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General Stress Transcription Factor σ^B and Its Role in Acid Tolerance and Virulence of *Listeria monocytogenes*

MARTIN WIEDMANN,[1] TOREY J. ARVIK,[1] RICHARD J. HURLEY,[2] AND KATHRYN J. BOOR[1]*

Department of Food Science, College of Agriculture and Life Sciences,[1] and Center for Research Animal Resources, College of Veterinary Medicine,[2] Cornell University, Ithaca, New York 14853

Received 6 April 1998/Accepted 19 May 1998

The gene encoding the general stress transcription factor σ^B in the gram-positive bacterium *Listeria monocytogenes* was isolated with degenerate PCR primers followed by inverse PCR amplification. Evidence for gene identification includes the following: (i) phylogenetic analyses of reported amino acid sequences for σ^B and the closely related σ^F proteins grouped *L. monocytogenes* σ^B in the same cluster with the σ^B proteins from *Bacillus subtilis* and *Staphylococcus aureus*, (ii) the gene order in the 2,668-bp portion of the *L. monocytogenes sigB* operon is *rsbU-rsbV-rsbW-sigB-rsbX* and is therefore identical to the order of the last five genes of the *B. subtilis sigB* operon, and (iii) an *L. monocytogenes* σ^B mutant had reduced resistance to acid stress in comparison with its isogenic parent strain. The *sigB* mutant was further characterized in mouse models of listeriosis by determining recovery rates of the wild-type and mutant strains from livers and spleens following intragastric or intraperitoneal infection. Our results suggest that σ^B-directed genes do not appear to be essential for the spread of *L. monocytogenes* to mouse liver or spleen at 2 and 4 days following intragastric or intraperitoneal infection.

Regulation of gene expression in response to environmental stress conditions is essential for bacterial survival (57). Host-imposed stress conditions include the acidic pH of the stomach for orally transmitted pathogens and the acidic pH and oxidative stress inside the host cell vacuole for intracellular pathogens. The gram-positive facultative intracellular pathogen *Listeria monocytogenes* is subjected to both classes of stress during the course of a food-borne infection. The association of alternative sigma factors with core polymerase provides a mechanism for alterations in gene expression by directing transcription of new regulons in response to cellular signals (27). Well-characterized stress responses regulated by alternative sigma factors include sporulation in *Bacillus subtilis* (41) and the stationary phase (57) and heat shock responses (62) in *Escherichia coli*.

In some gram-negative pathogens, the stress-responsive alternative sigma factor RpoS has been shown to contribute to virulence. For example, RpoS regulates the expression of the plasmid virulence genes *spvABCD* in *Salmonella* and of the virulence gene *yst* in *Yersinia enterocolitica* (14, 30). *Salmonella typhimurium* and *Salmonella dublin rpoS* mutants have increased susceptibility to nutrient deprivation, oxidative stress, and acid stress and significantly reduced virulence in mice (14, 21, 55). An altered *rpoS* allele in *S. typhimurium* contributes to avirulence in the laboratory strain LT2 (55).

In contrast, little is known regarding the contribution of stress-responsive sigma factors to virulence in gram-positive organisms. One well-studied example of such a sigma factor is σ^B, which has been predominantly characterized for *Bacillus subtilis* (9–11, 19, 25, 26) but has also been reported for *Staphylococcus aureus* (58). σ^B-dependent transcription in *B. subtilis* is activated upon entry into stationary phase or following exposure to various environmental stress and growth-limiting conditions, including heat shock, oxygen limitation, or exposure to ethanol and high salt concentrations (6, 8, 10, 12, 52). While disruption of *sigB* in *B. subtilis* has no apparent effect on the organism's ability to sporulate or to grow under many conditions (19, 25, 29, 32), σ^B mutants have been shown to be sensitive to oxidative stress (4, 20).

As a facultative intracellular pathogen, *L. monocytogenes* provides a model system for studying the role of alternative sigma factors, specifically σ^B, in the virulence of gram-positive bacteria. We report the identification of a σ^B homolog in *L. monocytogenes*. Our results indicate that although loss of σ^B function diminishes acid resistance in *L. monocytogenes*, σ^B-directed genes do not appear to be essential for the spread of the organism to mouse liver and spleen 2 and 4 days after intragastric or intraperitoneal infection.

MATERIALS AND METHODS

Strains. *L. monocytogenes* 689426 was used to determine the *sigB* sequence as well as a partial sequence of the *sigB* operon. Furthermore, *sigB* was sequenced from *L. monocytogenes* 2289 and 10403S and from *Listeria innocua* DD 680. *L. monocytogenes* 10403S was used to generate the *sigB* mutant.

