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# Reactivation of the *Bacillus subtilis* Anti-$\sigma^B$ Antagonist, RsbV, by Stress- or Starvation-Induced Phosphatase Activities

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**$\sigma^B$ is a secondary $\sigma$ factor that controls the general stress regulon in *Bacillus subtilis*. The regulon is activated when $\sigma^B$ is released from a complex with an anti-$\sigma^B$ protein (RsbW) and becomes free to associate with RNA polymerase. Two separate mechanisms cause $\sigma^B$ release: an ATP-responsive mechanism that correlates with nutritional stress and an ATP-independent mechanism that responds to environmental insult (e.g., heat shock and ethanol treatment). ATP levels are thought to directly affect RsbW's binding preference. Low levels of ATP cause RsbW to release $\sigma^B$ and bind to an alternative protein (RsbV), while high levels of ATP favor RsbW-$\sigma^B$ complex formation and inactivation of RsbV by an RsbW-dependent phosphorylation. During growth, most of the RsbV is phosphorylated (RsbV-P) and inactive. Environmental stress induces the release of $\sigma^B$ and the formation of the RsbW-RsbV complex, regardless of ATP levels. This pathway requires the products of additional genes encoded within the eight-gene operon (*sigB*) that includes the genes for $\sigma^B$, RsbW, and RsbV. By using isoelectric focusing techniques to distinguish RsbV from RsbV-P and chloramphenicol treatment or pulse-chase labeling to identify preexisting RsbV-P, we have now determined that stress induces the dephosphorylation of RsbV-P to reactivate RsbV. RsbV-P was also found to be dephosphorylated upon a drop in intracellular ATP levels. The stress-dependent and ATP-responsive dephosphorylations of RsbV-P differed in their requirements for the products of the first four genes (*rsbR*, *-S*, *-T*, and *-U*) of the *sigB* operon. Both dephosphorylation reactions required at least one of the genes included in a deletion that removed *rsbR*, *-S*, and *-T*; however, only an environmental insult required RsbU to reactivate RsbV.**

$\sigma^B$ is a secondary sigma factor of *Bacillus subtilis* (9, 16, 18, 20) that directs RNA polymerase (RNAP) to promoters of general stress response genes (5, 6, 10–12, 22, 23, 30, 40). Control of $\sigma^B$-dependent transcription occurs at the level of $\sigma^B$ activation. $\sigma^B$ is one of several sigma factors known to be held in an inactive state by the binding of a specific anti-$\sigma$ factor protein (2, 7, 17, 27, 35, 37). In the case of $\sigma^B$, this protein (RsbW) appears to block the ability of $\sigma^B$ to associate with RNAP (7). Metabolic or environmental stresses trigger RsbW to bind to an alternative protein (RsbV) and release $\sigma^B$ (7, 14, 15, 39). This leads to the formation of a $\sigma^B$-containing RNAP holoenzyme and the activation of stress response genes (7, 14, 39). RsbW is a protein kinase in addition to being an RsbV and $\sigma^B$-binding protein (14). RsbW and another well-studied *B. subtilis* anti-$\sigma$ factor, SpoIIAB, can phosphorylate the protein to which they bind in lieu of binding to their cognate $\sigma$ factors (1, 2, 14, 32). The phosphorylated forms of RsbV (RsbV-P) and the RsbV counterpart in the SpoIIAB ($\sigma^F$) system (SpoIIAA) appear to be incapable of binding to their respective anti-$\sigma$ factors (1, 2, 12, 13, 29).

There are at least two pathways which lead to $\sigma^B$ release from RsbW. The first involves a direct effect of intracellular ATP levels on RsbW's binding preference (1, 42). This is similar to an ATP-dependent pathway that was first proposed to control $\sigma^F$ activation (2). RsbW phosphorylates RsbV and binds to $\sigma^B$ when ATP levels are high and fails to phosphorylate RsbV efficiently when ATP levels are low, forming instead stable RsbW-RsbV complexes which leave $\sigma^B$ free and active (1).

A decline in intracellular ATP parallels the increase in $\sigma^B$-dependent transcription during carbon limitation but not under $\sigma^B$ activation conditions that involve induction by environmental insult (42). Environmental stress (e.g., heat shock and ethanol treatment) allows the release of $\sigma^B$ from RsbW and the formation of RsbW-RsbV complexes by an ATP-independent pathway (42). This pathway requires the products of additional genes encoded within the eight-gene operon (*sigB*) that includes the genes for $\sigma^B$, RsbW, and RsbV (15, 25, 39, 42, 43). The *sigB* operon is expressed constitutively from a $\sigma^A$-dependent promoter, with an internal $\sigma^B$-dependent promoter that enhances the expression of the downstream four genes (*rsbV*, *rsbW*, *sigB*, and *rsbX*) during periods of $\sigma^B$ activation (43). RsbX and the products of at least two of the four upstream genes (*rsbR*, *rsbS*, *rsbT*, and *rsbU*) are involved in the stress activation pathway (15, 25, 34, 39, 42). Disruption of *rsbU*, or a deletion that removes *rsbR*, *rsbS*, and *rsbT*, renders $\sigma^B$ activation uninducible by environmental stress but still activatable by a drop in intracellular ATP (15, 39). RsbS has been reported to be a negative regulator of $\sigma^B$ activity and RsbT, a positive regulator that is needed if a loss of RsbS is to stimulate $\sigma^B$ activity (25, 34). The loss of *rsbX* results in very high level of constitutive $\sigma^B$ activity (4, 12, 22, 24), but only in *B. subtilis* strains with intact *rsbU* or *rsbRST* regions (15, 39). Thus, RsbU and the product of at least one of the genes encoded within the $\Delta$*rsbRST* deletion are essential for $\sigma^B$ activation by stress, and RsbX is a negative regulator of this pathway.

