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Stress Triggers a Process That Limits Activation of the *Bacillus subtilis* Stress Transcription Factor σ^B

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Stress-induced activation of the *Bacillus subtilis* transcription factor σ^B is transitory. To determine whether the process that limits σ^B activation is itself triggered by stress, *B. subtilis* strains in which the stress pathway was artificially activated by the induced expression of a positive regulatory protein (RsbT) were exposed to ethanol stress and were monitored for the persistence of σ^B activity. Without ethanol treatment, the induced cultures displayed continuously high σ^B activity. Ethanol treatment restricted ongoing σ^B activity, but only in strains with intact *rsbX* and *-S* genes. The loss of other gene products (RsbR and Obg) known to participate in the stress activation pathway had little influence in blocking the ethanol effect. The data argue that stress upregulates the activity of the RsbX-S regulatory pair to restrict σ^B induction following stress.

σ^B is a transcription factor that controls the general stress regulon of *Bacillus subtilis*, a collection of genes whose products aid the bacterium in surviving any of a number of environmental traumas (10, 21, 23). Induction of the σ^B regulon occurs by the activation of σ^B itself, a process that is triggered by entry of *B. subtilis* into the stationary phase of growth or by the onset of environmental stress (e.g., heat, salt, acid, or ethanol) (12, 24, 25). A current model for σ^B regulation is depicted in Fig. 1. σ^B is present, but inactive, in the prestressed cell due to an association with the anti-σ^B protein RsbW. σ^B release from RsbW is effected by an additional protein (RsbV) which binds to RsbW in lieu of σ^B (4, 5, 7). The abundance of active RsbV determines the level of free σ^B (24). In unstressed cells, RsbV is largely inactive due to RsbW-catalyzed phosphorylation (7, 25). When *B. subtilis* enters stationary phase, unphosphorylated RsbV accumulates, likely due to the effects of an RsbV-P phosphatase (YvfP) as well as inefficient phosphorylation under the stationary-phase condition of low ATP (2, 24; K. Vijay, M. S. Brody, E. Fredlund, and C. W. Price, submitted for publication). As a result, RsbV is available to displace σ^B from the RsbW-σ^B complex and to induce the σ^B regulon. Environmental stress also activates rsbV, but does so using a separate collection of Rsb proteins (1, 6, 8, 12, 25, 27, 28). RsbT is the most upstream effector in this pathway (28). Following exposure to stress, RsbT, normally inactive and complexed to RsbS, phosphorylates RsbS and becomes free to activate the stress-specific RsbV-P phosphatase, RsbU (28). RsbU can then activate RsbV. Obg, a GTP binding protein (14, 19, 26), is also needed for stress triggering of σ^B activity; however, its explicit role in this process is unknown (17). Negative regulation is reestablished when RsbX, a RsbS-P phosphatase, dephosphorylates RsbS-P, thereby enabling RsbS to again inactivate RsbT (28).

The genes for σ^B and seven of its regulators (RsbR, -S, -T, -U, -V, -W, and -X) are cotranscribed as an eight-gene operon from a promoter (P_A) that is recognized by the *B. subtilis* housekeeping σ factor (σ^A) (11, 27). An internal σ^B-dependent promoter (P_B) enhances the expression of the four downstream genes when σ^B is active (11). Thus, the levels of σ^B, its principal regulators (RsbV and -W), and the RsbX phosphatase are elevated following activation of σ^B.

