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Regulation of σ^B Levels and Activity in *Bacillus subtilis*

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The *sigB* operon of *Bacillus subtilis* encodes σ^B plus three additional proteins (RsbV, RsbW, and RsbX) that regulate σ^B activity. Using an anti-σ^B monoclonal antibody to monitor the levels of σ^B protein, P_{SPAC} to control the expression of the *sigB* operon, and a *ctc-lacZ* reporter system to monitor σ^B activity, we observed that the *rsbV* and *rsbW* products control σ^B activity at the *ctc* promoter independently of their effects on σ^B levels. In contrast, RsbX was found to have no effect on expression of *ctc* when the *sigB* operon was controlled by P_{SPAC}. The data are consistent with RsbV and RsbW being regulators of σ^B activity and RsbX acting primarily as a negative regulator of *sigB* operon expression. Evidence that stationary-phase induction of the σ^B-dependent *ctc* promoter is accomplished by a reduction in RsbW-dependent inhibition of σ^B activity is also presented. In addition, Western blot (immunoblot) analyses of *sigB* operon expression demonstrated that σ^B accumulation is coupled to the synthesis of its primary inhibitor (RsbW). This finding is consistent with RsbW and σ^B being present within the cell in equivalent amounts, a circumstance that would permit RsbW to directly influence σ^B activity by a direct protein-protein interaction.

Ten different sigma factors have been identified in *Bacillus subtilis*. Each of these proteins confers a novel promoter specificity on the RNA polymerase holoenzyme which carries it. Four of the sigma factors (σ^E, σ^F, σ^G, and σ^K) are synthesized only when *B. subtilis* undergoes sporulation (16–18, 27, 28, 32, 38, 40, 41). They are essential for the expression of sporulation genes but are dispensable to vegetatively growing bacteria. The remaining six sigma factors (σ^A, σ^B, σ^C, σ^D, σ^H, and σ^L) are found in vegetatively growing bacteria, in which they participate in the expression of genes involved in diverse activities. The most abundant of the vegetative sigma factors is σ^A. σ^A transcribes the bulk of the cell's housekeeping genes as well as several early sporulation genes (12, 21, 29). σ^H is required for the transcription of competence and early sporulation genes (1, 13). σ^D and σ^L direct the transcription of chemotactic and degradative enzyme operons, respectively (10, 22, 34). The functions of σ^B and σ^C are unknown, but their low abundance argues that they are likely to participate in the expression of very specialized regulons. σ^C is known only by its activity in in vitro transcription reactions (25). In contrast, σ^B has been characterized both biochemically and genetically and shown to be nonessential for both growth and sporulation (6, 15, 19, 20).

An analysis of the DNA region encoding σ^B (*sigB*) revealed that *sigB* is the third gene of a four-gene operon: *rsbV rsbW sigB rsbX* (the *rsb* genes were previously designated *orf*), principally transcribed from a promoter that is recognized by σ^B-containing RNA polymerase ($E\sigma^B$) (15, 26). The downstream gene of the *sigB* operon (*rsbX*) is believed to be a negative regulator of either σ^B activity or its synthesis in that null mutations in this gene heighten expression from σ^B-dependent promoters (4, 7, 23, 26). The *rsbV* and *rsbW* products are also regulators of σ^B (4, 7). These proteins have significant homology to the predicted amino acid sequence of SpoIIAA and SpoIIAB, regulators of the *B. subtilis* sporulation-specific sigma factor σ^F (26). Genetic evidence suggests that RsbW and SpoIIAB are inhibitors of their respective sigma factors (4, 7, 37), with the other member of the regulatory pair (RsbV and SpoIIAA) functioning in the release of its particular sigma factor from this inhibition. In vitro reconstitution experiments indicate that RsbW inhibits σ^B by binding to it and preventing its association with RNA polymerase (5). Similar findings were obtained by Duncan and Losick in a study of SpoIIAB inhibition of σ^F (14).

The effects of null mutations in *rsbV*, *rsbW*, and *rsbX* on the activity of σ^B-dependent promoters suggested a model in which RsbW is the primary inhibitor of σ^B-dependent transcription with RsbV capable of counteracting RsbW's negative control but normally prevented from doing this by the *rsbX* gene product (4).

In the present study, we continue our examination of the roles of RsbV, RsbW, and RsbX in controlling the levels and activity of σ^B through experiments in which *sigB* operon expression is controlled by an inducible promoter (P_{SPAC}), σ^B abundance is directly monitored by Western blot (immunoblot) analyses using an anti-σ^B monoclonal antibody as a probe, and σ^B activity is measured at the σ^B-dependent *ctc* promoter by means of a *ctc::lacZ* reporter system. In this experimental system, we confirmed that it is the activity of RsbW and not the level of σ^B that is the critical element influencing the level of σ^B-dependent promoter activity. Consistent with our biochemically defined mechanism for RsbW control of σ^B (5) and the previous in vivo experiments of Boylan et al. (7), we observed that RsbV and RsbW regulate the expression of *ctc* independently of their influence on σ^B levels. This is not true of RsbX. The loss of RsbX resulted in elevated σ^B levels and heightened *ctc* transcription when the *sigB* operon was expressed from its normal σ^B-dependent promoter but had no effect on *ctc* expression when the *sigB* operon was expressed from P_{SPAC}. These data suggest that the RsbV-RsbW and RsbX regulators control different aspects of σ^B. RsbV and RsbW participate in directly regulating σ^B's activity, while RsbX appears to

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function primarily as a negative regulator of *sigB* operon expression.

MATERIALS AND METHODS

Bacterial strains and culture media. All *B. subtilis* strains constructed for this project were derived from PY22, which was originally obtained from P. Youngman (University of Georgia). *Escherichia coli* BL21(DE3)(p*Lys*S), constructed by F. W. Studier and B. A. Moffatt, (39), was obtained from V. Deretic (University of Texas Health Science Center, San Antonio). Cells were grown in Luria broth (LB) (33) or Difco sporulation medium (DS medium) (36) as previously described (4). Plasmid pSI-1 (P_{SPAC}) was from D. Henner (Genentech, South San Francisco, Calif.). pTet-I is pSI-1 (44), cut with *Eco*RI and *Pst*I to remove the *SPAC* promoter and carrying instead a 4.6-kbp DNA fragment of Tn*916* (9) at this site. The fragment contains the *tetM* gene of Tn*916* which was initially cloned into the multiple cloning site of pUC19 (43) as a *Hin*cII fragment by D. LeBlanc (University of Texas Health Science Center, San Antonio). The 4.6-kbp segment was cut out of this intermediate plasmid (pTC-2) as an *Eco*RI-*Pst*I fragment for cloning into pSI-1. pUS19 was constructed by cloning a 1.1-kbp *Cla*I-*Nde*I DNA fragment encoding spectinomycin resistance from pDL273 (31) into *Acc*I-*Nde*I-cut pUC19. pUS19 replicates in *E. coli* and confers spectinomycin resistance (100 μg/ml) on *E. coli* and *B. subtilis*. Stable maintenance of spectinomycin resistance in *B. subtilis* requires recombination of the plasmid into the *B. subtilis* chromosome.

