

Published in final edited form as:

*Mol Microbiol.* 2011 May ; 80(3): 798–810. doi:10.1111/j.1365-2958.2011.07609.x.

## ***In vivo* phosphorylation patterns of key stressosome proteins define a second feedback loop that limits activation of *Bacillus subtilis* $\sigma^B$**

Christine Eymann<sup>1,\*</sup>, Stephan Schulz<sup>1,†</sup>, Katrin Gronau<sup>1</sup>, Dörte Becher<sup>1</sup>, Michael Hecker<sup>1</sup>, and Chester W. Price<sup>2,\*\*</sup>

<sup>1</sup>Institute for Microbiology, Ernst-Moritz-Arndt-University Greifswald, F.-L.-Jahn-Str. 15, D-17487 Greifswald, Germany

<sup>2</sup>Department of Microbiology, University of California, One Shields Avenue, Davis, CA 95616 USA

### Summary

The *Bacillus subtilis* stressosome is a 1.8 MDa complex that orchestrates activation of the  $\sigma^B$  transcription factor by environmental stress. The complex comprises members of the RsbR co-antagonist family and the RsbS antagonist, which together form an icosahedral core that sequesters the RsbT serine-threonine kinase. Phosphorylation of this core by RsbT is associated with RsbT release, which activates downstream signaling. RsbRA, the prototype co-antagonist, is phosphorylated on T171 and T205 *in vitro*. In unstressed cells T171 is already phosphorylated; this is a prerequisite but not the trigger for activation, which correlates with stress-induced phosphorylation of RsbS on S59. By contrast, phosphorylation of RsbRA T205 has not been detected *in vivo*. Here we find (i) RsbRA is additionally phosphorylated on T205 following strong stresses; (ii) this modification requires RsbT; and (iii) the phosphorylation-deficient T205A substitution greatly increases post-stress activation of  $\sigma^B$ . We infer that T205 phosphorylation constitutes a second feedback mechanism to limit  $\sigma^B$  activation, operating in addition to the RsbX feedback phosphatase. Loss of RsbX function increases the fraction of phosphorylated RsbS and doubly phosphorylated RsbRA in unstressed cells. We propose that RsbX both maintains the ready state of the stressosome prior to stress, and restores it post-stress.

### Keywords

SigB; RsbR; signal transduction; protein phosphorylation; proteome

### Introduction

Bacteria manifest diverse adaptive responses to adjust their physiology to changing surroundings. In *Bacillus subtilis* and the related pathogens *Listeria monocytogenes* and *Staphylococcus aureus*, the general stress regulon controlled by  $\sigma^B$  plays a prominent role in this strategy, interacting with other response systems to form flexible adaptive networks (reviewed in Hecker *et al.*, 2007; Hecker *et al.*, 2009; Price, 2010). Our focus here is the signal transduction pathway that activates *B. subtilis*  $\sigma^B$  in response to environmental stresses, and the feedback mechanisms that damp this activation. Key elements of this pathway are also found in a variety of signaling contexts, and in phylogenetically diverse

\*\*\* For correspondence: eyman@uni-greifswald.de; Tel. +49 3834 864227; Fax +49 3834 864202. cwprice@ucdavis.edu; Tel. (+1) 530 752 1596; Fax (+1) 530 752 9014.

† Present address: Chair of Soil Ecology, Technological University München, Ingolstädter Landstr. 1, D-85764 Neuherberg, Germany

bacteria (Pané-Farré *et al.*, 2005). Our study is therefore relevant to understanding the operation of a versatile and widespread bacterial signaling module.

In *B. subtilis* and related organisms,  $\sigma^B$  activity is regulated by a signaling network that employs the partner switching mechanism, in which interactions between alternative binding partners are governed by serine and threonine phosphorylation (Hecker *et al.*, 2007; Price, 2010). The *B. subtilis* network includes two independent branches that converge on the RsbV and RsbW regulators of  $\sigma^B$  (Fig. 1A). Each of these independent branches terminates with a differentially regulated serine phosphatase required for response to a discrete class of stress signal: RsbU for environmental or RsbP for energy stresses. In unstressed cells  $\sigma^B$  is held inactive by the RsbW anti- $\sigma$  factor, which also acts as a serine kinase to phosphorylate and inactivate the RsbV anti-anti- $\sigma$ . Following stress, one or another of the signaling phosphatases removes this modification from RsbV, allowing it to bind the RsbW anti- $\sigma$ . Thus dephosphorylation of RsbV is the key event forcing RsbW to switch partners and release  $\sigma^B$ .

Genetic, biochemical and structural studies support a model in which upstream regulators in the environmental pathway activate the RsbU phosphatase by means of a second partner switch, defined by the 1.8 MDa stressosome complex (Hecker *et al.*, 2007; Marles-Wright *et al.*, 2008; Price, 2010). Due to the greater number of components in this second switch, the role of phosphorylation in controlling its operation is less well understood. As shown in Fig. 1B, the switch comprises the RsbT serine kinase and the RsbS antagonist, which are paralogs of RsbW and RsbV, respectively, and also one or more members of the RsbR co-antagonist family: RsbRA, RB, RC or RD (Akbar *et al.*, 2001; Chen *et al.*, 2003; Kim *et al.*, 2004a; Delumeau *et al.*, 2006; Reeves *et al.*, 2010). The switch target is the RsbU phosphatase, and RsbT serves as a positive regulatory subunit that activates RsbU by direct protein-protein interaction (Yang *et al.*, 1996; Delumeau *et al.*, 2004).

Structural studies have shown that the RsbS antagonist and the RsbR co-antagonists have related Sulfate Transporter Anti-Sigma Antagonist (STAS) domains that form the core of the stressosome complex, onto which the RsbT kinase binds (Marles-Wright *et al.*, 2008). RsbS consists of a single STAS domain (Fig. 1C). In contrast, each RsbR co-antagonist also possesses an N-terminal, non-heme globin domain; these form projections from the surface of the stressosome and are thought to sense stress signals, which are presumably conveyed to the C-terminal STAS domains via a 13 residue helical linker. RsbRA, the prototype of the RsbR co-antagonist family, has both negative and positive roles (Akbar *et al.*, 1997). The negative role reflects the requirement for RsbRA to act as a co-antagonist with RsbS to complex RsbT in the absence of stress; RsbS alone is unable to bind RsbT (Chen *et al.*, 2003; Kim *et al.*, 2004a). The positive role is thought to reflect the ability of RsbRA to enhance the phosphorylation of RsbS by RsbT (Gaidenko *et al.*, 1999; Chen *et al.*, 2004), an event associated with the stress-induced release of RsbT from the stressosome core and efficient activation of the environmental response (Kang *et al.*, 1996; Chen *et al.*, 2003; Kim *et al.*, 2004b). Expression of a separate RsbX feedback phosphatase is under  $\sigma^B$  control. It dephosphorylates RsbS-P *in vitro*, and is proposed to complete a negative feedback loop that returns the system to its initial state (Yang *et al.*, 1996; Voelker *et al.*, 1997).

How the phosphorylation state of the RsbR co-antagonists might influence signal transduction is less clear. Biochemical analysis has shown that, in addition to its action toward RsbS on S59, RsbT also phosphorylates RsbRA on both T171 and T205, with T171 being the more readily modified *in vitro* (Gaidenko *et al.*, 1999; Chen *et al.*, 2004). The *in vivo* phenotypes elicited by phosphomimetic or phosphorylation-deficient substitutions at T171, together with analysis of the *in vivo* phosphorylation states of the RsbR family and RsbS, have been interpreted as evidence that RsbRA T171 phosphorylation is an important

prerequisite but is not the trigger for signaling (Kim *et al.*, 2004a; Kim *et al.*, 2004b; Eymann *et al.*, 2007). Instead, signaling appears to correlate with phosphorylation of RsbS on S59.

By contrast, T205 phosphorylation has not been unambiguously detected *in vivo*, and its physiological role remains unknown. Biochemical analysis found that stressosomes formed from doubly phosphorylated versions of RsbRA cannot bind RsbT *in vitro*, leading Chen *et al.* (2004) to suggest that simultaneous phosphorylation of T171 and T205 leads to robust  $\sigma^B$  activation. However, this proposal is inconsistent with the *in vivo* phenotypes of T205 substitutions: the phosphomimetic T205D decreases  $\sigma^B$  activation while the phosphorylation-deficient T205A increases it, suggesting that T205-P has a negative effect on signaling (Akbar *et al.*, 1997; Kim *et al.*, 2004a). To resolve these uncertainties, we sought conditions under which T205 becomes phosphorylated *in vivo*. Here we report this modification occurs when exponentially growing cells are exposed to strong environmental stresses. Based on mutant analysis, we propose that T205 phosphorylation represents a feedback mechanism that inhibits signaling under extreme conditions.

