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Four Additional Genes in the *sigB* Operon of *Bacillus subtilis* That Control Activity of the General Stress Factor σ^B in Response to Environmental Signals

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σ^B of the gram-positive bacterium *Bacillus subtilis* is an alternative transcription factor activated by a variety of environmental stresses, including the stress imposed upon entry into the stationary growth phase. Previous reports have shown that this stationary-phase activation is enhanced when cells are grown in rich medium containing glucose and glutamine. The σ^B structural gene, *sigB*, lies in an operon with three other genes whose products have been shown to control σ^B activity in response to environmental stress. However, none of these is sufficient to explain the enhanced stationary-phase activation of σ^B in response to glucose. We show here that the four genes previously identified in the *sigB* operon constitute the downstream half of an eight-gene operon. The complete *sigB* operon is preceded by a σ^A-like promoter (P_A) and has the order P_A-orfR-orfS-orfT-orfU-P_B-*rsbV-rsbW-sigB-rsbX*, where *rsb* stands for regulator of sigma-B and the previously identified σ^B-dependent promoter (P_B) is an internal promoter preceding the downstream four-gene cluster. Although the genes downstream of P_B were also transcribed by polymerase activity originating at P_A, this transcription into the downstream cluster was not essential for normal induction of a σ^B-dependent *ctc-lacZ* fusion. However, deletion of all four upstream open reading frames was found to interfere with induction of the *ctc-lacZ* fusion in response to glucose. Additional deletion analysis and complementation studies showed that orfU was required for full glucose induction of σ^B-dependent genes. orfU encodes a *trans*-acting, positive factor with significant sequence identity to the RsbX negative regulator of σ^B. On the basis of these results, we rename orfU as *rsbU* to symbolize the regulatory role of its product.

The purpose of many prokaryotic signal transduction pathways is to change the level of transcription of a set of target genes in response to a particular environmental signal. Stress and starvation signals elicit particularly dramatic changes in bacterial gene expression (15, 19, 21, 27, 35, 46). In a number of cases, these stress and starvation signals have been shown to activate alternative σ factors that associate with RNA polymerase and reprogram the promoter recognition specificity of the enzyme, thus allowing the expression of new sets of target genes (18, 19, 32, 48). Because σ factors are such powerful regulators of bacterial gene expression, the signal transduction pathways that control their activation are an area of vigorous investigation.

The alternative transcription factor σ^B of *Bacillus subtilis* is activated upon entry into the stationary growth phase, and this stationary-phase activation is substantially enhanced when cells are grown in a rich medium containing high levels of glucose and glutamine (7, 9, 25). Significantly, recent work has shown that σ^B is also activated in logarithmically growing cells by a variety of environmental stresses that limit growth, including hyperosmosis, heat, oxidative stress, and ethanol shock (4, 6, 51). These findings have led to the proposal that σ^B controls a general stress regulon (6).

Some signals of environmental stress are conveyed to σ^B by a regulatory network encoded by the three genes adjacent to the σ^B structural gene in the *sigB* operon (2, 6, 8). This operon is transcribed from a σ^B-dependent promoter and has the gene order *rsbV-rsbW-sigB-rsbX* (29). Genetic analysis has shown that the RsbW protein is a key negative regulator responsible for rendering σ^B inactive during logarithmic growth (8). Subsequent biochemical analysis has shown that RsbW is an anti-σ factor that binds directly to σ^B and controls its activity by preventing its association with the RNA polymerase core enzyme (3). The RsbV protein counters the effect of RsbW and thus acts as a positive regulator that is normally required for σ^B activity to appear in stationary phase, or during logarithmic growth in response to salt or ethanol stress (2, 6, 8, 13). The role of the RsbX negative regulator is less well understood, but genetic analysis indicates that it acts before the RsbV-RsbW pair in a regulatory hierarchy (2, 8, 24, 29).

During characterization of the RsbV-RsbW-RsbX hierarchy, it became evident that none of the known regulators of σ^B activity was sufficient to fully account for the enhanced stationary-phase activation of σ^B in response to glucose, suggesting that additional regulatory elements remained to be discovered (8). We earlier presented evidence that a second, σ^B-independent promoter must exist for the *sigB* operon and that this promoter must lie more than 1,100 bp upstream from the σ^B-dependent promoter immediately preceding *rsbV* (29). Here we locate this upstream promoter and show that it controls an eight-gene operon, the downstream four genes of which comprise the previously identified *rsbV-rsbW-sigB-rsbX* cluster. We further show that one of the newly identified genes in the *sigB* operon encodes a *trans*-acting factor required for full stationary-phase induction of σ^B-dependent genes in rich medium containing glucose.

(A preliminary report of this work was presented at the 11th International Spores Conference [52].)

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TABLE 1. *B. subtilis* strains used

Strain	Genotype	Reference or construction[a]
PB2	*trpC2*	Wild-type Marburg strain
PB118	*rsbV*::pMD6 *trpC2*	29
PB197	SPβ*ctc-lacZ trpC2*	25
PB198	*amyE*::*ctc-lacZ trpC2*	8
PB241	*rsbV*::pAW18 *rsbV*::pMD6 *trpC2*	pAW18→PB118
PB242	*rsbV*::pAW18 *trpC2*	pAW18→PB2
PB243	*rsbV*::pAW18 *amyE*::*ctc-lacZ trpC2*	PB198→PB242
PB244	Δ*rsbU1*::*ermC amyE*::*ctc-lacZ trpC2*	pAW5→PB198
PB248	*amyE*::P_A-*lacZ trpC2*	pAW12→PB2
PB252	*amyE*::P_A-*lacZ trpC2*	pAW14→PB2
PB286	*amyE*::P_B-*lacZ trpC2*	pAW20→PB2
PB288.5	Δ(orfR-*rsbU*)1::*ermC trpC2*	pAW52→PB2
PB288	Δ(orfR-*rsbU*)1::*ermC trpC2* SPβ*ctc-lacZ*	PB197→PB288.5
PB289	Δ*rsbU1*::*ermC trpC2*	pAW5→PB2
PB291	Δ*rsbU1*::*ermC* SPβ*ctc-lacZ trpC2*	PB197→PB289
PB293.5	Δ*rsbU1*::*ermC amyE*::(P_A-orfR-orfS-orfT-*rsbU*) *trpC2*	pAW70→PB289
PB293.6	Δ*rsbU1*::*ermC amyE*::(P_A-orfR-orfS-orfT-*rsbU*) *trpC2*	pJL62[b]→B293.5
PB293	Δ*rsbU1*::*ermC* SPβ*ctc-lacZ amyE*::(P_A-orfR-orfS-orfT-*rsbU*) *trpC2*	PB197→PB293.6
PB294.5	Δ*rsbU1*::*ermC amyE*::(P_A-orfR-orfS-orfT) *trpC2*	pAW90→PB289
PB294.6	Δ*rsbU1*::*ermC amyE*::(P_A-orfR-orfS-orfT) *trpC2*	pJL62[b]→PB294.5
PB294	Δ*rsbU1*::*ermC* SPβ*ctc-lacZ amyE*::(P_A-orfR-orfS-orfT) *trpC2*	PB197→PB294.6
PB296	Δ*rsbU1*::*ermC amyE*::P_B-*lacZ trpC2*	pAW20→PB289

[a] Arrow indicates transformation from donor to recipent.
[b] pJL62 converts the construction at the *amyE* locus from *cat* to *spc* (31).

MATERIALS AND METHODS

Bacterial strains and genetic methods. *B. subtilis* strains are listed in Table 1. *B. subtilis* PB2 and its derivatives were made competent for transformation by the method of Dubnau and Davidoff-Abelson (12). Chromosomal DNA was isolated by the method of Ferrari et al. (16); the plasmids used in strain constructions and isolation of plasmid DNA are described below. Transformants were selected for drug resistance on tryptose blood agar plates (Difco Laboratories) containing either 5 μg of chloramphenicol per ml (for *cat*), 100 μg of spectinomycin per ml (for *spc*), or a combination of 0.5 μg of erythromycin and 12.5 μg of lincomycin per ml (for *erm*C).

