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Stress Activation of *Bacillus subtilis* σ^B Can Occur in the Absence of the σ^B Negative Regulator RsbX

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Environmental stress activates σ^B, the general stress response σ factor of *Bacillus subtilis*, by a pathway that is negatively controlled by the RsbX protein. To determine whether stress activation of σ^B occurs by a direct effect of stress on RsbX, we constructed *B. subtilis* strains which synthesized various amounts of RsbX or lacked RsbX entirely and subjected these strains to ethanol stress. Based on the induction of a σ^B-dependent promoter, stress activation of σ^B can occur in the absence of RsbX. Higher levels of RsbX failed to detectably influence stress induction, but reduced levels of RsbX resulted in greater and longer-lived σ^B activation. The data suggest that RsbX is not a direct participant in the σ^B stress induction process but rather serves as a device to limit the magnitude of the stress response.

σ^B, the general stress response σ factor of *Bacillus subtilis* (20), is activated by a drop in intracellular ATP levels (1, 22) or by any of a number of diverse environmental insults (e.g., heat shock, ethanol treatment, or salt shocks) (5, 7, 22, 23). σ^B is held inactive by a binding protein (RsbW) which can form a complex with either σ^B or an alternative target (RsbV) (6, 8, 9). RsbW is also a protein kinase which can phosphorylate RsbV and convert it into a form (RsbV-P) that no longer binds RsbW (8). The relatively high intracellular ATP levels that occur during growth favor the phosphorylation of RsbV, the formation of RsbW-σ^B complexes, and the inhibition of σ^B-dependent transcription (1, 22). Upon entry into stationary phase, a drop in intracellular ATP levels leads to ineffective phosphorylation of RsbV, the formation of RsbV-RsbW complexes, and the release of σ^B (1, 22). Environmental stress activation of σ^B occurs irrespective of intracellular ATP levels and the activity of the RsbW kinase (22). Instead, it involves the reactivation of RsbV-P by an RsbV-P-specific phosphatase (21). The existence of such a phosphatase was anticipated following the discovery that a phosphatase (SpoIIE) is involved in the reactivation of the phosphorylated form of the RsbV homolog (SpoIIAA) in the σ^F system (2, 10). RsbV-P phosphatases participate in both stress-induced and stationary-phase activation of σ^B (21). Phosphatase activity is essential for stress activation of σ^B; however, it merely enhances the stationary-phase response (21). Both the stationary-phase- and stress-induced dephosphorylation reactions require one or more of the products of the σ^B regulators RsbR, RsbS, and RsbT (13, 21, 23). The RsbU protein is also needed for stress-dependent, but not stationary-phase-dependent, RsbV-P dephosphorylation (21). Recent experiments have shown that RsbU can dephosphorylate RsbV-P in vitro and that this activity is enhanced by RsbT (15, 24). It is likely that RsbU is a stress-activated phosphatase with RsbT providing the activation signal (24). The stationary-phase-specific phosphatase is unknown.

An additional protein (RsbX) is a negative regulator of RsbU-dependent RsbV-P dephosphorylation. *B. subtilis* strains with null mutations in RsbX have high, growth-inhibiting levels of active σ^B (3, 7, 11, 12). The growth inhibition due to loss of RsbX can be suppressed by mutations in *sigB* (the σ^B structural gene), *rsbV*, or either of the two genes (*rsbT* and *rsbU*) known to be needed for the stress-induced phosphatase activity (3, 7, 11, 13, 16, 18). Given that RsbX is a negative regulator in the stress-induced phosphatase pathway, it seemed possible that stress activation could occur through a release from the negative regulation of RsbX. To test this possibility, we constructed strains in which the absence of RsbX is not lethal and examined the effects of RsbX levels on σ^B activity and the stress inducibility of σ^B.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation of bacteria. The *B. subtilis* strains and the plasmids used in this study are listed in Table 1. All BSA strains are derivatives of PY22, which was obtained from P. Youngman (University of Georgia). *B. subtilis* strains were routinely grown at 37°C with vigorous agitation in Luria broth (LB) (17). Bacteria were exposed to ethanol stress during exponential growth by adding ethanol to a final concentration of 4% (vol/vol). *Escherichia coli* TG2 and GM2163 (17) were used as hosts for cloning.

