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Isolation and Characterization of Dominant Mutations in the *Bacillus subtilis* Stressosome Components RsbR and RsbS[▽]

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The general stress response of *Bacillus subtilis* is controlled by the activity state of the σ^B transcription factor. Physical stress is communicated to σ^B via a large-molecular-mass (>10^6-Da) structure (the stressosome) formed by one or more members of a family of homologous proteins (RsbR, YkoB, YojH, YqhA). The positive regulator (RsbT) of the σ^B stress induction pathway is incorporated into the complex bound to an inhibitor protein (RsbS). Exposure to stress empowers an RsbT-dependent phosphorylation of RsbR and RsbS, with the subsequent release of RsbT to activate downstream processes. The mechanism by which stress initiates these reactions is unknown. In an attempt to identify changes in stressosome components that could lead to σ^B activation, a DNA segment encoding these proteins was mutagenized and placed into *B. subtilis* to create a merodiploid strain for these genes. Eight mutations that allowed heightened σ^B activity in the presence of their wild-type counterparts were isolated. Two of the mutations are missense changes in *rsbR*, and six are amino acid changes in *rsbS*. Additional experiments suggested that both of the *rsbR* mutations and three of the *rsbS* mutations likely enhance σ^B activity by elevating the level of RsbS phosphorylation. All of the mutations were found to be dominant over wild-type alleles only when they are cotranscribed within an *rsbR rsbS rsbT* operon. The data suggest that changes in RsbR can initiate the downstream events that lead to σ^B activation and that RsbR, RsbS, and RsbT likely interact with each other concomitantly with their synthesis.

The general stress regulon of *Bacillus subtilis* consists of over 200 genes whose products confer resistance to multiple forms of stress (18, 27, 28, 33). The general stress regulon is controlled by the activity state of σ^B, a stress-activated secondary sigma factor (6–8) (Fig. 1). In the absence of stress, σ^B is held inactive, complexed with the anti-σ^B protein RsbW (5). σ^B is released from RsbW when a second protein (RsbV) binds to RsbW in lieu of σ^B (13, 14). RsbV is unable to catalyze σ^B release in unstressed *B. subtilis* due to an inactivating RsbW-dependent phosphorylation (14). The dephosphorylation and reactivation of RsbV-P is catalyzed by either of two stress-responsive phosphatases (RsbP and RsbU) (20, 32, 36). The RsbP phosphatase responds to nutritional stress (glucose or PO_4 starvation or azide treatment), while RsbU is activated by physical stress (heat shock, osmotic shock, or ethanol) (1, 20, 32, 38, 40, 41). RsbP is cotranscribed with an additional protein (RsbQ) that is needed for its activity (9, 32). The metabolic inducer of RsbP/RsbQ is unknown, but RsbP/RsbQ activation occurs coincidently with a drop in cellular ATP levels (43, 44).

The physical stress phosphatase (RsbU) also requires an additional protein (RsbT) for activity (39, 41). In the absence of stress, RsbT is unavailable to RsbU, bound to an inhibitory protein (RsbS) in large multiprotein complexes (>10^6 Da) termed "stressosomes" (11, 24). The stressosome incorporates RsbS and RsbT in a structure formed from one or more members of a family of homologous proteins (RsbR, YkoB, YojH, YqhA) (2, 11, 24). The RsbR proteins are needed for proper interaction between RsbS and RsbT (1, 2). In their absence, RsbS is unable to inhibit the RsbT-dependent activation of RsbU. The specific roles of each of the RsbR family members are unknown, but their functions appear to be at least partially redundant (2, 24). The loss of any one RsbR family member does not significantly affect the activity of σ^B. Only when multiple members of the RsbR family are lost is the ability of RsbS to inhibit RsbT compromised (24).

Exposure to physical stress triggers RsbT to phosphorylate both RsbR and RsbS (23, 41). Phosphorylation of RsbS is the key event that allows RsbT access to RsbU. Biochemical evidence suggests that the phosphorylation of RsbR, and presumably its paralogs, facilitates the subsequent phosphorylation of RsbS by RsbT (11, 24). This result suggests a sequential series of events in which stress triggers the phosphorylation of RsbR as a prerequisite for the phosphorylation of RsbS (11, 24). Assuming that this progression occurs in vivo, both RsbR and RsbT represent plausible targets for stress-dependent changes that could trigger σ^B activation. Presumably, stress-induced factors could initiate changes in RsbR to make it more amenable to phosphorylation or alter RsbT in ways that empower its kinase activity.

σ^B activity levels elevated by the physical stress pathway are restored to prestress levels by RsbX, a phosphatase that dephosphorylates and reactivates both RsbR and RsbS. This allows RsbT to again be sequestered in an inactivating complex (10, 41). RsbX levels increase following σ^B activation; however, it is not clear whether the increase in RsbX per se is responsible for the reduction in σ^B activity (15). Artificial manipulation of RsbX levels from an inducible promoter has little effect on σ^B inducibility (35). Only when RsbX is eliminated does σ^B activity rise to very high levels (4, 8, 35). The question

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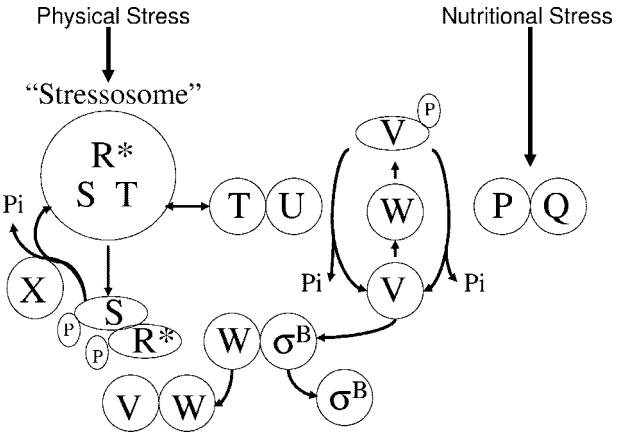


FIG. 1. Model of σ^B control. As depicted in the lower portion of the diagram, σ^B is normally inactive, complexed with the anti-σ^B protein RsbW (W). σ^B is freed from RsbW when a release factor, RsbV (V) binds RsbW in lieu of σ^B. In the absence of stress, RsbV is unable to trigger σ^B release due to an RsbW-catalyzed phosphorylation. RsbV is dephosphorylated by one of two stress-responsive phosphatases that uniquely respond to physical or nutritional stress. The nutritional stress phosphatase, RsbP (P), requires a coexpressed protein, RsbQ (Q), for activity. The RsbQ/RsbP trigger is unknown, but its activation coincides with conditions that cause a drop in ATP. The physical stress phosphatase, RsbU (U), is activated by a second protein, RsbT (T), that is ordinarily bound to a negative regulator, RsbS (S), in a large (>10^6-Da) complex (stressosome) formed from RsbR and a family of paralogous proteins (R*). Following exposure to physical stress, an unknown mechanism allows RsbR and RsbS to become phosphorylated by RsbT. This frees RsbT to activate the RsbU phosphatase. RsbR-P and RsbS-P are dephosphorylated and reactivated by RsbX (X), a phosphatase whose levels increase following σ^B activation. This model is based on references given in the text.

of whether the inherent activity of RsbX is regulated and, if so, what role this might play in σ^B induction is unresolved.

The components of the physical stress pathway are encoded by the first four genes of the eight-gene *sigB* operon (39). The operon appears to be constitutively expressed from a promoter (P_A) that is likely recognized by the cell's housekeeping sigma factor (σ^A), with a σ^B-dependent promoter (P_B) within the operon to upregulate the downstream four genes when σ^B becomes active (i.e., P_A *rsbR rsbS rsbT rsbU*, P_B *rsbV rsbW sigB rsbX*) (8, 15, 39). In an attempt to identify changes in stressosome components that could lead to σ^B activation, a DNA segment encoding RsbR, RsbS, RsbT, and RsbU along with the P_A promoter element was mutagenized and transferred into *B. subtilis* in such a way as to create merodiploid strains with two copies of this region. Eight mutations that allowed heightened σ^B activity in the presence of wild-type copies of the stress pathway genes were isolated. Two of the mutations were missense changes in *rsbR*, and six were amino acid changes in *rsbS*. Additional experiments suggest that both *rsbR* mutations and three of the six *rsbS* mutations enhance σ^B activity by allowing a higher background level of RsbS phosphorylation.