Recombinant DNA methods. *Escherichia coli* DH5α was host for all plasmid constructions. Plasmid DNA was isolated either by alkaline lysis (5) or by boiling (22). Standard methods were used for restriction digests, ligations, and transformation of *E. coli* (10, 43). PCR was done as described by Innes et al. (26), using primers from Operon Technologies (Alameda, Calif.). DNA sequencing was done by the dideoxynucleotide chain termination method with reactions primed on double-stranded DNA templates, using the Sequenase enzyme and protocols from U.S. Biochemical Corp. (Cleveland, Ohio). We sequenced 2,850 bp of the *sigB* region on both strands and through all restriction endpoints used for subcloning into pUC18.

Isolation of the upstream half of the *sigB* operon by plasmid excision. We used the integration and excision method of Youngman (53) to isolate two plasmids bearing the chromosomal region upstream from the previously characterized genes of the *sigB* operon (29). pAW10 contained a 5-kb DNA fragment extending from the *Eco*RV site within *rbsU* (orfU) to an upstream *Hin*dIII site, whereas pAW50 contained a 3.4-kb fragment extending from the *Afl*II site within *rsbV* to the *Cla*I site near the upstream promoter of the *sigB* operon (all restriction sites other than the upstream *Hin*dIII site are shown Fig. 1). Both pAW10 and pAW50 were derivatives of the integrational vector pCP115 (42). However, the *Bsm*I and *Eco*RI sites had been removed from the vector portion of pAW50 to facilitate later construction of deletion mutants.

Construction of operon fusions. We indirectly measured σ^B activity at the well-characterized σ^B-dependent *ctc* promoter (24, 38), using either a translational fusion to a *lacZ* reporter gene carried in single copy by the SPβ prophage of strain PB197 (constructed by Igo and Losick [25]) or a transcriptional fusion carried in single copy by the pDH32 vector integrated at the *amyE* locus of strain PB198 (constructed by Boylan et al. [8]). We also used the pDH32 vector (41) to construct a new transcriptional fusion to the σ^B-dependent P_B promoter of the *sigB* operon (29). This promoter was carried on a 327-nucleotide (nt) fragment extending from the *Pst*I site in *rsbU* to the *Afl*II site in *rsbV* (Fig. 1). The fragment was subcloned into the *Sma*I-*Bam*HI sites of pDH32, just upstream from the reporter gene fusion, to create pAW20. pAW20 was then linearized and transformed into *B. subtilis*, where it integrated by homologous recombination between the *amyE* "front" and "back" sequences provided by the vector and the *amyE* locus on the chromosome (41). In this single-copy state, the *B. subtilis* ribosomal binding site and initiation codon are supplied by the *B. subtilis spoVG* gene fused to *E. coli lacZ*. The timing and amount of β-galactosidase expression therefore relies on the transcriptional elements carried by the subcloned fragment.

The search for promoter activity in the newly discovered upstream half of the *sigB* operon was conducted by using pDH32 as a promoter-probe vector. As shown in Fig. 1, appropriate upstream fragments were subcloned, and the vectors were integrated at *amyE*. The initial assay for promoter activity was on tryptose blood agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Two strains showing promoter activity were examined in greater detail. Strain PB248 carried at its *amyE* locus a linear pAW12 (with a 2.1-kb *Eco*RV fragment fused to *spoVG-lacZ*), whereas strain PB252 carried a linear pAW14 (with a 104-nt PCR fragment fused to *spoVG-lacZ*). The DNA sequence of the 104-nt fragment carried by pAW14 was found to be the same as that of the wild-type *B. subtilis* chromosome (between nt 261 and 364 in Fig. 2), indicating that no mutations had been introduced by the PCR amplification.

Construction of mutations within the upstream half of the *sigB* operon. We made two plasmids, pAW5 and pAW52, in order to construct strains with large deletion-insertion mutations within the upstream reading frames of the *sigB* region. pAW5 was derived from pSK20 (28), a pUC19 derivative that carries a 2.3-kb insert extending from the *Eco*RI site just upstream from *rsbU* to the *Eco*RI site within *sigB*. pSK20 was digested with *Bsm*I to remove two adjacent fragments from within the *rsbU* coding sequence (indicated by Δ*rsbU1* in Fig. 1). This 694-bp deletion was replaced by a 1.4-kb *Taq*I fragment from pSK1 that bears the *ermC* gene (28). The linearized form of pAW5 was used to transform various recipients to macrolide resistance, creating the Δ*rsbU1*::*ermC* mutation on the *B. subtilis* chromosome. The resulting strains are listed in Table 1. We then made pAW52 to construct strains with a deletion-insertion mutation that removed most of the upstream reading frames. pAW50 (on which the upstream region had been initially isolated by excision from the *B. subtilis* chromosome) was digested with *Eco*RI and *Bsm*I to remove a 2.2-kb fragment that extended from the upstream *Eco*RI site in orfR to the downstream *Bsm*I site in *rbsU* [indicated by Δ(orfR-*rsbU*)1 in Fig. 1]. This deletion was replaced by the *ermC* gene to yield pAW52. Transformation of the linearized form of pAW52 was used to create the Δ(orfR-*rsbU*)1::*ermC* mutation on the *B. subtilis* chromosome (Table 1).

To test the ability of the *rsbU* reading frame to complement the Δ*rsbU1*::*ermC* mutation in *trans*, we made two additional plasmids, pAW70 and pAW90. The chromosomal inserts carried by these plasmids are shown in Fig. 1. Both plasmids relied on the *amyE* front and back sequences of a pDH32 derivative, pAW60, to insert a single copy of the upstream reading frames into the *amyE* chromosomal locus. We made pAW60 by removing a 1.1-kb *Cla*I-*Sst*I fragment from within the *lacZ* gene of pDH32. The resulting plasmid could be then be used to create partial diploid strains and, because it lacked a functional reporter gene, would not interfere with the assay of the *ctc-lacZ* fusion borne by the SPβ prophage. pAW53 served as the source of the fragments bearing the upstream promoter and reading frames that were subcloned into pAW60. pAW53 is essentially the same pAW50, which carried the excised upstream region, but with the *Cla*I site at nt 77 of the chromosomal insert removed and the *Hpa*I site at nt 155 (Fig. 2) replaced by a *Bam*HI site. To make pAW70, a 3.1-kb *Bam*HI fragment was cut

