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Relative Levels and Fractionation Properties of *Bacillus subtilis* σ^B and Its Regulators during Balanced Growth and Stress

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σ^B is a secondary sigma factor that controls the general stress response in *Bacillus subtilis*. σ^B-dependent genes are activated when σ^B is released from an inhibitory complex with an anti-σ^B protein (RsbW) and becomes free to associate with RNA polymerase. Two separate pathways, responding either to a drop in intracellular ATP levels or to environmental stress (e.g., heat, ethanol, or salt), cause the release of σ^B from RsbW. *rsbR*, *rsbS*, *rsbT*, and *rsbU* are four genes now recognized as the upstream half of an operon that includes *sigB* (σ^B) and its principal regulators. Using reporter gene assays, we find that none of these four genes are essential for stationary-phase (i.e., ATP-dependent) activation of σ^B, but *rsbU* and one or more of the genes contained within an *rsbR,S,T* deletion are needed for stress induction of σ^B. In other experiments, Western blot (immunoblot) analyses showed that the levels of RsbR, RsbS, RsbT, and RsbU, unlike those of the *sigB* operon's four downstream gene products (RsbV, RsbW, RsbX and σ^B), are not elevated during σ^B activation. Gel filtration and immunoprecipitation studies did not reveal the formation of complexes between any of the four upstream *sigB* operon products and the products of the downstream half of the operon. Much of the detectable RsbR, RsbS, RsbT, and RsbU did, however, fractionate as a large-molecular-mass (approximately 600-kDa) aggregate which was excluded from our gel filtration matrix. The downstream *sigB* operon products were not present in this excluded material. The unaggregated RsbR, RsbS, and RsbU, which were retarded by the gel matrix, eluted from the column earlier than expected from their molecular weights. The RsbR and RsbS fractionation profile was consistent with homodimers (60 and 30 kDa, respectively), while the RsbU appeared larger, suggesting a protein complex of approximately 90 to 100 kDa.

σ^B is a secondary sigma factor of *Bacillus subtilis* (7, 14, 15). σ^B-containing RNA polymerase (E-σ^B) transcribes a large group of genes that become active when the bacterium is exposed to any of a number of environmental stresses (e.g., heat, salt, ethanol, and acid) or ceases exponential growth under conditions which block sporulation (5, 8–10, 16, 17, 20, 32). σ^B, like at least two other *B. subtilis* sigma factors (σ^F and σ^G) (2, 13, 19, 22, 26, 28), is negatively regulated by a specific sigma factor-binding protein (anti-sigma factor) that blocks σ^B's association with RNA polymerase (6). The anti-σ^B protein (RsbW) is encoded by the second gene of a σ^B-controlled operon (P_B-*rsbV-rsbW-sigB-rsbX*) whose third member (*sigB*) is the structural gene for σ^B itself (18). The remaining two genes (*rsbV* and *rsbX*) encode additional regulators of σ^B (3, 4, 8, 10, 11, 14, 16). RsbV is a positive regulatory protein that is needed for the release of σ^B from RsbW, while RsbX is a negative regulator of the stress-induced σ^B activation pathway (3, 4, 10, 11, 31).

RsbW can form mutually exclusive complexes with either RsbV or σ^B (6, 11). In the absence of RsbV, RsbW remains associated with σ^B and blocks σ^B-dependent transcription (6, 11). RsbW is not only a σ^B/RsbV-binding protein but also a kinase that can transfer a PO_4 group from ATP to RsbV (11). Both RsbV and its homolog in the σ^F/σ^G system, SpoIIAA, can be phosphorylated by its respective anti-sigma factor partner (RsbW or SpoIIAB) (1, 2, 11, 22). The phosphorylated form of RsbV does not bind to RsbW (11).

There are two known routes by which environmental signals influence RsbW's binding decision. The first appears to depend on the cell's intracellular ATP concentration and is similar to an ATP-dependent pathway originally proposed to control σ^F activity (1, 2, 34). A correlation exists in vitro between an effect of ATP levels on RsbW-mediated phosphorylation of RsbV and a coincident inhibition of both RsbW-RsbV complex formation and σ^B-dependent transcription (1). At high ATP levels, RsbV is efficiently phosphorylated and inactivated by RsbW, thereby favoring RsbW-σ^B complex formation. Conversely, at low ATP levels, phosphorylation of RsbV is inefficient, leaving RsbV unphosphorylated and bound to RsbW. It is probable that a drop in intracellular ATP levels can also trigger σ^B activation in vivo. ATP levels fall coincident with the increase in σ^B-dependent transcription that occurs during carbon limitation (34).

Aside from the ATP-dependent pathway of σ^B activation, there is a second mechanism by which σ^B is released from RsbW. This pathway is ATP independent, is negatively regulated by RsbX, and requires the products of at least one member (*rsbU*) of a group of four genes (*rsbR-rsbS-rsbT-rsbU*) that lies immediately upstream of the original *sigB* operon (31, 34, 35). The upstream genes are under the control of a σ^A-dependent promoter, with no obvious termination site between this transcription unit and the previously recognized *sigB* operon (35). Thus, *rsbV*, *rsbW*, *sigB*, and *rsbX* are expressed with the upstream genes by the principal *B. subtilis* holoenzyme, E-σ^A, and independently activated by E-σ^B. Potential roles for the four most upstream gene products in σ^B activation are not well defined; however, it has been reported that RsbT is a positive stress regulator of σ^B activity and that RsbS acts as a negative regulator of σ^B in unstressed cells (25). The RsbU-dependent pathway is dispensable for σ^B activation during carbon limitation or stationary phase (i.e., ATP-dependent σ^B activation) but is needed for the activation of σ^B in response to environmental insult (ethanol treatment, heat shock, salt stress, etc.)

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TABLE 1. *B. subtilis* strains used

Strain	Relevant genotype	Reference or source
BSA46	SPβ *ctc*::*lacZ*	3
BSA177	*rsbU*::*kan rsbX*::pWH25[a] SPβ *ctc*::*lacZ*	3
BSA140	*rsbU*Δ*Nde*I *rsbX*::pWH25 SPβ *ctc*::*lacZ*	30
BSA158	*rsbU*Δ*Nde*I *rsbX*::pWH25	33
BSA199	*rsbU*::pAL265	This study
BSA206	*rsbR*::pAL330	This study
BSA219	*rsbR*::pAL330 *kan* upstream of *spoIIGA*	This study
BSA220	*rsbR*::*rsbT* Δ*rsbS kan* upstream of *spoIIGA*	This study
BSA221	*rsbR*::*rsbT* Δ*rsbS kan* upstream of *spoIIGA* SPβ *ctc*::*lacZ*	This study
BSA222	*rsbR*::*rsbT* Δ*rsbS spoIIGB*::pHP388.5	This study
BSA322	*rsbR*::*rsbT* Δ*rsbS spoIIGB*::pHP388.5::pAL430	This study
BSA323	*rsbR*::*rsbT* Δ*rsbS spoIIGB*::pHP388.5::pAL430 SPβ *ctc*::*lacZ*	This study
HP91	*kan* upstream of *spoIIGA*	Laboratory strain
SEP388.5	*spoIIG*::pHP388.5	24

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

(34). Although RsbU-dependent σ^B activation is not an ATP-dependent process, it is similar to the ATP-dependent pathway in that it requires the binding of unphosphorylated RsbV to RsbW as a prerequisite for σ^B release (33, 34). The precise mechanism by which this is accomplished is unclear; however, it may involve the induction of a phosphatase which can reactivate RsbV-P, thereby providing RsbV to bind RsbW and free σ^B (33).

