APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2004, p. 5349–5356 Vol. 70, No. 9
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.9.5349–5356.2004

RsbT and RsbV Contribute to σ^B-Dependent Survival under Environmental, Energy, and Intracellular Stress Conditions in *Listeria monocytogenes*

Soraya Chaturongakul and Kathryn J. Boor*

Department of Food Science, Cornell University, Ithaca, New York

Received 14 January 2004/Accepted 17 May 2004

Sigma B (σ^B) is a stress-responsive alternative sigma factor that has been identified in various gram-positive bacteria. Seven different regulators of sigma B (Rsbs) are located in the *sigB* operons of both *Bacillus subtilis* and *Listeria monocytogenes*. In *B. subtilis*, these proteins contribute to regulation of σ^B activity by conveying environmental and energy stress signals through two well-established branches of a signal transduction pathway. RsbT contributes to regulation of σ^B activity in response to environmental stresses, while RsbV contributes to σ^B activation under both environmental and energy stresses in *B. subtilis*. To probe *L. monocytogenes* Rsb roles in σ^B-mediated responses to various stresses, in-frame deletions were created in *rsbT* and *rsbV*. Phenotypic characterization of the *L. monocytogenes rsbT* and *rsbV* null mutants revealed that both mutants were similar to the Δ*sigB* strain in their abilities to survive under environmental stress conditions (exposure to synthetic gastric fluid, pH 2.5, acidified brain heart infusion broth [BHI], or oxidative stress [13 mM cumene hydroperoxide]). Under energy stress conditions (carbon starvation in defined media, entry into stationary phase, or reduced intracellular ATP), both Δ*rsbT* and Δ*rsbV* showed survival reductions similar to that of the Δ*sigB* strain. These observations suggest that the pathways for Rsb-dependent regulation of σ^B activity differ between *L. monocytogenes* and *B. subtilis*. As σ^B also activates transcription of the *L. monocytogenes prfA*P2 promoter, we evaluated virulence-associated characteristics of Δ*prfA*P1*rsbT* and Δ*prfA*P1*rsbV* double mutants in hemolysis and tissue culture assays. Both double mutants showed identical phenotypes to Δ*prfA*P1P2 and Δ*prfA*P1*sigB* double mutants, i.e., reduced hemolysis activity and reduced plaque size in mouse fibroblast cells. These findings indicate that RsbT and RsbV both contribute to σ^B activation in *L. monocytogenes* during exposure to environmental and energy stresses as well as during tissue culture infection.

Listeria monocytogenes, a gram-positive, non-spore-forming rod-shaped bacterium, is recognized as a foodborne pathogen. This organism is capable of surviving in a broad range of ecological niches (e.g., in farm environments and food processing plants) and in a wide range of hosts, including humans and many species of animals. In *L. monocytogenes*, the alternative sigma factor B (σ^B) contributes to survival under stressful environmental conditions, such as exposure to low pH, oxidizing conditions, and starvation (17, 18). Loss of σ^B also reduces *L. monocytogenes* virulence in a murine model (32, 44).

The *sigB* gene, which encodes σ^B, lies seventh in the *sigB* operon. This operon also includes seven additional genes, which encode the following regulator of sigma B proteins: RsbR, RsbS, RsbT, RsbU, RsbV, RsbW, and RsbX (4, 19, 25, 44, 45). In *B. subtilis*, activation of σ^B by the Rsb proteins is achieved through a complex phosphorylation/dephosphorylation cascade in response to various cellular stimuli, which have been categorized into two general types: environmental or metabolic (1, 6, 7, 9, 15, 21, 26, 27, 29, 42, 43, 46). In *Bacillus subtilis*, these two types of cellular stimuli are conveyed to σ^B through two interconnected but separate pathways. The environmental stimulus pathway is transmitted by regulatory proteins encoded in the *sigB* operon. The "metabolic" or energy stimulus pathway is signaled by proteins encoded in a two-gene operon (*rsbQ-rsbP*) that is physically distant from the *sigB* operon (9, 41). The presence of this *rsbQ-rsbP* operon is not evident in *L. monocytogenes*. The two primary regulators of *B. subtilis* σ^B activity are RsbV and RsbW. Under exponential growth conditions, RsbW, an anti-σ factor, binds directly to σ^B and blocks association between σ^B and RNA polymerase. RsbV is inactive as an anti-anti-σ factor when it has been phosphorylated on a conserved serine residue by the kinase activity of RsbW. However, *B. subtilis* RsbV is dephosphorylated by the phosphatase activity of RsbU or of RsbP under conditions of environmental (43) or energy (41) stress, respectively. The phosphatase activity of RsbU is activated upon protein-protein interaction with serine kinase RsbT (27) and that of RsbP by α/β hydrolase RsbQ (9). After dephosphorylation, RsbV binds to RsbW, thus freeing σ^B, which then becomes available to bind RNA polymerase core enzyme. In summary, the phosphorylation status of RsbV determines whether σ^B is bound to RsbW or is free to interact with core polymerase (16).

Expression of the majority of recognized *L. monocytogenes* virulence genes is regulated by positive regulatory factor A (PrfA). PrfA regulates expression of a set of virulence factors, including listeriolysin O (LLO), actin polymerization protein ActA, phospholipases (PlcA and PlcB), and internalins (30, 40). Transcription of *prfA* is initiated from three promoters: *prfA*P1, *prfA*P2, and a promoter upstream of *plcA*. In vivo, loss of either *prfA*P1 or -P2 appears to be compensated for by the remaining promoter; loss of both *prfA* promoters results in

* Corresponding author. Mailing address: Department of Food Science, 413 Stocking Hall, Cornell University, Ithaca, NY 14853. Phone: (607) 255-3111. Fax: (607) 254-4868. E-mail: kjb4@cornell.edu.

TABLE 1. *L. monocytogenes* strains used in this study[a]

Strain	Characteristics	Source or reference
DP-L1956	Δ*prfA*P1 (−10 promoter deletion)	20
DP-L1957	Δ*prfA*P2 (−10 promoter deletion)	20
DP-L1964	Δ*prfA*P1P2 (−10 deletion each in P1 and P2)	20
DP-L2161	Δ*hly*	24
FSL A1-254	Δ*sigB*	44
FSL B2-002	Δ*prfA*P1*sigB*	32
FSL C3-015	Δ*rsbT*	This study
FSL C3-047	Δ*prfA*P1*rsbT*	This study
FSL C3-049	Δ*prfA*P2*rsbT*	This study
FSL C3-053	Δ*prfA*P2*sigB*	This study
FSL C3-057	Δ*rsbV*	This study
FSL C3-091	Δ*prfA*P1*rsbV*	This study
FSL C3-095	Δ*prfA*P2*rsbV*	This study
10403S	Serotype 1/2a wild-type strain	5

[a] All strains are derivatives of 10403S.

virulence attenuation (20). Our group has shown that the *prfA*P2 promoter is σ^B dependent and that a combined loss of *prfA*P1 and σ^B also results in reduced virulence-associated characteristics (32). From this evidence, we conclude that, in addition to contributing to survival under environmental stress, σ^B also plays a role in *L. monocytogenes* virulence.