***B. subtilis* transformations.** Plasmid and chromosomal DNA was transformed into *B. subtilis* which had been made competent by the method of Yasbin et al. (45). Transformants were selected on LB agar plates containing 5 μg of chloramphenicol per ml, 5 μg of kanamycin per ml, 1 μg of erythromycin per ml, or 100 μg of spectinomycin per ml where appropriate.

Preparation of σ^B antigen for antibody production. In order to overexpress σ^B antigen for antibody production, a 700-bp DNA fragment that encodes the carboxy-terminal two-thirds of σ^B (4) was cloned into an *E. coli* expression plasmid (pRSET; Invitrogen, San Diego, Calif.). This resulted in a translational fusion of *sigB* at codon 85 to a phage T7 gene which had been modified by the manufacturer to include a polyhistidine metal binding domain in its product. The recombinant plasmid (pRSET::Δ*sigB*) was transformed into an *E. coli* strain (BL21) which carries the gene for T7 RNA polymerase under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *lac* UVR-5 promoter. T7 RNA polymerase is rifampin resistant. Figure 1A depicts an autoradiogram of sodium dodecyl sulfate (SDS)-lysed pRSET::Δ*sigB* cells that had been fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) following pulse-labeling for 5 min with 50 μCi of [^{35}S]methionine per ml in M-9 glucose minimal medium (33). The synthesis of the T7 RNA polymerase was either uninduced (lane 3) or induced in the presence of rifampin (lanes 1 and 2). Figure 1B illustrates a Coomassie blue-stained SDS-polyacrylamide gel of total protein present in BL21 carrying pRSET::Δ*sigB* either uninduced (lane UI) or 2 h after IPTG induction (lane I). A single protein with the anticipated mobility of the σ^B antigen was synthesized in the rifampin-treated cultures (Fig. 1A, lanes 1 and 2). A protein of this mobility also became the principal protein in the induced cell extract (Fig. 1B). This extract was centrifuged for 15 min at 10,000 × *g*, whereby the bulk of the σ^B antigen was found to pellet with

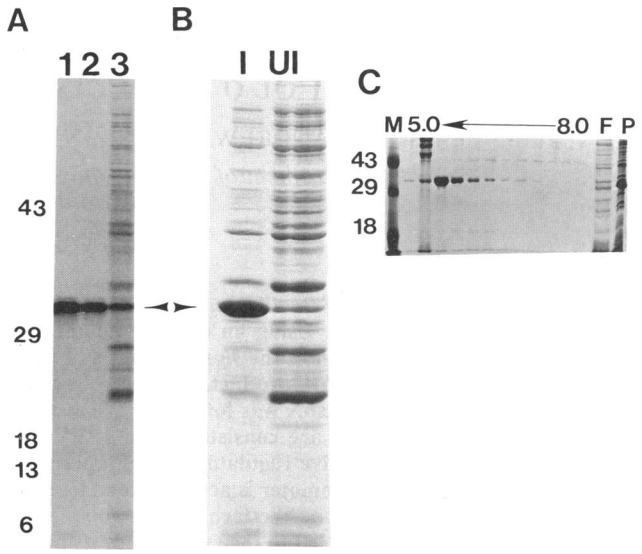

FIG. 1. Synthesis and purification of σ^B antigen. (A) *E. coli* BL21 carrying pRSET::Δ*sigB* was grown in minimal medium and pulse-labeled with [^{35}S]methionine as described in Materials and Methods either before induction of T7 RNA polymerase (lane 3) or 5 min (lane 2) and 20 min (lane 1) after induction in the presence of rifampin (200 μg/ml). Cell lysates were fractionated by SDS-PAGE, and radioactively labeled proteins were detected by autoradiography. The migration of molecular weight standards in this gel system is shown on the left. The arrowhead to the right of the panel indicates the position of the σ^B antigen. (B) The protein profile of BL21(pRSET::Δ*sigB*) cells was determined by fractionating cell lysates by SDS-PAGE and staining the resulting gels with Coomassie blue. Cells were harvested for analysis either prior to (UI) or 2 h after (I) the addition of IPTG to induce T7 RNA polymerase. The arrowhead indicates the position of the σ^B antigen. (C) SDS-PAGE analysis of fractions obtained during the purification of the σ^B fusion protein from the pellet fraction of a crude lysate (lane P) of BL21(pRSET::Δ*sigB*). The fusion protein was adsorbed to a nickel-nitrilotriacetic acid column in buffer containing 6 M guanidine, washed with buffer containing 8 M urea, and eluted from the column with a pH gradient from 8.0 to 5.0 in 8 M urea. Material which failed to bind to the column is shown in lane F. Molecular weight markers are shown in lane M.

the cell debris. The pellet from an induced 1-liter culture was resuspended in 10 mM Tris (pH 8.0)–0.1 M NaH_2PO_4–6 M guanidine HCl and applied to a 2-ml nitrilotriacetic acid resin-Ni^{2+} column (Qiagen, Studio City, Calif.). The column was washed with 10 mM Tris (pH 8.0)–0.1 M NaH_2PO_4–8 M urea (pH 8.0) and eluted with a pH gradient (pH 8.0 to 5.0) in the 8 M urea buffer. Figure 1C illustrates the column purification step. The σ^B antigen in the crude cell pellet (P) is significantly reduced in the column flowthrough (F) but is recovered from the column by low pH elution as a virtually homogeneous protein. On the basis of Coomassie blue protein assays, we estimate that 5 to 7 mg of σ^B antigen is obtained from a 1-liter culture.

Monoclonal antibody production. Hybridomas were produced in BALB/c mice as was done previously (42). Supernatants from wells containing proliferating hybridomas were screened for antibody production in enzyme-linked immunosorbent assays (ELISAs) using purified σ^B antigen in the assay. Hybridomas demonstrating antibody activity for σ^B protein were further screened in Western blot analyses by using the supernatants to probe crude *B. subtilis* cell extracts prepared from strains that either synthesized (PY22) or

could not synthesize (BSA80 *sigB314*) σ^B (4). Hybridomas which produced an antibody that bound to a protein of the proper size in the extract from the *sigB* strain but not in that from the mutant (*sigB314*) strain were single cell cloned by limiting dilution.