In this report, we describe experiments designed to better define the activities and possible associations of RsbR, RsbS, RsbT, and RsbU. We found that a deletion which removes the three promoter-proximal genes (*rsbR*, *rsbS*, and *rsbT*) eliminates stress-dependent, but not ATP-dependent, σ^B activation. Immunological probes were used to examine the levels and fractionation properties of the *sigB* operon's eight products. We observed that environmental conditions which enhance the accumulation of RsbV, RsbW, σ^B, and RsbX have little effect on the levels of RsbR, RsbS, RsbT, or RsbU. The abundance of this latter group of proteins remains relatively constant during σ^B activation. In addition, fractionation of crude cell extracts by techniques which we had previously used to establish the associations between RsbV, RsbW, and σ^B (3, 6, 11) failed to show simple complexes of RsbR, RsbS, RsbT, and RsbU with themselves or the better-characterized σ^B regulators. The fractionation analysis did, however, uncover curious properties of several of these proteins. RsbU, RsbS, and RsbR fractionated as proteins of larger size than anticipated on the basis of their predicted molecular weights. In addition, a significant portion of RsbR, RsbS, RsbT, and RsbU, but not the products of the downstream half of the *sigB* operon, was found in a large-molecular-mass (ca. 600-kDa) aggregate.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. The *B. subtilis* strains used in this study are listed in Table 1. All of the BSA strains are derivatives of PY22 (*trpC2*), which was obtained from P. Youngman (University of Georgia). *Escherichia coli* TG2 (27) and BL21 (DE3) containing pLysS (29) were used as hosts for the cloning experiments and for the overexpression of fusion proteins, respectively. *B. subtilis* and *E. coli* strains were routinely grown under vigorous agitation at 37°C in Luria broth (LB) (27). In some experiments, *B. subtilis* strains were cultivated in LB supplemented with 5% glucose and 0.2% glutamine (LBGG). Stress was imposed by adding ethanol to a final concentration of 4% (vol/vol). For the selection in *B. subtilis*, the antibiotics were used at the following concentrations: spectinomycin, 200 μg/ml; kanamycin, 20 μg/ml; chloramphenicol, 5 μg/ml; erythromycin, and 1 μg/ml; 100 μg of ampicillin per ml was used for *E. coli*. pBluescriptKS (27), pUS19 (4), and pRSET A and pRSET C (Invitrogen) were used as vectors. The inserts of the recombinant plasmids are shown Fig. 1.

PCRs. PCRs were routinely performed with the AmpliTaq DNA polymerase (Perkin-Elmer) according to standard protocols (27). When the amplified fragments were to be cloned, the UlTma DNA polymerase (Perkin Elmer) was used as recommended by the manufacturer. The sequences of the primers were as follows: universal primer, 5′-GTAAAACGACGGCCAGT-3′; reverse primer; 5′-GAGCGGATAACAATTTCAC-3′; ALPC3, 5′CTCAGAGGATCCATATG ATGTCGAACCAGACTG-3′; ALPC4, 5′-CCAAAG-AAGCTTATTCCCCCA ATGAAACG-3′; ALPC5, 5′-CCCTAAGGATCCATATGAGACATCCGAAA ATCCC-3′; ALPC6, 5′GTTTCCGGATCCATATGAACGACCAATCCTGTG-3′; ALPC7, 5′ATACCCCTGCAGCTACCGAAGCCATTTGATG-3′; and ALSEQ5, 5′-GAGGAATTTGGTGCTCG-3′. The universal and reverse primers are complementary to sequences of pUS19 and pBluescript. ALPC3 and -4 are at the 5′ and 3′ ends of *orfR*, respectively. ALPC5 is at the 5′ end of *orfS*. ALPC6 and -7 are at the 5′ and 3′ ends of *orfT*, respectively. ALSEQ5 is internal to *rsbU*.

Construction of a strain with a deletion of *orfR*, *orfS*, and *orfT*. We constructed pAL265 to clone the *rsbU* operon and upstream sequences by plasmid rescue. pAL265 was obtained by subcloning the 2-kb *Hin*dIII-*Eco*RI fragment from pV312 (3) into pBluescript KS. The insert includes the kanamycin resistance gene (*kan*), the 3′ half of *rsbU*, the promoter of the *sigB* operon, and the first two codons of *rsbV* (Fig. 1). Following the Campbell-like integration of pAL265 into the chromosome of PY22, the chromosomal DNA was extracted from the resulting strain (BSA199) and digested with either *Cla*I or *Hin*dIII. Both of these enzymes cut between the vector and the *kan* gene. The DNA was self-ligated and transformed into *E. coli* TG2 by electroporation, selecting with ampicillin. pAL301 and pAL322 were obtained from the *Cla*I and the *Hin*dIII digests, respectively. pAL301 contains a 3-kb insert that covers the entire *rsbU* operon (Fig. 1). pAL322 harbors a 6-kb insert composed of the complete *rsbU* operon plus 3.3 kb of upstream sequences. The identity of the *rsbU* operon in pAL301 and pAL322 was confirmed by restriction analysis and PCR amplification of *rstR* with the primers ALPC3 and ALPC4.

A deletion encompassing *rsbS* and a large part of *rsbR* and *rsbT* was constructed in pAL322 as follows. pAL301 was digested by *Spe*I and religated in order to eliminate the *Eco*RI site of the polylinker. The resulting plasmid, pAL343 (Fig. 1), was cut by *Eco*RI and self-ligated to create pAL348, a plasmid lacking the three *Eco*RI fragments of *rsbR*, *rsbS*, and *rsbT* (Fig. 1). As a result, the 5′ end of *rsbR* (33% of the coding sequence) and the 3′ end of *rsbT* (15% of the coding sequence) were fused in frame (*rsbR*::*rsbT*), and *rsbS* was completely deleted (Δ*rsbS*). The *Hpa*I-*Spe*I fragment of the insert of pAL322 was substituted by the *Hpa*I-*Spe*I fragment from pAL348, leading to pAL374. The latter contains thus (i) the *rsbU* operon deleted of its three internal *Eco*RI fragments and (ii) 3.3 kb of upstream DNA sequences (Fig. 1). The *Eco*RI deletion could not be made directly in pAL322 since two additional *Eco*RI sites were found in the upstream sequences.