The mechanism by which environmental stress can trigger the release of $\sigma^B$ from RsbW is unknown; however, formation of an RsbW-RsbV complex is required (42). During growth, most of the RsbV is phosphorylated and unable to bind to RsbW (14, 39). A plausible mechanism for the stress activation of $\sigma^B$ could involve the reactivation of preexisting RsbV-P by a stress-dependent phosphatase. A phosphatase of this kind

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TABLE 1. Characteristics of the *B. subtilis* strains used in this study

| Strain | Relevant genotype | Source or reference |
|---|---|---|
| PY22 | Wild type | |
| BSA46 | SPβ*ctc*::*lacZ* | 4 |
| BSA70 | *rsbU*::*kan* SPβ*ctc*::*lacZ* | 4 |
| BSA140 | *rsbU*ΔNdeI *rsbX*::pWH25[a] SPβ*ctc*::*lacZ* | 39 |
| BSA158 | *rsbU*ΔNdeI *rsbX*::pWH25[a] | 42 |
| BSA159 | *rsbV312 rsbX*::pWH25[a] | 42 |
| BSA160 | *rsbU*::*kan* | BSA70→PY22[b] |
| BSA220 | *rsbR*::*rsbT* Δ*rsbS kan* upstream of *spoIIGA* | 15 |
| BSA221 | *rsbR*::*rsbT* Δ*rsbS kan* upstream of *spoIIGA* SPβ*ctc*::*lacZ* | 15 |

[a] The integrative plasmid pWH25 contains a 2-kb *Eco*RI-*Sph*I fragment, including the 3′ end of *rsbX* and 1.9 kb downstream of *rsbX*. Strains transformed with this plasmid are RsbX$^+$.

[b] An arrow indicates the construction of the strain by transformation.

has been reported in the $\sigma^F$ system, where SpoIIE, known to be essential for $\sigma^F$ activation, was shown to dephosphorylate SpoIIAA, the RsbV homolog (16). By using isoelectric focusing (IEF) techniques to distinguish RsbV from RsbV-P and either chloramphenicol treatment or pulse-chase labeling, we have been able to detect formation of RsbV from RsbV-P after both stress and exposure to conditions that bring about a drop in intracellular ATP levels. The stress-dependent and ATP-dependent dephosphorylations of RsbV-P differed in their requirements for the products from the upstream half of the *sigB* operon. Both dephosphorylation reactions require at least one of the genes contained within the *rsbRST* deletion; however, only the pathway induced by an environmental insult requires RsbU to reactivate RsbV.

## MATERIALS AND METHODS

**Bacterial strains and cultivation conditions.** The *B. subtilis* strains used in this study are listed in Table 1. All strains are derived from PY22, which was originally obtained from P. Youngman (University of Georgia). The strains were cultivated with vigorous agitation (250 rpm) at 37°C in Luria-Bertani medium (LB) (36) or in a synthetic medium described previously (38). Stresses were imposed by adding ethanol, NaCl, or $MnCl_2$ to final concentrations of 4%, 2.5%, or 1 mM, respectively, or by shifting the culture from 37°C to 48°C. The availability of oxygen was restricted by reducing the shaking frequency from 250 to 50 rpm. Glucose limitation was accomplished by cultivating the bacteria in a synthetic medium with 0.05% glucose. In one set of experiments, the synthesis of new proteins was prevented by the addition of chloramphenicol (100-μg/ml final concentration) to the culture prior to the exposure to stress or limitations.

**ATP assay.** The level of ATP was determined as described previously (30) with a kit from Boehringer Mannheim.

**Two-dimensional protein electrophoresis, horizontal IEF, SDS-PAGE, and Western blot (immunoblot) analysis.** The cells were disrupted by passage through a French press. Protein concentrations were determined by a Bio-Rad protein assay according to the manufacturer's instructions. Identical amounts of protein were loaded onto each lane or tube gel. The two-dimensional electrophoresis of protein extracts with a pH range of 2.5 to 6 in the first dimension and a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% acrylamide in the second dimension was carried out as described earlier (21). The horizontal IEF and all additional steps of the Western blot analysis were carried out as described previously (5, 14). The monoclonal antibodies raised against RsbV, RsbW, SigB, and RsbX have been described previously (5, 14).

**Labeling techniques.** Bacteria were grown in a synthetic medium (38) and labeled with 20 μCi of a mix of L-[$^{35}$S]methionine and L-[$^{35}$S]cysteine per ml for the time indicated in the legend to Fig. 3. The incorporation of radioactive L-methionine and L-cysteine was stopped by the addition of an $10^5$-fold excess of the two unlabeled amino acids.

**RNA isolation and slot blot analysis.** Cells were harvested on ice, resuspended in TE (0.1 M Tris [pH 8.0], 10 mM EDTA), and disrupted by passage through a French press. After centrifugation (12,000 × *g*, 4°C, 10 min), 100 μl of the supernatant was used for a purification of the total RNA with RNeasy cartridges from Quiagen according to the manufacturer's instructions. Equal amounts (2 μg) of RNA were transferred onto a positively charged nylon membrane by slot blotting and hybridized with digoxigenin-labeled RNA synthesized in vitro from the linearized plasmids pKSctc8 and pKSgsiB5 (30). The specific hybrids were visualized with an alkaline phosphatase-conjugated anti-digoxigenin antibody and CDP-Star as a substrate.

**Assay of β-galactosidase activity.** Cells were harvested by centrifugation and frozen at −20°C. β-Galactosidase assays were done according to the method of Kenney and Moran (26) with chloroform and SDS to permeabilize the bacteria. The activity was expressed in Miller units [$10^3 \times A_{420}$/(volume × time × $A_{540}$)] (31).

