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Mutational Analysis of RsbT, an Activator of the *Bacillus subtilis* Stress Response Transcription Factor, σ^B

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σ^B, the stress-activated σ factor of *Bacillus subtilis*, requires the RsbT protein as an essential positive regulator of its physical stress pathway. Stress triggers RsbT to both inactivate the principal negative regulator of the physical stress pathway (RsbS) by phosphorylation and activate a phosphatase (RsbU) required for σ^B induction. Neither the regions of RsbT that are involved in responding to stress signaling nor those required for downstream events have been established. We used alanine scanning mutagenesis to examine the contributions of RsbT's charged amino acids to the protein's stability and activities. Eleven of eighteen *rsbT* mutations blocked σ^B induction by stress. The carboxy terminus of RsbT proved to be particularly important for accumulation in *Bacillus subtilis*. Four of the five most carboxy-terminal mutations yielded *rsbT* alleles whose products were undetectable in *B. subtilis* extracts. Charged amino acids in the central region of RsbT were less critical, with four of the five substitutions in this region having no measurable effect on RsbT accumulation or activity. Only when the substitutions extended into a region of kinase homology was σ^B induction affected. Six other RsbT variants, although present at levels adequate for activity, failed to activate σ^B and displayed significant changes in their ability to interact with RsbT's normal binding partners in a yeast dihybrid assay. These changes either dramatically altered the proteins' tertiary structure without affecting their stability or defined regions of RsbT that are involved in multiple interactions.

σ^B is a *Bacillus subtilis* transcription factor that controls the bacterium's general stress regulon. This is a collection of more than 200 genes whose transcription is elevated after exposure to physical (e.g., heat shock, ethanol, or osmotic shock) or nutritional (e.g., glucose limitation, phosphate limitation, or azide treatment) stress (7, 18, 24, 25, 31). Induction of the general stress regulon is triggered by the activation of σ^B (4–6, 8, 9). σ^B is encoded by the seventh gene of an eight-gene operon, with the remaining genes specifying regulators of σ^B activity (20, 36). All eight genes are constitutively expressed from a promoter (P_A) that is likely recognized by the cell's principal σ factor (σ^A). An internal σ^B-dependent promoter (P_B) elevates the expression of the *sigB* operon's downstream four genes during periods of σ^B activity (i.e., P_A Δ*rsbR rsbS rsbT rsbU* P_B Δ*rsbV rsbW sigB rsbX*) (4, 8, 9, 20).

As illustrated in Fig. 1, the primary regulators of σ^B are RsbV and RsbW. RsbW is an anti-σ^B protein that binds σ^B and makes it unavailable to RNA polymerase (6, 14). RsbV is the σ^B release factor (6, 8). RsbW forms mutually exclusive complexes with either the RsbV protein or σ^B (13, 14). RsbV availability determines the amount of σ^B that is released from RsbW, with the phosphorylation state of RsbV controlling its activity (14). RsbW is both an RsbV/σ^B binding protein and an RsbV-specific kinase. In unstressed *B. subtilis*, an RsbW-dependent phosphorylation of RsbV blocks its ability to extract σ^B from RsbW (3, 14). RsbV-P is reactivated by either of two stress-specific phosphatases: one in a pathway that responds to nutritional stress and the other responsive to physical stress (21, 30, 32, 33, 37). Either of these enzymes can dephosphorylate RsbV-P and allow it to again displace σ^B from the RsbW-σ^B complex.

The nutritional stress phosphatase (RsbP) is cotranscribed with a predicted hydrolase or acryltransferase (RsbQ) that is needed for RsbP activity (10, 30). The metabolic inducers of the RsbP/Q phosphatase are unknown, but the conditions that trigger this pathway are associated with a decrease in the cell's ATP levels, suggesting that change in nucleotide pools may be involved in triggering the activation (33, 39).

The phosphatase that responds to physical stress (RsbU) requires an additional factor, the RsbT protein, for activity (37). In unstressed *B. subtilis*, RsbT is held inactive in a complex with its negative regulator, RsbS. Exposure to stress empowers RsbT to phosphorylate and inactivate RsbS and then activate RsbU (1, 12, 35, 37). Interactions between RsbS and RsbT are believed to be modulated by RsbR and a family of related proteins (1, 2, 16). Recent experiments (12) have demonstrated that RsbR, and presumably its homologs, can self-associate into large-molecular-mass complexes (~10^6 Da) that can incorporate RsbS and RsbT. These complexes may represent the normal state of RsbR, RsbS, and RsbT proteins in unstressed *B. subtilis*. In vitro, the phosphorylation state of RsbR, which, like RsbS can be phosphorylated by RsbT, determines the effectiveness with which RsbT can phosphorylate and escape from RsbS (12, 16). σ^B activity returns to its prestress levels through the activity of RsbX, a RsbS-P-specific phosphatase that reactivates RsbS and allows it to again sequester RsbT into an inactivating complex (32, 37). The mech-

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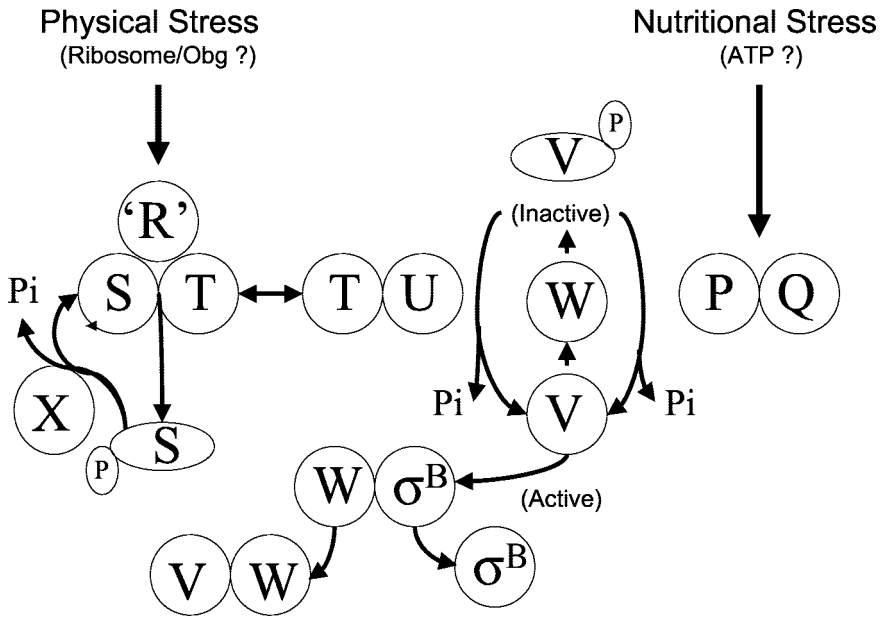

