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Temporal regulation of σ^B by partner-switching mechanism at a distinct growth stage in *Bacillus cereus*

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ABSTRACT

The alternative transcription factor σ^B in *Bacillus cereus* governs the transcription of a number of genes that confer protection against general stress. This transcription factor is regulated by protein-protein interactions among RsbV, RsbW, σ^B, RsbY, RsbM and RsbK, all encoded in the *sigB* cluster. Among these regulatory proteins, RsbV, RsbW and σ^B comprise a partner-switching mechanism. Under normal conditions, σ^B remains inactive by associating with anti-sigma factor RsbW, which prevents σ^B from binding to the core RNA polymerase. During environmental stress, RsbK activates RsbY to hydrolyze phosphorylated RsbV, and the dephosphorylated RsbV then sequesters RsbW to liberate σ^B from RsbW. Although the σ^B partner-switching module is thought to be the core mechanism for σ^B regulation, the actual protein-protein interactions among these three proteins in the cell remain to be investigated. In the current study, we show that RsbW and RsbV form a long-lived complex under transient stress treatment, resulting in high persistent expression of RsbV, RsbW and σ^B from mid-log phase to stationary phase. Full sequestration of RsbW by excess RsbV and increased RsbW:RsbV complex stability afforded by cellular ADP contribute to the prolonged activation of σ^B. Interestingly, the high expression levels of RsbV, RsbW and σ^B were dramatically decreased beginning from the transition stage to the stationary phase. Thus, protein interactions among σ^B partner-switching components are required for the continued induction of σ^B during environmental stress in the log phase and significant down-regulation of σ^B is observed in the stationary phase. Our data show that σ^B is temporally regulated in *B. cereus*.

1. Introduction

B. cereus, a food-borne pathogen, can cause human illness involving vomiting and diarrhea (Jensen et al., 2003). The alternative transcription factor σ^B is encoded in low-GC Gram-positive bacteria, such as members of the *B. cereus* family, *Bacillus subtilis*, *Staphylococcus aureus* and *Listeria monocytogenes* (Kazmierczak et al., 2005; van Schaik and Abee, 2005). It is also found in high GC Gram-positive bacteria, including *Mycobacterium tuberculosis* and *Streptomyces* species (Mittenhuber, 2002). The transcription factor σ^B controls the general stress regulon to enable bacteria to withstand environmental fluctuation caused by heat stress, osmotic shock and ethanol treatment (van Schaik et al., 2005; Wang et al., 2009). The mechanism involved in σ^B regulation is well established in *B. subtilis*, in which σ^B activity is regulated by a σ^B partner-switching mechanism, with the basic core members comprising RsbV/RsbW/σ^B. In unstressed cells, RsbV loses ability to sequester anti-σ factor RsbW due to RsbW-dependent phosphorylation, σ^B remains inactive by forming a noncovalent complex with the free RsbW (Benson and Haldenwang, 1993a; Dufour and Haldenwang, 1994). Two PP2C-type phosphatases, RsbU and RsbP, are activated during physical stress and nutritional stress, respectively, and they hydrolyze phosphorylated RsbV (Vijay et al., 2000; Voelker et al., 1996; Yang et al., 1996). The dephosphorylated RsbV then forms an alternative complex with RsbW, which liberates σ^B from RsbW (Dufour and Haldenwang, 1994). Thus, RsbV has dual roles as an antagonist and as the substrate of RsbW. The model of σ^B partner-switching has been confirmed via genetic approaches and biochemical characterization of the components as well as kinetic assays and size exclusion chromatography analysis, all of which showed the formation of an inactivated complex between Rsb regulators (e.g., RsbW-σ^B, RsbV-RsbW) (Kuo et al., 2004).

B. cereus and *B. subtilis* share partner-switching mechanisms that

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Table 1
Bacterial strains and plasmids.

Strain or plasmid	Genotype and/or description	Source or reference
E. coli strains		
DH5α	For general purpose cloning	Invitrogen
BL21 (λDE3)	For protein expression	Novagen
B. cereus strains		
ATCC14579	Wild type	ATCC[a]
$rsbV_{HA}$-$rsbW_{HA}$	ATCC14579 *$rsbV_{HA}$-$rsbW_{HA}$: rsbV-rsbW* using pMAD-*$rsbV_{HA}$-$rsbW_{HA}$*	This work
$rsbY_{HA}$	ATCC14579 *$rsbY_{HA}$:spnR* using pMAD-*$rsbY_{HA}$*	This work
ΔclpX	ATCC14579 *clpX:spnR* using pMAD-Δ*clpX*	This work
Δclpp1	ATCC14579 *clpp1:spnR* using pMAD-Δ*clpp1*	This work
Δclpp2	ATCC14579 *clpp2:tcR* using pMAD-Δ*clpp2*	This work
Plasmids		
pMAD	Integration plasmid; *ermC*, *bgaB*	Arnaud et al. (2004)
pMAD-*$rsbV_{HA}$-$rsbW_{HA}$*	Recombinant plasmid for deletion of *rsbKM*	This work
pMAD-Δ*clpX*	Recombinant plasmid for deletion of *clpX* (*bc4479*)	This work
pMAD-Δ*clpp1*	Recombinant plasmid for deletion of *clpp1* (*bc2793*)	This work
pMAD-Δ*clpp2*	Recombinant plasmid for deletion of *clpp2* (*bc5152*)	This work
pDG1728	Source of *spnR*	BGSC[b]
pHY300PLK	Source of *tcR*	Takara
yT & A	TA cloning vector, ampR	Yeastern Biotech
pET14b-rsbV-rsbW	Expression vector, native form RsbV and His_6-tagged RsbW, ampR	This work
pET14b-rsbV	Expression vector, His_6-tagged RsbV, ampR	This work
pET14b-rsbY	Expression vector, His_6-tagged RsbY, ampR	This work