**General methods.** Transformation of competent *B. subtilis* cells was performed by the method of Yasbin et al. (44).

## RESULTS

**Stress-induced $\sigma^B$ activation is accompanied by the appearance of unphosphorylated RsbV.** RsbV exists in both phosphorylated and unphosphorylated forms (14). Unphosphorylated RsbV is associated with RsbW, while RsbV-P is free within the cell (14). We interpreted this as evidence that unphosphorylated RsbV is the active form of the protein and that RsbV-P does not form a stable complex with RsbW. In growing cells, virtually all of the detectable RsbV is phosphorylated and unbound to RsbW (39). Stress activation of $\sigma^B$ (e.g., ethanol treatment) results in the appearance of RsbV-RsbW complexes and the formation of $\sigma^B$-containing RNAP (39). Thus, stress should generate unphosphorylated RsbV to bind to RsbW and drive the release of $\sigma^B$. To test this notion, we ethanol treated actively growing *B. subtilis* cells and analyzed them for changes in the abundance and phosphorylation state of RsbV. The downstream half of the *sigB* operon is under the control of a $\sigma^B$-dependent promoter (43). Thus, the levels of RsbV and the three other downstream gene products (RsbW, $\sigma^B$, and RsbX) rise after $\sigma^B$ activation. Figure 1A illustrates a Western blot in which monoclonal antibodies specific for each of the four $\sigma^B$-controlled *sigB* operon products were used to probe crude extracts of the ethanol-treated culture. A clear increase in all of the proteins is evident by 5 min after ethanol exposure, with the elevated levels persisting for at least 30 min.

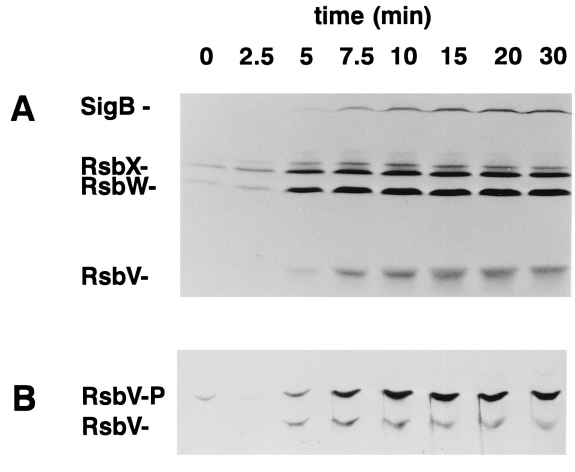


FIG. 1. Influence of ethanol stress on the level of *sigB* operon products and the phosphorylation profile of RsbV. Wild-type *B. subtilis* (PY22) was grown in LB and exposed to 4% ethanol (final concentration) during exponential growth. The bacteria were harvested before and at intervals after stress. Crude extracts were prepared as described in Materials and Methods. (A) One hundred-microgram protein samples were analyzed by Western blotting with monoclonal antibodies raised against RsbV, RsbW, SigB, and RsbX as probes. (B) Aliquots (100 μg of protein) of the extracts used in panel A were subjected to IEF. The proteins were transferred to nitrocellulose, and the membrane was probed with an anti-RsbV monoclonal antibody.

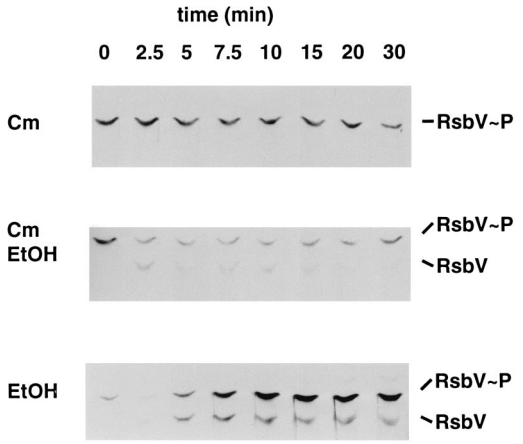


FIG. 2. IEF analysis of RsbV after treatment with chloramphenicol (Cm) and/or ethanol (EtOH). PY22 was grown in LB and treated with either ethanol (4% final concentration) or chloramphenicol (100 μg/ml) or treated with chloramphenicol for 5 min and then exposed to ethanol (Cm EtOH). Crude extracts were prepared from bacteria that were harvested either immediately before or at different times after the additions. The crude extracts were subjected to IEF and analyzed by Western blotting as described in the legend to Fig. 1B.

When samples of the same extracts were subjected to IEF to resolve RsbV from RsbV-P, the RsbV profile changed at the earliest time examined (2.5 min) after ethanol application (Fig. 1B). In the untreated culture, all of the detectable RsbV focused as RsbV-P (Fig. 1B, time zero); however, by 2.5 min after the application of ethanol, RsbV-P levels decreased and a faint band of RsbV became detectable (Fig. 1B, time 2.5). There was a substantial increase in both forms of RsbV by 5 min (Fig. 1B). This increase paralleled the general increase in $\sigma^B$-controlled gene products (Fig. 1A). Ethanol therefore induced activation of $\sigma^B$ coincident with the formation of unphosphorylated RsbV. The RsbV appeared to accumulate initially at the expense of RsbV-P. Thus, an early step in the stress-induced activation of $\sigma^B$ could be the dephosphorylation of preexisting RsbV-P.

**Ethanol can induce the dephosphorylation of RsbV-P and the activation of $\sigma^B$ in the absence of protein synthesis.** The initial drop in RsbV-P levels accompanying the ethanol-induced synthesis RsbV suggests that RsbV could arise from dephosphorylation of preexisting RsbV-P. We tested this idea by treating a *B. subtilis* culture with chloramphenicol, before ethanol exposure, to activate $\sigma^B$ in the absence of new protein synthesis. Figure 2 illustrates the effects of these treatments on the level and phosphorylation state of RsbV. Addition of chloramphenicol had no appreciable effect on RsbV in the absence of ethanol stress. Only RsbV-P, which persisted at a relatively constant level throughout the experiment (30 min), was detected in such a culture. Exposure of the chloramphenicol-treated culture to ethanol resulted in a rapid decrease in RsbV-P and the appearance of unphosphorylated RsbV. Because of the block in protein synthesis, the amounts of both RsbV species were substantially smaller than the amounts observed in the culture treated with ethanol alone (Fig. 2).