Cloning and sequencing of *sigB*. Based on the reported *sigB* sequences for *B. subtilis* (19) and *S. aureus* (58), two degenerate primers (LmsigB-1 and LmsigB-2 [Table 1]) were designed to amplify an internal *sigB* fragment from *L. monocytogenes*. These two primers were used in a touchdown PCR protocol (47) with an initial annealing temperature of 62°C, which was decreased by 0.5°C/cycle for 20 cycles, followed by 20 cycles with an annealing temperature of 52°C. This reaction led to amplification of a strong, single DNA fragment of the expected size (403 bp) from *L. monocytogenes* 689426. With BLASTN (3), this fragment shared 66 and 65% identities with the corresponding *sigB* regions from *B. subtilis* and *S. aureus*, respectively. This sequence was used to design primers LmsigB-3 and LmsigB-4 for inverse PCR amplification of the region adjacent to the 5′ end of the initial fragment. For inverse PCR, 10 μg of chromosomal DNA, isolated as described by Flamm et al. (23), was digested with selected restriction enzymes. Self-ligation of the digested DNA was performed at several DNA concentrations (0.5, 1.0, 2.25, 5, and 15 μg/μl) with a 50-μl reaction volume with 1 U of T_4 DNA ligase (Gibco BRL, Gaithersburg, Md.). Subsequent PCR was performed with 5 ng of the self-ligated DNA per reaction. Primers LmsigB-3 and LmsigB-4 yielded a PCR product of approximately 415 bp with *Sau*3AI-digested chromosomal DNA. The degenerate primer LmrsbW-1 was designed based on the reported *rsbW* sequences for *B. subtilis* and *S. aureus* to allow amplification of DNA sequences 5′ to those previously obtained. PCR amplification with LmrsbW-1 and LmsigB-10 yielded a PCR product of approximately 715 bp, providing an additional 112 bp 5′ of the *Sau*3AI inverse PCR product. Further inverse PCR amplification with LmrsbW-3 and LmrsbW-4 and with LmsigB-17 and

* 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. PCR primers used in this study

Primer	Sequence (5′→3′)
LmsigB-1	AC(A/G) TGC ATT TGA (C/G)(A/T)T A(A/T)A CCG A
LmsigB-2	G(C/T)G T(A/T)C ATG T(G/T)C CGA GAC G
LmsigB-3	TTT GCG GCG AGC TTT GTA AT
LmsigB-4	CAT CGG TGT CAC GGA AGA AGA A
LmsigB-10	CCC GTT TCT TTT TGA CTG C
LmsigB-12	CAT TTA TTG AAA ATC GCA GTC
LmsigB-15	AAT ATA TTA ATG AAA AGC AGG TGG AG
LmsigB-16	ATA AAT TAT TTG ATT CAA CTG CCT T
LmsigB-17	ACT TGA TAT ACT TCA AGT GCT
LmsigB-18	GGG TAT AGT ACT TCG AAT CG
LmsigB-19	CGA ATA TTA CCC ATA CCA G
LmsigB-20	TAT GAT TGG GAT GGA AG
LmrsbW-1	AAG GG(A/T) GAC AG(C/T) TTT GA(C/T) T(A/T)T GA
LmrsbW-3	GGC ATT CTC CTC CAC CTG
LmrsbW-4	CTC AAC CTG ATA AAG AGG CG
SOE-A[a]	GGA ATT CCA GGT GGA GGA GAA TGC
SOE-B[b]	CAC ACG TTC AAA TCC ATC ATC TGC AGC AAA TGC TTC AAA G
SOE-C	GAT GAT GGA TTT GAA CGT GTG
SOE-D[c]	GCT CTA GAA CAT CCC CGC AGT ATT G

[a] The *Eco*RI restriction site incorporated into this primer to facilitate cloning is underlined.
[b] The overhang complementary to SOE-C is underlined.
[c] The *Xba*I restriction site incorporated into this primer to facilitate cloning is underlined.

LmsigB-18 on *Sau*3AI- and *Hin*dIII-digested chromosomal DNA, respectively, yielded an additional 1,195 bp of sequence information. With two primer sets (LmsigB-8 and LmsigB-12 along with LmsigB-19 and LmsigB-20), a 1,000-bp fragment and a 460-bp fragment were amplified from *Hin*dIII- and *Nla*III-digested chromosomal DNAs, respectively, yielding sequence information 3′ of the initially amplified internal *L. monocytogenes sigB* fragment.

For sequencing, PCR products were cloned into the pCR 2.1 vector with the Original TA Cloning kit (Invitrogen, San Diego, Calif.) according to the manufacturer's recommendations. Plasmids were purified with the QIAquick Plasmid Purification kit (Qiagen, Chatsworth, Calif.) and used for DNA sequencing. To compare *sigB* allelic variations among *L. monocytogenes* strains, PCR primers LmsigB-15 and LmsigB-16 (Table 1) were used to amplify the complete *sigB* open reading frames (ORFs) from two additional *L. monocytogenes* strains and from *L. innocua*. The PCR products were purified with the QIAquick PCR Purification kit (Qiagen) and then sequenced directly with the same primers.

