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A *Bacillus*-Specific Factor Is Needed to Trigger the Stress-Activated Phosphatase/Kinase Cascade of σ^B Induction

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The general stress regulon of *Bacillus subtilis* is controlled by the transcription factor σ^B. Environmental stress activates σ^B via a phosphatase/kinase cascade that triggers σ^B's release from an anti sigma factor complex. To determine if the members of the phosphatase/kinase cascade are sufficient to detect environmental stress and activate σ^B, we expressed σ^B and its regulators in *E. coli*. In *E. coli*, as in *B. subtilis*, the intact collection of regulators silenced σ^B, while allowing σ^B to be active if the cascade's most upstream negative regulator was deleted. The regulators could not, however, activate σ^B in response to ethanol treatment or heat shock. In other experiments, the GroEL and DnaK chaperones, known to be important in controlling stress σ factors in *E. coli*, were found to be unimportant for σ^B activity in *B. subtilis*. The findings argue that stress induction of σ^B requires novel factors that are *B. subtilis* specific.

σ^B, a general stress response transcription factor of *Bacillus subtilis*, is activated by any of a number of environmental insults (e.g., heat shock, ethanol treatment, acid, salt shock) (1, 2, 3, 4, 5, 6). σ^B is present in the pre-stressed cell, but is held inactive by RsbW, an anti-σ^B protein which blocks σ^B's ability to form an RNA polymerase holoenzyme (E-σ^B) (7, 8). σ^B is released from RsbW, when a second regulatory protein (RsbV) binds to RsbW in lieu of σ^B (8). RsbV, -W and σ^B, as well as five additional σ^B regulatory proteins, are cotranscribed as an eight gene operon from a promoter (P_A) that is recognized by the *B. subtilis* housekeeping σ factor (σ^A) (9). An internal σ^B-dependent promoter (P_B) enhances expression of the downstream four genes during periods of σ^B activity (*i.e.*, $\mathbf{P_A}$ *rsbR -S -T -U* $\mathbf{P_B}$ *rsbV* -W *sigB rsbX*) (1, 2, 10). The mechanism by which the Rsb proteins cooperate to control σ^B activity is illustrated in Fig. 1. During normal growth, RsbV, the effector of σ^B activation, is inactive due to phosphorylation by RsbW (6, 8). Environmental stress initiates a sequence of events in which several of the Rsb proteins interact to reactivate RsbV. In stressed *B. subtilis*, RsbT inactivates its own inhibitor, RsbS, by phosphorylation and activates the RsbV-P phosphatase, RsbU (9). Once activated, RsbU removes the phosphate group from RsbV-P, which then displaces σ^B from RsbW. Negative regulation is reestablished when RsbX, an RsbS-P phosphatase, dephosphorylates RsbS-P (11), enabling RsbS to again bind and inactivate RsbT. There is respectable biochemical and genetic evidence for this model (4, 6, 11, 12, 13, 14, 15, 16, 17); however, the nature of the stress-induced signal that triggers the response and the receptor for this signal are unknown. In *Escherichia coli*, stress-induced changes in protein folding and/or chaperonin activity are thought to control both intercellular and extracytoplasmic stress activated σ factors (*i.e.*, σ^H and σ^E, respectively) (18, 19, 20). It is plausible that stress-induced changes in protein stability and/or chaperone activity could directly alter the Rsb proteins and lead to σ^B activation by similar means.

We chose to examine this possibility by placing the genes for σ^B and its known regulators into *E. coli*. The effects of environmental stress on σ^B activity in this alien host would allow us to determine whether the signals that trigger the Rsb proteins to activate σ^B are *Bacillus*-specific or features of stressed bacteria in general. The GroE chaperone systems of *E. coli* and *B. subtilis* are known to be functionally exchangeable (21). If stressed-induced changes in chaperone activity, protein stability, or a factor common to bacteria communicate directly to σ^B or its principal regulators, we would expect the SigB system to still respond to stress in *E. coli*. Alternatively, if σ^B is not activated by stress

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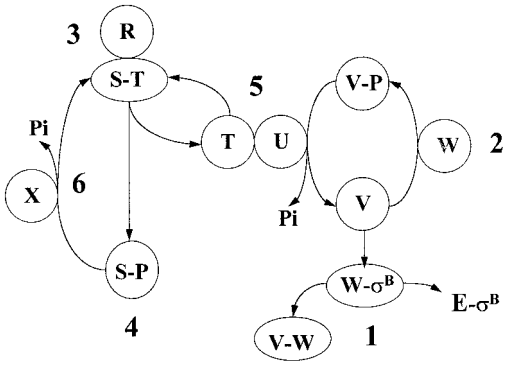