Construction of a strain with *rsbX* expressed from P_{SPAC}. Plasmid pUC19X (Table 1) was cut with *Bam*HI and then partially digested with *Hin*dIII. An 856-bp fragment containing *rsbX* was cloned in pHV501 that had been previously digested with the same enzymes. After integration into the *B. subtilis* chromosome, the resulting plasmid, pHV501X, permits the expression of *rsbX* from the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter P_{SPAC}. A suitable recipient strain for the recombination of pHV501X at a site other than the *sigB* operon was obtained by transformation of PY22 with plasmid pFP1. pFP1 is a 0.42-kbp *Hin*dIII fragment containing the *dacF* promoter cloned into *Hin*dIII-cut pUS19. The integration of pFP1 into *dacF* to create strain BSA334 was verified by PCR. BSA334 was transformed with pHV501X into BSA335. Integration of pHV501X into the pFP1 sequences was confirmed by the cotransformability of both plasmid antibiotic resistances (erythromycin and spectinomycin). A translational fusion between the σ^B-dependent *ctc* gene and *lacZ* was introduced into BSA335 by transduction with a SPβ-based specialized transducing phage (11), yielding BSA336. Finally, the wild-type *rsbX* gene in the *sigB* operon of BSA336 was inactivated by transformation with *Eco*RI-linearized pAK24, which carries an *rsbX* gene disrupted by the insertion of a kanamycin resistance cassette. In the resulting strain (BSA337), *rsbX* is expressed only from P_{SPAC}.

To maximize RsbX expression in *B. subtilis*, *rsbX* was also cloned downstream of P_{SPAC} into multicopy shuttle vector pDG28. An 856-bp *Sal*I-*Xba*I fragment from pAL365 was cloned into pDG28 that had been digested with the same enzymes. The resulting plasmid, pDG28X, was introduced into PY22 via transformation and selection for erythromycin resistance. One of the transformants (BSA359) was transduced with the *ctc-lacZ* SPβ transducing phage to allow monitoring of σ^B activity.

Selection of a mutation in *rsbT* which suppressed the loss of RsbX. Disruption of *rsbX* by integration of a spectinomycin resistance cassette following transfor-

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TABLE 1. *B. subtilis* strains and plasmids used in this study