[a] ATCC: ATCC Biological Resource Center.
[b] BGSC: Bacillus Genetic Stock Center.

serve as the downstream regulatory module for σ^B. Different gene organizations for the *sigB* operon (cluster) refer to discrete environmental stress receptors and upstream signaling pathways required to propagate the environmental stimuli to reach σ^B in these two closely related species. In *B. subtilis*, RsbS and one or more of the RsbR paralogues (YkoB, YqhA and YojH) form a supramolecular stressosome, which functions as a signaling hub to integrate complicated stress signals, to bind a dissociable positive regulator RsbT and as a kinase for RsbR and RsbS (Chen et al., 2003; Chen et al., 2004; Marles-Wright et al., 2008). Upon physical stress, RsbT is released form the stressosome due to RsbT-dependent phosphorylation of RsbS and subsequently stimulates RsbU through interaction with the N-terminal domain of RsbU (Delumeau et al., 2004). In contrast, RsbQ α/β hydrolase is needed for the activation of RsbP in response to nutritional stress, which coincides with decreased ATP levels (Zhang and Haldenwang, 2005). In *B. cereus*, σ^B activation is accomplished by the coupling of an upstream two-component system (TCS) sensory module to the downstream partner-switching mechanism. TCS consists of a transmembrane hybrid sensor histidine kinase RsbK with multiple functional domains, a cognate response regulator (RR) RsbY and a methyltransferase RsbM (Chen et al., 2012). Upon receiving environmental stimuli, the RsbK initiates autophosphorylation of a conserved histidine residue in the H-box and transfers the phosphate to N-terminus of the CheY response regulator receiver domain (REC) of RsbY by direct phosphoryl transfer or through a more complex, multi-step His-Asp-His-Asp transfer via the histidine containing phosphotransferase (Hpt) protein (Bijlsma and Groisman, 2003; de Been et al., 2010; Hoch and Silhavy, 1995; Stock et al., 2000). RsbY is homologous to RsbU and RsbP in *B. subtilis* (de Been et al., 2010; van Schaik et al., 2005). The phosphorylation of the REC domain activates the C-terminal phosphatase domain of RsbY, which is required to trigger the σ^B-mediated stress response. RsbM has been shown to specifically methylate the S-helix of RsbK in order to negatively regulate the activity of RsbK (Chen et al., 2012). Subdomain interaction between C-terminal REC (receiver) domain and the methylated S-helix in RsbK is critical to regulate histidine kinase activity of RsbK and thereby convey stress signals (Chen et al., 2015). The RsbK-M-Y regulatory module participates in the regulation of σ^B in *B. cereus* as well as in a broad range of biological functions in other microbial phyla (Chen et al., 2012; de Been et al., 2010).

The activation of σ^B of *B. subtilis* during physical stress in the log phase reaches a peak 20–30 min after imposition of the stress, and σ^B levels, thereafter, decline (Boylan et al., 1993; Voelker et al., 1995). The limited scope of the σ^B-mediated stress response is likely associated with stress-induced phosphatase activity of RsbX, which can dephosphorylate RsbS-P and RsbR-P, thereby acting as a negative feedback loop (Voelker et al., 1997; Yang et al., 1996). The feedback circuit to down-regulate the σ^B response after stress is not well understood in *B. cereus*. In a number of stress-induced systems, turnover of the triggering factors serves to limit the response and allow bacteria to resume balanced growth (Gottesman, 2003). It is plausible that similar proteolytic processes contribute to the transient nature of the σ^B response. Among the proteases in *B. subtilis*, ClpP is a particularly interesting candidate as a potential modulator of σ^B activity. ClpP is required for the proper functioning of a number of regulated processes in *B. subtilis*. These processes include competence development, motility, synthesis of degradation enzymes, growth at high temperatures and sporulation (Msadek et al., 1998). Interplay between ClpP and σ^B has been suggested because of the observation that levels of most of the *B. subtilis* proteins induced by stress or starvation, and several σ^B-dependent gene products, are elevated in $ClpP^-$ strains (Reeves et al., 2007). Few observations have shown proteolysis involved in the modulation of σ^B in *B. cereus*.

In this study, we investigated the expression levels during log phase through stationary phase. We examined the molecular size and distribution of RsbV, RsbW, RsbY and σ^B with or without the presence of environmental stress. We also assessed the effect of ADP on kinase activity of RsbW in order to characterize the σ^B partner-switching system. Environmental stress can result in long-lasting σ^B activation throughout the log phase; however, the persistent high levels of σ^B can be significantly down-regulated from the transition stage. σ^B of *B. cereus* was temporally regulated by partner-switching mechanisms and an unknown feedback circuit at a distinct growth stage.

2. Materials and methods

2.1. Bacterial strains and growth

The genotypes and sources of the bacterial strains and plasmids used