Listeria monocytogenes σ^B Has a Small Core Regulon and a Conserved Role in Virulence but Makes Differential Contributions to Stress Tolerance across a Diverse Collection of Strains[▽][†]

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Listeria monocytogenes strains are classified in at least three distinct phylogenetic lineages. There are correlations between lineage classification and source of bacterial isolation; e.g., human clinical and food isolates usually are classified in either lineage I or II. However, human clinical isolates are overrepresented in lineage I, while food isolates are overrepresented in lineage II. σ^B, a transcriptional regulator previously demonstrated to contribute to environmental stress responses and virulence in *L. monocytogenes* lineage II strains, was hypothesized to provide differential abilities for *L. monocytogenes* survival in various niches (e.g., food and human clinical niches). To determine if the contributions of σ^B to stress response and virulence differ across diverse *L. monocytogenes* strains, Δ*sigB* mutations were created in strains belonging to lineages I, II, IIIA, and IIIB. Paired parent and Δ*sigB* mutant strains were tested for survival under acid and oxidative stress conditions, Caco-2 cell invasion efficiency, and virulence using the guinea pig listeriosis infection model. Parent and Δ*sigB* mutant strain transcriptomes were compared using whole-genome expression microarrays. σ^B contributed to virulence in each strain. However, while σ^B contributed significantly to survival under acid and oxidative stress conditions and Caco-2 cell invasion in lineage I, II, and IIIB strains, the contributions of σ^B were not significant for these phenotypes in the lineage IIIA strain. A core set of 63 genes was positively regulated by σ^B in all four strains; different total numbers of genes were positively regulated by σ^B in the strains. Our results suggest that σ^B universally contributes to *L. monocytogenes* virulence but specific σ^B-regulated stress response phenotypes vary among strains.

Listeria monocytogenes is a Gram-positive, rod-shaped bacterium that can cause listeriosis, a life-threatening invasive disease in humans and animals. While listeriosis is rarely diagnosed in healthy individuals, the elderly, the immunocompromised, and pregnant women and their fetuses are particularly at risk of infection. The vast majority (99%) of listeriosis infections are food borne, and approximately 20% of diagnosed infections result in death (36).

Increasing evidence indicates that *L. monocytogenes* strains belong to multiple lineages that appear to differ in their abilities to be transmitted to humans (20). Characterization of *L. monocytogenes* isolates from a variety of hosts and environments by multiple subtyping methods (35, 63), including multilocus enzyme electrophoresis (MLEE) (47), has shown that strains comprising the species *L. monocytogenes* belong to two major divisions designated lineages I and II and at least one additional distinct genetic lineage. The third lineage, lineage III, can be subdivided into lineages IIIA/C and IIIB (lineage IIIB has recently been classified as lineage IV [65]), based on results obtained with a number of molecular subtyping strategies, including pulsed-field gel electrophoresis (PFGE) (20) and virulence gene sequencing (20, 68). While different nomenclatures have been used for these *L. monocytogenes* lineages (47, 53), the main groups described in multiple studies consistently include the same *L. monocytogenes* serotypes (20, 53, 66, 68). Based on the most commonly applied lineage designations (20), lineage I includes predominantly strains with the 1/2b, 3b, 3c, and 4b serotypes and lineage II includes primarily strains with the 1/2a, 1/2c, and 3a serotypes (38). Strains associated with human clinical listeriosis cases are significantly overrepresented in lineage I compared to strains associated with animal listeriosis cases or contaminated foods (20). Lineage I isolates appear to have significantly greater pathogenic potential than lineage II strains, as suggested by their relatively enhanced ability to spread to neighboring host cells in a cell culture plaque assay (20, 68). On the other hand, lineage II strains are significantly more common among food isolates than among isolates from human listeriosis cases (20). However, preferential recovery of lineage II strains over lineage I strains may occur with at least some selective media and isolation protocols (5), which may have contributed to this observation. Lineage III includes predominantly strains with the 4a and 4c serotypes, as well as some serotype 4b strains that are distinct from the strains grouped in lineage I (20, 26). Strains classified in lineage III appear to be associated with isolation from animals, occasionally with isolation from human listeriosis cases, and only rarely with isolation from foods (20).