MATERIALS AND METHODS

Bacterial strains and plasmids. All of the *B. subtilis* strains (Table 1) are derivatives of PY22. BSJ43 is BSA46 (4) transformed with a linearized *Escherichia coli* plasmid carrying the 1.2-kbp *rsbP* gene plus the *spc* cassette from pDG1726 (17) inserted into the unique HindIII site of *rsbP*. Plasmid pARE7 contains the 2.8-kbp DNA segment (P_A *rsbR rsbS rsbT rsbU*) of pRU13 (31), cloned as a BamHI/SphI fragment into pUK19 (19). The cloned P_A *rsbR rsbS rsbT rsbU* DNA was mutagenized in *E. coli* by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, as described by Autret et al. (3). *B. subtilis* strains BAR6 and its variants (BAR6-5 to BAR6-13) are BSJ43 (*rsbP*::*spc*) transformed with untreated or mutagenized (mut) pARE7 and selected for the plasmid-encoded antibiotic resistance. These strains carry integrated plasmids and are merodiploid for P_A *rsbR rsbS rsbT rsbU*.

To obtain *B. subtilis* strains in which the mutant *rsb* alleles are present in single copy (i.e., BAR11 to BAR15, BARM1, BARM15, BARM24, and BARM42), each of the two P_A *rsbR rsbU* regions of the mutant strains was separately amplified with oligonucleotides that hybridize to chromosomal sequences outside of those contained in the originally cloned *B. subtilis* DNA paired with oligonucleotides that hybridize to the vector sequence at either side of the cloned DNA (i.e., the M13 Fwd and Rev sequencing primers). The amplified DNAs were separately transformed into *B. subtilis* strain BSA46 (*ctc*::*lacZ*) (4) in a congression with chromosomal DNA from BSJ43 (*rsbP*::*spc ctc*::*lacZ*). Spc^r clones were screened for the mutant phenotype (i.e., blue colony color) on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Chromosomal DNAs from presumptive mutant strains were analyzed by PCR to verify the presence of the P_A *rsbR rsbS rsbT rsbU* region in single copy. The P_A *rsbR rsbS rsbT rsbU* elements carrying the mutant alleles were cloned into pCR2.1-T0P0 (Invitrogen, Carlsbad, CA) to create plasmids pARE30 to pARE65.

To construct strains in which the mutant *rsbR* or *rsbS* genes were expressed as P_A *rsbR* or P_A *rsbR rsbS* transcription units separately from the other genes of the *sigB* operon (i.e., BAR48, BAR50, BAR54, BAR59, BAR61, BAR62, BAR63, BAR68, and BAR70), DNA segments encoding these alleles were amplified from chromosomal DNA of *B. subtilis* strains that carry them in single-copy *sigB* operons. An oligonucleotide primer that hybridized 156 bp upstream of *rsbR* was used in conjunction with one that hybridized either 42 bp downstream of *rsbR* or 31 bp downstream of *rsbS* to amplify P_A *rsbR* or P_A *rsbR rsbS*, respectively. The amplified DNAs were cloned into pUK19 as BamHI/SphI DNA fragments. The resulting plasmids (pARE83 to pARE109) were then transformed into BSJ43 (*rsbP*::*spc* SPβ *ctc*::*lacZ*). Clones with plasmid integrations were selected on the basis of the plasmid-encoded Kan^r. Depending on the site within the P_A *rsbR* or P_A *rsbR rsbS* elements at which recombination with the *sigB* operon occurred, the mutant *rsbR* or *rsbS* alleles would either be expressed separately from the *sigB* operon or be exchanged for their wild-type counterparts within the *sigB* operon. When plated on media with X-Gal, two colony types were evident. One had the blue colony color characteristic of the parental mutant strain, while the other had the white phenotype of BSJ43. Chromosomal DNA was extracted from clones of each colony type. The P_A *rsbR* or P_A *rsbR rsbS* regions, both upstream (separate from the *sigB* operon) and downstream (within the *sigB* operon) of the integrated vector, were separately amplified with oligonucleotide primers that hybridized either 456 bp upstream of *rsbR* or 399 bp downstream of *rsbS* in concert with the M13 Fwd and Rev sequencing primers. This allowed amplification of the *rsbR rsbS* regions that were either upstream or within the *sigB* operon, respectively. The identity of the *rsbR* or *rsbS* allele in each of these sites was determined by DNA sequencing. In each case, the chromosomal DNA from the blue (mutant phenotype) colonies had a mutant *rsbR* or *rsbS* allele as part of their *sigB* operons, while the white (parental recipient phenotype) colonies contained wild-type *sigB* operons with the mutant alleles within the DNA element upstream of the integrated plasmid.

