10/pYtvA, with only RsbRD).

proteins. However, wild-type expression levels of *ytvA* might be insufficient to sequester RsbT, and the light conditions of these previous experiments were not controlled.

Therefore, we transformed strain MA11 (which lacks all four *rsbR* genes) with pYtvA. When overproduction of YtvA was induced the level of GSR activation decreased (Figure 4A), but it is unclear whether this is related to a general effect of overproduction or to a partial ability of an excess of YtvA to capture RsbT and lower the stress response. However, there was no additional induction of the GSR in the light, suggesting that YtvA is non-functional in this strain with respect to transmission of a light signal (Figure 4A). The addition of 300 mM NaCl also did not further activate the GSR, regardless of the light conditions (Figure 4B), which further suggests that if any stressosome-like structures with only YtvA and RsbT are formed at all, they are nonfunctional. There was also no transient induction of the GSR in response to light (Figure 4C). In all experiments with this strain the level of GSR activation rose as growth progressed from early to late exponential growth phase (e.g. Figure 4C), which might be related to various levels of overproduction of YtvA.

Taken together, these data show that YtvA is unable to form active stressosomes (capable of sequestering RsbT and transmitting a stress signal) without the presence of at least one RsbR protein, independent of the light conditions.

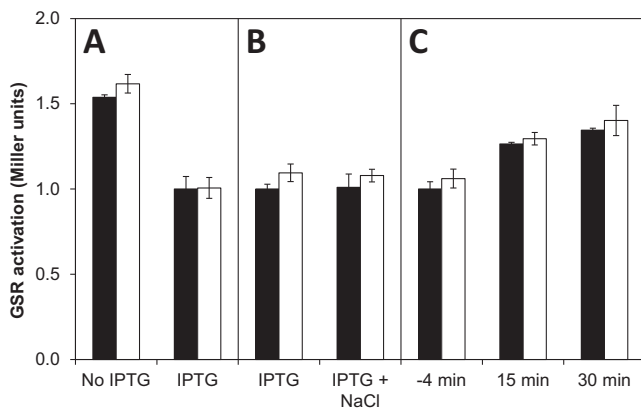


Figure 4: Effect of overproduction of YtvA in a strain lacking all four *rsbR* genes (MA11/pYtvA). Filled bars correspond to dark conditions; open bars correspond to light conditions. The data from each panel were normalized to the activity of the IPTG-induced strain in the dark for easy comparison. **A.** With or without IPTG induction of overproduction of YtvA after continuous exposure to light (1.75 h). **B.** 30 min after addition of 300 mM NaCl in cultures kept in the dark or in the light. **C.** Before and after turning on the light (at 0 min).

2.4.5. The observed negative effect of RsbRB is not due to a defect in the environmental stress pathway

We wondered if the striking negative effect of RsbRB on signals generated by YtvA was in fact a general effect on the activity of the entire environmental branch of the GSR. First, we probed the four strains with a single RsbR protein on a Western blot with polyclonal anti-N-terminal RsbRA (Figure 5) and observed a band that was present in all triple knockout mutants but not in the quadruple mutant that lacks all RsbR proteins. This suggests that the antibody recognizes an epitope common to all RsbR proteins. The intensities of the bands for all mutants were similar, hinting at similar protein levels and an expression-level-control mechanism for the total amount of RsbR protein in the cell.

We then stressed strains with either RsbRA (MA7) or RsbRB (MA8) as the only RsbR protein with 4% (685 mM) and 2% (342 mM) ethanol, 300 mM NaCl, and with water as a control. The entire experiment was performed in strict darkness, to prevent light

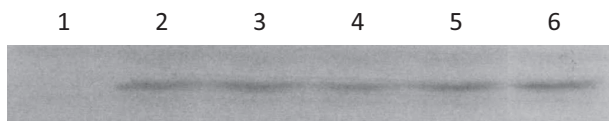


Figure 5: Western blot with anti-N-terminal RsbRA in: a strain lacking all RsbR proteins (MA11) (lane 1), a strain with RsbRA as the only RsbR protein (MA7) (lane 2) a strain with RsbRB as the only RsbR protein (MA8) (lane 3), a strain with RsbRC as the only RsbR protein (MA9) (lane 4), a strain with RsbRD as the only RsbR protein (MA10) (lane 5), a strain with all four RsbR proteins (PB198) (lane 6).

