

Stressosomes Formed in *Bacillus subtilis* from the RsbR Protein of *Listeria monocytogenes* Allow σ^B Activation following Exposure to either Physical or Nutritional Stress[∇]

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The general stress regulon of *Bacillus subtilis* is controlled by σ^B , a transcription factor that is activated by physical or nutritional stress. In *B. subtilis*, each of these two stresses is communicated to the primary σ^B regulators by distinct pathways. Physical stress activation of σ^B involves a large-molecular-mass (>10⁶-Da) structure (stressosome) formed by one or more homologous proteins (RsbRA, -B, -C, and -D) onto which the pathway's principal regulators are bound. The RsbR proteins are thought to be potential receptors for stress signaling. *Listeria monocytogenes* encodes orthologs of σ^B and its principal regulators; however, unlike *B. subtilis*, *L. monocytogenes* appears to use the stressosome pathway for both physical and nutritional stress activation of σ^B . In the current work, a *B. subtilis* strain that expressed *L. monocytogenes* *rsbR* (*rsbR_{Lm}*) in lieu of *B. subtilis* *rsbR* (*rsbR_{Bs}*) was created and was found to display the *Listeria* phenotype of σ^B activation following exposure to either physical or nutritional stress. *B. subtilis* expressing either the RsbR paralog *rsbRC* or *rsbRD*, but not *rsbRA* or *rsbRB*, as the sole source of RsbR also allowed σ^B induction following nutritional stress. It is unclear whether the nutritional stress induction seen in these strains is the result of a direct effect of nutritional stress on stressosome activity or a consequence of the background levels of σ^B activation in these strains and the effects of diminished ATP on the downstream phosphorylation reaction needed to reinactivate σ^B .

σ^B is an alternative sigma factor of *Bacillus subtilis* that directs RNA polymerase to the promoters for the more than 150 genes that make up the bacterium's general stress regulon (GSR) (23, 24, 32, 33, 40). The GSR is activated when exposure to physical (e.g., ethanol or heat or osmotic shock) or nutritional (e.g., glucose or phosphate starvation or O₂ limitation) stress initiates a series of reactions which frees σ^B from an inhibitory association with the anti- σ^B protein (RsbW) (6, 7, 8, 9). Release of σ^B from RsbW requires the binding of an additional protein (RsbV) to RsbW (16, 17). In unstressed *B. subtilis*, RsbV is not able to trigger σ^B release, due to an RsbW-dependent phosphorylation (17). Phosphorylated RsbV (RsbV-P) is dephosphorylated and reactivated by either of two stress-responsive phosphatases (RsbP or RsbU) (25, 39, 41, 42, 44).

The RsbP phosphatase and an additional protein (RsbQ) are required for nutritional stress activation of σ^B in wild-type *B. subtilis* (10, 39). The inducer of RsbPQ phosphatase activity is unknown. Recently, red light has also been shown to be a potential RsbP-dependent activator of σ^B ; however, the details of this activation and its relationship to nutritional stress activation remain to be resolved (5). RsbU, the phosphatase that reacts to physical stress, also requires an additional protein (RsbT) for activity (44). In unstressed *B. subtilis*, RsbT is se-

questered with its primary negative regulator (RsbS) in a large (1.8-MDa) complex formed by a family of homologous proteins: RsbR (RsbRA), YkoB (RsbRB), YojH (RsbRC), and YqhA (RsbRD) (1, 2, 14, 15, 28, 30). This complex of RsbR, -S, and -T proteins has been termed the "stressosome" (28, 30). Physical stress is believed to trigger an RsbT-dependent phosphorylation of both RsbR and RsbS, which allows the release of RsbT and its activation of RsbU (14, 20, 22, 44). The system is reset by RsbX, an additional phosphatase that can dephosphorylate RsbR-P and RsbS-P, allowing their reactivation and the potential resequestration of RsbT (13, 15, 44).

rsbRA is cotranscribed in an 8-gene operon with the σ^B structural gene and other key σ^B regulators, while the *rsbR* paralogs are expressed from diverse sites along the *B. subtilis* chromosome (2, 43). A fifth RsbR-like paralog has been described (YtvA); it can cofractionate with stressosomes but is unique in that it lacks the RsbT-dependent phosphorylation sites found on the other RsbR paralogs (4, 19). Instead, YtvA carries a light, oxygen, or voltage (LOV) domain. YtvA has been found to enhance σ^B activation in the presence of blue light, although the mechanism involved is unknown (4, 19, 38).

In vitro, the phosphorylation of RsbR promotes the phosphorylation of RsbS (13, 14, 20). This observation suggests that the stress activation process *in vivo* could proceed through RsbR to RsbS. As such, the RsbR proteins themselves might serve as targets for intracellular signals that might promote their susceptibility to phosphorylation by RsbT (18, 27). Cryo-electron microscopy (cryo-EM) analyses of stressosomes revealed a structure formed of multiple RsbR molecules with their C-terminal regions arranged as a base onto which RsbS and RsbT are bound (30). Studies of stressosome assembly and composition indicate that individual stressosomes are likely to