The deletion was introduced into the *B. subtilis* chromosome by congression. A recipient and a suitable source of a selection marker for the congression were obtained as follows. Plasmid pAL330, containing the 5′ end of *rsbR*, was constructed by subcloning the *Bam*HI-*Eco*RI fragment from pAL285 (see below for the construction of pAL285) into pUS19 (Fig. 1). pAL330 was integrated via a Campbell-like mechanism into the chromosome of *B. subtilis* PY22, creating BSA206, in which *rsbR*, *rsbS*, *rsbT*, and *rsbU* are separated from their promoter by the insertion of pUS19. BSA206 was used as a recipient for the congression. A suitable source of a selection marker for the congression was generated by transforming BSA206 with chromosomal DNA from *B. subtilis* HP91, which contains the *kan* gene linked to the *spoIIG* operon. The chromosomal DNA of a Km^r Sp^r transformant (BSA219) was used for congression performed as follows. BSA206 was transformed with a mix of approximately 1 μg of chromosomal

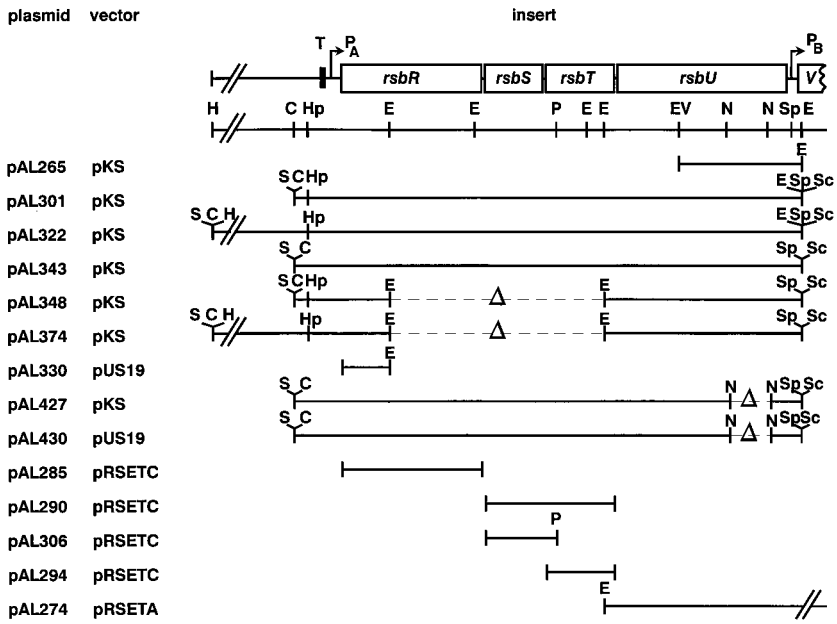

FIG. 1. Physical map of the upstream half of the *sigB* operon and recombinant plasmids. The open rectangles show the four open reading frames (*rsbR*, *rsbS*, *rsbT*, and *rsbU*) of the upstream half of the *sigB* operon and the beginning of *rsbV* (3, 31, 35). P_A and P_B indicate the σ^A- and σ^B-dependent promoters, respectively. T represents the terminator lying upstream of P_A. The broken line represents deleted regions. Only the restriction sites used in the construction of the recombinant plasmids are given. Two additional *Eco*RI sites are found in the most upstream *Hin*dIII-*Cla*I fragment. The inserts of the recombinant plasmids are shown, and the vectors used for each construct are indicated. Abbreviations: C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I; N, *Nde*I; P, *Pvu*II; S, *Sal*I; Sc, *Sac*I; Sp, *Spe*I; pKS, pBluescript KS.

DNA from BSA219 and 25 μg of pAL374 digested by *Hin*dIII and *Sac*I to release the insert. Transformants were selected on kanamycin-containing LB agar plates. Upon a successful congression event, pAL330 would be eliminated from the recipient bacteria, resulting in the loss of spectinomycin resistance. The individual Km^r colonies were thus screened for spectinomycin sensitivity. The integration of the *Eco*RI deletion in one Sp^s colony was confirmed by two different PCRs, using the following primer pairs: reverse primer-ALSEQ5 and ALPC3-ALSEQ5. The mutant strain was designated BSA220. BSA221 was obtained by transducing BSA220 with SP *ctc*::*lacZ*.

Complementation of the deletion of *orfR*, *orfS*, and *orfT*. pAL343 was digested by *Nde*I and relegated. The resulting plasmid, pAL427 (Fig. 1), harbors the *Nde*I deletion in *rsbU* which was previously used to generate an inactive form of RsbU (RsbUΔ*Nde*I) (31). The insert of pAL427 was excised by a *Sal*I-*Sac*I digestion and was subcloned into the integrative vector pUS19, creating pAL430 (Fig. 1).

BSA220 was transformed with chromosomal DNA from SEP388.5, selecting for chloramphenicol resistance. SEP388.5 contains pHP388.5, a Cm^r derivative of pUC18, integrated at *spoIIG* (23). pHP388.5 provides a homology to the pUC part of pUS19 and thus allows the Campbell-like integration of pAL430 in *trans* into the chromosome of *B. subtilis*. The Cm^r transformants of BSA220 were screened for the loss of the *kan* gene, and such a clone (BSA222) was transformed with pAL430, selecting for spectinomycin resistance. To isolate a strain which had integrated pAL430 in *spoIIG* and not in the *sigB* operon, PY22 was transformed with chromosomal DNA from different transformants of BSA222 carrying pAL430, and the linkage of the *spc* and *cm* markers was examined. BSA323 was obtained by transducing a strain (BSA322) in which the two markers were linked with SPβ *ctc*::*lacZ*.

Construction of RsbX mutants. RsbX mutants of *B. subtilis* were obtained by transformation with either *Pvu*I-digested pML7/X::Spc (4) or pAK24 linearized with *Sca*I (31). Both pML7/X::Spc and pAK24 contain an antibiotic resistance gene (*spc* and *kan*, respectively) inserted into the *Cla*I site of *rsbX*.

Preparation of RsbR, RsbS, RsbT, and RsbU antigens and antibody production. *orfR* was amplified by PCR, using the primers ALPC3 and ALPC4. pAL285 was constructed by cloning the amplified DNA into pRSET C, digested with *Bam*HI and *Hin*dIII. *rsbS* and *rsbT* were amplified as a single fragment with oligonucleotides ALPC5 and ALPC7. This fragment was inserted into the *Bam*HI-*Pst*I sites of pRSET C. The resulting plasmid, pAL290, was cut by *Pvu*II and self-ligated, in order to eliminate a large part (85%) of *rsbT*, creating pAL306. *rsbT* was amplified with the primers ALPC6 and ALPC7 and cloned at the *Bam*HI-*Pst*I sites of pRSET C, leading to pAL294. The 2.2-kb *Eco*RI fragment from pML7 (7), containing the 3′ end of *rsbT*, *rsbU*, *rsbV*, *rsbW*, and a part of *sigB*, was subcloned at the *Eco*RI site of pRSET A in the positive orientation, creating pAL274. pAL285, pAL306, pAL294, and pAL274 each fuse one of the structural genes of interest, in frame, with the 5′ end of a phage T7 gene that had been modified by the manufacturer to include a stretch of six histidine residues in its product.

