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Activation of *Bacillus subtilis* Transcription Factor σ^B by a Regulatory Pathway Responsive to Stationary-Phase Signals

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Alternative transcription factor σ^B of *Bacillus subtilis* controls a stationary-phase regulon induced under growth conditions that do not favor sporulation. Little is known about the metabolic signals and protein factors regulating the activity of σ^B. The operon containing the σ^B structural gene has the gene order *orfV-orfW-sigB-rsbX*, and operon expression is autoregulated positively by σ^B and negatively by the *rsbX* product (*rsbX* = regulator of sigma B). To establish the roles of the *orfV* and *orfW* products, *orfV* and *orfW* null and missense mutations were constructed and tested for their effects on expression of the σ^B-dependent genes *ctc* and *csbA*. These mutations were tested in two contexts: in the first, the *sigB* operon was under control of its wild-type, σ^B-dependent promoter, and in the second, the *sigB* operon promoter was replaced by the inducible P_{spac} promoter. The principal findings are that (i) the *orfV* (now called *rsbV*) product is a positive regulator of σ^B-dependent gene expression; (ii) the *orfW* (now called *rsbW*) product is a negative regulator of such expression; (iii) σ^B is inactive during logarithmic growth unless the *rsbW* product is absent; (iv) the *rsbX*, *rsbV*, and *rsbW* products have a hierarchical order of action; and (v) both the *rsbV* and *rsbW* products appear to regulate σ^B activity posttranslationally. There are likely to be at least two routes by which information can enter the system to regulate σ^B: via the *rsbX* product, and via the *rsbV* and *rsbW* products.

As the enzyme of central importance in bacterial gene expression, DNA-dependent RNA polymerase is the common target of diverse regulatory mechanisms. Among the most potent of these is the association of core RNA polymerase with alternative sigma (σ) factors, which confer different promoter recognition specificities on the polymerase holoenzyme and reprogram the pattern of gene expression in response to environmental, cell cycle, and morphological signals (18). Because alternative σ factors are such powerful regulatory effectors, the molecular mechanisms controlling their synthesis or activity in response to such signals are of fundamental importance.

In *Bacillus subtilis*, a cascade of at least five different alternative σ factors controls the sporulation process, which is a complex program of morphological and structural change occurring under certain stationary-phase growth conditions (30). The regulation of both the synthesis and activity of these σ factors is chiefly responsible for coupling developmental gene expression to morphological events (46). But sporulation is only one of several patterns of gene expression manifested by *B. subtilis* in stationary phase. Other examples include development of genetic competence, production of antibiotics and extracellular enzymes, and the necessary adaptation to nutrient limitation (43). The important question remains how the cell senses and integrates the information required to achieve the appropriate balance of these myriad stationary-phase activities.

Our approach to understanding how stationary-phase signals are conveyed to the transcription apparatus is to identify the protein and metabolic effectors that regulate the activity of the alternative transcription factor σ^B. σ^B, formerly called σ^{37}, was originally discovered biochemically through its association with a unique RNA polymerase activity present in early-stationary-phase cells (17). The exact physiological role of σ^B has not yet been established, and genetic analysis has shown that σ^B is one of two known stationary-phase σ factors that is not essential for sporulation (4, 15, 23, 27). (The other is σ^D, which controls the chemotaxis-motility regulon [32].) Rather, σ^B is required for maximal expression of a class of genes called *csb* (for controlled by sigma B), which constitute a large regulon expressed in early stationary phase under conditions not conducive to sporulation or to formation of tricarboxylic acid cycle enzymes (6, 8, 23, 24).

We previously reported that the σ^B structural gene (*sigB*) lies third in a four-gene operon which is subject to complex regulation (27). The genetic organization of the *sigB* operon is shown in Fig. 1. σ^B positively regulates expression of its own operon from a σ^B-dependent promoter immediately upstream, whereas the product of the fourth gene (*orfX*) is a negative regulator of operon expression. Our most striking finding was that the predicted products of the first two genes of the *sigB* operon (*orfV* and *orfW*) have significant sequence identity with the products of the first two genes of the *B. subtilis spoIIA* operon (*spoIIAA* and *spoIIAB*), the third gene of which (*spoIIAC*) encodes the sporulation-essential σ^F. On the basis of this clear evolutionary relationship and from the known phenotypes of *spoIIAA* and *spoIIAB* mutants (38, 50), we suggested that σ^B and σ^F might be regulated by a common mechanism involving the products of their respective upstream open reading frames.

Evidence confirming part of this suggestion was reported by Schmidt et al. (42), who demonstrated that the *spoIIAA* and *spoIIAB* products are potent effectors of σ^F-dependent gene expression. Here we present genetic evidence that the *orfV* product is a positive regulator and the *orfW* product is a negative regulator of σ^B-dependent gene expression and that the *orfX*, *orfV*, and *orfW* products form a hierarchical regulatory pathway. Related experiments conducted independently by Benson and Haldenwang (3) are consistent with these data. We have further shown that both the *orfV* and *orfW* products act at the posttranslational level to control σ^B activity, that σ^B is activated by cellular or

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TABLE 1. *B. subtilis* strains

Strain	Relevant genotype[a]	Reference or construction[b]
PB2	*trpC2*	Wild-type Marburg strain (37)
PB79	*lys*::Tn*917 trpC2*	5
PB114	*sigB*::pMD10	27
PB155	*csbA*::Tn*917lacZ trpC2*	8
PB197	SPβ*ctc-lacZ*[c] *trpC2*	SPβ*ctc-lacZ*→PB2[c]
PB198	*amyE*::pDH32-*ctc trpC2*	pDH32-*ctc*→PB2
PB199	*rsbVΔ1* SPβ*ctc-lacZ*	This study
PB200	*rsbV42* SPβ*ctc-lacZ*	This study
PB201	*rsbW1 amyE*::pDH32-*ctc*	This study
PB202	*sigBΔ2*::*cat dal-1 trpC2*	AG232→PB2
PB203	*rsbV42*	This study
PB204	*rsbV42 amyE*::pDH32-*ctc*	pDH32-*ctc*→PB201
PB205	*rsbVΔ1*	This study
PB206	*rsbVΔ1 amyE*::pDH32-*ctc*	pDH32-*ctc*→PB203
PB207	*amyE*::pDH32-*ctc trpC2* pDG148-*rsbW*	pDG148-*rsbW*→PB198
PB208	*amyE*::pDH32-*ctc trpC2* pDG148	pDG148→PB198
PB209	*socB trpC2*	EU100→PB202
PB210	*socB amyE*::pDH32-*ctc trpC2*	pDH32-*ctc*→PB209
PB212	P_{spac} (*rsbV*$^+$ *rsbW*$^+$ *sigB*$^+$ *rsbX*$^+$) *amyE*::pDH32-*ctc trpC2*	pAR7→PB198
PB213	P_{spac} (*rsbV*$^+$ *rsbWΔ1 sigB*$^+$ *rsbX*$^+$) *amyE*::pDH32-*ctc trpC2*	pAR7→PB198
PB214	P_{spac} (*rsbVΔ1 rsbW*$^+$ *sigB*$^+$ *rsbX*$^+$) *amyE*::pDH32-*ctc trpC2*	pAR6→PB198
PB216	P_{spac} (*rsbV*$^+$ *rsbW*$^+$ *sigB*$^+$ *socB*) *amyE*::pDH32-*ctc trpC2*	pAR7→PB210
PB217	*rsbVΔ1 rsbX*::*ery amyE*::pDH32-*ctc trpC2*	pSK15→PB206
PB218	*rsbX*::*ery amyE*::pDH32-*ctc trpC2*	PB217→PB198
PB219	P_{spac} (Δ[*rsbV-rsbW*] *sigB*$^+$ *rsbX*$^+$) *amyE*::pDH32-*ctc trpC2*	pAR8→PB198
PB221	P_{spac} (*rsbV*$^+$ *rsbW*$^+$ *sigB*::pMD10)	pAR7→PB114
PB222	P_{spac} (*rsbVΔ1 rsbW*$^+$ *sigB*::pMD10)	pAR6→PB114
PB226	*amyE*::pAWB *trpC2*	pAWB→PB2
PB238	*amyE*::pAWB *trpC2* pDG148-*rsbW*	pDG148-*rsbW*→PB226
PB239	*csbA*::Tn*917lacZ trpC2* pDG148-*rsbW*	pDG148-*rsbW*→PB155
PB240	*spoVG*$^+$::pZL207 *spo0J93 pheA1 trpC2* pDG148-*rsbW*	pDG148-*rsbW*→ZB223
AG232	*sigBΔ2*::*cat dal-1 pheA1 trpc2*	Alan Grossman
EU100	*socB pheA1 trpC2*	23
ZB223	*spoVG*$^+$::pZL207 *spo0J93 pheA1 trpC2*	51

[a] P_{spac} refers to the replacement of the wild-type sigma B operon promoter by the inducible P_{spac} promoter from pAG58 (26).
[b] The arrow indicates transformation from donor to recipient.
[c] SPβ*c2 del2*::Tn*917*(*cat*) *ctc*::*lacZΔ798* (24).

environmental signals present at the end of logarithmic growth, and that inactivation of the *orfW* product is a principal mechanism by which these signals are transmitted to σ^B. Because the *orfV*, *orfW*, and *orfX* products all control σ^B activity, we have renamed these genes *rsbV*, *rsbW*, and *rsbX* (for regulator of sigma B).