σ^B-dependent transcription is only transiently activated by stress (18, 25), declining by 20 to 30 min after its initial induction (Fig. 2). The observation that RsbX, the most upstream negative regulator of the stress pathway, is expressed at higher levels when σ^B becomes active suggested that the transience of the σ^B stress response could be attributed to an effect of elevated RsbX protein levels on the phosphorylation state of RsbS (28). Although the persistence of σ^B activity following stress induction of mutant strains lacking RsbX indicated that RsbX had a role in this process, manipulating RsbX levels by its expression from inducible promoters failed to show a cred-

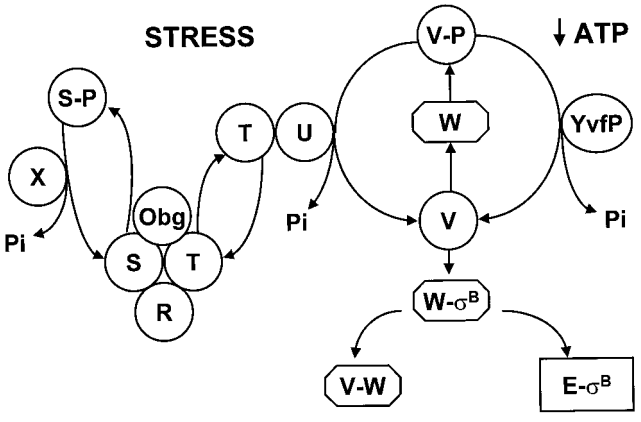

FIG. 1. Model of σ^B regulation. Active σ^B holoenzyme (E-σ^B) forms when the RsbV protein (V) binds to the anti-σ^B protein RsbW (W) to free σ^B (2, 7). RsbV is normally inactive (V-P) due to phosphorylation by RsbW but is reactivated by stationary-phase or stress-activated phosphatases, YvfP and RsbU (U), respectively (7, 20, 24, 28; Vijay et al., submitted). The stress phosphatase RsbU is activated by RsbT (T) (28). RsbT is normally inactive due to an association with its negative regulator RsbS (S). Upon exposure to stress, RsbT phosphorylates and inactivates RsbS (S-P) and activates RsbU (28). RsbR (R) is believed to facilitate the RsbT-RsbS interaction (1, 9). Obg, a GTP binding protein, is necessary for stress activation of RsbT, but its role is unknown (17). Negative control is resumed when RsbX (X), a phosphatase, dephosphorylates and reactivates the RsbS phosphate (28).

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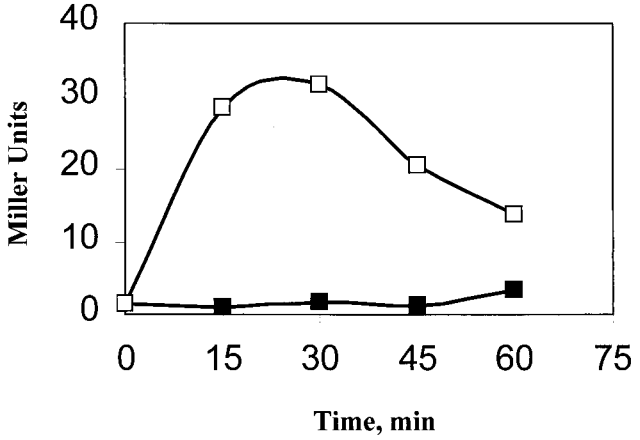

FIG. 2. Ethanol induction of σ^B in wild-type *B. subtilis*. BSA46 (*ctc*::*lacZ*) was grown in Luria broth (LB) (16) at 37°C. At an optical density at 540 nm (OD_{540}) of 0.15, ethanol (4% vol/vol) was added to half of the culture (0 time). Samples were taken at 15-min intervals and were analyzed for β-galactosidase (13). The data is given in Miller units (15).

ible correlation between the absolute levels of RsbX and the degree of σ^B activation in stressed cells (22). These results suggested that the RsbX protein was necessary, but not sufficient, to limit the induction of σ^B following stress.