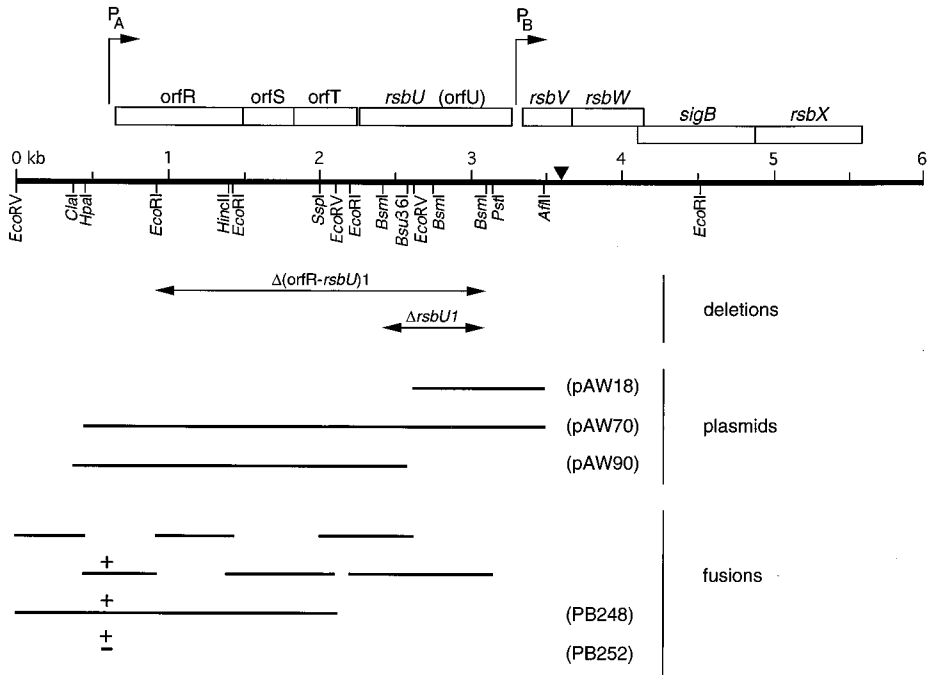


FIG. 1. Physical map of the eight-gene *sigB* operon. The chromosome in the *sigB* region is represented by the kilobase scale, and the locations of the four upstream open reading frames [orfR, orfS, orfT, and *rsbU* (orfU)] are indicated by the open rectangles above the physical map. The locations of the four downstream genes that encode σ^B and its regulatory network (*rsbV*, *rsbW*, *sigB*, and *rsbX*) are from Kalman et al. (29); the filled triangle within the *rsbV* coding region symbolizes the location of the *rsbV-lacZ* translational fusion construction carried by PB118. The *sigB* operon is transcribed from two promoters, a σ^A-like promoter (P_A) preceding orfR and a σ^B-dependent internal promoter (P_B) preceding *rsbV*. The restriction map shows only those sites used in the recombinant DNA constructions described in Materials and Methods; other sites recognized by these same enzymes are omitted. The regions of the chromosome used in some of these constructions are indicated beneath the restriction map. The regions labeled "deletions" were removed to make the Δ(orfR-*rsbU*)1 and Δ*rsbU1* deletion-insertion mutations. The regions labeled "plasmids" represent the chromosomal inserts carried by three of the plasmids used in this study. pAW18 was used to insert plasmid sequences between the upstream and downstream halves of the operon on the *B. subtilis* chromosome, thereby preventing transcription initiating at P_A from entering the downstream four genes. pAW70 and pAW90 were used for complementation studies, placing the indicated regions in *trans* at the *amyE* locus. Other plasmids used in this study are described in Materials and Methods. The fragments labeled "fusions" were subcloned into the single-copy transcriptional fusion vector pDH32 (41) in order to locate the upstream P_A promoter; the three fragments conferring promoter activity are labeled (+). Two of these fusions, carried by strains PB248 and PB252, were studied in detail.

from pAW53 and subcloned into the *Bam*HI site of pAW60, between the *amy* front and back sequences. This subcloned fragment extended from the newly constructed *Bam*HI site at nt 155 to a *Bam*HI site in the vector downstream of the *B. subtilis* chromosomal insert and therefore includes the promoter for the upstream region together with the complete orfR, orfS, orfT, and *rsbU* reading frames (Fig. 1). The resulting pAW70 vector was linearized and transformed into *B. subtilis*, and its integration into the *amyE* locus was detected by loss of amylase activity in the recipient strain. To make pAW90, a 2.2-kb fragment of pAW53 was subcloned into the *Bam*HI site of pAW60. This fragment extended from an *Sph*I site in the pAW53 vector upstream from the *B. subtilis* chromosomal DNA to a *Bsu*36I site within *rsbU* and therefore includes the promoter for the upstream region together with all of orfR, orfS, and orfT reading frames but only the 5′ third of *rsbU* (Fig. 1). pAW90 was linearized and transformed into the *amyE* locus. To allow introduction of the *ctc-lacZ* translational fusion on the SPβ prophage, the *cat* markers of the integrated pAW70 and pAW90 vectors were converted to *spc* by transformation with pJL62, a gift of John LeDeaux and Alan Grossman (31).

To test whether transcription originating in the upstream half of the *sigB* region extended into the downstream half, and whether this transcription was important for σ^B-dependent gene expression, we constructed the integrative plasmid pAW18. As shown in Fig. 1, the 833-nt fragment of *B. subtilis* chromosomal DNA carried by this plasmid extended from the *Eco*RV site within *rsbU* to an *Afl*II site within *rsbV*. This fragment was subcloned into pRS13, a gift of Ruth Schmidt and Richard Losick (44), to make pAW18. Upon transformation and selection for the macrolide resistance, pAW18 integrated into the *rsbU-rsbV* region of the *B. subtilis* chromosome. Following this integration event, the upstream orfR-orfS-orfT-*rsbU* reading frames were separated from the downstream *rsbV-rsbW-sigB-rsbX* cluster by intervening plasmid sequences that terminate transcription. We used PCR to establish that all of these constructions, including pAW18, the deletion-insertion mutations carried by pAW5 and pAW52 and the complementing upstream fragments carried by pAW70 and pAW90, had integrated into the chromosome in the predicted configuration.

Enzyme assays. *B. subtilis* strains were grown to late logarithmic stage either in unsupplemented Luria broth (LB) (10) or in LB supplemented with 5% glucose and 0.2% L-glutamine (25) and were then diluted 1:25 into fresh medium. Samples were taken throughout the logarithmic and stationary phases of growth. Cells were collected by centrifugation and then frozen at −70°C. Thawed samples were assayed for β-galactosidase activity by the method of Miller (36), using sodium dodecyl sulfate and chloroform to permeabilize the cells. Activity was expressed in Miller units, defined as $\Delta A_{420} \times 1{,}000$ per minute per milliliter per optical density at 600 nm.

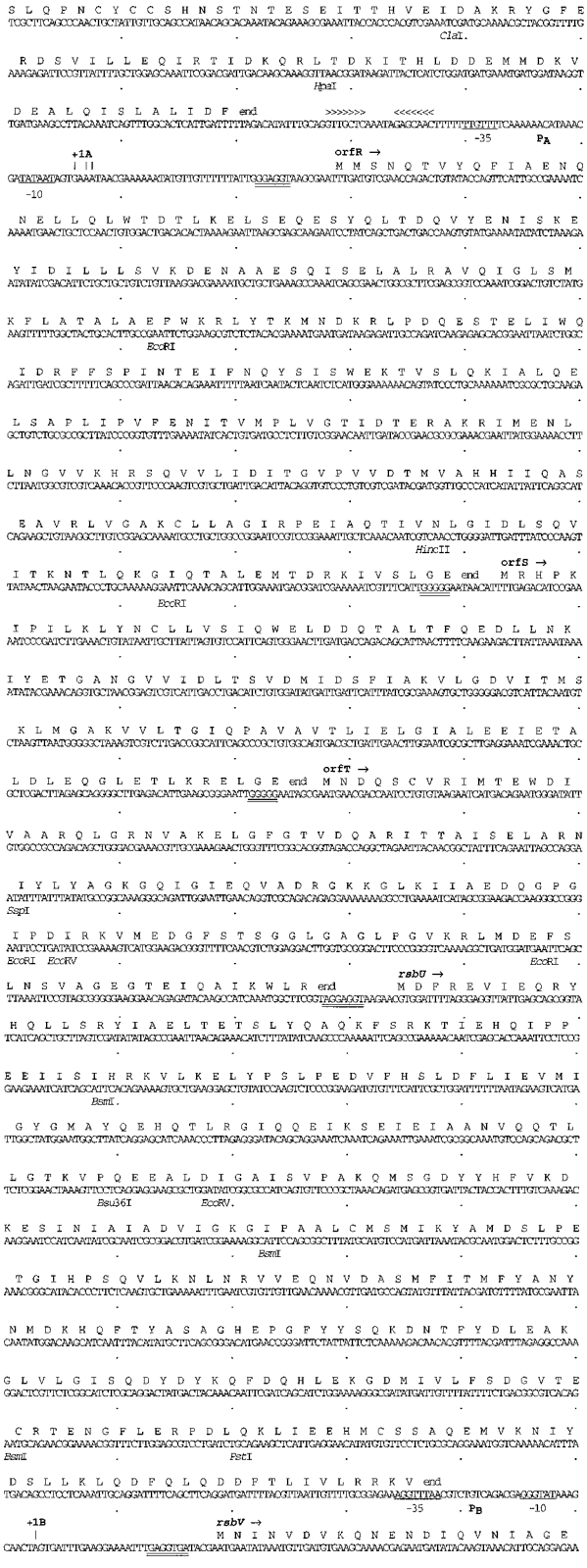
Mapping of the 5′ end of *sigB* operon message by primer extension. Parallel cultures of strain PB2 were grown in either LB or LB supplemented with 5% glucose and 0.2% glutamine. Cells from both cultures were harvested 30 min after entry into the stationary growth phase, and RNA was extracted by the method of Igo and Losick (25), using the modifications previously described (49). A 21-mer oligonucleotide primer (5′-TGGTATACAGTCTGGTTCGAC-3′, complementary to nt 363 to 383 within the orfR reading frame in Fig. 2) was 5′ end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) and T4 polynucleotide kinase (Promega), and primer extension reactions were done as previously described (49).