FIG. 1. Stress activation of σ^B. **1 -** The anti-σ^B protein RsbW (W) can form mutually exclusive complexes with either σ^B or its antagonist RsbV (V) (7, 8). When bound to RsbW, σ^B is unable to form an RNA polymerase holoenzyme (E-σ^B) (7). **2 -** In unstressed *B. subtilis*, RsbW phosphorylates and inactivates RsbV (8, 6). **5 -** Environmental stress activates an RsbV-P phosphatase, RsbU (U), which reactivates RsbV. **3 -** The RsbU activator, RsbT (T), is normally inactive due to an association with its negative regulator RsbS (S) (11). RsbR (R), an additional regulatory protein, can bind to RsbS and -T and may play a structural role in the stress pathway (33, 16). When *B. subtilis* is exposed to stress, **4 -** RsbT phosphorylates and inactivates RsbS and activates the RsbU phosphatase (11). **6 -** RsbS is reactivated to bind to RsbT and terminate the reaction by a phosphatase, RsbX (X) (11), whose levels increase with increasing σ^B activity (15).

in *E. coli*, additional factors, unique to *Bacillus*, would be implicated.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Plasmids encoding *sigB* operon products were constructed using PCR and standard cloning techniques. DNA fragments beginning approximately 150bp upstream of the start point of *sigB* operon transcription (22) and ending either at a PstI site in the amino terminal half of *rsbX* or at an SphI site several hundred bp downstream of the *sigB* operon (23) were cloned into pUK19 (24) to create pUKσ^B and pUKσ^Bx, respectively. pUKσ^Bx carries the entire *sigB* operon, including both of the σ^A-and σ^B-dependent promoters (*i.e., P_A rsbR -S -T -U P_B rsbV -W sigB rsbX*), while pUKσ^B is similar but lacks the capacity to synthesize RsbX. In order to create a reporter system for σ^B activity in *E. coli*, a 500bp PstI fragment, containing the promoter element of the σ^B-dependent *ctc* gene (13), was joined to a 3.4 Kbp PstI/BamH1 fragment from pEL-1, which carries the ribosomal binding site of the *B. subtilis sigE* gene fused to the *E. coli lacZ* gene (24). The resulting *ctc::lac* transcriptional fusion was then cloned into pWKS30 (25), an *E. coli* plasmid with a pUC-compatible pSC101 replicon, and transformed into the pUKσ^Bx and pUKσ^B containing strains.

Cultivation and labeling of bacteria. Bacteria were grown in LB (26) at 37°C. Cells were stressed during exponential growth by adding ethanol to a final concentration of 4% or a shift to 48°C. *E. coli* was pulse-labeled in LB with ^{35}S-Met/Cys (1uCi/ml) and analyzed as previously described (1).

General methods. SDS-polyacrylamide gel electrophoresis (PAGE), Western blot analyses, and β-galactosidase assays were performed as previously described (12). All DNA manipulations and transformation of *E. coli* were done according to standard protocols. Transformation of competent *B. subtilis* was carried out as described by Yasbin (27).

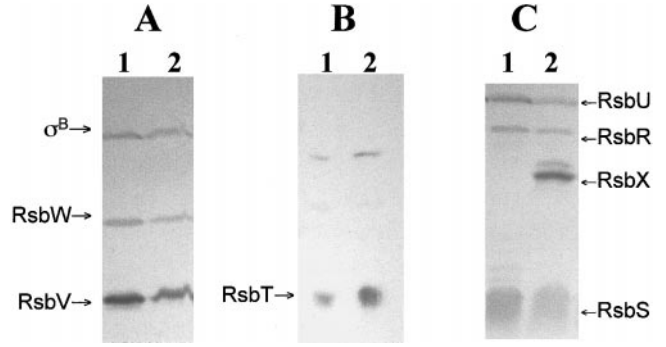

FIG. 2. Western blot analyses of *sigB* operon products expressed in *E. coli*. 200ul samples of *E. coli* strain TG-2 (OD 0.5) carrying either pUKσ^B (lanes 1) or pUKσ^BX (lanes 2), were lysed in SDS sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis (17.5% acrylamide), and analyzed by Western blot using monoclonal antibodies against σ^B, RsbV, -W (A), RsbT (B), or RsbU, -R, -X and -S (C) (12). Bound antibody was visualized with an alkaline phosphatase-conjugated goat immunoglobulin against mouse immunoglobulin (American Qualex) using an alkaline phosphatase substrate kit (Bio-Rad). The bands corresponding to the *sigB* operon products are indicated on the figure.