## Results

### In vivo phosphorylation of RsbRA T171 and the corresponding residues of its paralogs depends on the RsbT kinase

The paralogous proteins RsbRA (formerly RsbR), RsbRB (YkoB), RsbRC (YojH), and RsbRD (YqhA) possess two conserved threonine residues in their STAS domains (Fig. 1C). Our goals were to determine the growth conditions under which these conserved residues were phosphorylated *in vivo*, the contribution of the RsbT kinase and RsbX phosphatase, and the effects of these modifications on signaling. The four co-antagonists are functionally similar but are not identical (Kim *et al.*, 2004a). We therefore focused on RsbRA, extending the analysis where possible to determine which characteristics were applicable to other members of the RsbR family.

Based on analysis of the *in vivo* phosphorylation state of wild and mutant forms of RsbRA, Kim *et al.* (2004a, 2004b) proposed that T171 is ordinarily phosphorylated in unstressed cells. In support of this proposal, a mass spectrometric analysis showed that in exponentially growing cells the proteins RsbRA, RB, and RD are phosphorylated on T171, T186, and T181, respectively (Eymann *et al.*, 2007). RsbRC was also phosphorylated under these conditions, but its amount was too low for verification of the residue. However, in a separate study employing gel-free analysis after biochemical enrichment to detect less abundant phosphoproteins, phosphorylation of RC on T186 was also verified by mass spectrometry (Macek *et al.*, 2007).

Here we demonstrate that these initial phosphorylation events, which take place in the absence of external stress, depend on the action of the RsbT kinase. Comparison of the proteomes of exponentially growing wild type and mutant cells, in which phosphorylated proteins were visualized by ProQ-Diamond<sup>®</sup>-staining, revealed the presence of all four phosphorylated RsbR paralogs in the wild type and their absence in the *rsbT* mutant (Fig. 2). Because RsbT phosphorylates all four RsbR paralogs *in vitro* (Akbar *et al.*, 2001), the simplest interpretation of our results is that RsbT directly phosphorylates the RsbR paralogs *in vivo*.

### Occurrence of new RsbRA and RB isoforms in response to strong stresses

RsbT is known to phosphorylate RsbRA on T205 as well, but only from *in vitro* analysis (Gaidenko *et al.*, 1999; Chen *et al.*, 2004). We therefore looked for *in vivo* conditions under which RsbRA or its paralogs were phosphorylated on both conserved threonine residues.

Notably, after exposure of exponentially-growing cells to strong, growth-preventing stresses (54°C or 10% ethanol), additional ProQ-stained isoforms of RsbRA and RB appeared at a more acidic position, suggesting the occurrence of an additional phosphorylation on each (Fig. 3). No additional isoforms of RsbRC or RD were detected. We therefore turned our attention to the new isoforms of RsbRA and RB.

Mass spectromic analysis confirmed that the second isoform of RsbRA (RsbRA-2) was phosphorylated on both T171 and T205 after strong stress. As shown in Table 1, both phosphorylations were found in two separate peptides of this second RsbRA protein spot, with high confidence. By contrast, for the second isoform of RsbRB (RsbRB-2), phosphorylation could be confirmed on T186 but not T220. This was due to the complete absence of the T220-containing peptide from our tryptic digests. The basis of the altered mobility of the second RsbRB spot is therefore not yet established.

Regarding RsbRA, it is worth mentioning that both its ProQ-stained isoforms sometimes manifested vertical double spots that differed in size, in which the double phosphorylated isoform had a higher molecular weight than the single phosphorylated one (Fig. 3). This phenomenon suggests that additional posttranslational modifications of RsbRA occur *in vivo*. The nature and significance of these additional modifications remain to be investigated.

### Three distinct RsbRA or RsbRB isoforms detected by isoelectric focusing and Western blotting

We were unable to detect non-phosphorylated RsbRA or RsbRB on 2D gels after Flamingo staining, suggesting these forms were present in only low amounts. In order to engage all the isoforms and quantitate the non-phosphorylated and phosphorylated fractions under different stress conditions, we separated the proteins in cell extracts by isoelectric focusing (IEF) and detected the isoforms by Western blotting with antibodies raised against RsbRA or RsbRB. Proteins were isolated from *B. subtilis* wild type cells cultivated under qualitatively and quantitatively different stresses. These included (i) heat shock (50 or 54°C); (ii) ethanol stress (4 or 10%, v/v); and (iii) sodium chloride stress (4 or 8%, w/v). The “mild” stress did not affect growth rate (50°C) and the “moderate” stresses reduced it by about half (4% ethanol, 4% NaCl). By contrast, the “strong” stresses stopped growth completely (54°C, 10% ethanol, 8% NaCl; growth data not shown). All these conditions induced  $\sigma^B$  activity to varying degrees, as measured by Northern blotting with RNA probes against the  $\sigma^B$ -dependent genes *ctc* and *sigB* (shown in Fig. 4 for 54°C, 4% and 10% ethanol stress).

Proteins isolated from cells before and at different times after stress were separated on IEF gels; these contained pharalytes based on the pI value of RsbRA (4.73) or RsbRB (4.79). As shown in Fig. 5A and B, Western blotting with antibody against RsbRA or RsbRB detected three significant signals, representing three different isoforms for each paralog. In previous work with this IEF system (Kim *et al.*, 2004b), the three RsbRA signals were identified as non-phosphorylated (0), singly phosphorylated on T171 (1), and doubly phosphorylated on T171 and T205 (2). The evidence used in this earlier study included (i) incubations with  $\lambda$  phosphatase; (ii) analysis of a null mutant missing the RsbT kinase; and (iii) analysis of strains encoding mutant forms of RsbRA (T171 and T205 substitutions). However, the presence of some singly-phosphorylated T205 in the (1) signal of Fig. 5A cannot be precluded. The negative controls in Fig. 5A and B gave the patterns expected: only the non-phosphorylated (0) form of RsbRA or RsbRB appeared in extracts from an *rsbT* null mutant, and no signals were apparent in extracts from *rsbRA* or *rsbRB* null mutants.

## Analysis of RsbRA isoforms and phosphorylation patterns in response to stress

Under non-stress conditions two forms of RsbRA were detected *in vivo* (Fig. 5A). The first was the non-phosphorylated form (0), which was the only signal detected in the *rsbT* mutant. The second was the single phosphorylated form (1), most of which should correspond to the ProQ-spot modified on T171 (Fig. 3). Under stress conditions a third RsbRA isoform appeared as a significant signal. This was the doubly phosphorylated form (2), which should correspond to the ProQ-spot modified on T171 and T205 (Fig. 3). These latter forms (1 and 2) were undetectable in extracts of the *rsbT* mutant (Fig. 5A), indicating their RsbT dependence. To confirm the identity of the RsbRA isoforms, a strain encoding a T205A substitution was used for a further IEF experiment. As shown in Fig. 5C, in the T205A mutant no significant signal was detected for RsbRA isoform (2), either in unstressed cells or following a strong ethanol stress.

Quantification of the isoform signals from Fig. 5A revealed that in unstressed cells about 20% of the RsbRA was non-phosphorylated and about 70% was singly phosphorylated (see Fig. 5D). Under these conditions the doubly phosphorylated form represented only about 10% of total cellular RsbRA. However, as inspection of Fig. 5A shows, this doubly phosphorylated isoform significantly increased in response to those moderate or strong stresses that caused either growth rate reduction (4% EtOH, 4% NaCl) or growth rate stop (54°C, 10% EtOH, 8% NaCl). Such an increase did not occur in response to the mild 50°C stress, which did not affect growth rate. In the case of 10% ethanol stress, the fraction of doubly phosphorylated isoform increased to about 30% of the total while the non-phosphorylated fraction decreased (Fig. 5D). We obtained similar results with RsbRB following 54°C and 10% ethanol stresses (Fig. 5B): a decrease of the non-phosphorylated isoform (0); an increase of the presumed doubly phosphorylated isoform (2); and disappearance of isoform (2) in a mutant bearing an alanine substitution at the T220 position (data for the latter not shown).

We should note that the earlier study of Kim *et al.* (2004b) did not detect the doubly phosphorylated form of RsbRA, even though these authors employed a moderate 4% ethanol stress similar to the one shown in Fig. 5A. Two differences between the studies might account for this. First, we used a different mix of phosphatase inhibitors in the harvest buffer, including NaN<sub>3</sub>. And second, we used a synthetic minimal medium for cell growth rather than Luria broth, which may have rendered the 4% ethanol stress relatively stronger in our study.