Each of the fusion proteins was overexpressed in *E. coli* BL21(DE3)(pLysS) as follows. The recombinant strains were grown in ampicillin-containing LB to an optical density at 540 nm of ~0.7; 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 100 μg of ampicillin per ml were added, and the culture was incubated for an additional 4 to 5 h. The cells were harvested, resuspended in buffer A (6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris [pH 8.0]), and disrupted by passage through a French press cell. The debris were eliminated by two centrifugations of 10 min each at 10,000 × *g*. The fusion proteins were purified from the supernatant by adsorption to Ni^{2+}-nitrilotriacetic acid (Ni-NTA) resin (Qiagen) in buffer A for 1 h at room temperature. The resin was washed successively with buffer A, buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris [pH 8.0]), and buffer C (same composition as buffer B, but pH 6.3). The fusion proteins were eluted from the Ni-NTA resin with buffers D and E (same as buffer B, but pH 5.9 and 4.5, respectively). The bulk of the fusion proteins eluted in buffer E, as determined sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the different fractions. It was necessary to add 0.1% Triton X-100 to buffer E in order to release the RsbS protein fusion from the Ni-NTA resin. The antigens were extensively dialyzed versus phosphate-buffered saline (pH 7.4) and used to immunize BALB/c mice. The antibodies were produced as previously described (4, 30), using the non-immunoglobulin-secreting SP2/0-Ag14 NS1 BALB/c myeloma cell line to produce hybridomas.

Gel filtration chromatography. Bacteria were harvested and immediately chilled by being poured into centrifuge bottles containing equal volumes of ice chips. Cells were concentrated 200-fold in resuspension buffer (10 mM Tri-HCl [pH 8.0], 1 mM EDTA, 50 mM NaCl, 10 mM $MgCl_2$, 0.3 g of phenylmethylsulfonyl fluoride per liter, 3 mM dithiothreitol). Extracts were prepared and fractionated on Sephacryl matrices as described previously.

Immunoprecipitation. Crude cell extracts of *B. subtilis* were incubated overnight at 4°C with the respective antisera in binding buffer (10 mM Tris [pH 8.0], 1% Triton, 10 mM EDTA, 0.3 mg of phenylmethysulfonyl fluoride per ml). Immune complexes were bound to protein A immobilized to Sepharose CL4B, precipitated, washed, and separated by SDS-PAGE.

General methods. SDS-PAGE, Western blotting (immunoblotting), and the assays of β-galactosidase activity were performed as previously described (21, 34). DNA manipulations were performed according to standard protocols (27). *E. coli* strains were transformed by electroporation in an Electroporator II (Invitrogen) as described by the manufacturer. Transformation of natural competent *B. subtilis* cells was carried out as described by Yasbin et al. (36).

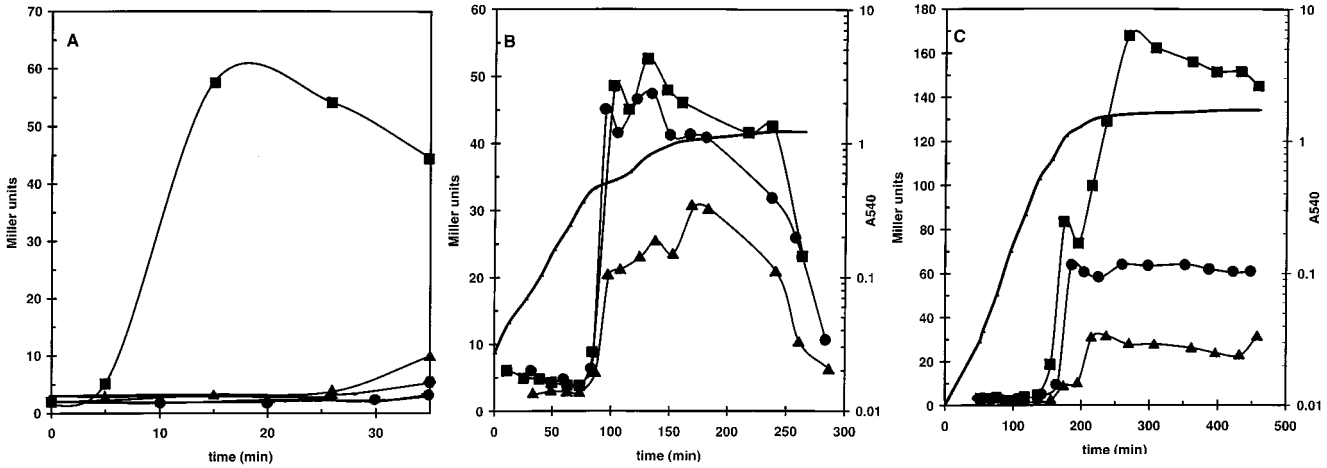

FIG. 2. Stress-induced expression of *ctc*::*lacZ*. *B. subtilis* strains carrying SPβ *ctc*::*lacZ* were grown in LB (A and B) or LBGG (C). Cells were either exposed to 4% ethanol during exponential growth at time zero (A) or allowed to enter stationary phase (B and C). The levels of β-galactosidase were determined as described in Materials and Methods. ■, BSA46 (wild type); ●, BSA140 (*rsbU*Δ*Nde*I); ▲, BSA221 (*rsbR*::*rsbT*Δ*rsbS*). In panel A, these symbols represent stressed cultures, with ● indicating an unstressed control culture (BSA46) whose levels were typical of the unstressed levels in all three strains. In panels B and C, these symbols represent these same strains during growth and entry into stationary phase. The solid thick lines in panels B and C portray growth of BSA46 (wild type). The growth rates of BSA140 and BSA221 were similar.

RESULTS

Effects of the loss of RsbR, RsbS, and RsbT on σ^B activation. The RsbU-dependent (stress activated) pathway of σ^B is negatively regulated by RsbX (31, 34). Introduction of an *rsbX* null allele into *B. subtilis* containing *lacZ* joined to a σ^B-dependent promoter (SPβ *ctc*::*lacZ*) yields growth-inhibited, dark blue colonies on medium that contains 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (3, 16, 18, 31). In contrast, the loss of RsbX has no effect on σ^B activity or colony growth in an RsbU$^-$ strain. RsbU$^-$ RsbX$^-$ strains form large light blue colonies on X-Gal-containing media (31). To determine if any of the genes cotranscribed with *rsbU* contribute to σ^B regulation in one or both of σ^B's two known activation pathways, we constructed a *B. subtilis* strain in which the structural genes for most of RsbR and RsbT and all of RsbS were deleted (*rsbR*::*rsbT*Δ*rsbS*) but their transcription unit, which includes *rsbU* and extends into the former *sigB* operon, remained intact. When an RsbX$^-$ mutation (*rsbX*::*spc*) was placed in a strain containing the *rsbR*::*rsbT* mutation, the transformants formed large light blue colonies characteristic of a strain without a stress-activatable σ^B pathway. To verify that this phenotype was due to a loss of RsbR, RsbS, and RsbT and not an unforeseen effect of the deletion on *rsbU* on the downstream *sigB* operon, we introduced a copy of *rsbR*, *rsbS*, and *rsbT* under the control of their normal promoter into the *rsbR*::*rsbT* strain at an alternative site (*spoIIG* locus) in the *B. subtilis* chromosome (see Materials and Methods). *rsbR*::*rsbT* strains with copies of *rsbR*, *rsbS*, and *rsbT* at *spoIIG* formed pinpoint, dark blue colonies when transformed to RsbX$^-$. This result is consistent with the absence of a RsbX$^-$ phenotype in the *rsbR*::*rsbT rsbX*::*spc* strain being due to a loss of one or more of the gene products encoded within the region lost in the *rsbR*::*rsbT* deletion.