To further investigate the mechanism responsible for the transience of the stress induction of σ^B, we sought to separate the effect of stress in triggering the pathway from its possible effect in limiting the duration of the response. To accomplish this, we took advantage of the finding that the enhanced synthesis of RsbT, relative to its negative regulator, RsbS, is sufficient to induce the σ^B stress pathway in the absence of stress (17, 28). This allowed us to artificially activate the pathway and then test the effects of stress and the need for particular *rsb* gene products on the duration of the response.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Construction[a] or source
Bacillus strains		
PY22	*trpC2*	P. Youngman
BSA46	*trpC2* SPβ *ctc*::*lacZ*	3
XS352	*trpC2 aph3′5″*/*sigB rsbST*	18
BSA419	*trpC2* P_{spac}::*rsbT* SPβ *ctc*::*lacZ*	17
BSJ13	*trpC2* P_{spac}::*rsbT* P_{xyl}::*obg* SPβ *ctc*::*lacZ*	17
BSJ38	*trpC2* P_{spac}::*rsbT rsbX*::*spec* SPβ *ctc*::*lacZ*	BSA625 → BSA419
BSJ39	*trpC2 aph3′5″*/*sigB* Δ*rsbST*	XS352 → PY22
BSJ40	*trpC2 aph3′5″*/*sigB* Δ*rsbST* P_{spac}::*rsbT* SPβ *ctc*::*lacZ*	pHV501T → BSJ39
BSJ41	*trpC2 aph3′5″*/*sigB* Δ*rsbST* P_{spac}::*rsbT* SPβ *ctc*::*lacZ*	BSA46 → BSJ40
BSJ42	*trpC2 aph3′5″*/*sigB rsbR*Δ*5* P_{spac}::*rsbT* SPβ *ctc*::*lacZ*	pJM49 → BSA419
Plasmids		
pHV501T	Ap^r Em^r P_{spac}::*rsbT*	17
pRS11	Ap^r Kan^r P_A::*rsbR rsbS*	18
pJM49	Ap^r Kan^r P_A::*rsbR*Δ*5 rsbS*	This study
pML7/X::spec	Ap^r Cm^r Sp^r P_B*rsbVW sigB rsbX*::*spec*	4

[a] Transformations were performed as described by Yasbin et al. (29).

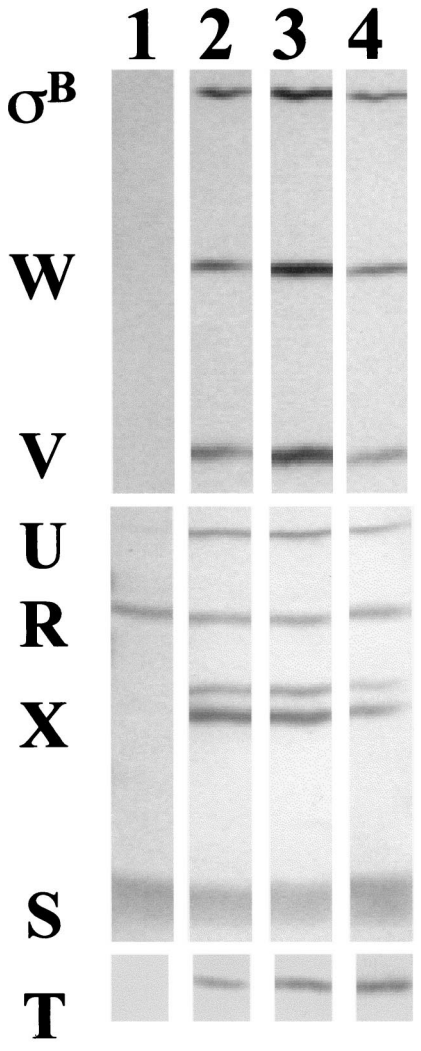

FIG. 3. Western blot analysis of BSA419 after treatment with ethanol. Cells were grown as described in the legend to Fig. 4, with samples harvested 30 min after induction by pouring over ice chips. Following centrifugation, the cells were resuspended in buffer (50 mM Tris-HCl [pH 8.0], 0.1 mM ETDA, 0.03% phenylmethylsulfonyl fluoride) and were disrupted by passage through a French press. The extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were transferred to nitrocellulose, and were probed by Western blotting by using monoclonal antibodies raised against RsbV, -W, -X, -R, -S, -T, and -U and σ^B (8). The anti-RsbX antibody detects doublet bands of unknown significance (24). Lane 1, cells immediately before addition of IPTG; lane 2, 30 min after IPTG induction; lane 3, 90 min after addition of IPTG, without ethanol treatment; lane 4, 90 min after addition of IPTG, with ethanol treatment (60 min).

