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Comparative Analysis of the σ^B-Dependent Stress Responses in *Listeria monocytogenes* and *Listeria innocua* Strains Exposed to Selected Stress Conditions▽†

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The alternative sigma factor σ^B contributes to transcription of stress response and virulence genes in diverse gram-positive bacterial species. The composition and functions of the *Listeria monocytogenes* and *Listeria innocua* σ^B regulons were hypothesized to differ due to virulence differences between these closely related species. Transcript levels in stationary-phase cells and in cells exposed to salt stress were characterized by microarray analyses for both species. In *L. monocytogenes*, 168 genes were positively regulated by σ^B; 145 of these genes were preceded by a putative σ^B consensus promoter. In *L. innocua*, 64 genes were positively regulated by σ^B. σ^B contributed to acid stress survival in log-phase cells for both species but to survival in stationary-phase cells only for *L. monocytogenes*. In summary, (i) the *L. monocytogenes* σ^B regulon includes >140 genes that are both directly and positively regulated by σ^B, including genes encoding proteins with importance in stress response, virulence, transcriptional regulation, carbohydrate metabolism, and transport; (ii) a number of *L. monocytogenes* genes encoding flagellar proteins show higher transcript levels in the Δ*sigB* mutant, and both *L. monocytogenes* and *L. innocua* Δ*sigB* null mutants have increased motility compared to the respective isogenic parent strains, suggesting that σ^B affects motility and chemotaxis; and (iii) although *L. monocytogenes* and *L. innocua* differ in σ^B-dependent acid stress resistance and have species-specific σ^B-dependent genes, the *L. monocytogenes* and *L. innocua* σ^B regulons show considerable conservation, with a common set of at least 49 genes that are σ^B dependent in both species.

A sigma factor is a dissociable protein subunit that directs bacterial RNA polymerase holoenzyme to recognize a promoter sequence upstream of a gene prior to transcription initiation. New associations between alternative sigma factors and core RNA polymerase essentially reprogram promoter recognition specificities of the enzyme in response to changing environmental conditions, thus allowing expression of new sets of target genes appropriate for the conditions. The alternative sigma factor σ^B, encoded by *sigB*, has been identified as contributing to the general stress response in several gram-positive bacteria, including *Bacillus subtilis* (31), *Bacillus licheniformis* (7), *Bacillus anthracis* (24), *Bacillus cereus* (68), *Listeria monocytogenes* (2, 70), *Staphylococcus aureus* (75), and *Corynebacterium glutamicum* (47).

L. monocytogenes is a non-spore-forming facultative intracellular pathogen that causes listeriosis, a serious invasive disease in both animals and humans. To establish a food-borne bacterial infection, *L. monocytogenes* must have the ability to survive under a variety of stress conditions, including those encountered in a wide range of nonhost environments and food matrices, as well as under rapidly changing conditions encountered during gastrointestinal passage (exposure to organic acids, bile salts, and osmotic gradients) and subsequent stages of infection (e.g., in the intracellular environment). *L. monocytogenes* σ^B is activated following exposure to a number of environmental stress conditions (2) and contributes to bacterial survival under acid and oxidative stresses and during carbon starvation (13, 21, 22, 70). In addition, transcription of several virulence genes, including *prfA*, *bsh*, *inlA*, and *inlB*, is at least partially σ^B dependent (41–43, 53, 58, 59, 65, 66). Further, a Δ*sigB* null mutant has reduced invasiveness in human intestinal epithelial cells (42) and reduced virulence in intragastrically inoculated guinea pigs (27). A previous study using a subgenomic microarray comprised of 208 *L. monocytogenes*-specific probes (41) identified 55 σ^B-dependent *L. monocytogenes* genes with ≥1.5-fold-higher transcript levels in the parent strain than in an isogenic *sigB* null mutant. However, as both the *B. subtilis* (33) and the *S. aureus* (4) σ^B regulons appear to be comprised of at least 150 genes, we hypothesized that the initial efforts to characterize the *L. monocytogenes* σ^B regulon (41) likely revealed less than half of the genes comprising the regulon in this species. A more complete identification of the *L. monocytogenes* σ^B regulon will enhance our understanding of the contributions of this key regulator to stress response and virulence in this important food-borne pathogen.

The nonpathogenic *Listeria innocua* is the *Listeria* species most closely related to *L. monocytogenes* (56). Previous comparative studies of these two species have already furthered our understanding of *L. monocytogenes* virulence. For example, while comparisons of these two species revealed a high level of genome synteny (29), identification and characterization of genes found in *L. monocytogenes*, but absent from *L. innocua*, have led to the discovery of virulence genes not previously recognized as such in *L. monocytogenes* (e.g., *aut* [9]).

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We thus reasoned that comparative transcriptome analyses of the σ^B regulons in *L. monocytogenes* and *L. innocua* would provide further insight into the role of σ^B in virulence and stress response. For example, we hypothesized that the organisms may have evolved different σ^B regulons and/or σ^B-dependent gene expression patterns to enable appropriate responses to the different sets of stress conditions likely to be encountered by an orally transmitted pathogen (*L. monocytogenes*) or a nonpathogenic saprophyte (*L. innocua*). We thus used an *L. monocytogenes* whole-genome microarray, which was modified to include additional probes targeting putative σ^B-dependent *L. innocua* genes, to identify additional members of the *L. monocytogenes* σ^B regulon and to initially characterize σ^B-dependent *L. innocua* genes, using a previously reported *L. monocytogenes* Δ*sigB* strain (70) as well as a newly constructed *L. innocua* Δ*sigB* strain. The isogenic parent and Δ*sigB* strain pairs for both species were also assayed for stress survival to allow comparison of the phenotypic consequences of *sigB* deletions in *L. monocytogenes* and *L. innocua*.

MATERIALS AND METHODS

Media, bacterial strains, and mutant construction. *L. monocytogenes* serotype 1/2a strain 10403S (5) and its isogenic *sigB* null mutant (FSL A1-254 [70]) as well as *L. innocua* strain FSL C2-008 (73), which was isolated in the year 2000 from smoked fish plant A, as described by Hoffman et al. (35), and its isogenic *sigB* null mutant (FSL R4-009) were used throughout this study. The *L. innocua* Δ*sigB* strain was created by using essentially the same method and primers previously used to create the *L. monocytogenes* Δ*sigB* strain (70). Briefly, a *sigB* fragment with a 297-bp in-frame deletion was created from *L. innocua* FSL C2-008 using splicing by overlap extension PCR and cloned into the shuttle vector pKSV7. The resulting plasmid (pSR2) was electroporated into *L. innocua*, and transformants were serially passaged at 40°C in brain heart infusion broth (BHI; Difco, Detroit, MI) containing 7.5 μg of chloramphenicol/ml to select for chromosomal integration of the plasmid. Chromosomal integrants were serially passaged in BHI at 30°C to allow excision of the plasmid to yield a single copy of *sigB*. Five passages were required for chromosomal integration of the plasmid and for subsequent excision. Retention of the desired Δ*sigB* allele was confirmed by PCR using primers LmsigB-15 and LmsigB-16 (as previously described [70]) and sequencing of the PCR products using the Big Dye terminator system and an Applied Biosystems 3700 sequencer at the Cornell University BioResource Center (Ithaca, NY).

Stock cultures were stored at −80°C in BHI containing 15% glycerol. Cultures were streaked onto BHI agar and incubated at 37°C for 24 h to obtain isolated colonies for inoculation of overnight cultures. Cells were grown in BHI broth at 37°C with shaking (250 rpm; series 25 incubator shaker; New Brunswick Scientific, Edison, NJ) unless otherwise indicated. Preliminary growth experiments in BHI at 37°C showed no growth pattern differences between parent and Δ*sigB* strains for either species.

HMM profiling. Hidden Markov models (HMMs) were built using HMMER version 2.3.2 (http://hmmer.janelia.org) with the default HMMER null model settings. The −10 and −35 sequences for 34 σ^B-dependent promoters from *L. monocytogenes* (see Fig. S1 and Table S1 in the supplemental material) were aligned manually and used to train the HMM; separate alignments were created using spacers of 13 to 16 bases between the −35 and −10 regions. Separate models were built for the forward and reverse orientations. The calibrated HMM was used to perform an HMM σ^B consensus promoter search against the genomic sequences for *L. monocytogenes* EGD-e and *L. innocua* CLIP 11262 (29) using the default null model. While the genome sequence for *L. monocytogenes* 10403S, the parent strain used here, has recently become available (*Listeria monocytogenes* Sequencing Project [http://www.broad.mit.edu]), the sequence has not been completely closed or annotated and was thus not used for the initial HMM search. PERL scripts were employed to identify coding sequences located between 20 and 350 bases downstream of a predicted σ^B-dependent promoter. High-scoring putative σ^B-dependent promoters, as predicted by HMM (e-value, ≤0.01), were identified 20 to 350 nucleotides (nt) upstream of 225 *L. monocytogenes* open reading frames (ORFs) (see Table S2 in the supplemental material).

A second HMM was built with the HMMER null model settings modified to reflect the GC content of the *L. monocytogenes* genome (A/T = 0.31, G/C = 0.19). When the p1 parameter was lowered from 0.999 to 0.966, 10 times more putative σ^B motifs (domains) were returned than had been generated by the default HMMER settings, which enabled an additional comparison with the σ^B promoter sequences previously identified and with the whole-genome microarray data. All other steps of the HMM search were performed as described above.

The results of these two HMM searches, in conjunction with visual evaluation of sequences upstream of genes identified as σ^B dependent by the microarray, were used to identify genes or operons preceded by putative σ^B promoters. Transcriptional terminators as defined by de Hoon et al. (17) were used to delimit operons. Promoters identified by different approaches were classified into one of seven different categories depending on the approach that had been used to identify a given σ^B-dependent promoter (see Table S3 in the supplemental material for details).