As the organization and components of the *B. subtilis* and *L. monocytogenes sigB* operons are identical (19), we hypothesized that the regulatory network that determines σ^B activity in *B. subtilis* could be used as a model for stress signal transduction by Rsb proteins in *L. monocytogenes*. To investigate the role of these Rsbs in stress resistance and virulence-associated characteristics in the bacterial pathogen *L. monocytogenes*: (i) in-frame deletion mutations were created in *rsbT* and *rsbV* and (ii) stress survival and PrfA-mediated virulence-associated characteristics of the Δ*rsbT* and Δ*rsbV* strains were compared with those of the Δ*sigB* and wild-type strains.

MATERIALS AND METHODS

Bacterial strains. *L. monocytogenes* 10403S and its derivatives were used throughout this study (Table 1). In comparison to the wild-type strain, all mutant strains had identical culture characteristics, including growth at 37°C with shaking (250 rpm) for at least 120 h in brain heart infusion (BHI) broth (Difco, Sparks, Md.) (data not shown). Stock cultures were stored at −80°C in BHI broth with 15% glycerol and streaked onto BHI agar plates prior to each experiment.

Mutant construction. *rsbT* and *rsbV* alleles with in-frame internal deletions were created in the *Escherichia coli-L. monocytogenes* shuttle vector pKSV7 (10) by SOE (splicing by overlap extension) PCR (23) and introduced into *L. monocytogenes* 10403S by allelic exchange mutagenesis (44). For the *rsbT* mutation, SOE PCR primers were designed to amplify two ~400-bp DNA fragments, one comprising the 5′ end of *rsbT* (amplified by primers SOE-rsbTA and SOE-rsbTB [Table 2]) and one comprising the 3′ end of *rsbT* (amplified by primers SOE-rsbTC and SOE-rsbTD [Table 2]). Subsequent PCR amplification of the two PCR products with SOE-rsbTA and SOE-rsbTD created an in-frame 267-bp deletion within the *rsbT* open reading frame. The resulting fragment was purified with the QIAquick PCR Purification kit (QIAGEN Inc., Valencia, Calif.) and subsequently digested with KpnI and XbaI. The resulting fragment was cloned into pKSV7 and transformed into *E. coli* DH5α. The resulting plasmid, pSC1, was electroporated into *L. monocytogenes* 10403S. Transformants were selected on BHI agar plates containing 10-μg/ml chloramphenicol. A transformant was serially passaged in BHI-chloramphenicol at 41°C to direct chromosomal integration of the plasmid by homologous recombination. Confirmation of chromosomal integration was done by PCR and sequencing. A single colony with a chromosomal integration was serially passaged in BHI at 30°C and screened for loss of chloramphenicol resistance. Allelic exchange mutagenesis was confirmed by PCR amplification and direct sequencing of the PCR product with primers CHK-ΔrsbTF and CHK-ΔrsbTR. Similar procedures were performed to create an in-frame 303-bp deletion in the *rsbV* gene by using the primers shown in Table 2.

Growth and stress conditions. For environmental stress survival, *L. monocytogenes* 10403S, FSL A1-254 (Δ*sigB* mutant), FSL C3-015 (Δ*rsbT* mutant), and FSL C3-057 (Δ*rsbV* mutant) were tested under three different conditions. Acid survival assays were performed by using synthetic gastric fluid (pH 2.5) (12) or acidified BHI (pH 2.5). Overnight cultures were inoculated into 10 ml of BHI broth (1:100 dilution). The cultures were grown at 37°C with shaking (250 rpm) to an optical density at 600 nm (OD_{600}) of 0.4, and then 0.1 ml of each culture was reinoculated into 10 ml of BHI broth. From this point, to prepare mid-log cells, 1 ml of each culture was collected when these cultures reached an OD_{600} of 0.4. Samples were centrifuged and resuspended in 1 ml of synthetic gastric fluid (pH 2.5) or BHI-HCl (pH 2.5). The assay tubes were incubated at 37°C with shaking (250 rpm). Immediately after acid challenge ($t = 0$) and 10 min after acid challenge ($t = 10$), 100-μl aliquots were removed, serially diluted, and plated on BHI agar plates for enumeration. To prepare stationary-phase cells, overnight cultures were inoculated into 10 ml of BHI broth (1:100). Following 12 h of incubation at 37°C with shaking (250 rpm), 1 ml of each culture was centrifuged and resuspended in 1 ml of synthetic gastric fluid (pH 2.5) or BHI-HCl (pH 2.5). Immediately after acid challenge ($t = 0$) and 60 min after acid challenge ($t = 60$), 100-μl aliquots were removed, serially diluted, and plated. Plates were incubated at 37°C for 48 h prior to enumeration.

TABLE 2. PCR primers used in this study

Primer	Sequence (5′→3′)
SOE-rsbTA[a]	GGG GTA CCG TGA ATA CTG TGG GGA TAC
SOE-rsbTB[b]	GGA TTC AAT ATC AAA ACT GTC GTT CAT TCC CCC AAT TCC TG
SOE-rsbTC	GAC AGT TTT GAT ATT GAA TCC
SOE-rsbTD[c]	GCT CTA GAT CAC TAC GTA ATT CTT TTT GC
CHK-ΔrsbTF[e]	GAG ATA GTA GAA AAA GAG GG
CHK-ΔrsbTR[e]	GCA TAT CTT CTG CTT GAG C
SOE-rsbVA[a]	GGG GTA CCT ATG TCA GAT GGT GTA ACA G
SOE-rsbVB[d]	CAT TCA TTT CAC CCT CTA CAA TAT TCA TCA CTT CAC CCC
SOE-rsbVC	GTA GAG GGT GAA ATG AAT G
SOE-rsbVD[c]	GCT CTA GAC AAT TAA AAA TAA ACC TAG ACC
CHK-ΔrsbVF[f]	GAT AAA AAT GCT GTC TAC CG
CHK-ΔrsbVR[f]	TAA TAA AGT TTC ACG TCA TCC

[a] The KpnI restriction site incorporated into this primer to facilitate cloning is underlined.
[b] The overhang complementary to SOE-rsbTC is underlined.
[c] The XbaI restriction site incorporated into this primer to facilitate cloning is underlined.
[d] The overhang complementary to SOE-rsbVC is underlined.
[e] Forward (F) and reverse (R) primers for in-frame *rsbT* deletion confirmation.
[f] Forward (F) and reverse (R) primers for in-frame *rsbV* deletion confirmation.