L. monocytogenes survival and persistence in diverse environments, including food processing plants, are facilitated by its ability to survive and grow at wide ranges of temperatures (0 to 45°C) (14), pH values (pH 4.4 to 9.4) (14), and other environmental conditions. The σ^B general stress regulator, which is conserved across many Gram-positive bacteria, includ-

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TABLE 1. Strains used in this study

Lineage	Strain	Serotype	Ribotype	Origin	Reference(s)
I	FSL J1-194	1/2b	DUP-1042B	Human clinical isolate	50
I	FSL C6-001 (ΔsigB)	1/2b	DUP-1042B	FSL J1-194	This study
II	10403S	1/2a	DUP-1030A	Laboratory type strain	2
II	FSL A1-254 (ΔsigB)	1/2a	DUP-1030A	10403S	15, 67
IIIA	FSL J2-071	4c	DUP-1061A	Bovine clinical isolate	68
IIIA	FSL O1-006 (ΔsigB)	4c	DUP-1061A	FSL J2-071	This study
IIIB	FSL J1-208	4a	DUP-10142	Caprine clinical isolate	68
IIIB	FSL O1-005 (ΔsigB)	4a	DUP-10142	FSL J1-208	This study

ing *Staphylococcus aureus* (69), *Bacillus anthracis* (16), and *Bacillus licheniformis* (3), has been shown to contribute to bacterial survival following exposure to environmental stresses, including acid, oxidative, and energy stresses (9, 15, 67). In the *L. monocytogenes* strains evaluated to date, σ^B positively regulates at least 160 genes directly and as many as 200 genes both directly and indirectly (48), including transcription of a number of virulence genes (e.g., *prfA*, *bsh*, *inlA*, and *inlB*) (30, 31, 33, 44, 48, 49). An *L. monocytogenes* 10403S ΔsigB null mutant has reduced invasiveness in human intestinal epithelial cells (32) and reduced virulence in intragastrically inoculated guinea pigs (17).

The contributions of σ^B to *L. monocytogenes* stress responses and virulence have been characterized most thoroughly for lineage II strains, including strains 10403S (44, 48) and EGD-e (21, 53). For example, the relative virulence characteristics of the lineage II strain 10403S and its isogenic ΔsigB mutant have been evaluated in the guinea pig model, but contributions of σ^B to gastrointestinal infection have not been reported for other *L. monocytogenes* lineages. Information about the contributions of σ^B to regulation of gene expression in strains belonging to lineages I, IIIA, and IIIB is just emerging. A recent comparative transcriptomic study found that *sigB* (lmo0895) was expressed at higher levels in lineage II strains than in lineage I strains (53). Also, a number of previously identified σ^B-regulated genes (30, 48) were expressed at higher levels in lineage II strains than in lineage I strains; these genes include *opuCA* and lmo1421, which encode a known compatible solute transporter protein and a putative compatible solute transporter protein, respectively. With respect to the stress response phenotype, Moorhead and Dykes (37) reported that the relative importance of σ^B in stress responses was not the same in all strains of *L. monocytogenes*, as assessed by comparison of the survival of two ΔsigB mutants having different serotypes under various stress conditions. Specifically, there were greater differences between a serotype 1/2a strain and its ΔsigB mutant than between a serotype 4a wild-type strain and its ΔsigB mutant.

We hypothesized that the inherent differences in the abilities of *L. monocytogenes* strains to survive under stress conditions and subsequently cause disease may be at least partially attributable to differences in the contributions of σ^B among strains. To determine if the contributions of σ^B to stress responses and virulence differ across diverse *L. monocytogenes* strains, we evaluated *L. monocytogenes* lineage I, II, IIIA, and IIIB strains using a combination of phenotypic and transcriptomic analyses, including a multigenome microarray to characterize differences in the σ^B regulons. Each pair of isogenic parent and ΔsigB strains was also assayed for survival under acid and oxidative stress conditions, invasiveness in a Caco-2 cell model, and virulence in the guinea pig listeriosis infection model.