FIG. 1. Model of σ^B activation. σ^B is held inactive in unstressed *B. subtilis* as a complex with an anti-σ^B protein, RsbW (W). σ^B is freed from RsbW when a release factor, RsbV (V), binds to RsbW. In unstressed *B. subtilis*, RsbV is inactive due to an RsbW-catalyzed phosphorylation (V-P). Physical stress activates an RsbV-P phosphatase, RsbU (U), which reactivates RsbV. RsbT (T) is the RsbU activator. RsbT is normally bound to a negative regulator RsbS (S), which inhibits its activity. RsbR and a family of related proteins ("R") also bind to RsbS and RsbT and are believed to facilitate their interactions. Upon exposure to stress, RsbT phosphorylates and inactivates RsbS then activates the RsbU phosphatase. A ribosome-mediated process and Obg, a ribosome-associated GTPase that can bind to RsbT, play essential, but unknown roles in the activation of RsbT. RsbS-P is dephosphorylated and reactivated by a phosphatase RsbX (X) that is encoded by one of the genes downstream of the *sigB* operon's σ^B-dependent promoter. RsbX levels become elevated when σ^B is active, which may facilitate a return of RsbT to an inactive complex with RsbS. Energy depletion activates a separate pathway in which a novel RsbV-P phosphatase (P) and an associated protein (Q) are triggered, by unknown means, to reactivate RsbV. The model is based on references (1, 4, 6, 8, 14, 20, 27, 35–37, 40).

anisms by which the presence of physical stress is conveyed to RsbR, RsbS, and RsbT are unknown. There is evidence, however, that a ribosome-associated event may contribute to the process. σ^B activation by physical stress fails to occur in *B. subtilis* strains that are either missing ribosome protein L11 or are deficient in a ribosome-associated GTP-binding protein (Obg) (27, 28, 40).

Although RsbT is a critical positive regulator of the physical stress pathway, neither the regions that are involved in responding to stress nor those which catalyze downstream events have been established. In the present study we use alanine scanning mutagenesis to examine the contributions of RsbT's charged amino acids to its stability and activities.

MATERIALS AND METHODS

Bacterial strains, plasmids and cultivation. The *B. subtilis* strains and the plasmids used for the present study are listed in Table 1. All BSA and BSW strains are derivatives of PY22. Bacteria were routinely grown in LB (26) at 37°C. Physical stress induction of σ^B was triggered by exposure of *B. subtilis* to ethanol during exponential growth at a final concentration of 4% (35). P_{SPAC} promoter fusions were induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to 0.1 mM.

PCR mutagenesis of *rsbT*. Site-directed PCR mutagenesis was performed on *rsbT* by using high-fidelity *Taq* polymerase (Life Technologies, Rockville, Md.) to replace charged amino acids along the length of the protein with alanine. To prepare a substrate for *rsbT* mutagenesis and subsequent expression, the P_A*rsbR-S* and *rsbT* regions of the *sigB* operon were amplified in sequential steps from PY22 chromosomal DNA. Two sets of primers were used. The most upstream primer, which hybridizes 5′ to the *sigB* operon's promoter (P_A) and incorporates a BamHI site, was paired for PCR with a primer containing the restriction site NdeI and designed to hybridize to the 3′ end of *rsbS*. A second pair of primers was designed to amplify *rsbT*, with the upstream primer containing the restriction site NdeI and the downstream primer, designed to hybridize to the 3′ end of *rsbT*, incorporating the restriction site SphI. Two separate PCR fragments were produced: P_A*rsbR-S* (1,359 bp) and *rsbT* (449 bp). The two fragments were mixed and used as templates for an additional amplification with the two most outboard primers from the original separate amplifications, i.e., the oligonucleotide that hybridized immediately upstream of P_A and downstream of *rsbT*. This "long PCR" product contained P_A*rsbR-S* and *rsbT* as a single fragment, with a unique NdeI site at the 5′ end and SphI at the 3′ end of *rsbT*. The NdeI/SphI sites were to be used to introduce the mutant *rsbT* alleles into plasmid vectors for transfer to *B. subtilis*.

The PCR product containing P_A*rsbR-S* and *rsbT* was cloned into a variant of pUS19 from which the NdeI site, normally present in the vector, had been deleted. As a consequence, the only NdeI site remaining is the one engineered upstream of *rsbT*. By using this plasmid (pDRNT) as a template, unique primers were designed to introduce the alanine substitutions throughout the length of the protein.

