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The *Bacillus subtilis rsbU* Gene Product Is Necessary for RsbX-Dependent Regulation of σ^B

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σ^B is a secondary σ factor of *Bacillus subtilis*. σ^B-dependent transcription is induced when *B. subtilis* enters the stationary phase of growth or is exposed to any of a number of different environmental stresses. Three genes (*rsbV*, *rsbW*, and *rsbX*), which are cotranscribed with the σ^B structural gene (*sigB*), encode regulators of σ^B-dependent gene expression. RsbW and RsbV have been shown to control σ^B activity, functioning as an inhibitory σ^B binding protein and its antagonist, respectively. Using the *SPAC* promoter (P_{SPAC}) to control the expression of the *sigB* operon, a *ctc*::*lacZ* reporter system to monitor σ^B activity, and monoclonal antibodies to determine the levels of *sigB* operon products, we have now obtained evidence that RsbX is an indirect regulator of σ^B activity. Genetic data and in vivo measurements argue that RsbX negatively regulates an extension of the RsbV-RsbW pathway that requires the product of an additional regulatory gene (*rsbU*) which lies immediately upstream of the *sigB* operon. The results are consistent with RsbU, or a process dependent on RsbU, being able to facilitate the RsbV-dependent release of σ^B from RsbW but normally prevented from doing this by RsbX.

σ^B is a secondary sigma factor of *Bacillus subtilis* (14). Genes that rely on σ^B for their expression are induced when *B. subtilis* ceases exponential growth in rich, sporulation-suppressing media or is exposed to any of several environmental insults (e.g., heat, salt, or ethanol) (2, 4, 7–10, 16, 25, 26). Although the circumstances which activate σ^B-dependent transcription argue that σ^B plays a role in *B. subtilis*'s ability to adapt to various stresses, it is not essential for the bacterium's survival under any of these conditions (4, 6, 9, 13). Null mutations in the σ^B structural gene confer no obvious phenotype on the strains which carry them (6, 13). Even though σ^B is not needed for any vital *B. subtilis* activity under laboratory conditions, proper control of σ^B is critical to the bacterium's survival. Release of σ^B from the negative regulation that keeps its activity in check is lethal to the cell (2, 9).

Negative regulation of σ^B, like that of at least two other *B. subtilis* σ factors (σ^F and σ^G) (12, 19, 21, 22), occurs by a specific binding protein (anti-σ factor) that blocks σ^B from joining to RNA polymerase (5). The anti-σ^B protein (RsbW) is encoded by the second gene of the four-gene operon that includes *sigB* as its third member (17). The remaining two genes (*rsbV* and *rsbX*) of the *sigB* operon encode additional σ^B regulators. RsbV, the product of the promoter-proximal gene, is a positive regulatory protein that antagonizes the RsbW block on σ^B (2, 9). Available evidence suggests a model whereby σ^B activity is controlled by the differential association of RsbW with either σ^B or RsbV (5, 11). The factors determining whether RsbW will bind to either RsbV or σ^B are largely unknown; however, two conditions have been proposed to influence the RsbW association decision. The first circumstance was suggested by in vitro binding studies of the RsbW homolog SpoIIAB, the anti-σ^F factor (1). These experiments showed that SpoIIAB preferentially binds to either SpoIIAA (an RsbV counterpart) or σ^F in response to the ratio of ATP to ADP present in the reaction mixture. A high ATP/ADP ratio favors SpoIIAB binding to σ^F, while a low ratio results in preferential association of SpoIIAB with SpoIIAA (1). RsbW is hypothesized to react similarly to ATP/ADP ratios, with stressors triggering a drop in ATP, a shift of RsbW to RsbV, and the release of σ^B (1). A second factor that has been suggested to influence RsbW's binding decision is the phosphorylation state of RsbV. Both RsbV and SpoIIAA can be phosphorylated by their respective anti-σ partners (RsbW or SpoIIAB) (11, 21). The phosphorylated form for RsbV does not appear to bind to RsbW (11). It is likely that RsbV's phosphorylation state contributes to σ^B regulation; however, the agents that determine whether RsbW phosphorylates or stably associates with RsbV, as well as the conditions under which RsbV phosphorylation is important for regulating σ^B activity, are not known.

RsbX, the product of the promoter-distal gene of the *sigB* operon, is also a negative regulator of σ^B. Although *rsbX* was the first gene shown to encode a regulator of σ^B-dependent transcription, its target has been elusive (15). Immunological studies failed to detect associations between RsbX and either RsbV, RsbW, or σ^B (11). Genetic analyses placed the site of RsbX inhibition upstream of the RsbV-RsbW regulatory pair, i.e., the loss of RsbX fails to stimulate σ^B-dependent transcription in $RsbV^-$ strains (2, 9). In addition, we and others had observed that σ^B activity is released from RsbX control but not from control by RsbV-RsbW when the *sigB* operon's σ^B-dependent promoter is replaced by an inducible σ^A-dependent promoter (P_{SPAC}) (3, 9). This result implied that RsbX is a specific regulator of the *sigB* operon's promoter rather than a participant with RsbV and RsbW in controlling σ^B activity. The idea that RsbX controls σ^B synthesis but not its activation state is difficult to reconcile with the observation that σ^B-dependent promoters are expressed at high levels in $RsbX^-$ $RsbW^+$ $RsbV^+$ strains (2, 9). It would be expected that if the loss of RsbX merely elevated the expression of the *sigB* operon, the coordinately enhanced synthesis of RsbW would continue to inhibit σ^B.

In the present communication, we resolve this apparent paradox with genetic evidence that RsbX regulates σ^B activity, albeit indirectly, by inhibiting a previously unrecognized extension of the σ^B activation pathway. This activation of σ^B is dependent on the product of an open reading frame (*rsbU*)

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

Strain	Relevant genotype[a]	Source or reference
BSA46	SPβ *ctc*::*lacZ*	2
BSA70	*rsbU*::*kan* (a) SPβ *ctc*::*lacZ*	2
BSA75	*rsbV312 rpsL* SPβ *ctc*::*lacZ*	2
BSA113	*rsbU*::*kan* (a) $P_BΔ28::P_{SPAC}$ (c) pTet-I SPβ *ctc*::*lacZ*	This study
BSA114	*rsbU*::*kan* (a) $P_BΔ28::P_{SPAC}$ (c) *rsbV312* pTet-I SPβ *ctc*::*lacZ*	This study
BSA115	*rsbU*::*kan* (a) $P_BΔ28::P_{SPAC}$ (c) *rsbW313* pTet-I SPβ *ctc*::*lacZ*	This study
BSA123	*rsbU*::*kan* (a) $P_BΔ28::P_{SPAC}$ (c) *rsbX*::*spc* (e) pTet-I SPβ *ctc*::*lacZ*	3
BSA131	*rsbV312 rpsL rsbX*::pWH25[b] (g) SPβ *ctc*::*lacZ*	This study
BSA132	*rsbV312 rsbX*::pWH25[b] (g) SPβ *ctc*::*lacZ*	This study
BSA133	*rsbV312 rsbX*::pWH25[b] (g) *kan* upstream of *spoIIGA* SPβ *ctc*::*lacZ*	This study
BSA135	*rsbUΔNde*I (b) *rsbX*::pWH25[b] (g) SPβ *ctc*::*lacZ kan* upstream of *spoIIGA*	This study
BSA136	*rsbX*::pWH25[b] (g) SPβ *ctc*::*lacZ*	This study
BSA140	*rsbUΔNde*I (b) *rsbX*::pWH25[b] (g) SPβ *ctc*::*lacZ*	This study
BSA141	*rsbU*::pAL127[c] (d)	This study
BSA142	*rsbU*::pAL127[c] (d) SPβ *ctc*::*lacZ*	This study
BSA143	*rsbUΔNde*I (b) *rsbX*::*kan* (f) *rsbX*::pWH25[b] (g) SPβ *ctc*::*lacZ*	This study
BSA147	*rsbU*::*kan* (a) $P_BΔ28::P_{SPAC}$ (c) *rsbX*::pWH25[b] (g) pTet-I SPβ *ctc*::*lacZ*	This study
BSA148	*rsbU*::*kan* (a) $P_BΔ28::P_{SPAC}$ (c) *rsbV312 rsbX*::*spc* (e) pTet-I SPβ *ctc*::*lacZ*	This study
BSA151	*rsbU*::*kan* (a) *rsbX*::*spc* (e) SPβ *ctc*::*lacZ*	This study
BSA152[d]	$P_BΔ28::P_{SPAC}$ (c) *rsbX*::pWH25[b] (g) pTet-I SPβ *ctc*::*lacZ*	This study
BSA155[d]	$P_BΔ28::P_{SPAC}$ (c) *rsbV312 rsbX*::*spc* (e) pTet-I SPβ *ctc*::*lacZ*	This study
BSA156[d]	$P_BΔ28::P_{SPAC}$ (c) *rsbX*::*spc* (e) pTet-I SPβ *ctc*::*lacZ*	This study
HP91	*kan* upstream of *spoIIGA*	Laboratory strain