Strain or plasmid	Relevant genotype or plasmid feature(s)	Construction, source, or reference[a]
Strains		
PY22	*trpC2*	P. Youngman[b]
BSA46	*trpC2* SPβ *ctc*::*lacZ*	3
BSA272	*trpC2 sigB*::*Hin*dIII-*Eco*RV::*cat*	22
BSA334	*trpC2 dacF*::pFP1	pFP1→PY22
BSA335	*trpC2 dacF*::pFP1::pHV501X	pHV501X→BSA334
BSA336	*trpC2 dacF*::pFP1::pHV501X SPβ *ctc*::*lacZ*	SPβ *ctc*::*lacZ* BSA335
BSA337	*trpC2 dacF*::pFP1::pHV501X *rsbX*::*kan* SPβ *ctc*::*lacZ*	pAK24→BSA336
BSA348	*trpC2 rsbT151S rsbX*::*spc* SPβ *ctc*::*lacZ*	This study
BSA354	*trpC2 sigB*::*Hin*dIII-*Eco*RV::*cat* SPβ *ctc*::*lacZ* pDG1481	pDG1481→BSA358
BSA358	*trpC2 sigB*::*Hin*dIII-*Eco*RV::*cat* SPβ *ctc*::*lacZ*	SPβ *ctc*::*lacZ** BSA272
BSA359	*trpC2* pDG28X	pDG28X→PY22
BSA360	*trpC2* SPβ *ctc*::*lacZ* pDG28X	SPβ *ctc*::*lacZ** BSA359
Plasmids		
pAK24	Ap^r *rsbX*::*kan*	19
pAL365	Ap^r 856-bp *Hin*cII fragment with *rsbX* in pBluescript KS	This study
pDG1481	Ap^r Km^r *oriBS*[c] P_{SPAC}::*sigB lacI*	P. Stragier[d]
pDG28	Ap^r Em^r *oriBS* P_{SPAC} *lacI*	P. Stragier
pDG28X	Ap^r Em^r *oriBS* P_{SPAC}::*rsbX lacI*	This study
pFP1	Ap^r Spc^r *dacF* in pUS19	J. Ju[e]
pHV501	Ap^r Em^r P_{SPAC}[f] *lacI*	S. D. Ehrlich[g]
pHV501X	Ap^r Em^r P_{SPAC}::*rsbX lacI*	This study
pML7/X::Spc	Ap^r Spc^r *rsbX*::*spc*	4
pUS19	Ap^r Spc^r	4
pUC19X	Ap^r Spc^r 856-bp *Hin*cII fragment with *rsbX* in pUC19	This study

[a] An arrow indicates construction of the strain by transformation, and an asterisk indicates construction of the corresponding strain by transduction with an SPβ-based specialized transducing phage.
[b] University of Georgia.
[c] *oriBS* indicates that the plasmid is able to replicate in *B. subtilis*.
[d] Institut de Biologie Physico-Chimie, Paris, France.
[e] University of Texas Health Science Center at San Antonio.
[f] This plasmid allows expression of target genes from IPTG-inducible promoter P_{SPAC}.
[g] Institut National de la Recherch Agronomique, Jouy-en-Josas, France.

mation of wild-type *B. subtilis* (BSA46) with linearized plasmid pML7/X::Spc triggers hyperactivation of σ^B. This results in growth inhibition and the formation of pinpoint, dark blue colonies on media containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (22). Spontaneous suppressor mutants, arising as large, light blue colonies, were analyzed (18). One suppressor clone (BSA348) was found by Western blot analysis to contain low levels of RsbT. Sequencing of the region coding for RsbT revealed a T-to-G base substitution in *rsbT* which changed the isoleucine at position 15 of RsbT to serine (18).

General methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blot analysis, and β-galactosidase assays were performed as previously described (14, 22). All DNA manipulations and the transformation of *E. coli* were done in accordance with standard protocols (16). Transformation of natural competent *B. subtilis* cells with plasmid and chromosomal DNAs was carried out as described by Yasbin et al. (25), and transformants were selected on LB agar plates containing kanamycin at 10 μg/ml, spectinomycin at 200 μg/ml, erythromycin at 1 μg/ml, or chloramphenicol at 5 μg/ml.