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TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Relevant genotype
Plasmids	
pARE241	Ap ^r Cm ^r P _A Δ <i>rsbRA1</i> <i>rsbS</i> <i>rsbT</i>
pLAM2	Ap ^r Cm ^r P _A <i>rsbR_{Lm}</i> <i>rsbS</i> <i>rsbT</i>
<i>B. subtilis</i> strains	
PY22	Wild type
BAR340	Δ <i>rsbRB2</i> Δ <i>rsbRC1</i> :: <i>ery</i> <i>rsbP</i> :: <i>spc</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BAR343	Δ <i>rsbRA1</i> (Cm) Δ <i>rsbRBI</i> :: <i>kan</i> Δ <i>rsbRC1</i> :: <i>ery</i> <i>rsbP</i> :: <i>spc</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSA70	SPβ <i>ctc</i> :: <i>lacZ</i> (Cm Ery)
BSH80	SPβ <i>ctc</i> :: <i>lacZ</i> (Tet Ery)
BSH176	<i>rsbQ</i> :: <i>ery</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSH192	<i>rsbQ</i> ::TnYLB-1 SPβ <i>ctc</i> :: <i>lacZ</i>
BSH304	Δ <i>rsbRB2</i> Δ <i>rsbRC1</i> :: <i>ery</i> Δ <i>rsbRD1</i> :: <i>spc</i> <i>rsbQ</i> ::TnYLB-1 SPβ <i>ctc</i> :: <i>lacZ</i>
BSH305	Δ <i>rsbRA1</i> (Cm) Δ <i>rsbRC1</i> :: <i>ery</i> Δ <i>rsbRD1</i> :: <i>spc</i> <i>rsbQ</i> ::TnYLB-1 SPβ <i>ctc</i> :: <i>lacZ</i>
BSH306	Δ <i>rsbRA1</i> (Cm) Δ <i>rsbRBI</i> :: <i>kan</i> Δ <i>rsbRD1</i> :: <i>spc</i> <i>rsbQ</i> :: <i>ery</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSH308	Δ <i>rsbRA1</i> (Cm) Δ <i>rsbRBI</i> :: <i>kan</i> <i>rsbQ</i> :: <i>ery</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSH311	Δ <i>rsbRA1</i> (Cm) Δ <i>rsbRB2</i> Δ <i>rsbRD1</i> :: <i>spc</i> <i>rsbQ</i> :: <i>ery</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSH312	Δ <i>rsbRA1</i> (Cm) Δ <i>rsbRB2</i> Δ <i>rsbRD1</i> :: <i>spc</i> <i>rsbQ</i> :: <i>ery</i> <i>rsbU</i> :: <i>kan</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSH313	Δ <i>rsbRA1</i> (Cm) Δ <i>rsbRB2</i> Δ <i>rsbRC1</i> :: <i>ery</i> <i>rsbP</i> :: <i>spc</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSH314	Δ <i>rsbRA1</i> (Cm) Δ <i>rsbRB2</i> Δ <i>rsbRC1</i> :: <i>ery</i> <i>rsbP</i> :: <i>spc</i> <i>rsbU</i> :: <i>kan</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSH315	Δ <i>rsbRA1</i> (Cm) Δ <i>rsbRD1</i> :: <i>spc</i> <i>rsbQ</i> :: <i>ery</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSH316	Δ <i>rsbRB2</i> Δ <i>rsbRD1</i> :: <i>spc</i> <i>rsbQ</i> :: <i>ery</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSH317	Δ <i>rsbRB2</i> Δ <i>rsbRC1</i> :: <i>ery</i> <i>rsbP</i> :: <i>spc</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSH318	P _{SPAC} <i>rsbT</i> <i>rsbP</i> :: <i>spc</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSJ43	<i>rsbP</i> :: <i>spc</i> SPβ <i>ctc</i> :: <i>lacZ</i>
BSL20	<i>ycdE</i> ::Cm <i>rsbQ</i> ::TnYLB-1 SPβ <i>ctc</i> :: <i>lacZ</i>
BSL24	P _A <i>rsbRD</i> (Δ <i>rsbRA</i>) Δ <i>rsbRB2</i> Δ <i>rsbRC1</i> :: <i>ery</i> Δ <i>rsbRD1</i> :: <i>spc</i> <i>rsbQ</i> ::TnYLB-1 SPβ <i>ctc</i> :: <i>lacZ</i>
BSL26	P _A <i>rsbRA</i> :: <i>rsbR_{Lm}</i> (Cm) Δ <i>rsbRB2</i> Δ <i>rsbRC1</i> :: <i>ery</i> Δ <i>rsbRD1</i> :: <i>spc</i> <i>rsbQ</i> ::TnYLB-1 SPβ <i>ctc</i> :: <i>lacZ</i>
BSL28	P _A <i>rsbR</i> :: <i>rsbR_{Lm}</i> (Cm) <i>rsbQ</i> ::TnYLB-1 SPβ <i>ctc</i> :: <i>lacZ</i>

be mosaics of multiple RsbR paralogs; however, the explicit functions of the multiple RsbR proteins are unclear (15, 28, 35). The N-terminal portions of these proteins, which appear to project outward from the stressosome, are much less conserved than their C-terminal regions (2, 30). This heterogeneity raised the possibility that each of the RsbR paralogs could serve as a receptor for a unique stress signal; however, a study examining the effects of the loss of one or more of the paralogs failed to demonstrate selective responsiveness. The loss of one or several of the RsbR-encoding genes did not prevent σ^B activation by any of the usual assortment of environmental inducers (2). Only when all of the *rsbR* genes were deleted did σ^B activity become unresponsive to physical stress (2). The persistent ability of σ^B to be induced by diverse physical stresses in the absence of one or more of the RsbR paralogs was interpreted as evidence that the responsiveness of the RsbR proteins to physical stress is overlapping. Given the heterogeneity of the amino termini of the RsbR proteins, their common responsiveness to the same physical stresses could be a consequence of each RsbR paralog responding to one of multiple signals generated by exposure to a given stress. In this view, each of the RsbR proteins would respond to a novel activator, although not in an obvious stress-specific manner.

Listeria monocytogenes encodes a σ^B ortholog, as well as counterparts of the regulatory proteins that control *B. subtilis* σ^B activation in response to physical stress (11, 12, 23, 31). *L. monocytogenes* σ^B is activated by both physical and nutritional stress; however, the essential components of *B. subtilis* σ^B nutritional stress activation (RsbPQ) are lacking in *L. monocytogenes* (11, 12). In contrast to *B. subtilis*, *L. monocytogenes* is thought to use the components of the physical stress pathway

to activate σ^B following nutrient deprivation (11, 12). To ask whether this unique responsiveness rests with the *L. monocytogenes* RsbR protein (RsbR_{Lm}), we constructed a *B. subtilis* strain in which the *rsbR_{Lm}* gene was placed within the *sigB* operon in lieu of the *B. subtilis* ortholog as the strain's sole source of RsbR. The resulting strain allowed σ^B activation following either physical or nutritional stress. This induction of σ^B following nutritional stress, but not physical stress, was blocked if the *B. subtilis* RsbR (RsbR_{Bs}) paralogs were also present. Testing of individual RsbR paralogs for similar properties revealed that RsbRC and RsbRD also permitted σ^B induction under conditions that normally activated the nutritional stress pathway. As with RsbR_{Lm}, σ^B was not induced by nutritional stress if other RsbR proteins (RsbRA, RsbRB) were present.