MATERIALS AND METHODS

Bacterial strains and genetic methods. We used *Escherichia coli* TG-1 (Amersham) as the host for pDG148 (44) and its derivatives and *E. coli* DH5α (Bethesda Research Laboratories) as the host for all other plasmid constructions. The *B. subtilis* strains used are listed in Table 1. For strain constructions, *B. subtilis* PB2 and its derivatives were the recipients for natural transformation with linear and plasmid DNA (13). Transformation selections for drug-resistant *B. subtilis* strains were done on tryptose blood agar plates (Difco Laboratories) containing either chloramphenicol (5 μg/ml), kanamycin (5 μg/ml), or erythromycin (0.5 μg/ml) plus lincomycin (12.5 μg/ml). The minimal medium used for *B. subtilis* was that of Anagnostopoulos and Spizizen (1), and Luria broth (LB) and M9 minimal glucose medium were from Davis et al. (12).

DNA methods. All standard recombinant DNA methods have been described previously (7), and polymerase chain reactions (PCRs) were done by standard protocols (25). DNA sequencing reactions were done by the dideoxynucleotide chain termination method (41) with appropriate restriction fragments cloned into M13mp19 and pUC19. We used Sequenase enzyme (U.S. Biochemical) and [α-^{35}S]dATP (Amersham) to label sequencing reaction mixes. Priming was done with custom oligonucleotide primers (from Operon Technologies, Alameda, Calif.) or standard forward and reverse primers (from U.S. Biochemicals).

σ^B-dependent promoter fusions. Five different σ^B-dependent *lacZ* fusions were used to monitor σ^B synthesis or activity in wild-type and mutant strains. We primarily used a single-copy transcriptional fusion of the *ctc* promoter inserted at the *amyE* locus of strain PB198. This strain was constructed by moving a 149-bp *Eco*RI-*Hin*dIII fragment containing the *ctc* promoter from pLC4 (39) into the *Eco*RI site of pDH32, immediately upstream from the promoterless, hybrid *spoVG-lacZ* reporter gene of the vector (20). pDH32-*ctc* was cut with *Sca*I and transformed into a PB2 recipient, in which the linear transcriptional fusion was incorporated into the *amyE* chromosomal locus via a double crossover event, with regions of *amyE* homology carried by the pDH32 vector. pDH32 was also the basis of the second transcriptional fusion, pAWB, which was constructed by Arlene Wise by using the 830-bp *Eco*RV-*Afl*II fragment carrying the *sigB* operon promoter (27) and borne at the *amyE* locus of strain PB226. The third transcriptional fusion was the recently described *csbA*::Tn*917lacZ* in strain PB155 (8). The fourth

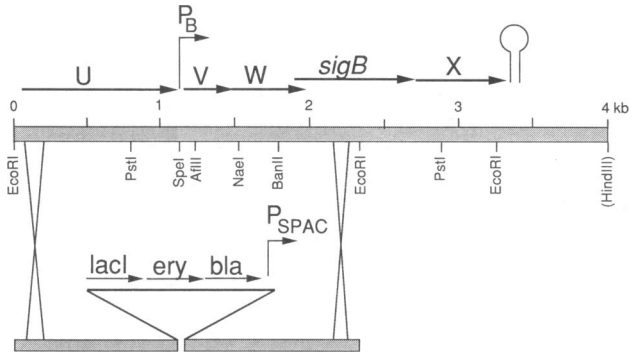


FIG. 1. *sigB* operon and strategy to replace the *sigB* operon promoter. Physical map of the *sigB* operon (27) showing the σ^B-dependent operon promoter (P_B), the *rsbV*, *rsbW*, *sigB*, and *rsbX* coding regions (arrows), and the operon terminator (stem-loop). The *orfU* coding region lies immediately upstream from the P_B promoter (49). The restriction sites shown in the 2.3-kb *Eco*RI fragment containing P_B were used to construct in-frame deletions of *rsbV* and *rsbW* and to replace P_B with the inducible P_{spac} promoter cassette, shown at the bottom (not to scale). For the P_{spac} construction, regions from the *B. subtilis* chromosome up- and downstream from P_B were subcloned into pAR4, flanking the *E. coli* lactose repressor gene (*lacI*), the pE194 erythromycin resistance gene (*ery*), the pBR322 ampicillin resistance gene (*bla*), and the SPO1 phage promoter controlled by the *E. coli lac* operator (P_{spac}). We transformed *B. subtilis* with the linearized construction, replacing P_B with P_{spac} by the double crossover event shown. Similar P_{spac} constructions were used to move *rsbV* and *rsbW* deletions into the chromosome by linked transformation with the *ery* marker of the P_{spac} cassette (see text).

fusion was the *ctc-lacZ* translational fusion constructed by Igo and Losick (24) and carried on the resident SPβ prophage of strain PB197. The fifth was a translational fusion between codon 45 of *sigB* and codon 8 of *lacZ*, integrated in single copy into the *sigB* locus and carried in strain PB114 (27).

Construction of missense and deletion mutations. We made missense mutations in *rsbV* and *rsbW* by oligonucleotide mutagenesis in vitro, using the *dut-ung E. coli* strain (29) as described in the Muta-Gene kit (Bio-Rad). For the following constructions, all nucleotide (nt) numbers refer to Fig. 2 of Kalman et al. (27). The 1,396-bp *Pst*I-*Eco*RI fragment containing *rsbV*, *rsbW*, and part of *sigB* (Fig. 1) was cloned into M13mp19. Mutagenic primers included a 21-mer complementary to nt 467 to 487, containing a transition at nt 477 (G to A), to generate the *rsbV42* mutation (Gly-95 to Asp in Fig. 2A) and a 22-mer complementary to nt 555 to 576, containing a transition at nt 566 (C to T), to generate the *rsbW1* mutation (Ala-14 to Val). We used the same method to generate the 258-bp in-frame deletion in *rsbV* (*rsbVΔ1*) via a 50-mer complementary to nt 211 to 235 and 494 to 518. The mutational alterations were confirmed by DNA sequence analysis, and the mutant *Pst*I-*Eco*RI fragments were cloned into pUC19.

Restriction digestion was used to make an in-frame deletion within *rsbW* (Fig. 2B) as well as a second in-frame deletion removing most of *rsbV* and *rsbW*. pSTR1 carried the *rsbV*, *rsbW*, and part of the *sigB* coding regions on the 1,391-bp *Pst*I-*Eco*RI fragment cloned into the *Pst*I and *Eco*RI polylinker sites of pUC19. pSTR1 was digested with *Nae*I and *Ban*II (Fig. 1), treated with T4 DNA polymerase to