RESULTS AND DISCUSSION

E. coli (TG-2) (28) carrying plasmids that encoded either the entire *sigB* operon (pUKσ^Bx) or one that lacked *rsbX* (pUKσ^B) were analyzed by Western blot for *sigB* operon products. The P_A promoter of the *sigB* operon (−35 TTGTTT; −10 TATAAT) is very similar to an *E. coli* σ^{70} consensus promoter (9). We therefore anticipated that the operon's products would be expressed in *E. coli*. This is proved to be true. Each of the two plasmid-containing *E. coli* strains accumulated the *sigB* operon products that were encoded on the plasmids (Fig. 2).

RsbX is the most upstream of the negative regulators in the stress activation cascade (Fig. 1). The loss of RsbX causes a dramatic increase in σ^B activity in unstressed *B. subtilis* due to the inability of such strains

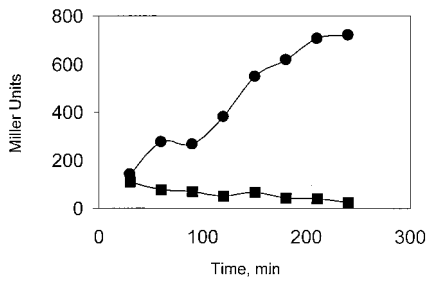

FIG. 3. *ctc::lacZ* expression in *E. coli*. Stationary phase cultures of TG-2, carrying pWKSZ1 (*ctc:lacZ*) and either pUKσ^B (●) or pUKσ^BX (■), were diluted 1:100 into LB medium and incubated with shaking at 37°C. When growth resumed, samples were taken at the indicated time points and analyzed for β-galactosidase synthesis as described previously (34).

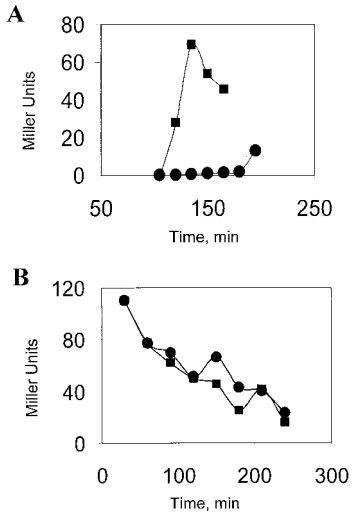

FIG. 4. Effects of ethanol treatment on *ctc::lacZ* expression in *B. subtilis* and *E. coli*. *B. subtilis* strain BSA46 (A) and *E. coli* strain TG-4 (pUKσ^BX, pWKSZ1) (B), growing in LB medium, were treated with ethanol (final concentration 4%) at an early stage in exponential growth (OD_{540} approx. 0.2) (■) or left untreated (●) and analyzed for σ^B-dependent β-galactosidase expression as in Fig. 3.

to reactivate RsbS, the inhibitor of the RsbV-P phosphatase, RsbT/-U (2, 13, 14, 17, 22, 23). If the *sigB* operon products could properly interact with each other in *E. coli*, we anticipated that σ^B activity would be higher in the $RsbX^-$ strain than in the *E. coli* strain that could synthesize RsbX. To assess σ^B activity in these strains, we introduced a σ^B-dependent *lacZ* fusion (pWKSZ1) and assayed the resulting β-galactosidase levels during growth. As expected from the findings in *B. subtilis*, β-galactosidase levels were 20 fold greater in the *E. coli* strain without RsbX than they were in the $RsbX^+$ strain (Fig. 3). This result demonstrates not only that σ^B can direct *E. coli* RNA polymerase to a σ^B-dependent promoter, but also that the Rsb proteins can interact with each other in *E. coli* as they do in *B. subtilis* (*i.e.*, in the presence of RsbX, RsbT/-U is inhibited, RsbV is largely phosphorylated, and σ^B is blocked by RsbW, while in the absence of RsbX, the RsbT/-U phosphatase is free to create sufficient unphosphorylated RsbV to drive the release of at least a portion of the σ^B from RsbW).