MATERIALS AND METHODS

Bacterial strains and plasmids. All of the *B. subtilis* strains are derivatives of PY22 (6). The strains and their relevant genotypes are listed in Table 1. Plasmid pARE241 (P_A Δ*rsbRA1* *rsbS* *rsbT*) (35) was used to construct pLAM2, a plasmid formed by placing an *L. monocytogenes* *rsbR* gene fragment (amplified from *L. monocytogenes* LM1061 DNA, a gift of A. Benson, University of Nebraska) into a unique SalI site created immediately downstream of the initiation codon of *rsbRA* and 3 codons before the *rsbRA* termination codon. The amplified *rsbR_{Lm}* was cloned "in frame" with the residual *B. subtilis* *rsbRA* sequence, adding a Met-Ser-Thr element to its N terminus and a Val-Asp-Leu-Gly-Glu sequence to its C terminus. BSH80 has been described previously (34). BSJ43 (*rsbP*::*spc* SPβ *ctc*::*lacZ*) is BSA46 (6) transformed with a DNA fragment carrying *rsbP* disrupted at an internal HindIII site by Spc^r (21). BSH163 (*ycdE*::Cm SPβ *ctc*::*lacZ*) is BSH80 transformed with plasmid pARE212 (35). BSH176 (*rsbQ*::*ery* SPβ *ctc*::*lacZ*) is BSH80 transformed to *rsbQ*::*ery* by a DNA fragment carrying *rsbQ* with an internal NdeI 563-bp segment deleted and replaced by an Ery^r cassette (21). BSH192 (*rsbQ*::TnYLB-1 SPβ *ctc*::*lacZ*) is BSH80 with a TnYLB-1 (Kan^r)

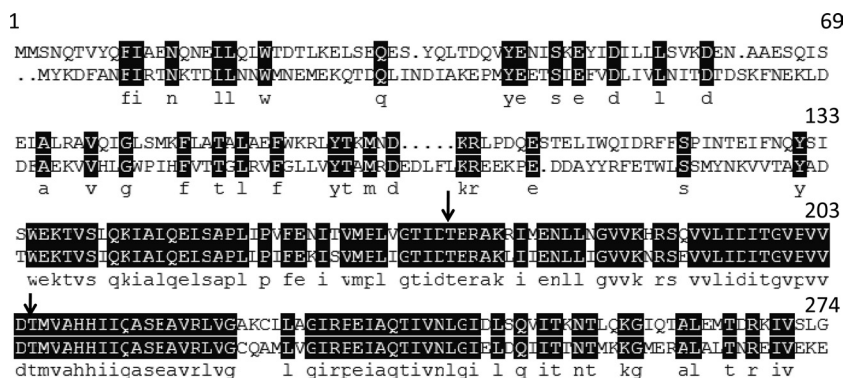


FIG. 1. Sequence comparison of RsbR of *L. monocytogenes* and RsbRA of *B. subtilis*. The sequence of the *L. monocytogenes* RsbR protein is given below that of the *B. subtilis* ortholog. The numbering is that of the *B. subtilis* protein. Identical amino acids are shaded. The conserved threonine sites for phosphorylation (T₁₇₁ and T₂₀₅) are indicated by arrows.

transposon inserted into *rsbQ* (29). BAR340 (Δ *rsbRB2* Δ *rsbRC1::ery rsbP::spc* SP β *ctc::lacZ*) and BAR343 [Δ *rsbRA1*(Cm) Δ *rsbRB1::kan* Δ *rsbRC1::ery rsbP::spc* SP β *ctc::lacZ*] are BAR203 (35) and BAR205 (35), respectively, each transformed to *rsbP::spc* with DNA from BSH343. BSH304 (Δ *rsbRB2* Δ *rsbRC1::ery* Δ *rsbRD1::spc* *rsbQ::TnYLB-1* SP β *ctc::lacZ*) and BSH305 [Δ *rsbRA1*(Cm) Δ *rsbRC1::ery* Δ *rsbRD1::spc* *rsbQ::TnYLB-1* SP β *ctc::lacZ*] are BAR298 (35) and BAR204 (35), respectively, transformed to *rsbQ::TnYLB-1* with DNA from BSH192. BSH306 [Δ *rsbRA1*(Cm) Δ *rsbRB1::kan* Δ *rsbRD1::spc* *rsbQ::ery* SP β *ctc::lacZ*] was constructed by transformation of BSH176 (*rsbQ::ery* SP β *ctc::lacZ*) with DNA from BAR199 [Δ *rsbRA1*(Cm) Δ *rsbRB1::kan* Δ *rsbRD1::spc* SP β *ctc::lacZ*] and selection first for Cm^r to create BSA217 [Δ *rsbRA1*(Cm) *rsbQ::ery* SP β *ctc::lacZ*], then for Kan^r to form BSH308 [Δ *rsbRA1*(Cm) Δ *rsbRB1::kan* *rsbQ::ery* SP β *ctc::lacZ*], and finally for Spe^r as the final step in the construction. BSH311 [Δ *rsbRA1*(Cm) Δ *rsbRB2* Δ *rsbRD1::spc* *rsbQ::ery* SP β *ctc::lacZ*] was formed in two steps. First, *rsbQ::ery* was transformed into BAR230 (Δ *rsbRB2*) from BSH176. The resulting strain, BSA309 (Δ *rsbRB2* *rsbQ::ery*), was then transformed to Δ *rsbRA1*(Cm) Δ *rsbRD1::spc* using chromosomal DNA from BSH214 [Δ *rsbRA1*(Cm) Δ *rsbRB1::kan* Δ *rsbRC1::ery* Δ *rsbRD1::spc* SP β *ctc::lacZ*] and selection for Cm^r and Spe^r. BSA312 [Δ *rsbRA1*(Cm) Δ *rsbRD1::spc* *rsbQ::ery* *rsbU::kan* SP β *ctc::lacZ*] is BSA311 transformed to *rsbU::kan* with DNA from BSA70 (6). BSH313 [Δ *rsbRA1*(Cm) Δ *rsbRB2* Δ *rsbRC1::ery* *rsbP::spc* SP β *ctc::lacZ*] was made by first transforming BAR230 (Δ *rsbRB2*) to Δ *rsbRC1::ery* with DNA from BSH214, followed by transformation to SP β *ctc::lacZ*, using Tet^r selection. The resulting strain, BSH310 (Δ *rsbRB2* Δ *rsbRC1::ery* SP β *ctc::lacZ*) was then transformed to Δ *rsbRI*(Cm) *rsbP::spc* with DNA from BAR343. BSH314 [Δ *rsbRA1*(Cm) Δ *rsbRB2* Δ *rsbRC1::ery* *rsbP::spc* *rsbU::kan* SP β *ctc::lacZ*] is BSH313 transformed to *rsbU::kan* with DNA from BSA70 (5). BSH315 [Δ *rsbRA1*(Cm) Δ *rsbRD1::spc* *rsbQ::ery* SP β *ctc::lacZ*] is BSA217 [Δ *rsbRA1*(Cm) *rsbQ::ery* SP β *ctc::lacZ*] transformed to Δ *rsbRD1::spc* with DNA from BSH214. BSH316 (Δ *rsbRB2* Δ *rsbRD1::spc* *rsbQ::ery* SP β *ctc::lacZ*) is BSH309 (Δ *rsbRB2* *rsbQ::ery*) transformed to Δ *rsbRD1::spc* SP β *ctc::lacZ* with DNA from BSH214. BSH317 (Δ *rsbRB2* Δ *rsbRC1::ery* *rsbP::spc* SP β *ctc::lacZ*) and BSH318 (*P*_{SPAC} *rsbT* *rsbP::spc* SP β *ctc::lacZ*) are BSH310 (Δ *rsbRB2* Δ *rsbRC1::ery* SP β *ctc::lacZ*) and BSA419 (*P*_{SPAC} *rsbT* SP β *ctc::lacZ*) (37), respectively, transformed to *rsbP::spc* with DNA from BSH343. BSL20 (*ydcE::Cm* SP β *rsbQ::TnYLB-1* *ctc::lacZ*) and BSL24 [*P*_A *rsbRD* (Δ *rsbRA*) Δ *rsbRB2* Δ *rsbRC1::ery* Δ *rsbRD1::spc* *rsbQ::TnYLB-1* SP β *ctc::lacZ*] are BSH163 (*ydcE::Cm* SP β *ctc::lacZ*) and BAR308 [*P*_A *rsbRD* (Δ *rsbRA*) Δ *rsbRB2* Δ *rsbRC1::ery* Δ *rsbRD1::spc* SP β *ctc::lacZ*], respectively, transformed to *rsbQ::TnYLB-1* with DNA from BSH192. BSL26 [*P*_A *rsbRA::rsbR_{Lm}*(Cm) Δ *rsbRB2* Δ *rsbRC1::ery* Δ *rsbRD1::spc* *rsbQ::TnYLB-1* SP β *ctc::lacZ*] is BSH304 transformed with plasmid pLAM2, replacing *B. subtilis* *rsbRA* with *rsbR* from *L. monocytogenes*. BSL28 [*P*_A *rsbRA::rsbR_{Lm}*(Cm) *rsbQ::TnYLB-1* SP β *ctc::lacZ*] is BSH80 transformed with DNA from BSL26, selecting for Cm^r (*P*_A *rsbRA::rsbR_{Lm}*) and Kan^r (*rsbQ::TnYLB-1*).