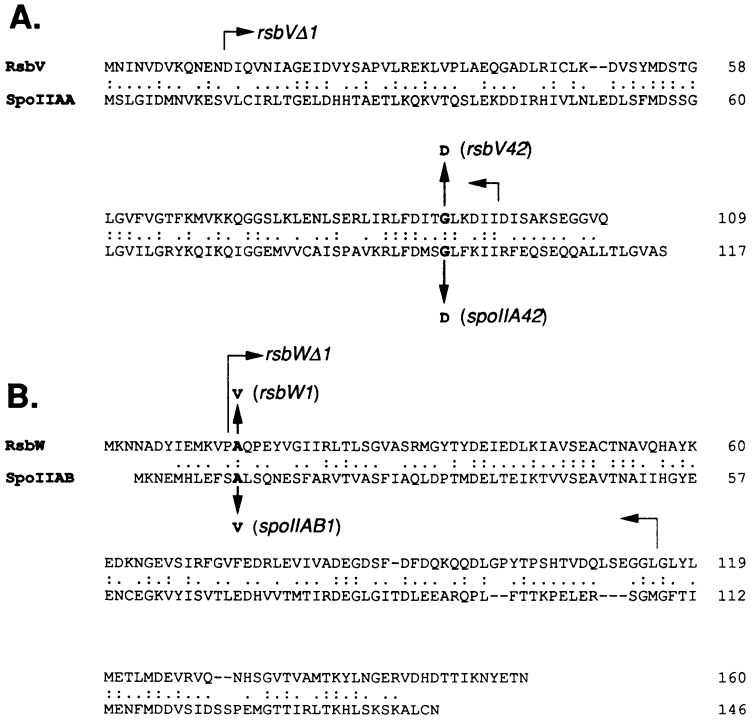


FIG. 2. Mutations introduced into the *rsbV* and *rsbW* coding regions. The alignments of the *rsbV* and *spoIIAA* products and of the *rsbW* and *spoIIAB* products are from Kalman et al. (27). Sequences are given in the single-letter code, with identical residues indicated by two dots and conserved substitutions by a single dot. (A) Gly-95 to Asp (*spoIIAA42*) alteration of SpoIIAA (50) and the corresponding Gly-93 to Asp (*rsbV42*) change we made in RsbV (see Materials and Methods). Also shown are the residues removed by the *rsbVΔ1* deletion, enclosed by arrows. (B) Ala-11 to Val (*spoIIAB1*) alteration of SpoIIAB (38) and the equivalent Ala-14 to Val (*rsbW1*) mutation we made in RsbW. The residues removed by the *rsbWΔ1* deletion are enclosed by arrows.

make blunt ends, and then ligated to make pSTR2. DNA sequence analysis indicated that the *rsbWΔ1* mutation removed 306 bp (nt 564 to 869) from within *rsbW*, deleting the region coding for residues most similar to SpoIIAB but leaving intact the region of dyad symmetry at the 3′ end of *rsbW* (27). The Δ(*rsbV-rsbW*) mutation was similarly made by removal of the 539-bp *Afl*II-*Ban*II fragment (Fig. 1; nt 331 to 869) to make pSTR3. This deletion removed 66 codons from the 3′ end of *rsbV* and 115 codons from the 5′ end of *rsbW*.

Replacement of chromosomal *rsbV* and *rsbW* alleles by congression. We replaced the wild-type chromosomal copies of *rsbV* or *rsbW* with the appropriate *rsbVΔ1*, *rsbV42*, and *rsbW1* alleles by congression (cotransformation) with the unlinked *trpC*$^+$ marker. In all constructions, the presence of each mutant allele in the chromosome was confirmed by DNA sequence analysis of PCR products amplified from the *sigB* chromosomal locus. The *trpC2* recipients PB197 (SPβ*ctc-lacZ*) and PB198 (pDH32*ctc-lacZ*) were transformed with a mixture of 0.1 μg of linearized pTrp-H3 plasmid DNA (2) per ml and 200 μg of linearized pUC19 plasmid containing the *rsbV* or *rsbW* mutation per ml. Trp$^+$ transformants were selected on minimal glucose plates; these plates also contained X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 80 μg/ml) to allow screening for colonies which differed in color from the parent. The *rsbVΔ1* mutation introduced into PB197 resulted in colonies that were white compared with the medium blue of the parent, whereas the *rsbV42* allele rendered the colonies light blue. These strains were called PB199 and PB200, respectively. The *rsbW1* transformants of PB198 had darker blue colonies than the parent; this strain was called PB201. The frequency of congression of these mutant colony phenotypes ranged from 2 to 5%.

We placed the *rsbV42* and *rsbVΔ1* alleles in backgrounds containing the pDH32*ctc-lacZ* transcriptional fusion as follows. For the *rsbV42* allele, PB200 was cured of its resident SPβ*ctc-lacZ* fusion by overnight growth in LB at 50°C; loss of the prophage was scored by chloramphenicol sensitivity. The cured strain, PB203, was transformed with linearized plasmid pDH32*ctc-lacZ* to make PB204. For the *rsbVΔ1* allele, we took advantage of the linkage of the *sigB* locus to the *dal-1* marker (4, 15). Strain PB202 (*dal-1 sigBΔ2::cat*) was transformed with chromosomal DNA from PB199 (*rsbVΔ1*), selecting for Dal$^+$. Scoring for chloramphenicol sensitivity allowed identification of transformants in which the *sigBΔ2::cat* region of PB202 had been replaced by the *sigB* region of PB199. The presence of the *rsbVΔ1* mutation in the chromosome was confirmed by DNA sequence analysis of PCR-amplified chromosomal products in six of six chloramphenicol-sensitive colonies tested. One of these strains, PB205, was transformed with linearized pDH32*ctc-lacZ* to yield PB206.

PB217, carrying the double mutation *rsbVΔ1 rsbX::ery*, was constructed by transforming PB206 (*rsbVΔ1* pDH32*ctc-lacZ*) with linearized pSK15, which contains the insertion-deletion mutation *rsbX::ery* (27). Transformants were selected for erythromycin-lincomycin resistance and screened by PCR to confirm the presence of both the *rsbVΔ1* and *rsbX::ery* mutations. The single *rsbX::ery* mutation was also moved into the PB198 (pDH32*ctc-lacZ*) background by transforming PB198 with chromosomal DNA from PB217.

Replacement of chromosomal *rsbV* and *rsbW* alleles by linked transformation with P_{spac}. As shown in Fig. 1, we placed the chromosomal copy of the *sigB* operon under control of the inducible P_{spac} promoter by using pAR7, a derivative of plasmid pAG58 (26). pAR7 and similar constructions also allowed us to use linked transformation rather than congression to replace the chromosomal copies of *rsbV* and *rsbW* with their mutant alleles. To begin the construction of pAR7, we first made pAR4, in which the 1,443-bp *Taq*I fragment encoding the macrolide resistance gene from pE194 (21) was substituted for the 1,587-bp *Stu*I-*Ava*I fragment containing the chloramphenicol resistance gene in pAG58; restriction sites generating protruding ends were blunted before ligation.

The σ^B-dependent promoter of the *sigB* operon has an *Spe*I site at the exact position of transcription initiation (27). We took advantage of this site to flank the P_{spac} promoter of pAR4 with *sigB* operon fragments from up- and downstream of the transcription initiation region. The upstream fragment came from pSK21, a pUC19 derivative in which the 2.3-kb *Eco*RI fragment containing the 5′ half of the *sigB* operon (27) was subcloned into the *Eco*RI site so that the polylinker region lay upstream from the operon. A 1,129-bp *Sma*I-*Spe*I fragment was removed from pSK21, the *Spe*I end was filled in, and the fragment was ligated into the flush *Sph*I site of pAR4 to create pAR5. This *Sma*I-*Spe*I fragment contained part of the pSK21 polylinker region, the 3′ end of an upstream open reading frame, *orfU* (49), and the *sigB* operon promoter. A 920-bp *Spe*I-*Eco*RI fragment from pSTR2—beginning at the +1 transcription initiation site of the *sigB* operon, containing the *rsbWΔ1* deletion, and extending well into the *sigB* coding region (Fig. 1)—was blunt-end ligated into the filled *Sal*I site of pAR5 to make pAR7.

We linearized pAR7 at the *Kpn*I site introduced via the pSK21 polylinker and transformed strain PB198 (containing the pDH32*ctc-lacZ* fusion). As shown in Fig. 1, selection for erythromycin-lincomycin resistance forced the linearized P_{spac} construction into the chromosome via a double crossover event. Because the *Spe*I-*Eco*RI fragment lacks the *sigB* operon promoter, this construction placed the *sigB* operon under the control of P_{spac}. We used PCR to choose two transformants for further study. In strain PB212, the second crossover occurred between P_{spac} and *rsbWΔ1*, regenerating an operon wild type for all four gene products but under P_{spac} control. In strain PB213, the second crossover occurred promoter-distal to *rsbWΔ1*, transferring the deletion to the chromosome. A similar strategy was used for the two other mutant replacement transformations, substituting the *Spe*I-*Eco*RI region from the following plasmids for the corresponding region of pAR7: pAR6 (*rsbVΔ1*), to make PB214; and pAR8 [Δ(*rsbV-rsbW*)], to make PB219. pAR6 and pAR7 were also transformed into strain PB114, placing the *sigB-lacZ* translational fusion at the *sigB* locus under P_{spac} control. In strain PB221, the second crossover occurred between P_{spac} and *rsbWΔ1*, to regenerate wild-type *rsbV* and *rsbW* preceding the *sigB-lacZ* translational fusion, and in PB222, the promoter-distal second crossover transferred *rsbVΔ1* into the chromosome of the fusion, replacing the wild-type *rsbV*. We confirmed the expected structure of each construction and mutation by PCR amplification of the *sigB* chromosomal locus.