DNA and protein sequence analyses were performed with Lasergene software (DNAStar, Madison, Wis.). Alignments were performed by the Clustal method (MEGALIGN). Phylogenetic analyses of σ^B and σ^F amino acid sequences were performed with Seqboot, Protdist, Neighbor, Consense, and Drawtree in the software package PHYLIP, version 3.57c (22).

Generation of an *L. monocytogenes sigB* mutant. A nonpolar internal deletion mutant allele of *sigB* was created in the *E. coli-L. monocytogenes* shuttle vector pKSV7 by SOE (splicing by overlap extension) PCR (28) and was introduced into *L. monocytogenes* 10403S by allelic exchange mutagenesis. SOE PCR primers were designed to amplify two ~300-bp DNA fragments, one comprising the 5′ end of *sigB* (nucleotides [nt] 1217 to 1490, amplified by primers SOE-A and SOE-B [Table 1]) and one comprising the 3′ end of *sigB* (nt 1788 to 2087, amplified by primers SOE-C and SOE-D [Table 1]). Subsequent PCR amplification with SOE-A and SOE-D created a 600-bp *sigB* fragment with an in-frame 297-bp deletion. This fragment was purified with the QIAquick PCR Purification kit (Qiagen) and was then digested with *Xba*I and *Eco*RI. The purified fragment was cloned into pKSV7 and transformed into *E. coli* DH5-α. The resulting plasmid, pTJA-57, was subsequently electroporated into *L. monocytogenes* 10403S as previously described (13), and transformants were selected on brain heart infusion (BHI) agar plates containing 10 μg of chloramphenicol per ml. A transformant was serially passaged at 42°C to direct chromosomal integration of the plasmid by homologous recombination. A single colony with a chromosomal integration was serially passaged in BHI and replica plated to obtain an allelic exchange mutant. Allelic exchange mutagenesis was confirmed by PCR amplification and direct sequencing of the PCR product (data not shown).

Acid tolerance assay. The ability of *L. monocytogenes* to survive acid stress was evaluated as described by Wilmes-Riesenberg et al. (55), with some minor modifications. Briefly, 1 ml of *L. monocytogenes* cells grown overnight in BHI broth was pelleted and then resuspended in 10 ml of BHI agar (pH 2.5). Aliquots were removed immediately and at 30, 60, and 120 min for plating on BHI agar plates.

Mouse virulence assays. Lightly anesthetized BALB/c mice (approximately 6 weeks old) were infected either intraperitoneally with approximately 2×10^4 bacteria (37) or intragastrically with approximately 2×10^9 bacteria in 0.9% saline (5). The mice were housed in an AAALAC-International accredited facility, and animal experiments were reviewed and approved by Cornell University's Institutional Animal Care and Use Committee. Food was withheld from the mice for 5 to 6 h prior to infection. Bacterial numbers in spleens and livers were determined at days 2 and 4 postinoculation. The results are expressed as mean values and standard deviations for five mice.

Nucleotide sequence accession numbers. The nucleotide sequence of the *L. monocytogenes* 689426 *sigB* region has been assigned GenBank accession no. AF032444. The *sigB* sequences of *L. monocytogenes* 2289 and 10403S and *L. innocua* DD 680 have been assigned GenBank accession no. AF032445, AF032446, and AF032447, respectively.

RESULTS

Cloning and sequencing of *sigB* and the partial *sigB* operon. The complete DNA sequence obtained by PCR with degenerate primers followed by inverse PCR amplification was deposited in GenBank under accession no. AF032444. Because cloned PCR products are potentially subject to sequence alterations due to PCR misincorporation, our *sigB* DNA sequence was confirmed by directly sequencing a PCR product comprising the complete *sigB* open reading frame (ORF). Furthermore, overlapping sequences obtained by independent inverse PCR amplifications showed no sequence variations. DNA sequence analyses revealed one partial and four complete ORFs with significant predicted amino acid identities to RsbU, RsbV, RsbW, σ^B, and RsbX in *B. subtilis* and RsbU, RsbV, RsbW, and σ^B in *S. aureus* (Fig. 1). The *rsbV* ORF is preceded by a possible σ^B promoter site. Alignments of the *L. monocytogenes* predicted amino acid sequences with RsbU, RsbV, RsbW, and σ^B from *B. subtilis* and *S. aureus* and RsbX from *B. subtilis* are shown in Fig. 2.

B. subtilis residues previously shown to be important for the function of RsbV and RsbW (33) are also conserved in the predicted *Listeria* gene products (Fig. 2), providing further evidence of identification of the *sigB* operon in *L. monocytogenes*. For example, the conserved serine residue, which represents a phosphorylation site in RsbV, and the conserved amino acid residues, which are thought to be important for ATP binding in RsbW (and in histidine kinases), were conserved in the respective proteins among all sequenced *Listeria* strains.