Three individual PCR amplifications were used to create each *rsbT* mutant allele. First, a primer upstream of *rsbT* was paired with a mutagenic oligonucleotide designed to hybridize at the region within *rsbT* at which the mutation would be made. The mutagenic primer contained the sequence specifying the bases for the desired alanine substitutions. A separate PCR was set up by using a mutagenic oligonucleotide that was complementary to that used in the first amplification paired with a primer downstream of *rsbT*. These two fragments were then mixed together as a template for a third reaction in which the two outboard primers—one upstream and the other downstream of *rsbT*—were used to create a "long" PCR product encoding upstream DNA, as well as all of *rsbT* with the specific alanine substitution(s) at the intended locations. After sequencing of the cloned PCR products to verify the correct *rsbT* sequence, the *rsbT* alleles were cloned as NdeI and SphI fragments into a variant of the plasmid pDRNT, in which the wild-type *rsbT* had been removed by using these same restriction endonucleases. This created a collection of plasmids capable of expressing *rsbR*, *rsbS*, and one of the *rsbT* variants from the *sigB* operon's P_A promoter in either *Escherichia coli* or *B. subtilis*.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype	Construction or source
Bacillus strains		
BSA46	*trpC2 SP*β *ctc*::*lacZ*	4
PY22	*trpC2*	29
XS352	*trpC2 aph3′ 5′′/sigB* Δ*rsbST SP*β *ctc*::*lacZ*	29
BSZ11	*trpC2 sigB* Δ*rsbST rsbX*::pWH25 *SP*β *ctc*::*lacZ*	pWH25 → XS352 → BSA46
BSA661-T0	*trpC2 aph3′5′′/sigB rsbT SP*β *ctc*::*lacZ*	pDRNT → XS352
BSA661-T1	*trpC2 aph3′5′′/sigB rsbT*$_1$ *SP*β *ctc*::*lacZ*	pT1 → XS352
BSA661-T2	*trpC2 aph3′5′′/sigB rsbT*$_2$ *SP*β *ctc*::*lacZ*	pT2 → XS352
BSA661-T3	*trpC2 aph3′5′′/sigB rsbT*$_3$ *SP*β *ctc*::*lacZ*	pT3 → XS352
BSA661-T4	*trpC2 aph3′5′′/sigB rsbT*$_4$ *SP*β *ctc*::*lacZ*	pT4 → XS352
BSA661-T5	*trpC2 aph3′5′′/sigB rsbT*$_5$ *SP*β *ctc*::*lacZ*	pT5 → XS352
BSA661-T6	*trpC2 aph3′5′′/sigB rsbT*$_6$ *SP*β *ctc*::*lacZ*	pT6 → XS352
BSA661-T7	*trpC2 aph3′5′′/sigB rsbT*$_7$ *SP*β *ctc*::*lacZ*	pT7 → XS352
BSA661-T8	*trpC2 aph3′5′′/sigB rsbT*$_8$ *SP*β *ctc*::*lacZ*	pT8 → XS352
BSA661-T9	*trpC2 aph3′5′′/sigB rsbT*$_9$ *SP*β *ctc*::*lacZ*	pT9 → XS352
BSA661-T10	*trpC2 aph3′5′′/sigB rsbT*$_{10}$ *SP*β *ctc*::*lacZ*	pT10 → XS352
BSA661-T11	*trpC2 aph3′5′′/sigB rsbT*$_{11}$ *SP*β *ctc*::*lacZ*	pT11 → XS352
BSA661-T12	*trpC2 aph3′5′′/sigB rsbT*$_{12}$ *SP*β *ctc*::*lacZ*	pT12 → XS352
BSA661-T13	*trpC2 aph3′5′′/sigB rsbT*$_{13}$ *SP*β *ctc*::*lacZ*	pT13 → XS352
BSA661-T14	*trpC2 aph3′5′′/sigB rsbT*$_{14}$ *SP*β *ctc*::*lacZ*	pT14 → XS352
BSA661-T15	*trpC2 aph3′5′′/sigB rsbT*$_{15}$ *SP*β *ctc*::*lacZ*	pT15 → XS352
BSA661-T16	*trpC2 aph3′5′′/sigB rsbT*$_{16}$ *SP*β *ctc*::*lacZ*	pT16 → XS352
BSA661-T17	*trpC2 aph3′5′′/sigB rsbT*$_{17}$ *SP*β *ctc*::*lacZ*	pT17 → XS352
BSA661-T18	*trpC2 aph3′5′′/sigB rsbT*$_{18}$ *SP*β *ctc*::*lacZ*	pT18 → XS352
BSW13B	*trpC2* P_{SPAC} *rsbT SP*β *ctc*::*lacZ*	pRW13 → BSA46
BSW14	*trpC2* P_{SPAC} *rsbT*$_3$ *SP*β *ctc*::*lacZ*	pRW10 → BSA46
BSW15	*trpC2* P_{SPAC} *rsbT*$_4$ *SP*β *ctc*::*lacZ*	pRW11 → BSA46
BSW16	*trpC2* P_{SPAC} *rsbT*$_5$ *SP*β *ctc*::*lacZ*	pRW12 → BSA46
BSW17	*trpC2* P_{SPAC} *rsbT*$_7$ *SP*β *ctc*::*lacZ*	pRW14 → BSA46
BSW18	*trpC2* P_{SPAC} *rsbT*$_{13}$ *SP*β *ctc*::*lacZ*	pRW15 → BSA46
BSW19	*trpC2* P_{SPAC} *rsbT*$_{16}$ *SP*β *ctc*::*lacZ*	pRW16 → BSA46
BSW44	*trpC2 aph3′5′′/sigB* P_A*rsbR*Δ*rsbS rsbT SP*β *ctc*::*lacZ*	pRW22 → XS352
BSW45	*trpC2 aph3′5′′/sigB* P_A*rsbR*Δ*rsbS rsbT*$_5$ *SP*β *ctc*::*lacZ*	pRW23 → XS352
BSW46	*trpC2 aph3′5′′/sigB* P_A*rsbR*Δ*rsbS rsbT*$_7$ *SP*β *ctc*::*lacZ*	pRW24 → XS352
BSW47	*trpC2 aph3′5′′/sigB* P_A*rsbR*Δ*rsbS rsbT*$_{16}$ *SP*β *ctc*::*lacZ*	pRW25 → XS352
BSW48	*trpC2* Δ*rsbST* P_{SPAC} *rsbT*$_3$ *SP*β *ctc*::*lacZ*	pRW10 → BSZ11
BSW49	*trpC2* Δ*rsbST* P_{SPAC} *rsbT*$_4$ *SP*β *ctc*::*lacZ*	pRW11 → BSZ11
BSW50	*trpC2* Δ*rsbST* P_{SPAC} *rsbT*$_5$ *SP*β *ctc*::*lacZ*	pRW12 → BSZ11
BSW13A	*trpC2* Δ*rsbST* P_{SPAC} *rsbT SP*β *ctc*::*lacZ*	pRW13 → BSZ11
BSW51	*trpC2* Δ*rsbST* P_{SPAC} *rsbT*$_7$ *SP*β *ctc*::*lacZ*	pRW14 → BSZ11
BSW52	*trpC2* Δ*rsbST* P_{SPAC} *rsbT*$_{13}$ *SP*β *ctc*::*lacZ*	pRW15 → BSZ11
BSW53	*trpC2* Δ*rsbST* P_{SPAC} *rsbT*$_{16}$ *SP*β *ctc*::*lacZ*	pRW16 → BSZ11
BSW69	*trpC2 aph3′5′′/sigB* P_A*rsbR*Δ*rsbS rsbT*$_3$ *SP*β *ctc*::*lacZ*	pRW36 → XS352
BSW70	*trpC2 aph3′5′′/sigB* P_A*rsbR*Δ*rsbS rsbT*$_4$ *SP*β *ctc*::*lacZ*	pRW37 → XS352
BSW71	*trpC2 aph3′5′′/sigB* P_A*rsbR*Δ*rsbS rsbT*$_{13}$ *SP*β *ctc*::*lacZ*	pRW38 → XS352
Plasmids		
pACT-2	Ap^r vector with AD of GAL4	Clontech
pAS2-1	Ap^r vector with BD of GAL4	Clontech
pUV70	Ap^r protein fusion of RsbS with AD of GAL4	34
pUV134	Ap^r protein fusion of RsbR with AD of GAL4	34
pUV166	Ap^r protein fusion of RsbT with AD of GAL4	34
pUV187	Ap^r protein fusion of RsbU with AD of GAL4	34
pJMObg	Ap^r protein fusion of Obg with AD of GAL4	27
pWH25	Ap^r Spec^r *rsbX*	29
pUK19	Ap^r Kan^r	19
pUK19-1.1	Ap^r Kan^r *spoIIB*	This study
pDG148	Ap^r Kan^r *lacI* P_{SPAC}	P. Stagier
pRW10	Ap^r Kan^r *lacI* P_{SPAC} *rsbT*$_3$	This study
pRW11	Ap^r Kan^r *lacI* P_{SPAC} *rsbT*$_4$	This study
pRW12	Ap^r Kan^r *lacI* P_{SPAC} *rsbT*$_5$	This study
pRW13	Ap^r Kan^r *lacI* P_{SPAC} *rsbT*	This study
pRW14	Ap^r Kan^r *lacI* P_{SPAC} *rsbT*$_7$	This study
pRW15	Ap^r Kan^r *lacI* P_{SPAC} *rsbT*$_{13}$	This study
pRW16	Ap^r Kan^r *lacI* P_{SPAC} *rsbT*$_{16}$	This study
pRW22	Ap^r Spec^r P_A*rsbR*Δ*rsbSrsbT*	This study
pRW23	Ap^r Spec^r P_A*rsbR*Δ*rsbSrsbT*$_5$	This study
pRW24	Ap^r Spec^r P_A*rsbR*Δ*rsbSrsbT*$_7$	This study
pRW25	Ap^r Spec^r P_A*rsbR*Δ*rsbSrsbT*$_{16}$	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant genotype	Construction or source
pRW26	Ap^r protein fusion of *rsbT*$_3$ with BD of GAL4	This study
pRW27	Ap^r protein fusion of *rsbT*$_4$ with BD of GAL4	This study
pRW28	Ap^r protein fusion of *rsbT*$_5$ with BD of GAL4	This study
pRW29	Ap^r protein fusion of *rsbT*$_7$ with BD of GAL4	This study
pRW30	Ap^r protein fusion of *rsbT*$_8$ with BD of GAL4	This study
pRW31	Ap^r protein fusion of *rsbT*$_{13}$ with BD of GAL4	This study
pRW32	Ap^r protein fusion of *rsbT*$_{15}$ with BD of GAL4	This study
pRW33	Ap^r protein fusion of *rsbT*$_{16}$ with BD of GAL4	This study
pRW34	Ap^r protein fusion of *rsbT*$_{18}$ with BD of GAL4	This study
pRW36	Ap^r Spec^r *P*$_A$*rsbRΔrsbSrsbT*$_3$	This study
pRW37	Ap^r Spec^r *P*$_A$*rsbRΔrsbSrsbT*$_4$	This study
pRW38	Ap^r Spec^r *P*$_A$*rsbRΔrsbSrsbT*$_{13}$	This study
pUS19	Ap^r Spec^r	5
pUSD19-1	Ap^r Spec^r NdeI removed	This study
pDRNT	Ap^r Spec^r *P*$_A$*rsbRST*	This study
pT$_1$	Ap^r Spec^r *P*$_A$*rsbRST*$_1$	This study
pT$_2$	Ap^r Spec^r *P*$_A$*rsbRST*$_2$	This study
pT$_3$	Ap^r Spec^r *P*$_A$*rsbRST*$_3$	This study
pT$_4$	Ap^r Spec^r *P*$_A$*rsbRST*$_4$	This study
pT$_5$	Ap^r Spec^r *P*$_A$*rsbRST*$_5$	This study
pT$_6$	Ap^r Spec^r *P*$_A$*rsbRST*$_6$	This study
pT$_7$	Ap^r Spec^r *P*$_A$*rsbRST*$_7$	This study
pT$_8$	Ap^r Spec^r *P*$_A$*rsbRST*$_8$	This study
pT$_9$	Ap^r Spec^r *P*$_A$*rsbRST*$_9$	This study
pT$_{10}$	Ap^r Spec^r *P*$_A$*rsbRST*$_{10}$	This study
pT$_{11}$	Ap^r Spec^r *P*$_A$*rsbRST*$_{11}$	This study
pT$_{12}$	Ap^r Spec^r *P*$_A$*rsbRST*$_{12}$	This study
pT$_{13}$	Ap^r Spec^r *P*$_A$*rsbRST*$_{13}$	This study
pT$_{14}$	Ap^r Spec^r *P*$_A$*rsbRST*$_{14}$	This study
pT$_{15}$	Ap^r Spec^r *P*$_A$*rsbRST*$_{15}$	This study
pT$_{16}$	Ap^r Spec^r *P*$_A$*rsbRST*$_{16}$	This study
pT$_{17}$	Ap^r Spec^r *P*$_A$*rsbRST*$_{17}$	This study
pT$_{18}$	Ap^r Spec^r *P*$_A$*rsbRST*$_{18}$	This study