Overexpression of the *rsbW* product in *B. subtilis*. We made pDG148-*rsbW* to place *rsbW* under control of the inducible P_{spac} promoter of the multicopy expression vector pDG148 (44). The 676-bp *Spe*I-*Hin*cII fragment (nt 166 to 1099) containing the *rsbVΔ1* mutation was subcloned from pUC19 into the *Xba*I and *Sma*I sites of pMTL23 (10). The cloned fragment was cut at the flanking *Sal*I-*Sph*I sites and moved downstream of the *spoIIIG* gene between the *Sal*I and *Sph*I sites of pDG298 (47) to obtain pDG67. We then deleted the

spoIIIG coding region from pDG67 by cutting with *Hin*dIII. The resulting plasmid, pDG148-*rsbW*, retained the *rsbV* ribosome-binding site together with a large, in-frame deletion of *rsbV*, in order to support *rsbW* expression if the frames are translationally coupled, as suggested by the DNA sequence (27).

Enzyme assays. *B. subtilis* strains were grown to the late logarithmic stage and diluted 1:25 into fresh medium. Samples taken in both logarithmic-growth and stationary phases were assayed for β-galactosidase assay by the method of Miller (34), using sodium dodecyl sulfate and chloroform to permeabilize the cells. Activity was expressed in Miller units, defined as 10^3 ΔA_{420} per minute per milliliter per unit of optical density at 600 nm.

RESULTS

Experimental design. Our genetic analysis of σ^B regulation was complicated by three features of *sigB* operon organization. First, because σ^B is required for transcription of its own structural gene, it is ordinarily difficult to determine whether regulatory mutations affecting σ^B-dependent gene expression act at the level of σ^B synthesis or σ^B activity (27). We therefore carried out our analysis in two different genetic backgrounds, one containing the wild-type, σ^B-dependent *sigB* operon promoter and another in which the autocatalytic regulatory circuit was broken by replacing the wild-type promoter with the inducible P_{spac} promoter (19, 26).

Second, an operon just upstream from the *sigB* operon might influence σ^B-dependent gene expression, either by readthrough transcription or by the potential *trans*-acting factors it might encode (49). It was therefore important to compare the effect of selected mutations in the wild-type context with the effect of the same mutations in the P_{spac} construction, in which readthrough transcription was prevented. The P_{spac} promoter construction was also designed to maintain the integrity of the coding regions in both the *sigB* and upstream operons.

Third, expression of the coding regions in the *sigB* operon—the *rsbV* and *rsbW* regions in particular—appears to be translationally coupled (27). We therefore studied the effects of both missense and null mutations in *rsbV* and *rsbW*, and the null mutations we constructed were large, in-frame deletions which should have little effect on translational expression of downstream genes.

We generally used the well-characterized σ^B-dependent *ctc* promoter (36) to assay the effect of the various mutations on σ^B activity in vivo. Promoter activity was measured by using a transcriptional fusion of the *ctc* promoter and a *lacZ* reporter gene in the single-copy vector pDH32, integrated at the *amyE* locus. Although the function of *ctc* remains unknown (22), the interactions of σ^B-containing holoenzyme with the *ctc* promoter are well defined, and some of the metabolic signals important for *ctc* expression are known. *ctc* is highly expressed early in stationary phase when cells are grown in rich medium containing high levels of glucose and glutamine, conditions which repress both the synthesis of tricarboxylic acid cycle enzymes and the sporulation process (24). Expression of a *ctc-lacZ* fusion is abolished in a *sigB* null mutant (23), and DNA methylation protection experiments have defined σ^B holoenzyme contacts within the *ctc* promoter (35). Mutational analysis has found that many of the bases identified by these contacts are important for transcription initiation in vitro and in vivo (39, 48). In some experiments, we also used transcription fusions of the *csbA* and *sigB* operon promoters (8, 27) to establish that the tested mutations globally affected σ^B-dependent gene expression and were not restricted to *ctc*. A sigma-H-dependent *spoVG-lacZ* fusion (9, 14, 51) was used to determine that the effects observed were specific to σ^B-dependent genes.

Effect of *rsbV* and *rsbW* mutations on σ^B-dependent gene expression. The *rsbV* and *rsbW* products have significant sequence identity with their counterparts in the *spoIIA* operon, SpoIIAA and SpoIIAB, and residues important for SpoIIAA and SpoIIAB function are conserved in the RsbV and RsbW proteins (27). The *spoIIAA42* mutation is an alteration of Gly-95 to Asp, which decreases sporulation frequency by 10^3 (16, 50). We made the corresponding Gly-93 to Asp change in *rsbV* (*rsbV42*) to determine whether this residue was also important for *rsbV* function (Fig. 2A). Similarly, the *spoIIAB1* mutation is a change of Ala-11 to Val, which increases expression of forespore-specific genes when cells are grown in medium that does not support sporulation (38). We therefore made the corresponding Ala-14 to Val change in *rsbW* (*rsbW1*; Fig. 2B).

In order to test the possible regulatory role of the *rsbV* and *rsbW* products, we replaced their wild-type chromosomal alleles with the *rsbV42* and *rsbW1* missense alleles by congression, as described in Materials and Methods. We also introduced the *rsbVΔ1* mutation, an in-frame deletion which removed 86 of the 109 codons from the *rsbV* coding region (Fig. 2A). In all three mutant strains, the presence of each mutation in the chromosome was confirmed by sequencing the amplified, subcloned PCR product of the region. The operon remained otherwise wild type and also remained under control of the wild-type *sigB* promoter. In contrast to our success with the *rsbV42*, *rsbVΔ1*, and *rsbW1* mutant alleles, we were unable to substitute the *rsbWΔ1* in-frame deletion for the *rsbW* wild-type allele. Rather, when using the *ctc-lacZ* fusion strain PB198 as the transformation recipient for the *rsbWΔ1* allele, we noted the appearance, at congression frequency, of pinpoint colonies of lysing cells. These lysing colonies were intensely blue on X-Gal plates compared with the medium blue of the PB198 parent. We infer from these results that the *rsbWΔ1* mutation is lethal in the context of the wild-type *sigB* operon promoter. We also infer that the *rsbWΔ1* mutation causes increased expression of the σ^B-dependent *ctc* fusion. These inferences were later confirmed in experiments which introduced the *rsbWΔ1* allele by linked transformation with the P_{spac} promoter, reported below.

We measured the effects of the *rsbV42*, *rsbW1*, and *rsbVΔ1* mutations on σ^B activity in the wild-type promoter context by monitoring expression of the *ctc-lacZ* transcriptional fusion in two different media. In unsupplemented LB, *ctc* transcription is relatively low, and in LB supplemented with glucose and glutamine, *ctc* transcription is normally induced fivefold (24). As shown in Fig. 3, results with the *rsbVΔ1* allele indicate that the *rsbV* product is a positive factor required for *ctc* expression in both media. In separate experiments, we also found that *rsbVΔ1* abolished σ^B-dependent expression of the *csbA* and *sigB* transcriptional fusions (not shown). Thus, the *rsbV* product is generally required for σ^B-dependent gene expression.