Mutagenesis. Oligonucleotide-directed mutagenesis was employed to remove the σ^B-dependent promoter of *sigB* in an M13mp18 clone (43) carrying either the *rsbV312* or the *rsbW313* mutation (4) on a 1.9-kbp *Pst*I fragment (Fig. 2). An oligonucleotide (TTCAAATCACTAGTTAACCTTTCTCC GCAA) complementary to the region 15 bases upstream and 15 bases downstream of nucleotides −5 to −33 relative to the *sigB* transcriptional start site was synthesized and used as described previously (4) to delete the intervening 28 nucleotides. The mutagenic oligonucleotide was also designed to create a unique *Hpa*I site at the junction of the deletion (Δ28*Hpa*I, Fig. 2). The identities of the mutant clones, selected by the method of Kunkel (30), were verified by restriction endonuclease mapping and DNA sequencing. The 1.9-kbp *Pst*I fragment from the mutant clones was ligated into the *Pst*I site of pAK39 (4) to form plasmids pV312Δ28 and pW313Δ28 (Fig. 2). The *SPAC* promoter (44), carried on a 134-bp *Eco*RV-*Sma*I DNA fragment, was cloned into the unique *Hpa*I site in these plasmids, forming pVSPAC and pWSPAC (Fig. 2). These plasmids now contain modified *sigB* operons in which the σ^B-dependent promoter has been replaced by P_{SPAC}. The *Eco*RI-*Pst*I region of pAK39 (Fig. 2) was originally constructed by inserting a kanamycin resistance gene into a fragment of *B. subtilis* DNA immediately upstream of the *sigB* operon (4). The *Pst*I site of pAK39 into which the 1.9-kbp *Pst*I fragments of P_BΔ28 DNA were inserted is the site at which these fragments are normally joined in the *B. subtilis* chromosome. We have therefore reconstructed the region upstream of the mutations, including approximately 1 kbp of DNA upstream of the kanamycin resistance gene. Linearization of each of these plasmids (pVSPAC and pWSPAC) with *Sca*I (Fig. 2), followed by its transformation into *B. subtilis*, yields kanamycin-resistant transformants which have a high probability of recombining both P_{SPAC} and the mutant *rsbV* and *rsbW* alleles into the *B. subtilis* chromosome. *rsbX*::*spec* was constructed by linearizing pML7 at its unique *Cla*I site within *rsbX* and cloning into it a Spec^r determinant that had been excised from pUS19 as a 1.4-kbp *Hpa*II DNA fragment. The resulting plasmid, pML7/X::Spec, was linearized with *Sca*I and transformed in *B. subtilis*, in which spectinomycin-resistant clones would carry the *rsbX*::*spec* allele.

Western blot analysis. Crude cell extracts were prepared from *E. coli* or *B. subtilis* and fractionated by SDS-PAGE (12.5% acrylamide) as previously described (42). Following electrophoretic transfer to nitrocellulose and blocking of the nitrocellulose with BLOTTO, the immobilized protein bands were probed with anti-σ^B monoclonal antibody. Bound antibody was visualized with an alkaline phosphatase-conjugated goat immunoglobulin against mouse immunoglobulin (HyClone Laboratories, Inc.).

β-Galactosidase assays. Cells were harvested from cultures and frozen at −70°C until further use. In some experiments, β-galactosidase activity was detected in lysates prepared by resuspending cell pellets in 5 ml of Z buffer (33) and passaging the cells through a French pressure cell at 15,000 lb/in^2. Debris was removed by centrifugation at 10,000 × *g* for 10 min. Dilutions of the supernatants were assayed for β-galactosidase activity by the method of Miller (33). The amount of protein present in the extracts was quantitated with the Bio-Rad protein assay reagent as recommended by the manufacturers. β-Galactosidase activity was calculated as the optical density at 420 nm × 100/mg of protein in a 30-min incubation at 37°C. In other studies, β-galactosidase activity was determined by the method of Kenney and Moran (28), with the levels calculated as Miller units.

DNA sequencing. DNA sequencing was performed by the Sanger method with Sequenase reagents (U.S. Biochemical Corp.) and the protocol provided by the manufacturer.

RESULTS

Specificity of anti-σ^B monoclonal antibody. As described above, σ^B antigen was synthesized in *E. coli* and used to immunize mice for hybridoma production. After spleen cell-myeloma fusion, selection, and screening by ELISA, supernatants from putative anti-σ^B-secreting clones were tested for specificity in Western blot analyses and single cells were cloned. Figure 3 illustrates the Western blot analyses for the antibody used in this study. Panel A depicts crude cell extracts from *E. coli* BL21 with or without pRSET::Δ*sigB*, fractionated by SDS-PAGE and either stained with Coomassie blue or transferred to nitrocellulose and probed with anti-σ^B antibody. In the data presented, 10 times more extract from the plasmidless strain than from the strain carrying pRSET::Δ*sigB* was analyzed. No detectable antibody bound to the proteins extracted from BL21 (Fig. 3A, lane 3); however, the antibody did react strongly to two protein bands in the extract from the pRSET::Δ*sigB*-containing strain (Fig. 3A, lane 4). The faster-migrating of the two bands had the mobility of the σ^B antigen. We suspect that the more slowly migrating band is an aggregated form of the σ^B antigen which was not disrupted during the sample preparation. We base this notion on its absence from the plasmidless *E. coli* extract and the observation that the σ^B antigen aggregates readily following induction in BL21.

We next tested our antibody on *B. subtilis* extracts to ensure that the probe recognizes an epitope on σ^B that is not shared with other *B. subtilis* proteins. To do this, we performed Western blot analyses on crude *B. subtilis* extracts prepared from cells that either synthesized σ^B (PY22) or contained a *sigB* allele (BSA80 *sigB314*) (4) with a frameshift mutation which terminates translation near the σ^B amino terminus. We observed that, although the monoclonal antibody detected no proteins in the extract from the *sigB314* strain (Fig. 3B, lane 3), it recognized a single protein with the apparent mobility of σ^B in the extract prepared from the wild-type strain (Fig. 3B, lane 4). We conclude that this antibody is highly specific for σ^B and can be used to monitor σ^B in *B. subtilis* extracts.