Growth conditions. *B. subtilis* strains were routinely grown in LB (36) with vigorous shaking (250 rpm) at 37°C. Physical stress was initiated by the addition of ethanol or NaCl to a final concentration of 4% or 2.5%, respectively. Nutritional stress was brought on by blocking the electron transport with the addition of sodium azide to 2 mM, by O₂ limitation through reducing the rate of culture shaking to 50 rpm, or by allowing the cultures to enter stationary phase in LB.

Glucose or phosphate limitation was induced by growth in a synthetic medium (42) with reduced glucose (0.05%) or KH₂PO₄ (0.18 mM). To elevate the background RsbU activity in the absence of stress, BSH318, carrying *rsbT* under the control of an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible promoter (*P*_{SPAC}), was grown and repeatedly diluted for 5 generations in LB with IPTG (0.025, 0.05, or 0.1 mM) before samples were taken for analysis of σ^B activity.

General methods. β -Galactosidase activities were determined using the chloroform-permeabilized technique of Kenney and Moran (26). *Bacillus* transformations were performed as described by Yasbin et al. (45).

RESULTS AND DISCUSSION

Expression of *rsbR_{Lm}* in *B. subtilis*. *L. monocytogenes* encodes a σ^B ortholog, as well as counterparts of the regulatory proteins that control *B. subtilis* σ^B activation in response to physical stress, in an operon that is structurally identical to the *B. subtilis* *sigB* operon (11, 12, 23, 31). As in *B. subtilis*, *L. monocytogenes* σ^B is activated by both physical and nutritional stress; however, the essential components of *Bacillus* σ^B nutritional stress activation (RsbPQ) are lacking in *L. monocytogenes* (11, 12). In contrast to *B. subtilis*, *L. monocytogenes* is thought to use the components of the physical stress pathway to activate σ^B following both physical stress and nutrient deprivation (11, 12). The RsbR protein encoded within the *Listeria* *sigB* operon is highly homologous to *B. subtilis* RsbRA in its C-terminal half (77% identical amino acids); however, at its N-terminal region, the RsbR segment proposed to be the protein's stress receptor element is much less conserved (22% identical amino acids) (Fig. 1). To ask whether *Listeria's* responsiveness to nutritional stress might rest with the RsbR_{Lm} protein, we constructed a *B. subtilis* strain in which the *rsbR_{Lm}* gene was placed within the *sigB* operon in lieu of the *B. subtilis* ortholog. The remainder of the *sigB* operon was left intact. The strain also carried disruptions in the paralogous *B. subtilis* *rsbR* genes, as well as RsbQ (*rsbQ::TnYLB-1*), a protein needed for the activity of the *B. subtilis* nutritional stress pathway's phosphatase. This left only the physical stress pathway intact, with the *Listeria* RsbR protein as its sole RsbR element.

Given the high degree of homology of the C-terminal region of RsbR_{Lm} with the corresponding regions of the *B. subtilis* RsbR proteins, it seemed likely that RsbR_{Lm} would be able to form stressosomes in *B. subtilis* that could incorporate the *B.*

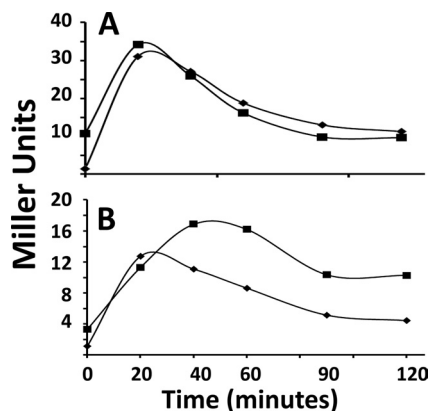


FIG. 2. σ^B induction by ethanol or salt in *B. subtilis* expressing either *rsbR_{Lm}* or *rsbRA_{Lm}*. *B. subtilis* strains BSH304 (Δ *rsbRB2* Δ *rsbRC1::ery* Δ *rsbRD1::spc* *rsbQ::TnYLB-1* SP β *ctc::lacZ*) (RsbRA⁺ strain) (\blacklozenge) and BSL26 [*P_A* *rsbRA::rsbR_{Lm}*(Cm) Δ *rsbRB2* Δ *rsbRC1::ery* Δ *rsbRD1::spc* *rsbQ::TnYLB-1* SP β *ctc::lacZ*] (RsbR_{Lm} strain) (\blacksquare) were grown in LB and subjected to 4% ethanol (A) or 2.5% NaCl (B) at the zero time point. Samples were taken at the indicated intervals and assayed for σ^B -dependent β -galactosidase activity.

subtilis regulators. To verify this, a preliminary experiment was undertaken in which crude extracts from the *rsbR_{Lm}*-expressing strain were examined by velocity centrifugation. As was done previously in similar experiments conducted on strains expressing other single *B. subtilis* *rsbR* genes (35), stressosome formation was estimated by the movement of RsbS into fast-sedimenting complexes. As anticipated, the *rsbR_{Lm}*-expressing strain displayed the presence of *B. subtilis* RsbS in high-molecular-weight fractions, indicative of stressosome formation (data not shown).