DNA segments in which *rsbR*(*171TA*) or *rsbS*(*59SA*) mutations were added to the previously isolated *rsbR* and *rsbS* mutations were constructed by site-directed mutagenesis (Gene Tailor; Invitrogen, Carlsbad, CA). The P_A *rsbR rsbS rsbT rsbU* regions, containing either the wild-type or mutant *rsbR* or *rsbS* alleles previously cloned in pUC19 (i.e., pARE30 to pARE65), were cut from these plasmids with BamHI/SphI and cloned downstream of the Kan^r gene in pUR1. pUR1 carries 520 bp of chromosomal DNA from the region immediately upstream of the *sigB* operon. This DNA is inserted next to the plasmid's Kan^r gene opposite the side at which the *rsbR rsbS rsbT rsbU* element is cloned. The resulting plasmids were mutagenized in vitro with oligonucleotides that would specifically add the *rsbR*(*171TA*) or *rsbS*(*59SA*) mutations. The presence of the desired changes in the mutagenized plasmid DNAs was determined by DNA sequencing. Representative plasmids (pARE114 to pARE142) were linearized with ScaI and transformed into BSJ43 (*rsbP*::*spc* SPβ *ctc*::*lacZ*). Kan^r clones arise by a double recombination between the homologous DNAs on both sides of the *kan* gene. The proximity of the *rsbR* and *rsbS* genes to the *kan* gene favored incorporation of the mutant alleles into the chromosomes of the transformants. Allelic replacement was verified by amplification of the region from the trans-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or features	Source, reference, or construction
Plasmids		
pUK19	Ap^r Kan^r	19
pUR1	Ap^r Kan^r 520 bp upstream of *sigB*	31
pARE7	Ap^r Kan^r P_A *rsbR rsbS rsbT rsbU*	This study
pARE30	Ap^r P_A *rsbR rsbS*(*66GR*) *rsbT rsbU*	This study
pARE31	Ap^r P_A *rsbR*(*225CY*) *rsbS rsbT rsbU*	This study
pARE33	Ap^r P_A *rsbR rsbS*(*22ER*) *rsbT rsbU*	This study
pARE34	Ap^r P_A *rsbR*(*136EK*) *rsbS rsbT rsbU*	This study
pARE41	Ap^r P_A *rsbR rsbS*(*83GD*) *rsbT rsbU*	This study
pARE42	Ap^r P_A *rsbR rsbS*(*23LF*) *rsbT rsbU*	This study
pARE43	Ap^r P_A *rsbR rsbS*(*76GR*) *rsbT rsbU*	This study
pARE65	Ap^r P_A *rsbR rsbS*(*86PL*) *rsbT rsbU*	This study
pARE83	Ap^r Kan^r P_A *rsbR*(*225CY*) *rsbS*	This study
pARE85	Ap^r Kan^r P_A *rsbR rsbS*(*66GR*)	This study
pARE86	Ap^r Kan^r P_A *rsbR rsbS*(*22EK*)	This study
pARE88	Ap^r Kan^r P_A *rsbR rsbS*(*76GR*)	This study
pARE89	Ap^r Kan^r P_A *rsbR*	This study
pARE91	Ap^r Kan^r P_A *rsbR rsbS*(*86PL*)	This study
pARE92	Ap^r Kan^r P_A *rsbR rsbS*(*23LF*)	This study
pARE93	Ap^r Kan^r P_A *rsbR*(*136EK*)	This study
pARE106	Ap^r Kan^r P_A *rsbR rsbS*(*83GD*)	This study
pARE109	Ap^r Kan^r P_A *rsbR rsbS*	This study
pARE114	Ap^r Kan^r pUR1::P_A *rsbR rsbS*(*22ER 59SA*) *rsbT rsbU*	This study
pARE116	Ap^r Kan^r pUR1::P_A *rsbR rsbS*(*86PL 59SA*) *rsbT rsbU*	This study
pARE122	Ap^r Kan^r pUR1::P_A *rsbR*(*136EK*) *rsbS*(*59SA*) *rsbT rsbU*	This study
pARE123	Ap^r Kan^r pUR1::P_A *rsbR*(*136EK 171TA*) *rsbS rsbT rsbU*	This study
pARE124	Ap^r Kan^r pUR1::P_A *rsbR*(*225CY*) *rsbS*(*59SA*) *rsbT rsbU*	This study
pARE125	Ap^r Kan^r pUR1::P_A *rsbR*(*225CY 171TA*) *rsbS rsbT rsbU*	This study
pARE126	Ap^r Kan^r pUR1::P_A *rsbR rsbS*(*66GR 59SA*) *rsbT rsbU*	This study
pARE129	Ap^r Kan^r pUR1::P_A *rsbR rsbS*(*83GD 59SA*) *rsbT rsbU*	This study
pARE131	Ap^r Kan^r pUR1::P_A *rsbR rsbS*(*76GR 59SA*) *rsbT rsbU*	This study
pARE132	Ap^r Kan^r pUR1::P_A *rsbR*(*171TA*) *rsbS rsbT rsbU*	This study
pARE133	Ap^r Kan^r pUR1::P_A *rsbR rsbS*(*59SA*) *rsbT rsbU*	This study
pARE142	Ap^r Kan^r pUR1::P_A *rsbR rsbS*(*23LF 59SA*) *rsbT rsbU*	This study
B. subtilis strains		
PY22	*trpC2*	4
BSA46	*trpC2* SPβ *ctc*::*lacZ*	4
BSJ43	*trpC2 rsbP*::*spc*	J. Scott
BAR6	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS rsbT rsbU* SPβ *ctc*::*lacZ*	pARE7-BSJ43
BAR6#5	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS*(*83GD*) *rsbT rsbU* SPβ *ctc*::*lacZ*	mutpARE7-BSJ43
BAR6#6	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS*(*23LF*) *rsbT rsbU* SPβ *ctc*::*lacZ*	mutpARE7-BSJ43
BAR6#7	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS*(*76GR*) *rsbT rsbU* SPβ *ctc*::*lacZ*	mutpARE7-BSJ43
BAR6#8	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS*(*86PL*) *rsbT rsbU* SPβ *ctc*::*lacZ*	mutpARE7-BSJ43
BAR6#9	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS*(*22ER*) *rsbT rsbU* SPβ *ctc*::*lacZ*	mutpARE7-BSJ43
BAR6#10	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR*(*136EK*) *rsbS rsbT rsbU* SPβ *ctc*::*lacZ*	mutpARE7-BSJ43
BAR6#11	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS*(*66GR*) *rsbT rsbU* SPβ *ctc*::*lacZ*	mutpARE7-BSJ43
BAR6#13	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR*(*225CY*) *rsbS rsbT rsbU* SPβ *ctc*::*lacZ*	mutpARE7-BSJ43
BAR11	*trpC2 rsbP*::*spc rsbS*(*22ER*) SPβ *ctc*::*lacZ*	BAR6#9-BSA46
BAR12	*trpC2 rsbP*::*spc rsbR*(*136EK*) SPβ *ctc*::*lacZ*	BAR6#10-BSA46
BAR14	*trpC2 rsbP*::*spc rsbS*(*66GR*) SPβ *ctc*::*lacZ*	BAR6#11-BSA46
BAR15	*trpC2 rsbP*::*spc rsbR*(*225CY*) SPβ *ctc*::*lacZ*	BAR6#13-BSA46
BARM1	*trpC2 rsbP*::*spc rsbS*(*83GD*) SPβ *ctc*::*lacZ*	BAR6#5-BSA46
BARM15	*trpC2 rsbP*::*spc rsbS*(*23LF*) SPβ *ctc*::*lacZ*	BAR6#6-BSA46
BARM24	*trpC2 rsbP*::*spc rsbS*(*76GR*) SPβ *ctc*::*lacZ*	BAR6#7-BSA46
BAR42	*trpC2 rsbP*::*spc rsbS*(*86PL*) SPβ *ctc*::*lacZ*	BAR6#8-BSA46
BAR48	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR*(*225CY*) SPβ *ctc*::*lacZ*	pARE83-BSJ43
BAR50	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS*(*83GD*) SPβ *ctc*::*lacZ*	pARE106-BSJ43
BAR54	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS*(*66GR*) SPβ *ctc*::*lacZ*	pARE85-BSJ43
BAR59	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS*(*86PL*) SPβ *ctc*::*lacZ*	pARE91-BSJ43
BAR61	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR* SPβ *ctc*::*lacZ*	pARE89-BSJ43
BAR62	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS*(*23LF*) SPβ *ctc*::*lacZ*	pARE92-BSJ43
BAR63	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS*(*76GR*) SPβ *ctc*::*lacZ*	pARE88-BSJ43
BAR68	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR*(*136EK*) SPβ *ctc*::*lacZ*	pARE93-BSJ43
BAR70	*trpC2 rsbP*::*spc aph3′5″*/P_A *rsbR rsbS* SPβ *ctc*::*lacZ*	pARE109-BSJ43
BAR85	*trpC2 rsbP*::*spc aph3′5″*/*rsbS*(*76GR 59SA*) SPβ *ctc*::*lacZ*	pARE131-BSJ43
BAR87	*trpC2 rsbP*::*spc aph3′5″*/*rsbS*(*22ER 59SA*) SPβ *ctc*::*lacZ*	pARE114-BSJ43

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant genotype or features	Source, reference, or construction
BAR89	*trpC2 rsbP*::*spc aph3′5″ rsbS*(*86PL 59SA*) SPβ *ctc*::*lacZ*	pARE116-BSJ43
BAR90	*trpC2 rsbP*::*spc aph3′5″*/*rsbR*(*136EK*) *rsbS*(*59SA*) SPβ *ctc*::*lacZ*	pARE122-BSJ43
BAR91	*trpC2 rsbP*::*spc aph3′5″*/*rsbR*(*136EK 171TA*) SPβ *ctc*::*lacZ*	pARE123-BSJ43
BAR92	*trpC2 rsbP*::*spc aph3′5″*/*rsbS*(*83GD 59SA*) SPβ *ctc*::*lacZ*	pARE129-BSJ43
BAR93	*trpC2 rsbP*::*spc aph3′5″*/*rsbS*(*66GR 59SA*) SPβ *ctc*::*lacZ*	pARE126-BSJ43
BAR95	*trpC2 rsbP*::*spc aph3′5″*/*rsbS*(*59SA*) SPβ *ctc*::*lacZ*	pARE133-BSJ43
BAR97	*trpC2 rsbP*::*spc aph3′5″*/*rsbR*(*171TA*) SPβ *ctc*::*lacZ*	pARE132-BSJ43
BAR99	*trpC2 rsbP*::*spc aph3′5″*/*rsbR*(*225CY 171TA*) SPβ *ctc*::*lacZ*	pARE125-BSJ43
BAR100	*trpC2 rsbP*::*spc aph3′5″*/*rsbR*(*225CY*) *rsbS*(*59SA*) SPβ *ctc*::*lacZ*	pARE124-BSJ43
BAR104	*trpC2 rsbP*::*spc aph3′5″*/*rsbR rsbS*(*23LF 59SA*) SPβ *ctc*::*lacZ*	pARE142-BSJ43
E. coli strains		
ARE7	P_A *rsbR rsbS rsbT rsbU*	pARE7-DH5α
ARE31	P_A *rsbR*(*225CY*) *rsbS rsbT rsbU*	pARE31-DH5α
ARE34	P_A *rsbR*(*136EK*) *rsbS rsbT rsbU*	pARE34-DH5α

formant clones' chromosomal DNA with primers that hybridized 456 bp upstream of *rsbR* and 31 bp downstream of *rsbS*, followed by DNA sequencing.