MATERIALS AND METHODS

Bacterial strains and storage. The strains selected for this study included *L. monocytogenes* lineage I, II, IIIA, and IIIB isolates (Table 1). FSL J1-194 (lineage I) was isolated from a sporadic human listeriosis case (50). It is serotype 1/2b, which is commonly associated with human disease (29), and ribotype DUP-1042B, which is considered a ribotype with enhanced virulence characteristics (20), as demonstrated by plaques in tissue culture plaque assays that are larger than the plaques formed by strains belonging to other ribotypes. 10403S is a laboratory type strain (2) and was selected (i) because it is a lineage II strain and (ii) to facilitate comparisons between this study and previous studies that also used this strain (10, 15, 17). FSL J2-071 and FSL J1-208 were selected from available lineage IIIA and IIIB strains, respectively. Both of these strains were isolated from clinical animal cases, and they are serotypes 4c and 4a, respectively, which are common in lineage III strains (38). While the strains selected for this study represent *L. monocytogenes* diversity, additional strains belonging to each lineage would have to be evaluated to determine if the strains used here are representative of the lineages (12). FSL A1-254, a 10403S isogenic ΔsigB mutant, was used in previous studies (9, 15, 67). Isogenic *sigB* null (ΔsigB) mutations were created in the other three strains using allelic exchange mutagenesis, as described previously (25, 67). Primers used for creation of mutants are shown in Table S1 in the supplemental material. Stock cultures were stored at −80°C in brain heart infusion (BHI) containing 15% glycerol. Cultures were streaked onto BHI agar (Difco, Detroit, MI) and incubated at 37°C for 24 h to obtain isolated colonies for inoculation of overnight cultures. The growth conditions for each experiment below are described.

Acid and oxidative stress survival assays. For acid and oxidative stress survival assays, strains were grown in 5 ml of BHI broth at 37°C with shaking (230 rpm) for 12 h. A 1% inoculum was transferred to 5 ml of preheated BHI broth and grown to an optical density at 600 nm (OD_{600}) of ~0.4. After growth to an OD_{600} ~0.4, a ~1% (vol/vol) inoculum was transferred to 50 ml of prewarmed (37°C) BHI broth in a 300-ml nephelo flask (Bellco, Vineland, NJ) to obtain a calculated OD_{600} of 0.004 (the volume transferred was adjusted based on the actual OD_{600} of the starting inoculum). Following the second passage, strains were grown to stationary phase (defined as 10 h after inoculation of the flask). Two 5-ml aliquots of stationary-phase cells were transferred to sterile 16-mm test tubes. For acid stress tests, one aliquot was used to determine the volume (in μl) of 12 N HCl (VBR, Westchester, PA) needed to reduce the pH of the culture to 2.5, as determined by direct measurement with a pH meter (Beckman, Coulter Inc., Fullerton, CA); the second aliquot was used for an experiment. Following addition of 12 N HCl to the experimental cultures, they were gently vortexed and immediately returned to incubation at 37°C with shaking. Aliquots of the acidified cultures were removed at 10, 30, and 60 min after acidification. Bacteria were quantified by serial dilution and standard plate counting. For oxidative stress tests, cumene hydroperoxide (CHP) (Sigma-Aldrich) that had been dissolved in dimethyl sulfoxide (DMSO) was added to 900-μl portions of 10-h cultures in 1.5-ml Eppendorf tubes to obtain a final CHP concentration of 13.0 mM. The tubes were incubated for 15 min at 37°C with shaking. An equal volume of DMSO was added to nonstressed control cultures; survival was assessed by serial dilution and standard plate counting. At least three independent replicates were performed for each *L. monocytogenes* strain tested under each condition.

Caco-2 cell invasion assay. Caco-2 invasion assays were performed as previously described (40). Confluent Caco-2 monolayers were inoculated with 10 μl of a stationary-phase culture (approximately 4.8×10^7 cells/well) grown as de-

scribed above for the acid and oxidative stress survival assays. Intracellular *L. monocytogenes* cells were enumerated by spiral plating (Autoplate 4000; Spiral Biotech, Norwood, MA) 10-fold dilutions of lysed Caco-2 cell suspensions in phosphate-buffered saline (PBS) on BHI agar plates. The invasion efficiency was expressed as the log ratio of the *L. monocytogenes* cells recovered to the cells in the initial inoculum. Three independent invasion assays were performed for each *L. monocytogenes* strain tested.