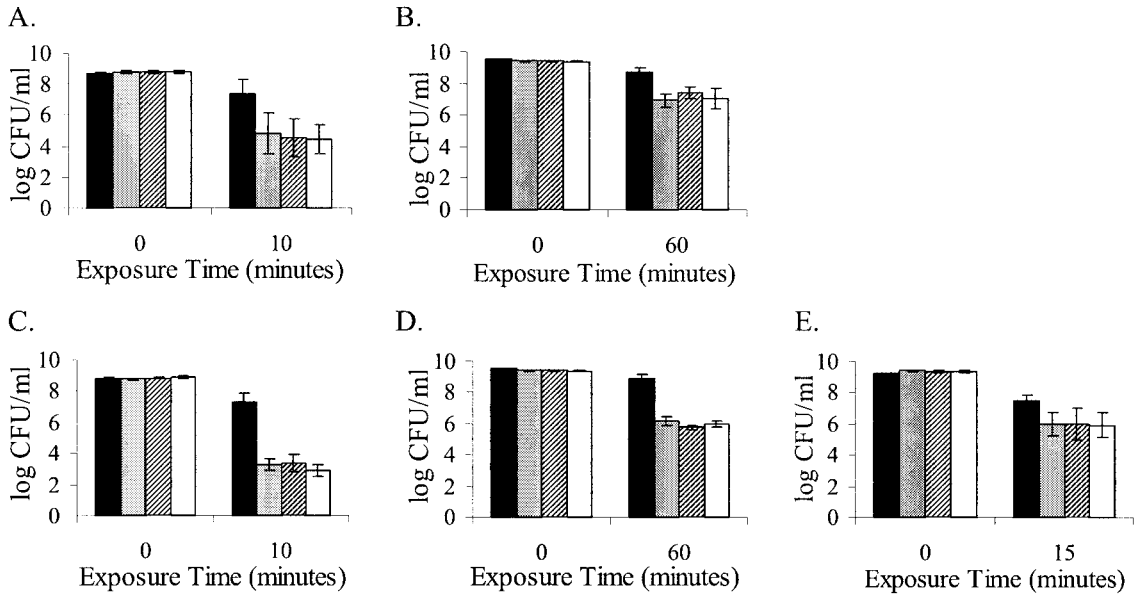

FIG. 1. Viabilities of *L. monocytogenes* wild-type 10403S (black), Δ*sigB* (gray), Δ*rsbT* (hatched), and Δ*rsbV* (white) strains following exposure of mid-log (A and C) or stationary-phase (B, D, and E) cultures to synthetic gastric fluid at pH 2.5 (A and B), acid (pH 2.5) (C and D), and CHP (13 mM) (E). The results shown are mean values from three independent experiments; error bars indicate standard deviations.

Oxidative stress survival was assessed with the oxidative agent cumene hydroperoxide (CHP) (Sigma, St. Louis, Mo.). CHP survival assays were performed as described by Antelmann et al. (3). Briefly, overnight cultures were inoculated into 10 ml of BHI broth (1:100). Following 12 h of incubation at 37°C, 1 ml of each culture was centrifuged and resuspended in 0.9 ml of dimethyl sulfoxide (Fisher Scientific, Fair Lawn, N.J.). A 100-μl aliquot of 130 mM CHP was added to yield a final CHP concentration of 13 mM. Assay tubes were incubated for 15 min at 37°C with shaking (250 rpm). Aliquots were removed for standard plate counts on BHI agar plates at $t = 15$ min. A 100-μl portion of a 12-h culture was serially diluted and plated to represent viable cells at $t = 0$ min (prior to CHP exposure).

For energy stresses, *L. monocytogenes* 10403S, FSL A1-254, FSL C3-015, and FSL C3-057 were tested under three different conditions. For the first condition, carbon starvation was induced with defined medium (DM) (36) with a growth-limiting concentration of glucose (0.04% [wt/vol]). Briefly, overnight cultures in BHI broth were inoculated into 10 ml of DM (1:100 dilution) supplemented with glucose (0.4% [wt/vol]). After 12 h of incubation with shaking (250 rpm) at 37°C, cultures were reinoculated into DM plus 0.04% glucose (1:100 dilution). A preliminary experiment showed that the ODs of cultures grown under these conditions reflected the viable counts of bacteria in the cultures. Thereafter, culture densities were monitored by measuring absorbance (OD_{600}) for up to 30 h. For the second energy stress condition, wild-type and mutant strains were monitored for survival during entry into stationary phase. Aliquots (100 μl) of overnight cultures from each strain were transferred into 10 ml of BHI (~1:100 dilution). All strains were incubated statically at 37°C. Bacterial numbers were measured by standard plate count procedures using 100-μl aliquots removed at various time points up to 72 h. Entry into stationary phase for these experiments was defined as the period during and after which bacterial numbers reached maximal levels (between 6 and 36 h postinoculation). For the third energy stress condition, the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used to induce energy stress (2, 42). The MIC of CCCP was determined for *L. monocytogenes* 10403S according to the procedures described by the National Committee for Clinical Laboratory Standards (33). A final CCCP concentration of 32 μg/ml (16 times the MIC) was added to exponentially growing cultures ($OD_{600} = 0.4$). In a preliminary experiment, the ODs of the cultures grown under these conditions were found to reflect the viable counts of bacteria in the cultures. Thereafter, culture densities were monitored by measuring absorbance (OD_{600}) over time.

Hemolysin assay. The enzymatic activity of LLO from each strain was measured as described previously (35), except that 0.5 mM dithiothreitol was used as a reducing agent instead of cysteine. Lysis of sheep red blood cells was measured as hemoglobin release at 420 nm in a Fusion Universal microplate analyzer (Packard, Meriden, Conn.). A hemolytic unit was defined as the reciprocal of the supernatant dilution at which 50% of the sheep red blood cells were lysed. The wild-type level, represented by *L. monocytogenes* 10403S, was set at 100% hemolysis.

Tissue culture plaque assay. Cytopathogenicities of different mutants were evaluated with a plaque assay performed with mouse L929 fibroblast cells as previously described (38). Briefly, overnight cultures (~12 h) grown statically at 30°C in BHI broth were centrifuged, resuspended, and serially diluted in phosphate-buffered saline (pH 7.4). Five microliters of a 10^2 dilution and 15 μl of a 10^3 dilution were used as inocula for two wells of a six-well plate containing monolayers of mouse L cells. Inocula were enumerated by plating serial dilutions onto BHI agar and incubating at 37°C overnight. Inocula were equivalent for all strains. For plaque visualization at 3 days postinfection, infected mouse L cells were overlaid with 2× Dulbecco's modified Eagle's medium (Difco) containing 1.4% Bacto agar (Difco) and neutral red solution (Sigma). For each assay, Sigmascan Pro 5.0 software (SPSS, Inc., Chicago, Ill.) was used to measure the areas of at least 25 plaques. Plaque area for each strain was expressed as a percentage of the *L. monocytogenes* 10403S plaque size, which was included as an internal standard for each assay and assigned a value of 100%.

Statistical analyses. All comparisons were evaluated by one-way analysis of variance. Statistical significance was established at $P < 0.05$. Statistical analyses were performed in Minitab (Statistical Software, State College, Pa.).