The effect of the *rsbV42* missense allele was both less severe than that of *rsbVΔ1* and more dependent on the growth medium. *ctc* expression in the *rsbV42* mutant was significantly lower than in the wild type in LB medium (Fig. 3A), whereas in LB containing glucose and glutamine, the difference between mutant and wild-type activity was less pronounced (Fig. 3B). These results indicate that the con-

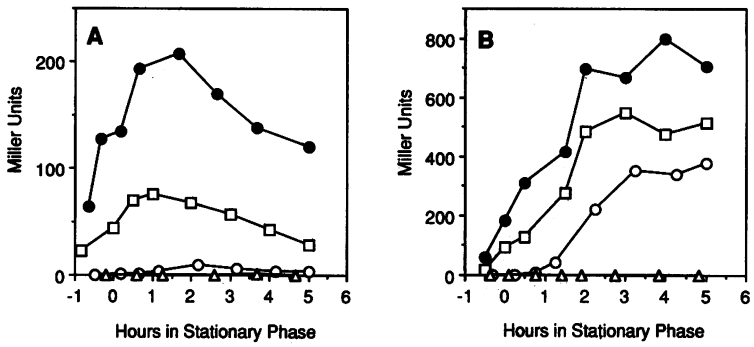


FIG. 3. Effect of *rsbV* and *rsbW* mutations on *ctc-lacZ* expression. σ^B activity was measured by monitoring β-galactosidase production from the single-copy pDH32-*ctc-lacZ* transcriptional fusion. Cultures were grown in LB (A) and in LB supplemented with 5% glucose and 0.2% glutamine (B). Samples were removed at various times during the logarithmic and stationary phases to assay for β-galactosidase activity; the activity of the PB2 negative control was subtracted for each time point. The strains used were PB198 (*rsbV*$^+$ *rsbW*$^+$) (□); PB201 (*rsbW1*) (●); PB204 (*rsbV42*) (○); and PB206 (*rsbVΔ1*) (△).

served Gly-93 residue is important for RsbV function, especially under noninducing growth conditions.

The effect of the *rsbW1* missense allele was also dependent on the growth medium. In LB medium, *ctc* expression in the *rsbW1* mutant was elevated three- to fivefold compared with that in the wild type (Fig. 3A). But the effect of *rsbW1* was less obvious when cells were grown in LB containing glucose and glutamine (Fig. 3B). Consistent with our results with the *rsbW1* allele, Rather et al. (38) found that the equivalent *spoIIAB1* mutation affected forespore-specific gene expression only when cells were grown under noninducing conditions for their system. We conclude that the conserved Ala-14 residue is important for RsbW function primarily under noninducing growth conditions.

Increased levels of *rsbW* inhibit σ^B-dependent gene expression. The effect of the *rsbW1* missense allele on *ctc* expression suggests that the *rsbW* product is a negative regulator of σ^B activity. However, it is possible that *rsbW1* causes an alteration rather than a loss of RsbW function. If RsbW were in fact a negative effector, we would predict that overproduction of the wild-type RsbW protein would inhibit expression of σ^B-dependent genes in *trans*.

This prediction was verified in the experiment shown in Fig. 4, for which we constructed a multicopy plasmid that placed *rsbW* expression under control of the inducible P_{spac} promoter. In this construction, we maintained the translational coupling of *rsbW* to the upstream *rsbV*, which was rendered null by the *rsbVΔ1* in-frame deletion (see Materials and Methods). We introduced this plasmid into strain PB198, which bore the *ctc-lacZ* transcriptional fusion, creating strain PB207. As shown in Fig. 4, when PB207 cells were grown without the IPTG (isopropyl-β-D-thiogalactopyranoside) inducer of the P_{spac} promoter, *ctc* was expressed as usual in the early stationary phase of growth. However, induction of *rsbW* completely abolished detectable *ctc* expression, both in LB medium supplemented with glucose and glutamine (Fig. 4) and in unsupplemented LB (not shown). *rsbW* induction had an equivalent negative effect on the σ^B-dependent *csbA* and *sigB* transcriptional fusions but had no influence on expression of a sigma H-dependent *spoVG-lacZ* fusion (not shown). Thus, an *rsbW* missense mutation and an *rsbW* overexpression system fulfill the predicted properties for a negative regulatory element which specifically affects σ^B-dependent gene expression. Because σ^B is responsible for transcription of its own structural gene, the RsbW product could exert its negative regulatory effect on either the synthesis or the activity of σ^B.

σ^B is activated by stationary-phase signals and factors. We replaced the wild-type *sigB* operon promoter with the inducible P_{spac} promoter for two reasons. First, this construction allowed us to break the autocatalytic transcriptional loop and analyze the level at which the *rsbV* and *rsbW* products regulated σ^B-dependent genes. Second, this construction allowed the introduction of potentially lethal mutations, such as *rsbWΔ1*, into the operon. As shown in Fig. 1, we essentially substituted the *E. coli lac* repressor-operator

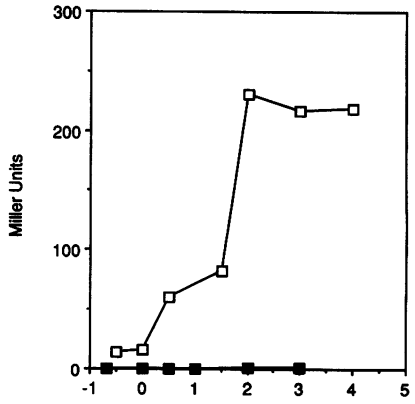


FIG. 4. Effect of increased RsbW levels on *ctc* expression. Strain PB207 contains the multicopy plasmid pDG148-*rsbW*, which places *rsbW* expression from the plasmid under control of the IPTG-inducible P_{spac} promoter. PB207 was grown until mid-log phase in LB medium supplemented with 5% glucose and 0.2% glutamine. The culture was then diluted 1:25 into fresh medium and split in half; one half received no IPTG (□), and the other received IPTG at 1 mM (final concentration) (■). β-Galactosidase activity from the resident pDH32-*ctc-lacZ* transcriptional fusion was measured at various times after the addition of IPTG. As a negative control, we followed the same growth and assay procedure with strain PB208, which contained the pDH32-*ctc-lacZ* transcriptional fusion and the parental plasmid pDG148, which carries no *B. subtilis* genes. β-Galactosidase activities measured in PB208 grown with or without IPTG were similar to those measured in PB207 grown without IPTG (data not shown).

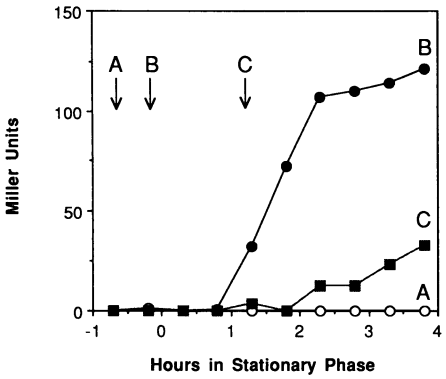


FIG. 5. σ^B activity with *sigB* operon expression under P_{spac} control. Plasmid pAR7 was integrated into the *sigB* operon locus by a double crossover event, replacing the σ^B-dependent *sigB* operon promoter with the IPTG-inducible P_{spac} promoter (see text). The resultant strain, PB212 (P_{spac} *rsbV*$^+$ *rsbW*$^+$ *amyE*::pDH32-*ctc*) was grown in triplicate in LB supplemented with 5% glucose and 0.2% glutamine. *sigB* operon transcription was induced by adding IPTG (1 mM, final concentration) to the cultures at −0.7 h (arrow A), −0.2 h (arrow B), or 1.25 h (arrow C); parallel control cultures were treated identically but were not induced with IPTG. The β-galactosidase activity shown for each culture is the difference between the activity in the induced culture and that in the uninduced control: A (○), B (●), and C (■).

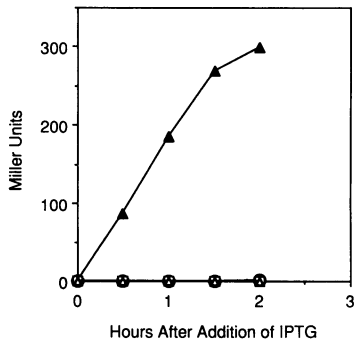


FIG. 6. Effect of *rsbV*, *rsbW*, and *rsbX* mutations on σ^B activity during logarithmic growth. Cultures were grown to the late logarithmic growth phase in unsupplemented LB. Cultures were then split and diluted 1:25 into fresh medium, and IPTG (1 mM, final concentration) was added to one half. Samples were removed from each culture at the indicated time after IPTG addition and assayed for β-galactosidase activity. For each point shown, the activity of the uninduced culture was subtracted from the activity of the corresponding induced culture. Symbols: □, PB212 (P_{spac} *rsbV*$^+$ *rsbW*$^+$ *amyE*::pDH32-*ctc*); ▲, PB213 (P_{spac} *rsbV*$^+$ *rsbWΔ1 amyE*::pDH32-*ctc*); △, PB214 (P_{spac} *rsbVΔ1 rsbW*$^+$ *amyE*::pDH32-*ctc*); ●, PB216 (P_{spac} *rsbV*$^+$ *rsbW*$^+$ *socB amyE*::pDH32-*ctc*). *socB* is a frameshift mutation early in the *rsbX* reading frame (23).

system for the *sigB* operon promoter, placing the P_{spac} promoter under *lacI* control. In this construction, the regions immediately up- and downstream from the *sigB* operon promoter flanked the *lac* control cassette of a pAG58 derivative (see Materials and Methods). When this construction was linearized and integrated into the *B. subtilis sigB* region by a double crossover event, the integrity of the *sigB* promoter was destroyed and the *sigB* operon was placed under P_{spac} control. The *orfU* reading frame encoded by the region upstream of the *sigB* operon (49) remained intact, as did the downstream *rsbV*, *rsbW*, *sigB*, and *rsbX* frames. If the downstream region of the linearized construction bore a mutant *rsbV* or *rsbW* allele, this allele was introduced into the *sigB* operon together with P_{spac} when the second crossover occurred downstream from the mutation. PCR experiments verified the presence of either the wild-type alleles or the *rsbVΔ1*, *rsbWΔ1*, or Δ(*rsbV-rsbW*) mutation in the *sigB* operon under P_{spac} control.