Gel filtration analysis. Gel filtration was performed as previously described (25). One-liter *Escherichia coli* cultures expressing the desired *rsb* alleles were grown to a mid-logarithmic stage (optical density at 540 nm [OD_{540}], 0.5), quickly chilled by the addition of ice, and harvested by centrifugation. Cells were washed, resuspended in 5 ml of a low-salt buffer (10 mM Tris [pH 8.0], 50 μM EDTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.03% phenylmethylsulfonyl fluoride), and disrupted with a French pressure cell. Cell debris was removed by centrifugation (5,000 × *g* for 10 min) at 4°C. Two milliliters of the supernatant was loaded onto a 120-ml Sephacryl S-300 column. Five-milliliter fractions were collected and precipitated with two volumes of ethanol for analysis by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (25).

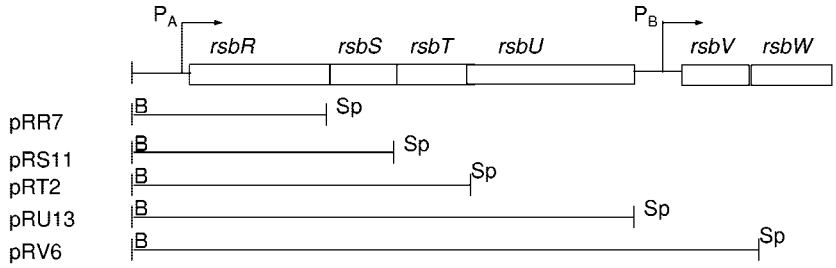
General methods. *B. subtilis* was grown with shaking in Luria-Bertani (LB) medium (29). Physical stress was imposed by the addition of ethanol (4% final concentration) to logarithmically growing cultures. β-Galactosidase assays were performed with chloroform-permeabilized cells, as described by Kenny and Moran (22). Western blot assays were undertaken as previously described with mouse monoclonal antibodies against RsbR and RsbS (15). *B. subtilis* transformation was carried out by the method of Yasbin et al. (42).

RESULTS

Isolation and identification of mutations in the physical stress pathway. The four principal components (RsbR, RsbS, RsbT, and RsbU) of the σ^B physical stress activation pathway are encoded by the first four genes of the *sigB* operon (39). We sought to identify changes in these proteins that lead to σ^B activation in the absence of stress. Such changes could offer clues into the properties of these proteins and the ways in which exposure to stress might alter them. The stress pathway components include both negative (RsbR, RsbS) and positive (RsbT, RsbU) regulators. To minimize activation of σ^B due to loss-of-function mutations in the negative regulators, the experiment was conducted with an *rsbR rsbS rsbT rsbU* merodiploid strain. The use of such a strain was anticipated to favor the isolation of dominant mutations that constitutively activate the σ^B physical stress pathway.

A DNA fragment containing the coding sequence for *rsbR*, *rsbS*, *rsbT*, and *rsbU* along with their normal promoter (P_A) was mutagenized and transformed into *B. subtilis* on an integrating plasmid vector. The resulting transformants contain two expressed copies of each of the four *rsb* genes. The recipient strain also carries a reporter gene (*lacZ*) under the control of a σ^B-dependent promoter (P_{ctc}) and a null mutation in the regulatory phosphatase (RsbP) required to activate σ^B in response to nutritional stress. Transformant clones that form blue colonies on media containing X-Gal are likely to include variant *B. subtilis* with dominant mutations in components in the *rsbR rsbS rsbT rsbU* element that allow σ^B to be active in the absence of stress and the presence of wild-type *rsbR rsbS rsbT rsbU* alleles. Eight clones with distinct blue colony phenotypes were selected for further study. DNA was extracted from these clones and transformed into a naïve $RsbP^-$ P_{ctc}::*lacZ* strain to verify linkage of the σ^B activation phenotype (i.e., blue colony color on X-Gal medium) with the antibiotic resistance of the integrated vector. Once the linkage was verified, we created strains in which the *rsbR rsbS rsbT rsbU* region and the mutant *rsb* alleles are present in single copy. To accomplish this, each of the two individual *rsbR rsbS rsbT rsbU* regions of the merodiploid strains was independently amplified from chromosomal DNA with oligonucleotide primers that specifically hybridize to the *rsbR rsbS rsbT rsbU* region that lies either upstream or downstream of the integrated plasmid vector. The amplified DNAs were separately transformed into *B. subtilis* by a DNA congression technique. Chromosomal DNA from a *B. subtilis* parental strain containing an *rsbP*::*spc* disruption was added to each of the PCR-amplified DNAs. The mixtures were then transformed into wild-type *B. subtilis* that carried the P_{ctc}::*lacZ* reporter system. Transformants selected on the basis of Spc^r were screened for the blue colony phenotype associated with heightened σ^B activity. As anticipated, only one of the two amplified DNAs yielded transformants that included clones with heightened β-galactosidase activity. PCR analysis of chromosomal DNA from representative clones verified the presence of a single copy of the *rsbR rsbS rsbT rsbU* region in these strains (data not shown).

A series of mapping plasmids (31) was then used to localize the position of *rsb* mutations within the *rsbR rsbS rsbT rsbU* gene cluster (Fig. 2). Each plasmid in the series contains a Kan^r gene adjoining a DNA segment that is homologous with the chromosomal region immediately upstream of the *sigB* operon. On the other side of the Kan^r gene is the *sigB* operon promoter and increasingly longer segments of the *sigB* operon. Each plasmid adds a *sigB* operon gene to the genes that are present



% wild type transformants					Gene	Allele
pRR7	pRS11	pRT2	pRU13	pRV6		
1.5%	36%	51%	59%	62%	*rsbS*	*rsbS23ER*
59%	62%	69%	84%	87%	*rsbR*	*rsbR136EK*
0%	64%	59%	69%	64%	*rsbS*	*rsbS66GR*
51%	65%	68%	64%	75%	*rsbR*	*rsbR225CY*
8%	20%	33%	56%	60%	*rsbS*	*rsbS83GD*
7%	35%	33%	42%	31%	*rsbS*	*rsbS22LF*
0%	44%	40%	51%	60%	*rsbS*	*rsbS76GR*
6%	85%	88%	82%	81%	*rsbS*	*rsbS86PL*

FIG. 2. Transformation mapping.

on the plasmid that precedes it in the series. Linearization of the plasmids, transformation into the mutant strains, and selection for Kan^r results in transformant clones in which the Kan^r cassette and increasing lengths of the *sigB* operon are introduced into the recipient cell's chromosome by homologous recombination. The promoter-distal gene on the smallest plasmid in the series that reversed the blue colony phenotype was inferred to be the likely site of the mutation. A variable number of white colonies (<10%) normally appeared among the transformant clones due to loss of the reporter system during the transformation process. Based on this analysis (Fig. 2), two of the mutations mapped to *rsbR* and the remaining six mapped to *rsbS*.