Computer analysis. DNA sequence assembly and analysis of the predicted gene products was done by using MacVector 4.1.4 software (Kodak Scientific Imaging Systems, New Haven, Conn.). Protein sequence alignments were done with the FASTA program of Pearson and Lipman (40), using the National Biomedical Research Foundation Protein Identification Resource database and VAX computer.

Nucleotide sequence accession number. The nucleotide sequence of the upstream half of the *B. subtilis sigB* operon has been assigned GenBank accession number L35574.

RESULTS

Kalman et al. (29) established that the σ^B structural gene lies third in a four-gene operon with the order *rsbV-rsbW-sigB-rsbX* and that this operon is transcribed from two different promoters. One of these is a σ^B-dependent promoter, P_B, that immediately precedes *rsbV*. The second is a σ^B-independent pro-



moter that allows continued expression of *lacZ* translational fusions to the *rsbV*, *rsbW*, and *sigB* reading frames in genetic backgrounds that are effectively *sigB* null mutants. Further studies to determine the location of this second promoter indicated that it must lie more than 1,100 bp upstream from P_B, but its precise location was not determined (29).

We inferred from these results that the *rsbV-rsbW-sigB-rsbX* gene cluster may be part of a larger operon. We therefore began an investigation of the upstream promoter and gene products with regard to their potential control of the general stress factor σ^B. As described in Materials and Methods, we used plasmid integration and excision to isolate an additional 5 kb of DNA upstream from P_B and the *rsbV-rsbW-sigB-rsbX* gene cluster. In experiments described below, we first located a strong promoter activity about 2.7 kb upstream from P_B. We then determined the nucleotide sequence of the intervening region as a preface to investigating whether the regulatory elements of the upstream region also controlled σ^B expression or activity.

The region between the upstream promoter and *rsbV* contains four open reading frames. As shown in Fig. 2, the region between the upstream promoter activity and P_B contained four open reading frames, in the order orfR-orfS-orfT-orfU, each preceded by a possible ribosome binding sequence appropriately spaced from a potential initiation codon (17). The predicted amino acid sequence of orfR suggested that it encodes a protein of 274 residues (31.0 kDa), and the hydropathy profile of the orfR product indicated that it might be membrane associated. In contrast, the hypothetical proteins encoded by orfS, orfT, and orfU are 121 residues (13.3 kDa), 133 residues (14.3 kDa), and 335 residues (38.6 kDa), respectively, and all had hydropathy profiles characteristic of soluble proteins (data not shown).

Notably, the predicted orfU product had significant identity to the RsbX negative regulator of σ^B activity. As shown in Fig. 3, the C-terminal portion of OrfU shared 24.7% identity with the smaller RsbX protein in a 146-residue overlap. Two other upstream products also have regions resembling those found in known regulators of σ activity. The predicted orfT product shared 29.3% identity with RsbX over a 58-residue overlap (not shown). This similarity is intriguing because of the close linkage of orfT and orfU, the significant similarity between OrfU and RsbX, and the known regulatory role of RsbX. Also intriguing is the resemblance of the hypothetical orfS product to the SpoIIAA protein of *Bacillus licheniformis*, with which it shares 25.5% identity in a 94-residue overlap (not shown). In *B. subtilis*, the 117-residue SpoIIAA protein is required for activation of the sporulation-essential σ^F (1, 45), and SpoIIAA is a homolog of RsbV, the positive activator of σ^B (8, 29).

FIG. 2. Nucleotide sequence of the upstream half of the *sigB* operon. Nucleotides are numbered from the 5′ end of the nontranscribed strand, with intervals of 20 nt indicated by a dot. The predicted amino acid sequence for each open reading frame is given in single-letter code above the DNA sequence, beginning with an unidentified open reading frame at nt 1. This open reading frame is separated from the *sigB* operon by a sequence resembling a factor-independent terminator ($\Delta G = -10$ kCal; 1 cal = 4.184 J), with a region of dyad symmetry at nt 256 to 274 (indicated by >) and a run of seven thymidines at nt 275 to 281. Within the *sigB* operon, the name of each open reading frame is shown above the initiating methionine, and the proposed ribosomal binding sites are double underlined. The 5′ ends of *sigB* operon message, determined by the primer extension experiment shown in Fig. 5, are labeled +1A at nt 312, 314, and 315, and the proposed −35 and −10 sequences of the σ^A-like P_A promoter are underlined at nt 280 to 285 and 303 to 308, respectively. The 5′ end found by Kalman et al. (29) is labeled +1B at nt 3006, and the proposed −35 and −10 sequences of the σ^B-dependent P_B promoter are underlined at nt 2969 to 2976 and 2991 to 2996. The sequence of nt 2839 to 3100 was reported previously (29).

However, the predicted orfS product has less resemblance to RsbV, and the function of orfS remains to be established. The orfR product had no significant similarity to any protein in the PIR database of the National Biomedical Research Foundation, release 42.

Additional features of the nucleotide sequence provided clues regarding the relationship between the upstream promoter and the previously identified genes in the downstream *sigB* cluster. In particular, the termination codon of the last open reading frame, orfU, was located within the previously identified −35 sequence of the σ^B-dependent P_B promoter (Fig. 2), and no sequence resembling a factor-independent termination signal was found between the upstream promoter region and *rsbV*. These observations suggested that transcription from the upstream promoter region might extend past P_B and into the downstream *sigB* cluster.

Location of the upstream promoter which controls the eight-gene *sigB* operon. After isolating the upstream region, we searched for promoter activity by using the single-copy transcriptional fusion vector pDH32. Strong promoter activity was found associated with a 104-bp fragment that lies 2.7 kb upstream from the σ^B-dependent P_B promoter, and no other promoter activity was detectable in the intervening region (Fig. 1). To determine whether transcription from the upstream promoter extended into the downstream four genes of the *sigB* cluster, we used the *lacZ* translational fusion to the *rsbV* reading frame in strain PB118 as an assay for downstream expression. In this strain, the fusion construction prevents expression of the downstream *sigB* gene, and the σ^B-dependent promoter immediately upstream from *rsbV* is therefore silent (29). As described in Materials and Methods, we constructed plasmid pAW18, which, upon integrating into the *sigB* region by a Campbell crossover, would insert plasmid sequences between the upstream promoter and the downstream four genes of the *sigB* operon, thus blocking transcription while leaving the upstream and downstream reading frames intact. The *B. subtilis* chromosomal DNA carried by this plasmid is shown in Fig. 1. Upon transformation of PB118 by pAW18, expression of the *rsbV-lacZ* fusion was abolished. We conclude that transcription from the upstream promoter extends at least into the *rsbV* reading frame. These results are consistent with the absence of an obvious termination sequence within the upstream region. Thus, the previously identified *rsbV-rsbW-sigB-rsbX* gene cluster constitutes the downstream half of an eight-gene operon, and the σ^B-dependent promoter P_B that lies between orfU and *rsbV* is an internal promoter of this larger operon.