Phylogenetic analysis of σ^B and σ^F. Because of reported sequence heterogeneities in the stress-responsive RpoS pro-

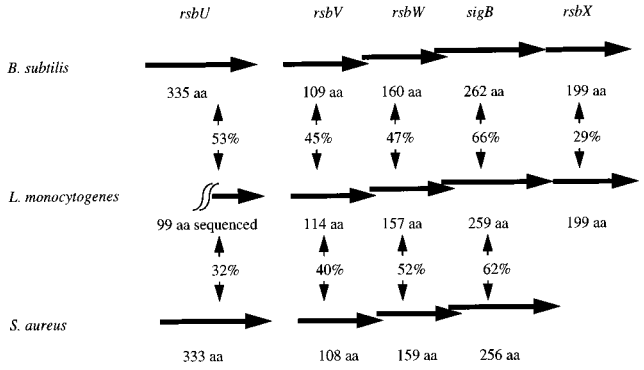
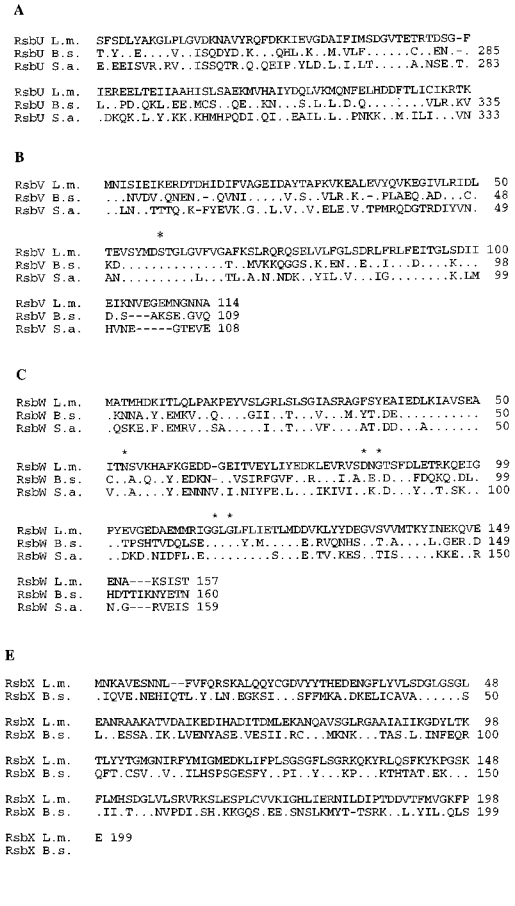


FIG. 1. Schematic of the organization of the *sigB* operon in *L. monocytogenes*, *B. subtilis*, and *S. aureus*. Predicted protein sizes and identities are indicated. For *L. monocytogenes* RsbU, a 99-aa C-terminal sequence was used for the calculation of identities.

tein of gram-negative organisms (31, 55), we investigated the phylogenetic diversity of σ^B among *Listeria* strains. We PCR amplified and sequenced complete *sigB* ORFs from three *L. monocytogenes* strains, each representing one of three major genetic lineages (54), and from one *L. innocua* strain. In addition to *L. monocytogenes* 689426 (lineage III), we sequenced *sigB* from strains 2289 (lineage I) and 10403S (lineage II). Alignment of the three *L. monocytogenes* sequences revealed a total of 31 polymorphic nucleotide sites, but only one predicted a polymorphic amino acid (aa) site, located at aa 216. Residue 216 is a phenylalanine in strains 689426 and 2289 but a tyrosine in strain 10403S. The addition of the *L. innocua sigB* sequence to the *L. monocytogenes* alignments identified an additional 43 polymorphic nucleotide sites for a total of 74 among the four sequences. Comparison of *L. innocua* σ^B with the predicted *L. monocytogenes* σ^B sequences identified only two polymorphic amino acid residues (Fig. 2D) in addition to the presence of a tyrosine at aa 216, as found in *L. monocytogenes* 10403S.

Previous reports (16, 17, 32, 44) and the results of our BLASTN analyses suggested phylogenetic relationships between sequences previously reported for σ^B and σ^F proteins. We probed these relationships by analyzing a multiple sequence alignment of the predicted amino acid sequences. This analysis revealed four σ factor clusters as follows: σ^B from *L. monocytogenes*, *B. subtilis*, and *S. aureus* (cluster A); σ^F from *Streptomyces* spp. (cluster B); putative σ^F factors from *Myco-*