Cell collection and growth for RNA isolation. *L. monocytogenes* and *L. innocua* parent and Δ*sigB* strains were exposed to different stress conditions before cells were collected for RNA isolation for subsequent microarray experiments. Stress conditions included (i) growth to early stationary phase or (ii) exposure of log-phase (optical density at 600 nm [OD_{600}] of 0.4) cells to salt stress (BHI with 0.3 M NaCl; an osmolarity level similar to that encountered by bacteria in the small intestine [66]). Optical density measurements were performed using a model DU640 spectrophotometer (Beckman, Fullerton, CA). For each strain, an isolated colony was inoculated into 5 ml of BHI broth and grown overnight; diluted 1:100 in 5 ml of fresh, prewarmed BHI; grown to an OD_{600} of 0.4; diluted 1:100 in 50 ml of fresh, prewarmed BHI (in a 300-ml sidearm flask); and then grown as follows. For early stationary phase, cells were grown to an OD_{600} of 1.0, followed by incubation for an additional 3 h. A total of 10 ml of stationary-phase culture was collected for RNA isolation. For exposure of log-phase cells to salt stress, cells were grown to an OD_{600} of 0.4, and then a 10-ml aliquot of these cells was added to an equal volume of prewarmed BHI containing 0.6 M NaCl to yield a final concentration of 0.3 M NaCl. After incubation at 37°C for 10 min with shaking, cells were harvested and used for RNA isolation. Three independent cell collections and RNA isolations were performed for each strain under each experimental condition; experiments were designated as LMO-NaCl and LIN-NaCl (i.e., exposure of *L. monocytogenes* or *L. innocua* to 0.3 M NaCl, respectively) or LMO-STAT and LIN-STAT (i.e., growth of *L. monocytogenes* or *L. innocua* to early stationary phase, respectively).

RNA isolation. RNA isolation was performed using the RNAprotect Bacteria reagent and the RNeasy Midi kit (Qiagen, Valencia, CA). Briefly, a 2× volume of RNAprotect Bacteria reagent was added to bacterial cells exposed to a given stress condition, followed by vortexing and incubation at room temperature for 5 min. Cells were then pelleted by centrifugation and stored at −80°C until RNA isolation. For each experimental stress condition, total RNA isolations, DNase treatments, and phenol-chloroform extractions were performed essentially as described in the work of Kazmierczak et al. (41) with the following modifications. Cells were treated with 20 mg of lysozyme/ml (Fisher, Hampton, NH) followed by sonication (Sonicator 3000; Misonix, Farmingdale, NY) on ice at 21 W (three 30-s bursts on ice). DNase treatment of RNA was performed with 40 units RQ1 DNase enzyme (Promega, Madison, WI) in the presence of 400 units RNasin Plus RNase inhibitor (Promega) for 1 h at 37°C. Following the final 100% chloroform extraction, RNA was ethanol precipitated and stored at −80°C overnight. Precipitated RNA was centrifuged, washed in 70% and 100% ethanol, and then resuspended in RNase-free water. RNA was checked for quality by agarose gel electrophoresis and UV spectrophotometer readings at 260 and 280 nm with the NanoDrop-1000 (NanoDrop Technologies, Wilmington, DE).

DNA isolation. Chromosomal DNA for use as positive-control spots on the microarray was isolated from overnight cultures of *L. monocytogenes* 10403S and *L. innocua* FSL C2-008 essentially as described by Flamm et al. (23). DNA was resuspended in printing buffer containing 1× SSC (0.15 M sodium chloride, 0.015 M sodium citrate) with 0.0025% Sarkosyl.

Microarray design and construction. DNA microarrays were designed to include all ORFs identified in the sequenced genome of *L. monocytogenes* EGD-e (29). Seventy-mer oligonucleotides representing 2,857 *L. monocytogenes* EGD-e ORFs were obtained from Qiagen Operon Array-Ready Oligo Sets. EGD-e and *L. monocytogenes* 10403S both represent the same *L. monocytogenes* lineage (II), serotype (1/2a), and ribotype (DUP-1039C); therefore, probes designed using the EGD-e genome were expected to hybridize well with 10403S genes (11). Using the unfinished genome sequence for strain 10403S (*Listeria monocytogenes* Sequencing Project [http://www.broad.mit.edu]), we verified cross-hybridization identities (CHIs) between the EGD-e probes and the target genes in strain 10403S; CHI is the percent identity of a given microarray probe to the corresponding homologous gene in another strain or species. Overall, 2,107, 2,578, and 2,695 of the EGD-e probes showed CHI values of 100, ≥95, and ≥90, respectively; only 45 probes showed CHI

values of <90. A total of 117 of the EGD-e-based microarray probes were not detected in the 10403S genome (11). We thus concluded that the array used here would allow comprehensive identification of differentially expressed genes in strain 10403S, with the possibility of some false negatives (i.e., inability to identify genes targeted by a probe with a low CHI or genes present in the 10403S genome and absent in the EGD-e genome).

For each 70-mer, the CHI of the probe to the corresponding ORF in *L. innocua* CLIP 11262 was provided by Qiagen. CHI values ranged from 32 (low identity to an *L. innocua* ORF) to 100 (complete identity to an *L. innocua* ORF). High-scoring putative σ^B-dependent promoters, as predicted by HMM (e-value, ≤0.01), were identified 20 to 350 nt upstream of 232 *L. innocua* ORFs (see Table S4 in the supplemental material). With the goal of achieving appropriate hybridization between microarray probes and the transcripts from these putative σ^B-dependent *L. innocua* genes, *L. innocua*-specific microarray probes (with a CHI of 100) were designed for 57 *L. innocua* ORFs that either had a CHI of <90 (28 genes) or were absent entirely from the *L. monocytogenes* microarray (29 genes). Probes were designed for an additional four genes with e-values slightly above the cutoff of 0.01. Probe design was performed using the ArrayOligoSelector program (http://arrayoligosel.sourceforge.net/) (6) and the *L. innocua* CLIP 11262 genome (29) (see Table S5 in the supplemental material). *L. innocua* probes were synthesized by Operon Biotechnologies (Huntsville, AL).

DNA microarrays were constructed to include 70-mer oligonucleotides representing (i) 2,857 ORFS from *L. monocytogenes* EGD-e, (ii) the 61 *L. innocua*-specific probes, and (iii) five yeast ORFS (*act1*, *mfa1*, *mfa2*, *ras1*, and *ste3*) as negative controls (74). Oligonucleotides were resuspended in printing buffer (1× SSC plus 0.0025% Sarkosyl) to a final concentration of 25 μM and spotted in duplicate onto UltraGAPs slides (Corning, Corning, NY) using a custom-built XYZ microarrayer at the Cornell University Microarray Core Facility (Ithaca, NY). Serial (1:2) dilutions in printing buffer of *L. monocytogenes* and *L. innocua* chromosomal DNA (representing 133.3 to 1.0 ng/μl) were spotted in duplicate on the microarray as positive controls. Salmon sperm DNA (0.1 μg/μl in 1× SSC plus 0.005% Sarkosyl) was also spotted as printing quality controls. Slides were UV cross-linked (Spectrolinker XL-1000 UV cross-linker; Spectronics Corporation, Westbury, NY) with 300 mJ energy and then stored under desiccation.

cDNA labeling and microarray hybridization. The SuperScript Plus Indirect cDNA Labeling System for DNA Microarrays (Invitrogen, Carlsbad, CA) was used according to the manufacturer's protocol to synthesize and differentially label cDNA from 10 μg total RNA. The QIAquick PCR purification kit (Qiagen) was used to purify cDNA after the overnight, 42°C reverse transcription reaction and the dye coupling reaction (an additional 35% guanidine-HCl wash step was included during purification). For each purification, RNase-free water was used for the final elution step. Purified, labeled cDNA was quantified with UV spectrophotometer readings (Nanodrop Technologies) at wavelengths appropriate to determine the frequency of incorporation, which is defined as the number of labeled nucleotides incorporated per 1,000 nt of cDNA. cDNA samples with frequencies of incorporation between 20 and 50 were used for microarray hybridization. Purified, labeled cDNAs for the parent strain and the corresponding Δ*sigB* strain (differentially labeled with fluorescent dyes) were combined and dried. The combined target was resuspended in 1× hybridization buffer containing 0.5× formamide, 5× SSC, 0.1% sodium dodecyl sulfate, 0.1 mM dithiothreitol, and 600 μg/ml salmon sperm DNA in diethyl pyrocarbonate-treated water (J. Gilbert, H. Hasseman, R. Cline, and J. Hnath, The Institute for Genomic Research Standard Operating Procedure #M008, "Hybridization of Labeled DNA Probes"). cDNA target samples were vortexed for 1 min and incubated at 95°C for 5 min (repeated twice), before a brief centrifugation to cool the sample.

Slide blocking, hybridization, and posthybridization washing were performed as previously described (49). Slides were centrifuged to dry and scanned with a GenePix 4000B Scanner (Molecular Devices, Sunnyvale, CA) at the Microarray Core Facility using the following general scanning parameters: pixel size, 10; focus position, 20; lines to average, 1; laser power, 100%. The auto-photomultiplier tube gain function was used to scan the slides with an acceptable photomultiplier tube gain range of 400 to 900.

Microarray replicates and statistical analysis. Raw TIFF images were automatically gridded and analyzed using the GenePix Pro 6.0 software. Spots flagged from the data and removed from the subsequent analysis included empty spots, spots saturated in both channels, and spots of poor morphology. Data were preprocessed (background corrected and normalized) using the LIMMA package (61) available from the BioConductor software project for the R programming environment (R version 2.2.1) (28).