## Consequence of the inability to phosphorylate RsbR on T205

The question arises regarding the role that phosphorylation of RsbRA T205 might play in the  $\sigma^B$  signaling network. Is sequential phosphorylation on T171 and T205, together with phosphorylation of RsbS on S59, responsible for releasing RsbT from the stressosome and inducing  $\sigma^B$  activity, as suggested by Chen *et al.* (2004)? To test this possibility, we compared  $\sigma^B$  activity in wild type with that of a strain encoding the T205A form of RsbRA, in which T205 cannot be phosphorylated. The assay shown in Fig. 6 was done by Northern blotting against *gsiB*, whose expression is wholly dependent on  $\sigma^B$  (Maul *et al.*, 1995). Following exposure of exponentially growing cells to 54°C or 10% ethanol -- conditions that promote T205 phosphorylation -- expression of *gsiB* message was greatly increased and sustained in the T205A mutant relative to wild type (Fig. 6). To support the idea that this difference reflected an inability to phosphorylate T205 and was not a general effect of the T205A substitution, we repeated the assay under conditions in which T205 would not be highly phosphorylated. Following exposure to 50°C or 2% ethanol, expression of *gsiB* message was only slightly elevated in the T205A mutant relative to wild type (Fig. 6).

Thus the T205A substitution did not diminish the stress response as predicted by the earlier *in vitro* analysis (Chen *et al.*, 2004), but in fact greatly enhanced it. We infer from these results that the second phosphorylation of T205 is not involved in mounting the response, but instead is responsible for its attenuation. In an attempt to extend these observations to another member of the RsbR family, we performed a similar assay using the T220A mutant of RsbRB, which bears a substitution at the likely (but not experimentally demonstrated) second site of phosphorylation of that co-antagonist. However, the T220A alteration had no influence on *gsiB* expression following 10% ethanol or 54°C stress (data not shown).

The *gsiB* assays reported here agree with results obtained on similar strains using a *ctc-lacZ* reporter assay (Akbar *et al.*, 1997; Kim *et al.*, 2004a). In these earlier studies, strains bearing a T205A alteration manifested elevated  $\sigma^B$  activity following 4% ethanol stress in an otherwise wild type background, in which all four co-antagonists were present, as was the case for our *gsiB* assays here. The phenotype of the T205A alteration was even more pronounced in a strain in which the other known paralogs (RsbRB, RC and RD) were absent, and co-antagonist function was solely dependent on the T205A form of RsbRA (Kim *et al.*, 2004a). Kim *et al.* also found that the RsbRB T220A alteration had no effect on  $\sigma^B$  activity, much as we observed in our assays. Thus the available data indicate that RsbRA and RsbRB have similar but not identical roles, and support the idea that phosphorylation of RsbRA T205 serves to attenuate  $\sigma^B$  activation in response to strong stress.

### Role of the RsbX phosphatase

The RsbX feedback phosphatase is thought to return the system to its pre-stress state by dephosphorylating RsbS-S59-P (Yang *et al.*, 1996; Voelker *et al.*, 1997; Smirnova *et al.*, 1998). The RsbRA co-antagonist and its paralogs are further potential substrates of RsbX; the conserved threonine residues in their STAS domains correspond to the D25 and S59 residues of RsbS (see Fig. 1C). In support of this notion, Chen *et al.* (2004) presented *in vitro* evidence that RsbX could dephosphorylate RsbRA-T205-P, but only when RsbRA was assembled into an intact stressosome with RsbS. These authors also found that RsbX manifested no activity toward RsbRA-T171-P under the same assay conditions.

To investigate the *in vivo* influence of RsbX on the phosphorylation state of RsbRA, we compared RsbRA isoforms in strains that were wild or mutant at the *rsbX* locus. Because *rsbX* null alleles cause severe growth and viability defects due to high  $\sigma^B$  activity, we suppressed these phenotypes by using strains that also bore an *rsbU* null allele (Voelker *et al.*, 1995). We first determined whether loss of RsbU phosphatase activity had any effect on the *in vivo* phosphorylation state of the RsbRA protein by comparing isoforms present in a wild type strain (BSA46) with those found in an *rsbU* null mutant (BSA70). As predicted by the model of the environmental signaling pathway (Fig. 1B), the phosphorylation pattern of RsbRA was not significantly affected in the *rsbU* null strain (data not shown). Because the *rsbU* alteration in these strains prevents induction of  $\sigma^B$  (and RsbX) synthesis in response to environmental stress, thereby breaking the negative feedback loop, we only examined the effects of the *rsbX* null allele in unstressed cells.

To test the activity of RsbX *in vivo*, proteins isolated from unstressed *rsbU* null (BSA70) and *rsbU/rsbX* double null mutants (BSA151) were separated by IEF and the different RsbRA isoforms were detected by Western blotting. As shown in Fig. 7A, in the absence of any imposed stress there were significant differences between the two strains. Nearly all of the RsbRA was phosphorylated in the *rsbX* null mutant, and a significant portion was in the doubly phosphorylated form. This result indicates that dephosphorylation of at least one of the threonine residues requires RsbX *in vivo*. Based on the *in vitro* data of Chen *et al.* (2004), we presume that RsbX can directly dephosphorylate T205-P *in vivo* but has little or no activity toward T171-P. To test this proposed specificity, we repeated the experiment

using strains bearing the T171A substitution. However, as others had observed *in vitro* (Gaidenko *et al.*, 1999), this substitution also prevented T205 phosphorylation *in vivo*, and we were unable to assay RsbX activity against T205-P (data not shown).

In parallel with our analysis of the RsbRA co-antagonist, we examined the influence of RsbX on the phosphorylation pattern of the RsbS antagonist, using IEF and Western blotting against monoclonal RsbS antibody. RsbX is known to dephosphorylate RsbS-S59-P *in vitro* (Yang *et al.*, 1996; Chen *et al.*, 2004), but this activity has not been tested *in vivo*. As shown in Fig. 7B, in unstressed cells RsbS was more highly phosphorylated in the *rsbU/rsbX* double null than in the *rsbU* single null control. Coupled with the available *in vitro* data, this result supports the hypothesis that RsbX directly dephosphorylates S59-P *in vivo*.

## Discussion

The stressosome is a large cytoplasmic complex that plays a central role in conveying the environmental cues that activate  $\sigma^B$ . However, the means by which the stressosome senses diverse signals as well as the mechanism of signal transduction within the stressosome itself are not well understood. These questions assume wider importance given the suggested role of RsbRST stressosome modules in controlling diverse signaling pathways in both Gram positive and Gram negative bacteria (Pané-Farré *et al.*, 2005). Because the physiological role of the stressosome is known only in *B. subtilis* and the related *L. monocytogenes*, analysis of the molecular basis of its function has focused on the *Bacillus* model system.

Previous *in vitro* and *in vivo* analysis has pointed to the importance of serine or threonine modification in the signal transduction process: phosphorylation of RsbRA T171 is an important prerequisite for the response, and phosphorylation of RsbS S59 correlates with the onset of signaling (Fig. 1). However, the role of RsbRA T205 was unknown. Here we have shown that T205 becomes phosphorylated under strong stress conditions that cause either a significant growth rate decrease or a complete growth stop, and that this modification requires the RsbT kinase. We have also shown that a mutant bearing the T205A substitution has greatly enhanced  $\sigma^B$  activity under the same stress conditions. These results lead us to propose that T205 phosphorylation acts as a negative feedback mechanism to attenuate excessive response in the face of severe stress.

One possible basis of this attenuation is suggested by earlier *in vitro* experiments. RsbRA has a positive role in enhancing the phosphorylation of RsbS by RsbT, but phosphorylation of RsbRA on both T171 and T205 was found to abolish this enhancing activity (Gaidenko *et al.*, 1999; Chen *et al.*, 2004). In light of the data reported here, we propose the new model for environmental signaling shown in Fig. 8. In unstressed cells, RsbT phosphorylates RsbRA on T171, and the stressosome is poised to activate the response. When a stress signal is perceived, RsbRA facilitates phosphorylation of RsbS, which is associated with the release of RsbT to activate the RsbU environmental phosphatase. However, if the stress signal is strong, RsbT additionally phosphorylates RsbRA on T205, preventing efficient phosphorylation of RsbS and thus attenuating the response.

This model is consistent with the *in vivo* phenotypes of D or A substitutions at T171 or T205, which are thought to mimic the phosphorylated or unphosphorylated states of these residues (Akbar *et al.*, 1997; Gaidenko *et al.*, 1999; Kim *et al.*, 2004a). It is also consistent with the strong negative effect the T205D substitution has on interaction of RsbRA and RsbS in the yeast two-hybrid system (Akbar *et al.*, 1997). RsbRA associates with RsbS in the stressosome (Chen *et al.*, 2003; Kim *et al.*, 2004a; Delumeau *et al.*, 2006), and phosphorylation of RsbRA on T205 does not disrupt integrity of the complex *in vitro* (Chen *et al.*, 2004). Thus the effect of T205D noted in the yeast two-hybrid system may reflect a

change in the interaction of RsbRA and RsbS within the confines of the stressosome, once RsbRA becomes phosphorylated on T205. It remains to be determined whether this postulated change in fact impinges upon the ability of RsbRA to facilitate phosphorylation of RsbS by RsbT or otherwise disrupts signal flux through the stressosome.