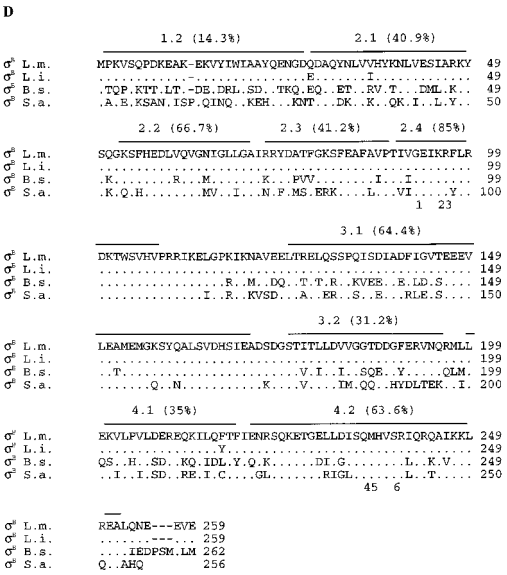


FIG. 2. Alignments of the deduced σ^B and Rsb amino acid sequences from *L. monocytogenes* (L.m.), *B. subtilis* (B.s.), and *S. aureus* (S.a.). The *L. monocytogenes* sequence is always shown on top; for the other species, amino acids are listed only when they differ from the *L. monocytogenes* sequence. Symbols: ., an amino acid that is identical to the *L. monocytogenes* sequence; −, a gap. (A) Alignment of the partial C-terminal *L. monocytogenes* RsbU sequence (99 aa) with the homologous regions of *B. subtilis* and *S. aureus*. (B) RsbV alignment. An asterisk above the alignment indicates a conserved serine residue that represents the site of phosphorylation by RsbW (33). (C) RsbW alignment. Asterisks above the alignment indicate conserved amino acid residues which are thought to be important for ATP binding in RsbW and in histidine kinases (33). (D) σ^B alignment. This alignment also includes the amino acid sequence from *L. innocua* DD 680 (L.i.). The regions and subregions of σ^B are indicated above the alignment (40). Amino acid conservation, calculated as the percent residues conserved in all four species relative to the number of residues in a given region, is indicated for each region. Numbers below the alignment indicate residues important for promoter recognition in region 2.4 (1 to 3) and in region 4.2 (4 to 6) for the following sigma factors (39): 1, Q-196 in *B. subtilis* σ^A (35), R-96 in *B. subtilis* σ^H (15), and Q-437 in *E. coli* RpoD (53); 2, T-440 in *E. coli* RpoD (48); 3, T-100 in σ^H (63) and M-124 in σ^E (50); 4, R-584 in *E. coli* RpoD (48); 5, mutations in this region switch promoter specificity among σ^B, σ^F, and σ^G in *B. subtilis* (39); and 6, R-588 in *E. coli* RpoD (24) and R-347 in *B. subtilis* σ^A (35, 36). (E) RsbX alignment. An RsbX homolog in *S. aureus* has not been identified (58).