To achieve robust ratios for low-intensity spots, "normexp" background correction was employed using the LIMMA package (with offset of 100). Print-tip loess normalization was performed to account for within-array spatial bias and intensity bias. To assist with the within-array normalization process, control spots known a priori to be nondifferentially expressed were given increased weight during normalization. Control spots were chosen to span the entire range of intensities (A values in a minus-versus-add plot of the data) and included genomic DNA spots (with dilutions spanning from middle to high intensities) and printing and negative controls (to account for low intensities). To enable comparison of results across arrays, scale normalization was performed.

The LIMMA package was also used for differential expression analysis (62). Briefly, a linear model was fitted to the normalized data, followed by empirical Bayes smoothing to calculate moderated t-statistics and B-statistics. To account for duplicate spots, the "duplicateCorrelation" function was used (63). *P* values were adjusted for multiple comparisons by controlling for the false discovery rate. Genes with an adjusted *P* value of <0.05 were considered statistically significant, and a change of ≥2.0-fold was used as a cutoff for identification of differentially expressed genes. Genes were considered to be positively regulated by σ^B if transcript levels were significantly and at least 2.0-fold higher in the parent strain than in the Δ*sigB* strain (represented as positive changes in Table S6 in the supplemental material). Genes were considered to be negatively regulated in a σ^B-dependent manner if transcript levels were significantly and at least 2.0-fold lower in the parent strain than in the Δ*sigB* strain (represented as negative changes in Table S7 in the supplemental material).

TaqMan quantitative reverse transcriptase-PCR (qRT-PCR). TaqMan probe and primer sets for *L. monocytogenes rpoB*, *gap*, *opuCA*, *gadA*, and *fri* have been reported previously (10, 40, 59, 66); TaqMan MGB (minor groove binder) probe and primer sets for *L. innocua* lin0285 (*rpoB*), lin0289, lin0942 (*fri*), lin1467 (*opuCA*), lin1992 (*cspD*), lin2528 (*gadA*), lin2553 (*gap*), and lin2891 and for *L. monocytogenes* lmo0265, lmo1879 (*cspD*), and lmo2748 (see Table S8 in the supplemental material) were designed using Primer Express 1.0 software (Applied Biosystems, Foster City, CA).

qRT-PCR was performed using TaqMan One-Step RT-PCR Master Mix Reagent, Multiscribe RT, and the ABI Prism 7000 Sequence Detection System (all from Applied Biosystems) as described by Sue et al. (66). Each qRT-PCR was run in duplicate on each reaction plate, and RT-negative reactions (excluding the Multiscribe RT) were run in parallel to account for any possible genomic DNA contamination in each qRT-PCR. Standard curves for each target gene were included for each assay to allow for absolute quantification of cDNA levels, and data were analyzed using the ABI Prism 7000 Sequence Detection System software as previously described (66). Normalization and log transformation were performed as described by Kazmierczak et al. (40). As microarray analyses indicated that transcript levels for *L. innocua gap* were lower in the Δ*sigB* strain, providing evidence of σ^B-dependent regulation for *gap* in the tested strain, statistical analyses of transcript levels for *L. innocua* genes were also repeated using transcript levels normalized to *rpoB* transcript levels only. With the exception of transcript levels for *L. innocua cspD* (see Results and Discussion for details), normalization of *L. innocua* gene transcript levels to either *rpoB* alone or both *rpoB* and *gap* identified the same differences as significant.

The same RNA that had been isolated for the microarray experiments was also used in the qRT-PCR assays, with the exception of one LIN-NaCl and one LIN-STAT replicate. RNA was isolated from an additional set of cells grown under each of these conditions to provide sufficient RNA for three replicate qRT-PCR assays.

Acid stress and ethanol stress experiments. *L. monocytogenes* and *L. innocua* parent and Δ*sigB* cultures were characterized in a series of stress survival assays, including (i) exposure to HCl-acidified BHI broth (pH 2.5) (21), (ii) exposure to synthetic gastric fluid (SGF; pH 2.5) (15), and (iii) exposure to BHI plus 16.5% (vol/vol) ethanol (21, 48). Exposure to BHI-HCl and SGF was performed using both log- and early-stationary-phase cells, while ethanol exposure was performed only with early-stationary-phase cells. Log-phase cells (i.e., OD_{600} of 0.4) were grown as detailed above for RNA isolation experiments. For early-stationary-phase cells, overnight cultures were subcultured 1:100 into 10 ml fresh BHI and grown to an OD_{600} of 0.8 for 1 h (a condition previously shown to activate σ^B [65]); this growth protocol was used to allow direct comparisons of results with previously reported survival experiments (13, 21). For acid exposure, 1 ml of culture was pelleted and resuspended in 1 ml of either BHI-HCl (pH 2.5) or SGF (pH 2.5), followed by incubation at 37°C with shaking for 10 min or 60 min for log- or early-stationary-phase cells, respectively. For ethanol survival, early-stationary-phase cells were pelleted and resuspended in 1 ml of BHI plus 16.5% (vol/vol) ethanol, followed by incubation at 37°C with shaking for 120 min. For all experiments, *Listeria* numbers were enumerated by plating appropriate serial dilutions in phosphate-buffered saline on BHI agar (in duplicate); enumeration was performed immediately prior to stress exposure and after stress exposure for the incubation times specified. Plates were incubated at 37°C for 48 h.

Swarming assay. Swarming abilities of *L. monocytogenes* and *L. innocua* strains were evaluated on semisoft agar; the *L. monocytogenes* Δ*flaA* strain (55)

TABLE 1. Functional categories of genes identified by microarray analyses as positively regulated by σ^B

Category	No. of σ^B-dependent genes with or without putative σ^B-dependent promoter[a,b]			
	L. monocytogenes (n = 168)		*L. innocua* (n = 64)	
	With promoter	Without promoter	With promoter	Without promoter
Stress	11 (***clpC***, ***ctc***, *sepA*)	1 (lmo2064)	7 (***clpC***, ***ctc***, *sepA*)	1 (*cspD*[c])
Virulence and virulence associated	5 (*bsh*, ***hfq***, *inlAB*)		1 (***hfq***)	
Transcriptional regulation	7 (***rsbV***, *hrcA*)	3 (lmo0402, lmo0445)	5 (***rsbV***, *rpoD*)	1 (lin0615)
Transport and transport systems	21 (*gbuA*, ***opuCA***)	5 (lmo0607, lmo0653)	6 (***opuCA***)	
Metabolism	37 (***ldh***, **lmo2571**)	8 (lmo0043, **lmo0913**)	14 (***ldh***, **lin2716**)	1 (**lin0913**)
DNA metabolism and transport	2 (lmo0996, **lmo1606**)		1 (**lin1647**)	
Protein synthesis and modification	3 (lmo1698, **lmo2511**)		1 (**lin2655**)	
Cell envelope and cellular processes	6 (lmo0610, **lmo0880**)	1 (lmo1291)	3 (lin0372, **lin0879**)	
Unknown and hypothetical	52 (**lmo0953**)	6 (lmo2671)	19 (**lin0952**)	4 (lin0483)

[a] Numbers of genes in a given category are indicated; examples of genes are listed in parentheses; boldface indicates genes identified as σ^B dependent in both *L. monocytogenes* and *L. innocua*.

[b] Putative σ^B-dependent promoters were identified by HMM or visual analyses as detailed in Table S3 in the supplemental material.

[c] Not confirmed as σ^B dependent by qRT-PCR.

was included as a negative control. Strains were initially grown for 24 h at 37°C on BHI agar and BHI agar containing 0.3 M NaCl. Colonies from BHI and BHI plus 0.3 M NaCl were used to stab-inoculate BHI semisoft agar (0.4%) and BHI plus 0.3 M NaCl semisoft agar (0.4%), respectively. Swarming ability was assessed by measuring colony area using SigmaScan Pro 5.0 (SPSS Inc., Chicago, IL) for each strain after incubation at 30°C and 37°C for 72 h. Growth area for each mutant strain was normalized to the growth area of the parent strain, which was set at 100%.

Statistical analyses. Statistical analyses were performed using SAS Version 9.1 (SAS Institute Inc., Cary, NC). Two-sided two-sample t tests were used for analysis of the qRT-PCR data, while a two-sided one-sample t test was used for analysis of the swarming data. Statistical analysis of the stress survival data was performed using a general linear model with Tukey's multiple comparison analysis.

Microarray data accession number. Raw and normalized microarray data in MIAME format are available at the NCBI Gene Expression Omnibus (GEO) data repository (20) under accession number GSE7492.

RESULTS AND DISCUSSION

Microarray, qRT-PCR, HMM, and phenotypic characterization of *L. monocytogenes* and *L. innocua* Δ*sigB* strains provided data indicating that (i) the *L. monocytogenes* σ^B regulon includes >140 genes that are both directly and positively regulated by σ^B, including genes encoding proteins with importance in stress response, virulence, transcriptional regulation, metabolism, and transport; (ii) a number of *L. monocytogenes* genes encoding flagellar proteins show higher transcript levels in a Δ*sigB* mutant, suggesting indirect effects of σ^B on motility and chemotaxis; and (iii) even though the *L. monocytogenes* and *L. innocua* σ^B regulons show considerable conservation with a core of at least 49 genes that appear to be σ^B dependent in both *Listeria* spp., *L. monocytogenes* and *L. innocua* both have species-specific σ^B-dependent genes and show differences in σ^B-dependent stress resistance phenotypes (i.e., acid stress resistance).