The appearance of RsbV in *B. subtilis* cells treated with both chloramphenicol and ethanol argues that ethanol can induce the dephosphorylation of preexisting RsbV-P. To both verify this conclusion and eliminate the possibility that chloramphenicol treatment was affecting our analysis in an unforeseen manner, we used pulse-chase labeling and two-dimensional gel electrophoresis to monitor the fate of preexisting RsbV-P after ethanol addition. Figure 3 illustrates this analysis. Column B represents an autoradiogram of the RsbV–RsbV-P region of a two-dimensional gel. It contains a crude protein extract that had been labeled with [$^{35}$S]methionine and [$^{35}$S]cysteine. A Western blot of the same area of the gel, probed with anti-RsbV antibody, is illustrated in Fig. 3A. Even in the minimal medium used for labeling, most of the labeled RsbV is in the form of RsbV-P during growth (Fig. 3, row 1). There is an increase in both labeled RsbV and RsbV-P when the labeling is continued after the addition of ethanol (Fig. 3, row 3). Consistent with ethanol stress inducing the dephosphorylation of RsbV-P, we detect a shift in radioactivity from the previously labeled RsbV-P to the position of RsbV after ethanol treatment of prelabeled cells in the presence of sufficient un-

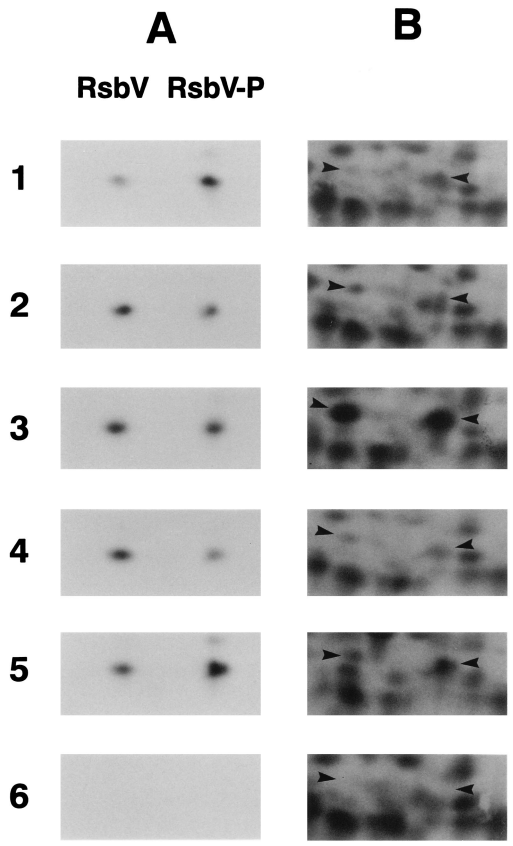


FIG. 3. Analysis of the phosphorylation profile of RsbV. The *B. subtilis* strains PY22 (wild type) and BSA159 (*rsbV312*) were grown in a synthetic medium and labeled with L-[$^{35}$S]methionine and L-[$^{35}$S]cysteine, and then crude cell extracts were prepared. Seventy five-microgram protein samples were separated by two-dimensional PAGE and transferred to nitrocellulose. The membranes were analyzed by Western blotting for RsbV and RsbV-P (A) and by autoradiography, with sections of the autoradiograms displaying the positions of both forms of RsbV (marked with arrowheads) shown in column B. Rows: 1 and 6, PY22 and BSA159, respectively, were labeled for three generations and immediately harvested; 2, a 100,000-fold excess of unlabeled L-methionine and L-cysteine was added to the PY22 culture depicted in row 1, and after 5 min, the bacteria were exposed to 4% ethanol for an additional 5-min period; 3, ethanol (4% final concentration) was added to PY22 during $^{35}$S-labeling with the bacteria harvested 5 min after ethanol treatment; 4, PY22, prelabeled with $^{35}$S as in row 1, was cultivated with growth-limiting amounts of glucose and a $10^5$-fold excess of unlabeled L-methionine and L-cysteine; 1 h after the exhaustion of glucose, the cells were harvested; 5, PY22 was cultivated with growth-limiting amounts of glucose and labeled with [$^{35}$S]methionine and [$^{35}$S]cysteine for 30 min after the entry into the stationary phase.

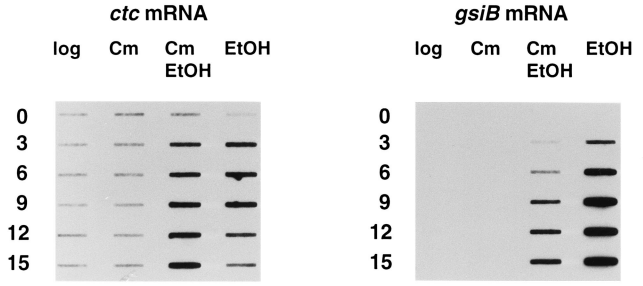


FIG. 4. Effect of ethanol and chloramphenicol on the level of *ctc* and *gsiB* mRNA. *B. subtilis* PY22 (wild type) was grown in LB and untreated (log), exposed to ethanol (4% final concentration) (EtOH), or chloramphenicol (100 μg/ml) (Cm), or pretreated with chloramphenicol for 5 min and then treated with ethanol (Cm EtOH). Samples were taken before (0 min) or at different times after the treatments (3, 6, 9, 12, and 15 min). RNA was prepared, and 2-μg aliquots of the RNA were transferred to a positively charged nylon membrane. The membrane was hybridized with digoxigenin-labeled probes specific for *ctc* and *gsiB*.

labeled methionine and cystine to block further incorporation of $^{35}S$ (Fig. 3, row 2).

To ask if the dephosphorylation of RsbV-P that we observed after ethanol stress provides sufficient RsbV to activate $\sigma^B$, we extracted RNA from cultures treated with chloramphenicol and ethanol and probed this RNA with DNA sequences from two E-$\sigma^B$-transcribed genes (*ctc* and *gsiB*). Figure 4 illustrates the resulting slot blot analysis. Both *ctc*- and *gsiB*-specific RNAs increased in abundance after ethanol exposure, regardless of whether or not chloramphenicol was present. *ctc* is expressed from both $\sigma^A$- and $\sigma^B$-dependent promoters (8, 23, 33). Hence, it is present prior to ethanol induction but induced to higher levels by ethanol. In the absence of chloramphenicol, *ctc* mRNA levels became most abundant 3 to 9 min after ethanol treatment and then decreased. The induced levels of *ctc* mRNA persisted if chloramphenicol was present. Apparently, there is a chloramphenicol-sensitive mechanism that restricts *ctc*-specific RNA accumulation under these conditions. *gsiB* transcription, being solely dependent on $\sigma^B$, was undetectable prior to ethanol treatment but accumulated rapidly once the culture was exposed to ethanol. Although induced in both the presence and absence of chloramphenicol, *gsiB* mRNA reached its highest levels in the culture lacking chloramphenicol, where $\sigma^B$ can accumulate. The ethanol-induced expression of *ctc* and *gsiB* in chloramphenicol-treated cultures demonstrates that $\sigma^B$ can be activated in the absence of new protein synthesis and that the dephosphorylation of preexisting RsbV-P forms adequate RsbV to activate $\sigma^B$.

