Regulation of $\sigma^B$ Levels and Activity in Bacillus subtilis

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The sigB operon of Bacillus subtilis encodes $\sigma^B$ plus three additional proteins (RsbV, RsbW, and RsbX) that regulate $\sigma^B$ activity. Using an anti-\(\sigma^B\) monoclonal antibody to monitor the levels of $\sigma^B$ protein, \(P_{SPAC}\) to control the expression of the sigB operon, and a ctc-lacZ reporter system to monitor $\sigma^B$ activity, we observed that the \(rsbV\) and \(rsbW\) products control $\sigma^B$ activity at the ctc promoter independently of their effects on $\sigma^B$ 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 $\sigma^B$ activity and RsbX acting primarily as a negative regulator of sigB operon expression. Evidence that stationary-phase induction of the $\sigma^B$-dependent ctc promoter is accomplished by a reduction in RsbW-dependent inhibition of $\sigma^B$ activity is also presented. In addition, Western blot (immunoblot) analyses of sigB operon expression demonstrated that $\sigma^B$ accumulation is coupled to the synthesis of its primary inhibitor (RsbW). This finding is consistent with RsbW and $\sigma^B$ being present within the cell in equivalent amounts, a circumstance that would permit RsbW to directly influence $\sigma^B$ 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 ($\sigma^B$, $\sigma^D$, $\sigma^O$, and $\sigma^K$) 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 ($\sigma^A$, $\sigma^B$, $\sigma^C$, $\sigma^D$, $\sigma^H$, and $\sigma^P$) 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 $\sigma^A$. $\sigma^B$ transcribes the bulk of the cell’s housekeeping genes as well as several early sporulation genes (12, 21, 29). $\sigma^B$ is required for the transcription of competence and early sporulation genes (1, 13). $\sigma^C$ and $\sigma^D$ direct the transcription of chemotactic and degradative enzyme operons, respectively (10, 22, 34). The functions of $\sigma^B$ and $\sigma^C$ are unknown, but their low abundance argues that they are likely to participate in the expression of very specialized regulons. $\sigma^C$ is known only by its activity in in vitro transcription reactions (25). In contrast, $\sigma^B$ 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 $\sigma^B$ (sigB) revealed that sigB is the third gene of a four-gene operon: \(rsbV\), \(rsbW\), sigB, and \(rsbX\) (the \(rsb\) genes were previously designated \(orf\)), primarily transcribed from a promoter that is recognized by $\sigma^B$-containing RNA polymerase (Es$\sigma^B$) (15, 26). The downstream gene of the sigB operon (\(rsbX\)) is believed to be a negative regulator of either $\sigma^B$ activity or its synthesis in that null mutations in this gene heighten expression from $\sigma^B$-dependent promoters (4, 7, 23, 26). The \(rsbV\) and \(rsbW\) products are also regulators of $\sigma^B$ (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 $\sigma^F$ (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 SpoIIA) functioning in the release of its particular sigma factor from this inhibition. In vitro reconstitution experiments indicate that RsbW inhibits $\sigma^B$ 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 $\sigma^D$ (14).

The effects of null mutations in \(rsbV\), \(rsbW\), and \(rsbX\) on the activity of $\sigma^B$-dependent promoters suggested a model in which RsbW is the primary inhibitor of $\sigma^B$-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 $\sigma^B$ through experiments in which sigB operon expression is controlled by an inducible promoter (\(P_{SPAC}\)). $\sigma^B$ abundance is directly monitored by Western blot (immunoblot) analyses using an anti-$\sigma^B$ monoclonal antibody as a probe, and $\sigma^B$ activity is measured at the $\sigma^B$-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 $\sigma^B$ that is the critical element influencing the level of $\sigma^B$-dependent promoter activity. Consistent with our biochemically defined mechanism for RsbW control of $\sigma^B$ (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 $\sigma^B$ levels. This is not true of RsbX. The loss of RsbX resulted in elevated $\sigma^B$ levels and heightened ctc transcription when the sigB operon was expressed from its normal $\sigma^B$-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 $\sigma^B$, RsbV and RsbW participate in directly regulating $\sigma^B$'s activity, while RsbX appears to
whereby the bulk antigen was uninduced in M-9 pulse-labeling for polyacrylamide gel. Cells subtilis B. subtilis. Stable polymerase metal lac UVR-5 plasmid autoradiogram of translational fusion E. fragment competent overexpress &B per mants confers by encoding this site. The site. (44). South Georgia). for this primarily as 2348 subtilis Health Science fragment a antibiotic LB and (4). Plasmid pSI-1 cut (PspAc) from this site. The site. The site. This site. The site. The site. IS10 (RpsAc) was cut into E. coli expression strain carrying pRSET::AsigB. The fusion protein was adsorbed to a nickel-nitriotriacetic 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.