Statistical analyses of stress and invasion assays. Statistical analyses were performed with Statistical Analysis Software (SAS) 9.0 (SAS Institute, Inc., Cary, NC). Regression analysis was used to calculate the death rate for cells exposed to pH 2.5, which was expressed as average log number of CFU that died per hour for each strain. Repeated-measures analysis of variance (ANOVA) was used to test if there was a significant difference in the death rate between a wild-type strain and its isogenic $\Delta sigB$ mutant. Two-sided *t* tests were used to test for differences in cell death due to oxidative stress and in invasion efficiency between wild-type and $\Delta sigB$ strains. For all statistical analyses a *P* value of < 0.05 was considered significant. ANOVA was also used to test for differences in stress survival and in invasion efficiency among the four wild-type strains.

Microarray, cDNA labeling, and microarray hybridization. Bacteria were initially grown in 5 ml of BHI broth at 37°C with shaking (230 rpm) for 15 h. A 1% inoculum was transferred to 5 ml of prewarmed BHI broth and grown to an OD_{600} of ~0.4. After growth to an OD_{600} of ~0.4, a ~1% (vol/vol) inoculum was transferred to 50 ml of prewarmed (37°C) BHI broth in a 300-ml nephelo flask (Bellco, Vineland, NJ) to obtain a calculated OD_{600} of 0.004 (the volume transferred was adjusted based on the actual OD_{600} of the starting inoculum). Following the second passage, cells were collected at stationary phase (defined as growth to an OD_{600} of 1.0, followed by incubation for an additional 3 h). Prior to centrifugation, RNAProtect bacterial reagent (Qiagen, Valencia, CA) was added to the cultures according to the manufacturer's instructions to stabilize the mRNA; pellets were stored at −80°C prior to RNA isolation. RNA was isolated as previously described (48). Briefly, bacterial cells were lysed enzymatically with lysozyme (Fisher Scientific, Pittsburg, PA) and mechanically using six bursts of sonication (Misonix, Farmingdale, NY) at 18 W on ice for 30 s. Total RNA was isolated and purified using an RNeasy midi kit (Qiagen) according to the manufacturer's protocol. RNA was eluted from the column using RNase-free water. Total RNA was incubated with RNasin (Promega, Madison, WI) to inhibit RNases and with RQ1 DNase (Promega) to remove contaminating DNA. After phenol-chloroform extraction, UV spectrophotometry (Nanodrop, Wilmington, DE) was used to quantify and assess the purity of the RNA. RNA integrity was assessed by agarose gel electrophoresis. Purified RNA was stored in RNase-free water at −80°C prior to reverse transcription (RT).

The Pathogen Functional Genomics Resource Center (PFGRC)/J. Craig Venter Institute (JCVI) *L. monocytogenes* microarray (version 2) was used to identify mRNA transcript level differences between wild-type strains and their $\Delta sigB$ mutants. PFGRC/JCVI microbial RNA aminoallyl labeling for microarray standard operating procedure M007 (http://pfgrc.jcvi.org/index.php/microarray /protocols.html) was used to reverse transcribe and label the total RNA. Spectrophotometry was used to quantify cDNA and the total numbers of picomoles of Cy3 or Cy5 (Amersham Biosciences, Piscataway, NJ) incorporated. Cy3-labeled cDNA and Cy5-labeled cDNA were combined and thoroughly dehydrated prior to hybridization. PFGRC/JCVI standard operating procedure M008 (http://pfgrc.jcvi.org/index.php/microarray/protocols.html) was used to hybridize the Cy3 and Cy5 dye-labeled cDNA to the microarray. Briefly, microarray slides were blocked in a prehybridization buffer supplemented with bovine serum albumen (BSA) (Sigma-Aldrich) and washed with deionized water and then with isopropyl alcohol. Dried Cy3- and Cy5-labeled cDNA was hydrated with hybridization buffer containing 0.6 mg/ml sheared salmon sperm DNA (Invitrogen), denatured twice at 95°C for 5 min, and briefly centrifuged. cDNA was hybridized to the microarray at 42°C for 16 h. Following hybridization, slides were washed using the PFGRC/JCVI M008 protocol and dried by centrifugation.

Microarray image processing, replicates, and statistical analysis. Image processing and analyses were performed as previously described (48). Data preprocessing and statistical analyses were performed using the LIMMA package available from the BioConductor software project for the R programming environment (18, 54). Background correction was performed using the "normexp" method to produce more robust ratios for low-intensity spots, and print tip normalization was used to correct for spatial effects and dye intensity bias (56). The empirical Bayes approach was used to assess differential expression (55). Three replicates were performed for each comparison of wild-type and isogenic $\Delta sigB$ strains. For each probe, fold changes, moderated t-statistics, and *P* values (adjusted for multiple tests by controlling for the false discovery rate) were calculated. Raw and normalized microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE21427.