**Multiple stresses induce dephosphorylation of RsbV-P.** Ethanol treatment is one of a number of environmental insults that induce the activation of $\sigma^B$. Heat, salt, acid shock, etc. all activate $\sigma^B$ by processes that are independent of ATP levels and that require the products of at least two of the four promoter-proximal genes of the *sigB* operon (15, 39, 42). To test whether the dephosphorylation of RsbV-P, triggered by ethanol treatment, is a common feature of stress-induced $\sigma^B$ activation, we subjected chloramphenicol-treated cultures of *B. subtilis* to heat shock and salt stress and monitored RsbV and RsbV-P changes by Western blot analyses of proteins fractionated by two-dimensional gel electrophoresis. Figure 5A compares the RsbV patterns of chloramphenicol-treated, unstressed cells with those of chloramphenicol-treated cultures that were subsequently exposed to ethanol, heat shock, or salt stress. In each case, there is a clear increase in RsbV at the expense of RsbV-P. We conclude that multiple environment stresses can activate the dephosphorylation of RsbV-P.

We had previously shown that the ATP-independent $\sigma^B$ activation requires RsbU and at least one of the genes encoded within the *rsbRST* region of the *sigB* operon. This result prompted us to ask whether any of these gene products are also needed for the RsbV-P dephosphorylation reaction. Strains with either a null mutation in *rsbU* (*rsbU*Δ*Nde*I) or a deletion that removed the RsbR, RsbS, and RsbT coding region (*rsbR*::*rsbT* Δ*rsbS*) were treated with chloramphenicol, exposed to ethanol, and analyzed by two-dimensional gel electrophoresis and Western blotting for their RsbV or RsbV-P profile. As can be seen in Fig. 5B, either mutation blocked the dephosphorylation of RsbV-P. Thus, genes known to be essential for stress-induced $\sigma^B$ activation are also required for RsbV-P dephosphorylation.

**Activation of $\sigma^B$ by the ATP-dependent pathway triggers dephosphorylation of RsbV-P.** Having determined that environmental stress can lead to the dephosphorylation of RsbV-P, we next asked whether the ATP-dependent pathway of $\sigma^B$ activation, thought to occur by a direct effect of ATP on RsbW (1, 42), might also have a dephosphorylation component. We chose oxygen limitation as the condition to activate $\sigma^B$ by a drop in ATP. This treatment induces $\sigma^B$-dependent transcription to a similar extent both in wild-type *B. subtilis* and in strains prevented by mutation (*rsbU*Δ*Nde*I) from responding to environmental stress (42). *B. subtilis* was subjected to $O_2$ limitation during growth or after exposure to chloramphenicol and analyzed by two dimensional gel electrophoresis and Western blotting for RsbV and RsbV-P. Figure 6A illustrates that $O_2$ limitation causes a readily detectable increase in both RsbV and RsbV-P in the strain that was not exposed to chloramphenicol and the appearance of RsbV at the expense of RsbV-P in the chloramphenicol-treated cultures. Similar results were observed in a pulse-chase experiment, in which labeled RsbV was seen to accumulate at the apparent expense of RsbV-P after oxygen limitation (Fig. 3, lane 5). When the $O_2$ limitation experiments were repeated with an RsbU$^-$ *B. subtilis* strain, the RsbV and RsbV-P patterns were virtually identical to those obtained from the RsbU$^+$ strain (Fig. 6A). The

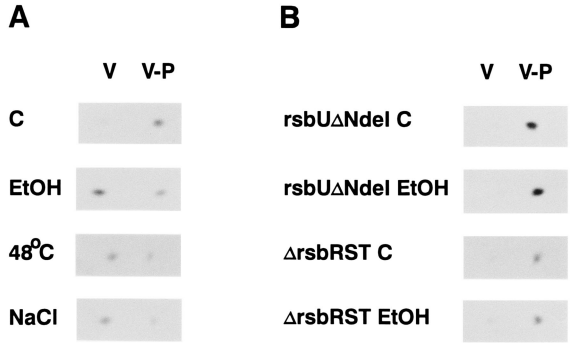


FIG. 5. IEF analysis of the dephosphorylation of RsbV. (A) Phosphorylation profile of RsbV after exposure to 4% ethanol (EtOH), heat shock (48°C), or 2.5% salt (NaCl). *B. subtilis* PY22 was grown in LB, pretreated with chloramphenicol (100 μg/ml) for 5 min, and then exposed to one of the stresses for 2.5 min. The cells were harvested before (C) or after exposure to the indicated stress. (B) Influence of mutations in *rsbU* and *rsbRST* on the stress-activated dephosphorylation of RsbV. *B. subtilis* BSA158 (*rsbU*Δ*Nde*I) and BSA220 (*rsbR*::*T*Δ*rsbS*) were grown in LB and exposed to ethanol as described for panel A. Crude extracts (100 μg of protein) were separated by two-dimensional PAGE and transferred to nitrocellulose and probed with a monoclonal antibody against RsbV.