With the wild-type *sigB* operon under P_{spac} control, the timing of induction was critical for σ^B activity (Fig. 5). When the operon was induced by adding IPTG to logarithmically growing cells, *ctc* expression was entirely lacking throughout the growth cycle, even though this experiment was conducted under growth conditions that maximize *ctc* expression. In contrast, when the operon was induced at the end of logarithmic growth, *ctc* was expressed during early stationary phase in a manner similar to that observed when the *sigB* operon was under control of its wild-type promoter (see Fig. 3). Induction of the *sigB* operon 1 h into stationary phase led to reduced expression of *ctc*, probably due to decreased permeability of the cells to IPTG (not shown).

We conclude from these results that σ^B activity requires signals or factors present in early-stationary-phase cells and that induction of the operon at an inappropriate growth stage cannot overcome the absence of these signals. Indeed, when the operon was induced during logarithmic growth, the lack of any σ^B activity, even in stationary phase, was particularly striking. One explanation of this result is that at least one of the *sigB* operon products inhibits σ^B activity during logarithmic growth and that the stationary-phase signals which normally activate σ^B cannot overcome early expression of this product.

To determine whether any of the *sigB* operon products was responsible for inhibiting σ^B activity during logarithmic growth, we measured *ctc* expression in various *sigB* operon mutants in which the operon was induced under P_{spac} control. This experiment was conducted under growth conditions in which *ctc* is not optimally induced in order to provide a stringent test of negative regulator function. As shown in Fig. 6, strains carrying null *rsbV* and *rsbX* mutations behaved essentially the same as the wild type, and the mutations did not relieve the block to σ^B activity in logarithmically growing cells. However, the *rsbWΔ1* mutation did dramatically reverse the inhibition of activity for at least 2 h after operon induction, at which point the cells stopped growing. We conclude that one role of the *rsbW* product is to prevent σ^B activity at inappropriate times and that the loss of *rsbW* is deleterious in logarithmically growing cells.

We also showed that the *rsbW* product has a comparable negative role in stationary-phase cells. In contrast to the induction of the *rsbWΔ1* mutant in logarithmically growing cells, induction of the P_{spac} construction at the end of logarithmic growth had no obvious deleterious effect on cell growth or viability. The experiment in Fig. 7 showed that in LB medium, the *rsbWΔ1* mutation strongly increased σ^B activity measured at the *ctc* promoter. The *rsbWΔ1* mutation had a similar but less potent effect on *ctc* expression when cells were grown in LB containing glucose and glutamine. In contrast, the phenotype of the *rsbVΔ1* mutation under P_{spac} control established that the *rsbV* product is an essential positive effector of σ^B activity in both media. Notably, the results shown in Fig. 7 were consistent with those in Fig. 3. Other than a change in the operon promoter, the principal difference between these experiments was that the *rsbW1* missense mutation was tested in one (Fig. 3) and the *rsbWΔ1*

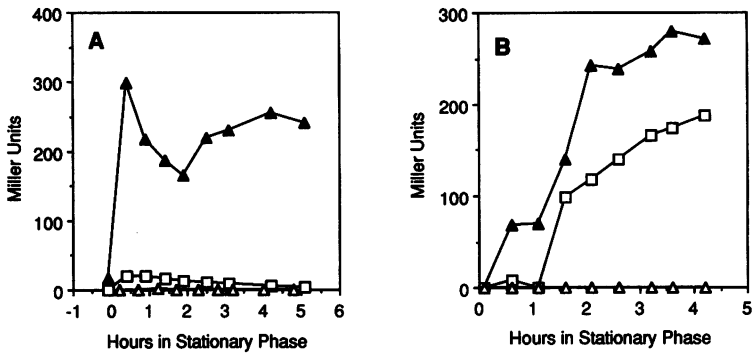


FIG. 7. σ^B activity in stationary phase with the *sigB* operon under P_{spac} control. IPTG (1 mM, final concentration) was added to one of two parallel cultures at the end of logarithmic growth (T_0). Samples were removed from each culture at the indicated times and assayed for β-galactosidase activity of the resident pDH32-*ctc-lacZ* transcriptional fusion. For each strain, β-galactosidase activities from the uninduced culture were subtracted from the activities of the corresponding induced culture. Symbols: □, PB212 (P_{spac} *rsbV*$^+$ *rsbW*$^+$ *amyE*::pDH32-*ctc*); ▲, PB213 (P_{spac} *rsbV*$^+$ *rsbWΔ1 amyE*::pDH32-*ctc*); △, PB214 (P_{spac} *rsbVΔ1 rsbW*$^+$ *amyE*::pDH32-*ctc*). (A) Cultures grown in unsupplemented LB; (B) Cultures grown in LB supplemented with 5% glucose and 0.2% glutamine.

mutation was tested in the other (Fig. 7). Together, the results of the experiments shown in Fig. 3 through 7 are in accord with the view that the *rsbW* product inhibits the activity of σ^B in both logarithmic- and stationary-phase cells. Because different promoters were used to express the *sigB* operon in the experiments shown in Fig. 3 and 7, we can rule out the possibility that RsbV and RsbW act primarily by controlling the rate of transcriptional initiation from the *sigB* operon promoter.

Relationship of *rsbV*, *rsbW*, and *rsbX* null mutations. We did tests of genetic epistasis to determine the relationships among the two regulatory gene products described here, RsbV and RsbW, and the *rsbX* gene product described earlier (15, 23, 27). Epistasis is the masking of the phenotype of one allele by the phenotype of another, and such tests allow inferences to be made about the order of action of regulatory elements from phenotypic comparison of a double mutation with the single mutations used in its construction.

As shown in Fig. 8A, we used strains bearing the wild-type *sigB* promoter to test the epistasis between *rsbVΔ1* and *rsbX*::*ery*, null regulatory mutations which had clear and opposite effects on the expression of σ^B-dependent gene expression. This experiment (and the ones shown in Fig. 8B and Fig. 9) were carried out in unsupplemented LB medium, a growth condition in which *ctc* ordinarily is not highly expressed. This condition gave a more pronounced difference in the effects of the single mutations, and thus the phenotype of a double mutation would be more readily apparent. σ^B activity in the *rsbVΔ1 rsbX*::*ery* double mutant was identical to that in the *rsbVΔ1* single mutant, indicating that *rsbVΔ1* was epistatic to *rsbX*::*ery* (Fig. 8A). The relationship of *rsbX*::*ery* and *rsbVΔ1* determined from the activity assays shown in Fig. 8A was the same as that deduced from the mutant growth phenotypes on LB plates. The *rsbX*::*ery* mutation causes a small-colony phenotype (27), and colony size was restored to wild type in the *rsbVΔ1 rsbX*::*ery* double mutant. These epistasis results imply that the *rsbX* product acts before the *rsbV* product in an ordered pathway regulating σ^B activity or that loss of the *rsbV* product negates any control exerted by the *rsbX* product.

As shown in Fig. 8B, we used the P_{spac} constructions to test the epistasis between the *rsbVΔ1* and *rsbWΔ1* null alleles, which also had opposite regulatory effects. The σ^B activity of the Δ(*rsbV-rsbW*) double mutant was very similar to that of the *rsbWΔ1* single mutant, indicating that *rsbWΔ1* was epistatic to *rsbVΔ1*. The relationship of *rsbVΔ1* and *rsbWΔ1* determined from the activity assays agreed with that inferred from the mutant growth phenotypes on LB plates.