RESULTS

Mutant survival under acid stress conditions. Two different acid stress conditions were used to study the roles of RsbT and RsbV in activation of σ^B in *L. monocytogenes*. First, wild-type 10403S and the Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains were exposed to synthetic gastric fluid (pH 2.5) (Fig. 1A and B). Within 10 min of exposure, numbers of exponential wild-type 10403S cells ($OD_{600} = 0.4$) were reduced by 1 log (relative to $t = 0$ counts) while all mutant strain numbers were reduced by approximately 4 logs. Numbers of wild-type survivors were significantly different from those of all mutant strains after 10 min in synthetic gastric fluid ($P = 0.007$). Following a 60-min exposure to synthetic gastric fluid, stationary-phase numbers of

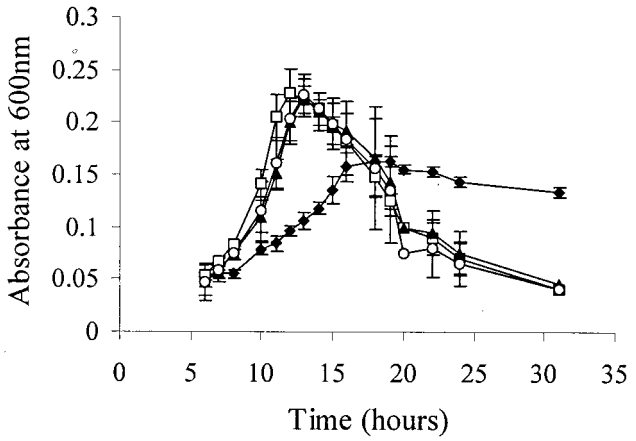

FIG. 2. Growth of *L. monocytogenes* 10403S (◆), Δ*sigB* (□), Δ*rsbT* (▲), and Δ*rsbV* (○) in a defined medium with a limiting amount of glucose (0.04% [wt/vol]). OD_{600} values were recorded. The results shown are mean values from three independent experiments; error bars indicate standard deviations.

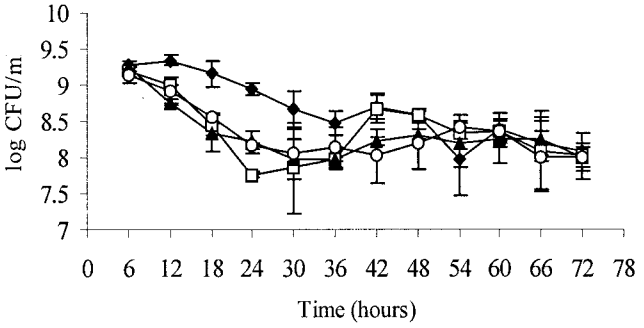

FIG. 3. Viabilities of *L. monocytogenes* 10403S (◆), Δ*sigB* (□), Δ*rsbT* (▲), and Δ*rsbV* (○) cultures that had been grown statically in BHI at 37°C. Bacteria were harvested at indicated times, serially diluted, and plated for enumeration on BHI agar plates. The results shown are mean values from three independent experiments; error bars indicate standard deviations.

the Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains were reduced by an average of 1.6 log more than those of the wild-type strain ($P = 0.007$). When mid-log wild-type and mutant strains were challenged with acidified BHI broth (pH 2.5) for 10 min (Fig. 1C and D), wild-type strain numbers were reduced by 1.5 log relative to $t = 0$, while all mutant strains were reduced by about 6 to 6.5 logs. Numbers of wild-type survivors were significantly different from those of the mutant strains ($P < 0.0001$). Bacterial numbers for wild-type stationary-phase cultures were approximately 2.9 logs higher than those for all mutant strains ($P < 0.0001$) following exposure to BHI-HCl (pH 2.5) for 60 min. Results from these experiments suggest that RsbT and RsbV contribute to acid stress survival of both exponential- and stationary-phase *L. monocytogenes* cultures, likely through σ^B activation.

Mutant survival under oxidative stress conditions. CHP (13 mM) was used to induce oxidative stress in this study. As shown in Fig. 1E, after 15 min of exposure, wild-type numbers were reduced by 2 logs; all mutant strains were reduced by 3 logs. Numbers of wild-type survivors were significantly different from those of the mutant strains ($P = 0.017$). These results show that wild-type *L. monocytogenes* 10403S is more resistant to oxidative stress imposed by 13 mM CHP than the Δ*sigB* strain or the strains lacking predicted σ^B regulatory proteins (Δ*rsbT* and Δ*rsbV*). Thus, RsbT and RsbV appear to contribute to σ^B-mediated survival following exposure to CHP stress.

Mutant survival under energy stress conditions. Energy stresses usually result from limitations in key metabolites such as carbon, phosphate, or nitrogen. For this study, we used an *L. monocytogenes* DM (36) supplemented with limiting glucose (0.04% [wt/vol]) or prolonged static incubation into the stationary phase to induce carbon starvation. As a third condition, the protonophore CCCP was added as a chemical agent to limit ATP synthesis, thereby resulting in an intrinsic energy stress. Figure 2 shows that the Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains entered exponential growth after 6 h, as compared to 10 h for the wild-type strain when all were grown under limiting glucose conditions. The mutant strains also grew more rapidly (average doubling times of approximately 180 min, between 8 and 13 h) than the wild type (doubling time = 360 min between 10 and 16 h). ODs of all three mutant strains reached higher maxima than the wild type; however, ODs declined rapidly for all three mutant strains, possibly due to cell lysis resulting from depletion of glucose (18). The wild-type strain, which grew more slowly and reached a lower maximum OD than those of the mutant strains, also declined more slowly than the mutant strains. From these data, we hypothesize that activated σ^B negatively affects growth in exponentially growing cells but positively contributes to maintenance of viability after glucose is depleted in defined media.

Survival following entry into stationary phase was tested by growing each of the four strains in BHI with static incubation at 37°C for at least 72 h. Cultures were sampled for enumeration every 6 h. As shown in Fig. 3, numbers of Δ*sigB*, Δ*rsbT*, and Δ*rsbV* declined more rapidly than those of the wild-type control following entry into stationary phase (between 6 and 36 h postinoculation). After 54 h, however, all cultures were present at similar numbers. σ^B appears to contribute to survival between 6 and 36 h under static growth in BHI.

The protonophore, CCCP, was added to exponentially growing cells ($OD_{600} = 0.4$) in BHI, and OD was monitored every 2 h for 24 h. OD_{600} slightly increased above 0.4 for all strains but then declined for all strains. ODs between the wild-type and mutant strains (Δ*sigB*, Δ*rsbT*, and Δ*rsbV*) were significantly different ($P = 0.01$) after 14 h, presumably following consumption of accumulated intracellular ATP (Fig. 4). σ^B appears to contribute to maintaining cell viability after ATP depletion.