[a] The letters in parentheses refer to the letters of the different mutations in the *sigB* region as illustrated in Fig. 1.
[b] 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*. It was integrated into the *B. subtilis* chromosome by a Campbell-like recombination that does not disrupt *rsbX* (see Fig. 1).
[c] The integrative plasmid pAL127 contains a 207-bp *Pst*I-*Eco*RI fragment that includes the 3′ end of *rsbU* and the *sigB* promoter. It was integrated into the *B. subtilis* chromosome by a Campbell-like recombination which does not disrupt *rsbU*.
[d] BSA152, BSA155, and BSA156 produce a RsbU protein with a C-terminal extension of 19 amino acids.

that is immediately upstream of the *sigB* operon. *rsbU* was disrupted in the strains used for some of our earlier experiments, thereby masking the effects of RsbX on σ^B activity (3). When *rsbU* is intact, inactivation of *rsbX* results in a dramatic increase in σ^B-dependent transcription, regardless of whether the *sigB* operon is transcribed from its normal promoter or P_{SPAC}. RsbU-dependent activation of σ^B also requires the RsbV product. The need for RsbV suggests that RsbU, or a process dependent on RsbU, facilitates the association of RsbW with RsbV and the release of σ^B.

MATERIALS AND METHODS

Bacterial strains, cultivation conditions, and vectors. The *B. subtilis* strains used in this study are listed in Table 1 and are all derivatives of PY22, which was obtained from P. Youngman (University of Georgia). The strains BSA113, BSA114, and BSA115 contain an insertion of a *kan* (*aph3′5″*) gene in the *Eco*RV site of *rsbU*, a substitution of the wild-type promoter of the *sigB* operon by the P_{SPAC} (30), and pTet-I (3), which carries the *lac*I gene for repression of P_{SPAC}. In addition, strains BSA114 and BSA115 carry null mutations in *rsbV* (*rsbV312*) and *rsbW* (*rsbW313*), respectively. The construction of these mutations has been described elsewhere (3). BSA151 was constructed by transforming BSA70 with pML7/X::*spc* (3), which had been previously linearized with *Sca*I. Spectinomycin-resistant (Sp^r) clones should carry the *rsbX*::*spc* allele, which is introduced by a double-crossover event. The construction of BSA151 was verified by PCR. *Escherichia coli* TG2 (23) was routinely grown in Luria broth (LB) (20) and was used as a host for DNA manipulation. *B. subtilis* strains were cultivated under vigorous agitation at 37°C in LB or in LB supplemented with 5% glucose and 0.2% glutamine. P_{SPAC} was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. pBluescriptSK (23) and pUS19 (3) were used as vectors.

PCRs. PCRs were performed according to standard protocols (23). The following primers were used to prove the presence or absence of the different mutations: (i) substitution of the wild-type promoter by P_{SPAC} and null mutation *rsbV312*, 5′-CTAGGACTGCAGAAGCTCATTGAGGAAC-3′ and 5′GCCT GCCTGCAGACAGTGCTTCCGTCCG-3′ (the presence of the null mutation in *rsbV* was verified by looking for the *Eco*RI site in *rsbV312* created by the null mutation); (ii) deletion of the internal *Nde*I fragment in *rsbU*, 5′-GTGCT GAAGGAGCTGTA-3′ (ALSEQ6) and 5′-TCCTGCGCTTGTTCATC-3′; (iii) integration of *kan* in the *Eco*RV site of *rsbU*, ALSEQ6 and 5′-GTCCCGCT GAAGCATAT-3′; (iv) integration of *spc* into the *Cla*I site of *rsbX*, 5′-TTGAT TCTAGATCCCTCGATGGAGTTA-3′ and 5′-ATCAGCCAATCTCCCTC-3′; and (v) Campbell-like integration of pAL127 near the 3′ end of *rsbU*, ALSEQ6 and the 17-mer M13-pUC sequencing primer (−20) 5′-GTAAAACGACGGC CAGT-3′.

Construction of the deletion mutation in *rsbU*. We cloned a 2.3-kb *Eco*RI fragment of pML7 (6) containing the 3′ end of *rsbU*, intact *rsbV* and *rsbW*, and the 5′ end of *sigB* into pBluescriptSK to yield plasmid pAL57. pAL57 was digested with *Nde*I, recircularized with DNA ligase, and transformed into TG2. The resulting plasmid, pAL102, which lacked the *Nde*I fragment internal to *rsbU*, was used for the congression experiment after linearization with *Sca*I.

A suitable recipient for the congression experiment was constructed in several steps. BSA75 (*rsbV312 rpsL*) was first transformed with the integrational plasmid pWH25, which carries a 2-kb *Eco*RI-*Sph*I fragment containing the 3′ end of *rsbX* and 1.9 kb of DNA downstream of the *sigB* operon inserted into pUS19. The Campbell-like recombination of pWH25 into the chromosome does not disrupt *rsbX* but places an *spc* marker downstream of the *sigB* operon (Fig. 1g). Chromosomal DNA from such a strain (BSA131) was transformed into BSA70, selecting for spectinomycin resistance. The transformants were screened for sensitivity to kanamycin and streptomycin. Loss of the streptomycin resistance mutation in *rpsL* was desirable, because preliminary data indicated that this mutation interferes with the induction of the *sigB* operon by stress. The resulting strain (BSA132) is $RsbU^+$ $RsbV^-$ and carries the *spc* marker downstream of the *sigB* operon. BSA132 was then transformed with chromosomal DNA from a *B. subtilis* strain (HP91), which contains the *kan* gene linked to the *spoIIG* operon. *kan* at this site has no effect on σ^B regulation or activity. A Km^r transformant of BSA132 was selected (BSA133) as a source of chromosomal DNA for the congression experiment. It contains a selectable marker (Km^r) as well as the *rsbU* and *rsbV312* alleles of the recipient strain. In the congression experiment, BSA132 was transformed with approximately 1 μg of chromosomal DNA from BSA133 and 200 μg of *Sca*I-linearized plasmid pAL102. Transformants were selected on LB agar plates containing 10 μg of kanamycin per ml and 100 μl of 2% 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal). RsbV, but not RsbU, is necessary for the induction of *ctc*::*lacZ* in stationary phase. Since both the BSA132 recipient and the BSA133 chromosomal DNA contain null mutations in *rsbV*, blue colonies could arise only if the wild-type *rsbV* allele of the linearized plasmid corrects the mutation in *rsbV*, an event that should carry the linked *rsbU* deletion mutation into the recipient cell's chromosome at high frequency. Chromosomal DNAs were isolated from several of the blue transformants and examined for the *rsbU* deletion by PCR. Chromosomal DNA of one of these transformants (BSA135) was then transformed into BSA46 to create a