We used a *B. subtilis* strain (BSA419), in which a P_{spac}::*rsbT* fusion plasmid (pHV501T) had entered the chromosome by a single-site recombination event at *rsbT* (Table 1). BSA419 contains a *sigB* operon in which *rsbR*, *-S*, and *-T* are expressed from the P_A promoter and a second copy of *rsbT* and the remaining downstream *sigB* genes, separated from P_A by the plasmid sequences, are expressed under the control of the inducible *spac* promoter (17). When P_{spac} is not induced, only RsbR and -S are evident in Western blots (Fig. 3, lane 1). *rsbT* is also expressed, but is difficult to detect in unstressed cells by Western blotting (8). Induction of P_{spac} with isopropyl-β-D-thiogalactopyranoside (IPTG) yields the anticipated increase in the

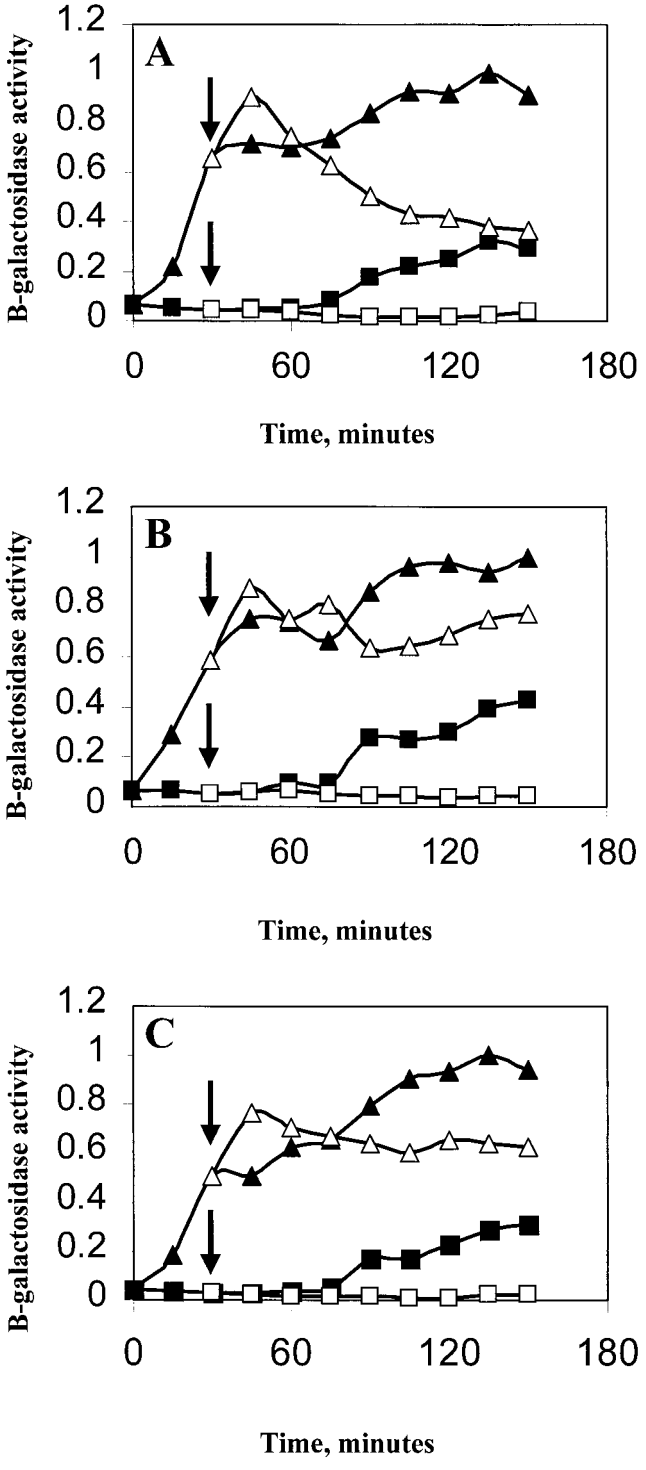

FIG. 4. Effect of ethanol on σ^B induction in P_{spac}::*rsbT* strains. *B. subtilis* strains carrying Spβ *ctc*::*lacZ* were grown at 37°C in LB to an OD_{540} of 0.1. The cultures were diluted 1:10 into fresh LB and were incubated further. When growth had recovered (OD_{540} of 0.05), portions of the culture were either left untreated (■) or were treated with 1 mM IPTG (▲). Thirty minutes later, as indicated by the arrows, ethanol (4%, vol/vol) was added to a portion of each of the cultures (open symbols). Samples from each of the cultures were taken every 15 min and were analyzed for β-galactosidase. Results are the averages of two experiments. The Miller unit values (15) were normalized to 1 by using the highest respective value of each strain. (A) BSA419 (P_{spac}::*rsbT*), 1 = 113 Miller units; (B) BSJ38 (P_{spac}::*rsbT rsbX*::*spec*), 1 = 128 Miller units; (C) BSJ41 (P_{spac}::*rsbT* Δ*rsbST*), 1 = 151 Miller units; (D) BSJ42 (P_{spac}::*rsbT rsbRT*Δ*5*), 1 = 163 Miller units.

products of the six genes that are downstream of P_{spac} (Fig. 3, lane 2).