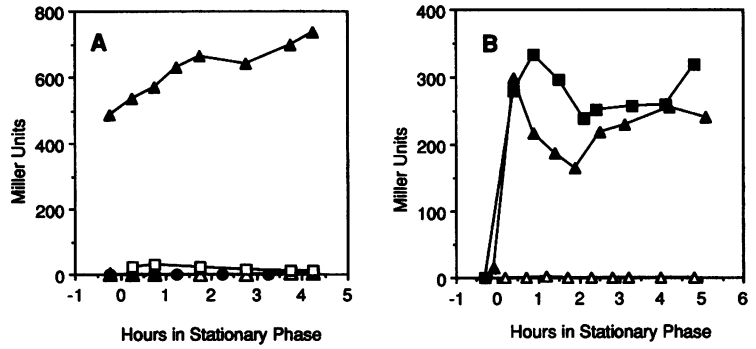


FIG. 8. Epistatic relationships among regulatory mutations in the *sigB* operon. (A) β-Galactosidase activities from strains PB198 (*rsbV*$^+$ *rsbX*$^+$ *amyE*::pDH32-*ctc*; □), PB206 (*rsbVΔ1 rsbX*$^+$ *amyE*::pDH32-*ctc*; △), PB217 (*rsbVΔ1 rsbX*::*ery amyE*::pDH32-*ctc*; ●), and PB218 (*rsbV*$^+$ *rsbX*::*ery amyE*::pDH32-*ctc*; ▲). Strains PB198 and PB206 were grown in LB, and strains PB217 and PB218 were grown in LB containing erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) to retain the *rsbX*::*ery* marker. (B) β-Galactosidase activities from strains PB213 (P_{spac} *rsbV*$^+$ *rsbWΔ1 amyE*::pDH32-*ctc*; ▲) and PB214 (P_{spac} *rsbVΔ1 rsbW*$^+$ *amyE*::pDH32-*ctc*; △) are from Fig. 7A. Strain PB219 (P_{spac} Δ[*rsbV-rsbW*] *amyE*::pDH32-*ctc*; ■) was grown in LB and assayed as described in the legend to Fig. 7.

Under conditions in which was P_{spac} was uninduced, the *rsbVΔ1*, *rsbWΔ1*, and Δ(*rsbV-rsbW*) mutants had no obvious growth defect. However, when P_{spac} was induced, both the *rsbWΔ1* mutant and the Δ(*rsbV-rsbW*) double mutant lysed (not shown), while the *rsbVΔ1* mutant grew normally. These epistasis results suggest that the *rsbV* product acts in a regulatory pathway before the *rsbW* product or, alternatively, that loss of the *rsbW* product overrides any control imposed through *rsbV*.

To further characterize the manner in which RsbW and RsbX inhibited σ^B activity, we found that the increase in activity caused by the simultaneous loss of *rsbW* and *rsbX* function was additive. If RsbX functions as a negative regulator in a pathway containing RsbW as the final negative effector of σ^B activity, then loss of *rsbX* function in an *rsbW* mutant should cause no additional increase in σ^B activity. However, if RsbX regulates σ^B activity by a mechanism independent of RsbW, then the increase in σ^B activity caused by loss of both RsbX and RsbW should be additive. We therefore attempted to construct a strain carrying the *rsbX*::*ery* null mutation together with the *rsbW1* missense mutation by transforming strain PB201 (*rsbW1 amyE*::pDH32-*ctc*) with plasmid pSK15, bearing the *rsbX*::*ery* insertion-deletion mutation. On plates selective for the *rsbX*::*ery* marker, we found only intensely blue, pinpoint colonies that proved inviable on restreaking. These results suggest that the increases in σ^B activity (and decreases in cell viability) caused by the loss of RsbX and RsbW function are additive. However, this result—implying independent action—must be interpreted with caution, because the *rsbW1* missense allele was not a total-loss-of-function mutation. Our preliminary results with an *rsbWΔ1 rsbX*::*ery* double null mutation under P_{spac} control confirm the additive effects of the two alleles, suggesting that the *rsbW* and *rsbX* products act independently (6).

***rsbV* and *rsbW* products regulate σ^B posttranslationally.** The *rsbV* and *rsbW* products could plausibly regulate σ^B-dependent gene expression by modulating either σ^B synthesis or σ^B activity. Our first indication of the level at which RsbV and RsbW act comes from the P_{spac} studies. The *rsbV* and *rsbW* mutations had a similar effect on σ^B-dependent gene expression whether the *sigB* operon was under control of the wild-type *sigB* promoter or P_{spac} (Fig. 3 and 7). Thus, RsbV and RsbW cannot act primarily through the region replaced in the P_{spac} construction, that is, through the *sigB* promoter sequences upstream from the *Spe*I site, the site of transcription initiation with the wild-type promoter (27).

We next tested whether the *rsbV* product regulated translation of the *sigB* message. We indirectly measured the effect of the *rsbVΔ1* allele on σ^B protein levels by using a *sigB-lacZ* translation fusion. This fusion was constructed by using the pJF751 vector, which upon integration into the *B. subtilis* chromosome both created the *sigB-lacZ* fusion and simultaneously abolished *sigB* and *rsbX* function (27). Thus, the PB114 strain carrying this fusion is a *sigB rsbX* double null mutant. We used the P_{spac} constructions both to place the *sigB-lacZ* fusion under the control of a functional promoter and to move the *rsbVΔ1* mutation into the fusion background. Because the epistasis results indicated that the nature of the *rsbX* allele had no effect on the phenotype of an *rsbV* null mutation (Fig. 8A), for the resolution of this experiment we could disregard the absence of a functional *rsbX* product in the fusion strain.

As shown in Fig. 9, there was no significant difference in expression of the *sigB-lacZ* fusion in the *rsbVΔ1* mutant compared with the strain that carried the wild-type *rsbV* allele. These results strongly suggest that the *rsbV* product acts on σ^B posttranslationally and thus works at the level of σ^B activity. Based on the epistatic relationship between *rsbV* and *rsbW* (Fig. 8B), we can also conclude that the *rsbW* product acts posttranslationally.

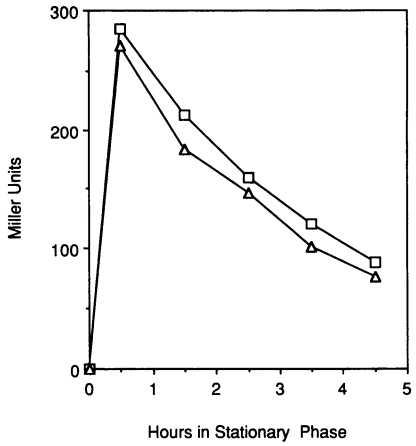


FIG. 9. Effect of *rsbVΔ1* mutation on expression of a *sigB-lacZ* translational fusion under P_{spac} control. β-Galactosidase activities of a *sigB-lacZ* translational fusion (pMD10) integrated at the *sigB* locus (27), with the σ^B-dependent promoter of the *sigB* operon replaced with the inducible P_{spac} promoter. For each strain, parallel cultures were grown in LB, and IPTG (1 mM, final concentration) was added to one of the cultures at the end of logarithmic growth. β-Galactosidase activities from the uninduced control were subtracted from the activities of the corresponding induced culture. Symbols: □, PB221 (P_{spac} *rsbV*$^+$ *rsbW*$^+$ *sigB*::pMD10); △, PB222 (P_{spac} *rsbVΔ1 rsbW*$^+$ *sigB*::pMD10).

DISCUSSION

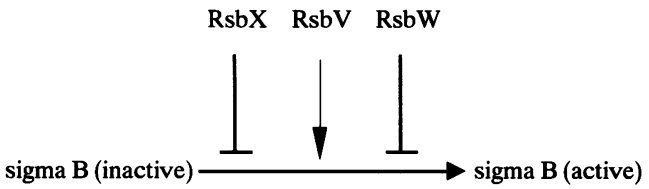
The genetic results reported here establish that all four genes in the *B. subtilis sigB* operon have important regulatory roles in exponential- and stationary-phase cells. Our experiments focused primarily on the roles of the first two genes of the operon, *rsbV* and *rsbW*, and their relationship to the last two genes, *sigB* and *rsbX*, which were previously shown to autoregulate operon expression (27). With regard to *rsbV* and *rsbW* function, our principal results are that (i) overexpression of the *rsbW* product blocks σ^B-dependent gene expression, whereas an *rsbW* null mutation increases expression; (ii) an *rsbV* null mutation prevents σ^B-dependent gene expression; and (iii) an *rsbW* null mutation reverses the block in σ^B-dependent gene expression caused by the loss of *rsbV*. From these genetic results, we conclude that RsbW is an inhibitor of σ^B-dependent gene expression and that RsbV is a positive effector which is required for σ^B activity only if RsbW is present. In addition, these results suggest that the function of RsbV is to counter the action of RsbW.