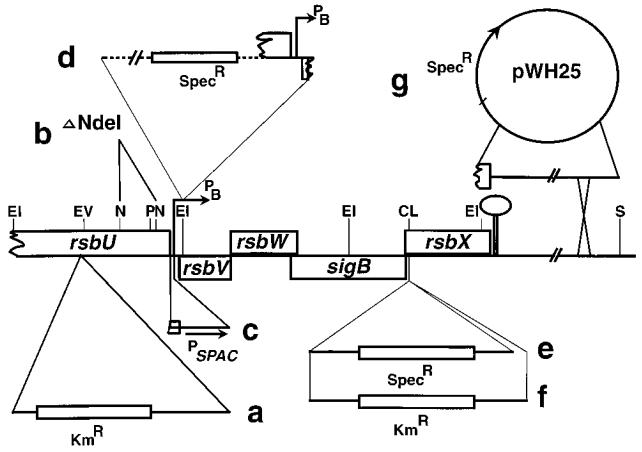

FIG. 1. Mutation map of the *B. subtilis sigB* region. The boxes indicate the locations of *rsbU*, *rsbV*, *rsbW*, *sigB*, and *rsbX*. The direction of transcription and the promoter are marked by an arrow. The stem-loop immediately downstream of *rsbX* represents the terminator of the *sigB* operon. The letters refer to the mutational changes introduced into the *sigB* region as follows: a, insertion of a 1.5-kb cassette conferring resistance to kanamycin into the *Eco*RV site of *rsbU*; b, deletion of a 236-bp *Nde*I fragment from *rsbU*; c, replacement of the wild-type promoter of the *sigB* operon by P_{SPAC}; d, disruption of read-through from *rsbU* into the *sigB* operon by inserting pAL127 by a Campbell-like recombination; e, insertion of a 1.4-kb cassette conferring resistance to spectinomycin into the *Cla*I site of *rsbX*; f, insertion of a 1.5-kb cassette conferring resistance to kanamycin into the *Cla*I site of *rsbX*; g, insertion of pWH25 downstream of the *sigB* operon by a Campbell-like recombination. Abbreviations: CL, *Cla*I; EI, *Eco*RI; EV, *Eco*RV; N, *Nde*I; P, *Pst*I; S, *Sph*I; P_B, wild-type *sigB* promoter.

strain without *kan* in front of *spoIIGA*. The transformants were selected on LB agar plates containing spectinomycin (200 μg/ml) and were screened for sensitivity to kanamycin. The presence of the deletion in *rsbU* was confirmed by PCR. A representative strain was designated BSA140.

Disruption of the potential *rsbU-sigB* transcription unit. pAL127 was constructed by cloning the 207-bp *Pst*I-*Eco*RI fragment from pV312 (2), which contains the 3′ end of *rsbU*, the *sigB* promoter, and the first two codons of *rsbV*, into pUS19. PY22 was transformed with pAL127, selecting for the vector-encoded spectinomycin marker. We verified by PCR the insertion of pAL127, via a Campbell-like recombination, between the 3′ end of *rsbU* and the *sigB* operon in the transformants. Chromosomal DNA of one of these transformants (BSA141) was used to transform BSA46 to Sp^r, creating BSA142.

Reconstruction of *rsbU* in the P_{SPAC} strains. As a first step toward creating P_{SPAC}-controlled *sigB* operons with intact *rsbU* alleles, chromosomal DNA from BSA132 (*rsbV312 rsbX*::pWH25) was transformed into BSA46. Transformants were selected on LB agar plates containing spectinomycin (200 μg/ml) and X-Gal. Blue colonies accounted for approximately 10% of all transformants. Chromosomal DNA was prepared from representative blue colonies, and the presence of the wild-type *rsbV* allele in these DNAs was verified by PCR. The chromosomal DNA of one of the transformants (BSA136) was used to introduce the *spc* gene downstream of the *sigB* operon in BSA113, generating BSA147. Sp^r could then be used in transformations as a selectable marker to separate P_{SPAC} from the *kan* gene in *rsbU*. Chromosomal DNA of BSA147 was prepared and transformed into BSA46 containing pTet-I. Transformants were selected on LB agar plates with spectinomycin. Colonies were screened for sensitivity to kanamycin, with the presence of P_{SPAC} being confirmed by PCR. BSA152 has a functional copy of *rsbU* and the *sigB* operon under the control of P_{SPAC}.

For the construction of an $RsbU^+$ strain with mutations in both *rsbV* and *rsbX*, BSA114 was transformed with the plasmid pML7/X::Spc linearized with *Pvu*I to yield BSA148 (*rsbU*::*kan* $P_B\Delta 28$::P_{SPAC} *rsbV312 rsbX*::*spc*). Chromosomal DNA from BSA148 was used to transform BSA46, containing pTet-I, to resistance for spectinomycin with transformants screened for sensitivity to kanamycin ($RsbU^+$). Chromosomal DNAs were prepared from several kanamycin-sensitive clones and were tested by PCR for the presence of P_{SPAC}, the *rsbV312*, allele and the insertion of *spc* in *rsbX*. This screening resulted in BSA155.

For the construction of BSA156 ($P_B\Delta 28$::P_{SPAC} *rsbX*::*spc*), chromosomal DNA of BSA123 (*rsbU*::*kan* $P_B\Delta 28$::P_{SPAC} *rsbX*::*spc*) was transformed into BSA46 pTet-I. Transformants were initially selected with spectinomycin and were screened for sensitivity to kanamycin and the presence of P_{SPAC}.

It should be noted that our replacement of the wild-type promoter of the *sigB* operon by P_{SPAC} involved a deletion of 28 bp which removed not only the *sigB* promoter but also the stop codon of *rsbU* (3). As a result, the reconstitutions of *rsbU* described above generate an *rsbU* allele which lacks its stop codon. Its product is an RsbU protein with a C-terminal extension of 19 amino acids. We do not know whether this extension influences any of RsbU's activities; however, it does not seem to impair its ability to activate σ^B (see Results).

DNA sequencing. A 0.9-kb *Eco*RI-*Pst*I fragment of pML7 (6) was cloned into pBluescript. Both strands of the insert were sequenced by the Sanger method with Sequenase reagents (U.S. Biochemical Corp.) and with the protocol provided by the manufacturer, with plasmid DNA as the template.

SDS-PAGE and Western blot (immunoblot) analysis. Bacteria were disrupted by passage through a French press. The protein concentration was determined by a Bio-Rad protein assay according to the manufacturer's instructions. For the detection of RsbV, RsbW, SigB, and RsbX or the Ctc-LacZ fusion protein, the extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on gels containing 17.5 or 10% acrylamide, respectively. All additional steps of the Western blot analysis were performed as described elsewhere (3).

Assay of β-galactosidase activity. Quantification of β-galactosidase fused to Ctc was used as an indirect measurement of the activity of σ^B. The cells were harvested by centrifugation and were frozen at −20°C. For the assay of β-galactosidase activity according to the method described by Kenney and Moran (18), bacteria were permeabilized with chloroform and SDS. The activity was expressed in Miller units $[10^3 \times A_{420}/(\text{volume} \times \text{time} \times A_{540})]$ (20).

Gel filtration chromatography. Exponentially growing cells were induced with IPTG for 30 min, immediately chilled by pouring the culture into centrifuge bottles containing equal volumes of ice chips, and harvested. Cells were concentrated 200-fold in resuspension buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 50 mM NaCl, 10 mM $MgCl_2$, 0.3 g of phenylmethysulfonyl fluoride per liter, 3 mM dithiothreitol). Extracts were prepared and analyzed on a Sephacryl S-200 column as described previously (11).