Response of σ^B levels to medium and growth state. The principal promoter of the *sigB* operon is transcribed by σ^B-containing RNA polymerase (4, 15). Two additional σ^B-dependent transcription units are known (*csbA* and *ctc*) (8, 24). Both *ctc* and *csbA* are induced approximately fivefold by a σ^B-dependent mechanism at the end of exponential growth in LB medium supplemented with glucose and glutamine but are elevated only slightly by entering stationary phase in DS medium (8, 24). Given the induction profile of *ctc* and *csbA*, it would be anticipated that *sigB* transcription, depending as it does on a σ^B-dependent promoter, would also fluctuate in response to growth state and medium. Our previous data suggested, however, that the *sigB* operon's σ^B-dependent promoter doesn't respond in an identical manner to the *ctc* and *csbA* promoters (4). We observed only a twofold increase in *sigB*-specific RNA in both LB and DS media when *B. subtilis* entered stationary

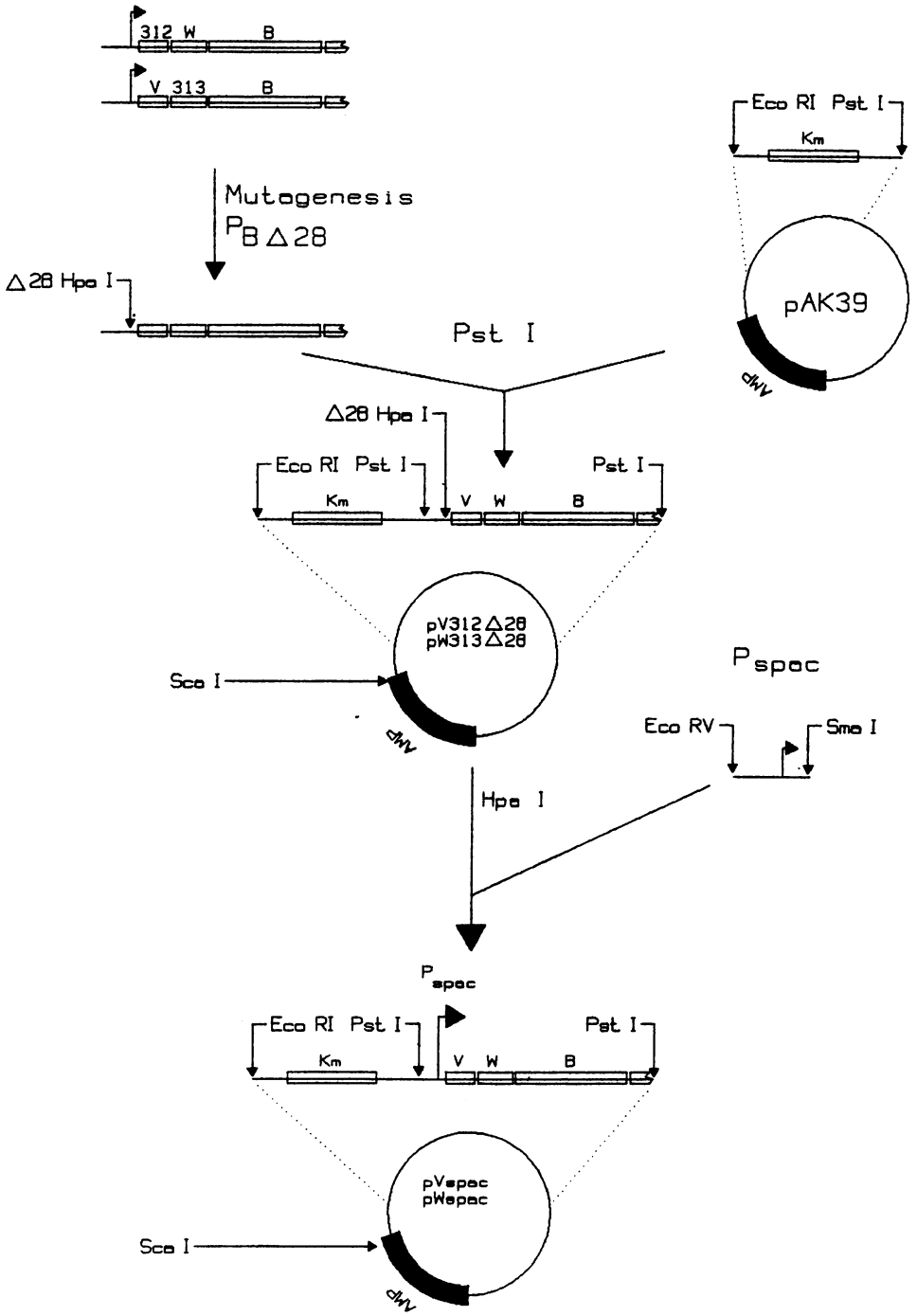

FIG. 2. Construction of plasmids pVSPAC and pWSPAC. *Pst*I-generated, 1.9-kbp DNA fragments, carrying the *sigB* operon with either the *rsbV312* or the *rsbW313* allele, were subjected to mutagenesis as described in Materials and Methods to delete the operon's σ^B-dependent promoter and cloned into the *Pst*I site of pAK39 (4). pAK39 contains an *Eco*RI-*Pst*I fragment that carries a Km^r gene within the DNA region immediately upstream of the 1.9-kbp *Pst*I fragment in the *B. subtilis* chromosome which becomes rejoined to the 1.9-kbp fragment in the resulting plasmids (pV312Δ28 and pW313Δ28). The plasmids were then opened at a unique *Hpa*I site that was placed in the *sigB* promoter region during the mutagenesis, and a 135-bp *Eco*RV-*Sma*I fragment containing P_{SPAC} (44) was cloned into this site. The resulting plasmids (pVSPAC and pWSPAC) contain mutant *sigB* operons under the control of P_{SPAC} with a selectable Km^r gene immediately upstream of the operon.

phase (4). The differences in the medium dependence and the degree of induction of the *sigB* and *ctc-csbA* operons suggest that it is unlikely that the profile of *ctc* and *csbA* induction merely reflects changes in σ^B levels. Instead, an additional level of control is likely to exist at *ctc* and *csbA*. The availability of an anti-σ^B antibody permitted us to monitor changes in the level of σ^B in response to medium or state-of-growth changes which affected *ctc* and *csbA* expres-