With regard to the regulatory relationships among the other products of the *sigB* operon, we first confirmed the finding of Igo et al. (23) that an *rsbX* null mutation increased the expression of σ^B-dependent genes and then determined that an *rsbV* null mutation reversed the effect of the *rsbX* mutation. Thus RsbX, RsbV, and RsbW form a sequential or hierarchical regulatory system which controls σ^B-dependent gene expression. Although our preliminary results suggest that RsbX and RsbW act separately, additional genetic analysis is needed to firmly establish whether the *rsbX*, *rsbV*, and *rsbW* products define independent regulatory

pathways or a single, dependent pathway (see reference 40 for an analysis which distinguishes dependent and independent pathways). In the absence of such analysis, we can construct two different types of models which are consistent with the available data. In one model, the three products form a dependent negative regulatory cascade, with RsbW inhibiting σ^B activity, RsbV inhibiting the action of RsbW, and RsbX inhibiting RsbV:

RsbX ⊣ RsbV ⊣ RsbW ⊣ sigma B

Alternatively, in the other model, the three products act sequentially yet independently to influence the activation of σ^B, with the absence of the downstream product overriding the need for the upstream product. In this model, the upstream product counteracts but does not need to exactly reverse the action of the downstream product:



Neither of these formal genetic models implies the molecular mechanism by which RsbX, RsbV, and RsbW act to control σ^B-dependent gene expression, and there is no biochemical evidence with respect to possible direct interactions of the four proteins in the system. We are therefore mindful of the possibility that RsbX, RsbV, and RsbW act indirectly on σ^B and that the σ^B system may include additional regulatory components.

Our results further show that RsbV and RsbW act posttranslationally, that RsbW is responsible for blocking σ^B-dependent gene expression during logarithmic growth, and that loss of this RsbW control is deleterious to the cell. The data indicating that RsbV and RsbW affect σ^B activity rather than synthesis comes from experiments in which a translational fusion was used to indirectly monitor σ^B protein levels. Therefore, we cannot rigorously rule out some forms of control, such as retroregulation (33), that might influence σ^B synthesis through sequences replaced by the *lacZ* fusion. However, for this discussion we assume the more likely explanation that RsbW inhibits σ^B activity. We can then imagine two broad classes for the mechanisms of RsbW action. In the first, the ultimate RsbW target is σ^B or σ^B-containing holoenzyme, and RsbW would modulate target activity either by direct protein-protein interaction, by catalytic addition or removal of a posttranslational modification, or by controlling the stability of the target protein. In the second class, the RsbW targets are σ^B-dependent promoters, at which RsbW would function directly as a repressor protein or indirectly by modulating such a protein. Arguing against the promoter-target mechanism are the lack of any obvious DNA-binding motifs in RsbW (27) and the lack of any common sequences which might define *cis*-acting RsbW binding sites in promoters known to be regulated by σ^B (8, 22, 27). If the final RsbW target is either σ^B or σ^B holoenzyme, this system—together with the analogous σ^F system (42)—would define a new mechanism for controlling sigma factor activity.

A significant finding of our current work is that σ^B and σ^F are regulated by similar molecular mechanisms. This conclusion is supported by three lines of evidence. First, the *sigB* and *spoIIA* (σ^F) operons have a similar genetic organization, with significant sequence identity between RsbV and SpoIIAA and between RsbW and SpoIIAB, the counterpart products of the two operons (27). Second, residues known to be important for SpoIIAA and SpoIIAB function are both conserved and important for RsbV and RsbW function (Fig. 2 and 3). Third, analysis of the σ^F system by Schmidt et al. (42) has shown that *spoIIAA* and *spoIIAB* mutations have regulatory consequences and epistatic relationships comparable to those of *rsbV* and *rsbW* mutations and that SpoIIAA and SpoIIAB likely affect σ^F activity at the posttranslational level.

Although both the σ^B and σ^F regulons are expressed in the stationary growth phase, at least some of the genes in each regulon have different physiological roles and most likely respond to different stationary-phase signals. σ^F activity is essential for successful completion of the sporulation process, and σ^F-directed transcription is thought to be crucial in establishing gene expression specifically in the forespore compartment (31). In contrast, σ^B is not required for sporulation (4, 15, 23, 27), and the genes it controls appear to be most highly expressed under stationary-phase growth conditions, in which sporulation is not favored (6, 8, 24).

A clear regulatory difference between the two operons is the presence of *rsbX* as a fourth gene in the *sigB* operon. The additional layer of negative control provided by RsbX may be required by the positive autoregulation that σ^B exerts over its own structural gene, a feature it shares with σ^K (28). σ^K controls essential sporulation genes expressed in the mother cell compartment, and the autocatalytic nature of *sigK* expression is thought to allow rapid amplification of σ^K activity, facilitating competition with σ factors from earlier stages of development. Like σ^B, the autocatalytic σ^K activity is subject to multiple levels of control, and disabling a key σ^K control leads to the production of defective spores by interfering with the timing of the sporulation process (11, 28, 45). In the case of σ^B, disabling the *rsbX* control leads to slow growth and a small-colony phenotype (15, 23, 27), while disabling *rsbW* control is apparently lethal to cells in logarithmic growth.

The additional complexity of the *sigB* operon also suggests at least two possible routes of information entry into the system: through RsbX and through the RsbV-RsbW pair. From our preliminary genetic results, we presume that RsbX and RsbW constitute independent regulatory pathways. And, although the absolute dependence of RsbV on RsbW remains to be established by genetic or biochemical criteria, our epistasis experiments and the apparent translational coupling of *rsbV* and *rsbW* expression suggest that RsbV acts with RsbW. Two lines of experiments indicate that the inferred RsbV-RsbW and RsbX pathways each respond to stationary-phase signals. First, with the wild-type *sigB* promoter, loss of RsbX leads to greatly increased expression of sigma B-dependent genes in exponential growth (27). Thus, in the wild-type context, RsbX is important for preventing the appearance of σ^B activity until the stationary phase. Second, experiments with the P_{spac} promoter (Fig. 6) showed that RsbW was responsible for preventing σ^B activity in exponentially growing cells. The inhibitory effects of RsbW and RsbX could be either potentiated by a metabolic signal or protein factor present in logarithmic growth or reversed by a signal or factor in stationary phase. RsbW and RsbX continue to affect σ^B activity in stationary phase, because loss of either factor increases stationary-phase expression of σ^B-dependent genes (Fig. 7 and 8). Thus,

RsbW and RsbX are important elements in regulating σ^B activity in both logarithmic- and stationary-phase cells.

To what signals and factors might the RsbV-RsbW and RsbX pathways respond? Although most σ^B-dependent genes are highly expressed in LB containing glucose and glutamine, the available data do not support the view that this response is mediated solely through RsbV, RsbW, or RsbX. A null *rsbX* mutant retains the glucose and glutamine regulation of *ctc* expression characteristic of the wild-type strain, and the null *rsbWΔ1* mutant in the P_{spac} background also retains the full glucose-glutamine response when partly induced (6). Because the *rsbV* null mutant lacks detectable σ^B activity, it is not useful in establishing the signals to which RsbV might respond. However, we did observe glucose-glutamine induction of *ctc* expression in the mutant carrying the *rsbV42* missense allele (Fig. 3). If the *rsbV42* allele is a loss-of-function mutation, as suggested by our data, this result argues that RsbV alone cannot mediate the glucose-glutamine response. Thus, additional regulatory elements remain to be discovered in the σ^B system.

Our results indicate that RsbW is a key element in σ^B regulation. Further analysis will determine whether signals that activate σ^B actually pass through RsbW or whether these signals act first on RsbV, which in turn transmits the signal to RsbW. Determination of the molecular mechanism by which RsbW regulates σ^B activity may well define a new means for controlling transcription in response to environmental or cell cycle signals.

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