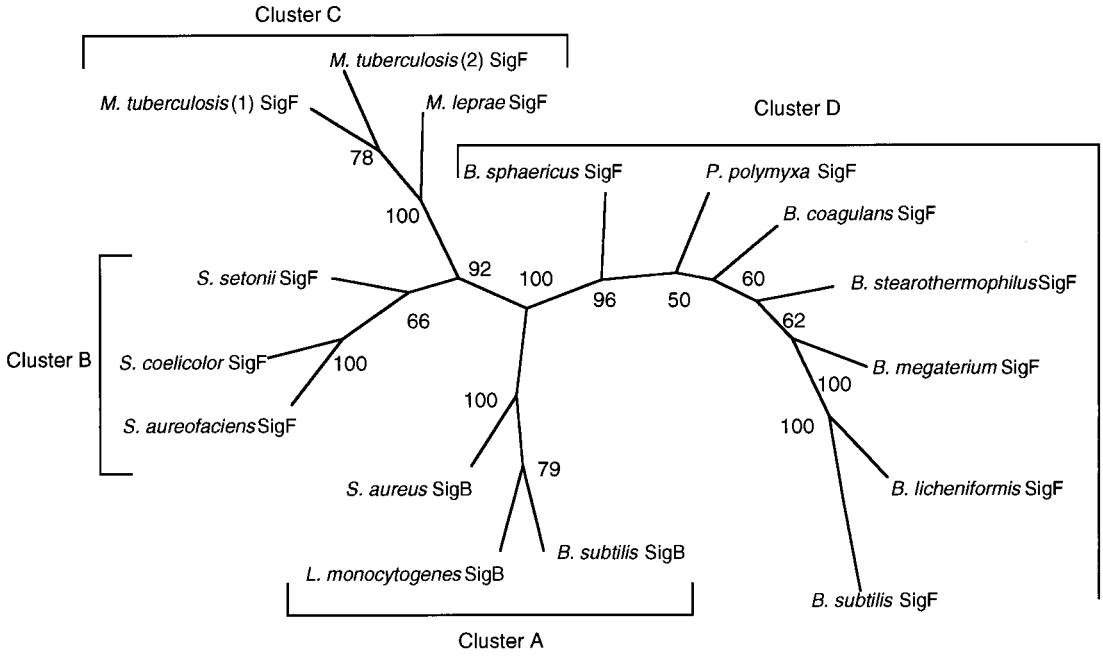


FIG. 3. Unrooted bootstrap tree (100 replicates) for σ^B and σ^F sequences constructed by the neighbor joining method. The tree was constructed with the Seqboot, Protdist, Neighbor, Consensus, and Drawtree programs in the software package PHYLIP (22). The numbers at the nodes of the tree represent the bootstrap values for each node. Sequences used for this analysis include σ^B from *L. monocytogenes*, *B. subtilis* (19) (GenBank accession no. M34995), and *S. aureus* (58) (GenBank accession no. Y09929) and σ^F from *Bacillus coagulans* (42) (GenBank accession no. Z54161), *Bacillus megaterium* (49) (GenBank accession no. X63757), *B. subtilis* (60) (GenBank accession no. M15744), *Bacillus stearothermophilus* (43) (GenBank accession no. L47360), *Bacillus licheniformis* (61) (GenBank accession no. M25260), *Bacillus sphaericus* (43) (GenBank accession no. L47359), *Paenibacillus polymyxa* (43) (GenBank accession no. L47358), *Streptomyces aureofaciens* (44) (GenBank accession no. L09565), *Streptomyces coelicolor* (44) (GenBank accession no. L11648), *Streptomyces setonii* (34) (GenBank accession no. D17466), and *M. leprae* (GenBank accession no. U00012) and from two *M. tuberculosis* isolates (16) (GenBank accession no. U41641 and Z92771).

bacterium tuberculosis and *Mycobacterium leprae* (cluster C); and σ^F from *Bacillus* spp. (cluster D). These clusters are displayed in a bootstrap tree in Fig. 3. Amino acid sequence identities among σ^B proteins (cluster A) ranged from 58 to 66%; sequence identities within clusters B and D ranged from 46 to 85% and from 57 to 90%, respectively. In cluster C, *M. tuberculosis* and *M. leprae* had 62% predicted sigma factor amino acid identity. Amino acid identities among the four clusters ranged from 23 to 41%.

Characterization of an *L. monocytogenes sigB* null mutant. To evaluate the function of σ^B in *L. monocytogenes*, we used allelic exchange mutagenesis to construct a nonpolar *sigB* mutant with an internal 99-aa deletion. Survival of *L. monocytogenes* σ^B mutant stationary-phase cells exposed to pH 2.5 for 1 or 2 h was significantly reduced ($P < 0.05$; *t* test) compared with that of its isogenic parent (Fig. 4). Survival after 1 or 2 h was 1.6 or 3.6 logs lower, respectively, for the σ^B mutant than for its isogenic parent.

The *sigB* mutant was further characterized with mouse models of listeriosis by determining recovery rates of wild-type and mutant strains from livers and spleens following intragastric or intraperitoneal infection (Table 2). The *sigB* mutant was recovered at slightly lower levels from livers at day 4 for intragastric inoculation and at day 2 for intraperitoneal inoculation ($P = 0.027$ and 0.029, respectively). In general, however, mutant and wild-type strains showed similar recovery rates at 2 and 4 days postinoculation. One of the mice infected intraperitoneally with the wild-type strain showed liver abscesses by macroscopic evaluation at day 4. No liver abscesses were observed in the animals infected with the *sigB* mutant.

DISCUSSION

The following evidence supports our identification of *sigB*, the gene encoding σ^B, in *L. monocytogenes*: (i) phylogenetic analyses of reported amino acid sequences for σ^B and the closely related σ^F proteins from various species grouped *L. monocytogenes* σ^B in the same cluster with the σ^B proteins from *B. subtilis* and *S. aureus*; (ii) *B. subtilis* residues previously shown to be important for the function of RsbV and RsbW (33) are also conserved in the predicted *Listeria* gene products (Fig. 2); (iii) the gene order in the 2,668-bp region of the *L. monocytogenes sigB* operon is *rsbU-rsbV-rsbW-sigB-rsbX* and is therefore identical to the order of the last five genes of the *sigB* operon in *B. subtilis*; and (iv) *L. monocytogenes* σ^B mutant cells are more sensitive to acid exposure than wild-type cells. Because stress-responsive sigma factors have been shown to be important for virulence among gram-negative bacteria (14, 21, 55), we tested the possible contribution of σ^B to *L. monocytogenes* virulence in a mouse model system. In mouse infection experiments, mutant and wild-type cells showed similar degrees of spreading to livers and spleens after either intragastric or intraperitoneal infection, although recovery of the mutant from livers was lower than that for the wild type at day 4 for intragastric inoculation and at day 2 for intraperitoneal infection ($P = 0.027$ and 0.029, respectively). Taken together, these findings suggest that σ^B-dependent proteins contribute to acid resistance in *L. monocytogenes* but are not essential for spreading of the organism to livers and spleens at 2 and 4 days postinoculation in this mouse model.