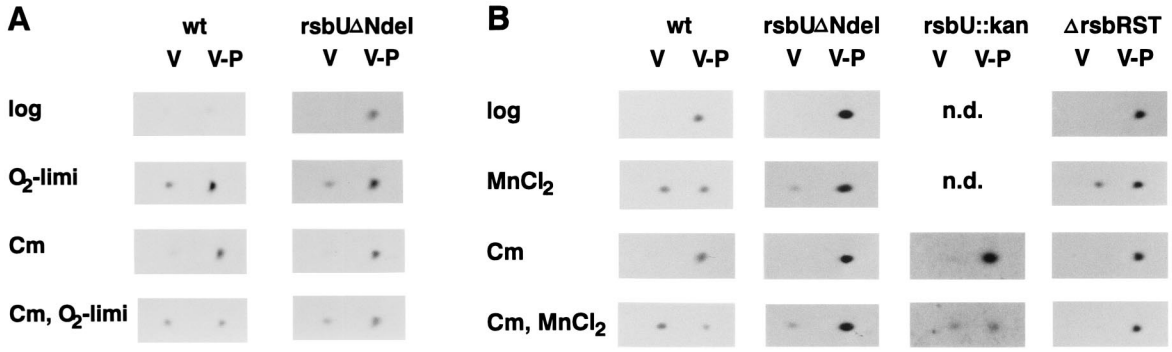


FIG. 6. Activation of the dephosphorylation of RsbV by oxygen limitation ($O_2$-limi) (A) and $MnCl_2$ (B). (A) Influence of oxygen limitation on the dephosphorylation of RsbV. *B. subtilis* PY22 (wild type [wt]) and BSA158 (*rsbU*Δ*Nde*I) were grown in LB, and the availability of oxygen was restricted by reduction of the shaking frequency from 250 rpm to 50 rpm. Samples were taken during exponential growth (log) or 40 min after the oxygen supply was restricted. (B) *B. subtilis* PY22 (wild type), BSA158 (*rsbU*Δ*Nde*I), BSA160 (*rsbU*::*kan*), and BSA220 (*rsbR*::*T*Δ*rsbS*) were grown in LB. Samples were taken during growth (log) or 10 min after the addition of 1 mM $MnCl_2$ (final concentration). The pretreatment with chloramphenicol (Cm), the sample preparation, and processing were carried out as described in the legend to Fig. 5 and Materials and Methods. V-P and V, phosphorylated and nonphosphorylated form of RsbV; n.d., not determined.

ATP-responsive pathway of $\sigma^B$ activation therefore also involves the dephosphorylation of RsbV-P; however, unlike the environmental stress-induced pathway, the dephosphorylation of RsbV-P does not require RsbU.

**Induction of the RsbU-independent pathway for $\sigma^B$ activation by $Mn^{2+}$.** Stress-induced activation of $\sigma^B$ can be easily accomplished by the addition of ethanol or salt. The ATP-dependent pathway is more difficult to manipulate. Reproducibly estimating the times at which intracellular ATP drops during growth in glucose-limiting media or by restricted aeration can be difficult. We and others have also used uncouplers of oxidative phosphorylation (e.g., carbonyl cyanide *m*-chloramphenylhydrazone) to induce this pathway, but have found them to be relatively poor inducers (2, 42). We therefore sought an alternative method for inducing the ATP-dependent pathway. $Mg^{2+}$ is an important cofactor in ATP-generating reactions (28). We therefore attempted to interfere with the availability of $Mg^{2+}$ as a means of reducing intracellular ATP. To this end, we challenged cells with high levels of $Mn^{2+}$ as an $Mg^{2+}$ competitor. As can be seen in Fig. 7A, addition of $Mn^{2+}$ to a growing culture of *B. subtilis* causes a rapid 10-fold increase in $\sigma^B$-dependent transcription. To determine whether $Mn^{2+}$ addition activates the ATP-dependent pathway and not the stress-dependent pathway, we treated $RsbU^-$ and $RsbRST^-$ strains with $Mn^{2+}$. Consistent with $Mn^{2+}$ treatment inducing the ATP-dependent pathway, $\sigma^B$ activity rose after $Mn^{2+}$ addition in both of these strains (Fig. 7A). We directly tested the notion that $Mn^{2+}$ caused a drop in ATP by measuring the intracellular ATP levels after exposure to $Mn^{2+}$ and found that the ATP levels fell to half that of the pretreatment levels within 20 min of $Mn^{2+}$ addition (Fig. 7B). This drop in ATP is similar to the drop in ATP that we had previously observed during $\sigma^B$ activation by glucose limitation (42). We assumed that $Mn^{2+}$ could drop ATP levels by interfering with $Mg^{2+}$-dependent reactions. This appears to be true. $Mn^{2+}$-dependent induction of $\sigma^B$-dependent transcription is prevented when cultures are grown in the presence of excess $Mg^{2+}$ (Fig. 7C).

Using $Mn^{2+}$ to induce ATP-dependent $\sigma^B$ activation, we examined the effects of the *rsbR*::*T*Δ*S* deletion and mutations in *rsbU* on RsbV-P dephosphorylation. Figure 6B shows that the addition of $Mn^{2+}$, like $O_2$ limitation (Fig. 6A), causes a dephosphorylation of preexisting RsbV-P, regardless of whether the strain carries the wild-type allele or the *rsbU*Δ*Nde*I mutation at *rsbU*. Although the *rsbU*Δ*Nde*I allele's product is inadequate for stress-dependent activation of $\sigma^B$, it is detectable by Western blotting in *B. subtilis* (15). It was therefore possible that the truncated RsbUΔ*Nde*I, was participating in RsbV-P dephosphorylation, even though it is unable to function in the stress pathway. We therefore repeated the experiment with a strain with a null mutation in *rsbU*. A strain that makes no detectable RsbU (*rsbU*::*kan*) still exhibited dephosphorylation of RsbV-P after $Mn^{2+}$ addition (Fig. 6B).

Although *rsbU* is dispensable for the ATP-dependent dephosphorylation of RsbV-P, at least one of the genes contained within the *rsbRST* deletion is required. RsbV failed to appear in the chloramphenicol-treated *rsbR*::*T*Δ*S* culture but was readily detectable after $Mn^{2+}$ treatment in the absence of chloramphenicol (Fig. 6B). Thus, one or more of the genes contained within the *rsbRST* region are needed for the dephosphorylation of RsbV-P in both the stress-dependent and ATP-dependent pathways. RsbU, in contrast, is only needed for the stress-induced dephosphorylation reaction.