Preparation of σB antigen for antibody production. In order to overexpress σB antigen for antibody production, a 700-bp DNA fragment that encodes the carboxy-terminal two-thirds of σB (4) was cloned into an E. coli expression plasmid (pRSET; Invitrogen, San Diego, Calif.). This resulted in a translation fusion of sigB at codon 85 to a phase T7 gene which had been modified by the manufacturer to include a polyhistidine metal binding domain in its product. The recombinant plasmid (pRSET::ΔsigB) was introduced 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::ΔsigB cells that had been fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) following pulse-labeling for 5 min with 50 μCi of [35S]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::ΔsigB either uninduced (lane UI) or 2 h after IPTG induction (lane I). A single protein with the anticipated mobility of the σB 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 σB antigen was found to pellet with the cell debris. The pellet from an induced 1-liter culture was resuspended in 10 mM Tris (pH 8.0)—0.1 M NaH2PO4—6 M guanidine HCl and applied to a 2-ml nitriotriacetic acid resin-Ni2+ column (Qiagen, Studio City, Calif.). The column was washed with 10 mM Tris (pH 8.0)—0.1 M NaH2PO4—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 σB 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 σB 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 σB antigen in the assay. Hybridomas demonstrating antibody activity for σB 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

**FIG. 1.** Synthesis and purification of σB antigen. (A) E. coli BL21 carrying pRSET::ΔsigB was grown in minimal medium and pulse-labeled with [35S]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 σB antigen. (B) The protein profile of BL21(pRSET::ΔsigB) 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 σB antigen. (C) SDS-PAGE analysis of fractions obtained during the purification of the σB fusion protein from the pellet fraction of a crude lysate (lane P) of BL21(pRSET::ΔsigB). The fusion protein was adsorbed to a nickel-nitriotriacetic 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.

**TABLE 1.** Materials and methods. 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)(pLysS), 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 (pSPAC) was from D. Henner (Genentech, South San Francisco, Calif.). pTet-1 is pSI-1 (44), cut with EcoRI and PstI to remove the SPAC promoter and carrying instead a 4.6-kbp DNA fragment of Tn916 (9) at this site. The fragment contains the tetM gene of Tn916 which was initially cloned into the multiple cloning site of pUC19 (43) as a HindII 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 EcoRI-PstI fragment for cloning into pSI-1. pUS19 was constructed by cloning a 1.1-kbp ClaI-Netd1 DNA fragment encoding spectinomycin resistance from pDL273 (31) into Accl-Ned1-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.
could not synthesize (BSA80 sigB314) \( \sigma^B \) (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 limited dilution.