Analysis of *rsbT* variants in *E. coli* and *B. subtilis*. pDRNT and its related plasmids (pT$_1$ to pT$_{18}$, Table 1) carry the *sigB* operon promoter (P_A) and the *rsbR*, *rsbS* and *rsbT* genes. The P_A promoter is recognized by *E. coli* RNA polymerase. Hence, *E. coli* strains carrying the pDNRT family of plasmids express the *B. subtilis rsbR*, *rsbS and rsbT* genes. Although the pDNRT plasmids cannot replicate in *B. subtilis*, their transformation into *B. subtilis*, followed by selection for a plasmid-encoded antibiotic (spectinomycin) resistance, results in the isolation of clones in which the plasmid integrates into the chromosome by Campbell-like homologous recombination at *sigB*. If the recipient strain lacks a source of RsbS and RsbT (i.e., XS352 [Δ*rsbS-T*]), the plasmid-encoded alleles become the sole source of RsbS and RsbT in the transformants. To assess the ability of each variant RsbT to activate σ^B, strains were grown in Luria broth and exposed to 4% ethanol during exponential growth (optical density at 540 nm [OD_{540}] = 0.2). The σ^B activities in these strains were monitored by measuring the accumulation of β-galactosidase from an *E. coli lacZ* gene fused to a σ^B-dependent promoter (*ctc*::*lacZ*).

To create *B. subtilis* strains that would express the *rsbT* variants but not RsbS, plasmids containing *P*$_A$*rsbRST*, with various *rsbT* alleles, were digested with HincII and NruI. HincII uniquely cleaves the plasmid at nucleotide 716 of the 821-nucleotide *rsbR* gene, whereas NruI cuts at nucleotide 186 of the 360-nucleotide *rsbS* gene. The resulting plasmid molecules, missing the 3′ end of *rsbR* and the 5′ region of *rsbS*, were religated and transformed into *E. coli*. Plasmids bearing the *rsbR*::*rsbS* deletion with either wild-type or variant *rsbT* alleles were transformed into a Δ*rsbS-T B. subtilis* strain (XS352). Integration of the plasmids into the *sigB* operon yields RsbR$^+$ RsbS$^-$ strains expressing the *rsbT* allele of the incoming plasmid DNA.

RsbT induction studies. *rsbT* expressed in excess to *rsbS* can activate σ^B in the absence of physical stress (21, 27). To test the ability of the variant rsbTs to activate in such a system, mutant and wild-type *rsbT* alleles were excised from their original plasmids with the restriction enzymes NruI and SphI and then cloned downstream of an IPTG-inducible promoter (P_{SPAC}) in a plasmid capable of autonomous replication in both *E. coli* and *B. subtilis* (pDG148). These plasmids were transformed into *B. subtilis* strains either lacking *rsbS* and *rsbT* (BSZ11) or containing an intact *sigB* operon (BSA46). Cultures of *B. subtilis* carrying the plasmids were grown to an early exponential phase of growth (OD_{540} = 0.2) and exposed to 0.1 mM IPTG to induce the SPAC promoter and initiate RsbT synthesis. Samples were collected after 1 h and then analyzed for σ^B-dependent reporter gene (*ctc*::*lacZ*) expression. To evaluate possible further induction of σ^B by ethanol stress in these strains, reporter gene (*ctc*::*lacZ*) expression was also analyzed in cultures grown in the presence or absence of IPTG and exposed to 4% ethanol during growth (OD_{540} = 0.2).

Analysis of RsbT interactions in the yeast dihybrid system. *rsbT* alleles were subcloned from the pDRNT/pT plasmids (Table 1) into the yeast "matchmaker" plasmid system in a two-step process. First, the pDRNT/pT plasmids were digested with SphI and the overhanging ends made blunt by using Klenow enzyme. The plasmids were then digested with NdeI to produce a *rsbT* DNA fragment suitable for cloning into the NdeI and SmaI sites of pAS2-1 (Clontech Laboratories, Inc., Palo Alto, Calif.). This created plasmids encoding translational fusions between the various *rsbT* alleles and the binding domain (BD) of the yeast GAL4 activator protein. These plasmids were transformed, according to established protocols (Clontech) into yeast strain Y190 harboring a resident plasmid that encoded the GAL4 activation domain fused to either RsbU, RsbR, RsbS, RsbT, or Obg (Table 1). Yeast strains carrying both plasmids were selected on the basis of plasmid-encoded prototrophic markers and screened for GAL4-dependent histidine prototrophy and β-galactosidase activity (34).

General methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and β-galactosidase activity assays were performed as previously described (14, 23). DNA manipulations followed standard protocols (26). Transformation of naturally competent *B. subtilis* cells was carried out as described by Yasbin et al. (38). Yeast β-galactosidase assays were performed as done previously (34). Sequencing of *rsbT* mutant alleles and plasmids was performed by the Center for Advanced DNA Technologies at the University of Texas Health Science Center. Quantitation of protein bands was performed on

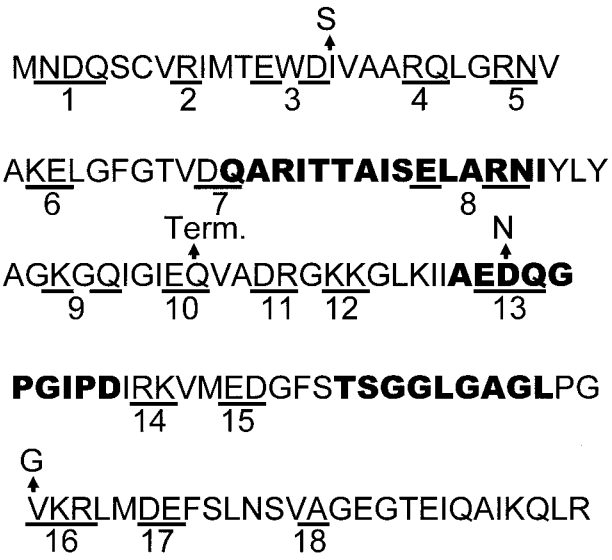

FIG. 2. RsbT mutations. The amino acid sequence of RsbT is illustrated (36). Selected amino acids (underlined) were replaced with alanine as described in Materials and Methods. Mutant designations are indicated below the unlined amino acids that were changed (i.e., 1 = $rsbT_1$, etc.). Mutations 1 to 17 are alanine substitutions, and mutation 18 introduced a termination codon at the underlined amino acid. Four previously described mutations in *rsbT* are indicated by the arrows at the sites affected. *rsbT15IS*, *rsbT63Qterm*, and, *rsbT107VG* have reduced RsbT activity and were isolated as suppressors of the heightened σ^B activity in RsbX$^-$. *B. subtilis* (29). *rsbT78DN* was a specific alteration created to disrupt RsbT's kinase activity (22). Boldface sequences delineate RsbT regions that are conserved among protein kinases (21).

digital images by using an Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, Calif.) and its associated software.

RESULTS AND DISCUSSION

Mutations in *rsbT*. RsbT, an essential component of the σ^B physical stress pathway, displays multiple interactions and activities. In the yeast dihybrid assay, RsbT binds to itself, RsbR, RsbS, and RsbU, as well as to the GTP-binding protein Obg, with sufficient affinity to activate Gal4-dependent transcription (27, 34). In vitro, RsbT can form a high-molecular-weight complex with RsbR and RsbS, phosphorylate either of these proteins with ATP as a $P0_4$ donor and activate the RsbU-dependent dephosphorylation of RsbV-P (12, 16, 37).

Previously, three spontaneous *rsbT* mutations had been isolated as *rsbT* alleles with reduced activity (29). These included an isoluecine-to serine change at position 15, a nonsense mutation at position 63, and a valine-to-glycine substitution at position 107. An additional *rsbT* mutation was created at a residue critical for RsbT's kinase activity (Asp_{78}) (22). This change, aspartate to asparagine, produced a mutant RsbT that was still able to activate RsbU but unable to inactivate RsbS by phosphorylation (22). The amino acid sequence of RsbT, including these four mutations, is illustrated in Fig. 2.

The small size of RsbT (133 amino acids) makes it suitable for a genetic analysis by alanine scanning mutagenesis. Relatively few directed changes could be used to span the entire length of the protein. In the present study, we replaced clusters of charged amino acids at sites throughout the length of the protein with alanine. Charged amino acids were chosen because they are likely residues to participate in protein-protein interactions, ligand binding, and/or catalysis. In addition, such amino acids have a high probability of being surfaced exposed and, as such, are less likely to be critical to the protein's general stability. Alanine was the chosen substitute because it is uncharged and compatible with most secondary structures.

A total of 17 dispersed positions (Fig. 2) where one or more charged amino acids are found along the length of RsbT were chosen as sites for alanine substitutions. The charged amino acids at each of these 17 sites were changed in unison to alanine by using standard PCR-based mutagenesis techniques (see Materials and Methods for details). In addition to the alanine substitutions, a termination codon was created at position 18 to remove the distal 14 amino acids and allow an assessment of the possible role of a putative carboxy-terminal alpha-helix on RsbT's activity. Substitutions 7, 8, and 13 fall within regions of the protein that are conserved among protein kinases (Fig. 2, boldface) and presumably could affect RsbT's ability to phosphorylate RsbR and RsbS.

Expression of the variant *rsbTs* in *E. coli* and *B. subtilis*. The *E. coli* plasmids that were created to transfer the mutant *rsbT* alleles to *B. subtilis* (see Materials and Methods) encode *rsbR* and *rsbS*, as well as *rsbT* downstream of the *sigB* operon's P_A promoter (i.e., P_A*rsbR-S-T*). P_A is recognized by both *B. subtilis* and *E. coli* RNA polymerase. This circumstance allows us to express the *rsbT* variants in *E. coli* and test their ability to accumulate in this bacterium. The resulting RsbT levels may serve as an indication of the inherent stability of the mutant proteins, separate from any potential regulation of RsbT stability in *B. subtilis*.

E. coli transformed with the *rsbT* plasmids were inoculated into Luria broth and grown overnight. Whole-cell lysates of each culture were analyzed by Western blotting with antibodies reactive to RsbT and RsbR proteins. The relative abundance of the RsbR protein serving as a standard for normalizing expression in the different isolates. Variations in RsbT levels, relative to that of RsbR, can then be taken as a measure of the changes in RsbT stability caused by the particular mutation that it carries.

The results of the Western blot analyses of the expression of wild-type RsbT and the 18 RsbT variants in *E. coli* are summarized in Table 2. RsbT abundance was reduced in strain T_3 and not detectable in strains T_{14} and T_{17}. The remaining RsbT variants accumulated at levels that were similar to that observed for the wild-type RsbT protein.

XS352 is a strain of *B. subtilis* with an *rsbS-T* deletion that removes the last 96 codons of the 121 codon *rsbS* gene and the first 16 codons of the 133 codon *rsbT* gene (data not shown). Neither RsbS nor RsbT is detectable in XS352 by Western blotting. pDRNT-based plasmids, encoding either the wild-type or altered *rsbT* alleles, were transformed into XS352 and plated under conditions for the selection of transformants in which the plasmids had integrated into the chromosome by single-site homologous recombination at *sigB*. Although the relative lengths of homologous DNA on the plasmids (ca. 1 kbp upstream and 350 bp downstream of the Δ*rsbS-T* deletion) should favor plasmid integration upstream of the deletion, reformation of a wild-type *rsbT* allele is possible if the recom-

TABLE 2. Accumulation and activity of RsbT variants[a]

rsbT allele	Accumulation and/or activity (%)		
	E. coli	*B. subtilis*	Inducibility
Wild type	+	+	+
T_1	+	+	+
T_2	+	± (38%)	+
T_3	+	± (51%)	−
T_4	+	+	−
T_5	+	+	−
T_6	+	+	+
T_7	+	+	−
T_8	+	± (20%)	−
T_9	+	+	+
T_{10}	+	± (24%)	+
T_{11}	+	± (42%)	+
T_{12}	+	+	+
T_{13}	+	± (34%)	−
T_{14}	−	−	−
T_{15}	+	−	−
T_{16}	+	+	−
T_{17}	−	−	−
T_{18}	+	−	−