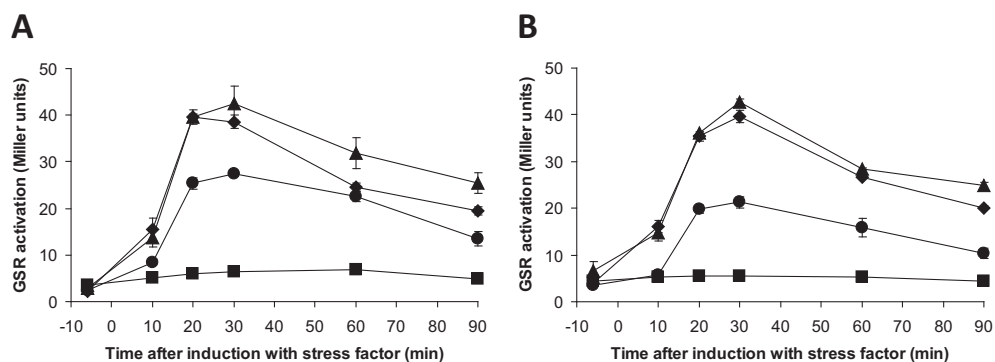


Figure 6: Response of strains containing only a single RsbR protein to environmental stresses other than light. At time point zero, the cultures were induced with 4% ethanol (triangles), 2% ethanol (diamonds), 300 mM NaCl (circles) or water as a control (squares). The experiments were carried out in strict darkness to prevent interference by the wild-type copy of *ytvA*. The data shown were acquired in the same experiment but split over two panels for clarity. **A.** Strain MA7, containing RsbRA as the only RsbR protein ($\Delta rsbRB \Delta rsbRC \Delta rsbRD$ mutants). **B.** Strain MA8, containing RsbRB as the only RsbR protein ($\Delta rsbRA \Delta rsbRC \Delta rsbRD$ mutants).

activation of YtvA. The results (Figure 6) show that both strains are very similar in both the dynamics and the absolute level of GSR activation (compare panels A and B). This observation confirms that both RsbRA and RsbRB are able to mediate a fully functional environmental stress response under our experimental conditions and that the results presented in Figure 3 are specific for the YtvA-mediated light-induced GSR.

2.4.6. An *rsbRB*-null mutation is sufficient to detect light activation of the GSR without overproduction of YtvA

The experiments with the triple null mutant with RsbRA as the only remaining RsbR protein produced an unexpected additional result: without overproduction of YtvA there was a highly reproducible difference between light and dark cultures (time point zero, Figure 3A). Previously, we and others had been unable to show a light-dependent activation of the GSR at wild-type expression levels of *ytvA* using the *lacZ* reporter, except when light stress was combined with an additional environmental stress, such as NaCl shock (16, 448). To exclude the possibility that the observed activation was caused by a leaky promoter in the pYtvA plasmid, the triple null mutant strains, with only RsbRA (MA7) or only RsbRB (MA8) and without the pYtvA plasmid, were grown in the dark and exposed to continuous illumination from time point zero onward. Upon illumination only MA7, and not MA8, showed an increase in GSR activation (Figure 7A), confirming that a strain with RsbRA as the only RsbR protein has a light-sensitive GSR without overproduction of YtvA.

The double-deletion mutant of *rsbRB* and *rsbRC* (MA5) and the single-deletion mutant of *rsbRB* (PB528) were also compared to the “wild-type” strain (PB198) in a similar assay without YtvA overproduction. The cultures were carefully kept in the dark during the

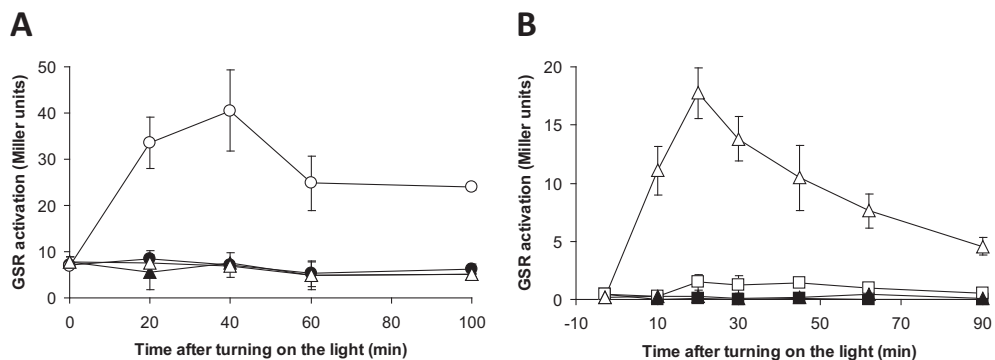


Figure 7: Light activation of the GSR without overproduction of YtvA. The β -galactosidase activity, a measure for GSR activation, was determined at various time points and is expressed in Miller units. Cultures were kept strictly in the dark until time point zero, at which time the light was switched on. Filled symbols represent cultures kept in the dark; open symbols represent illuminated cultures. **A.** Comparison of strains containing RsbRA (MA7, Δ *rsbRB* Δ *rsbRC* Δ *rsbRD* mutants; circles) or RsbRB (MA8, Δ *rsbRA* Δ *rsbRC* Δ *rsbRD* mutants; triangles) as the only RsbR proteins. **B.** Comparison of the single null mutant of *rsbRB* (PB528, triangles) and the “wild-type” control strain (PB198, squares).