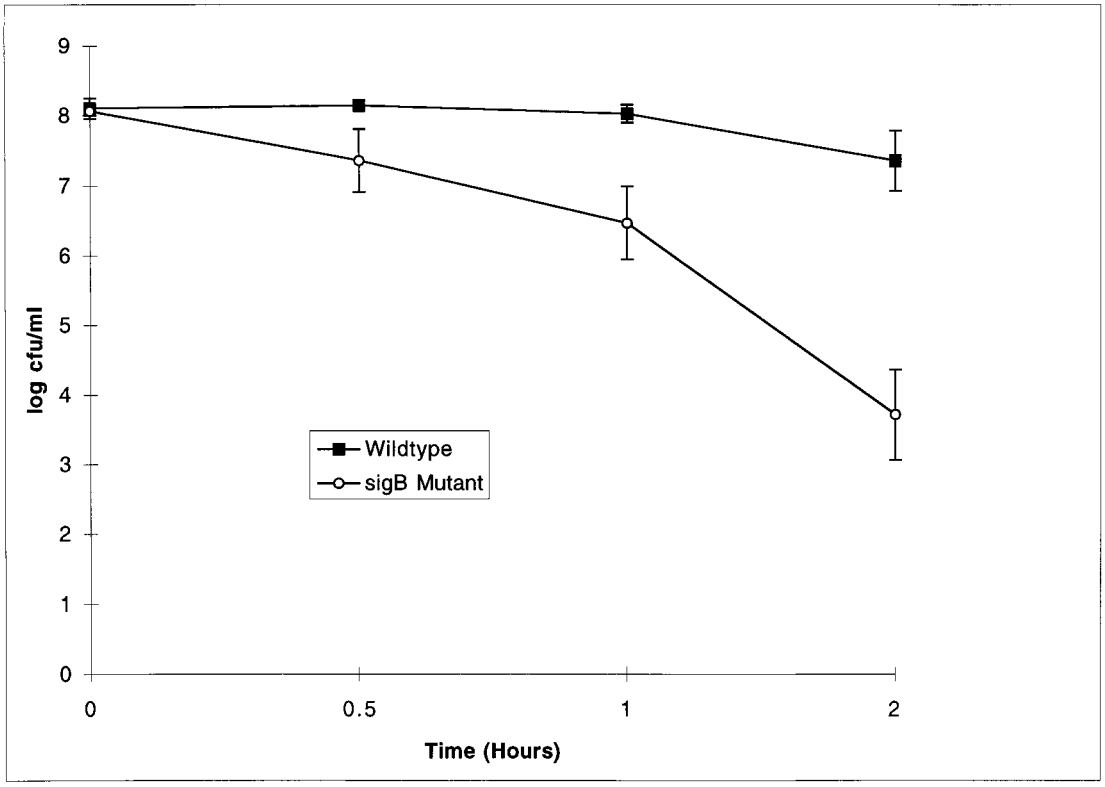


FIG. 4. Stationary-phase acid survival (pH 2.5) of *L. monocytogenes*. Values are the averages of two trials, each of which was performed in duplicate. Standard errors are given. The σ^B mutant showed significantly decreased survival compared to its isogenic parent at 1 and 2 h ($P < 0.05$).

***L. monocytogenes sigB* operon structure.** We have identified one partial and four complete ORFs in *L. monocytogenes* with significant predicted amino acid identities to RsbU, RsbV, RsbW, σ^B, and RsbX in *B. subtilis* and RsbU, RsbV, RsbW, and σ^B in *S. aureus* (Fig. 1). In *B. subtilis*, the *sigB* structural gene lies seventh in an operon which includes seven *rsb* genes, where *rsb* stands for regulator of σ^B. The *rsb* products regulate σ^B activity by means of coupled partner switching modules in response to signals of energy or environmental stress (1, 2, 6–8, 10, 12, 18, 33, 51, 56, 59). Each module is composed of three elements: a serine phosphatase (RsbU or RsbX), an antagonist protein (RsbS or RsbV), and a switch protein (RsbT or RsbW) (1, 33, 59). We speculate that the presence of RsbU and RsbX in *L. monocytogenes* predicts the existence of a dual-module σ^B regulatory network similar to that of *B. subtilis* and thus also predicts the presence of the σ^B regulatory proteins RsbR, RsbS, and RsbT in *L. monocytogenes*. In contrast, the gene order in the *S. aureus sigB* operon is *rsbU-rsbV-rsbW-sigB*, with an ORF (CTorf239) which shows no homology to *rsbX* immediately downstream of *sigB* (58). This finding suggests that the regulatory mechanisms controlling the activity of *S. aureus* σ^B may lack the environmental stress-responsive regulatory module further composed of RsbR, RsbS, and RsbT and thus may differ from the σ^B regulatory networks of *B. subtilis* and *L. monocytogenes*.