Hybridization index, identification of differentially expressed genes, and association of σ^B-dependent genes with JCVI role categories. The PFGRC/JCVI *L. monocytogenes* microarray contained multiple probes for certain loci that lacked a 70-mer consensus sequence for a given locus for the four strains represented on the microarray. When multiple probes for a single locus were present, the probe that best matched the sequence for a given strain in the present study was selected as the representative probe for subsequent analyses of that strain. BLASTN was used to calculate the hybridization index (HI), which was the level of identity (expressed as a percentage) between each probe and each strain; BLASTN results were parsed to compare levels of identity between each strain and the probes. EGD-e locus names were used to describe the corresponding open reading frames (ORFs) in the lineage I, IIIA, and IIIB strains as the genomes of these strains have not been annotated yet; EGD-e locus names were also used in lieu of 10403S locus names to facilitate comparisons among all of the strains in this study. The locus names of genes unique to F2365, F6852, and H7858 (i.e., genes with no EGD-e homologue) were used in this study. Genes whose transcript levels were higher in a wild-type strain than in the isogenic $\Delta sigB$ mutant (≥1.5-fold change) and whose adjusted *P* values were <0.05 were considered positively differentially expressed and thus σ^B dependent. A χ^2 test for trend was used to determine if there was an association between σ^B-dependent expression and JCVI role category. Subsequent Fisher's exact tests were used to determine which role categories were significantly associated with σ^B-dependent genes. *P* values of <0.05 were considered significant. The odds ratio (OR) for the presence of a σ^B-dependent gene in a given category rather than the other categories was determined.

TaqMan qRT-PCR and putative σ^B promoter comparisons. TaqMan quantitative reverse transcription-PCR (qRT-PCR) was used to confirm that there were lineage-specific σ^B-dependent genes. Total RNA was extracted from cells grown as described above for microarray analyses using an Ambion MicrobExpress kit (Ambion). RNA quality and integrity were assessed with an Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA), and the total RNA concentration was checked by spectrophotometry (Nanodrop). qRT-PCR was performed using an ABI Prism 7000 sequence detection system (Applied Biosystems) essentially as previously described (58), except that RNA was reverse transcribed to cDNA with cDNA reverse transcription reagents (Applied Biosystems) using random hexamers prior to quantification of transcript levels. To evaluate residual genomic DNA levels, the same reaction was performed with each sample without reverse transcriptase. In previous studies by our group (31, 58), the *rpoB* primers and probe were used to calculate the residual DNA copy number and to normalize transcript levels as there is minimal variation in the transcript level of this housekeeping gene under different conditions. Primers (IDT DNA, Coralville, IA) and probes (Applied Biosystems) used in this study (see Table S2 in the supplemental material) were designed with Primer Express (Applied Biosystems) based on a consensus sequence for the gene in the four wild-type strains. For some strains, the DNA sequence of a gene, including at least 200 nucleotides upstream of the coding region, was not available. To PCR amplify and sequence the region of interest for these strains, primers were designed based on the sequences of other wild-type strains. TaqMan primers and probes were designed based on the conserved sequence for all strains used in this study.

Guinea pig listeriosis infection model. The guinea pig listeriosis infection model was used to assess the virulence of *L. monocytogenes* parent and $\Delta sigB$ strains as previously described by Garner et al. (17). Bacteria were grown as described above for RNA isolation, except that cells were grown to early stationary phase (defined as growth to an OD_{600} of 0.8, followed by incubation for an additional 1 h) for consistency with previous guinea pig listeriosis infection model studies (17). Aliquots of early-stationary-phase bacterial cultures were concentrated by centrifugation and resuspended in 1 ml PBS (pH 7.4) containing 15% glycerol. Cells were frozen and stored in multiple aliquots at −80°C for use in three replicates; an aliquot of each strain was thawed, and the cells were enumerated by serial dilution and spiral plating to determine cell viability 1 day prior to inoculation of animals. An aliquot was thawed immediately before inoculation; the concentration of the inoculum was adjusted to 1.0×10^{10} CFU/ml, which was confirmed by serial dilution and plating immediately after inoculation of the guinea pig.