Exposure to ethanol is a potent inducer of RsbU-dependent σ^B activity (32, 34). Wild-type *B. subtilis* shows a 30-fold increase in σ^B-dependent transcriptional activity (*ctc*::*lacZ*) when it is exposed to 4% ethanol (Fig. 2A). As anticipated from the plate phenotype of the *rsbR*::*rsbT rsbX*::*spc* transformants, the strain lacking RsbR, RsbS, and RsbT resembled the RsbU$^-$ strain and failed to activate the *ctc* promoter when it was exposed to ethanol (Fig. 2A).

Although RsbU is essential for stress induced σ^B activation, it is dispensable for the σ^B activation that occurs when *B. subtilis* enters the stationary phase of growth in LB (34). The *rsbR*::*rsbT* strain behaved similarly and displayed substantial *ctc* induction in LB medium; however, this induction was approximately half of that seen in either wild-type or RsbU$^-$ strains (Fig. 2B). A reduced level of σ^B activity, compared with that found in a RsbU$^-$ strain, was also seen when the RsbRST$^-$ strain was allowed to enter stationary phase in LBGG (Fig. 2C). σ^B activation under these conditions has additive RsbU-dependent and -independent components (34). The RsbU-independent activation is likely due to a drop in ATP levels, while the RsbU-dependent activation appears to result from a lowering of culture pH (34). A strain lacking RsbU is unable to respond to the stress (acid shock)-induced stimulus and activates *ctc* transcription to only a third of the level seen in the wild-type strain (Fig. 2C). The RsbRST$^-$ strain, although RsbU$^+$, also failed to achieve the level of *ctc* expression of the wild-type strain. *ctc*-dependent β-galactosidase activity in the RsbRST$^-$ strain was substantially less then that seen in the wild-type strain and approximately half of that of the RsbU$^-$ culture (Fig. 2C). This level of *ctc* expression is similar to that seen when this strain enters stationary phase in LB (Fig. 2B). The results demonstrate that one or more of the gene products which were lost in the *rsbR*::*rsbT* deletion are essential for stress activation of σ^B and that at least one of these genes also provides an activity that is needed for maximum stationary-phase induction of σ^B.

Analysis of RsbR, RsbS, RsbT, and RsbU levels. The levels of σ^B and its previously known regulators (RsbV, RsbW, and RsbX) are dramatically elevated in response to conditions that activate σ^B (5, 34). As described in Materials and Methods, we prepared monoclonal antibodies which could be used as probes for RsbR, RsbS, RsbT, and RsbU. The specificity of the antibodies is illustrated in Fig. 3. Each of the antibodies detected a single protein band in Western blots of crude extracts from wild-type *B. subtilis* (Fig. 3, lanes 1, 3, 5, and 7) which was

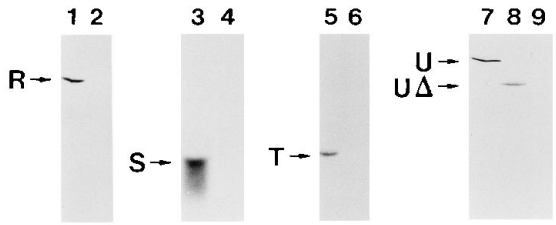

FIG. 3. Specificity of the anti-RsbR, -RsbS, -RsbT, and -RsbU antibodies. Crude *B. subtilis* extracts were prepared from stationary-phase cultures in LBGG. Sixty-microgram samples of protein were fractionated by SDS-PAGE and transferred to nitrocellulose, and the membrane was probed with one of the antibodies. The extracts were from PY22 (wild type; lanes 1, 3, 5, and 7), BSA220 (*rsbR*::*rsbT*; lanes 2, 4, and 6), BSA158 (*rsbU*Δ*Nde*I; lane 8), and BSA177 (*rsbU*::*kan*; lane 9). The membrane was probed with an anti-RsbR (lanes 1 and 2), an anti-RsbS (lanes 3 and 4), an anti-RsbT (lanes 5 and 6), or an anti-RsbU (lanes 7 to 9) antibody. Only the genotypes related to the *sigB* operon are given. Abbreviations: R, RsbR; S, RsbS; T, RsbT; U, RsbU; UΔ, RsbUΔ*Nde*I.

absent from mutant strains incapable of synthesizing these proteins (Fig. 3, lanes 2, 4, 6, and 9). The identity of the anti-RsbU-reacting protein was further substantiated by its disappearance, coincident with the appearance of a lower-molecular-weight protein, in a Western blot of an extract prepared from a strain with a truncated *rsbU* allele (*rsbU*Δ*Nde*I) (31) (Fig. 3, lane 8). In contrast to the sharp bands visualized by the antibodies raised against seven of the eight *sigB* operon products (11) (Fig. 3, lanes 1, 5, and 7), the RsbS band was quite broad on Western blot (Fig. 3, lane 3). The topmost portion of the band corresponds to the anticipated mobility of RsbS in this gel system. Apparently, RsbS is more sensitive than the other Rsb proteins to proteolysis or exists in multiple forms that cannot be resolved into tight bands.

Having established the specificity of our probes, we used these antibodies to examine the relative levels of the σ^B regulators in *B. subtilis* when σ^B activity was low (exponential growth) or when it was high (stationary phase). LB or LBGG was used as the culture medium to generate conditions for either RsbU-independent σ^B activation or RsbU-dependent and -independent σ^B activation, respectively. Neither inducing condition caused a marked increase in the abundance of RsbR, RsbS, RsbT, or RsbU relative to the levels that were seen during exponential growth (Fig. 4; compare lanes 3 and 5 with lanes 1 and 4). Curiously, the anti-RsbT antibody detected an additional protein (RsbT*) of approximately 40 kDa that appeared only in the extract from the LB stationary-phase culture

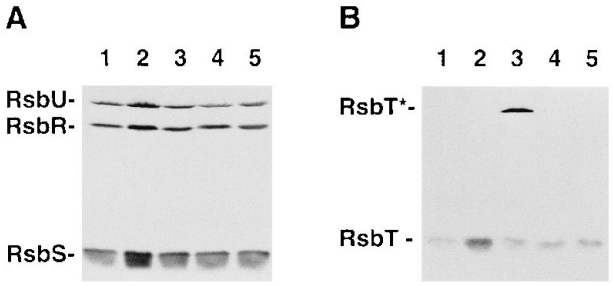

FIG. 4. Culture-dependent levels of RsbR, RsbS, RsbT, and RsbU in *B. subtilis*. BSA46 was grown in LB (lanes 1 to 3) or LBGG (lanes 4 and 5). Cells were harvested during exponential growth (lanes 1 and 4), after entry into stationary phase (lanes 3 and 5), or after treatment of exponentially growing bacteria with 4% ethanol for 30 min. Crude extracts were prepared, and 75-μg protein samples were subjected to Western blot analysis. The membrane was probed with antibodies raised against either RsbR, RsbS, and RsbU (A) or RsbT (B).

(Fig. 4B, lane 3). RsbT* is present in the *rsbR*::*rsbT* strain and therefore is not a variant form of RsbT but a second *B. subtilis* protein that carries the epitope recognized by our anti-RsbT antibody. Its significance is unknown.