BSA419 contains a *lacZ* reporter gene fused to a σ^B-dependent promoter (*ctc*::*lacZ*). Concomittant with induction of P_{spac}, there was a rapid rise in σ^B-dependent transcription, which remained high throughout the duration of the experiment (Fig. 4A). When the induced culture was exposed to ethanol stress 30 min after IPTG induction (Fig. 4A), reporter gene activity showed a small increase, followed by a decline in β-galactosidase activity that resembled the decline seen when σ^B is induced by stress in wild-type *B. subtilis* (Fig. 2). This difference in the activity of σ^B in stressed and unstressed cultures was also evident in the accumulation of the *sigB* operon products (Fig. 3). The *sigB* genes (*rsbV*, *rsbW*, *sigB*, and *rsbX*), controlled from P_B, continued to generate products in the absence, but not in the presence, of ethanol stress (Fig. 3, lane 3 versus lane 4). Ethanol treatment thus curtails the activity of σ^B, even when the activation of σ^B is independent of stress. The culture which was not IPTG treated did not show ethanol induction. This is likely due to the restricted expression of *rsbU*, which is downstream of the integrated plasmid. The uninduced culture did, however, display a modest increase in σ^B activity upon entry into stationary phase. Presumably, this occurred when σ^B, present at low levels in this strain, became active and triggered its further expression from P_B. The IPTG- and ethanol-treated cultures were growth impaired and did not enter stationary phase during the course of the experiment.

In previous studies, we noted that stress-induced σ^B activity did not decline in *B. subtilis* strains lacking RsbX (18, 22). We therefore tested whether the fall in σ^B activity, which occurred when the IPTG-induced culture was ethanol treated, also required RsbX. BSJ38 (Table 1) is a strain containing the P_{spac}::*rsbT* integration present in BSA419 plus a disruption of *rsbX* (*rsbX*::*spec*) (Fig. 5, lane 2). A culture of BSJ38 was induced with IPTG and a portion was exposed to ethanol stress. As was the case with the $RsbX^+$ strain, IPTG induction resulted in σ^B activation; however, unlike the $RsbX^+$ strain, ethanol treatment did not lead to a decline in σ^B reporter gene activity (Fig. 4B). Thus, the ethanol-dependent drop in σ^B activity requires functional RsbX.