Animal protocols (protocol 2002-0060) were approved by the Cornell University Institutional Animal Care and Use Committee prior to initiation of the experiments. Male Hartley guinea pigs (Elm Hill, Chelmsford, MA) weighing 348 ± 43 g that were 3 weeks old were housed individually, which allowed collection of each animal's feces. Animals were provided feed and water *ad libitum*. Cages were changed daily, and animal health and weight were monitored and recorded daily. Animals were acclimated for 5 days prior to inoculation; experiments were performed in triplicate using three animals per bacterial strain tested.

Intragastric inoculation of guinea pigs was performed as described previously

TABLE 2. Contributions of σ^B to survival under acid and oxidative stress conditions for all four *L. monocytogenes* lineages

Lineage	Death rate (log CFU/h) at pH 2.5			Death (log CFU) with 13 mM CHP		
	Wild type[a]	$\Delta sigB$ strain[b]	P value[c]	Wild type[d]	$\Delta sigB$ strain[e]	P value[f]
I	3.95 ± 0.93	6.62 ± 0.66	0.0004	1.27 ± 0.04	2.10 ± 0.23	0.0225
II	1.75 ± 0.49	6.76 ± 0.79	<0.0001	1.10 ± 0.08	2.57 ± 0.12	<0.0001
IIIA	4.02 ± 2.23	5.74 ± 1.89	0.2920	1.14 ± 0.22	1.74 ± 0.40	0.0827
IIIB	3.65 ± 0.77	6.37 ± 0.73	0.0047	1.38 ± 0.30	2.09 ± 0.22	0.0291

[a] Average death rate (± standard deviation) for wild-type parent strain exposed to pH 2.5 for 1 h.
[b] Average death rate (± standard deviation) (log CFU/h) for isogenic $\Delta sigB$ strain exposed to pH 2.5 for 1 h.
[c] P value for the time-strain interaction. A P value of <0.05 indicates there that was a significant difference in the average death rate between the wild-type and isogenic $\Delta sigB$ strains.
[d] Average death value (± standard deviation) (log CFU) for wild-type parent strain after 15 min of exposure to 13 mM CHP.
[e] Average death value (± standard deviation) (log CFU) for isogenic $\Delta sigB$ strain after 15 min of exposure to 13 mM CHP.
[f] P value for one-sided *t* test. A P value of <0.05 indicates that there was a significant difference in the average death value between the wild-type and isogenic $\Delta sigB$ strains.

(1). Briefly, feed was withheld for 12 h prior to inoculation. Animals were anesthetized with isoflurane administered via inhalation using oxygen as a carrier gas (17). *L. monocytogenes* was administered intragastrically by gavage. The stomach pH was neutralized with 1 ml of PBS containing 125 mg calcium carbonate (pH 7.4) prior to inoculation of *L. monocytogenes* (1.0×10^{10} CFU/ml). Each guinea pig was weighed daily and immediately prior to euthanasia by CO_2 at 72 h after inoculation. The brain, liver, spleen, mesenteric lymph nodes, and small intestine were harvested and evaluated individually for *L. monocytogenes* as previously described (17). Recovered organs were kept on ice until they were processed. All organs were weighed and visually inspected for lesions. A 20-cm portion of the small intestine distal to the cecum was harvested. After the contents were removed, the small intestine segments were rinsed twice in 20 ml PBS, incubated at room temperature for 90 min in 20 ml of Dulbecco modified Eagle medium (DMEM) (Invitrogen) containing 100 mg/ml gentamicin to kill extracellular bacteria, and then rinsed three times in PBS prior to homogenization. The liver was homogenized in 60 ml of sterile PBS in a small autoclaved blender unit for 30 s; the brain, spleen, mesenteric lymph nodes, and small intestine were homogenized in 30 ml of PBS for 30 s. Following homogenization, homogenates were directly spread plated in duplicate on BHI media; homogenates and dilutions of homogenates (in PBS) were also spiral plated on BHI agar. All samples were also enriched to enable detection of *L. monocytogenes* in all organs, as follows: 10 ml homogenate was added to 90 ml *Listeria* enrichment broth (LEB) (Difco, Sparks, MD), incubated at 30°C, and then plated (50 μl) on Oxford medium (ThermoFisher, Waltham, MA) after 24 and 48 h.