The *E. coli* strain that carried the intact *sigB* operon as well as the *ctc::lacZ* fusion and a *B. subtilis* strain with the same reporter gene fusion were exposed to ethanol (4%), an inductant of the σ^B stress response pathway (6, 17). The ethanol treatment resulted in a rapid, 10 fold, increase in σ^B activity in *B. subtilis* (Fig. 4A), but no change in the σ^B activity that was measured in the *E. coli* strain (Fig. 4B). This experiment was repeated using Western blot analyses to follow the abundance of three of the gene products (RsbV, -W and σ^B) that are controlled by the *sigB* operon's σ^B-dependent promoter and, as such, are elevated when σ^B is activated (29). Neither ethanol treatment nor heat shock (48°C), another σ^B activator in *B. subtilis* (1, 3, 5, 17), caused an increase in RsbV, -W or σ^B levels (Fig. 5A2, 3). To verify that the ethanol and heat shock treatments were sufficient to activate stress processes in *E. coli*, samples of the stressed *E. coli* cultures were pulse labeled with ^{35}S-Met/Cys and examined for changes in their protein profiles. When the proteins from the labeled *E. coli* culture were fractionated by SDS-polyacrylamide gel electrophoresis, proteins the size of the *E. coli* stress-induced Lon, DnaK, and GroEL proteins (19) increased in abundance following the stress treatments (Fig. 5B). We conclude that the stress conditions were adequate to activate *E. coli's* stress response, but not sufficient to induce σ^B in the *E. coli* system. Apparently, the known σ^B regulators, although functional in *E. coli*, are inadequate by them-

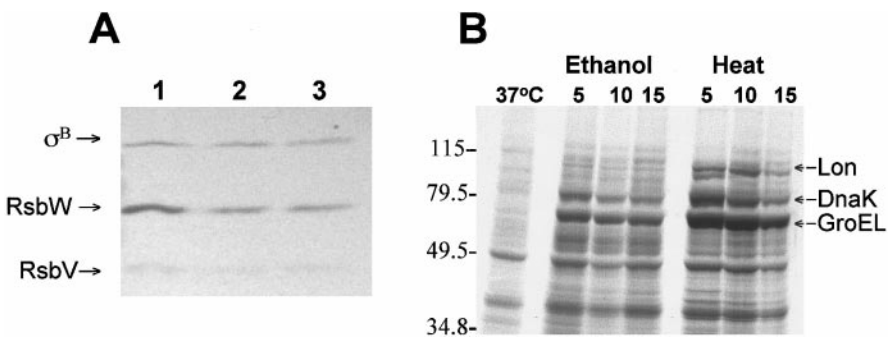

FIG. 5. Ethanol and heat shock treatment of *sigB*-expressing *E. coli*. Portions of a TG-2 (pUKσ^BX) culture grown to OD_{540} 0.3 in LB medium were either held untreated at 37°C (A1, B-37°C), or treated with ethanol to a final concentration of 4% (A2, B-Ethanol), or transferred to 48°C (A3, B-Heat). Samples were harvested at 20 minutes and analyzed by Western blot, as in figure 2 for the accumulation of RsbV, -W and σ^B (A), or pulse-labeled for 5 minutes with ^{35}S-Met/Cys (1 μCi/ml) at 5, 10 and 15 minutes after ethanol or heat shock treatments (B). The pulse labeled cultures were fractionated by SDS-PAGE (5-20% gradient of acrylamide) and analyzed by fluorography as described previously (1). The numbers to the left of the autoradiography depict the migration positions of protein size markers (Bio-Rad Broad Range). The arrows labeled Lon, DnaK and GroEL represent the positions to which *E. coli* proteins with the molecular weights of these stress inducible proteins (19) would migrate in our gel system.

TABLE 1

Strains and Plasmids Used

	Relevant genotype	Construction or source
Bacillus subtilis strains		
PY22	*trpC2*	P. Youngman
BSA46	*trpC2 SPB ctc::lacZ*	23
BT02	*dnaK::cat*	32, W. Schumann
AMX1	P_{xyl}*::groEL*	31, W. Schumann
RS374	*lon::kan*	30, C. Moran
BSJ-1	*trpC2 dnaK::cat*	BT02→PY22
BSJ-2	*trpC2* P_{xyl}*::groEL*	1012→PY22
BSJ-3	*trpC2 dnaK::cat SPB ctc::lacZ*	BSA46→BSJ-1
BSJ-4	*trpC2* P_{xyl}*::groEL SPB ctc::lacZ*	BSA46→BSJ-2
BSJ-7	*trpC2 lon::kan*	RS374→PY22
BSJ-8	*trpC2 lon::kan SPB ctc::lacZ*	BSA46→BSJ-7
Escherichia coli strains		
TG-2		28
Plasmids		
pUK19	*bla kan*	24
pUKσ^B	P_A*rsbRSTUP*$_B$*rsbVWsigB*	This study
pUKσ^BX	P_A*rsbRSTU* P_B*rsbVW sigB rsbX*	This study
pWKS30	pSC101 *ori*	25
pWKSZ1	pSC101*ori ctc::lacZ*	This study

selves to detect environmental stress and activate σ^B in this bacterium.