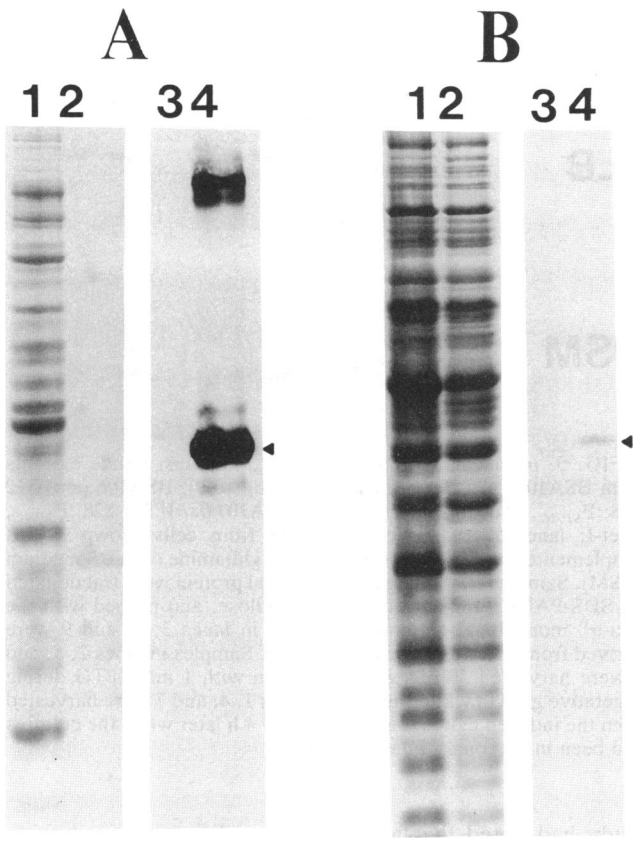

FIG. 3. Western blot reactions of the anti-σ^B monoclonal antibody. Extracts from *E. coli* (A) and *B. subtilis* (B) were prepared as described in Materials and Methods, fractionated by SDS-PAGE, and either stained with Coomassie blue (lanes 1 and 2) or transferred to nitrocellulose and probed with the anti-σ^B monoclonal antibody (lanes 3 and 4). For panel A, 100 μg of protein from BL21 (lanes 1 and 3) or 10 μg of protein from BL21(pRSET::Δ*sigB*) (lanes 2 and 4) was loaded. For panel B, 200 μg of protein from BSA80 (*sigB314*) (lanes 1 and 3) and 100 μg of protein from PY22 (lanes 2 and 4) was loaded. The arrowheads indicate the positions of the σ^B antigens.

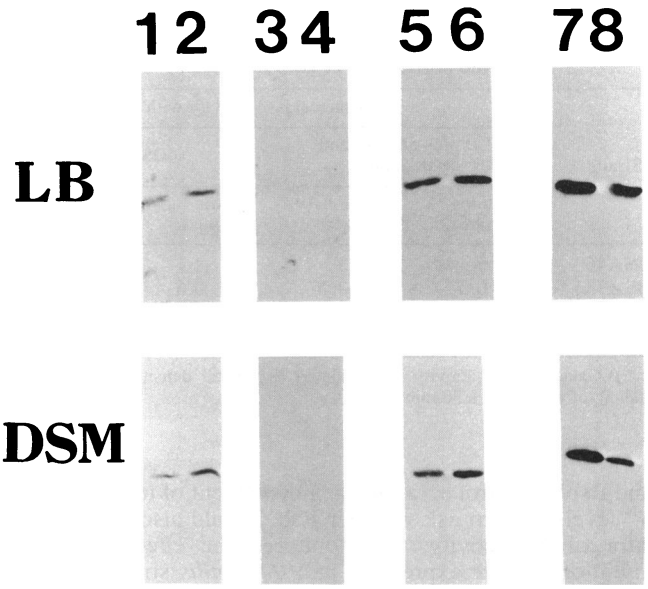

FIG. 4. Effects of the *orfV312*, *rsbW313*, and *rsbX*::pAK17 mutations upon accumulation of σ^B. Crude extracts containing 100 μg of total protein were prepared from strains BSA46 (wild type; lanes 1 and 2), BSA73 (*rsbV312*; lanes 3 and 4), BSA88 (*rsbW313*; lanes 5 and 6), and BSA64 (*rsbX*::pAK17; lanes 7 and 8); fractionated by SDS-PAGE; transferred to nitrocellulose; and probed with the anti-σ^B monoclonal antibody. The cells were grown in LB supplemented with 5% glucose and 0.2% glutamine or in DS medium (DSM) with samples harvested from logarithmically growing (lanes 1, 3, 5, and 7) and stationary-phase (lanes 2, 4, 6, and 8) cultures.

sion. We therefore measured the relative abundance of σ^B during vegetative growth and stationary phase in LB (glucose-glutamine) (*ctc*-inducing) or DS (non-*ctc*-inducing) medium. As can be seen in Fig. 4, the amounts of σ^B in both the DS medium- and the LB-grown cultures are very similar during vegetative growth (lane 1) with parallel small increases when the cells enter stationary phase (lane 2). On the basis of this experiment, σ^B appears to be most abundant when *B. subtilis* is cultured past the end of exponential growth and accumulates to similar levels regardless of whether the medium is sporulation inhibiting (LB with glucose-glutamine) or sporulation promoting (DS). The comparable levels of σ^B found in both LB and DS medium substantiate the previous finding (4) and demonstrate that the observed LB-dependent increase in *ctc* and *csbA* gene expression is unlikely to be solely due to increases in σ^B levels.

Accumulation of σ^B in *B. subtilis* strains with mutations in the *sigB* operon. We and others have observed that null mutations in two of the genes of the *sigB* operon (*rsbV* and *sigB*) eliminated expression from the *ctc* and *sigB* operons' σ^B-dependent promoters while null mutations in the remaining two genes (*rsbW* and *rsbX*) dramatically increased the activity of these promoters (4, 6, 7, 15, 23). Using the anti-σ^B antibody in Western blot analyses, we can directly observe the changes in σ^B abundance that occur in the *rsbV*, *rsbW*, and *rsbX* mutant strains and compare these changes with the effects of these mutations on *ctc* expression. Crude cell extracts were prepared from mid-log- or stationary-phase *B. subtilis* cultures and analyzed by Western blot for σ^B levels. Both the *rsbW* (BSA88; Fig. 4, lanes 5 and 6) and *rsbX* (BSA64; lanes 7 and 8) mutant strains accumulate substantially more σ^B protein than that found in the wild-type strain (BSA46; lanes 1 and 2), while the *rsbV312* strain (BSA93; lanes 3 and 4) lacks detectable σ^B. The results are similar regardless of whether the extracts were prepared from strains grown in LB or in DS medium (Fig. 4). This is not the case for *ctc* expression. The level of *ctc* promoter activity in the strains is presented in Table 1. Although there is a good correlation among the effects of the *rsbV*, *rsbW*, and *rsbX* mutations on σ^B abundance and *ctc* activity, there is an LB-dependent enhancement of *ctc* induction that was not seen as a change in σ^B protein levels. These results are consistent with RsbV, RsbW, and RsbX being regulators of σ^B synthesis and support the notion that there is an additional medium-dependent regulatory element operating at the σ^B-dependent *ctc* promoter that does not control the σ^B-dependent promoter of *sigB*.

Expression of σ^B from a P_{SPAC}-controlled *sigB* operon. Genetic and biochemical evidence argue that the RsbV-RsbW proteins compose a regulatory device for controlling σ^B activity and that RsbX is an additional regulator that functions at a point upstream of RsbV-RsbW in the pathway of σ^B control (4, 5, 7). In order to verify in vivo that RsbV

TABLE 1. *ctc*-dependent β-galactosidase activity in wild-type (BSA46), *rsbV312* (BSA73), *rsbW313* (BSA88), and *rsbX*::pAK17 (BSA64) strains

Strain	β-Galactosidase activity with[a]:			
	LB plus glucose and glutamine		DS medium	
	Mid-log phase	Stationary phase	Mid-log phase	Stationary phase
BSA46	5	23	4	8
BSA73	0.3	0.17	0.6	0.4
BSA88	51	129	18	35
BSA64	118	197	56	92

[a] β-Galactosidase activity is calculated as optical density at 420 nm × 100/mg of protein in a 30-min assay.