**Mutagenesis.** Oligonucleotide-directed mutagenesis was employed to remove the \( \sigma^B \)-dependent promoter of sigB in an M13mpl8 clone (43) carrying either the rsbW312 or the rsbW313 mutation (4) on a 1.9-kbp PstI fragment (Fig. 2). An oligonucleotide (TCTAAATCAGTTAACCCTTCGCA) 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 HpaI site at the junction of the deletion (Δ28HpaI, 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 PstI fragment from the mutant clones was ligated into the PstI site of pAK39 (4) to form plasmids pV312Δ28 and pW313Δ28 (Fig. 2). The SPA promoter (44), carried on a 134-bp EcoRV-SmaI DNA fragment, was cloned into the unique HpaI site in these plasmids, forming pVSPAC and pWSPAC (Fig. 2). These plasmids now contain modified sigB operons in which the \( \sigma^B \)-dependent promoter has been replaced by \( P_{SPA} \). The EcoRI-PstI region of pAK39 (Fig. 2) was originally constructed by inserting a kanamycin resistance gene into a fragment of \( B. subtilis \) DNA. An EcoRI/KpnI fragment containing a unique \( P_{SPA} \) site and the PstI site of pAK39 into which the 1.9-kbp PstI fragments of pVΔ28 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 ScaI (Fig. 2), followed by its transformation into \( B. subtilis \), yields kanamycin-resistant transformants which have a high probability of recombining both \( P_{SPA} \) and the mutant rsbW and rsbV alleles into the \( B. subtilis \) chromosome. rsbK::spec was constructed by linearizing pML7 at its unique ClaI site with EcoRI/kpnI to obtain a SpeI target site. The PstI fragment of pPS19 (4) cloned into the 1.4-kbp HpaI II DNA fragment. The resulting plasmid, pML7/X::Spec, was linearized with ScaI and transformed in \( B. subtilis \), in which spectinomycin-resistant clones would carry the rsbK::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-\( \sigma^B \) monoclonal antibody. Bound antibody was visualized with an alkaline phosphatase-conjugated goat immunoglobulin against mouse immunoglobulin (HyClone Laboratories, Inc.).

**\( \beta \)-Galactosidase assays.** Cells were harvested from cultures and frozen at −70°C until further use. In some experiments, \( \beta \)-galactosidase activity was detected in lysates prepared by resuspending cell pellets in 5 ml of Z buffer (33) and passing the cells through a French pressure cell at 15,000 lb/in². Debris was removed by centrifugation at 10,000 × g for 10 min. Dilutions of the supernatants were assayed for \( \beta \)-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. \( \beta \)-Galactosidase activity was calculated as the optical density at 420 nm × 100/mg of protein in a 30-min incubation at 37°C. \( \beta \)-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-\( \sigma^B \) monoclonal antibody.** As described above, \( \sigma^B \) 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-\( \sigma^B \)-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::ΔsigB, fractionated by SDS-PAGE and either stained with Coomassie blue or transferred to nitrocellulose and probed with anti-\( \sigma^B \) antibody. In the data presented, 10 times more extract from the plasmidless strain than from the strain carrying pRSET::ΔsigB 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::ΔsigB-containing strain (Fig. 3A, lane 4). The faster-migrating of the two bands had the mobility of the \( \sigma^B \) antigen. We suspect that the more slowly migrating band is an aggregated form of the \( \sigma^B \) 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 \( \sigma^B \) 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 \( \sigma^B \) 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 \( \sigma^B \) (FY22) or contained a sigB allele (BSA80 sigB314) (4) with a frameshift mutation which terminates translation near the \( \sigma^B \) 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 the \( \sigma^B \) in the extract prepared from the wild-type strain (Fig. 3B, lane 4). We conclude that this antibody is highly specific for \( \sigma^B \) and can be used to monitor \( \sigma^B \) in \( B. subtilis \) extracts.

**Response of \( \sigma^B \) levels to medium and growth state.** The principal promoter of the sigB operon is transcribed by \( \sigma^B \)-containing RNA polymerase (4, 15). Two additional \( \sigma^B \)-dependent transcription units are known (csbA and ctc) (8, 24). Both ctc and csbA are induced approximately fivefold by a \( \sigma^B \)-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 \( \sigma^B \)-dependent promoter, would also fluctuate in response to growth state and medium. Our previous data suggested, however, that the sigB operon's \( \sigma^B \)-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
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 σB levels. Instead, an additional level of control is likely to exist at ctc and csbA. The availability of an anti-σB antibody permitted us to monitor changes in the level of σB in response to medium or state-of-growth changes which affected ctc and csbA expres-
We therefore measured the relative abundance of \( \sigma^B \) 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 \( \sigma^B \) 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, \( \sigma^B \) 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 \( \sigma^B \) found in both LB and DS medium substantiate the previous finding (4) and demonstrate that the observed LB-dependent increase in ctc and ctaA gene expression is unlikely to be solely due to increases in \( \sigma^B \) levels.