The upstream promoter of the *sigB* operon is induced by glucose and glutamine. We examined the newly discovered upstream promoter of the *sigB* operon in greater detail to determine whether the smallest fragment which conferred promoter activity in the fusion assays contained the elements required for wild-type regulation. This 104-bp PCR-amplified fragment was designed so that its 5′ end began within a possible terminator sequence upstream from orfR and its 3′ end lay within the orfR coding sequence (from nt 261 to 364 in Fig. 2). We compared the β-galactosidase activity of strain PB252, which carried a single-copy *lacZ* transcriptional fusion made with this 104-bp fragment, with that of strain PB248, which carried the 2.1-kb *Eco*RV fragment from the upstream region. The locations of these two fragments are shown in Fig. 1. In both strains, β-galactosidase activity was induced during the late exponential growth phase in LB medium and was increased and sustained when cells were grown in LB with added glucose and glutamine (Fig. 4). The similar stationary-phase induction profiles for the two fusions shown in Fig. 4 suggested that under the conditions tested, the 104-bp fragment contained sequences sufficient for wild-type regulation of transcription from the upstream promoter.

To more precisely locate the upstream promoter within the 104-bp region, we mapped the 5′ end of the upstream message by primer extension. As shown in Fig. 5, the levels of upstream message increased in early-stationary-phase cells grown in glucose-supplemented medium. The locations of the three closely spaced 5′ ends detected in the primer extension experiment are indicated on the nucleotide sequence of the upstream region shown in Fig. 2. The 5′ ends mapping to the guanosine at nt 312, the adenosine at nt 314, and the adenosine at nt 315 are labeled +1A and are preceded by the sequence TTGTT T-17 bp-TATAAT (nt 279 to 308). This sequence and spacing closely match the consensus recognized by RNA polymerase holoenzyme containing the major sigma factor of *B. subtilis*, σ^A (18). We will therefore refer to the upstream promoter of the *sigB* operon as P_A. As shown in Fig. 2, the region upstream from P_A contains an unidentified open reading frame followed by a sequence that resembles a factor-independent transcription terminator, and the proposed −35 region of P_A begins within the run of seven thymidines following the stem-loop of the terminator. Thus, we presume that the eight-gene *sigB* operon is transcribed independently from any genes further upstream.

Aside from the σ^B-dependent genes identified thus far (7, 9, 24, 25), few *B. subtilis* genes are known to be induced by glucose. One such example is the *menCD* operon, which is involved in menaquinone biosynthesis. Like the *sigB* operon, *menCD* is transcribed during stationary phase from a glucose-inducible, σ^A-like promoter (37). One consequence of growth in glucose is the progressive acidification of the medium due to the production of acetate and pyruvate (47), and the *menCD* promoter has been shown to be responsive to changes in extracellular pH (20). Because σ^B-dependent genes are thought to comprise a general stress regulon (6, 51), the glucose-inducible P_A promoter of the *sigB* operon might also be expected to respond to acid stress.

To determine whether medium acidification or other general environmental stresses might influence expression of P_A and, by implication, the expression and activity of the downstream *rsbV-rsbW-sigB-rsbX* gene cluster, we compared expression of the P_A-*lacZ* fusion in strain PB248 when cells were grown under a variety of conditions. We saw no difference in P_A expression when cells were grown in buffered or unbuffered LB medium containing glucose or when 0.3 M NaCl or 4% ethanol was added to buffered LB medium to induce a stress response (not shown). In other experiments, P_A expression was also unaffected by null mutations in the *sigB* and *rsbX* genes that are known to affect P_B or by a null mutation in orfU that we show below affects σ^B-dependent gene expression under some growth conditions. We conclude that, in contrast to the case of the *menCD* promoter, glucose increases expression from P_A by a means other than medium acidification. We also provisionally conclude that P_A is not responsive to common signals of general stress.

Transcription from P_A into the *sigB* cluster is not required for induction of σ^B-dependent genes. The expression of a *sigB-lacZ* translational fusion at the *sigB* chromosomal locus (29), coupled with the fact that P_A was the only σ^B-independent promoter detected upstream from this fusion, indicated that transcription from P_A must extend into the *sigB* gene. Thus, a simple model to explain the glucose induction of the autocatalytic *sigB* gene—and, by extension, other σ^B-dependent genes—was that increased *sigB* expression was dependent on a priming transcript originating at the glucose-responsive P_A promoter. As an alternative model, the glucose induction of

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RsbU(111) -LLGTKVPQEEALDIGAISVPAKQMSGDYYHFVKDKESINIAIADVIGKGIPAALCMSMIK
                                 :| ::|| : :  |::::  |:|| :|:|  |: : : ||
RsbX(1)    MIQVEENEHIQTLVYQLNKEGKSICGDSFFMKADDKELICAVADGLGSGSLANESSAAIK

           YAMDSLPETGIHPSQVLKNKNRVVEQNVDASMFITMFYANYNMDKHQFTYASAGHEPGFY
             ::: ::::::   :::: |:::::: :|:  | ::    |::::|||| |:|: : :
           DLVENYASEDVE--SIIERCNQAMKNKRGATASILKI----NFEQRQFTYCSVGNVRFIL

           YSQKDNTFYDLEAKGLVLGISQDYDYKQFDQHLEKGDMIVLFSDGVTECRTENGFLERPD
           :|::::::|| |  :| : | :|  :||   :  |||:::::: :||:
           HSPSGESFYPLPISGYLSGKPQ--KYKTHTATYEKGSKFIIHTDGLNVPDIRSHLKKGQS

           LQKLIEEHMCSSAQEMVKNIYDSLLKLQDFQLQDDFTLIVLRRKV (335)