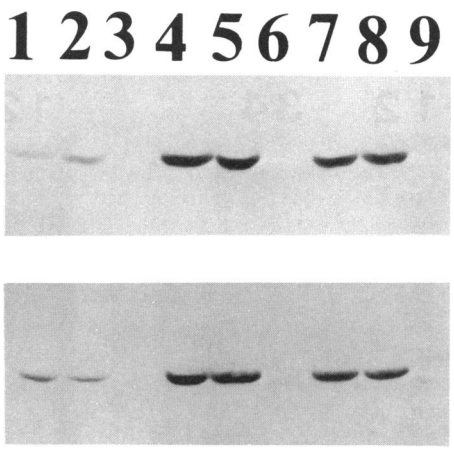

FIG. 5. σ^B accumulation in Δ28::P_{SPAC} strains. Crude extracts from BSA105 (Δ28::P_{SPAC}, pTet-I; lanes 7 to 9), BSA106 (*rsbV312* Δ28::P_{SPAC}, pTet-I; lanes 4 to 6), and BSA107 (*rsbW313* Δ28::P_{SPAC}, pTet-I; lanes 1 to 3) were prepared from cells grown in LB supplemented with 5% glucose and 0.2% glutamine or in DS medium (DSM). Samples containing 100 μg of total protein were fractionated by SDS-PAGE, transferred to nitrocellulose, and probed with the anti-σ^B monoclonal antibody. Samples in lanes 3, 6, and 9 were derived from cultures prior to induction. Samples in lanes 2, 5, and 8 were harvested 30 min after induction with 1 mM IPTG during vegetative growth while samples in lanes 1, 4, and 7 were harvested from the induced cultures approximately 4 h later when the cultures had been in stationary phase for 1.0 h.

and RsbW control σ^B activity, independent of their effect on σ^B levels, and to ask whether RsbX could also be a modulator of σ^B activity, we monitored the effects of these mutations on *ctc* expression in a *B. subtilis* strain in which *sigB* operon expression no longer depended on σ^B. To do this, we deleted a critical element of the σ^B-dependent promoter of *sigB* and replaced it with the *SPAC* promoter as described in Materials and Methods. The reengineered operons were then introduced by transformation into a *B. subtilis* strain (BSA46) which is lysogenic for an SPβ prophage bearing a *ctc*::*lacZ* translational fusion (24) and carries a source of the *E. coli lacI* gene product on a multicopy plasmid (pTet-I). In BSA46, P_{SPAC}, and hence *sigB*, is inducible by the addition of IPTG to the culture medium.

The termination codon of *rsbV* is superimposed on the initiation codon of *rsbW* (26). In addition, *rsbW* and *sigB* overlap by 13 codons (26). These overlapping regions prompted Kalman et al. to propose that translation of *sigB* operon genes might be coupled (26). Given the possibility of translational coupling, we performed a preliminary experiment to ask whether the *rsbV312* and *rsbW313* alleles, which contain frameshift mutations near the amino termini of their coding regions (4), affect the accumulation of σ^B in a *B. subtilis* strain in which their potential effects on *sigB* transcription would not mask a posttranscriptional influence. *B. subtilis* cultures containing P_{SPAC}-driven *sigB* operons, specifying wild-type or mutant *rsbV* or *rsbW* alleles, were induced with IPTG and analyzed for σ^B levels by Western blot. Figure 5 shows induction of σ^B protein from *sigB* operons with wild-type (BSA105, lanes 7 to 9), *rsbV312* (BSA106, lanes 4 to 6), or *rsbW313* (BSA107, lanes 1 to 3) alleles. There was induction in all three cases, but the level of σ^B protein found in the *rsbW313*-containing strain was less than 10% of that seen in the wild-type and *rsbV312* strains. If the *rsbW313* and wild-type extracts are probed with anti-RsbV antibody rather than anti-σ^B antibody, comparable levels of this operon product can be seen in both extracts (data not shown). The drop in σ^B but not RsbV in the *rsbW313*-containing strain is consistent with the low level of σ^B protein seen in this strain being due to a posttranscriptional effect of the *rsbW313* allele on σ^B accumulation rather than an unforeseen transcriptional defect at this strain's *SPAC* promoter. The latter circumstance would have reduced all of the operon's products. Efficient synthesis of σ^B apparently requires the translation of *rsbW* but not *rsbV*.

We next examined the effect of inducing *sigB* operons containing either wild-type or mutant alleles on the activity of the σ^B-dependent *ctc* promoter. Boylan et al. in a similar study had noted that appreciable levels of *ctc* expression were observed only if transcription of the *sigB* operon was initiated near the end of exponential growth (7). We therefore induced our cultures at different times during growth or stationary phase to ensure that the physiological state of the cultures was not contributing to the phenotype that we would attribute to the mutant allele under study. Figure 6 illustrates these experiments with the *ctc*::*lacZ* induction curves plotted above the growth curves of the cultures being induced. The points on the *ctc* expression plots at which the IPTG-induced cultures diverge from the uninduced culture correspond to the times at which IPTG was added to those cultures. Figure 6A depicts the expression of *ctc* in an IPTG-inducible strain (BSA105) that synthesizes wild-type *sigB* gene products. Consistent with the findings of Boylan et al. (7), *ctc* expression rose over the uninduced levels only when the *sigB* operon was induced at the end of exponential growth. Induction of the *sigB* operon 1 h after the onset of stationary phase gave no appreciable elevation of *ctc*-dependent β-galactosidase levels over that seen in the uninduced culture (data not shown). We next performed the experiment with a strain of *B. subtilis* in which the P_{SPAC}-driven *sigB* operon contained a null mutation in *rsbX* (BSA123; *rsbX*::*spec*). This experiment, illustrated in Fig. 6B, revealed that the absence of the *rsbX* gene product had no effect on *ctc* expression when the *sigB* operon was expressed from P_{SPAC}. This is in contrast with the dramatic increase in *ctc* expression following the loss of RsbX from a strain in which the *sigB* operon is expressed from its normal promoter (Table 1). The absence of such an increase in BSA123 argues that RsbX is a regulator of the *sigB* operon expression and not a significant controlling element in the activation of σ^B itself. In contrast to the result obtained with the *rsbX* mutant, the absence of RsbW had a marked effect on *ctc* expression (Fig. 6C). As was found in the previous study (7),