Tissue culture virulence and hemolysis phenotypes. Contributions of σ^B and putative σ^B activators to virulence-associated phenotypes were characterized in vitro by performing hemolysis assays and tissue culture plaque assays. Strains used in these assays included the Δ*sigB*, Δ*rsbT*, Δ*rsbV*, Δ*prfA*P1, and Δ*prfA*P2 strains and selected double mutants (Table 1). The rationale for our research hypothesis regarding the interplay between σ^B activation and PrfA-mediated virulence arose from the finding that the *prfA*P2 promoter is σ^B dependent. A combined loss of *prfA*P1 and σ^B results in reduced virulence (32). Therefore, we predicted that loss of *prfA*P1 and σ^B activity (through Δ*prfA*P1*rsbT* or Δ*prfA*P1*rsbV*) would also affect virulence-associated phenotypes.

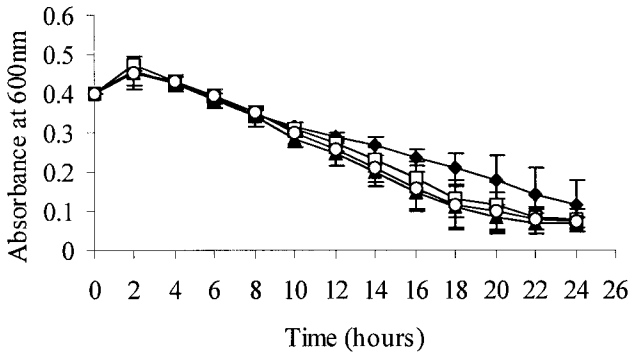

FIG. 4. OD_{600} values for *L. monocytogenes* 10403S (◆), Δ*sigB* (□), Δ*rsbT* (▲), and Δ*rsbV* (○) strains after exposure of mid-log cells (OD_{600} = 0.4) to 32-μg/ml CCCP. The results shown are mean values from three independent experiments; error bars indicate standard deviations.

A hemolysis assay was used to measure the hemolytic activity of LLO in the culture supernatant from each strain. The ability to lyse sheep red blood cells was compared among wild-type and mutant strains. Results are shown in Table 3. The Δ*prfA*P1*sigB*, Δ*prfA*P1*rsbT*, or Δ*prfA*P1*rsbV* strain showed reduced hemolytic activities. These results suggest that the activated σ^B has a negative effect on LLO activity or perhaps on the *prfA*P1 promoter.

A plaque assay was conducted in mouse fibroblast cells (L929). Cells were infected with *L. monocytogenes* 10403S, Δ*sigB*, Δ*rsbT*, or Δ*rsbV* mutants. The cytopathogenicity of each strain was inferred from bacterial ability to infect and disseminate among host cells, as measured by plaque sizes (Table 3). A combined loss of *prfA*P1 and *sigB*, *rsbT*, or *rsbV* led to a range in plaque sizes from wild type to very small. The Δ*sigB*, Δ*rsbT*, Δ*rsbV*, Δ *prfA*P2*sigB*, Δ*prfA*P2*rsbT*, and Δ *prfA*P2*rsbV* strains yielded similar plaque sizes as the wild-type strains, in concurrence with previous findings (20, 32).

TABLE 3. Virulence-associated phenotypes of *L. monocytogenes* strains used in this study

Genotype	% Hemolysis (mean ± SD)[a]	Plaque size relative to 10403S (mean ± SD)[a]
Wild type	100 ± 0	100 ± 0
Δ*sigB*	250 ± 122[b]	103 ± 6
Δ*rsbT*	250 ± 122[b]	105 ± 8
Δ*rsbV*	233 ± 82[b]	101 ± 9
Δ*prfA*P1	21 ± 6[b]	79 ± 12
Δ*prfA*P2	75 ± 27	92 ± 9
Δ*prfA*P1P2	5 ± 3[b]	45 ± 1[b]
Δ*prfA*P1*sigB*	6 ± 0[b]	62 ± 8[b]
Δ*prfA*P1*rsbT*	9 ± 3[b]	58 ± 8[b]
Δ*prfA*P1*rsbV*	10 ± 4[b]	63 ± 5[b]
Δ*prfA*P2*sigB*	175 ± 125	93 ± 4
Δ*prfA*P2*rsbT*	175 ± 125	90 ± 4
Δ*prfA*P2*rsbV*	133 ± 58	91 ± 4
Δ*hly*	0 ± 0[b]	NT[c]

[a] Data report triplicate experiments.
[b] $P < 0.05$ when data are compared with those from the wild type.
[c] NT, not tested.

DISCUSSION

Our overarching research hypothesis is that *L. monocytogenes* σ^B promotes bacterial survival under exposure to environmental stresses both outside and inside a host, thus contributing to bacterial pathogenicity. To begin to dissect σ^B-activating stress signaling pathways in *L. monocytogenes*, we created two *rsb* null mutants: one in *rsbT* and the other in *rsbV*. We hypothesized that, as with the *L. monocytogenes* Δ*sigB* strain, the Δ*rsbT* and Δ*rsbV* strains also would be more susceptible than the wild type to environmental stresses such as exposure to synthetic gastric fluid, reduced pH, and oxidative stresses. Based on the *B. subtilis* model (Fig. 5A), we predicted that only the Δ*rsbV* and Δ*sigB* strains would survive less well than the wild type under energy stress conditions such as carbon starvation or ATP depletion. However, we found that the Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains had identical phenotypes under all stress conditions, suggesting that stress signaling pathways that activate σ^B differ between *L. monocytogenes* and *B. subtilis*.

σ^B, RsbT, and RsbV enhance environmental and energy stress survivals. As predicted, the Δ*rsbT*, Δ*rsbV*, and Δ*sigB* strains survived at significantly lower levels than the wild-type strain after exposure to environmental stresses. Specifically, σ^B-mediated survival of the wild-type strain in synthetic gastric fluid (pH 2.5), acid (pH 2.5), and CHP (13 mM) appears to require σ^B activation. This activation requires both RsbT and RsbV, since loss of either resulted in the same phenotype as loss of σ^B. We further hypothesize that RsbT and RsbV contribute to σ^B activation and *L. monocytogenes* survival under environmental stresses such as those encountered during gastric passage (bile salt in the host gut) (28), or during acid and reactive oxygen intermediate exposure inside the host cell vacuole.