Our experiments also support the hypothesis that serine-59 phosphate of RsbS and at least one of the threonine phosphates of RsbRA are direct targets of the RsbX feedback phosphatase *in vivo* (see Fig. 6). Other considerations suggest that RsbRA T205-P (and not T171-P) is dephosphorylated by RsbX *in vivo*, including the correspondence of the S59 residue in RsbS with the T205 residue in RsbRA (Fig. 1C) and the specificity of RsbX toward T205-P *in vitro* (Chen *et al.*, 2004). The activity of RsbX is thought to be low in unstressed cells (Smirnova *et al.*, 1998; Chen *et al.*, 2004). This low activity is sufficient to maintain both RsbS and RsbRA in the ready but non-signaling state, with RsbS unphosphorylated and RsbRA phosphorylated mostly on T171 (Kim *et al.*, 2004b). For RsbRA this state likely reflects the balance of RsbT kinase activity, with its preference for T171, and RsbX phosphatase activity, with its specificity for T205-P (Chen *et al.* 2004). For RsbS, kinase activity is presumably too low in unstressed cells to overcome the RsbX phosphatase. We therefore propose that one significant role of RsbX is to maintain this ready state in unstressed cells, with full capacity for signalling (involving phosphorylation of RsbS-S59) and attenuation (involving phosphorylation of RsbRA-T205).

We further suggest that during the stress response the sensitivity of these substrates to phosphorylation and dephosphorylation is such that RsbX can perform additional roles, depending on signal strength. Following mild stress, RsbT kinase activity would modestly exceed RsbX activity against S59; RsbS becomes phosphorylated, and  $\sigma^B$  is efficiently activated. Because its structural gene is under  $\sigma^B$  control, RsbX levels would then increase, leading to the eventual dephosphorylation of RsbS and attenuation of the response. By contrast, following strong stress, RsbT kinase activity would significantly exceed RsbX phosphatase activity, and RsbS becomes phosphorylated on S59 and RsbRA on T205. T205 phosphorylation would limit  $\sigma^B$  activation to a tolerable level, and the resulting increase in RsbX levels would eventually return the system to the ready state. In this view, RsbX has three roles: (i) a maintenance phosphatase in the absence of stress; (ii) a feedback phosphatase following response to mild stress; and (iii) a reset phosphatase following response to strong stress.

The environmental signaling branch of the  $\sigma^B$  network thus has two negative feedback loops – the previously described RsbX loop and the T205 loop proposed here. These results therefore identify a mechanism consistent with a previous study, which suggested that RsbX was not the only source of feedback in the system (Smirnova *et al.* 1998). What is the physiological role of these two different feedback circuits? Network modelling suggests the RsbX loop allows a rapid initial activation of  $\sigma^B$  by environmental stress, with some overshoot, and a subsequent readjustment to match the new steady-state condition (Igoshin *et al.*, 2007). We now believe that this loop would function primarily under mild stress conditions. By contrast, the T205 loop would serve to attenuate the general stress response in the face of potentially lethal stresses. Under these conditions protein synthesis is restricted, and attenuation of the response may allow the cell to balance its resources among competing stress systems. The two feedback systems would thus have evolved to refine the interplay between them, allowing a flexible contribution from each to accommodate different stress levels.

Questions remain regarding the conserved threonine residues found in the RsbRB, RC and RD paralogs of *B. subtilis*, and the threonine (or serine) residues found in corresponding positions of the RsbR homologues of diverse bacteria (Krishnamurthy *et al.*, 2006). Their



preservation suggests functional importance, but the phenotypes of T186 and T220 substitutions in RsbRB indicate that their roles might differ in degree from T171 and T205 of RsbRA (Kim *et al.*, 2004a; our unpublished results). Nonetheless, the present study underscores the importance of the RsbX feedback phosphatase in maintaining the ready state of the RsbRST signaling module. In this regard, we note that diverse bacterial genomes encoding this module often possess a closely linked gene for an RsbX-like protein phosphatase (Pané-Farré *et al.*, 2005).

## Experimental procedures

### Bacterial strains and culture conditions

*B. subtilis* strains used in this study are listed in Table 2. Strains were cultivated under vigorous agitation (180 rpm) at 37°C in a synthetic minimal medium, as described previously (Stülke *et al.*, 1993). Stress experiments were performed by (i) adding ethanol to a final concentration of 4% or 10% (v/v); (ii) adding sodium chloride to a final concentration of 4% or 8% (w/v); or (iii) shifting the culture from 37°C to 50°C or 54°C. Samples were taken at different times after the stress.

### Sample preparation, two-dimensional protein electrophoresis

Bacterial cells were harvested and washed twice with killing buffer containing 20 mM Tris pH 7.5, 5 mM MgCl<sub>2</sub>, 20 mM NaN<sub>3</sub> and 10 mM NaF (18.300 × g, 4°C, 10 min). The pellet was resuspended in lysis buffer containing 10 mM Tris pH 7.5, 1 mM EDTA, 1 mM PMSF and 10 mM NaF. Cells were disrupted by ultrasonication and the soluble protein fraction separated from cell debris by centrifugation. Protein concentration was determined using Roti<sup>®</sup>-Nanoquant (Roth, Karlsruhe, Germany). 200 µg protein was separated by 2D PAGE as described previously (Eymann *et al.*, 2004). Isoelectric focusing was done with commercially available 18-cm-IPG strips in the pH ranges 4–7 or 4.5–5.5 (GE Healthcare, Munich, Germany). 2D gels from the narrow pI gradients 4.5–5.5 were exclusively used for protein identifications by mass spectrometry (see below). Staining procedures with the fluorescence stains ProQ Diamond<sup>®</sup> and Flamingo (Molecular Probes) and image scanning were performed as described previously (Eymann *et al.*, 2007).

### Identification of RsbRA and RsbRB proteins and phosphate-containing peptides by mass spectrometry

Depending on signal intensity, up to seven Pro-Q-stained gel spots from replicate gels were manually cut and pooled into one tube. The gel pieces were washed twice with 200 µl of 20 mM NH<sub>4</sub>HCO<sub>3</sub> in 30% (v/v) CH<sub>3</sub>CN for 30 min at 37°C, then dried in an Eppendorf Concentrator 5301 vacuum centrifuge. In-gel digestion with 10 ng/µl trypsin (Promega, Madison, WI) in 20 mM NH<sub>4</sub>HCO<sub>3</sub> was performed overnight at 37°C. Incubation of the gel pieces in an ultrasonic bath for 15 min in 20 µl HPLC-grade water resulted in peptide extraction. The supernatant was transferred into micro sample vials for mass spectrometric analysis. Peptides were separated and measured online by ESI-mass spectrometry using a nanoACQUITY UPLC<sup>™</sup> system (Waters, Milford, MA) coupled to an LTQ Orbitrap<sup>™</sup> mass spectrometer (Thermo Fisher Scientific, Waltham, MA). They were desalted onto a trap column (Symmetry<sup>®</sup> C18 5 µm, 180 µm inner diameter x 20 mm, Waters). Elution was performed onto an analytical column (BEH130 C18 1.7 µm, 100 µm inner diameter x 100 mm, Waters) by a binary gradient of buffer A (0.1% CH<sub>3</sub>COOH, v/v) and B (100% CH<sub>3</sub>CN in 0.1% CH<sub>3</sub>COOH, v/v) with a flow rate of 400 nl/min for 50 min. The LTQ Orbitrap was operated in data-dependent MS/MS mode, using multistage activation (MSA) for phospho relevant masses and the lockmass option for recalibration.

Proteins were identified by searching all MS/MS spectra in .dta format against all *Bacillus subtilis* proteins (extracted from the Uniprot-KB database: <http://www.uniprot.org/uniprot/?query=Bacillus+subtilis+168&sort=score>) using “Sorcerer™-SEQUEST®” (Sequest v. 2.7 rev. 11, Thermo Electron) including “Scaffold\_2\_05\_02” (Proteome Software Inc., Portland, OR). Sequest search was performed applying a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 10 ppm. Up to two tryptic miscleavages were allowed. Methionine oxidation (+15.99492 Da), cysteine carbamidomethylation (+57.021465 Da) and phosphorylation on serine, threonine and tyrosine (+79.966331 Da) were considered as variable modifications.

Proteins were identified by at least two peptides applying a probability score of greater than 99.9% (“Scaffold\_2\_02\_03”; Proteome Software Inc., Portland, OR). Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al.*, 2003). Phosphorylated peptides that have a peptide probability score greater than 95.0% as specified by the Peptide Prophet algorithm (Keller *et al.* 2002) were examined manually. They were only accepted when b- or y-ions confirmed the identification. Identified peptides are listed in Table 1.