TABLE 2. Recovery of *L. monocytogenes* 10403S and the isogenic *sigB* mutant from tissues of infected mice after intragastric or intraperitoneal infection

Type of inoculation and tissue	Log_{10} no. of bacteria/g of tissue (± SD) at the following day postinfection[a]:			
	2		4	
	10403S	*sigB* mutant	10403S	*sigB* mutant
Intragastric				
Liver	3.7 (1.2)	3.4 (1.3)	3.7 (0.5)[c]	2.9 (0.4)[c]
Spleen	4.0 (1.0)	4.1 (1.5)	4.9 (0.4)	4.5 (0.2)
Intraperitoneal				
Liver	4.3 (0.5)[b]	3.6 (0.3)[b]	3.9 (1.5)	4.3 (0.9)
Spleen	5.1 (0.4)	4.8 (0.4)	5.0 (1.5)	5.1 (0.5)

[a] Values are the averages of five inoculated animals, except for the intragastric-inoculation day-2 data, which represent the averages of four animals (one animal was excluded from each group because fewer bacteria than the detection limit [10 CFU/g for liver and $<10^2$ CFU/g for spleen] were recovered).
[b] Bacterial numbers differ significantly at $P = 0.029$.
[c] Bacterial numbers differ significantly at $P = 0.027$.

Phylogenetic analyses of σ^B and σ^F proteins. Multiple alignments of predicted σ^B and σ^F amino acid sequences from *Streptomyces* spp., *Mycobacterium* spp., *Bacillus* spp., and *L. monocytogenes* clustered the products in a manner suggesting that σ^B and σ^F proteins sequenced to date represent four phylogenetically distinct σ factor groups with a possible common ancestor (Fig. 3). These results are also consistent with the hypothesis that σ^B and σ^F in the genus *Bacillus* may have arisen by tandem duplication from a common ancestor (32).

Alignment of the predicted σ^B amino acid sequences from strains representing the three major genetic lineages of *L. monocytogenes* (54) and from an *L. innocua* strain identified 74 polymorphic nucleotide sites which predict only 3 polymorphic amino acid residues (Fig. 2D). This level of conservation is

noteworthy in comparison with observed allelic variations in other well-characterized *L. monocytogenes* genes. To illustrate, 22 of 53 polymorphic nucleotides in a 539-bp fragment of the *L. monocytogenes actA* virulence gene are predicted to result in amino acid changes (54) and 2 of 12 polymorphic nucleotides in a 150-bp fragment of the *hly* virulence gene yield predicted amino acid changes (45, 46, 54). Our findings strongly suggest that functional constraints within *Listeria* spp. limit evolutionary alterations in the σ^B protein.

Characterization of an *L. monocytogenes* σ^B mutant. Bacterial survival in the acidic environment of the stomach and in the vacuole of the macrophage is likely to be important for full virulence of an intracellular pathogen commonly transmitted by food, such as *L. monocytogenes*. We report a significant reduction in stationary-phase acid tolerance for our *L. monocytogenes* σ^B mutant in comparison with its isogenic parent (Fig. 4). Our finding of increased acid sensitivity in the σ^B mutant suggests that σ^B-dependent proteins provide some protection to *L. monocytogenes* cells exposed to lethal acidic conditions. *B. subtilis* σ^B mutant cells have been shown to be more sensitive than wild-type cells to oxidative stress, specifically exposure to cumene hydroperoxide and lethal doses of hydrogen peroxide (4, 20). Our demonstration of reduced tolerance to lethal acid stress for *L. monocytogenes* σ^B mutant cells provides phenotypic evidence supporting the role of σ^B-dependent proteins in response to conditions of environmental stress.

Based on the reduced acid resistance of the *L. monocytogenes sigB* mutant, we hypothesized that σ^B might play a role in *L. monocytogenes* virulence. Specifically, we speculated that σ^B may protect the organism from the acid stress encountered during stomach passage (~2 hours at pH 2 to 3), which may enhance its survival and passage into the intestinal tract, the site of systemic invasion. Therefore, we evaluated the effects of loss of *L. monocytogenes* σ^B function in mouse models of listeriosis.

Spreading of an *L. monocytogenes sigB* mutant to the liver 2 or 4 days after intragastric or intraperitoneal inoculation was only minimally impaired in comparison with that of its isogenic parent. The *sigB* mutant was recovered at slightly lower levels from livers 4 days after intragastric inoculation and 2 days after intraperitoneal inoculation ($P = 0.027$ and 0.029, respectively). These findings suggest that loss of σ^B function has only minimal effects on the early spread of *L. monocytogenes* in this mouse virulence assay. Our findings contrast with results obtained with the gram-negative enteric pathogen *S. typhimurium*, in which the general stress σ factor RpoS (38) is essential for full virulence (21, 55). While our results with the mouse model appear to rule out a significant contribution of *L. monocytogenes* σ^B to intracellular survival and spread, they do not rule out a contribution to the infection process. For example, it is possible that direct intragastric inoculation alters the stomach passage time normally encountered by ingested materials. If this proves to be the case, then our results do not rule out a direct contribution of σ^B to pathogenesis in food-borne infections. Furthermore, the experiments that we report do not fully address the role of *L. monocytogenes* σ^B in surviving environmental stress. Because loss of σ^B function leads to decreased resistance to acid stress, we consider it likely that loss of σ^B function will also lead to decreased resistance to other environmental stresses. Such environmental stress resistance may contribute to survival in foods and therefore indirectly to pathogenicity.

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