We defined energy stress as a condition in which bacteria are deprived of a carbon source or after depletion of intracellular ATP. Our results show that σ^B plays a positive role in maintaining cellular viability in glucose-limiting defined media after glucose is depleted. However, shortly after inoculation into fresh glucose-limiting media, the Δ*rsbT*, Δ*rsbV*, and Δ*sigB* strains grew more rapidly than the wild-type strain (Fig. 2), possibly by utilizing the carbon source more efficiently than the wild type. All three mutant cultures achieved higher numbers of cells more rapidly than the wild-type culture. Similar observations also have been reported with *B. subtilis* and *E. coli* strains that bear mutations in their respective general stress response sigma factors, σ^B or σ^S (34, 37). Schweder et al. (37) and Notley-McRobb et al. (34) have hypothesized that both *B. subtilis* and *E. coli* respond primarily to "hunger" rather than "general stress" during glucose-limited growth and suggest that a hunger signal may be perceived independently from a general stress signal. We hypothesize that at least some genes that are responsible for glucose transport or utilization are regulated by a sigma factor(s) other than σ^B in *L. monocytogenes*. For example, we identified putative σ^A-dependent promoters upstream of *lmo0169* and *lmo0176*, which encode putative glucose uptake proteins, in a preliminary examination of the *L. monocytogenes* genome (22) with the ListiList Web Server (http://genolist.pasteur.fr/ListiList) (31). It is possible that the loss of σ^B reduces overall competition among other sigma factors for core RNA polymerase. If this hypothesis is true and

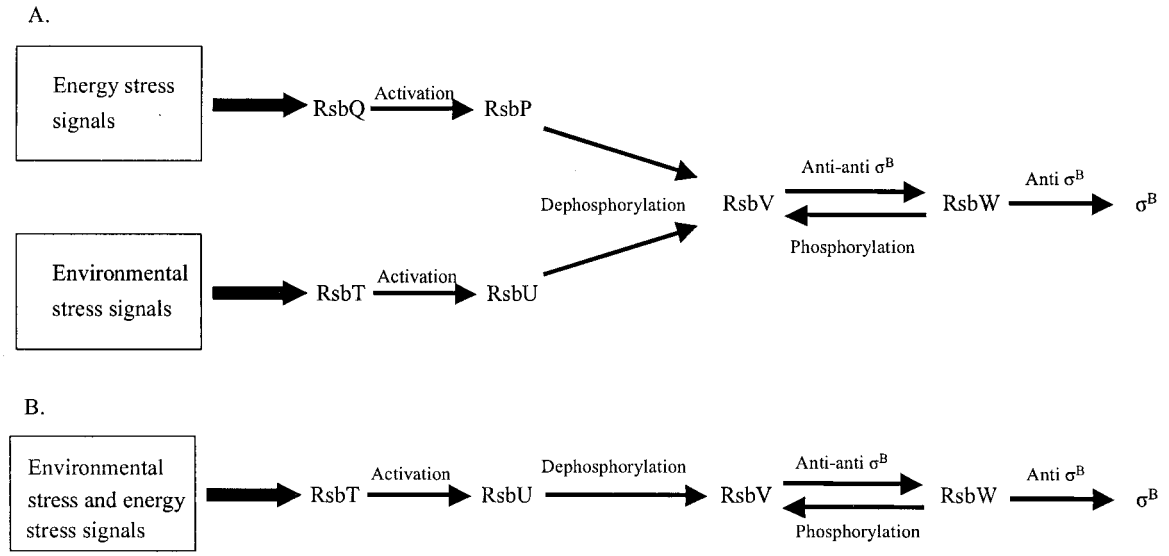

FIG. 5. Proposed model of Rsb-mediated σ^B activation in *L. monocytogenes* (B) in comparison to Rsb-mediated σ^B activation in *B. subtilis* (A).

if glucose transport is not σ^B regulated, an *L. monocytogenes* strain lacking σ^B (or an activated σ^B) may initially respond more efficiently to nutrient-limiting conditions than the wild-type strain. Another hypothesis is that σ^B and genes in its regulon may negatively regulate genes or proteins responsible for glucose transport (C. P. O'Byrne, personal communication) such that loss of activated σ^B results in very rapid uptake of glucose, leading to rapid growth followed by a dramatic dropoff after the glucose is completely consumed.

We also tested survival of the wild-type strain in comparison to the Δ*rsbT*, Δ*rsbV*, and Δ*sigB* mutants during prolonged static incubation at 37°C. The wild-type strain survived better following entry into stationary phase (6 to 36 h postinoculation), concomitantly with predicted activation of σ^B, which occurs in a growth phase-dependent manner (18). σ^B, RsbT, and RsbV are clearly important for survival in nutrient-limiting batch culture, particularly in early stationary phase.

CCCP is a protonophore that carries protons across the plasma membrane, which destroys the electron motive force, inhibits electron transport systems, and prevents proton flow back through ATPase, thereby limiting ATP synthesis. CCCP treatment has been shown to result in increased σ^B protein levels, while simultaneously reducing intracellular ATP in *Bacillus cereus* (39). As shown in Fig. 4, the ODs of all four *L. monocytogenes* strains increased slightly for approximately 2 h after addition of CCCP, possibly resulting from completion of cell division that had been initiated prior to CCCP exposure. After 2 h, the ODs of all four strains decreased at a similar rate until ~14 h postexposure. The ODs of the three mutant strains were significantly lower than that of the wild-type strain after 14 h. We hypothesize that accumulated intracellular ATP was depleted in all strains after 14 h. Thus, while insufficient for complete viability maintenance, the presence of active σ^B appears to reduce the rate of death in the presence of CCCP following ATP depletion.

Under the conditions selected for these studies, we did not distinguish phenotypic differences predicted by the *B. subtilis* model between the *L. monocytogenes* Δ*rsbT* and Δ*rsbV* mutants following energy stress (i.e., the *B. subtilis* model predicts that the Δ*rsbT* strain would be affected by environmental, but not energy, stresses) (Fig. 5A). Based on the evidence presented, we propose that, in contrast with *B. subtilis*, in *L. monocytogenes*, Rsb activation of σ^B by energy and environmental stresses is achieved through a single pathway (Fig. 5B). Both types of stresses appear to be conveyed to σ^B by RsbT, RsbU, RsbV, and RsbW. Our model does not rule out the existence of additional contributors to energy stress signaling pathways in *L. monocytogenes*. Additional σ^B-activating pathways have recently been proposed in *B. subtilis*. For example, Zhang et al. (48) suggest that, in addition to Rsb proteins, RelA also is associated with activation of σ^B under energy stresses in *B. subtilis*. Moreover, Brigulla et al. (8) put forward the possibility of the existence of yet another environmental stress signaling pathway responsible for σ^B-dependent contributions to low-temperature growth of *B. subtilis*. Given that *L. monocytogenes* is capable of growth at temperatures as low as 0°C (47), it is possible that a low-temperature σ^B activation pathway exists in this organism as well.

σ^B, RsbT, and RsbV contribute to virulence-associated phenotypes. In contrast with the *B. subtilis* system, the *L. monocytogenes* system also can be used to investigate the role of σ^B in bacterial virulence. Plaque assays and relative comparisons of hemolytic activities were used to examine the role of σ^B activation in *L. monocytogenes* virulence-associated characteristics. In the hemolysis assays, we observed that the loss of σ^B resulted in more than a twofold increase in apparent LLO activity relative to that measured in the wild-type strain. This finding is consistent with previous studies in which increased hemolysin activity was observed in a *Staphylococcus aureus sigB* mutant (11). We used sodium dodecyl sulfate-polyacrylamide gel electrophoresis to examine protein samples collected from wild-type, Δ*sigB*, Δ*rsbT*, and Δ*rsbV* culture supernatants. We found no gross differences in protein secretion patterns between the wild type and mutants (data not shown), suggesting

that the observed differences in hemolytic activities are not a consequence of vastly different quantities of hemolysin in the culture supernatants. As predicted, the Δ*prfA*P1*rsbT* and Δ*prfA*P1*rsbV* double mutants had dramatically reduced LLO activity, similar to the Δ*prfA*P1P2 and Δ*prfA*P1*sigB* double mutant phenotypes (32). We propose that the reduced LLO production phenotypes are likely a consequence of reduced *prfA* expression. We conclude that RsbT and RsbV contribute to σ^B activation required to reach the wild-type level of PrfA expression from the *prfA*P2 promoter, which leads to the wild-type level of LLO expression.