### IEF and Western blot (immunoblot) analysis

Cells were harvested and washed with killing buffer before ultrasonication in lysis buffer (buffer compositions given above). For IEF, 50 µg of protein extracts were mixed in a 1:1 volume with 2 × IEF sample buffer, loaded onto each lane of a mini-IEF-gel and separated as described by Kim *et al.* (2004b), with minor modifications. IEF sample buffer contained 2.6% (v/v) and the gel solution 4% (v/v) ampholytes (pH 3.5 – 5) or pharmalytes (pH 4.5 – 5.4 for RsbRA and RsbRB, or pH 4.2 – 4.9 for RsbS), together with 0.6% (v/v) pharmalytes (pH 3 – 10) and 1% (w/v) Triton X-100 (ampholytes and pharmalytes from GE Healthcare, Munich, Germany). After isoelectric focusing, gels were incubated for 30 minutes in solution 1: 50% (v/v) methanol, 1% (w/v) sodium dodecyl sulfate (SDS), 0.6 M Tris pH 8; then for 30 minutes in solution 2: 50% (v/v) methanol, 1% (w/v) SDS, 0.4 M Tris pH 9.5. Equilibrated proteins were electroblotted at 100 V for 3 h to Immobilon-P membrane (Millipore Corp., Billerica, MA), using transfer buffer containing 25 mM Tris pH 8.3, 193 mM glycine, 20% (v/v) methanol, 0.1% (w/v) SDS. Immunoblot detection has been described previously (Voelker *et al.*, 1995). Monoclonal anti-RsbRA and anti-RsbS antibodies (Dufour *et al.*, 1996) were kindly provided by Uwe Völker. The polyclonal anti-RsbRB antibody was described by Kim *et al.* (2004a); it was purified by adsorption against an acetone powder (Harlow and Lane, 1988) made from *rsbRB* deletion mutant PB545. Antibody specificity was confirmed by Western blot analysis of wild-type and mutant cell extracts separated on SDS-polyacrylamide gel electrophoresis. Detection and quantification of specific signals was done using the Roche Lumi-Imager™ F1 Workstation and Roche Lumi-Analyst software 3.0.

### Northern blot analysis

Total RNA isolation and Northern blot analyses were performed as described previously (Wetzstein *et al.*, 1992). RNA of *B. subtilis* wild-type and mutant strains was isolated during exponential growth (for control) and at different time points after exposure to ethanol or heat stress. Hybridizations specific for *sigB*, *ctc* and *gsiB* were conducted with digoxigenin-labelled RNA probes synthesized *in vitro* with T7 RNA polymerase from T7 promoter-containing internal PCR products of *sigB*, *ctc* or *gsiB*. The following primers were used for PCR, respectively (T7 polymerase promoter sequence underlined): *sigB*-for (5'-CACAACCATCAAAAACTACG-3') and *sigB*-rev (5'-CTAATACGACTCACTATAGGGAGATAAGGCTTGATAGCTTTTGC-3'); *ctc*-for (5'-AGAACGGACTTTACTCGTTC-3') and *ctc*-rev (5'-CTAATACGACTCACTATAGGGAGAAGGCTTGAAATATCAGCCTC-3'); *gsiB*-for (5'-

ATGGCAGACAATAACAAAATGAG-3') and *gsiB*-rev (5'-CTAATACGACTCACTATAGGGAGAGTCGTTGTTGCGGGCGTTTCCGCC-3'). A Roche Lumi-Imager™ F1 Workstation was used to detect *gsiB* message signals; these were quantified with ImageJ 1.42q (<http://rsb.info.nih.gov/ij/>) and expressed relative to the controls, whose signal intensities were taken as 1.

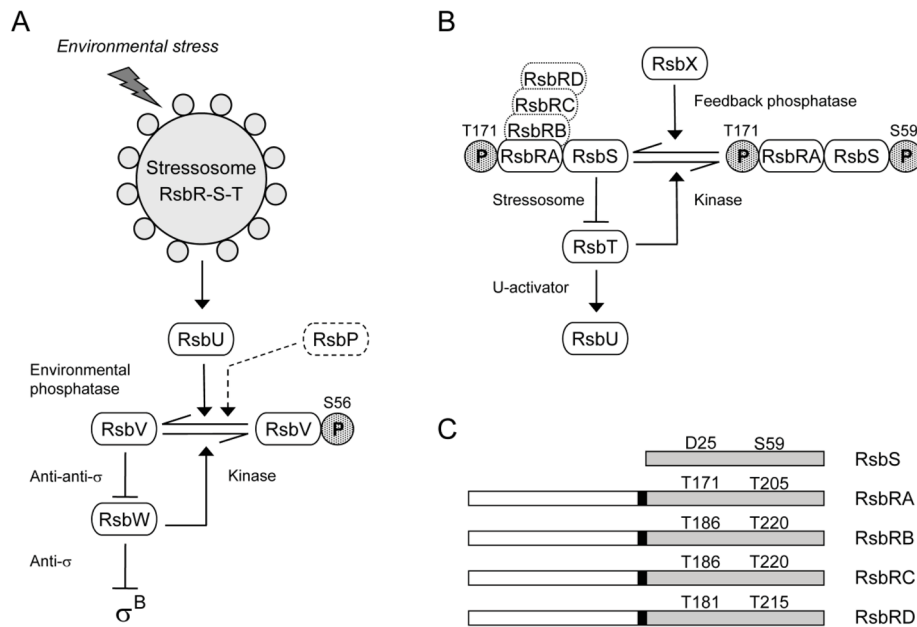
## Acknowledgments

We thank Uwe Völker and William Haldenwang for providing strains and monoclonal antibodies against RsbRA and RsbS; Jörg Bernhardt for creating Figure 2 by means of the software Delta 2D; and Tanya Gaidenko for her critical comments on the manuscript. This research was supported by Grants from the Deutsche Forschungsgemeinschaft (HE 1887/7-4; HE 1887/8-1) and the BMBF (0313978A) (to MH), and by Public Health Service Grant GM42077 from the National Institute of General Medical Sciences (to CWP).

## References

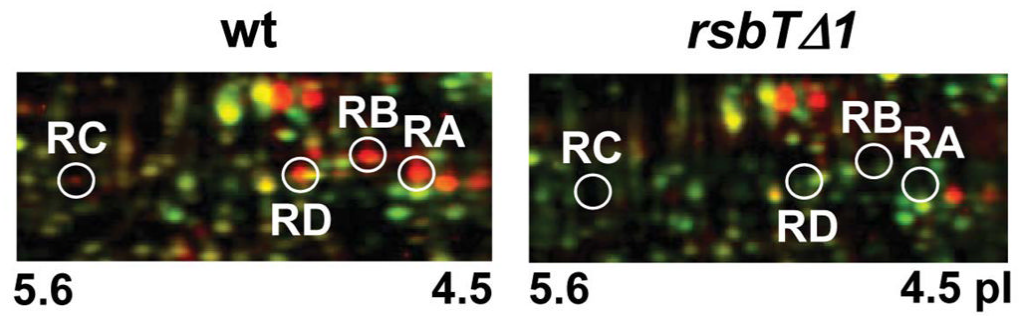
- Akbar S, Gaidenko TA, Kang CM, O'Reilly M, Devine KM, Price CW. New family of regulators in the environmental signaling pathway which activates the general stress transcription factor  $\sigma^B$  of *Bacillus subtilis*. *J Bacteriol.* 2001; 183:1329–1338. [PubMed: 11157946]
- Akbar S, Kang CM, Gaidenko TA, Price CW. Modulator protein RsbR regulates environmental signalling in the general stress pathway of *Bacillus subtilis*. *Mol Microbiol.* 1997; 24:567–578. [PubMed: 9179850]
- Chen CC, Lewis RJ, Harris R, Yudkin MD, Delumeau O. A supramolecular complex in the environmental stress signalling pathway of *Bacillus subtilis*. *Mol Microbiol.* 2003; 49:1657–1669. [PubMed: 12950928]
- Chen CC, Yudkin MD, Delumeau O. Phosphorylation and RsbX-dependent dephosphorylation of RsbR and paralogues in the RsbR-RsbS complex of *Bacillus subtilis*. *J Bacteriol.* 2004; 186:6830–6836. [PubMed: 15466036]
- Delumeau O, Chen CC, Murray JW, Yudkin MD, Lewis RJ. High-molecular-weight complexes of RsbR and paralogues in the environmental signaling pathway of *Bacillus subtilis*. *J Bacteriol.* 2006; 188:7885–7892. [PubMed: 16963570]
- Delumeau O, Dutta S, Brigulla M, Kuhnke G, Hardwick SW, Völker U, et al. Functional and structural characterization of RsbU, a stress signaling protein phosphatase 2C. *J Biol Chem.* 2004; 279:40927–40937. [PubMed: 15263010]
- Dufour A, Voelker U, Voelker A, Haldenwang WG. Relative levels and fractionation properties of *Bacillus subtilis*  $\sigma^B$  and its regulators during balanced growth and stress. *J Bacteriol.* 1996; 178:3701–3709. [PubMed: 8682769]
- Eymann C, Becher D, Bernhardt J, Gronau K, Klutzny A, Hecker M. Dynamics of protein phosphorylation on Ser/Thr/Tyr in *Bacillus subtilis*. *Proteomics.* 2007; 7:3509–3526. [PubMed: 17726680]
- Eymann C, Dreisbach A, Albrecht D, Bernhardt J, Becher D, Gentner S, et al. A comprehensive proteome map of growing *Bacillus subtilis* cells. *Proteomics.* 2004; 4:2849–2876. [PubMed: 15378759]
- Gaidenko TA, Yang X, Lee YM, Price CW. Threonine phosphorylation of modulator protein RsbR governs its ability to regulate a serine kinase in the environmental stress signalling pathway of *Bacillus subtilis*. *J Mol Biol.* 1999; 288:29–39. [PubMed: 10329124]
- Harlow, E.; Lane, D. *Antibodies: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1988. p. 632-633.
- Hecker M, Pané-Farré J, Völker U. SigB-dependent general stress response in *Bacillus subtilis* and related gram-positive bacteria. *Annu Rev Microbiol.* 2007; 61:215–236. [PubMed: 18035607]
- Hecker M, Reder A, Fuchs S, Pagels M, Engelmann S. Physiological proteomics and stress/starvation responses in *Bacillus subtilis* and *Staphylococcus aureus*. *Res Microbiol.* 2009; 160:245–258. [PubMed: 19403106]