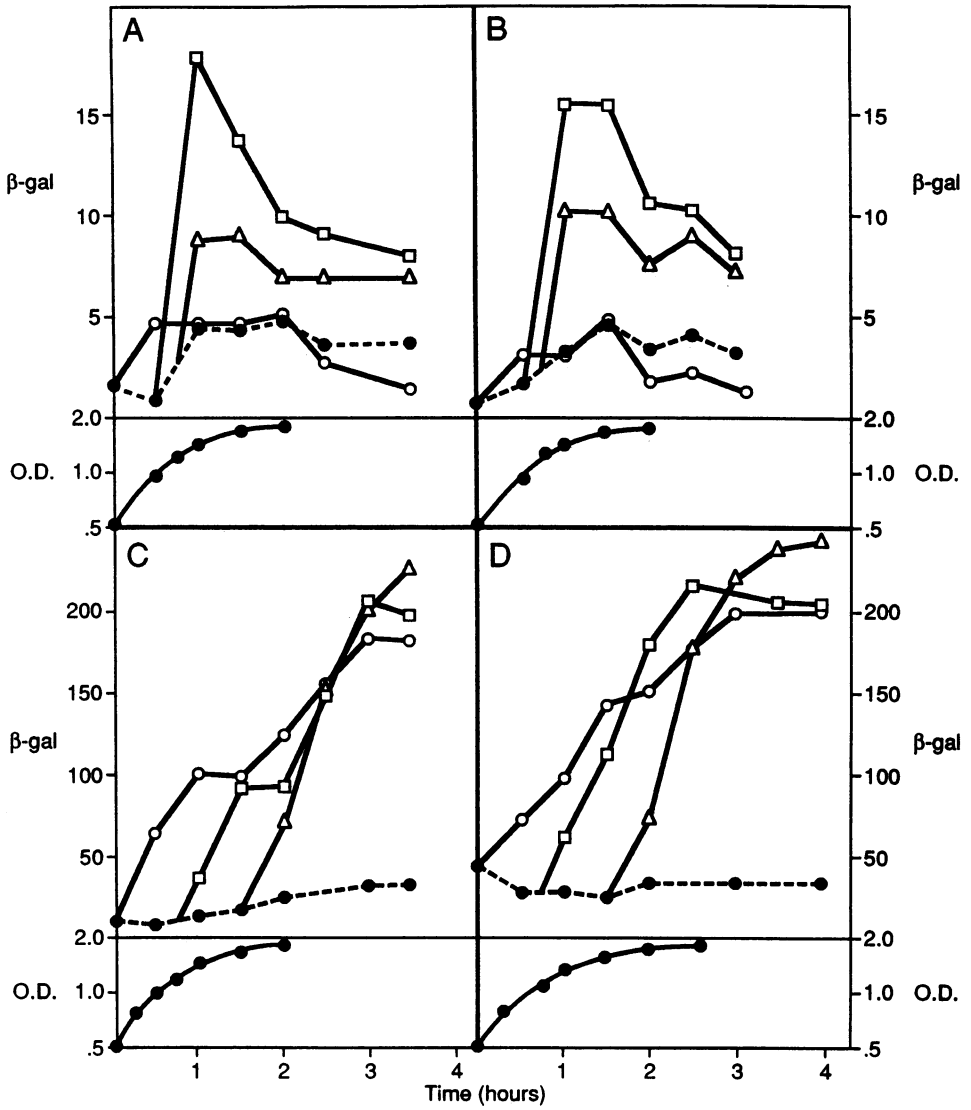

FIG. 6. *ctc* expression following induction of P_{SPAC}-controlled *sigB* operons. Cultures of BSA105 ($P_B\Delta28::P_{SPAC}$, pTet-I) (A), BSA123 (*rsbX*::*spec* $P_B\Delta28::P_{SPAC}$, pTet-I) (B), BSA107 (*rsbW313* $P_B\Delta28::P_{SPAC}$, pTet-I) (C), and BSA125 (*rsbW313 rsbX*::*spec* $P_B\Delta28::P_{SPAC}$, pTet-I) (D) were grown in LB supplemented with 5% glucose–0.2% glutamine. Samples were taken from uninduced cultures (— —) or portions of the cultures in which the P_{SPAC} promoter was induced by the addition of IPTG to 1 mM (○, □, and △) and assayed for *ctc*-dependent β-galactosidase production (28). IPTG was added to the cultures at the points on the graphs where the induced culture plots diverge from the plots of the uninduced cultures. The β-galactosidase levels are recorded as Miller units (33). The growth curve of the uninduced culture is depicted below each β-galactosidase expression plot to illustrate the growth stage of the culture at the time of IPTG addition. The optical densities of the cultures were measured at 540 nm.

induction of a *sigB* operon which lacks a functional *rsbW* gene results in heightened *ctc* expression that commences immediately after the induction of the *sigB* operon. The absence of RsbW thus frees *ctc* expression from the need to induce *sigB* at a particular time in the growth cycle. This was first reported by Boylan et al. (7), who suggested that stationary-phase induction of *ctc* is channeled through a release from RsbW-dependent inhibition. This would be the simplest interpretation of these results; however, it is formally possible that the stationary-phase activation of σ^B could occur by its release from an unknown inhibitor. The relatively modest induction which occurs at stationary phase (Fig. 6A) could still be occurring in the *rsbW313* mutant but be masked by the very high levels of σ^B activity in this strain (Fig. 6C). As expected from the previous result (Fig. 6B), the *ctc* induction profile of the *rsbW313 rsbX*::*spec* double mutant, BSA125 (Fig. 6D), is similar to that of the *rsbW313* single mutant. Induction of a P_{SPAC}-driven *sigB* operon in a strain (BSA106) containing a null *rsbV* allele results in no detectable *ctc* expression regardless of when in the growth cycle the *sigB* operon was induced (data not shown). Thus, the loss of RsbV or RsbW but not RsbX significantly affects the activity of σ^B at the *ctc* promoter when control of the *sigB* operon is removed from its influence.

In order to confirm that the changes in *ctc* expression that were observed in the experiments whose results are illustrated in Fig. 6 were due to differences in σ^B activity and not its abundance, samples of the cultures were taken before or

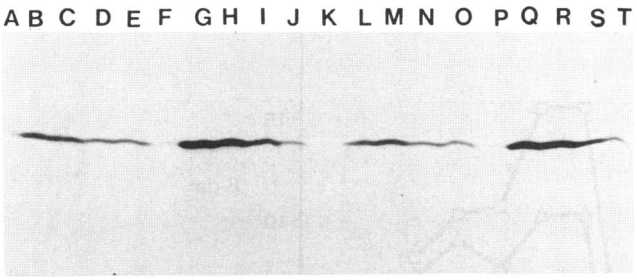

FIG. 7. σ^B in P_{SPAC}-controlled operons. One-milliliter samples of the cultures analyzed for Fig. 6 were lysed and processed for SDS-PAGE as described by Arnosti et al. (2) and analyzed for σ^B protein as for Fig. 3. Lanes A to E are samples from BSA106 (Fig. 6C), lanes F to J are samples from BSA105 (Fig. 6A), lanes K to O are samples from BSA125 (Fig. 6D), and lanes P to T are samples from BSA123 (Fig. 5B). The first sample in each series (i.e., lanes A, F, K, and P) was taken from an uninduced culture at an optical density at 540 nm of 1.0. The remaining four lanes represent samples from IPTG-induced cultures harvested 1 h after the addition of IPTG. The first three induced culture lanes in each series (i.e., B to D, G to I, L to N, and Q to S) represent the σ^B synthesis that followed the IPTG additions depicted in Fig. 6. The fourth lane in each series (E, J, O, and T) depicts the induced σ^B synthesis that occurs if IPTG is added 1 h later than the previous time of addition.