To verify the sites of the mutations and identify the changes responsible for the phenotype, the amplified DNA fragments that transformed wild-type *B. subtilis* to the mutant phenotype were cloned and sequenced. Each sequenced DNA contained a base change in the gene that had been identified in the mapping experiment. In all cases, the base changes substituted an alternative amino acid at the site of the change. These changes are listed in the last column of Fig. 2 and illustrated in Fig. 3. One of the mutations in *rsbR* results in a charge change

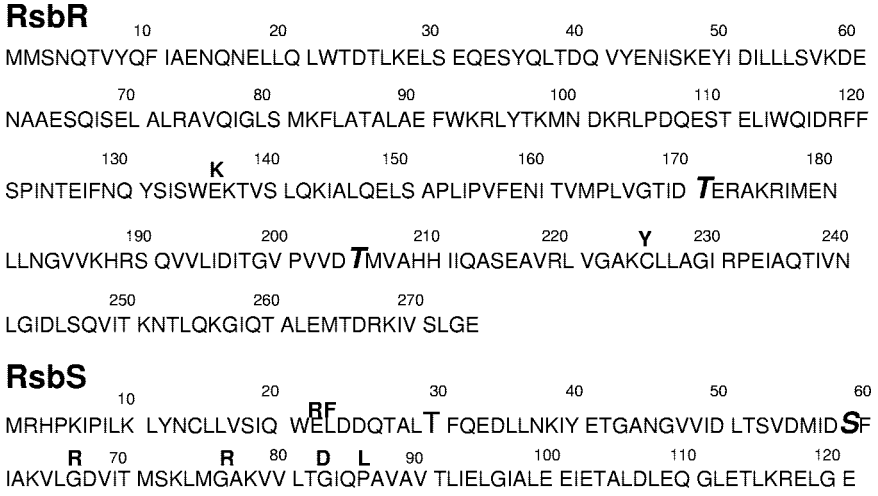


FIG. 3. RsbR and RsbS mutations. The amino acid sequences of RsbR and RsbS are illustrated. Sites of RsbR and RsbS phosphorylation (i.e., T_{171} and T_{205} of RsbR and S_{59} of RsbS) are in boldface type. Amino acid changes in RsbR and RsbS that were mapped and indicated in Fig. 2 are placed above the original residues in the sequence.

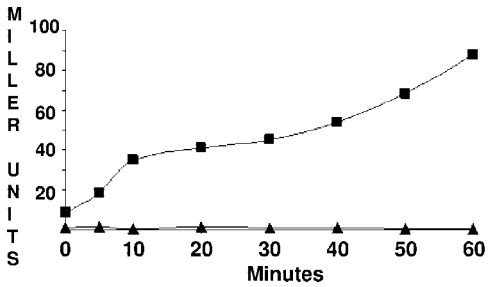


FIG. 4. σ^B activity in a representative mutant. *B. subtilis* strains BSJ43 (*rsbP*::*spc* SPβ *ctc*::*lacZ*) (triangles) and BAR12 [*rsbP*::*spc rsbR*(*136EK*) SPβ *ctc*::*lacZ*)] (squares) growing logarithmically in LB medium were sampled at the indicated times and analyzed for σ^B-dependent β-galactosidase levels (Miller units) as described in Materials and Methods.

(Glu→Lys) at residue 136. The second mutation leads to a replacement of the protein's sole cystine residue (Cys_{225}) with a tyrosine. Three of the *rsbS* mutations substituted charged amino acids for glycine residues in a glycine-rich region of the protein (i.e., Gly_{66}→Arg, Gly_{76}→Arg, Gly_{83}→Asp). The remaining mutations included two that were clustered near the amino terminus of RsbS, a substitution of Phe for Leu at position 22 and a charge change (Glu→Arg) at the adjoining position 23. The final *rsbS* mutation replaced a Pro residue at position 86 with Leu. All of the mutations in *rsbS*, as well as the Glu_{136}→Lys_{136} change in *rsbR*, are changes in amino acids that are invariant at these positions in sequenced *Bacillus* and *Listeria* species. The Cys changed to Tyr at position 225 of *rsbR* is less highly conserved but is the only Cys residue in the protein.

Effects of the *rsbR* and *rsbS* mutations on σ^B activity and product abundance. To quantitate the effects of the mutations in *rsbR* and *rsbS* on the background activity of the physical stress pathway, strains lacking the nutritional stress pathway (i.e., $RsbP^-$) and carrying either the wild-type or altered *rsbR*/*rsbS* alleles were grown in LB and analyzed for σ^B-dependent β-galactosidase activity. As was seen in previous studies wherein the activities of negative regulators of σ^B were compromised (4), β-galactosidase specific activity in the mutant cultures increased during growth. This presumably reflects the increase in σ^B from its autoregulated promoter under conditions where normal negative control is not fully present. A representative plot illustrating this phenomenon in one of the *rsbR* mutations is presented in Fig. 4. The σ^B activity levels of each of the mutant strains during mid-log phase (OD = 0.5) and 30 min after entry into stationary phase are illustrated in Table 2. The table, depicting the average values from three independent experiments, includes mid-log-phase data from both the merodiploid and single-copy strains. The effects of the mutations in *rsbR* or *rsbS* are not suppressed by the presence of their wild-type counterparts. In general, the σ^B activities of the mutant *rsbR* or *rsbS* strains are elevated to similar degrees regardless of whether or not the mutant genes are expressed as the strain's sole source of RsbR or RsbS.

As expected from the ongoing accumulation of β-galactosidase during growth in these mutant strains (Fig. 4), β-galactosidase levels are significantly lower during a mid-log phase of growth than during stationary phase. During growth, some mutants [e.g., *rsbS*(*23LF*) and *rsbS*(*83GD*)] exhibited σ^B activity that was barely above that seen in the wild-type parental strains, while others [e.g., *rsbR*(*136EK*), *rsbR*(*225CY*), and *rsbS*(*22ER*)] displayed σ^B activities more than 20-fold higher than that of their wild-type parent. By the time the cultures had entered stationary phase, this had increased to levels that were 7- to 100-fold higher than that seen in a stationary-phase culture of the wild-type parental strain.

We next asked whether any of the mutations, in altering the background activity of the physical stress pathway, might also prevent further induction of the pathway by physical stress. To this end, wild-type and mutant *B. subtilis* cells grown in LB were subjected to ethanol stress (4% ethanol) and analyzed for σ^B-dependent β-galactosidase activity 20 min after ethanol addition. In each instance, the addition of ethanol resulted in elevated σ^B activity (Table 2). The increase in σ^B activity following ethanol treatment in the *rsbR* mutant strain may be the result of either the residual activity in the mutant RsbR proteins or the compensating activities of the RsbR paralogs (2, 24). In the strains carrying mutant *rsbS* alleles, the products of these alleles are the strains' only source of RsbS. The stress inducibility of σ^B activity in these strains suggests that the altered RsbS proteins are still able to at least partially perform their roles as stress-modulated inhibitors of RsbT.

Western blot analyses were next conducted to determine whether the mutations in *rsbR* or *rsbS* alter the abundance of their products. Given that loss-of-function mutations in *rsbR* are essentially without effect on σ^B activity, it is unlikely that changes in RsbR levels per se could account for the *rsbR* alleles' mutant phenotype. The loss of RsbS does, however, elevate σ^B activity. As such, it is formally possible that some of the mutations in *rsbS* might elevate σ^B by reducing the levels of RsbS. As a means of standardizing the Western blot reactions, the relative abundance of RsbR and RsbS in each strain's extract was compared to that of the physical stress pathway phosphatase (RsbU) which is also present in the extracts.