[a] The columns represent detection by Western blotting of the various *rsbT* products in *E. coli* and *B. subtilis* and their abilities to activate σ^B after ethanol treatment. For *E. coli*, "+" represents a detectable product regardless of level, and "−" represents undetectable RsbT. For *B. subtilis*, quantitation of the Western blots was performed with an Alpha Imager 2000 and its associated software. RsbT variants that accumulated at levels approximating that of wild-type RsbT are designated "+"; those displaying background signal levels are designated "−" and those with intermediate accumulation of RsbT are depicted as "±," along with their relative abundances as a percentage of the wild-type RsbT levels. A "+" in the inducibility column reflects the ability of *B. subtilis* carrying the particular *rsbT* allele to display σ^B induction (5- to 10-fold increase in σ^B-dependent reporter gene activity) by 15 min after ethanol addition. The "−" strains displayed no difference in the activities of treated and untreated cultures.

bination event occurs within the residual *rsbT* sequences that were both downstream of the *rsbS-T* deletion in the chromosome and the upstream of the *rsbT* mutations on the plasmids. To preclude the inclusion of such a transformant in our analyses, the *sigB* regions of the transformants were examined by using a series of selective PCR analyses to verify that the plasmids had integrated upstream of the Δ*rsbS-T* deletion. All of the transformants that we analyzed contained the integrations at the upstream site (data not shown).

After transfer of the *rsbT* alleles to *B. subtilis*, Western blot analyses were undertaken on cultures of each transformant to determine whether the variant RsbTs would accumulate at levels similar to wild-type RsbT in *B. subtilis*. As was done with the *E. coli* extracts, the analyses included anti-RsbR antibody to normalize the Western blots of extracts with potentially different RsbT levels.

The results of an analysis on all 18 *rsbT* mutant strains and a wild-type *rsbT* control are shown in Fig. 3 and summarized in Table 2. RsbT is present at lower levels in T_2, T_3, T_8, T_{10}, T_{11}, and T_{13} and not detectable in mutant strains T_{14}, T_{15}, T_{17}, and T_{18}. The remaining eight alleles accumulated RsbT at approximately wild-type levels. The most notable finding from this analysis is the apparent importance of RsbT's carboxy terminus to its stability. The deletion of the terminal 14 amino acids (T_{18}) or substitutions in four of the five charged amino acid clusters that preceded the site of the deletion yielded RsbT variants that either failed to be detected (T_{14}, T_{15}, and T_{17}) or were present at a reduced level (T_{13}). The instability of RsbT 14 and 17 was common to both *E. coli* and *B. subtilis*; however, the reduced levels of the other RsbTs appeared to be *Bacillus* specific.

Specific *rsbT* alleles fail to respond to stress. Having determined the degree to which the products of the various *rsbT* alleles accumulate in *B. subtilis*, we examined their ability to activate σ^B after exposure to stress. *B. subtilis* strains expressing either a wild-type or mutant *rsbT* allele and a σ^B-dependent reporter system (*ctc*::*lacZ*) were grown in Luria broth and then exposed to ethanol at concentrations (4%) that normally activate σ^B. β-Galactosidase assay results for strains expressing each of the 18 *rsbT* mutants or a wild-type rsbT allele are summarized in Table 2. Strains containing 11 of the mutant alleles—T_3, T_4, T_5, T_7, T_8, T_{13}, T_{14}, T_{15}, T_{16}, T_{17}, and T_{18}—failed to induce reporter gene activity after ethanol stress. Three of the six strains that accumulated less RsbT (T_3, T_8, and T_{13}) and the four strains with undetectable RsbT (T_{14}, T_{15}, T_{17}, and T_{18}) in the Western blot analyses were included in this

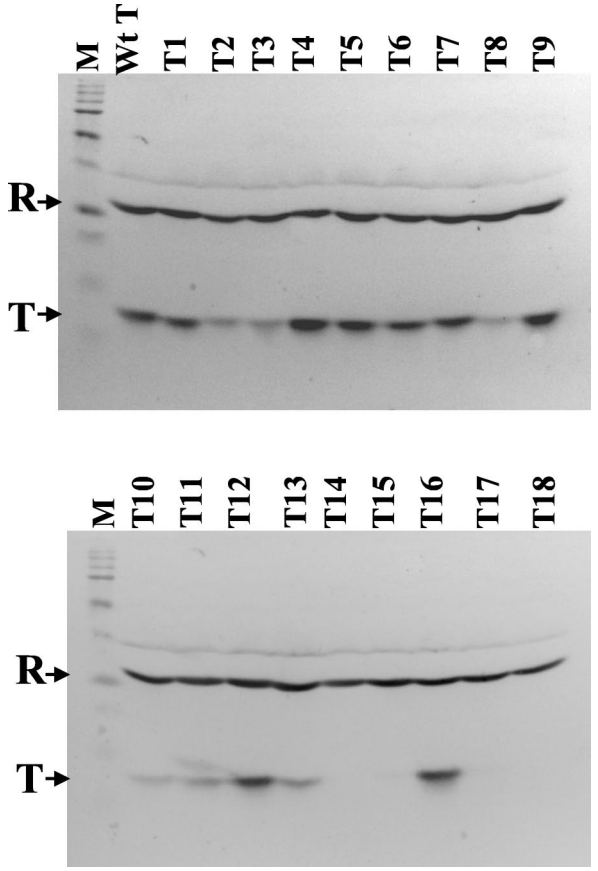

FIG. 3. Western blot analysis of RsbT variants in *B. subtilis*. Stationary-phase cultures of *B. subtilis* (BSA661 T_0 to T_{18}), expressing *rsbR-S* and either wild-type *rsbT* (wtT) or one of the *rsbT* variants (i.e., T_1, T_2, . . ., etc.) from a pDRNT plasmid integrated at *sigB* were analyzed by Western blotting as described in Materials and Methods with anti-RsbR and anti-RsbT monoclonal antibodies as probes. The positions of the RsbR (R) and RsbT (T) proteins are indicated. The band that migrated above RsbR is a cross-reactive *B. subtilis* protein (ca. 40 kDa) present in stationary-phase extracts (15). Lane M contains "broad-range" prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis molecular mass standards (Bio-Rad, Hercules, Calif.) that were electrophoresed and transferred with the crude extracts.