           VEEISNSLKMYTTSRKDDLTYILGQLS (199)
```

FIG. 3. FASTA alignment (40) of the C-terminal portion of the 335-residue *rsbU* (orfU) product with the 199-residue RsbX regulatory protein. The RsbU (OrfU) sequence is shown beginning at leucine 111 and extending to the C-terminal valine 335. Identical residues are indicated by |, and conserved substitutions (11) are indicated by :. Highly related sequences usually have an optimized alignment score greater than 100 and a *z* value greater than 10. This alignment has an optimized score of 116 and a *z* value of 10.2.

sigB could depend upon the action of one or more products of the four upstream reading frames under P_A control.

To test the priming transcript model, we assayed σ^B activity with a single-copy transcriptional fusion made between the σ^B-dependent *ctc* promoter and *lacZ*. We compared expression of this fusion in two different genetic backgrounds. In strain PB198, the *sigB* operon was wild type. However, in strain PB243, the *sigB* region carried an integrated copy of the pAW18 plasmid, which inserts plasmid sequences between the upstream and downstream halves of the *sigB* operon but which does not disrupt any of the reading frames or promoter regions. We had previously found that this construction prevents transcription originating in the upstream half of the operon (P_A-orfR-orfS-orfT-orfU) from extending into the downstream half (P_B-*rsbV-rsbW-sigB-rsbX*). As shown in Fig. 6, σ^B activity at the *ctc* promoter was the same in both the wild-type strain and the strain containing the integrated plasmid, regardless of whether the cells were grown in unsupplemented LB medium or in LB supplemented with glucose and glutamine. We conclude that the enhanced stationary-phase expression of σ^B-dependent genes in the presence of glucose does not demand that high levels of priming transcript originating at P_A enter the downstream half of the *sigB* operon. This experiment does not rule out the possibility that the priming transcript is physiologically important under other environmental conditions in which the cell becomes depleted for σ^B protein.

Deletion of the upstream half of the *sigB* operon interferes with glucose induction of σ^B-dependent gene expression. The data in Fig. 6 show that expression of σ^B-dependent genes does not require that transcription from P_A enter the downstream half of the operon. Our alternative model to explain how the glucose-inducible P_A might influence σ^B-dependent gene expression assumes that P_A controls the level of a *trans*-acting factor important for σ^B activity. To test this hypothesis, we constructed strain PB288, in which the orfR, orfS, orfT, and orfU reading frames had been deleted and replaced by the *ermC* gene. To facilitate strain construction with the available drug markers, we used the single-copy *ctc-lacZ* translational fusion in an SPβ prophage (25) to monitor σ^B activity in PB288. We then compared *ctc* expression in PB288 with that in strain PB197, which carried the same *ctc-lacZ* translational fusion in SPβ but which was wild type at the *sigB* operon.

As shown in Fig. 7, deletion of the four reading frames in the upstream half of the *sigB* operon significantly reduced *ctc* expression in LB medium supplemented with glucose and glutamine. In contrast, deletion of these four frames increased *ctc* expression three- to fivefold in unsupplemented LB. These results suggest that the region between P_A and P_B has a dual regulatory role: activation of σ^B-dependent gene expression under inducing growth conditions (supplemented LB) and repression of σ^B-dependent gene expression under noninducing growth conditions (unsupplemented LB). Coupled with the results of the experiment shown in Fig. 6, these findings lead us to conclude that it was the loss of the upstream reading frames rather than the loss of transcription from P_A that produced this regulatory effect.

orfU encodes a *trans*-acting factor that is required for full induction of σ^B-dependent genes. A more detailed examination of the regulatory role of the upstream portion of the *sigB* operon requires analysis of mutations within the individual orfR, orfS, orfT, and orfU reading frames. A null orfU mutation was an attractive candidate to begin this process for two reasons. First, the predicted OrfU sequence was similar to that of RsbX, a known regulator of σ^B activity. Second, the experiment shown in Fig. 6 indicated that transcription from P_A into the *rsbV-rsbW-sigB-rsbX* cluster was not required for full expression of σ^B-dependent genes, allowing us to analyze the phenotype of a polar orfU mutation without regard to its effect on transcription of the downstream cluster.

We therefore made a large deletion in orfU and replaced the deleted region with the *ermC* gene, assaying the effects of this alteration by using the same σ^B-dependent *ctc-lacZ* translational fusion employed for the assay of the orfR-orfU deletion shown in Fig. 7. The results shown in Fig. 8 indicate that the dual regulatory consequences we observed following loss of the entire orfR-orfU region could be largely explained by the loss of orfU alone. Compared with strain PB197, which is wild type at the *sigB* locus, expression of the *ctc-lacZ* translational fusion was reduced three- to fourfold when the orfU mutant strain PB291 was grown under inducing conditions (Fig. 8A) and was enhanced three- to fourfold when the mutant cells were grown under noninducing conditions (Fig. 8B).

To determine whether it was the orfU product itself or *cis*-acting sequences removed by the orfU deletion that caused the observed dual regulatory phenotype, we devised a complementation test that supplied a second copy of orfU in *trans* at the *amyE* locus. Because the arrangement of the four upstream reading frames suggested that their expression might be translationally coupled (Fig. 2), we provided the entire upstream region, P_A-orfR-orfS-orfT-orfU, at *amyE*. We then compared

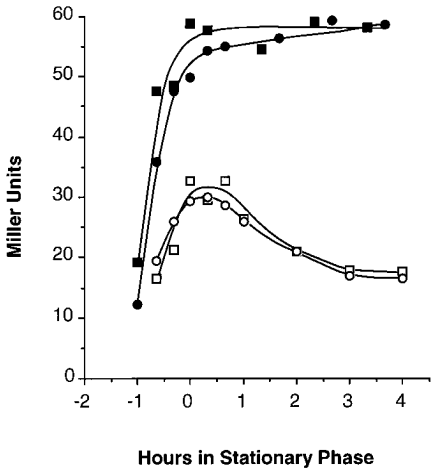

FIG. 4. The upstream promoter P_A is active in the stationary growth phase and is induced in rich medium containing glucose and glutamine. Expression was measured by monitoring β-galactosidase production from single-copy transcriptional fusions. *B. subtilis* strains harboring these fusions were grown in either unsupplemented LB medium or LB medium supplemented with 5% glucose and 0.2% L-glutamine. Samples were removed at the indicated times and assayed for β-galactosidase activity. Symbols represent PB248 (containing the 2.1-kb *Eco*RV fragment fused to *lacZ*) in supplemented (■) and unsupplemented (□) LB and PB252 (containing the 104-bp PCR fragment fused to *lacZ*) in supplemented (●) and unsupplemented (○) LB. Hour 0 is the end of logarithmic growth.

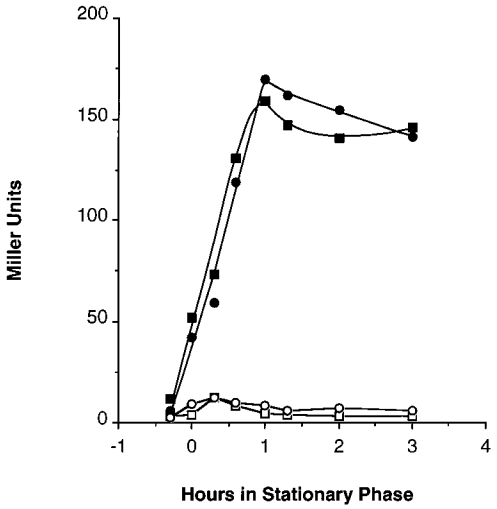

FIG. 6. Transcription of *sigB* from P_A is not required for induction of the σ^B-dependent *ctc-lacZ* transcriptional fusion. Cells were grown and assayed for β-galactosidase activity as described in the legend to Fig. 4. Symbols represent PB198 (*amyE*::*ctc-lacZ*) grown in LB supplemented with glucose and glutamine (■) and in unsupplemented LB (□) and PB243 (*amyE*::*ctc-lacZ rsbV*::pAW18) in supplemented (●) and unsupplemented (○) LB. Integration of pAW18 at the *rsbV* chromosomal locus prevents upstream transcription from reading through the *rsbV-rsbW-sigB-rsbX* cluster.