TABLE 2. σ^B activity in mutant *rsbR* and *rsbS* strains during growth and physical stress

Strain	β-Galactosidase (*ctc*::*lacZ*) activity (Miller units) under the indicated conditions[a]			
	Merodiploid[b]	Single copy[c]		4% ethanol[d]
		Growth (OD, 0.5)	Stationary phase	
Wild type ($RsbP^-$)	1.2 ± 0.3	1.0 ± 0.1	0.6 ± 0.7	37.7 ± 0.1
rsbR(*136EK*)	19.2 ± 0.6	21.4 ± 0.9	34.5 ± 2.6	98.4 ± 4.6
rsbR(*225CY*)	28.5 ± 2.2	42.3 ± 1.8	62.4 ± 4.0	97.6 ± 2.2
rsbS(*22ER*)	42.5 ± 7.2	60.7 ± 0.1	82.5 ± 3.7	119.0 ± 6.9
rsbS(*23LF*)	3.6 ± 0.9	1.2 ± 0.1	6.9 ± 1.3	52.8 ± 0.7
rsbS(*66GR*)	6.6 ± 1.0	13.2 ± 1.1	39.9 ± 3.3	117.0 ± 9.3
rsbS(*76GR*)	3.1 ± 1.1	5.3 ± 0.6	4.4 ± 0.3	75.7 ± 3.7
rsbS(*83GD*)	3.1 ± 0.4	1.4 ± 0.1	10.6 ± 2.4	69.5 ± 2.1
rsbS(*86PL*)	7.3 ± 2.9	11.6 ± 1.6	36.4 ± 2.2	77.1 ± 8.9

[a] Values are averages of three separate determinations ± standard deviations.
[b] Merodiploid strains contain two copies of the P_A *rsbR rsbS rsbT rsbU* element. Both copies are wild type in the first strain listed. The remaining strains contain the indicated allele of *rsbR* or *rsbS* in one of the two elements, with its wild-type counterpart in the other. All strains are $RsbP^-$.
[c] Strains are $RsbP^-$ and contain single copies of *sigB* operon genes with the indicated alleles. Stationary-phase values were obtained 30 min after the cessation of growth.
[d] Samples were taken from actively growing cultures at 20 min after ethanol addition.

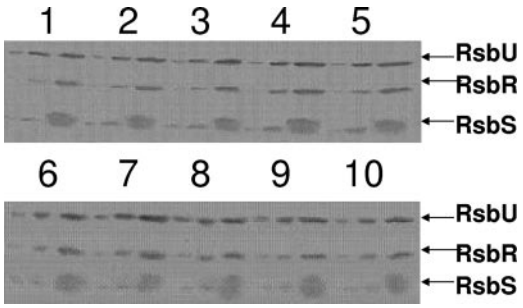


FIG. 5. Western blot analysis of RsbR and RsbS abundance in wild-type and mutant *B. subtilis*. *B. subtilis* strains BSJ43 (wild-type *rsbR* and *rsbS*) (lanes 1 and 6), BAR11 *rsbS*(*22ER*) (lane 2), BAR12 *rsbR*(*136EK*) (lane 3), BAR14 *rsbS*(*66GR*) (lane 4), BAR15 *rsbR*(*225CY*) (lane 5), BARM1 *rsbS*(*83GD*) (lane 7), BARM15 *rsbS*(*23LT*) (lane 8), BARM24 *rsbS*(*79GR*) (lane 9), and BAR42 *rsbS*(*86PL*) (lane 10) were grown to an OD of 0.5 in LB. Crude extract samples equivalent to 100, 300, and 900 μl of the original cultures were analyzed by Western blotting using monoclonal antibodies specific for RsbU, RsbR, and RsbS. The positions of each of these proteins are indicated.

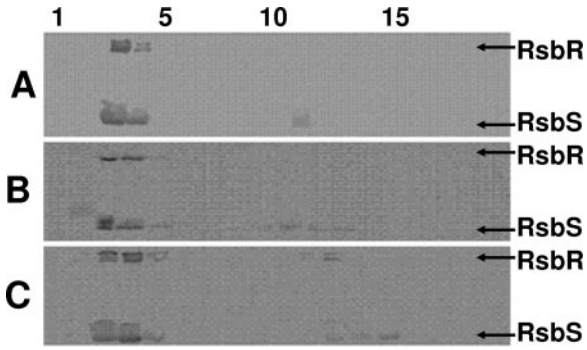


FIG. 6. Gel filtration chromatography of Rsb proteins in *E. coli* extracts. Crude extracts were prepared from *E. coli* strains carrying plasmids pARE7 (P_A *rsbR rsbS rsbT rsbU*) (A), pARE34 [P_A *rsbR*(*136EK*) *rsbS rsbT rsbU*] (B), or pARE31 [P_A *rsbR*(*225CY*) *rsbS rsbT rsbU*] (C) were fractionated through Sephacryl S-300. Samples from each fraction were analyzed by SDS-PAGE and Western blotting using monoclonal antibodies specific for RsbR and RsbS. Numbers at the top of the figure are fraction numbers, with fraction 1 being the earliest-eluting (high-molecular-mass) fraction. Coomassie-stained gels (not shown) indicate elution of ribosomes between fractions 1 and 5. The positions of RsbR and RsbS on the Western blots are indicated.

Figure 5 displays the Western blots. The levels of RsbR and RsbS in each of the mutant strain extracts are indistinguishable from those of their wild-type parent. Thus, the changes in σ^B activity are not the result of changes in the levels of either RsbR or RsbS but rather changes in their inherent activities.

RsbR and RsbS are negative regulators of the physical stress pathway (2, 20, 24). As such it could be envisioned that mutations that interfere with their ability to act as inhibitors might allow σ^B to become active even in the presence of a wild-type allele. Although this is at least plausible in the case of the *rsbS* mutations, where a loss of RsbS leads to a dramatic increase in σ^B activity, it is unlikely with respect to the *rsbR* mutations. *rsbR* deletion mutations have little effect on σ^B activity. Only when both RsbR and its paralogs are lost is the activity of σ^B markedly elevated. This suggests that either the changes in RsbR are somehow inhibiting the ability of both the mutant RsbRs and the RsbR paralogs to function in RsbS/RsbT sequestration or the changes in RsbR have created an RsbR variant that has become a positive effector of σ^B activation. The latter possibility could involve altering RsbR in ways that allow it to facilitate the phosphorylation of RsbS in the absence of stress.

A possible mechanism by which changes in RsbR could impair the ability of both the mutant RsbR and its paralogs to sequester RsbS and RsbT would be if the mutant protein fails to properly engage in stressosome formation and as a consequence disrupts the chimeric stressosome structure that is believed to form from RsbR and its paralogs (12, 24). To test whether the mutant RsbR proteins can form high-molecular-weight associations that are able to sequester RsbS, crude extracts were prepared from *E. coli* strains carrying DNA segments that express *rsbR rsbS rsbT rsbU* with either wild-type *rsbR* or the *rsbR*(*136EK*) or *rsbR*(*225CY*) alleles. High-molecular-mass RsbR/RsbS structures form when wild-type RsbR and RsbS are expressed in *E. coli* (25). *E. coli* extracts from strains expressing the Rsb proteins were fractionated by gel filtration and analyzed by Western blotting for the partitioning of RsbR and RsbS among the fractions. The use of *E. coli* in this experiment allows visualization of potential RsbR/RsbS complexes in the absence of the RsbR paralogs which might mask any deficiency of RsbR itself in sequestering RsbS. The results of the gel filtration analyses are illustrated in Fig. 6. *E. coli* extracts containing either the RsbR(136EK) or RsbR(225CY) proteins (Fig. 6B and C, respectively) contained large complexes that included RsbS (fractions 3 and 4). The fractionation properties of these complexes were indistinguishable from those formed by wild-type RsbR (Fig. 6A). Although this result does not rule out the possibility that the 136EK and 225CY mutations cause subtle changes in the interactions among the RsbR subunits or between RsbR and RsbS, the changes in RsbR caused by these mutations do not grossly alter the ability of the mutant proteins to form high-molecular-mass associations and sequester RsbS.