- Igoshin OA, Brody MS, Price CW, Savageau MA. Distinctive topologies of partner-switching signaling networks correlate with their physiological roles. *J Mol Biol.* 2007; 369:1333–1352. [PubMed: 17498739]
- Kang CM, Brody MS, Akbar S, Yang X, Price CW. Homologous pairs of regulatory proteins control activity of *Bacillus subtilis* transcription factor  $\sigma^B$  in response to environmental stress. *J Bacteriol.* 1996; 178:3846–3853. [PubMed: 8682789]
- Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem.* 2002; 74:5383–5392. [PubMed: 12403597]
- Kim TJ, Gaidenko TA, Price CW. A multicomponent protein complex mediates environmental stress signaling in *Bacillus subtilis*. *J Mol Biol.* 2004; 341:135–150. [PubMed: 15312768]
- Kim TJ, Gaidenko TA, Price CW. *In vivo* phosphorylation of partner switching regulators correlates with stress transmission in the environmental signaling pathway of *Bacillus subtilis*. *J Bacteriol.* 2004; 186:6124–6132. [PubMed: 15342582]
- Krishnamurthy N, Brown DP, Kirshner D, Sjölander K. PhyloFacts: an online structural phylogenomic encyclopedia for protein functional and structural classification. *Genome Biol.* 2006; 7:R83. [PubMed: 16973001]
- Macek B, Mijakovic I, Olsen JV, Gnäd F, Kumar C, Jensen PR, Mann M. The serine/threonine/tyrosine phosphoproteome of the model bacterium *Bacillus subtilis*. *Mol Cell Proteomics.* 2007; 6:697–707. [PubMed: 17218307]
- Marles-Wright J, Grant T, Delumeau O, van Duinen G, Firbank SJ, Lewis PJ, et al. Molecular architecture of the “stressosome,” a signal integration and transduction hub. *Science.* 2008; 322:92–96. [PubMed: 18832644]
- Maul B, Völker U, Riethdorf S, Engelmann S, Hecker M.  $\sigma^B$ -dependent regulation of *gsiB* in response to multiple stimuli in *Bacillus subtilis*. *Mol Gen Genet.* 1995; 248:114–120. [PubMed: 7651322]
- Murray JW, Delumeau O, Lewis RJ. Structure of a nonheme globin in environmental stress signaling. *Proc Natl Acad Sci USA.* 2005; 102:17320–17325. [PubMed: 16301540]
- Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem.* 2003; 75:4646–4658. [PubMed: 14632076]
- Pané-Farré J, Lewis RJ, Stülke J. The RsbRST stress module in bacteria: a signalling system that may interact with different output modules. *J Mol Microbiol Biotechnol.* 2005; 9:65–76. [PubMed: 16319496]
- Price, CW. General stress response in *Bacillus subtilis* and related Gram positive bacteria. In: Storz, G.; Hengge, R., editors. *Bacterial Stress Responses*. 2. Washington, DC: American Society for Microbiology Press; 2010. p. 301-318.
- Reeves A, Martinez L, Haldenwang W. Expression of, and *in vivo* stressosome formation by, single members of the RsbR protein family in *Bacillus subtilis*. *Microbiology.* 2010; 156:990–998. [PubMed: 20019076]
- Smirnova N, Scott J, Voelker U, Haldenwang WG. Isolation and characterization of *Bacillus subtilis* *sigB* operon mutations that suppress the loss of the negative regulator RsbX. *J Bacteriol.* 1998; 180:3671–3680. [PubMed: 9658013]
- Stülke J, Hanschke R, Hecker M. Temporal activation of  $\alpha$ -glucanase synthesis in *Bacillus subtilis* is mediated by the GTP pool. *J Gen Microbiol.* 1993; 139:2041–2045. [PubMed: 8245830]
- Voelker U, Dufour A, Haldenwang WG. The *Bacillus subtilis* *rsbU* gene product is necessary for RsbX-dependent regulation of  $\sigma^B$ . *J Bacteriol.* 1995; 177:114–122. [PubMed: 8002609]
- Voelker U, Luo T, Smirnova N, Haldenwang W. Stress activation of *Bacillus subtilis*  $\sigma^B$  can occur in the absence of the  $\sigma^B$  negative regulator RsbX. *J Bacteriol.* 1997; 179:1980–1984. [PubMed: 9068644]
- Wetzstein M, Völker U, Dedio J, Lobau S, Zuber U, Schiesswohl M, et al. Cloning, sequencing, and molecular analysis of the *dnaK* locus from *Bacillus subtilis*. *J Bacteriol.* 1992; 174:3300–3310. [PubMed: 1339421]
- Yang X, Kang CM, Brody MS, Price CW. Opposing pairs of serine protein kinases and phosphatases transmit signals of environmental stress to activate a bacterial transcription factor. *Genes Dev.* 1996; 10:2265–2275. [PubMed: 8824586]



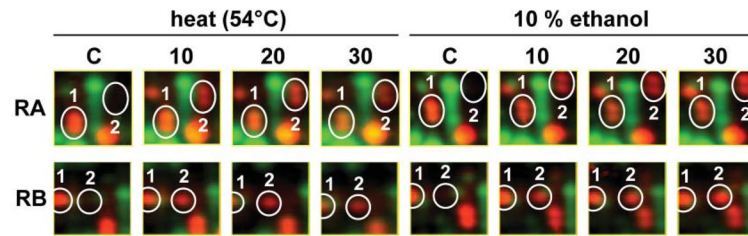
### Figure 1. The $\sigma^B$ regulatory network

(A) Model of signaling pathways that regulate  $\sigma^B$  (modified from Hecker *et al.*, 2007; Price, 2010). Environmental and energy pathways converge on RsbV and RsbW, which directly regulate  $\sigma^B$  activity (see text). The stressosome controls activation of the RsbU environmental phosphatase in response to diverse signals; the RsbP energy phosphatase is represented only in dotted outline. Horizontal arrows show conversion between RsbV and RsbV-P (with phosphate as stippled P). Full arrowheads indicate activating effects and T-headed lines inhibiting ones. (B) Model of stressosome control of RsbU phosphatase activity. The stressosome comprises the RsbRA, RB, RC, and RD co-antagonists and the RsbS antagonist, which together bind the RsbT kinase. In unstressed cells RsbT phosphorylates RsbRA on T171; we establish here that RsbT is also required for phosphorylation of RB on T186, RC on T186, and RD on T181. During the stress response, RsbRA-T171-P enhances the activity of the RsbT kinase, which inactivates its RsbS antagonist by phosphorylating S59; RsbT is released to bind and activate the RsbU environmental phosphatase. The RsbX feedback phosphatase dephosphorylates RsbS, damping continued signaling. (C) The RsbS antagonist consists of a single STAS domain (shaded), whereas the RsbR co-antagonists have an N-terminal, non-heme globin domain (open) connected to a C-terminal STAS domain by a 13 residue linker (black rectangle). Conserved threonine residues are indicated in the STAS domains of the RsbR proteins, together with the corresponding aspartate and serine residues of RsbS. These threonine and serine residues are the known or presumed sites of phosphorylation by RsbT.