ctc expression in strain PB293, which carries the *ctc-lacZ* translational fusion in the SPβ prophage, the orfU deletion at the *sigB* locus, and P_A-orfR-orfS-orfT-orfU at the *amyE* locus, with that of strain PB294, which, like PB293, carries the *ctc-lacZ* translational fusion and the orfU deletion but which has only P_A-orfR-orfS-orfT at *amyE*. Thus, PB293 and PB294 each lacked a functional orfU at the *sigB* locus, but in PB293 this was potentially complemented by the orfU copy at *amyE*. As shown in Fig. 8A, orfU was in fact able to complement the orfU deletion in *trans*, increasing *ctc* expression to normal levels under inducing conditions. However, as shown in Fig. 8B, the orfU copy at the *amyE* locus was unable to complement the regulatory defect observed under noninducing conditions, and *ctc* expression remained elevated.

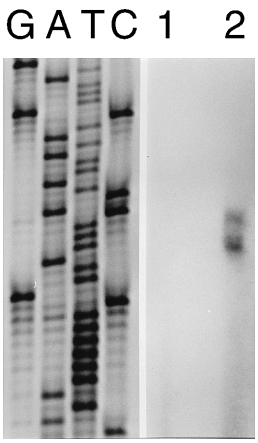

FIG. 5. Mapping the 5′ end of *sigB* operon message by primer extension. Wild-type strain PB2 was grown in either unsupplemented LB medium or LB medium supplemented with 5% glucose and 0.2% glutamine. Cells from both cultures were harvested 30 min after entry into stationary phase, the RNA was extracted, and primer extensions were done with a molar excess of a 21-mer synthetic primer complementary to nt 363 to 383 within the orfR coding region (Fig. 2). Samples containing 25 μg of RNA were loaded onto adjacent lanes of a sequencing gel. Lane 1, RNA from PB2 grown in unsupplemented LB; lane 2, RNA from PB2 grown in supplemented LB. A sequencing ladder was run in parallel, using the same 21-nt primer; the letters G, A, T, and C indicate the dideoxynucleotides used to terminate the reaction. The probable 5′ ends of the *sigB* operon message are indicated on the nontranscribed strand of Fig. 2 as the guanosine complementary to cytidine 312 and the adenosines complementary to thymidines 314 and 315.

Because these complementation experiments were assayed by using a *ctc-lacZ* translational fusion, we also examined the effect of the orfU deletion on two σ^B-dependent transcriptional fusions to ensure that the effect of orfU was in fact exerted at the transcriptional level. As shown in Fig. 9A, the orfU deletion had essentially the same regulatory consequences when it was assayed by using a *ctc-lacZ* transcriptional fusion, although the elevated expression under noninducing conditions was not as pronounced as with the translational fusion. As shown in Fig. 9B, the orfU deletion also had a dramatic effect on glucose induction of the P_B-*lacZ* transcriptional fusion. This latter result clearly shows that P_B itself is a glucose-inducible promoter and is at variance with an earlier report (29). Glucose inducibility of P_B may have been overlooked because the *sigB-lacZ* fusion strain previously used also contained *rsbV* and *rsbW* at the *amyE* locus and was therefore diploid for genes whose products are now known to be potent regulators of σ^B activity (2, 3, 6, 8).

Our transcriptional fusion experiments shown in Fig. 9 establish that the loss of orfU affects the expression of two different σ^B-dependent promoters, the *ctc* promoter and the internal promoter of the *sigB* operon, P_B. Because we had previously found that the loss of orfU had no effect on expression of the σ^A-like upstream promoter of the *sigB* operon, P_A, orfU appears to be specific for σ^B-dependent promoters. From these results we conclude that orfU has a role in the regulation of σ^B activity and that one physiological condition in which this role is manifest is in stationary phase when cells are grown in rich medium containing glucose and glutamine. On the basis of

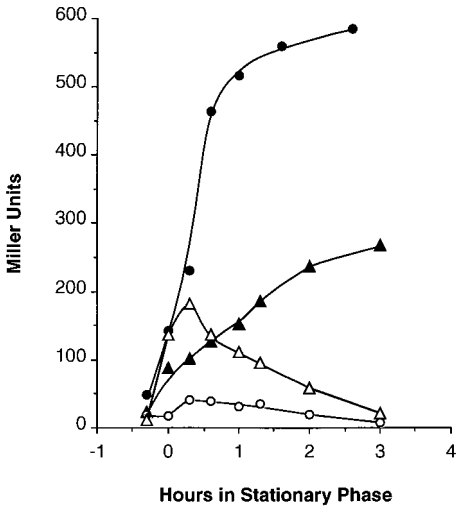

FIG. 7. Deletion of the four upstream open reading frames of the *sigB* operon interferes with glucose induction of the σ^B-dependent *ctc-lacZ* translational fusion. β-Galactosidase activities for PB197 (SPβ*ctc-lacZ*) grown in LB supplemented with glucose and glutamine (●) and in unsupplemented LB (○) and for PB288 [Δ(orfR-*rsbU*)1::*ermC* SPβ*ctc-lacZ*] in supplemented (▲) and unsupplemented (△) LB are shown.

the evident regulatory role of its product, we haved renamed orfU as *rsbU*, where *rsb* stands for regulator of sigma-B.

DISCUSSION

The metabolic signals by which bacteria sense stress and starvation and the signal transduction pathways that channel these signals to the transcription apparatus are only beginning to be understood (15, 21, 23, 27, 33, 39). The transcription factor σ^B of *B. subtilis* controls a set of genes that are induced both upon entry into stationary phase and by a variety of physiological stresses encountered during logarithmic growth (4, 6, 7, 9, 25, 29, 51). Previous genetic analysis indicated that the products of the *rsbV*, *rsbW*, and *rsbX* genes in the *sigB* operon form a hierarchical regulatory pathway that controls σ^B activity in response to stationary-phase signals as well as to signals of salt and ethanol stress (2, 6, 8, 24, 29).

One possible explanation for the response of σ^B to these diverse environmental signals comes from work on σ^F, a sporulation-specific transcription factor which is regulated by homologs to RsbV and RsbW (8, 14, 29, 34, 45). Alper et al. (1) have presented in vitro evidence that these homologs form a sensing system which regulates σ^F activity in response to changes in adenosine nucleotide levels. Consistent with this hypothesis, σ^F activity was modestly induced when an uncoupler of oxidative phosphorylation was added to growing cells. Significantly, Alper and colleagues also found that σ^B activity was strongly induced in a similar uncoupler experiment and that this induction was entirely dependent on the RsbV positive regulator which is required for the transmission of some stress signals to σ^B (6). It is therefore attractive to consider that the RsbV-RsbW proteins might also control σ^B activity in response to adenosine nucleotide levels, which could serve as the metabolic sensor of a variety of growth-limiting conditions, such as the entry into stationary phase or salt stress (1).

Based on the results presented here, it is clear that the RsbV-RsbW proteins are not the only regulators involved in sensing the critical transition to a growth-limiting condition. We have shown that the *sigB* operon contains four additional genes that lie upstream from the previously identified *rsbV-rsbW-sigB-rsbX* cluster and that one of these genes, *rsbU*, has a dual regulatory role in controlling σ^B activity at the onset of the stationary growth phase. In the presence of glucose and glutamine, loss of the *rsbU* reading frame led to decreased σ^B-dependent gene expression. This mutant phenotype could be complemented in *trans*, suggesting that RsbU is a positive factor that controls σ^B in response to glucose and glutamine. In contrast, in the absence of glucose and glutamine, loss of the *rsbU* reading frame led to increased σ^B-dependent gene expression. This phenotype could not be complemented, suggesting that the *rsbU* deletion removes a *cis*-acting negative regulatory site. An alternative interpretation of the complementation data is that RsbU must be translated near its target in order to negatively regulate σ^B in medium lacking glucose and glutamine. This target could be either a nucleic acid binding site or an interacting protein factor.