General genetic methods. Plasmid isolation, restriction enzyme analysis, and transformation of *E. coli* were performed according to the methods described by Sambrook et al. (23). Transformation of natural competent *B. subtilis* cells with plasmid and chromosomal DNAs was carried out according to the method described by Yasbin et al. (31). Transformants were selected on LB agar plates containing 10 μg of kanamycin or 200 μg of spectinomycin per ml. For the transformation of linearized pAK24 into BSA46 and BSA140, transformants were selected on LB agar plates with only 5 μg of kanamycin per ml, because the pinpoint colonies did not grow on plates with 10 μg of kanamycin per ml.

Computer analysis of sequence data. The sequence data manipulations were performed with the sequence analysis software package from the Genetics Computer Group Inc. (Madison, Wis.).

Nucleotide sequence accession number. The nucleotide sequence data reported here appear in the EMBL and GenBank nucleotide sequence databases under accession number X81652.

RESULTS

Sequences upstream of the *sigB* operon affect RsbX function. Previous data suggested that RsbX could be a specific regulator of the *sigB* operon's σ^B-dependent promoter (3); however, we have been unable to demonstrate binding of RsbX to this promoter (data not shown). To see whether the presence of a *kan* (*aph3′5″*) gene, inserted upstream of the *sigB* promoter (Fig. 1a) in some of the strains that were used in these studies of RsbX function, might have obscured the true role of RsbX, we created isogenic $RsbX^-$ strains that either carry or lack the upstream insertion. *B. subtilis* strains with *lacZ* joined to a promoter dependent on σ^B (SPβ *ctc*::*lacZ*) and either the *kan* insertion (BSA70) or an intact upstream region (BSA46) were transformed with *sigB* DNA (linearized plasmid pML7/X::*spc*) that included a spectinomycin resistance gene within *rsbX*. Sp^r transformants arose by replacement of the wild-type *rsbX* allele by *rsbX*::*spc* (Fig. 1e). As can be seen by the colony morphologies of the Sp^r transformants (Fig. 2), the presence or absence of the upstream interruption had a significant effect on the $RsbX^-$ phenotype. The bulk of the Sp^r clones that arose from the strain lacking the *kan* insertion formed dark-blue pinpoint colonies on medium that contained X-Gal (Fig. 2A). This is the anticipated $RsbX^-$ phenotype, caused by high levels of σ^B activity, which inhibits cell growth (2, 9, 15). Some large colonies appeared at a low frequency. These presumably contained additional mutations that suppressed the loss of σ^B control and allowed the cells to grow more normally (2, 9, 15). In contrast, the Sp^r transformants of the *kan* insertion strain formed only large, light-blue colonies on medium containing X-Gal (Fig. 2B). These differences in

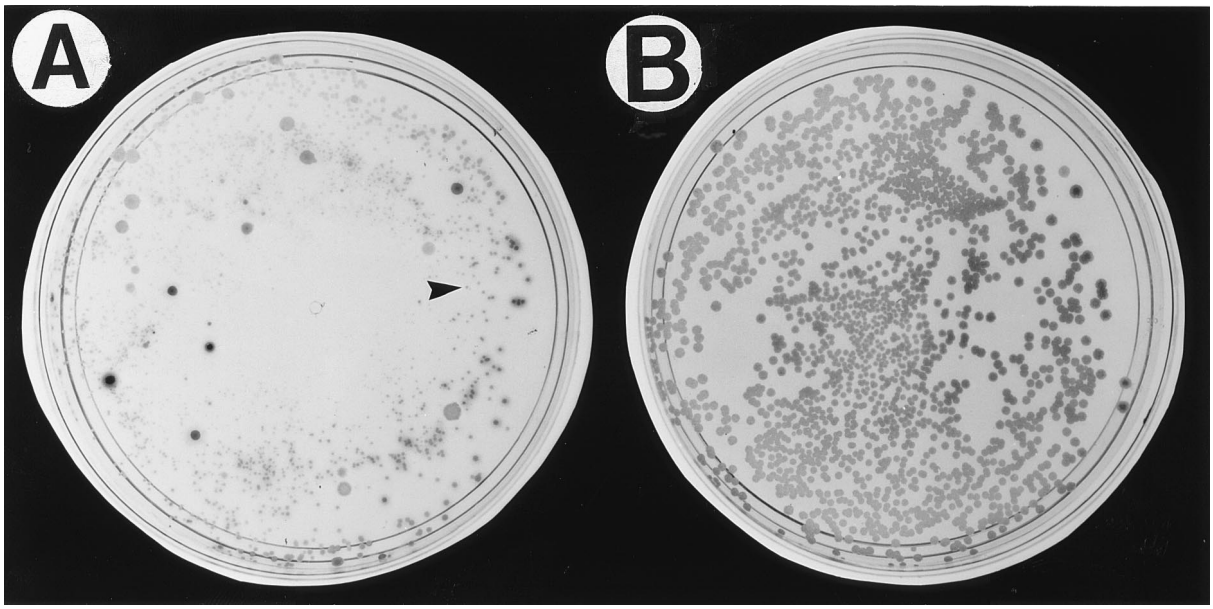

FIG. 2. Colony morphologies of Km^s $RsbX^-$ and Km^r $RsbX^-$ transformants. The photograph depicts BSA46 (wild type) (A) and BSA70 (*kan*) (B) transformed with *Pvu*I-digested pML7/X::*spc*. The transformants were selected on LB agar plates containing spectinomycin and X-Gal. The arrow points to a typical dark-blue pinpoint colony on the BSA46 transformation plate.

colony size and apparent σ^B activity suggested that the *kan* insertion was suppressing the $RsbX^-$ phenotype.

To test whether the large-colony phenotype of the Sp^r transformants of BSA70 is linked to the *kan* insertion and is not the result of an unknown mutation that occurred somewhere else on the chromosome, we prepared chromosomal DNA from BSA151 (*kan rsbX*::*spc*; Fig. 1a and e) and introduced this DNA into a wild-type *B. subtilis* strain (BSA46) selecting for either Km^r or Sp^r (*rsbX*::*spc*). We scored the transformants for coinheritance of the unselected marker (Km^r or Sp^r) and the large- and/or small-colony phenotype. The results are presented in Table 2. All of the Km^r clones displayed the large-colony phenotype, although 90.5% of them were also Sp^r ($RsbX^-$). Among the transformants selected for Sp^r, none of the pinpoint colonies (73 tested) was Km^r. The large colonies arising from this transformation (89.2% of the Sp^r transformants) were all Km^r. The frequency of pinpoint transformant colonies (10.8%) is not significantly different from the segregation frequency of the *kan* and *rsbX*::*spc* markers (9.5%). We interpret these results as evidence that the large-colony phenotype of the *kan rsbX*::*spc* transformants (BSA151) is caused by the *kan* insertion upstream of the *sigB* operon and that this insertion at least partially masks the effects of a loss of RsbX on σ^B regulation.

TABLE 2. Cotransformation of Km^r and Sp^r from BSA151 into BSA46[a]

Primary selection	Phenotype of colonies	Screening result		
		No. of colonies tested	No. of colonies resistant to second antibiotic	%[b]
Sp^r	Big, light blue[c]	184	183 Km^r	99.4[d]
Sp^r	Pinpoint, dark blue[c]	73	0 Km^r	0
Km^r	Big, light blue	610	552 Sp^r	90.5
Km^r	Pinpoint, dark blue[e]		None detected	

[a] The locations of the insertion of *kan* (Km^r) and *spc* (Sp^r) are indicated in Fig. 1 (insertions a and e, respectively).

[b] Frequency of cotransformation of both antibiotic resistance markers.