## DISCUSSION

Regardless of the inducing condition, activation of $\sigma^B$ involves its release from RsbW and the formation of an RsbW-RsbV complex. We had previously shown that RsbV exists in phosphorylated (RsbV-P) and unphosphorylated (RsbV) forms, with RsbV bound to RsbW and RsbV-P free within cell extracts (14). We concluded that the phosphorylation state of RsbV is critical to its ability to associate with RsbW and that RsbV-P is an inactive form of RsbV. It remained unresolved whether the RsbW-RsbV complexes that were formed after $\sigma^B$ activation were created exclusively from newly synthesized RsbV or whether preexisting RsbV-P could be dephosphorylated to again become an RsbW-binding protein. SpoIIAA-P, the anti-$\sigma^F$ factor antagonist, is reactivated by a phosphatase (16). In this system, the phosphatase (SpoIIE) is essential for $\sigma^F$ activation (16). We now show that at least a portion of the RsbV that appears during $\sigma^B$ activation also arises from a dephosphorylation reaction. In the $\sigma^B$ system, the phosphatase reaction is not essential for the ATP-dependent activation, but it may be a critical component of the stress-induced activation pathway. Mutations which remove either RsbU or RsbRST block stress-induced $\sigma^B$ activity and the dephosphorylation of RsbV-P. Thus, it is possible that the dephosphorylation of RsbV by the action of a stress-induced phosphatase

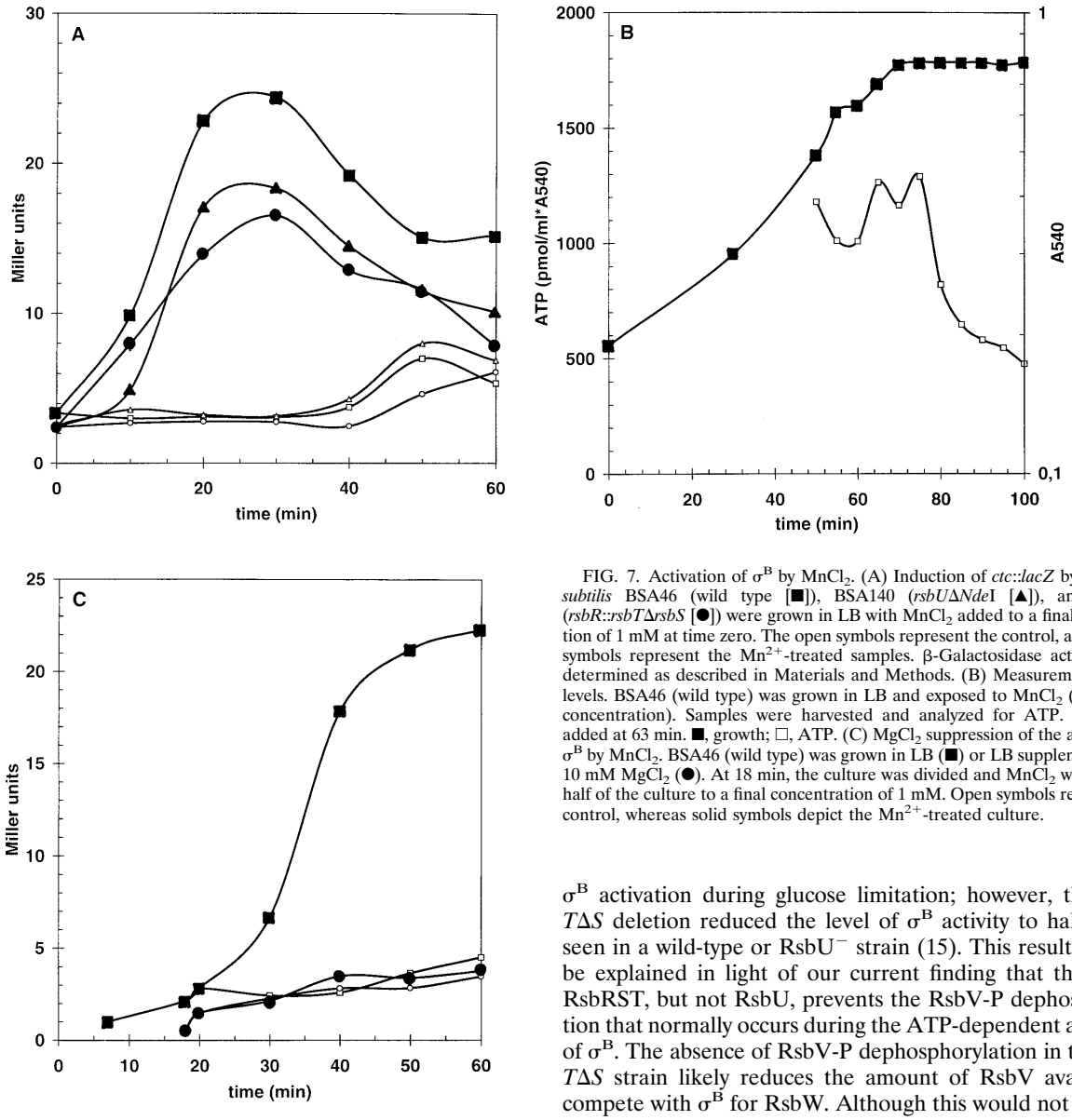


FIG. 7. Activation of $\sigma^B$ by $MnCl_2$. (A) Induction of *ctc*::*lacZ* by $MnCl_2$. *B. subtilis* BSA46 (wild type [■]), BSA140 (*rsbU*Δ*Nde*I [▲]), and BSA221 (*rsbR*::*rsbT*Δ*rsbS* [●]) were grown in LB with $MnCl_2$ added to a final concentration of 1 mM at time zero. The open symbols represent the control, and the solid symbols represent the $Mn^{2+}$-treated samples. β-Galactosidase activities were determined as described in Materials and Methods. (B) Measurement of ATP levels. BSA46 (wild type) was grown in LB and exposed to $MnCl_2$ (1 mM final concentration). Samples were harvested and analyzed for ATP. $MnCl_2$ was added at 63 min. ■, growth; □, ATP. (C) $MgCl_2$ suppression of the activation of $\sigma^B$ by $MnCl_2$. BSA46 (wild type) was grown in LB (■) or LB supplemented with 10 mM $MgCl_2$ (●). At 18 min, the culture was divided and $MnCl_2$ was added to half of the culture to a final concentration of 1 mM. Open symbols represent the control, whereas solid symbols depict the $Mn^{2+}$-treated culture.

may be the principal mechanism through which stress activates $\sigma^B$.