Accumulation of \( \sigma^B \) in \( B. \ subtilis \) strains with mutations in the \( \text{sigB} \) operon. We and others have observed that null mutations in two of the genes of the \( \text{sigB} \) operon (\( \text{rsbV} \) and \( \text{sigB} \)) eliminated expression from the ctc and \( \text{sigB} \) operons' \( \sigma^B \)-dependent promoters while null mutations in the remaining two genes (\( \text{rsbW} \) and \( \text{rsbX} \)) dramatically increased the activity of these promoters (4, 6, 7, 15, 23). Using the anti-\( \sigma^B \) antibody in Western blot analyses, we can directly observe the changes in \( \sigma^B \) abundance that occur in the \( \text{rsbV} \), \( \text{rsbW} \), and \( \text{rsbX} \) 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 \( \sigma^B \) levels. Both the \( \text{rsbW} \) (BSA46; Fig. 4, lanes 5 and 6) and \( \text{rsbX} \) (BSA64; lanes 7 and 8) mutant strains accumulate substantially more \( \sigma^B \) protein than that found in the wild-type strain (BSA46; lanes 1 and 2), while the \( \text{rsbV}312 \) strain (BSA93; lanes 3 and 4) lacks detectable \( \sigma^B \). 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 \( \text{rsbV} \), \( \text{rsbW} \), and \( \text{rsbX} \) mutations on \( \sigma^B \) abundance and ctc activity, there is an LB-dependent enhancement of ctc induction that was not seen as a change in \( \sigma^B \) protein levels. These results are consistent with RsbV, RsbW, and RsbX being regulators of \( \sigma^B \) synthesis and support the notion that there is an additional medium-dependent regulatory element operating at the \( \sigma^B \)-dependent ctc promoter that does not control the \( \sigma^B \)-dependent promoter of \( \text{sigB} \).

Expression of \( \sigma^B \) from a \( \text{P}_{\text{spac}} \)-controlled \( \text{sigB} \) operon. Genetic and biochemical evidence argue that the RsbV-RsbW proteins compose a regulatory device for controlling \( \sigma^B \) activity and that RsbX is an additional regulator that functions at a point upstream of RsbV-RsbW in the pathway of \( \sigma^B \) control (4, 5, 7). In order to verify in vivo that RsbV...
and RsbW control σB activity, independent of their effect on σA levels, and to ask whether RsbX could also be a modulator of σA 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 σA. To do this, we deleted a critical element of the σA-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 SPB 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, PspAC and hence σB, is inducible by the addition of IPTG to the culture medium.

The termination codon of rsbV is superimposed on the initiation codon of ctc (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 σA in a B. subtilis strain in which their potential effects on sigB transcription would not mask a posttranscriptional influence. B. subtilis cultures containing PspAC-driven sigB operons, specifying wild-type or mutant rsbV or rsbW alleles, were induced with IPTG and analyzed for σA levels by Western blot. Figure 5 shows induction of σA 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 σA 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-σA antibody, comparable levels of this operon product can be seen in both extracts (data not shown). The drop in σA but not RsbV in the rsbW313-containing strain is consistent with the low level of σA protein seen in this strain being due to a posttranscriptional effect of the rsbW313 allele on σA 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 σA 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 σA-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 PspAC-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 PspAC. 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 σA 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),

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>LB plus glucose and glutamine</th>
<th>DS medium</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mid-log phase</td>
<td>Stationary phase</td>
</tr>
<tr>
<td>BSA46</td>
<td>5.2</td>
<td>0.6</td>
</tr>
<tr>
<td>BSA73</td>
<td>3.0</td>
<td>0.17</td>
</tr>
<tr>
<td>BSA88</td>
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</tr>
<tr>
<td>BSA64</td>
<td>118</td>
<td>1.97</td>
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* β-Galactosidase activity is calculated as optical density at 420 nm x 100/mg of protein in a 30-min assay.