[c] The selection for Sp^r yielded big light-blue and small pinpoint dark-blue colonies on plates containing X-Gal. Of the 3,248 colonies counted, 2,898 were big and light blue and 350 were pinpoint and dark blue, which corresponded to 89.2 and 10.8%, respectively.

[d] The one colony which failed to grow on LB plates containing kanamycin is likely a *rsbX*::*spc* strain that contains a suppressor mutation. Suppressor mutations commonly arise in $RsbX^-$ *B. subtilis* (2, 15, 17) (Fig. 2A).

[e] The selection for Km^r generated only big and light-blue colonies and no pinpoint and dark-blue colonies.

The *kan* insertion disrupts an upstream gene (*rsbU*) needed for RsbX-dependent regulation. C. Price and colleagues have noted the existence of a four-gene cluster of undefined function upstream of the *sigB* operon (9, 28). To determine the position of the *kan* insertion relative to this cluster, we sequenced an *Eco*RI-*Pst*I fragment (947 bp) that lies immediately upstream of the previously published *sigB* operon sequence, using pML7 (6) subclones as sources of DNA. The sequence revealed a large open reading frame (>360 codons) which begins beyond our cloned DNA and extends into the *sigB* operon sequence (Fig. 3). A more extensive sequencing of this region, which is reported in the accompanying paper by Wise and Price, led to the identification of a translational termination sequence immediately upstream of our cloned DNA (29). On the basis of this result, it is likely that the amino terminus of RsbU, as indicated by Wise and Price, is at the GTG which is depicted as the 26th codon of our sequence (Fig. 3). The predicted *rsbU* product, inferred from its sequence, has no significant homology to any protein in the protein data bank, except for a limited homology with RsbX (34% identity in a 43-amino-acid overlap using the BLAST algorithm and 25% identity in a 146-amino-acid overlap using the FASTA algorithm, which allows the introduction of gaps). The *kan* insertion occurs at the *Eco*RV site, 212 codons from the predicted 3′ end of *rsbU* (Fig. 3). The 3′ end of *rsbU* overlaps the −35 box of the *sigB* operon promoter, with no obvious terminator between *rsbU* and the *sigB* operon. The potential *rsbU*-encoding transcript could therefore extend into the *sigB* operon. The idea that the previously recognized *sigB* operon

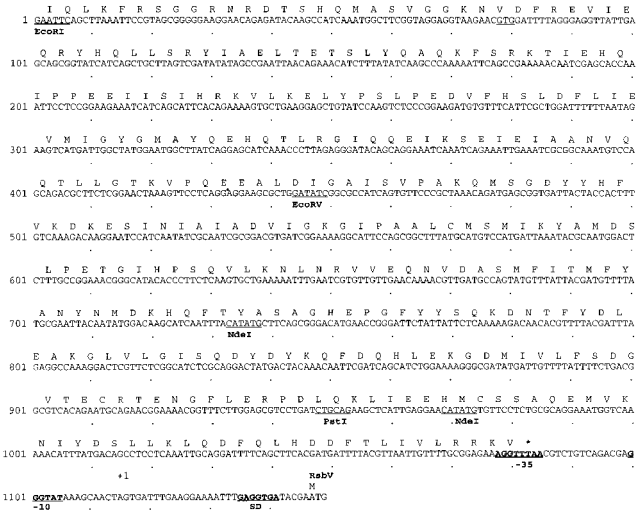

FIG. 3. Nucleotide sequence of *rsbU* region. Nucleotides are numbered from the 5′ end of the nontranscribed strand, and the predicted amino acid sequence of RsbU is given in the single-letter code above the DNA sequence. The newly determined sequence extends from nucleotides 1 to 947. The amino terminus of RsbU is likely to be codon 26 (underlined) of the illustrated open reading frame, because of a translational stop codon immediately upstream of our sequence (28). The reading frame extends into previously published sequence data (17), which are reproduced in the figure as nucleotides 948 to 1149, and terminates within the promoter of the *sigB* operon. The −35 and the −10 regions of the σ^B-dependent promoter of the *sigB* gene are displayed in boldface letters and are underlined. The potential Shine-Dalgarno (SD) sequence and the start of translation of *rsbV* are marked by boldface letters and are underlined. A number of restriction endonuclease sites used in the present study are also indicated.

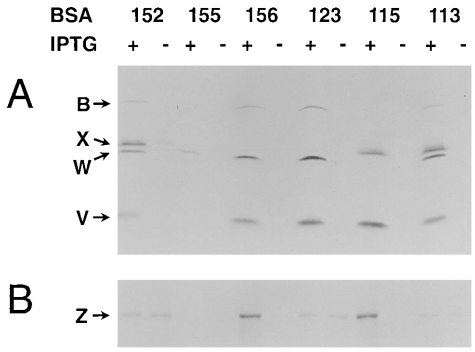

FIG. 4. Induction of the *sigB* operon products and a Ctc-LacZ fusion protein by IPTG. The *B. subtilis* strains indicated in the figure were incubated for 30 min with IPTG during exponential growth. All strains contain SPβ *ctc*::*lacZ*, in addition to an IPTG-inducible promoter at *sigB*, and are as follows: BSA152 (wild type), BSA155 (*rsbV312 rsbX*::*spc*), BSA156 (*rsbX*::*spc*), BSA123 (*rsbU*::*kan rsbX*::*spc*), BSA115 (*rsbU*::*kan rsbW313*), and BSA113 (*rsbU*::*kan*). Crude extracts were prepared as described in Materials and Methods from cultures exposed (+) or unexposed (−) to IPTG, and equal amounts of protein were analyzed by Western blotting. The nitrocellulose blots were probed with monoclonal antibodies raised against RsbV, RsbW, σ^B, and RsbX (A) or anti-β-galactosidase antibodies (B). Abbreviations: B, σ^B; V, RsbV; W, RsbW; X, RsbX; Z, Ctc-LacZ fusion protein.

could be part of a larger operon structure is also advanced by Wise and Price, who show that the upstream transcription unit which includes *rsbU* extends into the *sigB* operon (29). The presence of an additional transcription unit raises the possibility that the insertion of *kan* into the *Eco*RV site of *rsbU* affects σ^B regulation by either disrupting the *rsbU* reading frame or by blocking extension of the upstream transcript into *sigB*.

We designed two experiments to examine whether it is the loss of RsbU or the disruption of an extended transcript that suppresses the RsbX$^-$ phenotype of the *kan* insertion strain. To investigate the role of the RsbU protein, we introduced a 236-bp deletion (Δ*Nde*I; Fig. 1b) internal to *rsbU* into the *B. subtilis* chromosome, creating the strain BSA140. Δ*Nde*I alters the reading frame and leads to a translational stop 13 codons downstream of the religated *Nde*I site. The *rsbU*Δ*Nde*I product lacks the 115 C-terminal amino acids of the wild-type RsbU. BSA46 (intact *rsbU*) and BSA140 (*rsbU*Δ*Nde*I; Fig. 1b) were transformed with a linearized plasmid (pAK24) that contains an insertion of *kan* inside *rsbX* (Fig. 1f). The bulk of BSA46 transformants (*rsbU rsbX*::*kan*; Fig. 1f) formed dark-blue pinpoint colonies (data not shown). In contrast, all BSA140 (*rsbU*Δ*Nde*I *rsbX*::*kan*; Fig. 1b and f) transformants formed large light-blue colonies (data not shown). Deletion of the 115 C-terminal amino acids of RsbU is thus sufficient to suppress the RsbX$^-$ phenotype. This result argues that it is RsbU and not a putative extended transcript whose loss affects RsbX-dependent regulation of σ^B. We tested this idea in a second experiment, in which we separated the *sigB* operon from the upstream transcription unit while keeping *rsbU* intact. This involved a Campbell-like insertion of pAL127 (Fig. 1d), an integrative plasmid whose insert contains the 3′ end of *rsbU* and the *sigB* promoter, into the chromosome of BSA46. When the resulting strain (BSA142) was transformed with the linearized pAK24 (*rsbX*::*kan*; Fig. 1f), the bulk of the transformants formed dark-blue pinpoint colonies (data not shown). The RsbX$^-$ phenotype is thus not significantly impaired in the *rsbU*::pAL127 strain, although the potential *rsbU-sigB* transcription unit is disrupted. It is formally possible that the out-of-frame deletion has an unforeseen polar effect on downstream transcription and that the integrated plasmid provides a fortuitous promoter for read-through transcription into *sigB*; however, we consider this unlikely and interpret our results as evidence that it is the loss of RsbU function rather than an interruption of extended transcription from *rsbU* which is responsible for the suppression of the RsbX$^-$ phenotype in *rsbU*::*kan* strains. Wise and Price have also concluded that *rsbU* encodes a protein involved in the regulation of σ^B-dependent genes (29).