entire experiment (including all precultures) to prevent any unwanted preliminary activation of YtvA. Switching on the light at time point zero resulted in a clear and transient light-dependent activation of the GSR in both MA5 (data not shown) and PB528 (Figure 7B). To further confirm that the observed light-dependent induction was really due to YtvA and not due to an effect of the *rsbRB* mutation, the experiment was repeated with a strain with null mutations in both *rsbRB* and *ytvA* (JBS10011). In this strain, the observed light-dependent activation of the GSR was completely abolished, confirming the dependence of this effect on YtvA (0.0 \pm 0.0 Miller units for JBS10011, as compared to 16.8 \pm 2.5 for PB528, 15 min after turning on the light). Thus, these results clearly show that the loss of *rsbRB* is sufficient to make the light-sensitivity of the GSR observable in an otherwise wild-type background in exponentially growing cells.

2.5. Discussion

2.5.1. Differentiation of function among RsbR proteins with respect to light-induced activation of the stress response

It has generally been held that the four RsbR proteins are functionally very similar, albeit not identical (231), because early experiments already showed limited differences between them (5). In hindsight, the interpretation of these studies has become difficult because they were performed before YtvA was known to function as a photoreceptor in the GSR (16). Thus, illumination conditions were not controlled.

We have now presented clear evidence of a differentiation of function among the RsbR proteins with respect to YtvA-dependent light-induced stress. Using genetic studies

(Figure 1-3) we have established that the presence of RsbRA has a positive effect on the transmission of the light signal, whereas RsbRB has a clear negative effect. When RsbRB is the only RsbR protein present, no light effect is detectable at all (Figure 3B).

Our results and data available in literature show that the effect of RsbRB in a strain with none of the other RsbR proteins present is not due to differences in protein levels or due to a nonfunctional signaling pathway. First, no increased basal activation of the GSR is observed in this strain (see, for example, figure 6B and references (5, 231)), which suggests that there is sufficient protein to form enough stressosomes to capture all RsbT and prevent activation of the GSR. Second, the response to salt and ethanol in strains with either RsbRA or RsbRB as the only RsbR protein is similar (figure 6 and reference (231)), again indicating normal functionality of the stressosomes. Third, a recent study found the expression levels of *rsbRA* and *rsbRB* to be similar (392). The availability of sufficient protein is also suggested by the Western blot shown in figure 5. Taken together, these data show that RsbRB cannot simply be regarded as a general inhibitor of the environmental GSR, and its inhibitory effect appears to be exclusive to YtvA.

The roles of RsbRC and RsbRD are less distinct, but RsbRC clearly allows the transmission of the light signal (Figure 3C), while the results obtained for RsbRD are inconclusive (Figure 3D). The latter observation might be explained by a lower expression level of *rsbRD*, as has recently been suggested (392), although our Western blot does not suggest large differences in protein concentration. However, despite the minor roles of RsbRC and RsbRD, the additional presence of either one allows RsbRA to overcome the negative effect of RsbRB (Figure 1). Thus, the exact roles and interactions of the individual RsbR proteins are likely very complex.

In addition to the well-established light-dependent induction of the GSR by YtvA, we observed a small YtvA-dependent induction of the GSR in the dark in the strain with only RsbRA (Figure 3A). This may be caused by a lowered capacity to inactivate RsbT when stressosomes incorporate YtvA as an additional Rsb protein (see further below), partly replacing RsbRA.

2.5.2. Relevance of having four RsbR proteins

The four RsbR proteins have been assumed to be at least partially redundant (5, 231), which we have also found for at least RsbRA and RsbRB in the response to NaCl and ethanol shock (Figure 6). The two main hypotheses proposed for their presence are that (i) either one senses a different environmental stress or that (ii) either one senses a common signal(s) with a different sensitivity or threshold (231). The latter explanation has been favored because of the redundancy of the RsbR proteins and the fact that their transcriptional regulation is different. The results presented here show that an individual RsbR protein can modulate the response to a specific environmental stress (*i.e.*, RsbRB modulating light stress), which suggests that the biological rationale for the existence of four RsbR proteins may be a mix of both aforementioned hypotheses.