Microarray analyses identify >140 *L. monocytogenes* genes directly and positively regulated by σ^B. Whole-genome microarrays were used in competitive hybridization experiments with RNA isolated from *L. monocytogenes* 10403S and an isogenic Δ*sigB* strain to define the σ^B regulon under two conditions that result in high σ^B activity, including (i) exposure of log-phase cells to salt stress (65) and (ii) entry into stationary phase (2, 65). Statistical analysis was performed separately for the two data sets. Genes were identified as putatively σ^B regulated under at least one of these conditions if they met both of the following criteria: (i) an adjusted P value of <0.05 and (ii) an at least 2.0-fold difference in mRNA transcript levels between the Δ*sigB* mutant and parent strain. Using these criteria, 168 genes showed ≥2.0-fold-higher transcript levels in the *L. monocytogenes* parent than in the Δ*sigB* mutant (Table 1; see also Table S6 in the supplemental material); these genes are considered to be positively regulated by σ^B. A total of 128 genes showed ≥2.0-fold-higher transcript levels in the *L. monocytogenes* Δ*sigB* mutant than in the parent strain (see Table S7 in the supplemental material); these genes are considered to be negatively regulated by σ^B.

Among the 168 genes identified as positively regulated by σ^B through microarray analysis, 83 genes (49%) had an HMM-predicted putative σ^B-dependent promoter sequence either directly upstream of a specified ORF or upstream of the first gene of an operon, suggesting direct σ^B regulation of these genes. As the initial HMM-based approach used stringent criteria for promoter identification (i.e., e-value of ≤0.01 with distance from start codon of between 20 and 350 nt [see Table S2 in the supplemental material]), the upstream regions of the remaining 85 σ^B-dependent genes were reevaluated for evidence of a σ^B-dependent promoter sequence using both visual inspection and HMM-identified putative promoters with higher e-values and less-stringent distance criteria. These reevaluations identified a putative σ^B-dependent promoter upstream of an additional 62 genes to yield a total of 145 genes that appear to have a σ^B-dependent promoter and thus are predicted to be directly regulated by σ^B (see Tables S3 and S6 in the supplemental material).

A total of 72 genes (including 49 preceded by a σ^B-dependent promoter directly upstream either of a specified ORF or of the first gene of an operon) were positively regulated by σ^B in both salt-stressed and stationary-phase cells (see Fig. S2 in the supplemental material). A total of 58 and 38 genes showed σ^B-dependent transcript levels only in salt-stressed cells (e.g., the *inlAB* operon) or only in stationary-phase cells (e.g., the lmo0398-0400 operon, encoding a fructose-specific phosphotransferase system [PTS]), respectively (see Table S6 in the supplemental material). Interestingly, among the 26 σ^B-dependent *L. monocytogenes* genes related to transport, 11 genes

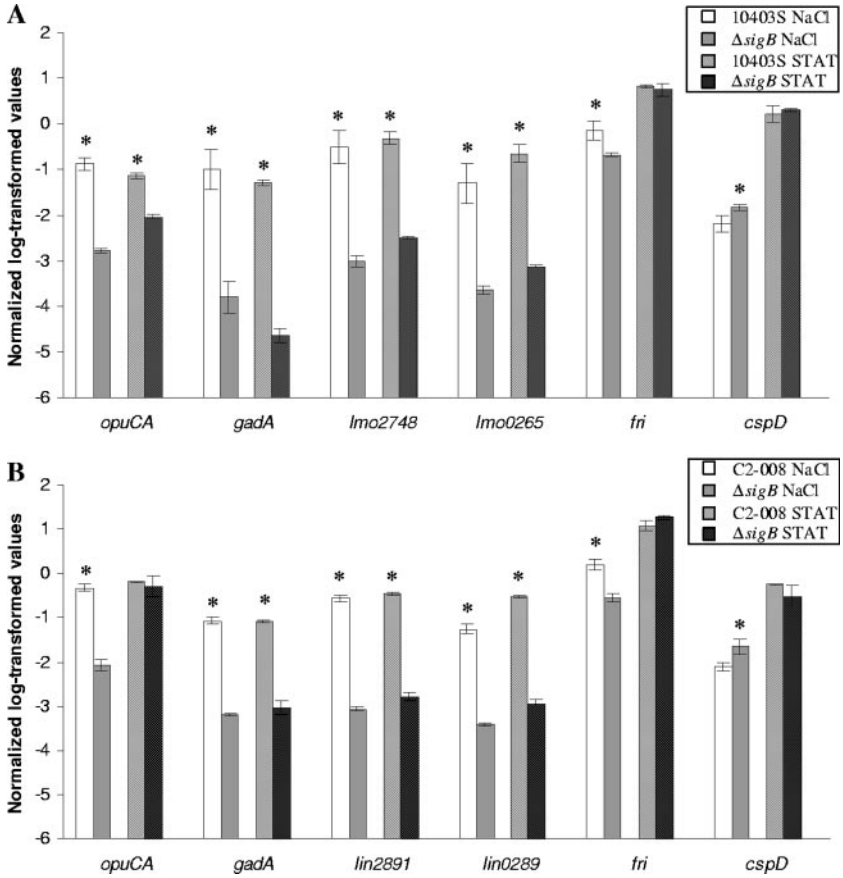

FIG. 1. qRT-PCR confirmation of selected genes identified by microarray data as σ^B dependent in *L. monocytogenes* 10403S (A) and in *L. innocua* FSL C2-008 (B). qRT-PCRs were designed to be specific for the six selected *L. monocytogenes* and *L. innocua* genes (see Table S8 in the supplemental material); correlation plots for microarray and qRT-PCR results are provided as Fig. S8 in the supplemental material. The six genes were selected to represent genes with homologues in both species; homologous genes are presented in the same order in each figure, e.g., lmo2748 and lin2891 are homologous. Transcript levels for the test genes were normalized to the geometric mean of the transcript levels for housekeeping genes *rpoB* and *gap*; an asterisk indicates that normalized transcript levels for the parent and Δ*sigB* strain differed significantly for cells exposed to a given stress condition (salt stress or stationary phase). As microarray analyses indicated that transcript levels for *L. innocua gap* were lower in the Δ*sigB* strain, statistical analyses of transcript levels for *L. innocua* genes were also repeated using transcript levels normalized to *rpoB* only; except for *cspD* (see text), these statistical analyses identified the same differences as significant as the analyses based on transcript levels normalized to the geometric mean of *rpoB* and *gap* transcript levels.

were induced in a σ^B-dependent manner only in salt-stressed cells, demonstrating the importance of σ^B in facilitating transport, e.g., of compatible solutes, during salt and/or osmotic stress. qRT-PCR of *fri* (lmo0943/lin0942), which encodes Fri, a nonheme iron-binding ferritin, confirmed that *fri* is positively regulated by σ^B in salt-stressed, but not stationary-phase, *L. monocytogenes* and *L. innocua* (Fig. 1). This is consistent with the microarray data, which showed higher transcript levels in both the *L. monocytogenes* and the *L. innocua* parent strain exposed to salt (compared to the Δ*sigB* strain; adjusted $P < 0.05$ for *L. innocua* and adjusted $P < 0.1$ for *L. monocytogenes*), while showing no differences in stationary-phase cells between the two species (see Table S6 in the supplemental material). qRT-PCR thus confirmed that some genes show σ^B-dependent transcription only under some stress conditions.

The genes positively regulated by σ^B were classified into nine functional categories (Table 1; see also Table S6 in the supplemental material). As expected, a number of genes related to stress response displayed σ^B-dependent transcription including the general stress response genes *ctc* and lmo1601, as well as *ltrC*, which is involved in cold stress response (76, 77), and *gadA*, which encodes a glutamate decarboxylase important for acid resistance (14). Several virulence and virulence-associated genes also showed σ^B-dependent expression including *bsh* (encoding the bile salt hydrolase), *hfq* (encoding an RNA-binding protein), and various internalin genes (e.g., the *inlAB* operon [see Fig. S3 in the supplemental material] and *inlD*). While the contributions of σ^B to the gastrointestinal stages of infection have been well documented (27), σ^B-dependent genes also appear to contribute to intracellular infection by *L. monocytogenes*. For example, of the 26 σ^B-dependent genes previously identified to be upregulated in either intravacuolar or intracytosolical *L. monocytogenes* (12), 23 were confirmed as σ^B dependent in our microarray analyses (see Table S9 in the supplemental material). Three members of the universal stress protein (Usp) family, found to be upregulated intracellularly by Chatterjee et al. (12), were also found to be σ^B dependent, including lmo2748, which was also confirmed as σ^B dependent

by qRT-PCR (Fig. 1; see also Table S6 in the supplemental material). Further analyses on the overlaps between the σ^B regulon identified here and *L. monocytogenes* genes that are upregulated during intracellular replication and survival (e.g., references 12 and 38) will likely shed additional insight on the contributions of σ^B to transcriptional regulation during infection.

The 168 genes positively regulated by σ^B included 26 operons with more than one gene identified as σ^B-dependent (these operons contained from two to eight genes identified as σ^B dependent; see Fig. S3 and S4 in the supplemental material), including a number of operons involved with carbohydrate metabolism. *L. monocytogenes* genes and operons positively regulated by σ^B and involved in carbohydrate metabolism include a mannose-specific PTS operon (lmo0784 to lmo0781; lmo0784 was previously identified as σ^B dependent), a fructose-specific PTS (lmo0398 to lmo0400, not previously reported as σ^B dependent), and an operon (lmo0348 to lmo0341) that includes genes encoding enzymes in the pentose phosphate pathway, as well as a gene involved in galactose metabolism (lmo0539). In addition, two two-gene operons encoding dihydroxyacteone kinases were also identified as positively regulated by σ^B (see Fig. S4 and Table S6 in the supplemental material). Except for the fructose-specific PTS operon, all other genes mentioned above were preceded by a putative σ^B-dependent promoter, suggesting direct regulation by σ^B. σ^B-dependent transcription of genes involved in carbohydrate metabolism suggests that σ^B may be important for energy metabolism, most likely during exposure to energy stress conditions, consistent with the observation that *B. subtilis* σ^B is activated during energy stress (8, 69).