RsbX regulates σ^B activity. The discovery that an RsbX$^-$ phenotype is observed only in RsbU$^+$ *B. subtilis* strains prompted us to reexamine the question of whether RsbX controls synthesis or activity of σ^B. Our previous study, in which a loss of RsbX appeared to have no effect on σ^B activity, was performed with P_{SPAC}-dependent *sigB* operons in strains that had acquired *kan* in *rsbU* during their construction (3). We now know that the absence of the RsbU function alone, rather than the substitution of the *sigB* promoter by P_{SPAC}, could account for the lack of an RsbX$^-$ phenotype in these strains. Therefore, we constructed RsbX$^+$ and RsbX$^-$ strains (BSA152 and BSA156, respectively) that have the *sigB* operon under the control of P_{SPAC} but lack *kan* within *rsbU* to determine if RsbX could influence σ^B activity when *rsbU* is intact. RsbU$^-$ (BSA113), RsbU$^-$ RsbW$^-$ (BSA115), and RsbU$^-$ RsbX$^-$ (BSA123) strains were also included in the experiments in order to allow a comparison with our previous investigation (3). Expression of the *sigB* operon was induced by the addition of IPTG during vegetative growth, and a *ctc*::*lacZ* reporter system was used to monitor σ^B activity. Figure 4 shows Western blot analyses of the four *sigB* operon products (Fig. 4A) and of a Ctc-LacZ fusion protein (Fig. 4B) produced with or without induction by IPTG. The *sigB* operon products are induced by IPTG in all strains, with the presence or absence of particular *sigB* operon products in individual strains

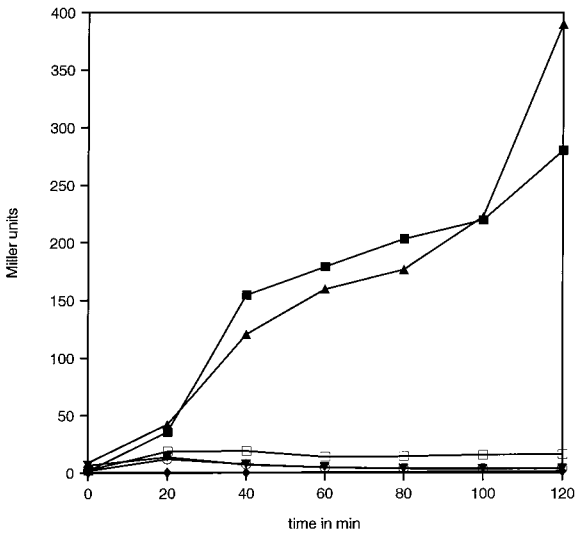

FIG. 5. *ctc*::*lacZ* activity in strains with P_{SPAC}-controlled *sigB* operons. Bacteria were grown in LB supplemented with 5% glucose and 0.2% glutamine and were induced with IPTG during exponential growth. The cells were harvested at the times indicated after the addition of IPTG (zero time), and the activity of β-galactosidase was assayed as described in Materials and Methods. □, BSA113 (*rsbU*::*kan*); ■, BSA115 (*rsbU*::*kan rsbW313*); ▼, BSA123 (*rsbU*::*kan rbsX*::*spc*); ○, BSA152 (wild type); ◆, BSA155 (*rsbV312 rsbX*::*spc*); ▲, BSA156 (*rsbX*::*spc*).

confirming the genotypes of those strains (Fig. 4A). It should be noted that the $RsbW^-$ strain (BSA115) accumulates much less σ^B, but not less RsbV and RsbX, than do the $RsbW^+$ strains (Fig. 4A). This has been seen in previous studies and apparently reflects a need for cosynthesis of RsbW and σ^B for σ^B accumulation (3).

In our previous study using $RsbU^-$ strains, only a *sigB* operon lacking a functional *rsbW* gene yielded high levels of active σ^B when its expression was induced in exponentially growing cultures (3). Using an anti-β-galactosidase antibody to monitor Ctc-LacZ accumulation by Western blotting (Fig. 4B), we verify this result, finding that the level of Ctc-LacZ is changed only slightly after induction of *sigB* operons in BSA113 ($RsbU^-$ $RsbX^+$) and BSA123 ($RsbU^-$ $RsbX^-$) but is significantly elevated in BSA115 ($RsbU^-$ $RsbW^-$). No appreciable change in Ctc-LacZ levels is seen following induction of the *sigB* operon of BSA152 ($RsbU^+$ $RsbX^+$); however, induction of the *sigB* operon of BSA156 ($RsbU^+$ $RsbX^-$) causes an accumulation of Ctc-LacZ fusion protein that is similar to the level seen in a strain lacking RsbW (BSA115, Fig. 4B). The degree of expression of the *ctc* promoter after P_{SPAC} induction was quantitated by colorimetric assaying of β-galactosidase activity (Fig. 5). The results of this assay correlate well with the Western blot data of Fig. 4B and show that the amounts of Ctc-LacZ accumulated in BSA115 and BSA156 are equivalent and substantially greater than those seen in the other strains. We conclude that induction of the *sigB* operons of BSA115 and BSA156 results in the formation of similar amounts of active σ^B. This result argues that RsbX, like RsbW and RsbV, is a regulator of σ^B activity; however, unlike RsbV and RsbW, RsbX requires a functional *rsbU* allele to display its activity. RsbU, or a process dependent on RsbU, appears to be RsbX's primary target. Presumably, an RsbU-dependent process can activate σ^B in the absence of RsbX.

RsbU acts through the RsbV-RsbW pathway. With the exception of heat shock activation, all known σ^B activation processes require RsbV (2, 3, 4, 7, 9). Therefore, we wished to test whether the RsbU-dependent activation that occurs in the absence of RsbX also requires RsbV or whether it defines a novel σ^B activation system. To address this, we constructed a $RsbU^+$ $RsbV^-$ $RsbX^-$ *sigB* operon under the control of P_{SPAC} (strain BSA155) and examined σ^B activity following its induction. IPTG induction of *sigB* in BSA155 results in the production of σ^B and RsbW (Fig. 4A); however, unlike the high levels of σ^B activity seen in the $RsbU^+$ $RsbX^-$ strain, BSA155 fails to elicit detectable σ^B activity (Fig. 4B and 5). The $RsbV^-$ phenotype is therefore epistatic to $RsbX^-$. We conclude that RsbU-dependent activation of σ^B requires RsbV.