**FIG. 5. σA accumulation in Δ28::PspAC strains.** Crude extracts from BSA105 (Δ28::PspAC, pTet-I; lanes 7 to 9), BSA106 (rsbV312 Δ28::PspAC, pTet-I; lanes 4 to 6), and BSA107 (rsbW313 Δ28::PspAC, 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-σA 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.
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 σB 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 σB 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 PspAC-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 σB 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 σB activity and not its abundance, samples of the cultures were taken before or
FIG. 7. \(\sigma^B\) 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 Arnott et al. (2) and analyzed for \(\sigma^B\) 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 \(\sigma^B\) synthesis that followed the IPTG additions depicted in Fig. 6. The fourth lane in each series (E, J, O, and T) depicts the induced \(\sigma^B\) 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-\(\sigma^B\) monoclonal antibody as a probe. In the absence of IPTG, the \(\sigma^B\) 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 \(\sigma^B\) 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 \(\sigma^B\) levels (e.g., lanes G to J versus lanes Q to T), but the presence of the rsbW313 mutation reduced the levels of \(\sigma^B\) 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 \(\sigma^B\) 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 \(\sigma^B\) (i.e., BSA105 induced during vegetative growth [lane G]) and at a high level in the culture with one of the lowest levels of \(\sigma^B\) (i.e., BSA106 induced at stationary phase [lane D]). It would appear that it is not the abundance of \(\sigma^B\) 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 \(\sigma^B\) activity while RsbX appears to be a negative regulator of the sigB operon and not a direct inhibitor of \(\sigma^B\) 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 \(\sigma^B\)-dependent transcription (4, 7). These analyses indicated that rsbW and rsbX encode negative regulators of \(\sigma^B\) while rsbV specifies a positive regulator which functions to counteract the negative regulation of RsbW. RsbW-dependent inhibition of \(\sigma^B\) appears to involve a direct association between RsbW and \(\sigma^B\) which prevents \(\sigma^B\) from binding to RNA polymerase (5). Given that RsbW and \(\sigma^B\) form a complex, RsbV presumably acts by disrupting or preventing formation of this complex.

The notion that RsbV and RsbW control \(\sigma^B\) 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 \(\sigma^B\)-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 \(\sigma^B\) protein were monitored, we found little correspondence between the expression of ctc and the levels of \(\sigma^B\) protein. In the absence of RsbW, high levels of ctc expression occur even at relatively low \(\sigma^B\) levels, while a lack of RsbV prevents the expression of ctc even when \(\sigma^B\) 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 \(\sigma^B\).

The previously noted difference in the activities of the \(\sigma^B\)-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 \(\sigma^B\) 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 \(\sigma^B\) levels may be due to reduced translation of sigB in the absence of a fully translated rsbW gene; however, other explanations are possible. \(\sigma^B\) and RsbW appear to form a complex within the cell (5). Therefore, in the absence of RsbW, \(\sigma^B\) may be more susceptible to degradation. Pulse-chase experiments will be needed to distinguish between potential roles for rsbW in \(\sigma^B\) synthesis or stabilization.

The function of RsbX in \(\sigma^B\) regulation is not clear. Unlike the RsbW-RsbV pair, RsbX has no apparent effect on \(\sigma^B\) activity when the sigB operon is expressed from \(P_{SPAC}\). Although this finding suggests that RsbX controls \(\sigma^B\) expression but not its activation state, such a notion is not easily reconciled with the observation that \(\sigma^B\)-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 \(\sigma^B\). The apparent failure of RsbW to inhibit \(\sigma^B\) 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 \(\sigma^B\) 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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REFERENCES