The *rsbR_{Lm}*-expressing strain and a congenic strain expressing *B. subtilis* *rsbRA* were next grown in LB and exposed to 4% ethanol or 2.5% NaCl, conditions which normally activate the *B. subtilis* stressosome-dependent pathway for σ^B induction (42). As illustrated in Fig. 2, σ^B -dependent reporter gene activity in both strains was initially low but rapidly increased following exposure to either stress. This reveals that the RsbR_{Lm} protein is able to function in *B. subtilis*, interacting with the *B. subtilis* σ^B regulators to restrict σ^B activity during growth and allow its activation when the bacterium is exposed to physical stress. Apparently, the stress signals to which the RsbR_{Lm} protein responds are also generated by stress in *B. subtilis*.

The *rsbR_{Lm}* and *rsbRA_{Lm}* strains were then either exposed to sodium azide or allowed to enter stationary phase, conditions which normally require the nutritional stress pathway for σ^B activation in wild-type *B. subtilis*. The *rsbR_{Lm}*-expressing strain, but not the strain expressing the *Bacillus* ortholog, displayed significant σ^B induction under these conditions (Fig. 3). The ability of stressosomes formed from *Listeria* RsbR, when present as the cell's sole RsbR protein, to allow σ^B activation under nutrient-limiting conditions invites the question of whether σ^B activation under these circumstances might be influenced by the presence of the *B. subtilis* paralogs, proteins that would presumably form composite stressosomes with *Listeria* RsbR. To examine this, the *Listeria* *rsbR* gene was substituted for *B. subtilis* *rsbRA* in a strain that still encoded the three

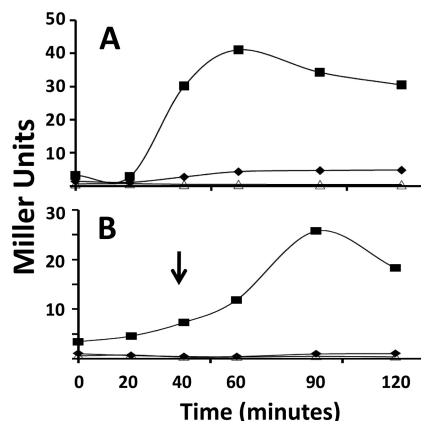


FIG. 3. σ^B induction following nutritional stress in *B. subtilis* expressing either *rsbR_{Lm}* or *rsbRA_{Bs}*. *B. subtilis* strains BSH304 (Δ *rsbRB2* Δ *rsbRC1::ery* Δ *rsbRD1::spc* *rsbQ::TnYLB-1* SP β *ctc::lacZ*) (RsbRA⁺ strain) (\blacklozenge), BSL26 [*P_A* *rsbRA::rsbR_{Lm}*(Cm) Δ *rsbRB2* Δ *rsbRC1::ery* Δ *rsbRD1::spc* *rsbQ::TnYLB-1* SP β *ctc::lacZ*] (RsbR_{Lm} strain) (\blacksquare), and BSL28 [*P_A* *rsbRA::rsbR_{Lm}*(Cm) *rsbQ::TnYLB-1* SP β *ctc::lacZ*] (\triangle) were treated with azide (2 mM) at time zero (A) or grown to stationary phase (arrow) in LB (B). Samples were taken at the indicated intervals and assayed for σ^B -dependent β -galactosidase activity.

RsbR paralogs but lacked the nutritional stress pathway phosphatase. When this strain was subjected to nutritional stress by azide treatment or entry into stationary phase, the previous RsbR_{Lm}-dependent induction of σ^B failed to occur (Fig. 3). Thus, the presence of the other RsbR proteins can block the σ^B induction observed in the strain that expressed RsbR_{Lm} alone.

RsbRC and RsbRD allow σ^B activation following nutritional stress. The observation that *Listeria* RsbR allows σ^B activation following nutritional stress when it is the sole RsbR protein present in *B. subtilis* raises the question of whether this is a unique characteristic of *Listeria* RsbR or whether other RsbR proteins share this property. Nutrient stress activation of σ^B via the physical stress pathway is not evident in *B. subtilis* expressing the full complement of RsbR proteins; however, as was seen with RsbR_{Lm}, the ability of a single RsbR protein to allow σ^B activation could be blocked in the presence of the other RsbR proteins within the cell.

In order to test the possibility that individual RsbR paralogs might be capable of allowing σ^B activation during nutritional stress, strains which carried a σ^B -dependent reporter gene (*ctc::lacZ* strains), lacked the nutritional stress phosphatase (i.e., *rsbP::spc* or *rsbQ::ery* strains), and carried disruptions within all but one of the *rsbR* genes were constructed. The *rsbR* gene that remained in each strain was expressed from its normal locus. These strains, as well as an RsbPQ⁻ strain expressing all of the *rsbR* genes, were grown in LB and exposed to ethanol to verify the presence of an intact physical stress pathway in each. All of the strains, as expected, showed induction of σ^B in response to ethanol (Fig. 4A). When these same strains were allowed to enter stationary phase, those expressing either *rsbRC* or *rsbRD* alone also displayed σ^B activation (Fig. 4B). Neither the parental strain nor the *rsbRA*- or *rsbRB*-expressing strains responded to entry into stationary phase with the induction of σ^B . The presence of both RsbRA and RsbRB is not necessary to prevent σ^B activation under pre-