1 h after induction of the *sigB* operon and subjected to Western blot analysis using the anti-σ^B monoclonal antibody as a probe. In the absence of IPTG, the σ^B level was below that detectable in the assay (Fig. 7, lanes A, F, K, and P). Induction of *sigB* during exponential growth resulted in a greater level of σ^B synthesis than that observed when the operon was induced during stationary phase (e.g., lanes G and H versus lanes I and J). The presence of the *rsbX*::*spec* mutation had no effect on σ^B levels (e.g., lanes G to J versus lanes Q to T), but the presence of the *rsbW313* mutation reduced the levels of σ^B below that seen in a strain with a functional *rsbW* allele (e.g., lanes B to E versus lanes G to J). When the level of σ^B protein present in each of the cultures is compared with the culture's level of *ctc* expression, there is little direct correlation. *ctc* expression is at its lowest in the culture with one of the highest levels of σ^B (i.e., BSA105 induced during vegetative growth [lane G]) and at a high level in the culture with one of the lowest levels of σ^B (i.e., BSA106 induced at stationary phase [lane D]). It would appear that it is not the abundance of σ^B itself but its activity state that determines the level of *ctc* induction. We conclude from these experiments and previous studies that RsbV and RsbW are regulators of σ^B activity while RsbX appears to be a negative regulator of the *sigB* operon and not a direct inhibitor of σ^B activity under the conditions that we employed.

DISCUSSION

In earlier studies, we and others generated null mutations in the four open reading frames of the *sigB* operon and analyzed the consequences of these mutations on σ^B-dependent transcription (4, 7). These analyses indicated that *rsbW* and *rsbX* encode negative regulators of σ^B while *rsbV* specifies a positive regulator which functions to counteract the negative regulation of RsbW. RsbW-dependent inhibition of σ^B appears to involve a direct association between RsbW and σ^B which prevents σ^B from binding to RNA polymerase (5). Given that RsbW and σ^B form a complex, RsbV presumably acts by disrupting or preventing formation of this complex.

The notion that RsbV and RsbW control σ^B activity and not merely its synthesis is supported by the present study and the experiments of Boylan et al. (7) in which *rsbV* and *rsbW* gene products are shown to affect the transcription from a σ^B-dependent promoter even when the expression of the *sigB* operon is placed under the control of P_{SPAC}. In our study, where the levels of σ^B protein were monitored, we found little correspondence between the expression of *ctc* and the levels of σ^B protein. In the absence of RsbW, high levels of *ctc* expression occur even at relatively low σ^B levels, while a lack of RsbV prevents the expression of *ctc* even when σ^B levels are abundant. We also verified the observation, initially made by Boylan et al. (7), that *ctc* is expressed during logarithmic growth only by the induction of a *sigB* operon that lacks a functional *rsbW* gene product. Presumably, a substance present in vegetative cells stimulates RsbW-dependent inhibition. In other studies (3), we found that dilution of a stationary-phase culture, which was expressing *ctc*, into fresh medium results in a rapid RsbW-dependent fall in the level of *ctc* expression. Apparently, the hypothetical substance that stimulates RsbW-dependent inhibition can inactivate previously active σ^B.

The previously noted difference in the activities of the σ^B-dependent promoters of *sigB* and *ctc* was also observed in the present study. *ctc* is induced approximately 5- to 10-fold at the end of the exponential-growth phase only in LB (glucose-glutamine) medium (4, 7, 24) while *sigB* is induced twofold at stationary phase regardless of the medium (4). These findings are consistent with two levels of regulation at the *ctc* promoter, a medium-dependent component that does not have an obvious counterpart in the regulation at *sigB* and a stationary-phase component that is shared by the *sigB* promoter.

Our Western blot analyses also revealed that the *rsbW313* mutation reduced σ^B abundance to less than 10% of that seen in a strain with a wild-type *rsbW* allele (Fig. 5). The overlap between the *rsbW* and *sigB* genes (26) suggests that this reduction in σ^B levels may be due to reduced translation of *sigB* in the absence of a fully translated *rsbW* gene; however, other explanations are possible. σ^B and RsbW appear to form a complex within the cell (5). Therefore, in the absence of RsbW, σ^B may be more susceptible to degradation. Pulse-chase experiments will be needed to distinguish between potential roles for *rsbW* in σ^B synthesis or stabilization.

The function of RsbX in σ^B regulation is not clear. Unlike the RsbW-RsbV pair, RsbX has no apparent effect on σ^B activity when the *sigB* operon is expressed from P_{SPAC}. Although this finding suggests that RsbX controls σ^B expression but not its activation state, such a notion is not easily reconciled with the observation that σ^B-dependent promoters are expressed at very high levels in $RsbX^-$ $RsbW^+$ $RsbV^+$ strains. It would be expected that if a loss of RsbX merely elevated the expression of the *sigB* operon, the coordinately enhanced synthesis of RsbW would continue to inhibit σ^B. The apparent failure of RsbW to inhibit σ^B in $RsbX^-$ strains could be rationalized by assuming a need for an additional factor to maintain the inhibition. If the *sigB* operon is expressed at high levels, as it is in $RsbX^-$ strains, there may be insufficient amounts of this factor present to prevent the RsbV-dependent inactivation of RsbW. Although such a possibility seems plausible, it is unlikely to be true in its simplest form. A comparison of the σ^B protein levels detectable by Western blot analysis reveals that a fully

induced P_{SPAC}-driven *sigB* operon produces approximately half as much σ^B as that formed in an RsbX$^-$ strain that expresses *sigB* from its normal σ^B-dependent promoter and 5- to 10-fold more σ^B than that seen in strains with a wild-type *sigB* locus (data not shown). This implies that high-level expression of the *sigB* operon alone is insufficient to account for the RsbX$^-$ phenotype. Models to account for these results would be highly speculative and should await more experimental data; however, it does appear that specific inhibition of *sigB* operon expression is unlikely to be the only function of RsbX. What its other functions may be and how they ultimately influence σ^B activation remain a mystery.

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