Interestingly, seven genes encoding transcriptional regulators were found to be positively regulated by σ^B, including lmo2460, which encodes CggR, a transcriptional regulator highly similar to the *B. subtilis* central glycolytic gene regulator (18); lmo2460 is preceded by a σ^B consensus promoter. *hrcA*, which encodes a negative regulator of chaperone proteins and is involved in resistance to heat stress (32) also showed lower transcript levels in the Δ*sigB* strain and is preceded by a σ^B-dependent promoter. In addition, the last two genes in the four-gene *clpC* operon (*ctsR-mcsA-mcsB-clpC*), which has a σ^B-dependent promoter preceding *mcsA*, also showed evidence of σ^B-dependent transcription in salt-stressed cells. HrcA and CtsR both regulate transcription of heat shock genes in *L. monocytogenes* (25, 32, 39, 54). Regulatory interactions between *L. monocytogenes* σ^B and PrfA, a transcriptional regulator of virulence gene transcription, have been reported previously (45), including coregulation of genes by both proteins (50, 52) and σ^B-dependent transcription of *prfA* (53, 58, 59). Our microarray data suggest additional important contributions of σ^B to multiple *L. monocytogenes* regulatory networks based on evidence of interactions between *L. monocytogenes* σ^B and other regulators.

We also confirmed that the *L. monocytogenes* σ^B regulon includes a number of stress response genes (e.g., *gadA*, *ctc*, and *clpC*) and virulence genes (e.g., *inlAB*, *inlD*, *bsh*, and *hfq*), as well as genes encoding transporters and cell surface proteins. The size of the *L. monocytogenes* σ^B regulon identified by our microarray analyses is consistent with the σ^B regulons identified in other bacteria including *B. subtilis* (127 genes) (57) and *S. aureus* (198 genes) (4). Previously only ~55 *L. monocytogenes* genes had been classified as σ^B dependent, predominantly through use of a subgenomic microarray targeting 208 *L. monocytogenes* genes (41). Our microarray results confirmed 45 of the 55 *L. monocytogenes* genes identified as σ^B dependent by Kazmierczak et al. (41). Six of the 10 genes not confirmed by the present study (lmo0524, lmo1539, lmo2389, lmo2399, *pdhA*, and *qoxA*) had statistically significant differences (adjusted $P < 0.05$) in transcript levels between the parent and Δ*sigB* strain in at least one data set, but the differences did not meet our minimum 2.0-fold criterion. While we conclude that we have identified the majority of the *L. monocytogenes* σ^B regulon, further studies using cells exposed to different stress conditions, followed by qRT-PCR and promoter mapping strategies, will likely reveal some additional members of the *L. monocytogenes* σ^B regulon. Genes identified here as showing significant evidence for σ^B dependence with changes below the 2.0-fold cutoff represent likely candidates for additional members of the σ^B regulon.

A number of *L. monocytogenes* genes encoding flagellar proteins show higher transcript levels in a Δ*sigB* mutant, suggesting indirect effects of σ^B on motility and chemotaxis. While σ^B clearly is important as a positive regulator of transcription in *Listeria* and in a number of other gram-positive bacteria, we also identified 128 genes that showed higher transcript levels in the *L. monocytogenes* Δ*sigB* strain (see Table S7 in the supplemental material), suggesting that σ^B also contributes to negative regulation of gene transcription, likely through indirect means, such as through positive regulation of a repressor. Interestingly, *hly* (lmo0202) showed significantly higher transcript levels in the *L. monocytogenes* Δ*sigB* strain, suggesting indirect negative regulation of this virulence gene by σ^B; these findings are consistent with previously reported results that showed increased hemolysis activity in the *L. monocytogenes* Δ*sigB* strain compared to that in the *L. monocytogenes* 10403S parent strain (13, 53). Similar to our findings, others have also reported genes that appear to be indirectly repressed by σ^B, including 53 genes in *S. aureus* (4).

A total of 91 genes showed lower transcript levels in the parent strain only in salt-stressed cells (LMO-NaCl), including 21 genes encoding different ribosomal subunit proteins; 11 of these genes are located in a 19-gene locus (lmo2614 to lmo2632). These data suggest that σ^B indirectly controls transcription of *L. monocytogenes* genes involved in translation, consistent with findings that *rpsJ*, which encodes the ribosomal protein S10, was found to be negatively regulated by σ^B in *B. subtilis* (57).

Interestingly, 16 genes located in a 28-gene locus (lmo0691 to lmo0718) that encodes flagellar structural components also showed higher transcript levels in the Δ*sigB* strain in cells exposed to NaCl; this locus is one of two large operons that encode the flagellar apparatus (72) (see Fig. S5 in the supplemental material). Specific comparisons between the previously reported *L. monocytogenes* DegU regulon (72) and the σ^B-repressed *L. monocytogenes* genes reported here identified a total of 23 genes in the DegU regulon that are repressed by σ^B. When the cutoff for genes differentially regulated by σ^B was relaxed from a difference of ≥2.0-fold to a difference of >1.3-fold (still with an adjusted $P < 0.05$), 41 genes determined to be positively regulated by the two-component response regu-

lator DegU at 24°C (including 18 flagellum-specific genes and three chemotaxis-specific genes) (72), were also found to be negatively regulated by σ^B in salt-stressed cells. lmo1699 (encoding a protein similar to methyl-accepting chemotaxis proteins) and lmo1700 (encoding an unknown protein), which represent an operon within the DegU regulon, also were found to be negatively regulated by σ^B in salt-stressed cells; these two genes displayed the greatest differences between the Δ*sigB* strain and the parent strain (−4.7 and −5.3 for lmo1699 and lmo1700, respectively). Phenotypic characterization of swarming ability of the *L. monocytogenes* and *L. innocua* Δ*sigB* strains supported the relevance of the transcriptome analyses and determined that in bacteria grown at 30°C in BHI with and without 0.3 M NaCl, the Δ*sigB* strain consistently showed small, but statistically significant, increases in swarming compared to the parent strain (see Fig. S6 in the supplemental material). When bacteria were grown at 37°C (a temperature that permits limited swarming in 10403S but no swarming in EGD-e [30]), the *L. innocua* Δ*sigB* strain still showed small, but statistically significant, increases in swarming compared to the isogenic parent strain (see Fig. S6 in the supplemental material), despite limited microarray-based evidence for significantly higher transcript levels for the flagellar genes in the *L. innocua* Δ*sigB*. This apparent contradiction is likely explained by the observation that these genes, which showed <2.0-fold difference, were targeted by probes with relatively low CHI values (see Fig. S5 in the supplemental material).

Overall, our data indicate that σ^B negatively regulates genes encoding proteins related to motility and chemotaxis in response to salt stress and that DegU and σ^B coregulate a number of genes, in particular genes related to motility and chemotaxis. Specifically, our data provide support for the hypothesis that σ^B controls transcription of a gene encoding a protein that suppresses transcription of selected motility and chemotaxis genes, although a specific gene(s) responsible for this effect remains to be identified. For example, microarray data did not show effects of the *sigB* deletion on transcript levels for *mogR*, which encodes a transcriptional repressor of flagellar and motility genes (30). While *hrcA*, which encodes a negative regulator, is positively regulated by σ^B, microarray experiments showed no evidence that HrcA regulates flagellar genes (37). Previous studies have shown that an *L. monocytogenes* Δ*degU* strain as well as a Δ*mogR* strain has attenuated virulence in mouse models of infection (30, 44, 71). As an *L. monocytogenes* Δ*sigB* null mutant also showed attenuated virulence in a guinea pig model beyond that caused by reduced transcription of the σ^B-dependent *inlA* (27), it is possible that interactions between DegU, MogR, and σ^B are important for transcription of virulence genes; experiments using appropriate *sigB*, *degU*, and *mogR* null mutants will be required to further test this hypothesis. In *B. subtilis*, the DegS/DegU two-component system has been shown to sense salt stress (16, 46, 64) and phosphorylated DegU is thought to repress transcription of motility genes through repression of *sigD*, which encodes the flagellum-specific sigma factor σ^D in *B. subtilis* (1, 67), which is absent in *L. monocytogenes*. These data support the idea that interactions among regulators contribute to regulation of flagellar proteins in different bacteria. Our results extend the list of possible regulators of flagellar protein expression in *L. monocytogenes* to include σ^B.

The *L. monocytogenes* and *L. innocua* σ^B regulons show considerable conservation with a core of at least 49 genes that appear to be σ^B dependent in both species. Initial HMM analysis using the *L. innocua* CLIP 11262 genome (29) identified 232 putative *L. innocua* σ^B promoter sequences with e-values of ≤0.01 located between 20 and 350 nt upstream of a start codon of a predicted ORF (see Table S4 in the supplemental material); 123 of these ORFs had homologues in both *L. monocytogenes* and *L. innocua* and were preceded by a putative σ^B-dependent promoter in both species. Interestingly, a total of 29 *L. innocua* ORFs with no homologue in *L. monocytogenes* (as determined by the ListiList server [http://genolist.pasteur.fr/ListiList/]) were predicted to have upstream σ^B-dependent promoters. Similarly, a total of 26 *L. monocytogenes* ORFs with no homologue in *L. innocua* (as determined by the ListiList server) were predicted to have upstream σ^B-dependent promoters (e.g., *prfA*, *sepA*, and *bsh*), providing preliminary evidence of diversification of the σ^B regulons between the two species. To enable sufficient hybridization between microarray probes and the transcripts from putative σ^B-dependent *L. innocua* genes, additional *L. innocua*-specific probes were designed to target genes that were predicted to be σ^B dependent by HMM but either (i) did not have a homologue in *L. monocytogenes* (29 genes) or (ii) were not expected to hybridize with the probe targeting the *L. monocytogenes* homologue due to sequence divergence, as the *L. monocytogenes*-specific probe showed a CHI of <90 with the *L. innocua* target gene based on the genome sequence for *L. innocua* CLIP 11262 (28 genes; see Materials and Methods for further explanation).