B. subtilis was next exposed to 4% ethanol, a σ^B activation condition which is entirely dependent on the RsbU pathway. There was a slight increase in the levels of RsbR, RsbS, and RsbU and a more substantial, although still small, increase in RsbT (Fig. 4, lane 2). The increases in RsbR, RsbS, RsbT, and RsbU were minor compared with the increases in RsbV, RsbW, RsbX, and σ^B that occurred under similar conditions. Thus, unlike the products of the downstream half of the *sigB* operon, the levels of RsbR, RsbS, RsbT, and RsbU do not vary with σ^B activity.

Gel filtration analysis of *sigB* operon products. As a means of detecting possible associations among the *sigB* operon products, we separated the components of cytosolic extracts on a Sephacryl S-200 column and probed the resulting fractions with monoclonal antibodies. The *sigB* operon products have the following predicted molecular masses: RsbR, 31 kDa; RsbS, 13.3 kDa; RsbT, 14.3 kDa; RsbU, 38.6 kDa; RsbV, 12 kDa; RsbW, 18 kDa; σ^B, 30 kDa; and RsbX, 22 kDa (18, 36). The Western blot data for the products of the downstream half of the *sigB* operon (Fig. 5A) are consistent with our previous findings that three (RsbV, RsbW, and σ^B) of these four proteins can be found in protein complexes (4, 6, 11, 31). As we described previously, a small portion of the σ^B elutes with the excluded proteins (fractions 1 to 5) and likely represents E-σ^B RNA polymerase holoenzyme (11, 31). The bulk of the σ^B coelutes with RsbW as a broad peak centered at fractions (15 to 17) in which proteins of 90 to 100 kDa elute. Presumably, these complexes are in the form of $RsbW_2$-$\sigma^B{}_2$ tetramers (96 kDa) (11). The broad RsbW peak (fractions 13 to 27) includes not only the RsbW-σ^B complexes but also associations of RsbW and RsbV (fractions 19 to 27). On the basis of the elution positions, at least some of these RsbW-RsbV complexes are likely to exist as $RsbW_2$-$RsbV_2$ tetramers (60 kDa) (11). Most of the RsbV is found in fractions (31 to 51) that do not contain RsbW. These fractions do contain RsbX; however, coelution of RsbX with RsbV is likely to be coincidental. We previously demonstrated that each of these proteins continues to elute in these fractions even when the other protein is absent from the extract (11). The RsbV elution profile is consistent with RsbV existing as monomers and higher-molecular-weight forms when not bound to RsbW. On the basis of the position at which the peak of RsbX elutes (anticipated molecular mass of 23 to 25 kDa), RsbX likely exists as a monomer (22 kDa) in the extract.

When a similar analysis is applied to the products of the upstream half of the *sigB* operon, (Fig. 5B), a significant portion of RsbR and RsbS and, to a lesser extent, RsbU was observed in the excluded fractions (1 to 5). When fractionated on a gel filtration matrix (Sephacryl S-300) with a useful working range of 10 to 800 kDa, the previously excluded RsbU, RsbR, and RsbS eluted as a single peak at the position anticipated for a 600-kDa protein complex (data not shown). We do not know whether this large potential complex has biological relevance or represents an artifactual aggregation of these proteins with themselves and/or other cell components.

Although a portion of RsbR, RsbS, and RsbU was excluded from the column, at least half of RsbR and RsbS and most of RsbU were retarded by the column matrix. RsbU eluted as a broad peak in the same fractions that contain the RsbW-σ^B complexes. Although this coelution suggests that RsbU could be in a complex with RsbW and/or σ^B, we consider this unlikely. The RsbW-σ^B complexes continue to elute in these

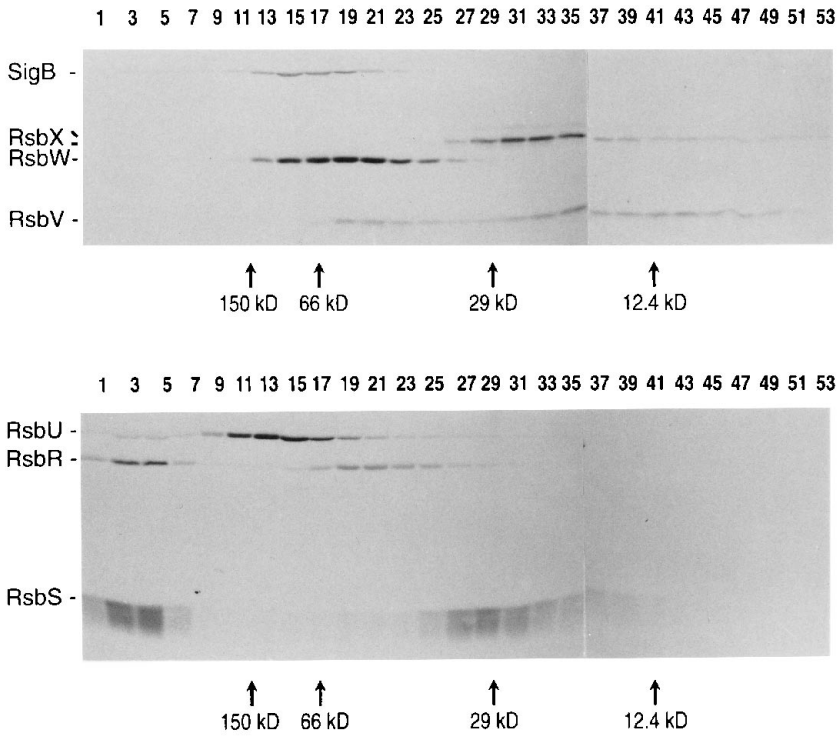

FIG. 5. Gel filtration fractionation of crude *B. subtilis* extracts. *B. subtilis* BSA46 (wild type) was grown in LB and exposed to 4% ethanol for 10 min during exponential growth. The cells were disrupted by pressing through a French press. Cell debris and the membrane fraction were removed by ultracentrifugation as described in Materials and Methods. The supernatant of the ultracentrifugation was fractionated on a Sephacryl S-200 column. Aliquots of the fractions were subject to SDS-PAGE and Western blot analysis using monoclonal antibodies raised against the products of the *sigB* operon as probes. The positions of the proteins and the elution peaks of the molecular mass markers are indicated.

same fractions in the absence of RsbU, and the elution profile of RsbU is unchanged in extracts prepared from strains of *B. subtilis* incapable of synthesizing σ^B (data not shown). The notion that RsbU is not associated with RsbW or σ^B is reinforced by immunoprecipitation studies (Fig. 6) which showed only trace amounts of RsbU coprecipitating with either σ^B or RsbW. Thus, although RsbU exited the gel filtration column at a position anticipated for proteins substantially larger (90 to 100 kDa) than its calculated size (38.6 kDa), this does not seem to be due to complex formation with any of the known σ^B regulators. Either RsbU is an irregularly shaped homodimer with unpredictable gel filtration properties or it is associated with proteins other than those encoded by the *sigB* operon. RsbR and RsbS also exited the column at positions larger than would be anticipated on the basis of their molecular weights. Their elution profiles overlap those of several other *sigB* operon products (i.e., RsbW, RsbV, and RsbX), but their peak elution positions are not coincidental (Fig. 5B), and attempts to coimmunoprecipitate RsbS and RsbR with any of these proteins were unsuccessful (Fig. 6 and data not shown). It is possible that RsbS and RsbR form nonprecipitable complexes with RsbW, RsbV, or RsbX or are associated with an unknown protein. It is equally plausible, however, that RsbR and RsbS exist as homodimers. Their elution positions are consistent with the fractions in which $RsbR_2$ (61 kDa) and $RsbS_2$ (26.6 kDa) might be expected to exit the column.