RsbV-dependent activation of σ^B is believed to involve the release of σ^B from σ^B-RsbW complexes coincident with the formation of RsbV-RsbW complexes (5, 11). The need for RsbV in RsbU-dependent σ^B activation suggests that RsbU participates in a process that facilitates RsbW-RsbV association. To test this idea, we determined the association state of *sigB* operon products in strains harboring P_{SPAC}-controlled *sigB* operons and that are $RsbU^-$ $RsbX^-$ (BSA123) or $RsbU^+$ $RsbX^-$ (BSA156). We predict that there would be an increase in RsbV-RsbW complexes in the extract from the strain with the active σ^B ($RsbU^+$ $RsbX^-$) compared with that present in the extract from the strain with the relatively inactive σ^B ($RsbU^-$ $RsbX^-$). As in previous experiments (11), we used gel filtration chromatography to fractionate crude *B. subtilis* extracts. The fractions were analyzed for their RsbV, RsbW, and σ^B contents by Western blotting. As anticipated, the distribution of RsbV in the gel filtration fractions is dependent on the RsbU phenotype of the strain from which the extract was prepared (Fig. 6A compared with B). RsbV from both extracts eluted in a form (Fig. 6A and B [peak fractions 24 to 26]) that had previously been identified as nonassociated RsbV (11); however, a second peak appeared in the $RsbU^+$ $RsbX^-$ extract but not in the $RsbU^-$ $RsbX^-$ extract (Fig. 6A). This second peak lies at the anticipated position of RsbV-RsbW complexes (Fig. 6B [fractions 6 to 12]) (11). RsbV-RsbW complexes, therefore, appear to be more abundant in the $RsbU^+$ extract than in the extract from the $RsbU^-$ strain.

We had previously determined that RsbW not only can associate with RsbV but also can phosphorylate RsbV. Furthermore, the phosphorylation state of RsbV was found to be a predictor of its association with RsbW. Only unphosphorylated RsbV was detected in RsbV-RsbW complexes. In keeping with this earlier finding, an analysis by isoelectrofocusing (11) of the phosphorylation state of RsbV from different gel filtration fractions showed that nonassociated RsbVs from both strains were phosphorylated, whereas RsbV complexed to RsbW in the $RsbU^+$ extract was unphosphorylated (data not shown). The formation of an RsbW-RsbV complex thus continues to correlate with the presence of unphosphorylated RsbV.

The amount of RsbV involved in the RsbW-RsbV complex of the $RsbU^+$ $RsbX^-$ strain is modest, compared with that found in the nonassociated state (Fig. 6B [compare fractions 6 to 12 with fractions 19 to 29]). This is likely the reason for the similar abundance of σ^B-RsbW complexes in the $RsbU^+$ and $RsbU^-$ extracts (Fig. 6A and B [fractions 1 to 8]). Apparently, a relatively small amount of σ^B is released from σ^B-RsbW complexes in the $RsbU^+$ strain, with the bulk of the σ^B still joined to RsbW. We attempted to detect the released σ^B by analyzing the gel filtration fractions containing proteins that were excluded from the gel matrix (>200 kDa) (Fig. 6C and D). These fractions contain RNA polymerase and should contain σ^B if it is now joined to this enzyme. Figure 6 illustrates that when similar amounts of extract were analyzed, σ^B was barely observed in RNA polymerase-containing fractions of the $RsbU^-$ extract (Fig. 6C) but was present in higher levels in

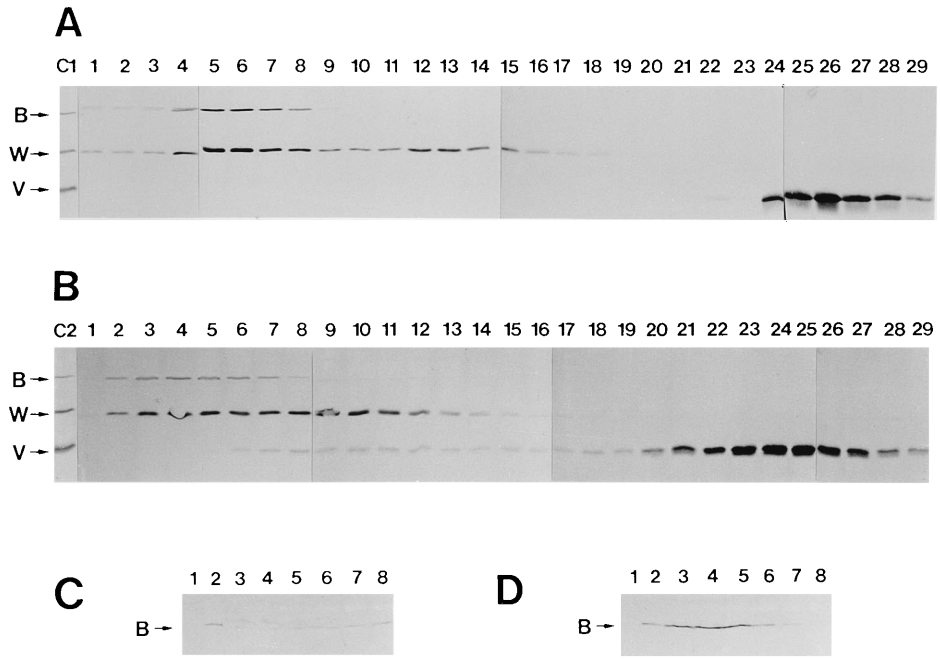

FIG. 6. Western blot analysis of gel filtration chromatography fractions of crude *B. subtilis* extracts. The *sigB* operon under the control of P_{SPAC} was induced by the addition of IPTG to exponentially growing *B. subtilis* cultures, and the cells were harvested 30 min later as described in Materials and Methods. Extracts were prepared and fractionated on a Sephacryl S-200 gel filtration column as described in Materials and Methods. Samples of the included fractions (A and B) and the excluded fractions (C and D) were ethanol precipitated and analyzed by Western blotting using anti-σ^B, -RsbW, and -RsbV monoclonal antibodies or anti-σ^B monoclonal antibody as a probe for the included or excluded fractions, respectively. Analyses of BSA123 (*rsbU*::*kan rsbX*::*spc*) (A and C) and BSA156 (*rsbX*:*spc*) (B and D) fractions are shown. Lanes C1 and C2 are unfractionated extracts from BSA123 and BSA156, respectively. The positions of σ^B (B), RsbW (W), and RsbV (V) are indicated.

similar fractions of the $RsbU^+$ extract (Fig. 6D). Thus, elevated σ^B in the RNA polymerase-containing fractions is coincident with the presence of demonstrable RsbV-RsbW complexes.

DISCUSSION

There is compelling evidence that the activities of at least three *B. subtilis* σ factors (σ^B, σ^F, and σ^G) are controlled by anti-σ factor proteins (5, 12, 19, 21, 22, 24). These regulators sequester their target σ's into complexes wherein they are unavailable to RNA polymerase (1, 5, 12, 21). In the cases of σ^F and σ^G, the known regulatory proteins are limited to a common anti-σ factor, SpoIIAB, and its antagonist, SpoIIAA (1, 12, 19, 21, 24). An attractive model has been offered to explain how these proteins interact to control σ activity. σ^F, and presumably σ^G, regulation is hypothesized to be effected by the binding of SpoIIAB, alternatively, to either a σ factor or SpoIIAA in response to changes in the cell's ATP/ADP ratio (1). A high ATP/ADP ratio is thought to favor the formation of the SpoIIAB-σ complex, while a low ATP/ADP ratio promotes the SpoIIAB-SpoIIAA complex. In addition to the hypothetical ATP/ADP-driven changes in SpoIIAB's binding preference, SpoIIAB can phosphorylate SpoIIAA (21). This reaction is thought to be important for proper σ^F regulation; however, its role in this process is not yet clear. The σ^B regulatory system appears to be more intricate than the corresponding system that controls σ^F and σ^G. Aside from an anti-σ^B protein (RsbW) and its antagonist (RsbV), σ^B activity is now known to be influenced by two additional proteins, RsbX and RsbU. As with the σ^F and σ^G systems, the principal σ^B regulator is the anti-σ factor; however, the inducing stimuli are more diverse. There is a growing list of conditions, including entry into stationary phase, heat shock, osmotic shock, ethanol treatment, and anaerobiosis, etc., which lead to activation of σ^B (2, 4, 7, 9, 16, 26). In each instance, activation of σ^B presumably involves its release from RsbW. It is possible, as has been suggested by Alper et al. (1), that all of these inducing conditions generate a common internal signal for σ^B release. They propose that the fluctuations in ATP/ADP ratios, which may be responsible for σ^F release, could occur following exposure of *B. subtilis* to different forms of stress, thereby inducing σ^B release by a similar mechanism (1). Alternatively, release of σ^B from RsbW may be accomplished by multiple means, depending on the initiating signal. Clearly, an alternative mechanism of σ^B activation occurs during heat shock. Unlike σ^B activation by the other known stressors, which is absolutely dependent on RsbV, at least a portion of the σ^B activation during heat shock is independent of RsbV, i.e., it can occur in an $RsbV^-$ background (4, 7). Either RsbW or the σ^B-RsbW complex could be inherently thermolabile, or there may be another factor in *B. subtilis* that can substitute for RsbV following heat shock. If the latter is true, RsbW may represent a common target at which distinct signals from separate effectors are integrated to modulate the σ^B response.