Application of our modified *L. monocytogenes* whole-genome array identified 64 genes that displayed significantly, and at least 2.0-fold-, higher expression in the *L. innocua* parent than in the isogenic Δ*sigB* null mutant under at least one of the two stress conditions (LIN-NaCl or LIN-STAT; see Table S6 in the supplemental material). Among the 64 genes positively regulated by σ^B in *L. innocua*, 28 genes showed σ^B-dependent transcription under both experimental conditions; 28 and 8 genes had higher transcript levels in the parent strain only in salt-stressed cells (e.g., *ctc* and *fri*) or only in stationary-phase cells (e.g., *pdhA* and *pdp*), respectively (see Fig. S2 and Table S6 in the supplemental material). Among these 64 genes, 42 were preceded by a putative σ^B-dependent promoter as identified by HMM (e-value of ≤0.01 with distance of 20 to 350 nt [see Table S6 in the supplemental material]). Reevaluation of the upstream regions of the remaining 22 genes with relaxed HMM criteria for σ^B promoter identification (i.e., higher e-values and less stringent distance requirement; see Table S3 in the supplemental material) identified an additional 13 genes with putative upstream σ^B-dependent promoters. Visual evaluation identified putative σ^B-dependent promoters upstream of an additional two genes to yield a total of at least 57 genes that appear to be directly positively regulated by σ^B in *L. innocua* (see Table S6 in the supplemental material).

Our approach of applying *L. monocytogenes* microarrays to *L. innocua* only partially identified the *L. innocua* σ^B regulon, since transcripts for some σ^B-dependent *L. innocua* genes were unlikely to have been detected due to low CHI of a given *L. innocua* gene for the *L. monocytogenes*-specific microarray probe. Of the 1,030 *L. innocua* genes targeted with a probe

with a CHI of ≥95, a total of 6% were identified as σ^B dependent by microarray analyses, while only 0.3% of *L. innocua* genes targeted with a probe with a CHI of <95 were identified as σ^B dependent. Genes targeted with a highly homologous probe (CHI of ≥95) were significantly more likely to be identified as σ^B dependent ($P < 0.001$; χ^2 test of independence). While our approach thus may have led to a number of false negatives in *L. innocua* (i.e., σ^B-dependent *L. innocua* genes were not identified as such), it likely generated very few false positives (i.e., non-σ^B-dependent *L. innocua* genes identified as σ^B dependent). Consequently, the σ^B-dependent *L. innocua* genes identified here should be considered a partial σ^B regulon with additional σ^B-dependent *L. innocua* genes likely to be identified in future experiments with *L. innocua*-specific microarrays.

The 64 *L. innocua* genes positively regulated by σ^B included nine operons with more than one gene identified as σ^B dependent; eight of these operons also showed clear evidence of σ^B dependence in *L. monocytogenes* (see Fig. S4 in the supplemental material). While the remaining operon (lin2550 to lin2552) was σ^B dependent in *L. innocua*, only one gene from the operon (*pgm*, homologous to lin2550) showed σ^B dependence in *L. monocytogenes*. This operon is situated in a six-gene locus (lin2549 to lin2554) that encodes proteins related to glucose metabolism (see Fig. S4 in the supplemental material). The first gene in the locus (lin2554 or lmo2460) is preceded by a predicted σ^B promoter in both species, supporting the idea that this operon is transcribed by σ^B in both species.

By comparing the 64 σ^B-dependent *L. innocua* genes with the *L. monocytogenes* σ^B regulon, we were able to identify a total of 49 σ^B-dependent genes common to both *L. monocytogenes* and *L. innocua* (representing genes that show higher transcript levels in the parent strain than in the Δ*sigB* strain under at least one stress condition in both species) (Table 2). A total of 20 genes (e.g., *hfq* and *ldh*) were found to be positively regulated by σ^B under both stress conditions in both *L. monocytogenes* and *L. innocua* (see Fig. S2 in the supplemental material). While the 49 conserved σ^B-dependent genes include a number of genes encoding hypothetical proteins, which we propose are important candidates for functionally important σ^B-dependent genes, they also include a number of genes encoding stress response proteins, transporters, and metabolic enzymes. For example, the homologues lin0674 and lmo0669 (which both encode an oxidoreductase [see Fig. S4 in the supplemental material]) as well as lin2332 and lmo2230 (which both encode an arsenate reductase) were σ^B dependent in both species and showed considerably lower transcript levels in the Δ*sigB* mutant in both species (Table 2). Some of the genes found to be σ^B dependent in both *L. monocytogenes* and *L. innocua* also have homologues previously found to be σ^B dependent in *B. subtilis* (e.g., *ctc* and *yvyD*) (57), *S. aureus* (e.g., N315-SAS0202, encoding a phophoglycerate mutase; N315-SA0163, encoding a cation efflux transport protein) (4), or both (e.g., *ywmG*/*csbD*; *yhxD*/*ydaD*, encoding a short-chain dehydrogenase or oxidoreductase) (4, 34, 57), indicating conservation of certain σ^B-dependent stress response mechanisms across gram-positive genera. Interestingly though, early-stationary-phase cells for both *L. monocytogenes* and *L. innocua* Δ*sigB* strains did not show reduced ability to survive ethanol stress (compared to the respective parent strains [see Fig. S7 in the supplemental material]); while bacterial numbers for all strains were reduced by ~5 logs, there were no significant ($P > 0.05$) differences among the four strains. While this is consistent with previous observations that ethanol resistance in stationary-phase *L. monocytogenes* cells is largely σ^B independent (21), ethanol resistance in *B. subtilis* has been shown to be σ^B dependent (36), supporting some diversification of σ^B-dependent stress response functions between the closely related genera *Bacillus* and *Listeria*.

***L. monocytogenes* and *L. innocua* include species-specific σ^B-dependent genes.** Identification of *L. monocytogenes* and *L. innocua* species-specific σ^B-dependent genes is likely to provide further insight into gene regulation during infection and adaptation of stress response systems to pathogenic and saprophytic lifestyles. Specifically, σ^B-dependent genes present in *L. monocytogenes* and absent in *L. innocua* are likely candidates for virulence genes, as σ^B has been shown to contribute to *L. monocytogenes* virulence (27). Overall, 10 of the σ^B-dependent *L. monocytogenes* genes identified do not have homologues in *L. innocua*, including *inlA*, *inlB*, *inlD*, *sepA*, lmo2085, *bsh*, lmo0445, lmo2671, lmo2290, and lmo2387. Five of these genes (*inlA*, *inlB*, *sepA*, lmo2085, and lmo0445) have previously been shown to be upregulated in intracellular bacteria (12), consistent with the well-defined virulence contributions of InlA and InlB (19, 26, 51). While no clear contributions to virulence have been ascribed to lmo2085, *sepA*, and lmo0445 or to the other five *L. monocytogenes*-specific σ^B-dependent genes, we hypothesize that these genes include candidate virulence genes. In particular, *sepA*, which encodes a protein of the metallo-beta-lactamase family (41), is likely to contribute to virulence as its transcription is regulated both by σ^B and by the virulence gene regulator PrfA (12, 52), similar to the σ^B-PrfA coregulation reported for two internalins, *inlA* and *inlB* (50). Interestingly, the only σ^B-dependent *L. innocua* gene with no clear homologue in *L. monocytogenes* (lin0372 [see Fig. S3 in the supplemental material]) also encodes a putative internalin, indicating that σ^B-dependent transcription of internalins is evolutionarily conserved among *Listeria* spp. This observation suggests that internalin expression may contribute to *Listeria* survival in nonhost environments, consistent with recent evidence that σ^B-dependent *L. monocytogenes* internalins are highly transcribed at temperatures below mammalian body temperatures (49).