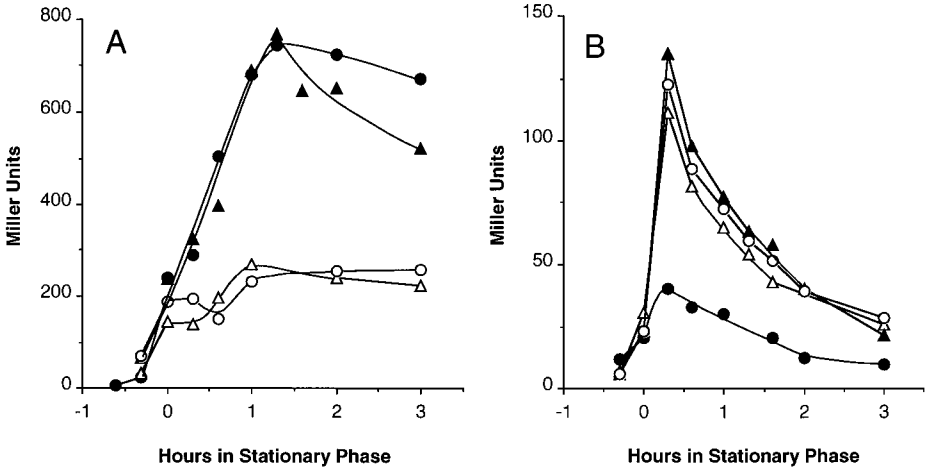

FIG. 8. Deletion of *rsbU* interferes with glucose induction of the σ^B-dependent *ctc-lacZ* translational fusion. (A) β-Galactosidase activities of fusion strains grown in LB supplemented with glucose and glutamine; (B) activities of strains grown in unsupplemented LB. ●, PB197(SPβ*ctc-lacZ*); △, PB291 (SPβ*ctc-lacZ* Δ*rsbU1*::*ermC*); ▲, PB293 [SPβ*ctc-lacZ* Δ*rsbU1*::*ermC amyE*::(P_A-orfR-orfS-orfT-*rsbU*)]; ○, PB294 [SPβ*ctc-lacZ* Δ*rsbU1*::*ermC amyE*::(P_A-orfR-orfS-orfT)].

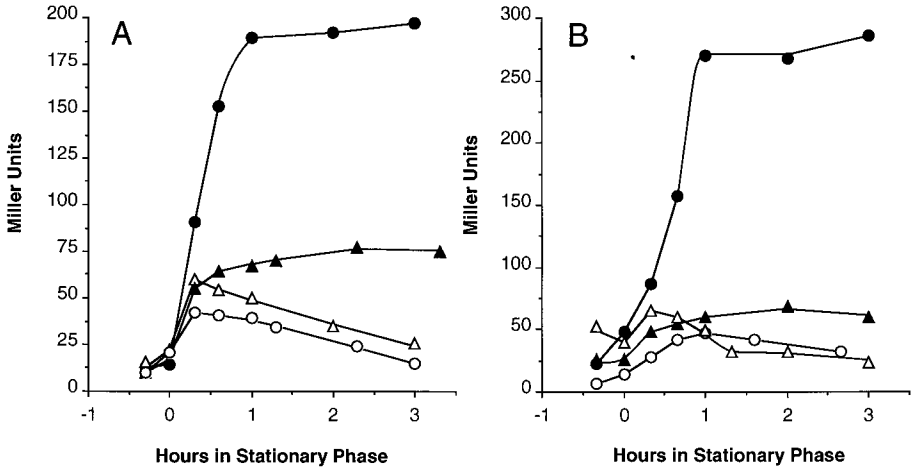

FIG. 9. Deletion of *rsbU* interferes with glucose induction of the σ^B-dependent *ctc-lacZ* and P_B-*lacZ* transcriptional fusions. (A) β-Galactosidase activities of PB198 (*amyE*::*ctc-lacZ*) grown in LB supplemented with glucose and glutamine (●) and in unsupplemented LB (○) and of PB244 (*amyE*::*ctc-lacZ* Δ*rsbU1*::*ermC*) in supplemented (▲) and unsupplemented (△) LB; (B) activities of PB286 (*amyE*::P_B-*lacZ*) in supplemented (●) and unsupplemented (○) LB and of PB296 (*amyE*::P_B-*lacZ* Δ*rsbU1*::*ermC*) in supplemented (▲) and unsupplemented (△) LB.

We have also extended the paradigm of a dual regulatory role for *rsbU* to include exponentially growing cells subjected to salt stress, one of the general stress conditions that activate σ^B (6, 51). Under conditions of salt stress, expression of a σ^B-dependent *ctc* fusion was reduced 10-fold in an *rsbU* null strain compared with the wild type (30). In contrast, in the absence of this stress, the basal level of *ctc* expression was enhanced fivefold in the *rsbU* mutant. The phenotype observed in salt-stressed cells parallels the effect of an *rsbU* null mutation in the experiments reported here, which use the presence of glucose and glutamine in the medium as an inducer. That is, loss of *rsbU* function decreases *ctc* expression under inducing conditions and increases expression under noninducing conditions. It was originally proposed that the addition of glucose and glutamine provides a nutritional signal that increases σ^B-dependent gene expression as cells enter the stationary growth phase (24, 25). However, our results for salt-stressed cells raise the possibility that high levels of glucose and glutamine may provide an osmotic rather than a nutritional signal (30) and that RsbU is involved in the transmission of general stress signals to σ^B.

FIG. 10. Model of the σ^B regulatory pathway. The products of the downstream five genes in the *sigB* operon are depicted in the order in which their structural genes appear in the operon. Transcription of the RsbU structural gene depends on the P_A promoter, which lies well upstream, whereas transcription of the RsbV-RsbW-σ^B-RsbX gene cluster is augmented by the autoregulated, σ^B-dependent P_B promoter, which lies just upstream from RsbV (see Fig. 1). RsbW is an anti-σ factor which binds directly to σ^B and prevents its association with RNA polymerase core; RsbV is an anti-anti-σ factor which counters the action of RsbW (2, 3, 8, 13). By analogy to the similar SpoIIAA-SpoIIAB system that regulates σ^F, the RsbV-RsbW system is thought to respond to changes in adenosine nucleotide levels (1). The RsbU positive regulator described here is also thought to control σ^B primarily at the level of activity, and this positive function is modulated by the negative action of the RsbX regulator (50). RsbX therefore provides a link between the two transcriptionally distinct halves of the *sigB* operon. Because an *rsbU* null mutant manifests opposite phenotypes under inducing and noninducing conditions (Fig. 8), we speculate that RsbU acts by potentiating the action of the RsbV-RsbW system.

A possible model to explain the dual phenotype of a *rsbU* null mutant is that RsbU serves to potentiate the action of the RsbV-RsbW system, sharpening its discrimination as cells encounter growth-limiting conditions. For example, RsbU could provide input from another type of signal in order to augment an adenosine signal from the RsbV-RsbW pair. How might this signal enter the σ^B system? Previous work established that RsbX is a negative regulator of σ^B whose effect is subordinate to the RsbV-RsbW system (2, 8, 24, 29). In an extension of these results, Voelker et al. (50) have shown that RsbX exerts its negative effect on σ^B by directly or indirectly modulating the positive control function of RsbU. A model of a σ^B regulatory pathway that is consistent with the available genetic and biochemical data is shown in Fig. 10.

Within the context of this model, there are two broad mechanisms by which RsbU might channel general stress signals to σ^B, and these mechanisms are not mutually exclusive. First, RsbU itself could sense and convey the signal, perhaps in conjunction with RsbX. Although the molecular mechanism by which RsbU and RsbX might transduce such a signal remains speculative, on the basis of the significant sequence similarity between them, we imagine that RsbU and RsbX interact with a common factor, which could be a protein, a small metabolite, or a specific nucleic acid sequence. A second, equally plausible mechanism relies on the unique transcriptional organization of the eight-gene *sigB* operon, in which expression of the upstream orfR-orfS-orfT-*rsbU* genes is controlled by the σ^B-independent promoter P_A and expression of the downstream *rsbV-rsbW-sigB-rsbX* genes is additionally autoregulated by the internal, σ^B-dependent promoter P_B. Although the RsbX regulator establishes an internal link between the two halves of the operon (Fig. 10), the P_A promoter may allow RsbU levels to be regulated differentially from the levels of the downstream gene products. A signal entering the system via P_A could therefore alter the balance of RsbU relative to other regulatory factors, such as RsbX.

In contrast to the clear regulatory role of RsbU, the physiological roles of the other three reading frames in the upstream half of the *sigB* operon have yet to be determined. Under the stationary-phase growth conditions tested here, the absence of all four upstream reading frames produced essentially the same phenotype as the loss of *rsbU* alone. Therefore,

if the orfR, orfS, and orfT reading frames are involved in σ^B regulation under such conditions, their effect is masked by the loss of *rsbU*. On the basis of the similarity of the orfS and orfT products to other regulators of σ factor activity, we speculate that these upstream genes may be responsible for sensing signals distinct from those detected by the RsbV-RsbW pair and that RsbU is the agent by which these signals are transmitted to σ^B. The emerging complexity of the σ^B regulatory network may therefore provide a means of integrating multiple environmental and metabolic signals to effect activation of a single transcription factor.

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