We also examined the role of σ^B in virulence-associated characteristics in a tissue culture plaque assay. Although the Δ*sigB*, Δ*rsbT*, or Δ*rsbV* and the Δ*prfA*P2*sigB*, Δ*prfA*P2*rsbT*, or Δ*prfA*P2*rsbV* strain had higher levels of LLO activity than the wild-type strain, these strains all yielded wild-type plaque sizes. Earlier studies have shown that LLO activity levels do not directly correlate with virulence in vivo (14). The *L. monocytogenes* requirement for LLO during intracellular infection appears to be minute and temporal (13). The wild-type plaque sizes among the Δ*sigB*-equivalent strains listed above suggest that while the increased LLO activity that we observed might possibly assist the bacterium in phagosomal escape, it does not appear to enhance bacterial ability to spread from cell to cell. We also show that σ^B, RsbT, and RsbV are not absolutely required for *L. monocytogenes* infection and dissemination in mouse fibroblast cells, as previously suggested for σ^B (32). However, the apparent contributions of these proteins to PrfA expression, which is important for cell-to-cell spread and overall virulence-associated phenotypes, are illustrated by the significantly reduced plaque sizes of the Δ*prfA*P1P2, Δ*prfA*P1*sigB*, Δ*prfA*P1*rsbT*, and Δ*prfA*P1*rsbV* strains in comparison to those of the wild-type strain.

In summary, we used a genetic approach to analyze the σ^B-activating stress signaling pathway in *L. monocytogenes*. We disproved our initial hypothesis that the σ^B activation pathways in *L. monocytogenes* are identical to those in *B. subtilis*. σ^B activation, which requires both RsbT and RsbV, contributes to *L. monocytogenes* survival under both environmental and energy stresses outside of the host as well as to virulence-associated characteristics in in vitro systems.

ACKNOWLEDGMENTS

We thank H. Marquis for assistance with the protein work and for intellectual discussions. We are grateful for the generosity of D. Portnoy for providing bacterial strains and for the valuable ideas of C. P. O'Byrne and M. Wiedmann. We truly appreciate the technical assistance of B. Bowen and T. Arvik with mutant strain creation.

This work was supported in part by National Institutes of Health award no. RO1-AI052151-01A1 (to K.J.B.). S.C. was supported by the Office of the Civil Service Commission (Thailand).

REFERENCES

1. **Akbar, S., C. M. Kang, T. A. Gaidenko, and C. W. Price.** 1997. Modulator protein RsbR regulates environmental signaling in the general stress pathway of *Bacillus subtilis*. Mol. Microbiol. **24:**567–578.
2. **Alper, S., L. Duncan, and R. Losick.** 1994. An adenosine nucleotide switch controlling the activity of a cell type-specific transcription factor in *B. subtilis*. Cell **77:**195–205.
3. **Antelmann, H., S. Engelmann, R. Schmid, and M. Hecker.** 1996. General and oxidative stress responses in *Bacillus subtilis*: cloning, expression, and mutation of the alkyl hydroperoxide reductase operon. J. Bacteriol. **178:** 6571–6578.
4. **Becker, L. A., M. S. Çetin, R. W. Hutkins, and A. K. Benson.** 1998. Identification of the gene encoding the alternative sigma factor σ^B from *Listeria monocytogenes* and its role in osmotolerance. J. Bacteriol. **180:**4547–4554.
5. **Bishop, D. K., and D. J. Hinrichs.** 1987. Adoptive transfer of immunity to *Listeria monocytogenes.* The influence of *in vitro* stimulation on lymphocyte subset requirements. J. Immunol. **139:**2005–2009.
6. **Boylan, S. A., A. Rutherford, S. M. Thomas, and C. W. Price.** 1992. Activation of *Bacillus subtilis* transcription factor σ^B by a regulatory pathway responsive to stationary-phase signals. J. Bacteriol. **174:**3695–3706.
7. **Boylan, S. A., A. R. Redfield, M. S. Brody, and C. W. Price.** 1993. Stress-induced activation of the σ^B transcription factor of *Bacillus subtilis*. J. Bacteriol. **175:**7931–7937.
8. **Brigulla, M., T. Hoffmann, A. Krisp, A. Völker, E. Bremer, and U. Völker.** 2003. Chill induction of the SigB-dependent general stress response in *Bacillus subtilis* and its contribution to low-temperature adaptation. J. Bacteriol. **185:**4305–4314.
9. **Brody, M. S., K. Vijay, and C. W. Price.** 2001. Catalytic function of an α/β hydrolase is required for energy stress activation of the σ^B transcription factor in *Bacillus subtilis*. J. Bacteriol. **183:**6422–6428.
10. **Camilli, A., L. G. Tilney, and D. A. Portnoy.** 1993. Dual roles of PlcA in *Listeria monocytogenes* pathogenesis. Mol. Microbiol. **8:**143–157.
11. **Cheung, A. L., Y.-T. Chien, and A. S. Bayer.** 1999. Hyperproduction of alpha-hemolysin in a *sigB* mutant is associated with elevated SarA expression in *Staphylococcus aureus*. Infect. Immun. **67:**1331–1337.
12. **Cotter, P. D., C. G. Gahan, and C. Hill.** 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Mol. Microbiol. **40:** 465–475.
13. **Dancz, C. E., A. Haraga, D. A. Portnoy, and D. E. Higgins.** 2002. Inducible control of virulence gene expression in *Listeria monocytogenes*: temporal requirement of listeriolysin O during intracellular infection. J. Bacteriol. **184:**5935–5945.
14. **Decatur, A. L., and D. A. Portnoy.** 2000. A PEST-like sequence in listeriolysin O essential for *Listeria monocytogenes* pathogenicity. Science **290:**992–995.
15. **Delumeau, O., R. J. Lewis, and M. D. Yudkin.** 2002. Protein-protein interactions that regulate the energy stress activation of σ^B in *Bacillus subtilis*. J. Bacteriol. **184:**5583–5589.
16. **Dufour, A., and W. G. Haldenwang.** 1994. Interactions between a *Bacillus subtilis* anti-σ factor (RsbW) and its antagonist (RsbV). J. Bacteriol. **176:** 1813–1820.
17. **Ferreira, A., C. P. O'Byrne, and K. J. Boor.** 2001. Role of σ^B in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. Appl. Environ. Microbiol. **67:**4454–4457.
18. **Ferreira, A., D. Sue, C. P. O'Byrne, and K. J. Boor.** 2003. Role of *Listeria monocytogenes* σ^B in survival of lethal acidic conditions and in the acquired acid tolerance response. Appl. Environ. Microbiol. **69:**2692–2698.
19. **Ferreira, A., M. Gray, M. Wiedmann, and K. J. Boor.** 2004. Comparative genomic analysis of the *sigB* operon in *Listeria monocytogenes* and in other Gram-positive bacteria. Curr. Microbiol. **48:**39–46.
20. **Freitag, N. E., and D. A. Portnoy.** 1994. Dual promoters of the *Listeria monocytogenes prfA* transcriptional activator appear essential in vitro but are redundant in vivo. Mol. Microbiol. **12:**845–853.
21. **Gaidenko, T. A., X. Yang, Y. M. Lee, and C. W. Price.** 1999. Threonine phosphorylation of modulator protein RsbR governs its ability to regulate a serine kinase in the environmental stress signaling pathway of *Bacillus subtilis*. J. Mol. Biol. **288:**29–39.
22. **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couv, A. D. Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K.-D. Entian, H. Fsihi, F. G.-D. Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L.-M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueo, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. D. Pablos, J.-C. Prez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J.-A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart.** 2001. Comparative genomics of *Listeria* species. Science **294:**849–852.
23. **Horton, R. M., Z. Cai, S. N. Ho, and L. R. Pease.** 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques **8:**528–534.
24. **Jones, S., and D. A. Portnoy.** 1994. Characterization of *Listeria monocytogenes* pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O. Infect. Immun. **62:**5608–5613.
25. **Kalman, S., M. L. Duncan, S. M. Thomas, and C. W. Price.** 1990. Similar organization of the *sigB* and *spoIIA* operons encoding alternate sigma factors of *Bacillus subtilis* RNA polymerase. J. Bacteriol. **172:**5575–5585.
26. **Kang, C. M., M. S. Brody, S. Akbar, X. Yang, and C. W. Price.** 1996. Homologous pairs of regulatory proteins control activity of *Bacillus subtilis* transcription factor σ^B in response to environmental stress. J. Bacteriol. **178:**3846–3853.
27. **Kang, C. M., K. Vijay, and C. W. Price.** 1998. Serine kinase activity of a *Bacillus subtilis* switch protein is required to transduce environmental stress