While gene homologues that were identified as σ^B dependent in *L. monocytogenes*, but not in *L. innocua*, are likely to include a number of false-negative *L. innocua* genes targeted by microarray probes with relatively low CHI values, the 14 genes that were initially identified as σ^B dependent in *L. innocua* but not identified as such in *L. monocytogenes* represent candidates for genes that have evolved to be regulated by different mechanisms in these two species. Further evaluation of these 14 genes showed that one *L. monocytogenes* gene (lmo0265, encoding a succinyldiaminopimelate desuccinylase) was targeted by a microarray probe with a low CHI (CHI of 67), as the DNA sequence of the lmo0265 allele in EGD-e differs from that in 10403S in the probe binding site. qRT-PCR clearly showed σ^B-dependent transcription of this gene in both *L. monocytogenes* and *L. innocua* (Fig. 1). An additional 10 *L. monocytogenes* genes (which showed σ^B-dependent transcription in *L. innocua*) showed lower transcript levels in the *L.*

TABLE 2. Genes identified by microarray analyses as positively regulated by σ^B in both *L. monocytogenes* and *L. innocua*

Function and *L. monocytogenes*[a]/*L. innocua*[b] name (gene name)	Fold change (parent/Δ*sigB*) for[c]:				σ^B promoter for[d]:		EGD-e probe CHI for[e]:		Description of protein[f]
	LMO-NaCl	LMO-STAT	LIN-NaCl	LIN-STAT	*L. monocytogenes*	*L. innocua*	*L. monocytogenes* 10403S	*L. innocua* CLIP 11262	
Stress									
lmo0211/lin0243 (*ctc*)	1.4	**2.1**	**2.4**	1.4 (NS)	+	+	97	100	Similar to *B. subtilis* general stress protein
lmo0232/lin0264 (*clpC*)	**2.5**	−1.2 (NS)	**2.2**	1.1 (NS)	+	+	100	98	Endopeptidase Clp ATP-binding chain C
lmo0669/lin0674	**34.4**	**34.4**	**19.1**	**15.1**	+	+	98	98	Similar to oxidoreductase
lmo1433/lin1472[g]	**2.7**	**2.6**	**7.3**	3.4 (NS)	+	+	100	100 (LI)	Similar to glutathione reductase
lmo1601/lin1642	**2.5**	**4.2**	**4.4**	**9.7**	+	+	100	98	Similar to general stress protein
lmo2230/lin2332	**33.9**	**21.8**	**11.6**	**37.4**	+	+	100	98	Similar to arsenate reductase
Virulence and virulence associated									
lmo1295/lin1333 (*hfq*)	**2.5**	**3.9**	**4.2**	**8.8**	+	+	100	98	Similar to host factor 1 protein
Transcriptional regulation									
lmo0893/lin0892 (*rsbV*)	**2.2**	1.5	**4.2**	**3.5**	+	+	100	98	Anti-anti-sigma factor (antagonist of RsbW)
lmo0894/lin0893 (*rsbW*)	**2.4**	1.3	**4.3**	3.4 (NS)	+	+	100	100	σ^B activity negative regulator RsbW
lmo0958/lin0957	**2.0**	1.5 (NS)	**2.3**	5.5 (NS)	+	+	100	98	Similar to transcription regulator (GntR family)
Transporter									
lmo0169/lin0212	**2.8**	**2.1**	**2.1**	1.2 (NS)	+	+	100	98	Similar to a glucose uptake protein
lmo0654/lin0657[g]	**2.2**	**2.1**	**15.4**	**39.7**	+	+	100	100 (LI)	Similar to ABC transporter, ATP-binding protein
lmo0782/lin0775	**10.3**	**12.6**	**5.2**	**8.5**	+	+	100	98	Similar to mannose-specific PTS component IIC
lmo1425/lin1464 (*opuCD*)	**7.0**	1.8	**5.6**	1.6 (NS)	+	+	100	97	Similar to betaine/carnitine/choline ABC transporter (membrane protein)
lmo1428/lin1467 (*opuCA*)	**10.1**	**2.9**	**13.6**	2.4 (NS)	+	+	98	98	Similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)
Metabolism									
lmo0210/lin0242 (*ldh*)	**2.0**	**3.1**	**2.0**	**3.7**	+	+	100	98	Similar to L-lactate dehydrogenase
lmo0231/lin0263 (*mcsB*)	**2.0**	1.1 (NS)	**2.4**	1.3 (NS)	+	+	100	98	Similar to arginine kinase
lmo0539/lin0543	**18.8**	**13.7**	**9.6**	**14.3**	+	+	98	98	Similar to tagatose-1,6-diphosphate aldolase

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TABLE 2—*Continued*

Function and *L. monocytogenes*[a]/*L. innocua*[b] name (gene name)	Fold change (parent/Δ*sigB*) for[c]:				σ^B promoter for[d]:		EGD-e probe CHI for[e]:		Description of protein[f]
	LMO-NaCl	LMO-STAT	LIN-NaCl	LIN-STAT	*L. monocytogenes*	*L. innocua*	*L. monocytogenes* 10403S	*L. innocua* CLIP 11262	
lmo0913/lin0913	**9.7**	**5.8**	**3.7**	**5.2**	−	−	100	91	Similar to succinate semialdehyde dehydrogenase
lmo1883/lin1996	**2.5**	**12.0**	1.9	**10.2**	+	+	100	100	Similar to chitinases
lmo2456/lin2550 (*pgm*)	**2.5**	−1.1 (NS)	**2.0**	2.4 (NS)	+	+	98	98	Phosphoglycerate mutase
lmo2571/lin2716	**6.2**	**4.0**	**4.3**	**8.4**	+	+	100	100	Similar to nicotinamidase
lmo2674/lin2821	1.8	**2.7**	1.6	**4.3**	+	+	92	92	Similar to ribose 5-phosphate epimerase
lmo2695/lin2843	**7.0**	1.3 (NS)	**8.0**	1.9 (NS)	+	+	98	98	Similar to dihydroxyacetone kinase
lmo2696/lin2844	**6.5**	1.5	**6.9**	2.0 (NS)	+	+	92	91	Similar to hypothetical dihydroxyacetone kinase
lmo2724/lin2872	**3.7**	1.8	**3.3**	**4.8**	+	+	100	100	Similar to unknown proteins
DNA metabolism and transport									
lmo1606/lin1647[g]	1.2 (NS)	**2.8**	1.9	**5.9**	+	+	97	100 (LI)	Similar to DNA translocase
Protein synthesis and modification									
lmo2511/lin2655	1.3 (NS)	**3.2**	**3.1**	1.3 (NS)	+	+	100	98	Similar to conserved hypothetical proteins similar to *B. subtilis* YvyD protein
Cell envelope and cellular processes									
lmo0880/lin0879[g]	**12.3**	**13.0**	**9.0**	**23.7**	+	+	100	100 (LI)	Similar to wall-associated protein precursor (LPXTG motif)
lmo1694/lin1802	**3.4**	**2.6**	**2.1**	**3.6**	+	+	100	98	Similar to CDP-abequose synthase
Unknown									
lmo0133/lin0180[g]	**2.4**	1.2 (NS)	**11.0**	**10.2**	+	+	94	100 (LI)	Similar to *Escherichia coli* YjdI protein
lmo0170/lin0213	**4.0**	1.9 (NS)	**4.7**	**3.0**	+	+	100	98	Hypothetical protein
lmo0291/lin0319	**2.0**	**2.1**	1.4 (BS)	**2.8**	+	−	98	94	Conserved hypothetical protein similar to *B. subtilis* YycJ protein
lmo0321/lin0346	**3.0**	**4.3**	**2.2**	1.5 (NS)	+	+	100	98	Similar to unknown proteins
lmo0589/lin0598	**3.9**	1.8	**3.7**	1.3 (NS)	+	+	100	92	Hypothetical protein
lmo0592/lin0601	**2.0**	1.5	**2.0**	1.1 (NS)	+	+	100	95	Hypothetical protein
lmo0596/lin0605	**17.7**	**12.3**	**14.5**	**13.2**	+	+	98	98	Similar to unknown proteins

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TABLE 2—*Continued*

Function and *L. monocytogenes*[a]/*L. innocua*[b] name (gene name)	Fold change (parent/Δ*sigB*) for[c]:				σ^B promoter for[d]:		EGD-e probe CHI for[e]:		Description of protein[f]
	LMO-NaCl	LMO-STAT	LIN-NaCl	LIN-STAT	*L. monocytogenes*	*L. innocua*	*L. monocytogenes* 10403S	*L. innocua* CLIP 11262	
lmo0628/lin0637	**2.2**	**3.8**	**2.3**	**3.9**	+	+	100	95	Hypothetical protein
lmo0647/lin0650	**4.8**	**3.6**	**8.9**	−2.1 (BS)	+	+	100	98	Hypothetical protein
lmo0670/lin0675	**12.8**	**8.3**	**4.0**	3.1 (NS)	+	+	98	95	Hypothetical protein
lmo0953/lin0952[g]	**3.0**	**3.0**	**9.9**	**19.5**	+	+	100	100 (LI)	Hypothetical protein
lmo0995/lin0994	**3.9**	**2.8**	**5.9**	**3.8**	+	−	95	95	Similar to *B. subtilis* YkrP protein
lmo1241/lin1205	**2.3**	**2.6**	**3.2**	**3.2**	+	+	100	98	Hypothetical protein
lmo1602/lin1643	**3.6**	**5.1**	**4.2**	12.4 (BS)	+	+	100	98	Similar to unknown proteins
lmo2158/lin2261	**17.8**	**15.6**	**5.1**	**6.8**	+	+	100	94	Similar to *B. subtilis* YwmG protein
lmo2191/lin2295	**3.5**	**2.7**	**2.1**	2.2 (NS)	+	+	100	98	Similar to unknown proteins
lmo2232/lin2334	**4.9**	1.6	1.5	**3.3**	+	+	100	98	Similar to unknown proteins
lmo2269/lin2370	**2.5**	**5.7**	**6.4**	**3.1**	+	+	100	100	Hypothetical protein
lmo2391/lin2490	**5.7**	**7.5**	**2.0**	**8.5**	+	+	100	91	Conserved hypothetical protein similar to *B. subtilis* YhfK protein

[a] Probe name based on *L. monocytogenes* EGD-e gene.
[b] *L. innocua* homologue as given by ListiList server (http://genolist.pasteur.fr/Listilist).
[c] Positive changes represent higher transcript levels in the parent strain than in the Δ*sigB* strain. Negative changes represent higher transcript levels in the Δ*sigB* mutant than in the parent strain. Bold indicates σ^B-dependent genes (adjusted $P < 0.05$; change, ≥2.0-fold). NS, not significant (adjusted $P > 0.05$); BS, borderline significant (adjusted P between 0.05 and 0.1).
[d] A plus sign indicates the presence of a σ^B-dependent promoter upstream of the ORF or the first gene of an operon. A minus sign denotes that no σ^B-dependent promoter was identified. See Tables S3 and S6 in the supplemental material for promoter descriptions and classifications of σ^B-dependent promoters, respectively.
[e] *L. monocytogenes* EGD-e probe CHI to *L. monocytogenes* 10403S and *L. innocua* CLIP 11262. LI denotes *L. innocua*-specific probe (CHI, 100) designed by ArrayOligoSelector.
[f] Description of protein as given by ListiList server (http://genolist.pasteur.fr/ListiList/).
[g] Denotes *L. innocua*-specific probe. Changes in LIN-NaCl and LIN-STAT reflect transcript levels from this probe.