Our current view of $\sigma^B$ activation is illustrated in Fig. 8. Stress is depicted reactivating RsbV to bind RsbW and free $\sigma^B$. We cannot distinguish whether formation of unphosphorylated RsbV is the only consequence of stress-activated RsbRST and RsbU action or whether these proteins have additional roles in facilitating the formation of RsbV-RsbW complexes and the freeing of $\sigma^B$. The dephosphorylation of RsbV-P accompanying the ATP-dependent activation of $\sigma^B$ is not essential. *B. subtilis* with the *rsbR*::*TΔS* deletion fails to appreciably dephosphorylate RsbV-P and yet is able to activate $\sigma^B$. Apparently, the effects of low ATP levels are able to overcome the absence of a phosphatase. We assume that RsbV, newly synthesized under conditions of low ATP, is inefficiently phosphorylated by RsbW and remains active to bind to RsbW. Although the dephosphorylation of RsbV-P is not essential to ATP-dependent $\sigma^B$ activation, it likely contributes to this process. In earlier work, we observed that a loss of RsbU had no effect on $\sigma^B$ activation during glucose limitation; however, the *rsbR*::*TΔS* deletion reduced the level of $\sigma^B$ activity to half of that seen in a wild-type or $RsbU^-$ strain (15). This result can now be explained in light of our current finding that the loss of RsbRST, but not RsbU, prevents the RsbV-P dephosphorylation that normally occurs during the ATP-dependent activation of $\sigma^B$. The absence of RsbV-P dephosphorylation in the *rsbR*::*TΔS* strain likely reduces the amount of RsbV available to compete with $\sigma^B$ for RsbW. Although this would not block $\sigma^B$ activation, it would lead to a reduction in the overall level of free $\sigma^B$.

It has been proposed, on the basis of sequence homologies between RsbU and SpoIIE, that RsbU may be a RsbV-P phosphatase (16). Assuming this to be true, RsbU's phosphatase activity must be more highly regulated than that of SpoIIE. Overexpression of *spoIIE* leads to heightened $\sigma^F$ activity (3, 19), while overexpression of *rsbU* has no detectable effect on $\sigma^B$ activity or inducibility (41). If RsbU is an RsbV-P phosphatase, it likely requires an additional activation component or signal. In vitro evidence for such a notion was recently presented by C. W. Price and his associates at the 12th International Spores Conference (5 to 8 June 1996, Madison, Wis.). Price reported that RsbU can dephosphorylate RsbV-P in an in vitro reaction that is stimulated by RsbT. It is plausible that RsbT transmits a stress activator signal to RsbU to provoke the dephosphorylation of RsbV-P.

The dephosphorylation of RsbV-P which occurs after ATP limitation is RsbU independent. Therefore, at least one additional RsbV-P phosphatase must be present in *B. subtilis*. Iden-

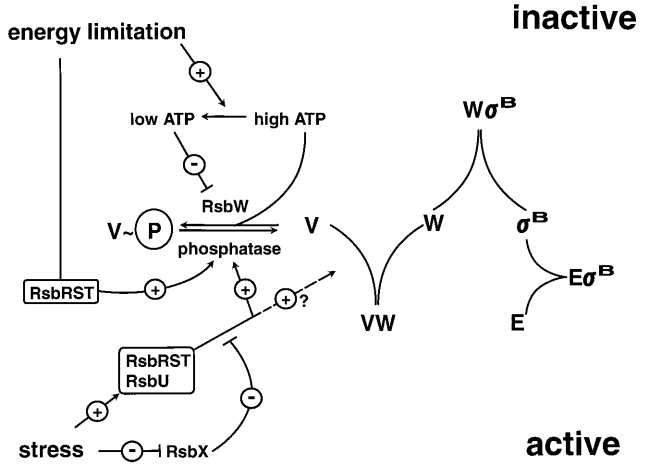


FIG. 8. Pathways of $\sigma^B$ activation. RsbW (W), the primary $\sigma^B$ regulator, can form complexes with either $\sigma^B$ or RsbV (V). Binding of RsbW to $\sigma^B$ blocks $\sigma^B$'s ability to associate with RNAP. If the RsbW-RsbV complex is formed, $\sigma^B$ remains free to become part of an RNAP holoenzyme (E-$\sigma^B$ [E$\sigma^B$]) RsbW's binding to $\sigma^B$ can be altered by energy limitation or environmental stress. Energy limitation leads to a drop in intracellular ATP levels, which directly influences RsbW's activity. Low levels of ATP both provoke the binding of RsbW to RsbV and restrict a kinase activity of RsbW which phosphorylates RsbV and converts it into an inactive form (RsbV-P). Energy limitation also activates a phosphatase in a reaction that requires one or more of the products of the *rsbR*, *-S*, and *-T* genes. The phosphatase elevates $\sigma^B$ induction, but it is not essential for low-ATP-dependent $\sigma^B$ activation. If energy is not limited, high intracellular ATP levels favor RsbW phosphorylation of RsbV and RsbW binding to $\sigma^B$. Environmental stress activates $\sigma^B$ in the presence of high intracellular ATP levels with a phosphatase to reactivate RsbV-P. The stress-induced phosphatase requires RsbU, in addition to one or more products of the *rsbRS* and *-T* gene cluster. Activation of a phosphatase to reactivate RsbV may be the only function of RsbRST and RsbU in the stress activation of $\sigma^B$; however, additional roles for these proteins in facilitating RsbV-RsbW complex formation have not been excluded. The stress-activated pathway is negatively regulated by RsbX. The mechanism by which RsbX influences this process and the signals to which RsbX responds are unknown.

tification of this enzyme and the circumstances that affect its activity may reveal additional pathways of $\sigma^B$ control.

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