The role of RsbX in the stress-induction pathway is thought to involve reactivation of RsbS, a negative regulator of RsbT (28). Given that the activation of σ^B in our artificial system was caused by the induced expression of RsbT rather than by a

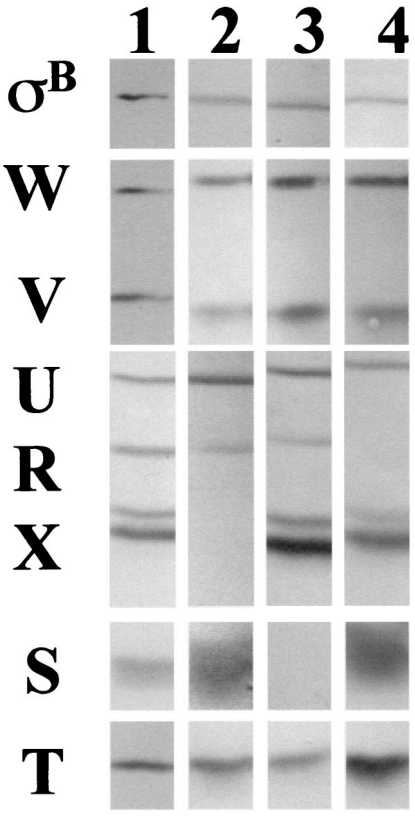

FIG. 5. Rsb profiles of BSA419 and mutant strains. *B. subtilis* strains were grown at 37°C in LB to an OD_{540} of 0.1, were treated with IPTG (1 mM) to induce *Pspac* upstream of *rsbT*, and were harvested 30 min after induction and processed as described in the legend to Fig. 3. Lane 1, BSA419 (P_{spac}::*rsbT*); lane 2, BSJ38 (P_{spac}::*rsbT rsbX*::*spec*); lane 3, BSJ41 (P_{spac}::*rsbT* Δ*rsbST*); lane 4, BSJ42 (P_{spac}::*rsbT rsbR*Δ*5*).

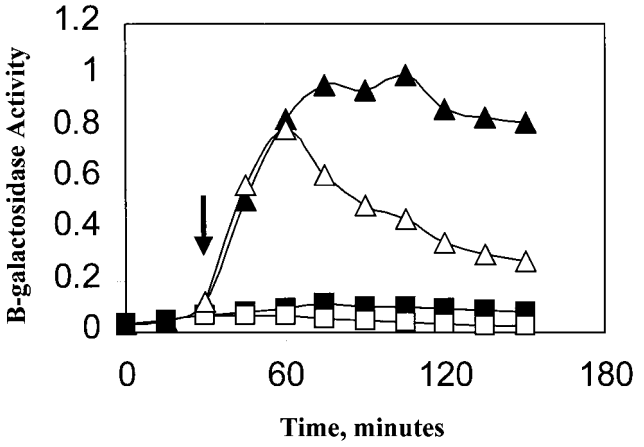

FIG. 6. Effect of ethanol on σ^B induction in Obg-depleted cells. BSJ-13 (P_{spac}::*rsbT* P_{xyl}::*obg*) was grown in LB without xylose in order to deplete Obg. When growth slowed (time 0), IPTG (1 mM) was added to a portion of the culture (triangles) to induce P_{spac} upstream of *rsbT*, while the remaining portion was left untreated (squares). Thirty minutes later, as indicated by the arrows, ethanol (4%, vol/vol) was added to a portion of each of the cultures (open symbols). Samples from each of the cultures were taken every 15 min and were analyzed for β-galactosidase (13). Results are the averages of two experiments. The Miller unit values were normalized to 1 by using the highest value for the strain (1 = 132 Miller units).