monocytogenes Δ*sigB* mutant, although at levels below the 2.0-fold criterion (adjusted $P < 0.05$ for eight genes; adjusted P between 0.05 and 0.1 for two genes), indicating either that these genes are σ^B dependent in both species or that quantitative differences in σ^B-dependent transcription for some of these genes are subtle. As these data suggested that few of the genes with homologues in both *L. monocytogenes* and *L. innocua* may have species-specific patterns of σ^B-dependent transcription, qRT-PCR was used to further evaluate σ^B-dependent transcription of an additional four genes with homologues in both *L. monocytogenes* and *L. innocua*. lmo2434/lin2528 (encoding GadA, a glutamate decarboxylase) and lmo2748/lin2891 (encoding a stress protein similar to *B. subtilis* YdaG) were included in the qRT-PCR experiments as microarray data showed positive σ^B-dependent regulation for the *L. monocytogenes*, but not the *L. innocua*, homologues, possibly because of the relatively low CHI of the probe for the *L. innocua* genes (CHIs of 94 and 91, respectively). qRT-PCR clearly showed σ^B-dependent transcription for both of these genes in both *L. monocytogenes* and *L. innocua* (Fig. 1), supporting the idea that probes with low CHIs may yield false-negative results. lmo1879/lin1992 (encoding CspD, a cold shock protein) was included in qRT-PCR experiments as the microarray data (based on a probe with CHIs of 100 and 98 for *L. monocytogenes* and *L. innocua*, respectively) showed lower transcript levels in the salt-stressed *L. monocytogenes* parent strain (compared to the Δ*sigB* strain), while showing higher transcript levels in the stationary-phase *L. innocua* parent strain (compared to the Δ*sigB* strain) (see Table S6 in the supplemental material). Initial analysis of qRT-PCR data for cells exposed to salt showed lower transcript levels in both the *L. monocytogenes* and *L. innocua* parent strain than in the respective *sigB* null mutants with no difference in transcript levels for stationary-phase cells (Fig. 1). When transcript levels for *L. innocua cspD* were normalized to *rpoB* only (rather than to the geometric mean of *rpoB* and *gap*, an approach chosen as *L. innocua gap* showed some σ^B-dependent transcription), *L. innocua cspD* showed higher transcript levels in the parent strain in stationary phase and no difference between parent and Δ*sigB* strain for salt-stressed cells, representing the same trends observed in the microarray data for *L. innocua*. While these findings illustrate the effect of different normalization

procedures on data interpretation, including the possibility that normalization to a large number of genes (as performed in a microarray) may provide for more robust data, the biological relevance of these findings remains to be determined. Importantly though, for all other genes where transcript levels were evaluated by qRT-PCR, normalization of *L. innocua* transcript levels to *rpoB* alone (rather than both *rpoB* and *gap*) did not change the conclusions (Fig. 1 legend). Finally, lmo1428/lin1467 (encoding OpuCA, a component of the carnitine transporter) were included in the qRT-PCR experiments, as *opuCA* has previously been shown to be σ^B dependent in *L. monocytogenes* (40, 66). qRT-PCR showed that *L. monocytogenes opuCA* transcription is σ^B dependent in both stationary-phase and salt-stressed cells, while *L. innocua opuCA* was σ^B dependent only in salt-stressed cells, consistent with microarray data (Fig. 1). These data support the idea that σ^B-dependent regulation of transcription may differ between *L. monocytogenes* and *L. innocua*, even for genes found in both species.

Phenotypic characterization reveals different stress survival patterns for *L. monocytogenes* and *L. innocua sigB* null mutants. Acid stress survival assays showed that exposure of log-phase cells to BHI broth acidified to pH 2.5 or SGF at pH 2.5 leads to a clear reduction of *L. monocytogenes* and *L. innocua* bacterial numbers, with the Δ*sigB* strains showing significantly ($P < 0.05$) (Fig. 2A) greater reduction after acid exposure than the respective parent strains, indicating the importance of σ^B for acid stress survival in log-phase *L. monocytogenes* and *L. innocua*. Exposure of stationary-phase cells to BHI-HCl or SGF for 60 min also led to a clear reduction of *L. monocytogenes* and *L. innocua* bacterial numbers; while the *L. monocytogenes* Δ*sigB* strain showed significantly ($P < 0.05$) (Fig. 2B) higher reduction than the parent strain after acid exposure, stationary-phase *L. innocua* Δ*sigB* and parent strains did not differ in their susceptibility to either BHI-HCl or SGF, suggesting that σ^B contributes less to stationary-phase acid stress survival in *L. innocua* than in *L. monocytogenes*, suggesting that the relative contributions of σ^B to acid stress survival differ between these species. The observed difference in acid resistance phenotype may relate to the fact that the *L. monocytogenes* σ^B stress response system has adapted to suit a pathogenic lifestyle (as also supported by σ^B-dependent virulence gene transcription), including the need to rapidly adapt to and subsequently survive in the low-pH environment of the mammalian stomach. This hypothesis is consistent with the emerging picture that *L. monocytogenes* σ^B is particularly critical during the gastrointestinal stages of infection, by regulating transcription of genes encoding bile-associated proteins (3, 65, 66), internalins important for attachment to intestinal epithelial cells and other cells (42, 43), and other proteins important for gastrointestinal infection (e.g., OpuC [60]). *L. innocua*, on the other hand, may have evolved a more constitutively expressed mechanism of stationary-phase acid resistance, which relies less on σ^B.

Conclusions. Overall, our data clearly support the importance of σ^B as a transcriptional regulator in both pathogenic and nonpathogenic *Listeria* species, as indicated by the considerable number of genes positively regulated as well as indirectly repressed by σ^B. Our data also suggest the involvement of σ^B in regulatory networks and interactions that appear to be critical to ensure appropriate gene expression in *Listeria* species under different environmental stress conditions as well as during host infections. In addition to the well-supported interactions between PrfA and σ^B, which appear to be critical to virulence and virulence gene regulation (26, 40), σ^B also appears to contribute to transcriptional regulation of genes encoding other regulators (e.g., *hrcA*) and appears to affect transcription of a number of genes, including flagellar genes, that are also regulated by the motility gene repressor MogR and the two-component response regulator DegU. The role of σ^B in gene regulation, including in regulatory networks, also appears to have adapted to the differing requirements for gene regulation in a pathogen versus a nonpathogen, as supported by pathogen-specific regulatory networks (i.e., the σ^B-PrfA network [40, 50]) and σ^B-dependent transcription of genes unique to the pathogen *L. monocytogenes* (e.g., *inlAB* and *bsh*) and of at least one internalin-like gene unique to the nonpathogen *L. innocua*.

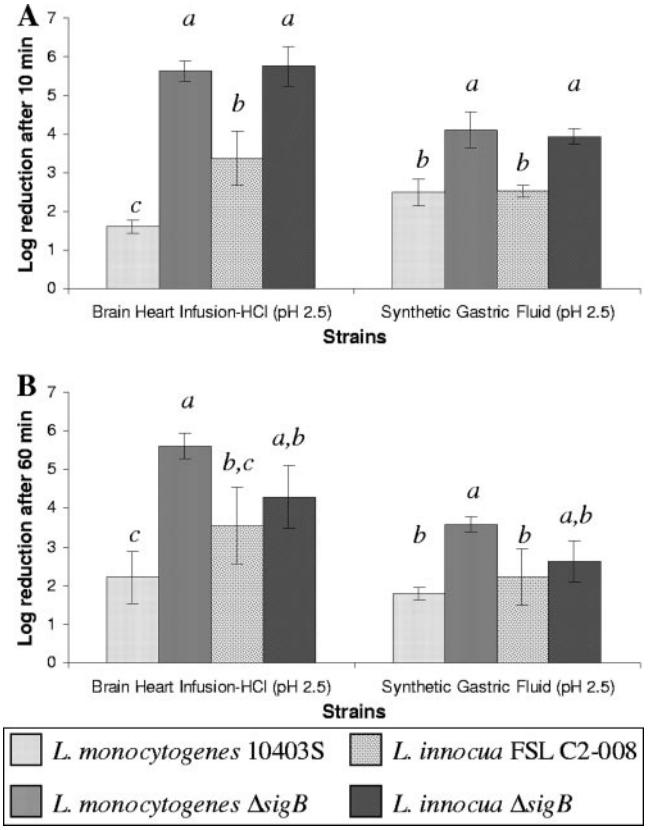

FIG. 2. Log reduction of *L. monocytogenes* and *L. innocua* parent and Δ*sigB* strains for log-phase cells (A) and stationary-phase cells (B) after exposure to acid stress. Strains with higher log reduction showed greater sensitivity to acid stress (e.g., *L. monocytogenes* Δ*sigB* showed a 5.5-log reduction while *L. monocytogenes* parent strain 10403S showed a 2-log reduction; the Δ*sigB* strain is thus more sensitive to acid stress). Experiments were performed in three independent trials, and data shown represent the averages of these trials; error bars show the standard deviations. Bacterial numbers after acid stress were analyzed using the general linear model with Tukey's multiple-comparisons procedure; bars labeled with different letters indicate bacterial numbers that differed significantly ($P < 0.05$), while bars labeled with identical letters indicate bacterial numbers that did not differ significantly.

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