If the total level of RsbR proteins in the cell is indeed approximately constant, as suggested by Figure 5, this does not necessarily diminish the relevance of the differential regulation of the *rsbR* genes. Regulation of the pool size of the RsbR proteins may be post-transcriptional, and the upregulation of one RsbR protein may result in a smaller contribution of other RsbR proteins to this pool. However, more extensive experiments are required before such interpretations can be confirmed.

While the present study was in preparation, Martinez *et al.* (294) reported that triple null mutants containing RsbRC or RsbRD as the sole RsbR protein were able to respond to energy stress, a stress which is normally not sensed by the stressosome in *B. subtilis*. These researchers could not exclude the possibility that the higher background activation observed in these strains (see also Figure 3C and D), in combination with a decreased ATP concentration during energy stress, might indirectly result in the inability to keep σ^B fully inactivated. In addition, the light conditions in that study were not specified. However, in light of our results, it appears probable that the absence of certain RsbR proteins renders the GSR sensitive to stresses that were not previously thought to activate the environmental GSR, which may well include starvation signals.

2.5.3. The loss of RsbRB is sufficient for a light-sensitive GSR

The loss of *rsbRB* is sufficient for a clearly observable light-dependent GSR in otherwise wild-type cells, *i.e.*, even without overproduction of YtvA (Figure 7B). Previously, no light-dependent effect of YtvA was observed without either overproduction or the imposition of an additional environmental stress (16, 448), which made it less obvious to conclude that YtvA has a significant biological role as a photoreceptor. However, our results now show that downregulation of *rsbRB* is enough to make the GSR of *B. subtilis* strongly light sensitive. It will, therefore, be very interesting to uncover the (transcriptional) regulation of *rsbRB*, because such experiments may reveal conditions in which light is perceived as a strong stress factor for the organism. Intriguingly, the light intensities needed for activation of the GSR through YtvA are far below ambient intensities at moderate latitude at midday.

2.5.4. Mechanism of inhibition of the YtvA-dependent light-induced GSR by RsbRB

Although interaction between YtvA and the stressosome has been shown previously (5, 144), it is far from clear how this interaction takes place. The most intuitive explanation would be that the C-terminal STAS domain of YtvA occupies a position similar to the STAS domains of the other RsbR proteins (which form the core of the stressosome (291)). However, direct evidence to support this notion is lacking, and it is not known whether the STAS domain of YtvA is equivalent in function to the STAS domains of the RsbR proteins. Also, the quadruple mutant, which lacks all *rsbR* genes but has YtvA (MA11/pYtvA), has a high basal level of GSR activation, which suggests that YtvA alone is unable to form stressosomes that can inactivate RsbT (Figure 4). Based on these results the following

hypothesis can be proposed: YtvA is part of the stressosome as an RsbR protein but needs at least one of the other RsbR proteins to either capture RsbT or maintain stressosome integrity. This hypothesis also offers a possible explanation for the observed negative role of RsbRB: stressosomes with (mostly) RsbRB might expel or exclude YtvA, thereby inhibiting the ability of YtvA to transmit the light signal. However, more work needs to be done to confirm these hypotheses.

Indirect support of YtvA's presence in the stressosome was obtained in an experiment in which we swapped the N-terminal domain of RsbRA for the LOV-domain of YtvA. Cells containing this fusion protein as the only RsbR paralog display light dependence of their salt-stress response, which shows that a LOV domain is capable of activating the stressosome (J.B. van der Steen *et al.*, unpublished results).

2.5.5. Light as a functional probe

The lack of knowledge on the nature of the direct input signals for the stressosome has hampered progress in research into the molecular mechanism by which the stressosome operates, in particular with respect to signal integration. We have shown here that the use of YtvA, a photoreceptor with a well-defined input signal, allows one to overcome such limitations. With the new observation that loss of *rsbRB* is enough to create a light-sensitive GSR, probing the parameters of stressosomes becomes much easier, *in vivo* as well as *in vitro* if stressosomes containing YtvA can be isolated or constructed. This is not only true for *B. subtilis*; the important pathogen *L. monocytogenes*, for example, also has a stressosome and a YtvA-homolog (279, 359), and a blue-light effect dependent on this homolog has been demonstrated (357), which shows that the approach presented here can be extended to other organisms as well.

2.6. Acknowledgements

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