signals but not to activate its target PP2C phosphatase. Mol. Microbiol. **30:**189–196.
28. **Kazmierczak, M. J., S. C. Mithoe, K. J. Boor, and M. Wiedmann.** 2003. *Listeria monocytogenes* σ^B regulates stress response and virulence functions. J. Bacteriol. **185:**5722–5734.
29. **Kovacs, T., A. Hargitai, K. L. Kovacs, and I. Mecs.** 1998. pH-dependent activation of the alternative transcriptional factor σ^B in *Bacillus subtilis*. FEMS Microbiol. Lett. **165:**323–328.
30. **Milohanic, E., P. Glaser, J. Y. Coppée, L. Frangeul, Y. Vega, J. A. Vázquez-Boland, F. Kunst, P. Cossart, and C. Buchrieser.** 2003. Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. Mol. Microbiol. **47:**1613–1625.
31. **Moszer, I., P. Glaser, and A. Danchin.** 1995. SubtiList: a relational database for the *Bacillus subtilis* genome. Microbiology **141:**261–268.
32. **Nadon, C. A., B. M. Bowen, M. Wiedmann, and K. J. Boor.** 2002. Sigma B contributes to PrfA-mediated virulence in *Listeria monocytogenes*. Infect. Immun. **70:**3948–3952.
33. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. Approved standard M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
34. **Notley-McRobb, L., T. King, and T. Ferenci.** 2002. *rpoS* mutations and loss of general stress resistance in *Escherichia coli* populations as a consequence of conflict between competing stress responses. J. Bacteriol. **184:**806–811.
35. **Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs.** 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. **167:**1459–1471.
36. **Premaratne, R. J., W. Lin, and E. A. Johnson.** 1991. Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. Appl. Environ. Microbiol. **57:**3046–3048.
37. **Schweder, T., A. Kolyschkow, U. Völker, and M. Hecker.** 1999. Analysis of the expression and function of the σ^B-dependent general stress regulon of *Bacillus subtilis* during slow growth. Arch. Microbiol. **171:**439–443.
38. **Sun, A. N., A. Camilli, and D. A. Portnoy.** 1990. Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. Infect. Immun. **58:**3770–3778.
39. **van Schaik, W., M. H. Tempelaars, J. A. Wouters, W. M. de Vos, and T. Abee.** 2004. The alternative sigma factor σ^B of *Bacillus cereus*: response to stress and role in heat adaptation. J. Bacteriol. **186:**316–325.
40. **Vázquez-Boland, J. A., G. Domínguez-Bernal, B. González-Zorn, J. Kreft, and W. Goebel.** 2001. Pathogenicity islands and virulence evolution in *Listeria*. Microbes Infect. **3:**571–584.
41. **Vijay, K., M. S. Brody, E. Fredlund, and C. W. Price.** 2000. A PP2C phosphatase containing a PAS domain is required to convey signals of energy stress to the σ^B transcription factor of *Bacillus subtilis*. Mol. Microbiol. **35:**180–188.
42. **Voelker, U., A. Voelker, B. Maul, M. Hecker, A. Dufour, and W. G. Haldenwang.** 1995. Separate mechanisms activate σ^B of *Bacillus subtilis* in response to environmental and metabolic stresses. J. Bacteriol. **177:**3771–3780.
43. **Voelker, U., A. Voelker, and W. G. Haldenwang.** 1996. Reactivation of the *Bacillus subtilis* anti-σ^B antagonist, RsbV, by stress- or starvation-induced phosphatase activities. J. Bacteriol. **178:**5456–5463.
44. **Wiedmann, M., T. J. Arvik, R. J. Hurley, and K. J. Boor.** 1998. General stress transcription factor σ^B and its role in acid tolerance and virulence of *Listeria monocytogenes*. J. Bacteriol. **180:**3650–3656.
45. **Wise, A. A., and C. W. Price.** 1995. Four additional genes in the *sigB* operon of *Bacillus subtilis* that control activity of the general stress factor σ^B in response to environmental signals. J. Bacteriol. **177:**123–133.
46. **Yang, X., C. M. Kang, M. S. Brody, and C. W. Price.** 1996. Opposing pairs of serine protein kinases and phosphatases transmit signals of environmental stress to activate a bacterial transcription factor. Genes Dev. **10:**2265–2275.
47. **Yuqian, L., and A. E. Yousef.** 1999. Characteristics of *Listeria monocytogenes* important to food processors, p. 131–224. *In* E. T. Ryser and E. H. Marth (ed.), *Listeria*, listeriosis, and food safety, 2nd ed. Marcel Dekker, Inc., New York, N.Y.
48. **Zhang, S., and W. G. Haldenwang.** 2003. RelA is a component of the nutritional stress activation pathway of the *Bacillus subtilis* transcription factor σ^B. J. Bacteriol. **185:**5714–5721.