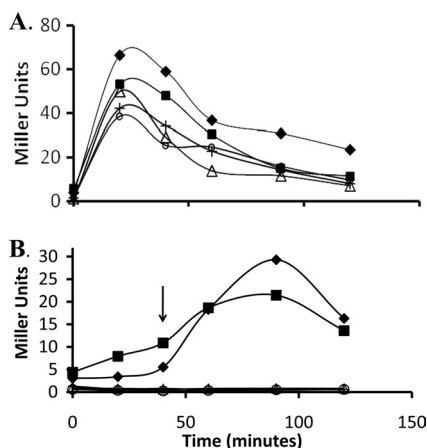


FIG. 4. σ^B induction by ethanol or entry into stationary phase in *B. subtilis* strains with single RsbR proteins. *B. subtilis* strains BSJ43 (*rsbP::spc SP β ctc::lacZ*) (○), BSH304 (*Δ rsbRB2 Δ rsbRC1::ery Δ rsbRD1::spc *rsbQ*::TnYLB-1 SP β ctc::lacZ*) (+), BSH305 [*Δ rsbRA1(Cm) Δ rsbRC1::ery Δ rsbRD1::spc *rsbQ*::TnYLB-1 SP β ctc::lacZ*] (△), BSH306 [*Δ rsbRA1(Cm) Δ rsbRB1::kan Δ rsbRD1::spc *rsbQ*::ery SP β ctc::lacZ*] (■), and BAR343 [*Δ rsbRA1(Cm) Δ rsbRB1::kan Δ rsbRC1::ery *rsbP*::spc SP β ctc::lacZ*] (◆) were grown in LB and either subjected to 4% ethanol (A) or allowed to enter stationary phase (B). Samples were taken at the indicated intervals and assayed for σ^B -dependent β -galactosidase activity. Ethanol was added at the zero time point in panel A. The entry into stationary phase is indicated by the arrow in panel B.

sumed nutritional stress. The stationary-phase induction in the *rsbRC*-expressing strains was blocked if either *rsbRA* or *rsbRB* alone was present (Fig. 5). Similar results were seen in the *rsbRD*-expressing strain (data not shown). Curiously, a strain which expresses both *rsbRC* and *rsbRD*, although still responsive to nutritional stress, was only half as effective in activating σ^B as a strain expressing each of these genes singly (Fig. 5). Apparently, the presence of both RsbRC and RsbRD forms a more effective barrier to σ^B activation following nutritional stress. Perhaps composite stressosomes, even when composed of RsbR paralogs that can singly permit σ^B activation under this condition, are less adept at allowing the response when these proteins are both present.

In order to verify that the stationary-phase induction of σ^B in the *rsbRC*- and *rsbRD*-expressing strains is dependent on the physical stress pathway, a disruption of this pathway's phosphatase (*rsbU::kan*) was introduced into the *rsbRC* and *rsbRD* strains, creating *rsbRC*- or *rsbRD*-expressing variants that lacked both the RsbU and RsbPQ phosphatases. When these strains were allowed to enter stationary phase in LB, σ^B -dependent reporter gene activity was no longer induced (data not shown).

RsbR_{Lm}, RsbRC_{Lm}, and RsbRD_{Lm} respond to multiple stimuli associated with nutritional stress activation. The ability of *B. subtilis* expressing *rsbR_{Lm}*, *rsbRC_{Lm}*, or *rsbRD_{Lm}* as the sole *rsbR* gene to activate σ^B upon entry into stationary phase may indicate that the strains are responding to nutritional stress or are being affected by an unappreciated stress signal that is generated at the end of exponential growth and not nutritional stress *per se*. The nutritional stress pathway in *B. subtilis*, defined by its dependence on the RsbPQ phosphatase, is acti-

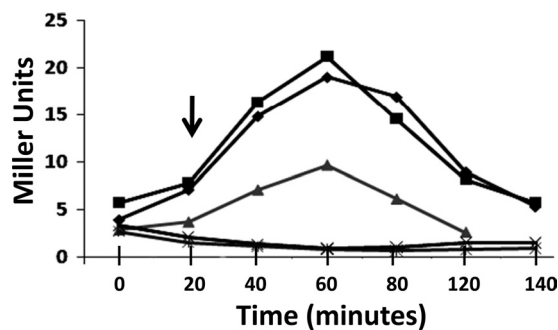


FIG. 5. RsbU-dependent σ^B activation in stationary phase by *B. subtilis* with novel RsbR combinations. *B. subtilis* strains BSH308 [*Δ rsbRA1(Cm) Δ rsbRB1::kan *rsbQ*::ery SP β ctc::lacZ*] (▲), BSH311 [*Δ rsbRA1(Cm) Δ rsbRB2 Δ rsbRD1::spc *rsbQ*::ery SP β ctc::lacZ*] (◆), BAR343 [*Δ rsbRA1(Cm) Δ rsbRB1::kan Δ rsbRC1::ery *rsbP*::spc SP β ctc::lacZ*] (■), BSH315 [*Δ rsbRA1(Cm) Δ rsbRD1::spc *rsbQ*::ery SP β ctc::lacZ*] (✱), and BSH316 [*Δ rsbRB2 Δ rsbRD1::spc *rsbQ*::ery SP β ctc::lacZ*] (×) were grown to stationary phase (indicated by the arrow) in LB. Samples were taken at the indicated intervals and assayed for σ^B -dependent β -galactosidase activity.

vated coincident with exposure to a number of agents or culture conditions that have as their common feature the ability to cause a drop in ATP levels (42, 46). To determine whether the induction of σ^B activity in the *rsbR_{Lm}*-, *rsbRC_{Lm}*-, and *rsbRD_{Lm}*-expressing strains is similarly affected by such treatments, σ^B -dependent reporter gene activity was examined following treatment with sodium azide, restrictive O₂ conditions, or limitation for glucose or phosphate. The ability of the *rsbR_{Lm}*-, *rsbRC_{Lm}*-, and *rsbRD_{Lm}*-expressing strains to respond to these potential inducers was compared to the response of similarly treated strains that expressed all of the RsbR proteins and lacked either the physical stress (*rsbU::kan*) or the nutritional stress (*rsbP::spc*) phosphatase. The results of this exercise are summarized in Table 2. As expected, the RsbP⁻ strain, lacking the nutritional stress pathway's phosphatase but otherwise wild type, displayed very low σ^B activity under all of the conditions that normally induce the nutritional stress pathway. In contrast, the RsbU⁻ strain, lacking the physical stress phosphatase but with an intact nutritional stress pathway, exhibited σ^B activity levels that were substantially greater than those seen in the RsbP⁻ strain under these same conditions. The strains lacking the nutritional stress phosphatase but expressing *rsbR_{Lm}*, *rsbRC_{Lm}*, or *rsbRD_{Lm}* as the sole RsbR protein displayed levels of σ^B activity that were similar to or greater than the levels seen in the strain with an intact nutritional stress pathway.