TABLE 3. σ^B activation after induced expression of *rsbT*[a]

Genotype	Mean β-galactosidase activity (Miller units) ± SD	
	Uninduced	Induced
rsbT/P_{SPAC}*rsbT*	7 ± 2	25 ± 2
rsbT/P_{SPAC}*rsbT*$_3$	5 ± 2	6 ± 2
rsbT/P_{SPAC}*rsbT*$_4$	3 ± 2	4 ± 3
rsbT/P_{SPAC}*rsbT*$_5$	5 ± 3	19 ± 2
rsbT/P_{SPAC}*rsbT*$_7$	4 ± 2	14 ± 2
rsbT/P_{SPAC}*rsbT*$_{13}$	6 ± 2	5 ± 2
rsbT/P_{SPAC}*rsbT*$_{16}$	7 ± 2	12 ± 2
Δ*rsbS-T*/P_{SPAC}*rsbT*	2 ± 2	19 ± 4
Δ*rsbS-T*/P_{SPAC}*rsbT*$_3$	3 ± 3	3 ± 2
Δ*rsbS-T*/P_{SPAC}*rsbT*$_4$	2 ± 2	2 ± 2
Δ*rsbS-T*/P_{SPAC}*rsbT*$_5$	1 ± 1	1 ± 1
Δ*rsbS-T*/P_{SPAC}*rsbT*$_7$	1 ± 1	1 ± 1
Δ*rsbS-T*/P_{SPAC}*rsbT*$_{13}$	1 ± 1	2 ± 2
Δ*rsbS-T*/P_{SPAC}*rsbT*$_{16}$	1 ± 1	1 ± 1

[a] *B. subtilis* strains with a wild-type *rsbT* allele at the *sigB* operon (*rsbT*) or a *rsbS-T* deletion at that locus (Δ*rsbS-T*) and one of the indicated *rsbT* alleles under the control of an IPTG-inducible promoter (P_{SPAC}) on a multicopy plasmid (Table 1) were grown in Luria broth with (induced) or without (uninduced) IPTG. Samples were taken 1 h after IPTG addition and then assayed for σ^B-dependent (*ctc*::*lacZ*) β-galactosidase activity. The values (Miller units) represent the average of three independent assays.

group. Mutants T_3, T_4, and T_5 lay within a region at the amino-terminal portion of the protein that, based on a GOR_4 analysis of its primary structure (http://abs.cit.nih.gov/gov/), is likely to form an alpha-helix. Mutants T_7, T_8, and T_{13} fall within regions of RsbT with high homology to protein kinases. Mutant T_{16} lies within a predicted alpha-helical domain near the carboxy-terminal portion of the protein, near where an RsbT-inactivating mutation (*rsbT*$_{107}$*VT*) was found in an earlier study (29).

Activation of σ^B by induced RsbT synthesis. Four of the *rsbT* mutants (T_4, T_5, T_7, and T_{16}) that made normal levels of RsbT in *B. subtilis* but did not respond to ethanol stress, as well as two additional nonresponsive mutants (T_3 and T_{13}) whose RsbT levels were greater than that of a stress-activatable rsbT variant (T_{10}) with a reduced level of RsbT, were selected for further study. Elevated expression of *rsbT* can activate σ^B in the absence of stress (21, 27). The reason for this phenomenon is not certain, but presumably it represents a failure of RsbR/S to effectively inhibit the higher levels of RsbT. Plasmids, capable of replication in *B. subtilis* and carrying one of the *rsbT* alleles under the control of an IPTG-inducible promoter were constructed. Wild-type *B. subtilis* (BSA46), transformed with these plasmids and growing in Luria broth, was exposed to IPTG to induce the expression of *rsbT*. Cells were harvested after 1 h and then examined for the effects of *rsbT* expression on σ^B-dependent reporter gene activity (*ctc*::*lacZ*). *B. subtilis* strains carrying three (T_5, T_7, and T_{16}) of the six *rsbT* variants activated σ^B when their expression was induced, although the degree of activation was still lower than that seen when the wild-type *rsbT* allele was expressed in this system (Table 3).

The strains used in the heightened expression assay have a wild-type copy of *rsbT* at the *sigB* operon, as well as the plasmid-encoded IPTG-inducible *rsbT* allele. As such, there are two plausible mechanisms by which heightened expression of the *rsbT* alleles could activate σ^B in this strain. The induced RsbTs could directly activate the RsbU phosphatase or, alternatively, they might compete for the negative regulators of RsbT (i.e., RsbR and RsbS), thereby allowing the wild-type RsbT protein to become free to activate RsbU. To distinguish between these possibilities, the *rsbT*-expressing plasmids were placed in the Δ*rsbS-T* strain of *B. subtilis* and reexamined for the ability of the *rsbT* variants to activate σ^B without the possibility of activation by wild-type RsbT or inhibition by RsbS. The cultures were grown, induced for *rsbT* expression by the addition of IPTG, and analyzed for σ^B-dependent reporter gene (*ctc*::*lacZ*) expression as described above. Unlike the previous experiment, in which several of the *rsbT* variants could activate σ^B when expressed in the presence of the wild-type RsbT, no induction of σ^B was evident when those alleles were expressed in its absence (Table 3). Thus, none of the *rsbT* variants that were unable to respond to stress appears to be capable of directly activating RsbU.

If the inability of the variant RsbTs to activate RsbU does not inhibit their RsbU binding, the presence of these proteins might interfere with the ability of the wild-type RsbT protein to interact with RsbU and activate σ^B in response to stress. To explore this possibility, *B. subtilis* strains expressing a wild-type *rsbT* allele from the *sigB* operon, as well as one of the mutant *rsbT* alleles from the IPTG-inducible promoter on a multicopy plasmid, were examined for σ^B activation after exposure to ethanol. *B. subtilis* strains, expressing additional wild-type or variant *rsbT* from the inducible promoter, had distinct background levels of σ^B activity, reflecting the inherent activity of the RsbT made in each strain. However, upon exposure to ethanol stress, each of the strains elevated σ^B-dependent β-galactosidase activity by approximately 30 to 40 Miller units (data not shown). Thus, none of the mutant RsbTs appears to inhibit wild-type RsbT's ability to trigger σ^B induction in response to stress. Either the mutant RsbTs do not bind to RsbU or RsbU's abundance is sufficiently high so that the binding of the variant RsbTs is insufficient to deny wild-type RsbT access to RsbU. In summary, none of the RsbT variants that accumulate, but fail to induce σ^B in response to stress, are able to activate RsbU or block wild-type RsbT's access to it. At least some of these (i.e., *rsbT*$_5$, *rsbT*$_7$, and *rsbT*$_{16}$) do, however, appear to retain the ability to compete for the negative regulators of RsbT and allow the wild-type allele to show activity when the mutant alleles are expressed at elevated levels in a merodiploid strain.

Analysis of RsbT interactions in the yeast dihybrid assay. Previously, the yeast dihybrid system demonstrated that RsbT is able to interact with itself, RsbR, RsbS, RsbU, and Obg, a ribosome-binding protein needed for stress activation of σ^B (27, 34). In an attempt to further characterize the six mutant RsbTs that accumulate but fail to activate σ^B, we used the yeast dihybrid system to examine the binding properties of the RsbT variants.

The *rsbT* alleles were cloned into plasmid vectors in such a way as to create translational fusions between *rsbT* and the DNA-binding domain of the GAL4 yeast gene activator protein. To verify that the *rsbT*::*GAL4* fusion proteins formed from each *rsbT* allele would be able to accumulate at comparable levels in yeast, Western blot analyses were performed on yeast strains carrying each of the *rsbT* fusions, with an *rsbT*-specific monoclonal antibody as a probe. A protein of the anticipated size of the RsbT::Gal4BD (30 kDa) was present at

TABLE 4. Activation of *lacZ* reporter gene by RsbT-Gal4 chimeras[a]

AD fusion	Reporter gene activity (Miller units) with BD fusion:							
	Vector (pAS2-1)	*rsbT*	*rsbT*$_3$	*rsbT*$_4$	*rsbT*$_5$	*rsbT*$_7$	*rsbT*$_{13}$	*rsbT*$_{16}$
Vector (pACT2)	W	0.19	W	W	0.11	0.75	W	W
rsbR	0.05	1.35	W	0.04	0.83	1.02	W	W
rsbS	0.07	50.14	W	0.05	0.14	1.27	W	W
rsbT	0.03	0.68	W	0.05	0.14	0.59	W	W
rsbU	0.05	0.50	W	W	0.55	2.29	W	W
obg	0.05	0.48	W	W	0.21	1.53	W	W