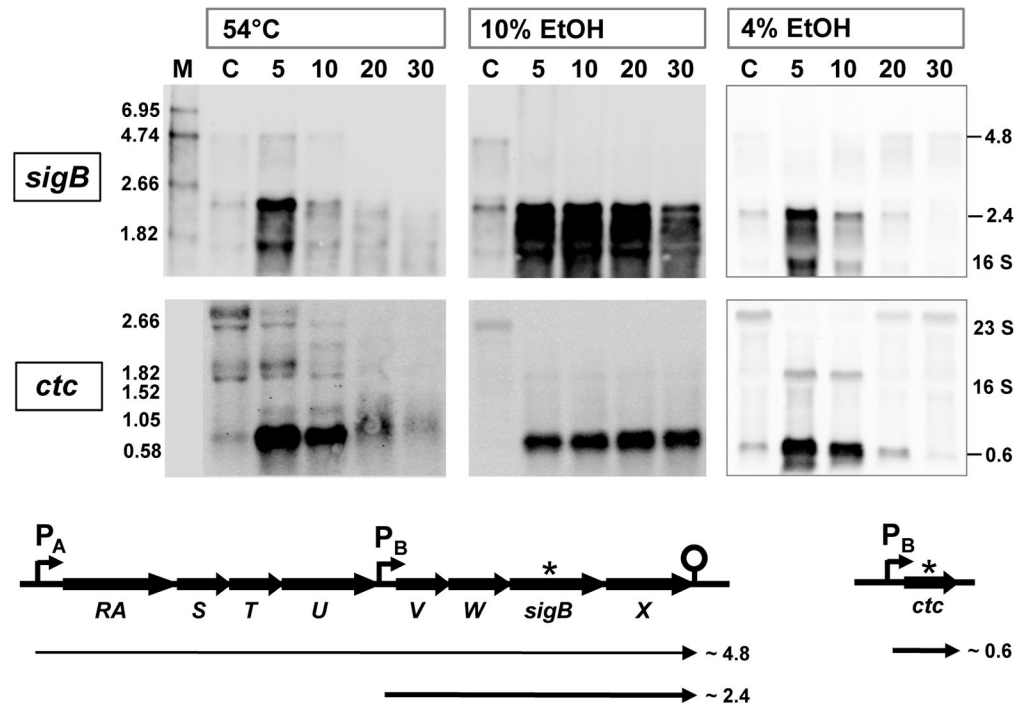


**Figure 2. RsbT kinase activity is required for phosphorylation of RsbR proteins in unstressed cells**

Sections of dual channel images of phosphor-proteomes are shown. Proteins from exponentially-growing wild type (wt; PB2) and mutant cells (*rsbT*Δ1; PB421) were separated on two-dimensional gels and stained with ProQ-Diamond® (phosphorylated proteins = red color channel), then with Flamingo (all proteins = green color channel).



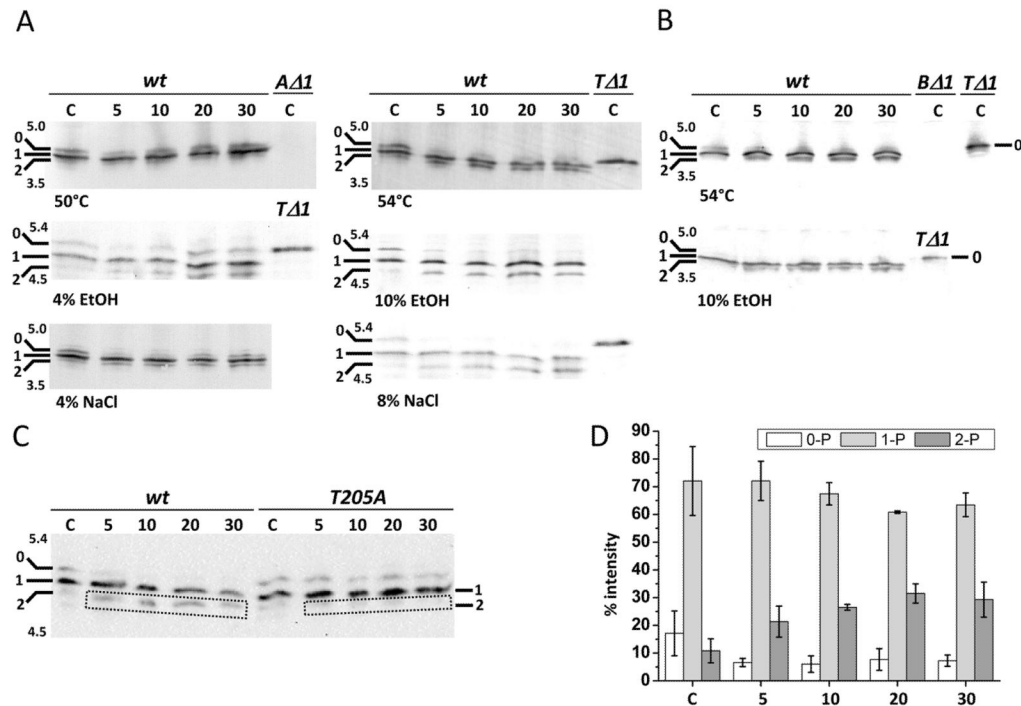
**Figure 3. New isoforms of RsbRA and RsbRB appear following strong heat or ethanol stress**  
 Relevant sections of dual channel images of the phosphor-proteome are shown. Time of stress is indicated in minutes; samples from wild type cells were separated and stained as in the Fig. 2 legend. For RsbRA (upper row) and RsbRB proteins (lower row), potential single- and double-phosphorylated isoforms are labelled (1) and (2), respectively. Note that the most acidic isoforms (double-phosphorylated, labelled 2) were detected only in response to stress.



**Figure 4. Induction of  $\sigma^B$ -dependent message from *sigB* or *ctc* in response to heat or ethanol stress**

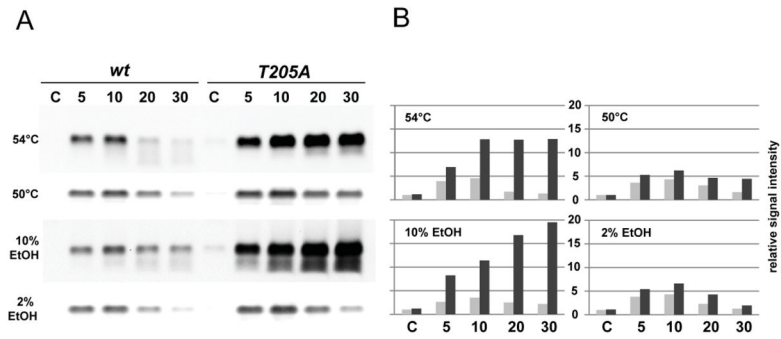
RNA probes were made against the  $\sigma^B$ -dependent genes *sigB* and *ctc*; these were used for Northern blot analysis of RNA isolated before (lane C) and at various times (in minutes) after stress, as described in *Experimental procedures*. Size standards (lane M) facilitated identification of gene-specific transcripts as well as rRNA species, indicated on the right. Organization of the *sigB* and *ctc* regions is shown beneath the blots, with  $\sigma^B$ -dependent promoters labelled  $P_B$ , the source of the probe indicated by \*, and the expected transcripts designated by bold arrows.





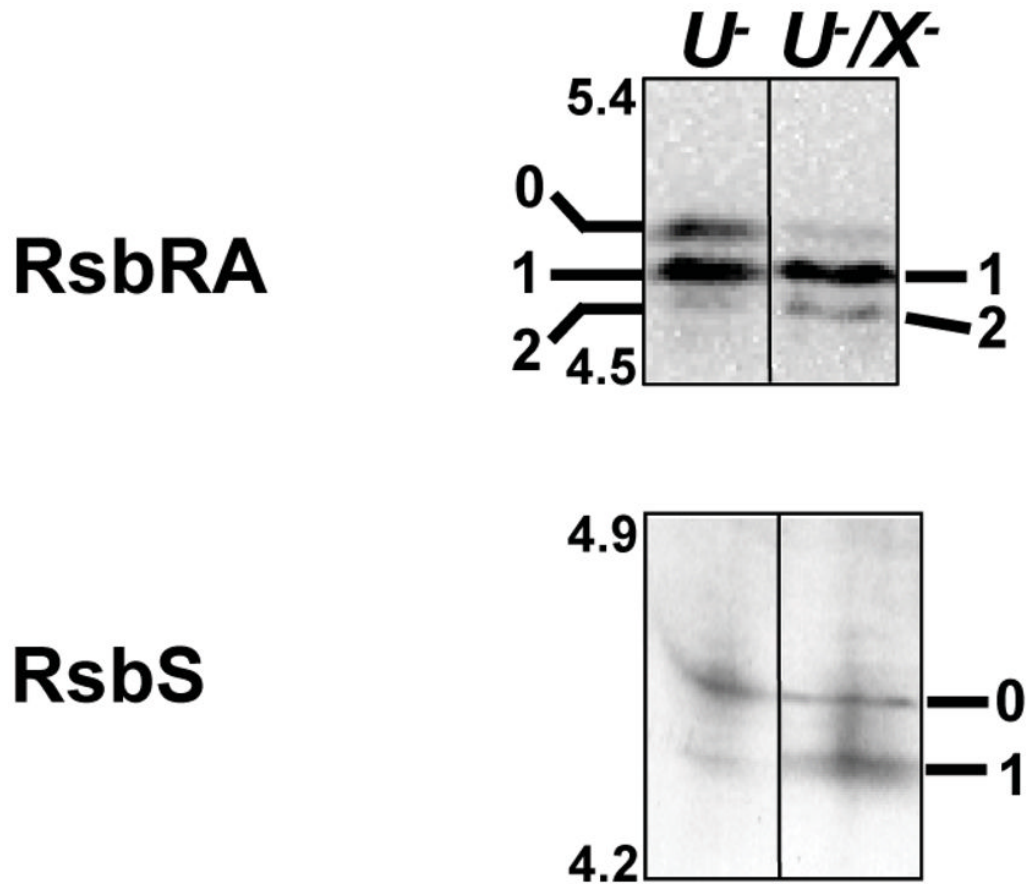
**Figure 5. Multiple isoforms are detected in crude cell extracts using anti-RsbRA or anti-RsbRB antibody**