Although these results are consistent with the notion that all three of these RsbR proteins (RsbR_{Lm}, RsbRC_{Lm}, and RsbRD_{Lm}) can allow σ^B induction following stresses that are similar to those that activate the σ^B nutritional stress pathway, a previous study revealed that *rsbRD* is expressed at a relatively low level compared to that of the other *rsbR* genes (35). This raises the formal possibility that if entry into stationary phase or other treatments which inhibit growth give rise to a physiological state in which protein turnover is accelerated, the strains that express solely *rsbRD* might be more susceptible than the other *rsbR*-expressing strains to becoming functionally RsbR⁻ and no longer able to form the stressosomes needed to

TABLE 2. σ^B -dependent β -galactosidase activity under the indicated conditions

Condition	β -Galactosidase activity (Miller units) ^a				
	RsbP ⁻ strain	RsbU ⁻ strain	RsbR _{Lm} strain	RsbRC ⁺ strain	RsbRD ⁺ strain
Stationary phase	0.6 ± 0.2	19.4 ± 5.6	25.7 ± 5.4	21.8 ± 2.0	22.1 ± 1.0
Sodium azide treatment	0.4 ± 0.4	4.7 ± 0.8	23.6 ± 1.4	47.9 ± 14.4	32.3 ± 8.9
O ₂ deprivation	2.5 ± 0.7	7.4 ± 2.9	26.4 ± 1.2	15.0 ± 2.4	27.0 ± 4.2
Glucose limitation	1.7 ± 0.5	43.3 ± 7.2	22.9 ± 1.3	29.0 ± 5.3	30.4 ± 4.9
Phosphate limitation	3.8 ± 1.3	45.8 ± 10.1	21.8 ± 1.2	48.1 ± 15.9	47.6 ± 7.4

^a Values are averages ± standard deviations from three separate determinations. RsbP⁻ strain, BSH306; RsbU⁻ strain, BSA70; RsbR_{Lm} strain, BSL26; RsbRC⁺ strain, BSH306 (stationary phase, sodium azide treatment, and O₂ deprivation) or BSH311 (glucose/phosphate limitation); RsbRD⁺ strain, BAR343 (stationary phase, sodium azide treatment, and O₂ deprivation) or BSH313 (glucose/phosphate limitation). Stationary-phase values were obtained at 30 min after exponential growth. Sodium azide treatment and O₂ deprivation readings were obtained 40 min after initiating the treatments. Glucose and phosphate limitation values were determined 60 min after growth slowed in glucose or phosphate limiting medium.

hold RsbT inactive. To address the possible complication raised by the low expression level of *rsbRD*, we repeated the σ^B induction analysis using a strain in which the *rsbRD* sequence had been recombined into the *B. subtilis* chromosome, in lieu of *rsbRA*, at the *sigB* operon. This created a strain that is similar to the strain that expressed *rsbR_{Lm}* from this site, allowing *rsbRD* expression under the control of the *rsbRA* regulatory elements as the cells' sole source of RsbR. In the previous study mentioned above (35), expression of *rsbRD* from this site increased RsbD-dependent stressosomes 3-fold, to a level that is over 60% of that seen when RsbRA-dependent stressosomes are formed. This is a level of stressosome formation equivalent to that seen when RsbRB is the sole RsbR source (35). Figure 6 depicts the σ^B -dependent reporter gene activity in three RsbPQ⁻ strains: one that expresses all of the *rsbR* genes and two others that express either *rsbRA* or *rsbRD* from within the *sigB* operon. The strain with *rsbRD* expressed from *sigB*, but neither of the other two strains, induced σ^B activity following azide treatment (Fig. 6A), O₂ limitation (Fig. 6B), or entry into stationary phase (Fig. 6C). The level of σ^B activity seen in the strain with *rsbRD* at *sigB* was equal to or

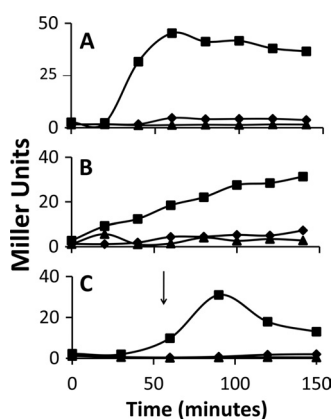


FIG. 6. RsbRD-dependent nutritional stress activation of σ^B . *B. subtilis* strains BSL20 (*ydcE::Cm rsbQ::TnYLB-1 SP β ctc::lacZ*) (\blacktriangle), BSH304 (Δ *rsbRB2* Δ *rsbRC1::ery* Δ *rsbRD1::spc rsbQ::TnYLB-1 SP β ctc::lacZ*) (\blacklozenge), and BSL24 [*P_A rsbRD* (Δ *rsbRA*) Δ *rsbRB2* Δ *rsbRC1::ery* Δ *rsbRD1::spc rsbQ::TnYLB-1 SP β ctc::lacZ*] (\blacksquare) were grown in LB and subjected to sodium azide (2 mM) (A) or O₂ limitation (B) at the beginning of the experiment (time zero) or allowed to enter stationary phase at the time point indicated by the arrow (C). Samples were taken at the indicated intervals and assayed for σ^B -dependent β -galactosidase activity.

greater than that seen in the strain with *rsbRD* expressed from its normal locus (Fig. 4; Table 2). Taken together, the data argue that all three of these RsbR proteins (RsbR_{Lm}, RsbRC_{Lm}, and RsbRD_{Lm}) can allow σ^B induction following exposure to any of a number of conditions that are normally associated with nutritional stress activation of σ^B .

σ^B activation during nutritional stress in a strain with elevated background σ^B activity. The activation of σ^B during nutritional stress in *B. subtilis* expressing *rsbR_{Lm}*, *rsbRC_{Lm}*, or *rsbRD_{Lm}* as its sole source of RsbR may be a direct result of nutritional stress on stressosome activity. However, an alternative explanation of the data, which invokes a more dynamic view of stressosome activity and σ^B regulation, is possible. It is known that σ^B activity is very high during growth in *B. subtilis* strains lacking RsbX, the phosphatase responsible for reactivating RsbR-P and RsbS-P (6). This observation suggests that even in the absence of overt stress there is likely to be a background level of RsbR/RsbS phosphorylation, RsbT release, and activation of σ^B . Such a circumstance would include the dephosphorylation of RsbV-P, which would then require phosphorylation, at the expense of ATP, to inactivate RsbV and allow the sequestration of σ^B into a complex with RsbW (3, 17).