The chaperone proteins of *B. subtilis* and *E. coli* are functionally exchangeable (21). Thus, we would have expected that if the activation of the σ^B stress regulators was chaperone-mediated, the similarity of the chaperones in these two bacteria should have allowed σ^B induction in *E. coli.* To examine the possibility that σ^B activation is chaperone-mediated but that subtle differences between the *B. subtilis* and *E. coli* chaperone proteins make σ^B's regulation by them *Bacillus*-specific, we assayed σ^B activity in several *B. subtilis* strains with defects in the principal chaperones (GroEL and DnaK), as well as the regulatory protease Lon. The Lon protease was included in this study because it is known to affect the activity of at least one *B. subtilis* σ factor, the sporulation-specific sigma factor, σ^G (30). GroEL, but not DnaK or Lon, is essential for *B. subtilis* viability under normal growth conditions (30, 31, 32). We therefore introduced a conditional mutant of *groEL* or null alleles of the genes for *dnaK* and *lon* into our *B. subtilis* reporter gene strain (BSA46). The *groEL* mutant was constructed by transforming BSA46 with chromosomal DNA which encodes *groEL* under the control of an inducible promoter (P_{xyl}) (Table 1) (31). In the resulting strain, GroEL levels varied in response to xylose in the media. The activity of σ^B was determined, using a *ctc::lacZ* reporter system, during growth in the *dnaK* and *lon* null mutants, and the P_{xyl} *groEL* strain in the presence or absence of xylose. Unlike the circumstance in *E. coli*, where the loss of DnaK leads to a toxic activation of the *E. coli* heat shock σ (σ^H) (18), the loss of DnaK, GroEL, or Lon did not enhance the activity of σ^B (Table 2). It therefore appears that the principal chaperone proteins of *B. subtilis* are unlikely to play a major role in restricting σ^B's activity. This notion is consistent with the observation of Mogk *et al.* (21), that the formation of nonnative proteins in *B. subtilis*, a condition which induced the expression of the chaperone genes *groEL*, *dnaK*, did not activate the σ^B-dependent regulon.

Our data imply that the known σ^B regulators, *i.e.*, the Rsb proteins encoded by the *sigB* operon, are in-

TABLE 2

Effect of DnaK, Lon, and GroEL Mutants on σ^B Activity

Strain	Average β-galactosidase activity (Miller Units)[a]
BSA46 (wildtype)[b]	3.76
BSJ-3 (*dnaK::cat*)[b]	2.74
BSJ-8 (*lon::kan*)[b]	4.1
BSJ-4 (P_{xyl}*::groEL*) with xylose[c]	1.70
BSJ-4 (P_{xyl}*::groEL*) without xylose[c]	1.47

[a] β-Galactosidase activity assays are presented in Miller units (26). The values represent the average of 7 samples collected from each of the strains.

[b] Cells were diluted 1:100 from an overnight culture into LB. Samples were collected every 15 minutes throughout log phase (OD_{540} 0.15-0.8) and stored at −20°C until use.

[c] Cells were grown overnight in LB supplemented with xylose (0.5%), washed with LB and diluted 1:100 into LB with and without xylose (0.5%). Cells grown with xylose were harvested every 15 minutes over log phase (OD_{540} 0.15-0.8). Cells grown in the absence of xylose were collected every 30 minutes for 3 hours as growth ceased due to depletion of GroEL and kept at −20°C until use.

sufficient to directly detect environmental stress and activate σ^B. In addition, the principal chaperone proteins, implicated in controlling stress-induced σs in *E. coli*, are unlikely to play similar roles in *B. subtilis* σ^B regulation. Although it is possible that stress generates a unique internal environment in *B. subtilis*, which activates the Rsb protein cascade, we consider it more likely that, σ^B induction requires novel *Bacillus*-specific effectors to sense environmental stress and convey its presence to the σ^B regulators. We are currently using genetic techniques to search for these putative signalers.

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