Activity of mutant RsbR proteins requires residues that are targets for stress-dependent phosphorylation. The activation of σ^B by physical stress is believed to involve an RsbT-dependent phosphorylation of RsbR and then RsbS, with the phosphorylation of RsbR accelerating the subsequent phosphorylation of RsbS (10, 11, 16, 24). In such a model, RsbR initially serves as a negative regulator of the stress pathway by allowing RsbS to sequester RsbT, but once phosphorylated, RsbR acts as a positive element for the reactions that follow. This raises the possibility that one or both of the mutations that we isolated in RsbR are activating σ^B by changing RsbR so that it either resembles the phosphorylated form of RsbR or is more readily phosphorylated. In either case, the downstream consequence should be σ^B activation that is dependent on the phosphorylation of RsbS. To determine whether the heightened σ^B activity seen in the *rsbR* mutant strains was occurring via RsbS phosphorylation, we created *B. subtilis* strains in which the mutant *rsbR* alleles were paired with an *rsbS* allele, *rsbS*(*59SA*), whose product cannot be phosphorylated by RsbT (21) and asked what effect this additional mutation had on the strain's original σ^B activity. As seen in Table 3, alteration of RsbS so that it can no longer be phosphorylated lowers the mutant

TABLE 3. σ^B activity in mutant *rsbR* and *rsbS* double mutants

Relevant allele	β-Galactosidase (*ctc*::*lacZ*) activity (Miller units)[a]
Wild type	1.03 ± 0.42
rsbR(*171TA*)	0.52 ± 0.14
rsbS(*59SA*)	0.62 ± 0.39
rsbR(*136EK*)	20.1 ± 0.67
rsbR(*136EK 171TA*)	0.80 ± 0.27
rsbR(*136EK*) *rsbS*(*59SA*)	0.85 ± 0.51
rsbR(*225CY*)	39.7 ± 2.42
rsbR(*225CY 171TA*)	0.39 ± 0.22
rsbR(*225CY*) *rsbS*(*59SA*)	0.41 ± 0.20
rsbS(*22ER*)	63.2 ± 3.46
rsbS(*22ER 59SA*)	50.3 ± 4.10
rsbS(*23LF*)	1.20 ± 0.225
rsbS(*23LF 59SA*)	0.49 ± 0.69
rsbS(*66GR*)	11.7 ± 0.75
rsbS(*66GR 59SA*)	4.51 ± 0.27
rsbS(*76GR*)	4.39 ± 1.76
rsbS(*76GR 59SA*)	1.70 ± 0.28
rsbS(*83GD*)	1.28 ± 0.12
rsbS(*83GD 59SA*)	0.47 ± 0.10
rsbS(*86PL*)	13.8 ± 0.08
rsbS(*86PL 59SA*)	0.42 ± 0.27

[a] Values are averages of three determinations ± standard deviations for cultures harvested during mid-logarithmic-phase growth.

strains' σ^B-dependent reporter gene activity to a level seen in a strain with the *rsbS*(*59SA*) allele alone. The data are consistent with the notion that the *rsbR* mutations do not elevate σ^B activity by preventing RsbS from binding RsbT but rather by altering RsbR in such a way as to allow RsbS to be more readily phosphorylated by RsbT.

In vitro phosphorylation of the threonine residues at position 171 or 205 of RsbR enhances the kinase activity of RsbT for RsbS (10, 11). Thr 171 is the site in RsbR that is preferentially phosphorylated by RsbT and the likely site in RsbR at which stress-dependent phosphorylation occurs to stimulate the phosphorylation of RsbS (10). If the mutant RsbR proteins facilitate the phosphorylation of RsbS via an RsbR-P intermediate, prevention of RsbR phosphorylation may block the activation. To test this idea, *rsbR* alleles were created in which a Thr-to-Ala substitution at position 171 was added to the 136EK and 225CY mutations. When σ^B activity was assayed (Table 3), the β-galactosidase levels in the strains carrying both mutations in *rsbR* had fallen to the level observed in a strain with the *rsbR*(*171TA*) mutation alone. Thus, the *rsbR*(*171TA*) mutation is dominant over both the *rsbR* 136EK and 225CY changes. Although it is possible that the Thr→Ala substitution may have altered properties of RsbR beyond merely preventing its phosphorylation (10, 16), it is clear that altering this residue abolishes the ability of the mutant *rsbR* alleles to activate σ^B.

We next turned to the mutations in *rsbS*. Given the role of RsbS as a direct inhibitor of RsbT, there are at least two possible ways that the mutations in *rsbS* could enhance σ^B activity. The changes could either reduce the ability of the mutant RsbS to bind RsbT and prevent the activation of RsbT or make the RsbS products more prone to phosphorylation and RsbT release. Although mutations of the former kind (i.e., those that inactivate RsbS) were not initially anticipated due to the presence of a potentially complementing wild-type *rsbS* allele, there is increasing evidence that RsbS predominantly regulates the RsbT protein with which it is cotranscribed (see below and reference 45). Thus, mutations that impair RsbS function could be included in our collection. In an attempt to distinguish changes in RsbS that impair its ability to bind and hold RsbT inactive from those which facilitate RsbS phosphorylation by RsbT, we added the *rsbS*(*59SA*) mutation to the altered *rsbS* alleles. Mutations that impair the ability of RsbS to inhibit RsbT should still allow σ^B to be active in the presence of the serine-to-alanine substitution, while those that promote σ^B activity via RsbS phosphorylation should be no more active than strains with the *rsbS*(*59SA*) mutation alone. When *B. subtilis* strains carrying both mutations in *rsbS* were assayed for σ^B-dependent activity (Table 3), three of the six *rsbS* alleles retained most [*rsbS*(*22ER*)] or part [*rsbS*(*66GR*) and *rsbS*(*76GR*)] of their original heightened σ^B activity. Presumably, these strains are compromised in the ability to inhibit RsbT and so their phosphorylation is not critical. The σ^B activity in the remaining three mutant strains fell to the level seen in a strain with the *rsbS*(*59SA*) mutation alone. This is particularly evident in the case of the *rsbS*(*86PL 59SA*)-expressing strain, where introduction of the *rsbS*(*59SA*) mutation caused a 30-fold drop in σ^B activity. These results suggest that the *rsbS* mutations include variants with impaired RsbT inhibition and others that are more readily phosphorylated.

***cis*/*trans* analyses of *rsbR* and *rsbS* alleles.** The observation that some of the *rsbS* mutations allow elevated σ^B activity when coupled with a mutation, *rsbS*(*59SA*), that normally prevents RsbT release/σ^B activation suggests that these *rsbS* variants encode RsbS proteins that are defective in binding and inhibiting of RsbT. It is curious that these mutant RsbS proteins are not complemented by a wild-type *rsbS* allele. A possible explanation for the failure of the wild-type *rsbS* allele to substitute for the impaired RsbS proteins is offered by our recent observation that RsbT activity is principally controlled by the product of the *rsbS* gene with which it is cotranscribed (45). Thus, the wild-type *rsbS* allele might fail to effectively compensate for the mutant *rsbS* allele when it is expressed in *trans* to the *rsbT* gene whose product must be controlled. This characteristic of RsbS/RsbT raises the possibility it might also apply to the *rsbR* mutations. In such a case, the failure of the mutant *rsbR* genes to be complemented by their wild-type counterpart might reflect a similar effect of their coexpression with *rsbS* and *rsbT*. To examine the possibility that the *rsbR* and *rsbS* mutants display their σ^B activation phenotypes only when cotranscribed with a subset of their potential binding partners, we created *B. subtilis* strains in which the mutant *rsbR* alleles or the mutant *rsbS* alleles plus wild-type *rsbR* were inserted with their promoters into the *B. subtilis* chromosome immediately upstream of the wild-type *sigB* operon. In the case of the *rsbS* allele, the operons also included a wild-type *rsbR* gene. This places the mutant *rsbR* alleles on transcription units separate from *rsbS*, *rsbT*, and *rsbU* and the *rsbS* alleles separate from *rsbT* and

TABLE 4. σ^B activity in merodiploid rsbR and rsbS strains during growth

rsbR/*rsbS* allele[a]	β-Galactosidase (*ctc*::*lacZ*) activity (Miller units) for indicated duplicated element[b]	
	P_A *rsbR rsbS rsbT rsbU*	P_A *rsbR*/P_A *rsbR rsbS*
Wild type	1.2 ± 0.3	0.64 ± 0.45/0.76 ± 0.38[c]
rsbR(*136EK*)	19.2 ± 0.6	1.2 ± 0.02
rsbR(*225CY*)	28.5 ± 2.2	1.0 ± 0.46
rsbS(*22ER*)	42.5 ± 7.2	0.67 ± 0.07
rsbS(*23LF*)	3.6 ± 0.9	0.57 ± 0.45
rsbS(*66GR*)	6.6 ± 1.0	0.38 ± 0.25
rsbS(*76GR*)	3.1 ± 1.1	0.40 ± 0.20
rsbS(*83GD*)	3.1 ± 0.4	0.53 ± 0.33
rsbS(*86PL*)	7.3 ± 2.9	0.62 ± 0.21

[a] Each strain contains a wild-type *sigB* operon plus the indicated *rsbR* or *rsbS* allele in a transcription unit containing *rsbR rsbS rsbT rsbU* or *rsbR* (for *rsbR* mutations), *rsbR rsbS rsbT rsbU* or *rsbR rsbS* or *rsbR rsbS* (for *rsbS* mutations).