The background level of σ^B activity in strains expressing single *rsbR* genes, particularly *rsbR_{Lm}*, is severalfold higher than that seen in the strain expressing all of the *B. subtilis* *rsbR* genes or *rsbRA* alone. Such heterogeneity might be a consequence of the inherent biochemical properties of each of these proteins and/or disparities in the background levels of the signaling molecules to which each responds. Regardless of the basis of these differences in σ^B activity, it is plausible that higher background levels of σ^B activity could have consequences when the strains with these higher levels experience nutritional stress. The strains with inherently higher levels of RsbV-P dephosphorylation might be more prone to activate σ^B when ATP levels fall and the cell's ability to phosphorylate RsbV would presumably be diminished. Early *in vitro* studies revealed a close correlation among the concentration of ATP required for efficient RsbW-mediated phosphorylation of RsbV, inhibition of RsbV/RsbW complex formation, and σ^B -directed transcription (3). Prior to the discovery of the RsbPQ phosphatase, the effect of a decline in ATP levels on RsbW kinase activity was, in fact, suggested as the device responsible for activation of σ^B by nutritional stress (3). In the alternative model, the signal to which *Listeria* RsbR responds is not gen-

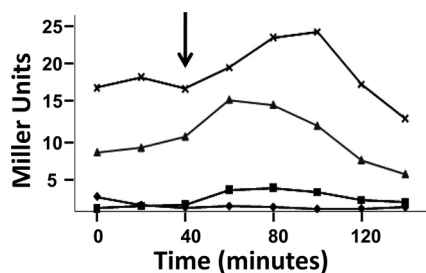


FIG. 7. RsbU-dependent σ^B activation in stationary phase by *B. subtilis* strains with induced RsbU activity. *B. subtilis* BSH318 (P_{SPAC} *rsbT* *rsbP*::*spc* SP β *ctc*::*lacZ*) was grown in LB without IPTG (◆) or supplemented with 0.025 mM (■), 0.05 mM (▲), or 0.1 mM (×) IPTG to stationary phase (arrow). Samples were taken at the indicated intervals and assayed for σ^B -dependent β -galactosidase activity.

erated by both physical and nutritional stress. Instead, RsbR_{Lm} might respond solely to signals generated by physical stress but exhibits a sufficiently high “steady-state” level of σ^B activation to allow σ^B activity to be more sensitive to changes in ATP levels than that seen in strains with RsbR proteins that have lower background levels of σ^B activity.

To test the possibility that elevated background activity in the physical stress pathway might allow heightened σ^B activity during periods of nutritional stress, a *B. subtilis* strain (BSH318) expressing the activator (RsbT) of the RsbU phosphatase under the control of an IPTG-inducible promoter (P_{SPAC} ::*rsbT*) was grown and allowed to enter stationary phase in the presence of various concentrations of IPTG. Induction of P_{SPAC} ::*rsbT* increases the expression of *rsbT* relative to that of its primary negative regulator (*rsbS*), thereby allowing enhanced activity of the physical stress pathway in the absence of stress (37). Figure 7 illustrates that the addition of increasing amounts of IPTG leads to corresponding increases in σ^B activity during growth, which rises further when the cultures enter stationary phase. Presumably, the stationary-phase elevation in σ^B activity is due to the decreased availability of ATP for rephosphorylation of the RsbV that had become dephosphorylated by the increased RsbU phosphatase activity. Although entry into stationary phase enhanced σ^B activity in this *B. subtilis* strain with heightened background levels of RsbR-P dephosphorylation, the degree of this enhancement, at least under the conditions used in this experiment, was relatively modest (Fig. 7) compared to that seen when strains with stressosomes formed from RsbR_{Lm}, RsbRC_{Lm}, or RsbRD_{Lm} entered stationary phase (Fig. 3, 4, and 6). If the nutritional stress induction of σ^B activity in the RsbR_{Lm}, RsbRC_{Lm}, or RsbRD_{Lm} strain is a consequence of heightened σ^B background activity, the robust induction seen in these strains, compared to that seen when σ^B activity is artificially elevated, suggests that there are additional properties associated with the stressosome-associated process that are lacking when the elevated σ^B activity is generated by merely raising RsbU phosphatase activity.

Regardless of whether nutritional stress allows σ^B activation by directly targeting the RsbR_{Lm} protein or the ability of RsbW to maintain RsbV's phosphorylation state in the presence of RsbR_{Lm}, the finding that the RsbR_{Lm} protein, but not its *B. subtilis* counterpart, allows σ^B activation during nutri-

tional stress offers the *Listeria* RsbR protein itself as a plausible basis for the observation that σ^B can be activated by the stressosome-associated pathway following nutritional stress in *Listeria* but not *B. subtilis*. Sorting out the target for the nutritional stress activation will ultimately require the development of assays to directly monitor the effects of nutritional stress on the phosphorylation state of the RsbR proteins themselves. If they are responding directly to signals generated by either physical or nutritional stress, their level of phosphorylation would be expected to increase under either of these conditions. However, if the RsbR proteins do not respond to signals generated directly by nutritional stress, their phosphorylation state should remain unchanged under this condition.

The *Listeria* RsbR protein, although highly homologous at its C terminus to *B. subtilis* RsbRA and its paralogs, has a novel N-terminal region. Assuming that this region is the target for stress signaling, its uniqueness suggests that it responds to a potentially novel input. The ability of the *Listeria* RsbR protein to function in *B. subtilis*, restricting σ^B activity during growth but allowing its activation following stress, argues both that it can productively interact with the *B. subtilis* regulators and that the signals to which it responds are generated by stress in *B. subtilis* as well as in *Listeria*. This opens the possibility that the signaling molecules that activate the *L. monocytogenes* stressosome could be sought and studied in the more experimentally tractable *B. subtilis*, rather than in *Listeria* itself. RsbR_{Lm}, as a novel RsbR variant that can apparently interact with the *B. subtilis* stressosome components, could also serve as an additional vehicle to study *B. subtilis* stressosome activity. If, for example, RsbR_{Lm} responds directly to a signal generated by nutritional stress, inhibition of its activation of σ^B by other RsbR proteins would support the notion of stressosomes as composites of multiple RsbR species in which the presence of each component influences the activities of the others (30). The stressosome then becomes a device for integrating multiple stress signals to allow σ^B activation only when a critical stress threshold is reached (30). Given the diversity of the amino termini of the RsbR paralogs, the specific signaling molecules to which each reacts may be unique and potentially generated by distinct stress-responsive elements within the cell. If this is so, the individual RsbR paralogs could then serve as a gauge of the effects of stress on multiple cell components. In this view, the stressosome may represent not only a device that responds to overall stress levels but also one that integrates regulatory inputs from diverse sources to allow σ^B activation only when each of several cell components has been affected by stress to provide its input. Uncovering the specific signals to which each of the RsbR proteins responds will be an important next step toward exploring this model.

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