RESULTS AND DISCUSSION

A *B. subtilis* strain (BSA337; Table 1) carrying an antibiotic resistance gene in *rsbX* and a second copy of *rsbX* under the control of P_{SPAC} was grown with or without IPTG and exposed to ethanol stress. The amount of IPTG present in the media influenced the level of RsbX (Fig. 1B). In the absence of IPTG, RsbX was barely detectable (Fig. 1B, lane 1), while at 1 mM IPTG, RsbX was at least as abundant as in wild-type *B. subtilis* during growth (Fig. 1B, lanes 3 and 4). Although the levels of RsbX were significantly different in some of these cultures, the σ^B activities under unstressed conditions were similar. This is illustrated by both the activity of the *ctc* promoter (Fig. 1A) and the σ^B-dependent accumulation of the *sigB* operon's RsbV, RsbW, and σ^B (Fig. 1B, lanes 1 to 4). Apparently, low levels of RsbX are sufficient to keep RsbU-dependent phosphatase activity contained in the absence of stress. The differences in RsbX levels did, however, influence the degree of σ^B activation following stress. This is seen in the inverse relationship between the amount of RsbX present and the activity of P_{ctc} after ethanol treatment (Fig. 1A). The lowest *ctc* induction occurred in the wild-type strain, where the stress-activated *sigB* operon promoter (Fig. 1B, lane 8) generated more RsbX than did the most fully induced *SPAC* promoter (Fig. 1B, lane 7). Both the level of σ^B activation and the profile of its persistence appeared to be affected by RsbX abundance. The level of σ^B-dependent reporter gene activity in the wild-type strain reached a maximum approximately 20 min after ethanol exposure and then slowly decreased, while β-galactosidase levels persisted or continued to increase in the cultures with lower levels of RsbX (Fig. 1A).

Given that low levels of RsbX allowed greater induction of σ^B activity by stress, we investigated whether higher RsbX levels during growth would restrict σ^B induction. We transformed *B. subtilis* with a replicating plasmid (pDG28X) that carries *rsbX* under the control of P_{SPAC} (Table 1). When grown in the presence of IPTG, the resulting strain (BSA360; Table 1) had approximately five times the amount of RsbX detectable in the parental strain (Fig. 2, lane 3 versus lane 1). This additional amount of RsbX was insufficient to limit the activation of σ^B by ethanol treatment. Stress-induced *ctc*::*lacZ* expression was similar in a wild-type strain and one preloaded with RsbX (Fig. 2). Although pDG28X could markedly enhance the amount of RsbX in unstressed cells, it did not sig-