(A & B) For IEF in the ranges indicated on the gel images, 50  $\mu$ g of protein from wild type (PB2) cells isolated before (control, leftmost lane C) or at various times after exposure to heat (50°C, 54°C), ethanol (4%, 10%) or sodium chloride (4%, 8%) were loaded into each lane. Samples from unstressed *rsbRA* ( $A\Delta 1$ ; PB427), *rsbRB* ( $B\Delta 1$ ; PB545), or *rsbT* ( $T\Delta 1$ ; PB421) null mutants were also analyzed (lane C on the right). Western blots were probed with anti-RsbRA (A) or anti-RsbRB (B); gel images are oriented with alkaline regions uppermost and with isoform positions (0, 1, 2) indicated on the left. (C) Blot using anti-RsbRA antibody to probe extracts of wild type (PB2) and *rsbRA* T205A mutant (PB505) cells isolated before (control, leftmost lane C) or at various times after exposure to 10% ethanol; isoform positions (0, 1, 2) indicated on the left. (D) Quantification of RsbRA isoform signals from three independent ethanol stress experiments. Isoforms (0-P, 1-P, 2-P) are shown as % of total RsbRA detected.

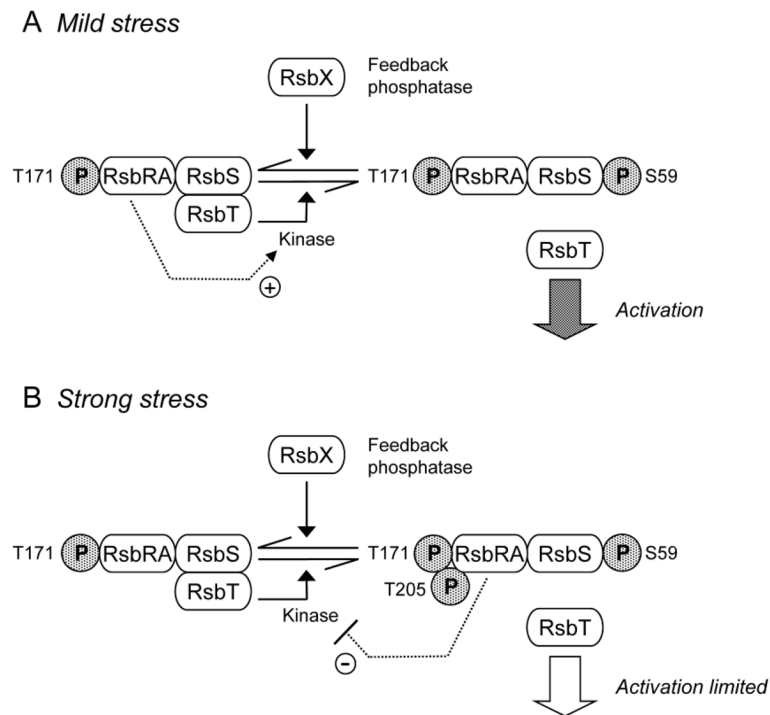


**Figure 6. T205A alteration in RsbRA greatly increases  $\sigma^B$ -dependent message levels during severe stress**

(A) Northern blot analysis of RNA isolated from the wild type (wt; strain PB2) or a mutant encoding the T205A alteration in *rsbRA* (T205A; strain PB505), subjected to either strong (54°C, 10% ethanol) or mild (50°C, 2% ethanol) stress for the indicated times (minutes). An RNA probe was used to monitor expression of the  $\sigma^B$ -dependent gene *gsiB*; control lane C indicates signal in unstressed cells. (B) Quantitation of the data shown in (A), expressed relative to that of the unstressed, wild-type control, taken as 1. Grey bars indicate *gsiB* expression in the wild type strain and black bars the T205A mutant.



**Figure 7. RsbRA and RsbS are substrates for the RsbX phosphatase in unstressed cells**  
 Western blot analyses using anti-RsbRA (*upper*) or anti-RsbS (*lower*) antibody against IEF-separated crude cell extracts of unstressed strains bearing either a null allele in *rsbU* ( $U^-$ ; BSA70) or null alleles in both *rsbU* and *rsbX* ( $U^-/X^-$ ; BSA151). Isoforms are 0 (unphosphorylated), 1 (singly phosphorylated) or 2 (doubly phosphorylated) for RsbRA; and 0 (unphosphorylated) or 1 (singly phosphorylated) for RsbS.



**Figure 8. Model of stressosome function in response to different levels of environmental stress**  
 In unstressed cells RsbT phosphorylates the RsbRA co-antagonist on T171. **(A)** Following mild environmental stress, RsbRA-T171-P enhances activity of the RsbT kinase (+ arrow), which phosphorylates the RsbS antagonist on S59. This event correlates with release of RsbT from the complex, and RsbT activation of the RsbU phosphatase by direct protein-protein interaction. RsbX feedback phosphatase subsequently removes phosphate from S59, damping the general stress response. **(B)** Following strong environmental stress, RsbT additionally phosphorylates RsbRA on T205. We propose that doubly phosphorylated RsbRA no longer functions as a kinase activator (- arrow), limiting the general stress response. RsbX phosphatase then removes the phosphate from both RsbRA-T205 and RsbS-S59, resetting the system to function anew in unstressed cells.

Table 1

MS/MS data of proteins identified after 10% ethanol stress

| Spot   | Accession Number | Protein Name | Molecular Mass (Da) | Protein Identification Probability (%) | Peptide Identification Probability (%) | Coverage (%) | Number of Unique Peptides | Phosphorylation site | Phosphopeptide sequence   |
|--------|------------------|--------------|---------------------|--|--|--------------|---------------------------|----------------------|---|
| RsbRA1 | P42409           | RsbRA        | 31050.70            | 100                                    | 95                                     | 64           | 12                        | T171                 | (K)IALQELSAPLIPVFENITV <sup>m</sup> PLVGTID <sup>p</sup> TERAK(R) |
| RsbRA2 | P42409           | RsbRA        | 31050.70            | 100                                    | 95                                     | 71           | 15                        | T171                 | (K)IALQELSAPLIPVFENITV <sup>m</sup> PLVGTID <sup>p</sup> TER(A)   |
| RsbRA2 | P42409           | RsbRA        | 31050.70            | 100                                    | 95                                     | 71           | 15                        | T205                 | (R)SQVVLIDITGYPVVVD <sup>p</sup> T <sup>m</sup> VAAHHIIQASEAVR(L) |
| RsbRB1 | O34860           | RsbRB        | 32387.60            | 100                                    | 95                                     | 53           | 13                        | T186                 | (K)STALLPLVGDID <sup>p</sup> TER(A)                               |
| RsbRB2 | O34860           | RsbRB        | 32387.60            | 100                                    | 95                                     | 63           | 14                        | T186                 | (K)STALLPLVGDID <sup>p</sup> TER(A)                               |

**pT** = phosphorylated Threonine

m = oxidized methionine

**Table 2***Bacillus subtilis* strains

| <i>B. subtilis</i> strain | Genotype   | Reference                    |
|---------------------------|--|------------------------------|
| BSA46                     | SP $\beta$ <i>ctc::lacZ</i>                            | Voelker <i>et al.</i> , 1995 |
| BSA70                     | <i>rsbU::kan</i> SP $\beta$ <i>ctc::lacZ</i>           | Voelker <i>et al.</i> , 1995 |
| BSA151                    | <i>rsbU::kan rsbX::spc</i> SP $\beta$ <i>ctc::lacZ</i> | Voelker <i>et al.</i> , 1995 |
| PB2                       | <i>trpC2</i>   | Marburg strain               |
| PB421                     | <i>rsbTA1 trpC2</i>                                    | Akbar <i>et al.</i> , 1997   |
| PB427                     | <i>rsbRAA1 trpC2</i>                                   | Akbar <i>et al.</i> , 1997   |
| PB545                     | <i>rsbRBA1::km trpC2</i>                               | Akbar <i>et al.</i> , 2001   |
| PB505                     | <i>rsbRAT205A trpC2</i>                                | Akbar <i>et al.</i> , 1997   |
| PB602                     | <i>rsbRBT220A trpC2</i>                                | Kim <i>et al.</i> , 2004a    |