RsbT proved difficult to analyze by gel filtration. The bulk of the RsbT that was loaded onto the gel filtration column could not be detected after fractionation. Either it was degraded, it diluted beyond detection over many fractions, or it bound the gel matrix. The RsbT that we could recover eluted in the excluded fractions where the putative RsbR-RsbS-RsbU aggregate was found (data not shown).

Immunoprecipitation studies. A peak of RsbU coincided with the peak of $RsbW_2$-$\sigma^B{}_2$ protein during gel filtration. To investigate potential complexes of RsbW, σ^B, or RsbU with each other or additional σ^B regulators, we immunoprecipitated each of these proteins from crude cell extracts and analyzed the precipitated material by Western blotting for other *sigB* operon products. Extracts were prepared from exponentially growing *B. subtilis* (σ^B inactive), ethanol-treated cells (σ^B activated by the RsbU-dependent pathway), and a stationary-phase culture (σ^B activated by the RsbU-independent pathway). The culture conditions used affect both the abundance of several of the proteins (i.e., RsbV, RsbW, σ^B, and RsbX) (Fig. 6, lanes 6) and the degree to which associations occur (e.g., RsbW-σ^B complexes predominate during growth, and RsbW-RsbV complexes become evident during stress) (Fig. 6, lanes 5). Polyclonal anti-σ^B, polyclonal anti-RsbW, monoclonal anti-RsbU, monoclonal anti-σ^B, and monoclonal anti-RsbW antibodies (Fig. 6, lanes 2, 3, 4, and 5, respectively) were used as the precipitating antibodies. Each of the precipitating antibodies brought down its target protein. In addition, the anti-σ^B and anti-RsbW antibodies, but not the anti-RsbU antibody, precipitated additional proteins. Coprecipitation varied with the particular antibody preparation used and the extract analyzed. The polyclonal anti-σ^B antibody (lane 1), and to a lesser extent the monoclonal anti-σ^B antibody (lane 4), coprecipitated RsbW with σ^B from extracts of ethanol-treated cultures but not from extracts of logarithmically growing or stationary-phase cells. The failure of the anti-σ^B antibodies to coprecipitate RsbW with σ^B from logarithmic-phase and stationary-

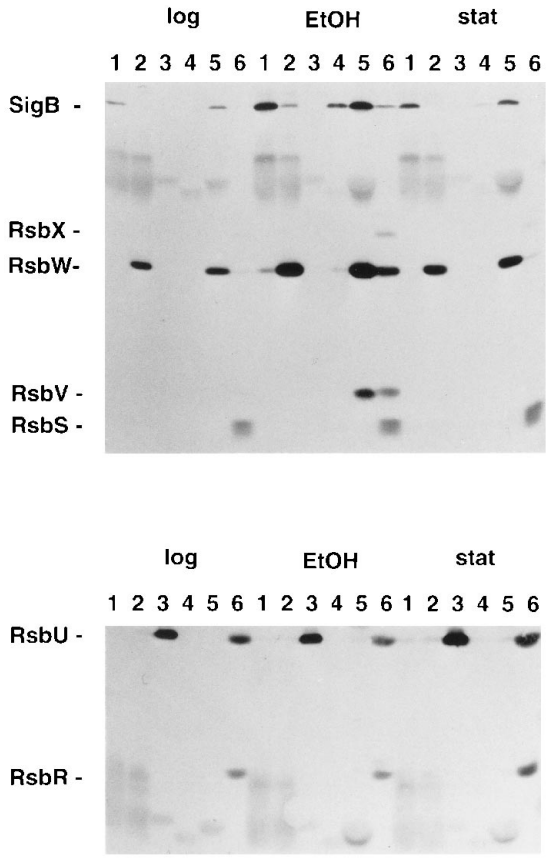

FIG. 6. Immunoprecipitation of *B. subtilis* extracts with anti-σ^B, anti-RsbW, and anti-RsbU antibodies. *B. subtilis* BSA46 (wild type) was grown in LB and harvested during exponential growth (log), 10 min after the addition of ethanol to a final concentration of 4% (EtOH), or 1 h after the entry into stationary phase (stat). Crude extracts were prepared as described in Materials and Methods. These extracts were incubated with polyclonal anti-σ^B (lanes 1) or anti-RsbW (lanes 2) antibodies or monoclonal antibodies raised against RsbU (lanes 3), σ^B (lanes 4), or RsbW (lanes 5). After precipitation, washing, and separation by SDS-PAGE, the proteins were visualized by Western blot analysis using monoclonal antibodies prepared against the products of the *sigB* operon. Lane 6 contains 10% aliquots of the crude extract used for the immunoprecipitation to allow a comparison of the abundance of the proteins during the different conditions. The positions of the proteins are marked. The diffuse unmarked bands represent the lighter chain of the precipitated antibodies.

phase extracts is likely due to their relative inefficiency as precipitating antibodies and the lower abundance of σ^B and RsbW in these extracts. The monoclonal anti-RsbW antibody (lane 5) proved to be an especially effective antibody for coprecipitation. It coprecipitated σ^B with RsbW from all three extracts and also brought down RsbV with RsbW from the ethanol-treated extract, where RsbW-RsbV complexes should be most abundant. Although the polyclonal anti-RsbW antibody precipitated RsbW as efficiently as did the monoclonal anti-RsbW antibody, it coprecipitated only a small amount of σ^B from the ethanol-treated cell extract and failed to precipitate RsbV from any of the tested extracts (lane 2). Either the polyclonal anti-RsbW antibodies predominantly recognize epitopes which are masked when RsbW is complexed to RsbV-σ^B or the antibody binding to RsbW instigates the release of RsbV and σ^B.

Both the anti-σ^B and the anti-RsbW antibodies coimmunoprecipitated small amounts of RsbU from the stationary-phase cell extract. Judging from the intensity of the Western blot bands, the amount of coprecipitated RsbU was approximately 1 to 2% of the amount of RsbU that was present in the extract. Although the specificity of the precipitation reaction argues that RsbU can associate with RsbW and/or σ^B, the small amount of RsbU coprecipitated and the failure of the anti-RsbU antibody to coprecipitate RsbW or σ^B (lanes 3) make it unlikely that significant amounts of RsbU are present in a RsbW-σ^B complex. RsbR and RsbS are present in readily detectable amounts in all of the extracts (lanes 5), but neither protein coprecipitated with any of the other *sigB* operon proteins. Attempts to coimmunoprecipitate *sigB* operon products with RsbX, RsbS, RsbR, and RsbT by using monoclonal antibodies specific for each of these proteins failed to reveal any detectable complexes between them and other *sigB* operon products (data not shown).