[a] The columns represent yeast clones carrying the Gal4 DNA-binding domain (BD) vector pAS2- 1 either without insert DNA (vector) or with one of the *rsbT* alleles depicted, translationally fused to the Gal4 DNA-binding domain. The rows represent the Gal4 activation domain (AD) vector pACT2 with or without translational fusions to the RsbT regulators that are listed. Data depict reporter gene (*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ*) activity in yeast cells cotransformed with both of the plasmids indicated by the rows. W, pairings in which the yeast clones failed to give a blue color after freeze-thawing and exposure to X-Gal. The numbers represent a measurement of *lacZ* activity in those clones that displayed a blue colony phenotype after exposure to X-Gal. The values represent the average of duplicate assays of three independent clones for each plasmid pair. Aside from a Gal4-dependent *lacZ* gene, the host yeast strain (Y190) also contains *GAL1*$_{UAS}$-*HIS3*$_{TATA}$-*HIS3*, allowing histidine prototrophy if an active GAL4 regulator is formed. All of the blue colonies (i.e., pairings represented by numbers), but none of the white colonies (W), could form colonies on media lacking histidine. X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

similar levels in extracts of yeast strains that expressed either the wild-type *rsbT* or the *rsbT* variant fusion proteins (data not shown). Plasmids carrying the *rsbT* fusions were then transformed into yeast strains expressing the GAL4 activating domain fused to RsbU, RsbR, RsbS, or Obg. The recipient yeast strains lacked an intact *GAL4* gene but carried both a gene needed for histidine prototrophy (*GAL1*$_{UAS}$-*HIS3*$_{TATA}$-*HIS3*) and an *E. coli lacZ* gene (*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ*) expressible from GAL4-activated promoters. Plasmid-containing yeast were selected on the basis of Trp and Leu prototrophy, (markers carried on each of the plasmids) and screened for GAL4-dependent histidine prototrophy and β-galactosidase activity (34). The Gal4 activation data are summarized in Table 4.

Although one of the mutant *rsbT* alleles (T_7) showed an enhanced activity with two of the RsbT binding partners (Obg and RsbU), a common feature of the six mutant *rsbT* alleles is a reduced activity, with most of the proteins typically bound by wild-type RsbT. This is quite evident in the pairings with RsbS, normally one of the strongest of the interactions between wild-type RsbT and its partners but markedly reduced in all six of the mutant *rsbT* alleles. This is curious given that a decreased affinity for the RsbS, the principal negative regulator of RsbT, might be expected to enhance RsbT's activity, and yet all of these RsbT variants, including T_7, an RsbT with heightened RsbU binding, are inactive.

At least two possibilities can be suggested. One possibility is that RsbT might require an interaction with RsbR and RsbS in order to be properly configured to activate RsbU and that without this interaction RsbU will remain in an inactive state. Such a model is not supported, however, by the observation that RsbT-dependent σ^B activity is very high in *B. subtilis* lacking RsbS (21). An alternative possibility is that similar regions of RsbT are required for proper interaction with both RsbS and RsbU. Common sites of contact between alternative binding partners have, in fact, been documented in the partner switching system responsible for the control of the *Bacillus* transcription factor σ^F (11, 17). In that instance, the regions of the σ^F inhibitor (SpoIIAB) that participate in its binding to σ^F are also involved in its association with the competing release factor, SpoIIAA (11, 17). A similar circumstance would explain why there were no constitutively active mutant RsbTs among our collection. If the same RsbT domains were involved in both its negative control by RsbS and its ability to activate RsbU, highly specific mutations might be needed to disrupt one activity without influencing the other.

The three *rsbT* alleles, whose products presumably compete for RsbS and allow the wild-type *rsbT* to display activity (Table 3), are poor RsbS binding partners in the yeast dihybrid assay. This suggests that the interactions that occur between these proteins in *B. subtilis* may not be mirrored in the yeast system. Chen et al. (12) have recently demonstrated that RsbR must be present if RsbS is to function as an effective inhibitor of RsbT. Perhaps the binding reaction that is observed between RsbS and RsbT in the yeast system only partially reflects the binding that occurs between RsbT and the RsbR/S complex in *B. subtilis*. If this is true, our "activating" *rsbT* alleles might still interact with RsbS in *B. subtilis* but not display significant binding in yeast. Alternatively, the apparent loss of interaction between the RsbT variants and RsbS in the yeast system may be a true reflection of the state of their binding capacities. If, as the yeast dihybrid data also suggest (Table 4) (34), RsbT can interact with itself, RsbT may normally exist in a multimeric form. If this is so, the ability of some of the RsbT variants to activate σ^B when expressed in the presence of wild-type RsbT, but not when expressed alone, might be a consequence of each of these RsbTs acting in concert as RsbT heterodimers. It is possible that RsbT heterodimers, consisting of an RsbT variant with impaired RsbS binding and wild-type RsbT capable of activating RsbU, could escape RsbS inhibition and activate σ^B.

Previous studies have shown that the loss of RsbX, the σ^B regulator that reestablishes negative control over RsbT by the dephosphorylation of RsbS-P, results in constitutively high levels of σ^B activity in unstressed *B. subtilis* (4, 9, 29). This implies that even in the absence of obvious stress, *B. subtilis* RsbT is phosphorylating RsbR/S and being released from the RsbR/S complex. Thus, the binding of RsbT and the RsbR/S complex can be viewed as transient. This likely accounts for the ability of the RsbT variants, unable in themselves to activate RsbU, to compete for the RsbR/S complex and permit the wild-type RsbT to activate RsbU when their synthesis is induced from P_{SPAC}. If the RsbR/S/T complexes were stable, competition by the newly synthesized RsbTs for the complex might be difficult.

The substitution of the groups of charged amino acids along the length of RsbT by alanine and the deletion of RsbT's carboxy terminus revealed several general features of RsbT. Most notable is the importance of carboxy terminus of RsbT on its ability to accumulate in *B. subtilis*. This is demonstrated by the observation that four (T_{14}, T_{15}, T_{17} and T_{18}) of the five carboxy-terminal mutations yielded *rsbT* alleles whose products are not detectable in *B. subtilis* extracts. The accumulation defect for two of these proteins was less obvious in *E. coli*, suggesting a *Bacillus*-specific proteolytic event. It is also noteworthy that four (T_9, T_{10}, T_{11}, and T_{12}) of the five substitutions in the central region of the protein had no measurable effect on RsbT's activity. Only when a substitution (T_{13}) extended into a

region of kinase homology was RsbT activity affected. Apparently, the presence of charged amino acids in this region does not contribute significantly to RsbT's functions. Finally, in cases in which the substitutions disrupted RsbT's ability to respond to stress while still allowing the mutant allele's products to accumulate (T_3, T_4, T_5, T_7, T_{13}, and T_{16}), the effects of the mutations were not limited to a single RsbT property. These mutations altered most of the variant proteins' binding properties in the yeast dihybrid system, as well as affecting both their ability to activate RsbU and their ability to compete for the RsbR/S complex. Presumably, these changes either dramatically altered the proteins' tertiary structure without dramatically affecting their stability or define particular regions of RsbT that are involved in multiple interactions.

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