[b] All strains are RsbP$^-$. The indicated allele is present in the diploid element outside of the *sigB* operon.

[c] Values represent two copies of wild-type *rsbR*/*rsbR rsbS*.

rsbU. As seen in Table 4, removal of the mutant alleles from the other genes of the *sigB* operon lowered σ^B activity to the level seen in a wild-type strain. Thus, the heightened σ^B activity caused by each of the mutations is dependent on cotranscription of the mutant alleles with other stressosome components. In the case of *rsbS*, this is consistent with the notion that RsbS and RsbT function as a unit upon synthesis; however, given that the RsbR paralogs, transcribed from diverse sites, can substitute for RsbR in properly regulating RsbS/RsbT interactions, this was not expected of *rsbR*. The finding that the *rsbR* alleles must also be joined to the *sigB* operon for heightened σ^B activity suggests that RsbR, RsbS, and RsbT rapidly interact following synthesis and that RsbS and RsbT may join RsbR prior to association within the stressosome.

DISCUSSION

The *B. subtilis* stressosome is a large protein assemblage that couples the occurrence of physical stress to the activation of σ^B (11, 12, 24). The principal structural components of the stressosome are a family of homologous proteins (the RsbR family) that are needed for proper interactions between the other regulatory components. Central to σ^B induction is the release of RsbT, a positive regulatory protein trapped within the stressosome, from an association with its primary negative regulator (RsbS). Release of RsbT follows its phosphorylation of RsbR and then RsbS (10, 12, 24). The mechanism by which stress empowers RsbT to initiate these phosphorylations is unknown.

In the present work we describe missense changes in RsbR and RsbS that allow σ^B to be active in the absence of applied stress. Previous mutations in *rsbS* which enhanced σ^B activity during growth consisted either of deletions of the *rsbS* gene itself or a serine-to-aspartate change at the amino acid (Ser 59) that is believed to be phosphorylated by RsbT (39, 41). The latter mutation is believed to mimic RsbS phosphorylation. Both mutations eliminate or reduce the ability of RsbS to sequester RsbT in an inhibitory complex. Several of the RsbS mutations that were isolated in the present study are also likely to compromise the ability of RsbS to bind RsbT. These are the RsbS variants that allow residual enhanced σ^B activity when paired with a mutation, *rsbS*(*59SA*), that normally blocks the release of RsbT from RsbS. Among the variants are an RsbS protein with a glutamate-to-arginine substitution at position 22 and two others with glycine-to-arginine changes at positions 66 or 76. A third glycine substitution (Gly 83→Glu 83) in the same general region also elevates σ^B activity; however, this mutation failed to elevate σ^B activity when paired with the *rsbS*(*59SA*) change. Although such a result would be consistent with a change in *rsbS* that elevated σ^B activity via altered RsbS phosphorylation, this particular mutant allele, as well as another, *rsbS*(*23LF*), causes relatively modest increases in σ^B activity. The addition of the *rsbS*(*59SA*) mutation reduces σ^B activity as much as 40% even in *rsbS* variants where substantial σ^B activity persists [e.g., *rsbS*(*76GR*)]. It thus appears possible that in the case of the two *rsbS* alleles with relatively low increases in σ^B activity, the smaller reduction in their products' ability to bind and hold RsbT may be masked by the secondary effects of the *rsbS*(*59SA*) mutation. This makes their dependence on RsbS phosphorylation ambiguous. Ambiguity is not evident, however, in the instance of the *rsbS*(*86PL*) mutation. Addition of the Ser→Ala substitution to this allele leads to a 30-fold drop in σ^B activity. The ability of this variant to be phosphorylated is clearly important for activation of σ^B. The RsbS(59SA) protein, believed to bind RsbT in a complex from which it is not released, still requires an interaction with the RsbR proteins to effect this inhibition. The RsbS(59SA) variant is unable to block σ^B activation in the absence of the RsbR proteins (24). Thus, the loss of elevated σ^B activity caused by the RsbS(86PL) mutation upon the addition of the *rsbS*(*59SA*) mutation argues that this variant RsbS still interacts with the RsbR proteins and that the heightened σ^B activity is not due to a failure to be sequestered within the stressosome. It seems more likely that this mutation heightens the phosphorylation state of RsbS. Presumably the Pro→Leu change at residue 86 creates an RsbS variant that is more accessible to phosphorylation by RsbT, resistant to dephosphorylation by RsbX, or both.

The σ^B-activating mutations in *rsbR* are particularly interesting. Null *rsbR* mutations have little effect on σ^B activity in strains that still express the RsbR paralogs (1, 24). Both the $rsbR_{136}EK$ and $rsbRT_{225}CY$ mutations elevate σ^B activity in the presence of both wild-type *rsbR* and its paralogs. The ability of the RsbR variants to form high-molecular-weight associations that sequester RsbS lessens the possibility that their phenotype is a consequence of stressosome disruption. Instead, the observation that the *rsbR* alleles allow σ^B to be active only if both their products and RsbS carry the amino acid residues that are phosphorylated during activation argues that these *rsbR* mutations increase the likelihood that RsbR will be phosphorylated, with heightened phosphorylation of RsbS as a consequence. As with the *rsbS* allele [*rsbS*(*86PL*)], which also requires its phosphorylation site for σ^B activity, it is unresolved whether the mutations in *rsbR* alter their products' phosphorylation rate by RsbT or dephosphorylation by RsbX.

The finding that modifications of RsbR can alter σ^B activity lends credence to the notion that RsbR and its paralogs may be potential targets for stress induction. It is unlikely, however, even if the RsbR proteins are the stressosome components that receive stress signals, that the RsbR proteins themselves undergo stress-induced changes to initiate the response. The

known Rsb proteins, expressed and functional in *E. coli*, fail to activate σ^B in response to applied stress (31). It appears more likely that if the RsbR proteins are a target for stress activation, unknown *Bacillus*-specific factors interact with them to modify their rate of phosphorylation or dephosphorylation.

An additional interesting observation coming from the present work is the finding that mutations in either *rsbR* or *rsbS* are dominant only when coexpressed with other *rsb* genes. We had previously noted that *rsbT* is unable to complement an *rsbT* deletion unless it is cotranscribed with other *rsb* genes (45). It was also determined that an *rsbS* variant, *rsbS*(*59SD*), that normally allows heightened σ^B activity during growth must be cotranscribed with *rsbT* to exert this effect (45). These results were interpreted as evidence that RsbS and RsbT interact concomitantly with their synthesis to form a stable association that is critical to RsbT activity. In such a model, RsbS principally controls the activity of the RsbT protein with which it is cosynthesized. If this is true, it is not surprising that our mutant *rsbS* alleles must also be cotranscribed with *rsbT* for them to display their phenotypes. The observation that our mutant *rsbR* genes fail to activate σ^B unless cotranscribed with *rsbS* and *rsbT* suggests that all three of these stress pathway components interact soon after their synthesis. This implies that RsbS and RsbT may bind to RsbR prior to RsbR becoming part of the stressosome and that RsbR, RsbS, and RsbT could, in fact, enter the stressosome as a preformed complex. The significance of this to stressosome assembly is not known.

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