DISCUSSION

Activation of σ^B occurs when environmental or metabolic stimuli cause RsbW, the anti-σ^B protein, to release σ^B and form a stable complex with an alternative protein, RsbV (6, 11, 31). RsbV is essential for σ^B activation under all known inducing conditions (3, 8, 34). There are at least two mechanisms for σ^B activation (34). The simplest mechanism may involve a direct response of RsbW to intracellular ATP concentrations (1, 2). High levels of ATP appear to cause RsbW to phosphorylate RsbV and bind to σ^B, while low ATP levels favor the formation of RsbW-RsbV complexes and the release of σ^B (1). This mechanism is thought to activate σ^B during glucose limitation and the onset of stationary phase in LB (34). A second mechanism, activated by environmental stress and independent of ATP levels, was previously shown to require at least one additional protein, RsbU (31, 34). RsbU is encoded by one of four cotranscribed genes (*rsbR*, *rsbS*, *rsbT*, and *rsbU*) that lie immediately upstream of original *sigB* operon (35). Our current findings now argue that at least one of the upstream gene products, in addition to RsbU, is involved in stress activation of σ^B, but none of these genes is essential for the stationary-phase induction of σ^B. Although RsbR, RsbS, and RsbT are not essential for ATP-dependent activation of σ^B, one or more of these proteins is required for this induction to reach its full level. Unlike the loss of RsbU which has virtually no effect on stationary phase σ^B induction, the loss of RsbR, RsbS, and RsbT halves the stationary-phase activity of σ^B (Fig. 2). Apparently, at least one of these proteins provides a function which allows RsbW to more readily release σ^B and bind RsbV.

It is tempting to speculate that the genes in the upstream half of the *sigB* operon encode proteins which convey stress response signals to the RsbW-RsbV regulatory pair, allowing RsbW to alter its binding preferences regardless of ATP levels. One of the *sigB* operon products, RsbR, has a hydropathy profile consistent with a membrane protein (35). This finding and the possibility that several of the stresses (e.g., salt and pH) that activate σ^B could exert their effects by stimulating membrane-associated surface receptors prompted us to examine whether any of the σ^B regulators would partition as membrane-associated proteins during ultracentrifugation. When fractionated extracts were probed with antibodies against σ^B and its regulators, all were found to be cytosolic (33). If there is a surface receptor responding to stress signals, it is unlikely to be one of the known σ^B regulators.

Two or more genes within the extended *sigB* operon's upstream half are involved in stress activation of σ^B; nevertheless, the levels of their products do not fluctuate in response to σ^B-activating conditions. This contrasts with the dramatic increase in the level of the four downstream gene products which

occurs following σ^B activation (Fig. 4). The reason for this difference likely lies in the promoter elements controlling each of these transcription units. The upstream half of the *sigB* operon is transcribed from a σ^A-dependent promoter (35). This generates a transcript that extends through the entire eight gene *sigB* operon and probably constitutes the principal means by which the downstream half of the operon is expressed during noninducing conditions. Upon σ^B induction, a σ^B-dependent promoter between *rsbU* and *rsbV* is activated, thereby enhancing the expression of the downstream portion of the operon (4, 10). As a consequence of this, RsbV, RsbW, σ^B, and RsbX levels rise abruptly whereas RsbR, RsbS, RsbT, and RsbU levels are unchanged. Thus, the ratio of the upstream to downstream operon products becomes altered following σ^B activation. This circumstance may have regulatory significance. Stress induction of σ^B is a transient response. The σ^B-activated half of the operon includes the coding sequence for a negative regulator (RsbX) of the stress-induced pathway (18, 31). Perhaps placement of the coding sequence for RsbX in the σ^B-controlled region of the operon represents a device to limit the σ^B stress response. By raising the abundance of RsbX relative to the stress activator products that are encoded in the upstream portion of the operon, stress induction may be quelled. We are currently testing this notion by genetic and biochemical means.

Although most of the products of the upstream half of the extended *sigB* operon do not change in response to σ^B activation conditions, RsbT levels increased modestly following ethanol treatment. RsbT abundance rose, while the levels of the other gene products in its transcription unit remained static. It is possible that *rsbT* is subject to translational regulation; however, there is no obvious sequence element in *rsbT* to suggest that its translation is unique compared with that of the other genes of its operon. Its increase following ethanol treatment may instead reflect an enhanced stabilization of the protein, perhaps by altered associations in response to stress.

The unusual width of the protein band that reacted with the anti-RsbS antibody suggests that RsbS has unusual electrophoretic properties. The broadness of the RsbS band is not a function of RsbS's primary sequence. RsbS that is synthesized in *E. coli* migrates as a relatively tight band (data not shown). Finding the diffusely migrating RsbS in extracts from *B. subtilis*, but not *E. coli*, implies that RsbS can carry *B. subtilis*-specific modifications. Partial degradation of RsbS in *B. subtilis* would represent a trivial explanation for the faster-migrating forms of RsbS; however, if proteolysis is responsible, we would expect the faster-migrating, and presumably smaller, RsbS proteins to have been displaced to the lower-molecular-weight fractions during our gel filtration study (Fig. 5). This did not occur. The appearance of the broad RsbS band was unchanged among the fractions in which RsbS eluted from the gel filtration column (Fig. 5). The elution pattern is consistent with an RsbS modification that alters Rsb's electrophoretic mobility rather than its size. The nature of the RsbS modification is under investigation.

The gel filtration experiment, in conjunction with the coimmunoprecipitation study, indicated that none of the products of the upstream half of the *sigB* operon are likely to form stable complexes with each other or the products of the operon's downstream half. These experiments did, however, suggest that RsbU, RsbR, and RsbS are probably in multimeric complexes with themselves or unknown additional proteins. Analysis of the gel filtration fractions also revealed that a portion of all four of the upstream, but not the downstream *sigB* operon gene products, can be found in an aggregate that is largely excluded from the gel filtration matrix (Fig. 5). If this association is artifactual, it must be due to a property of RsbR, RsbS, RsbT, and RsbU that is not shared by the other products of the operon. Alternatively, given that these proteins are likely to be the sensors and transmitters of stress response signals to RsbW, it is not unreasonable to speculate that they could be part of a large biologically relevant complex that includes other cellular components.

The identification of additional gene products that are needed for stress-induced activation of σ^B further complicates an already complex pathway. Although the mechanisms by which the newly described *sigB* operon proteins convey the stress signal for σ^B activation are unclear, the ultimate consequence of this activity is known. Stress leads to an increase in the ratio of active RsbV to inactive RsbV-P accompanied by a shift in RsbW binding, from σ^B to RsbV. In other studies (33), we have determined that stress activates the dephosphorylation of RsbV-P in a reaction that depends on RsbRST and RsbU. It has been proposed, on the basis of sequence homologies between RsbU and a phosphatase that functions in the σ^F system (SpoIIE), that RsbU could be a phosphatase (12). A plausible model would then envision RsbU as a phosphatase that is controlled by RsbR, RsbS, RsbT, and RsbX and activated by stress. It is possible that the dephosphorylation of RsbV by the action of a stress-induced phosphatase is the principal mechanism by which stress activates σ^B.

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