Multiple inducing signals for σ^B activation are suggested by our current data. The known negative regulator of σ^B, RsbX, is now shown to participate in a previously unrecognized extension of the RsbV-dependent pathway of σ^B activation, which appears to be dispensable under some circumstances. RsbU is a potent activator of σ^B in the absence of RsbX; however, we did not detect an $RsbU^-$ phenotype in our previous studies. We believe that this is likely due to the conditions under which RsbU contributes to σ^B activation. Our earlier experiments were largely limited to σ^B activation upon entry of *B. subtilis* cultures into the stationary phase of growth (2, 3), and, in our hands, RsbU is not a major effector of σ^B

activation during stationary phase. In the accompanying paper (29), Wise and Price present evidence that the expression of the σ^B-dependent *ctc* promoter is enhanced severalfold by a *rsbU* mutation when the strain which carries it enters stationary phase in LB. We fail to find a significant difference in the activity of σ^B-dependent promoters in our RsbU$^+$ and RsbU$^-$ strains under this condition. There may be additional unknown features of each of the strains that are contributing to the minor differences that are appearing in the results. Our failure to find an important role for RsbU in the stationary-phase activation of σ^B argues that the RsbU-RsbX pathway is responding to signals which are distinct from those employed to activate σ^B when exponential growth ceases. We have data that show that although the RsbU-dependent pathway is relatively unimportant for stationary-phase activation of σ^B in LB, it is important for σ^B activation in response to other environmental stresses (e.g., ethanol and salt, etc.) (27). This idea that RsbU may participate in the activation of σ^B following stress is also proposed by Wise and Price (29). These authors also present evidence that RsbU is required for full glucose-glutamine induction of σ^B-dependent genes (29). We see a similar need for RsbU in the full induction of *ctc* under these conditions; however, we believe that the role of RsbU in this induction is restricted to σ^B activation as a stress response to the pH drop that occurs in this medium and not part of the stationary-phase response per se (27).

In previous experiments, we were able to separate free RsbV from RsbV-RsbW complexes by gel filtration chromatography of crude *B. subtilis* cell extracts (11). We noted that two prominent forms of RsbV are present in crude cell extracts of *B. subtilis*. These RsbV proteins differ in their phosphorylation states, with only the unphosphorylated form of RsbV found to be associated with RsbW (11). This result suggested that the differential binding of RsbW to either RsbV or σ^B could be at least partially controlled by phosphorylation of RsbV; however, we were not able to identify an induction condition under which changes in σ^B activity could be correlated with changes in the RsbW-RsbV association. We now believe that this failure was likely due to the conditions under which the cells were harvested and extracts were prepared. During the present study, we discovered that unless the *B. subtilis* cultures are rapidly chilled by mixing with ice chips, RsbV can become associated with RsbW during harvesting. Apparently, the stresses incurred by the harvesting procedure induce RsbV-RsbW complex formation and σ^B activation. Using a modified harvesting procedure, we now observe a direct correlation between the presence of RsbV-RsbW complexes in cell extracts and the in vivo activity of σ^B in the cells from which these extracts were prepared (Fig. 6).

The phenotypes of single and multiple mutations in σ^B regulators and the biochemical evidence for their interactions can be used to formulate the model illustrated in Fig. 7. As in previous models, RsbW is viewed as the principal regulator of σ^B activity, with RsbV antagonizing RsbW's sequestration of σ^B into an inactivating complex. If the RsbW-RsbV complex is produced, σ^B remains free to form an active RNA polymerase holoenzyme. RsbV can also be phosphorylated by RsbW. RsbV is present in phosphorylated (RsbV-P) and unphosphorylated (RsbV) states in *B. subtilis* extracts. RsbV is found in RsbV-RsbW complexes, while RsbV-P is unassociated. RsbV phosphorylation may be a consequence of RsbW's binding decision. We have not distinguished between the phosphorylation of RsbV as a device to prevent RsbV's future binding to RsbW or as the result of a reaction that RsbW must catalyze in order to bind to σ^B. RsbU and RsbX can now be added to the model. On the basis of the phenotypes of the mutants constructed in the present study, RsbU or a factor dependent on RsbU can be viewed as a positive regulator of σ^B activity that is normally prevented from acting by RsbX or a factor under RsbX's control. The finding that RsbU-dependent activation requires a functional *rsbV* allele places RsbU in the RsbV-RsbW pathway. RsbU, directly or indirectly, facilitates the RsbV-RsbW association, leading to the release of σ^B.

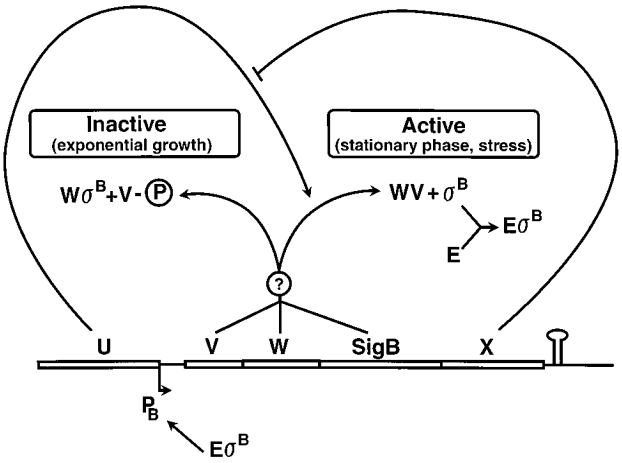

FIG. 7. Model for the regulation of σ^B. RsbW is the principal regulator of σ^B. In response to environmental stimuli, RsbW is proposed to form mutually exclusive complexes between itself and either RsbV or σ^B. If the RsbW-RsbV complex is formed, σ^B remains free to form an active RNA polymerase holoenzyme (Eσ^B). If the RsbW-σ^B complex forms, σ^B is sequestered into an association that blocks its availability to RNA polymerase. RsbV can also be phosphorylated by RsbW. RsbV is present in phosphorylated (RsbV-P) and unphosphorylated states (RsbV) in *B. subtilis* extracts. RsbV is found in RsbW-RsbV complexes, while RsbV-P is unassociated. Phosphorylation of RsbV may be a device to prevent future binding of RsbV to RsbW or may be a result of a reaction that RsbW undergoes in order to bind to σ^B. RsbU is viewed as a component of a pathway that facilitates the formation of the RsbW-RsbV complex under a subset of activating conditions. This pathway is negatively regulated by RsbX. Finally, the *sigB* operon is autoregulated, with its primary promoter being transcribed by Eσ^B. Other abbreviations are as in the legend to Fig. 6.

Although additional factors have now been added to the σ^B story, the mechanisms by which σ^B is released from RsbW and the intracellular cues involved remain a mystery.

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