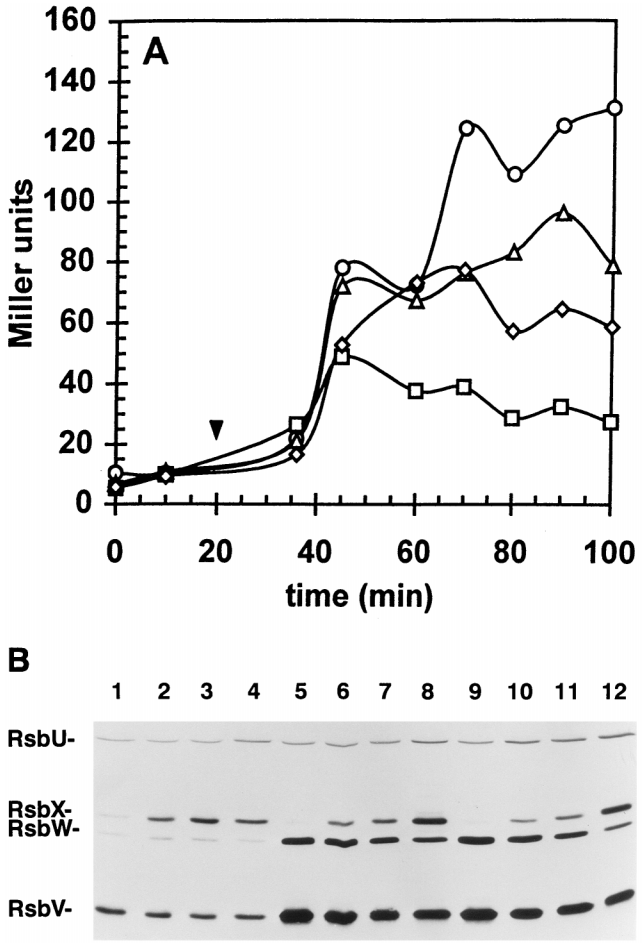

FIG. 1. Effect of reduced levels of RsbX on the stress activation of σ^B. *B. subtilis* BSA46 (wild type) and BSA337 (P_{SPAC} *rsbX*) were cultivated in LB. BSA337, grown in the presence of 1 mM IPTG, was diluted into fresh, prewarmed LB containing different concentrations of IPTG. (A) Induction of *ctc*::*lacZ* by ethanol stress. At the time indicated by the arrowhead, cells were exposed to 4% (final concentration) ethanol. β-Galactosidase activities were determined as described in Materials and Methods. Symbols: □, BSA46; ◇, BSA337 plus 1.0 mM IPTG; △, BSA337 plus 0.1 mM IPTG; ○, BSA337 with no IPTG. (B) Western blot analysis of RsbU, RsbV, RsbW, and RsbX. Samples were taken prior to (lanes 1 to 4) and 25 (lanes 5 to 8) or 80 (lanes 9 to 12) min after treatment with ethanol. Crude protein extracts (75 μg) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting with RsbU-, RsbV-, RsbW-, and RsbX-specific antibodies. Lanes: 1, 5, and 9, BSA337 with no IPTG; 2, 6, and 10, BSA337 plus 0.1 mM IPTG; 3, 7, and 11, BSA337 plus 1 mM IPTG; 4, 8, and 12, BSA46.

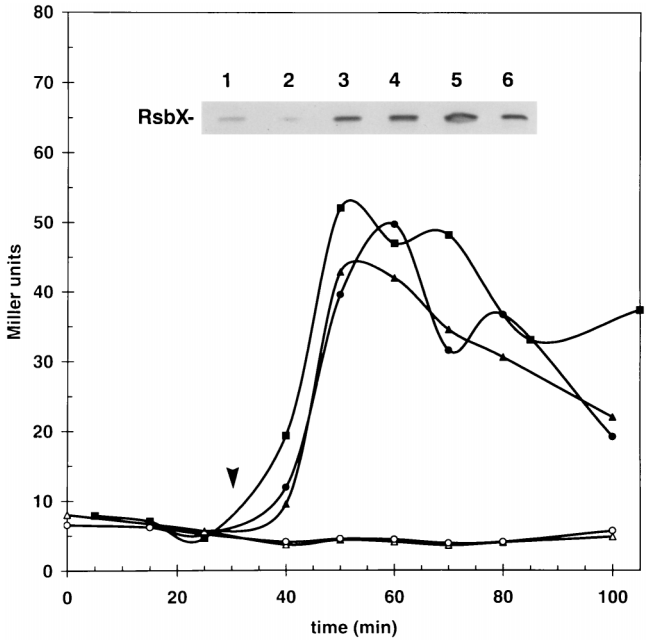

FIG. 2. Stress activation of σ^B in cells preloaded with RsbX. *B. subtilis* BSA46 (wild type) or BSA360 (RsbX$^+$ P_{SPAC} *rsbX*) was grown in LB with or without IPTG and subjected to ethanol stress (4% final concentration) at the time indicated by the arrowhead. Samples were taken and analyzed for β-galactosidase as described in the legend to Fig. 1. Open symbols represent the control, and filled symbols depict stressed samples. The levels of RsbX (inset) were visualized by Western blot analysis using an RsbX-specific monoclonal antibody. Samples for Western blot analysis were taken immediately before (lanes 1 to 3) or 25 min after exposure to ethanol stress. BSA46 (□), lanes 1 and 4; BSA360 with no IPTG (○), lanes 2 and 5; BSA360 with 1 mM IPTG (△), lanes 3 and 6.

nificantly contribute to the amount of RsbX that ultimately accumulated once the cultures were exposed to ethanol. Both the pDG28X-containing strain and its parent had similar levels of RsbX following stress (Fig. 2, lanes 3, 4, and 5). Apparently, the *sigB* operon's σ^B-dependent promoter becomes sufficiently active under this condition to become the cell's principal source of RsbX. The comparable prestress σ^B activities in this (Fig. 2) and our previous experiment (Fig. 1) show that relatively low levels of RsbX are sufficient to silence the stress activation pathway in the absence of an induction signal. Within the limits of the amounts of RsbX synthesized by the strains used in our study, our data suggest that preinduction levels of RsbX have little effect on σ^B activation by stress but that the levels of RsbX present after induction influence the magnitude and persistence of the activation response. This can be interpreted as evidence that RsbX acts to limit the extent of the activation response rather than play a direct role in the induction process itself.