putative stress-triggered inactivation of RsbS by RsbT, we asked whether the fall in σ^B activity following ethanol treatment required RsbS. The $RsbS^-$ strain was constructed by transforming the P_{spac}::*rsbT* plasmid into BSJ39, a strain containing a deletion in the *rsbS* and *-T* region of the *sigB* operon (Table 1). The resulting strain (BSJ41) has an inducible source of RsbT but lacks RsbS (Fig. 5, lane 3). As was also observed with the $RsbX^-$ strain, the strain lacking RsbS failed to restrict σ^B activity after stress (Fig. 4C). This result is consistent with the notion that gratuitous expression of *rsbT* results in an inactivation of RsbS, which can be at least partially reactivated by RsbX in stressed *B. subtilis* but not in unstressed cells.

Recently, Gaidenko et al. found that RsbR could influence the ability of RsbT to phosphorylate RsbS (9). They proposed that RsbR modulated the inactivation of RsbS by RsbT, either in response to environmental signals or as part of a feedback mechanism to prevent continued stress signaling (9). This result prompted us to ask whether RsbR played a role in the stress-dependent restriction of σ^B activity which we observed in our present experiments. A $RsbR^-$ mutation was constructed by deleting a 500-bp *Eco*RI fragment from the interior of *rsbR* on the plasmid pRS11 (18). The resulting plasmid (pUM49) was then linearized with *Sca*I and was transformed into BSA419 to generate BSA42 (*rsbR*Δ*5* P_{spac}::rsbT) (Fig. 5, lane 4). When BSA42 was induced with IPTG and treated with ethanol, its σ^B activity profile (Fig. 4D) resembled that of the parent strain (Fig. 4A). There was a small reproducible difference (15% lower) in the degree to which σ^B activity fell in the $RsbR^-$ strain compared to the decline in the $RsbR^+$ strain; however, given that the principal pattern of decline was still evident, we conclude that RsbR is not an important component of this process. Thus, RsbX and -S, but not RsbR, are essential for the stress-activated drop in σ^B activity. Presumably, stress influences the activation state of the RsbX phosphatase and its ability to reactivate RsbS-P.

In earlier studies, we discovered that an essential GTP binding protein of *B. subtilis*, Obg, is needed for σ^B activation by stress (17). Obg was also found to interact with RsbT, -W, and -X in the yeast dihybrid system (17). Given the possible interaction of Obg with RsbX, we tested whether the stress-dependent stimulation of RsbX is affected by Obg. *B. subtilis* BSJ13 (Table 1), which carries the P_{spac}::*rsbT* construction within *rsbT*, as well as a second inducible promoter (P_{xyl}) driving the expression of *obg*, was used for this experiment. By withholding xylose, we can deplete Obg from the culture. This depletion of Obg causes a slowing of growth and a failure of stress to induce σ^B (17). After culturing BSJ13 in a medium without xylose to a point where growth had slowed and σ^B could no longer be activated by stress, we induced the stress pathway with IPTG and examined the ability of ethanol to restrict σ^B activity in these Obg-depleted cells. As is seen in Fig. 6, ethanol treatment could still curtail σ^B activity in the absence of Obg. Thus, the putative stress activation of RsbX appears to be independent of Obg.

The data presented herein argue that, aside from inducing σ^B activity, ethanol stress activates a process that limits this induction. Although ethanol treatment was the only stress examined in the present study, other stresses (e.g., acid shock and salt stress) also induce σ^B transiently and likely engage in a similar process. The ethanol-responsive process requires RsbX and RsbS and presumably involves the ability of RsbX to dephosphorylate and reactivate RsbS-P. The limiting factor in this reaction is not the RsbX protein, but rather is its activation. RsbX was present at higher levels in the culture that was not ethanol treated than in the ethanol-treated culture (Fig. 3,

lane 3 versus lane 4) and yet was relatively ineffective in curtailing σ^B activity. We conclude that either stress activates RsbX directly or there are additional stress-responsive factors which modulate the activity of RsbX.

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