To test whether stress activation of σ^B can occur in the absence of RsbX, we constructed a strain in which σ^B synthesis is divorced from σ^B activity. In wild-type *B. subtilis*, σ^B is autoregulated with active σ^B driving the transcription of its own operon (12). In the absence of RsbX, this results in the accumulation of lethal levels of active σ^B (3, 12). The test strain (BSA354; Table 1) contains an antibiotic resistance gene within the *sigB* structural gene which results in loss of both σ^B and RsbX, the downstream gene product. In addition, BSA354 carries a replicating plasmid with a copy of *sigB* under P_{SPAC} control. When grown in the presence of IPTG, BSA354 displays high levels of σ^B activity (Fig. 3). Presumably, this is due to the uncoupling of σ^B synthesis from that of its regulators and a higher level of free σ^B. Although already high, this level of σ^B activity rises even further when ethanol is added to the culture (Fig. 3). Although the disruption of the *sigB* operon by placement of the antibiotic resistance gene in *sigB* results in the loss of both detectable σ^B and RsbX in Western blot analyses, it is formally possible that a small amount of RsbX persists to respond to stress. We therefore constructed a strain in which a second antibiotic resistance gene was placed into *rsbX* in BSA354. This *sigB*::*cat rsbX*::*spc* P_{SPAC} *sigB* strain behaved as did BSA354 in inducing σ^B activity in response to stress (data not shown). We conclude that at least some stress-induced σ^B activation can occur in the absence of RsbX.

Further support for the idea that stress activation of σ^B does not involve RsbX comes from experiments with a mutant *B. subtilis* strain that had been isolated on the basis of suppression

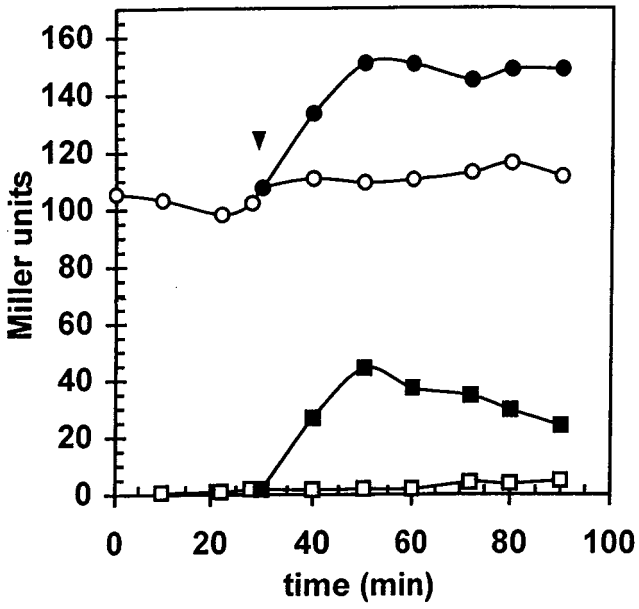

FIG. 3. Activation of σ^B by ethanol stress in the absence of RsbX. *B. subtilis* BSA46 (wild type) and BSA354 (*sigB*::*Hin*dIII-*Eco*RV::*cat* P_{SPAC} *sigB*) were cultivated in LB with or without IPTG and exposed to 4% (final concentration) ethanol at the time indicated by the arrowhead. Samples were taken at the times indicated and analyzed for β-galactosidase as described in the legend to Fig. 1. Open symbols represent the control, and filled symbols depict the culture exposed to 4% ethanol. Symbols: □, BSA46; ○, BSA354 plus 1 mM IPTG.

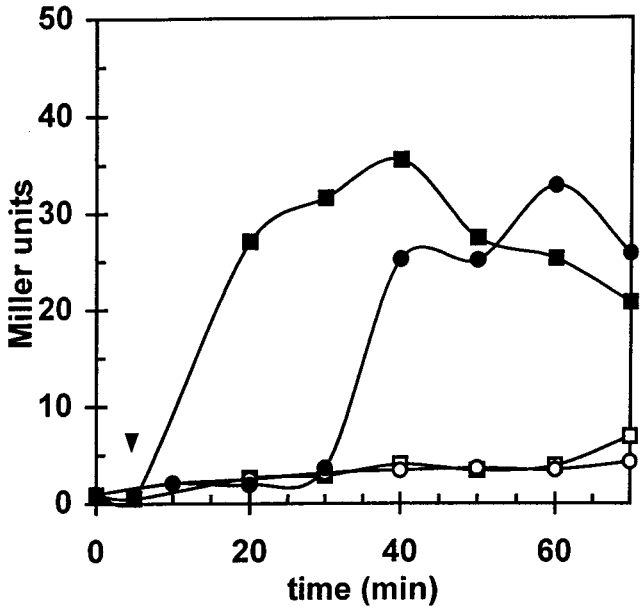

FIG. 4. Induction of *ctc*::*lacZ* by ethanol stress in an RsbX$^-$ suppressor strain of *B. subtilis*. *B. subtilis* BSA46 (wild type) and BSA348 (*rsbX*::*spc rsbT15IS*) were grown in LB and exposed to 4% (final concentration) ethanol at the time indicated by the arrowhead. Samples were taken at the times indicated and analyzed for β-galactosidase as described in the legend to Fig. 1. Open symbols represent the untreated control, and filled symbols depict the stressed cultures. Symbols: □, BSA46; ○, BSA348.

of the growth inhibition normally associated with the loss of RsbX (18). This strain (BSA348; Table 1) has an antibiotic resistance gene (*spc*) inserted into *rsbX* and a compensating mutation in *rsbT* (*rsbT15IS*) which permits the strain to grow at near normal levels in the absence of RsbX. The mutant RsbT may be less able than its wild-type counterpart to activate the RsbU phosphatase. When BSA348 is exposed to ethanol, σ^B activation occurs, although it is delayed relative to that seen in a wild-type strain (Fig. 4). Thus, RsbX is not essential for stress-dependent σ^B activation in this strain.

The observations that stress induction of σ^B can occur in the absence of RsbX and that the degree of σ^B activation is greater when RsbX levels are low suggest that RsbX is involved in limiting the magnitude of stress-dependent σ^B activation rather than as a direct participant in the activation process (e.g., a target for stress induction). This notion is consistent with the profile of RsbX synthesis. Unlike the other regulators (*rsbR*, *-S*, and *-T*) of the stress activation pathway, *rsbX* is not constitutively expressed but is, instead, encoded within part of the *sigB* operon whose transcription is enhanced by a σ^B-dependent promoter (4, 9, 23). Thus, RsbX abundance rises following σ^B activation. This could provide the stressed cell with high levels of RsbX to modulate further σ^B activation. A mechanism by which this could occur is suggested by biochemical experiments in the Price laboratory (15, 24) that showed that RsbX has a phosphatase activity which can dephosphorylate RsbS in vitro. RsbS was previously shown to be a negative regulator of RsbT, a protein needed to activate the stress-inducible phosphatase (RsbU) that ultimately frees σ^B (13). Presumably, this RsbX-dependent dephosphorylation of RsbS could allow it to inactivate RsbT. It will be interesting to learn whether the poststress levels of RsbX are, in themselves, sufficient to inhibit σ^B or whether additional controls are placed on RsbX's activity.

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