After inoculation, feces were collected from all guinea pigs daily and processed as described by Garner et al. (17). A total of 0.5 g of feces was homogenized in 4.5 ml of PBS. Homogenized samples were serially diluted in PBS; homogenates were spread plated on Oxford medium (Oxoid, Basingstoke, United Kingdom), and subsequently dilutions in PBS were spiral plated on Oxford medium and grown for 24 h at 35°C. *L. monocytogenes* colonies were enumerated and confirmed on *L. monocytogenes* plating medium (LMPM) (Biosynth, Naperville, IL).

Statistical analyses were performed with Statistical Analysis Software (SAS) 9.0 (SAS Institute, Inc., Cary, NC). The levels of recovery (in log CFU/g) of *L. monocytogenes* from organs (i.e., liver, spleen, mesenteric lymph nodes, and small intestine) were used as the main measure of virulence. For specimens that were negative as determined by direct plating and positive as determined by enrichment, numbers of bacteria were conservatively estimated to represent the mean between the direct plating and enrichment detection limits; for specimens that were negative by both direct plating and enrichment, bacterial numbers were conservatively estimated to represent the mean between the enrichment detection limit and 0 CFU/g. One-sided *t* tests were used to determine whether the number *L. monocytogenes* bacteria (log CFU/g) recovered from a given organ or feces was higher for the wild-type strain than for the isogenic $\Delta sigB$ mutant. A P value of <0.05 was considered significant.

RESULTS

Contributions of σᴮ to survival of stationary-phase cells at pH 2.5 and under oxidative stress conditions are different for different strains. For both survival under acid stress conditions and survival under oxidative stress conditions, there were no significant differences in the death rates among the four wild-type strains. While the death rates of the wild-type lineage I, lineage II, and lineage IIIB strains under acid stress conditions differed significantly from those of their isogenic $\Delta sigB$ mutants ($P = 0.0004$, $P < 0.0001$, and $P = 0.0047$, respectively) (Table 2), the death rate of the lineage IIIA wild-type strain and the death rate of its isogenic $\Delta sigB$ mutant did not differ significantly ($P = 0.2920$). The largest difference in death rates (~5 log CFU/h) was observed for the lineage II wild-type strain and its $\Delta sigB$ mutant, suggesting that σᴮ is more important for survival under acid stress conditions in the lineage II strain than in the other strains. Further, while oxidative stress assays showed that there were significant differences in the numbers of cells killed between lineage I, lineage II, and lineage IIIB wild-type strains and their $\Delta sigB$ mutants ($P = 0.0225$, $P < 0.0001$, and $P = 0.0291$, respectively) (Table 2), σᴮ did not contribute significantly to the survival of the lineage IIIA strain under these conditions ($P = 0.0827$). σᴮ apparently played the largest role in survival under oxidative stress conditions in the lineage II strain, as shown by the largest difference (~1.4 log CFU) between the lineage II wild-type strain and its $\Delta sigB$ mutant. σᴮ contributed significantly to the survival of the lineage I, II, and IIIB strains at pH 2.5 and under oxidative stress conditions but apparently contributed little to the survival of the lineage IIIA strain under the same conditions.

Contributions of σᴮ to invasion of Caco-2 cells by stationary-phase *L. monocytogenes* cells are different for strains belonging to different lineages. Assays of invasion of Caco-2 epithelial cells by stationary-phase *L. monocytogenes* cells (Fig. 1) showed that there were significant differences in invasion efficiency between wild-type strains and their isogenic $\Delta sigB$ mutants for lineage I ($P = 0.0004$), lineage II ($P = 0.0319$), and lineage IIIB ($P = 0.0192$) strains. Specifically, lineage I, II, and IIIB wild-type strains had higher invasion efficiencies than their isogenic $\Delta sigB$ mutants. No significant difference in invasion efficiency was found between the lineage IIIA wild-type strain and its $\Delta sigB$ mutant ($P = 0.1991$). The lineage IIIA parent strain showed the lowest invasion efficiency of the four parent strains; ANOVA showed that there was a significant effect ($P = 0.0126$) of the factor "strain" on the invasion efficiencies of the four parent strains, and a Tukey's post hoc test showed that the lineage IIIA wild-type strain was significantly less invasive than the lineage IIIB wild-type strain ($P = 0.0097$), while no other wild-type strains differed significantly in
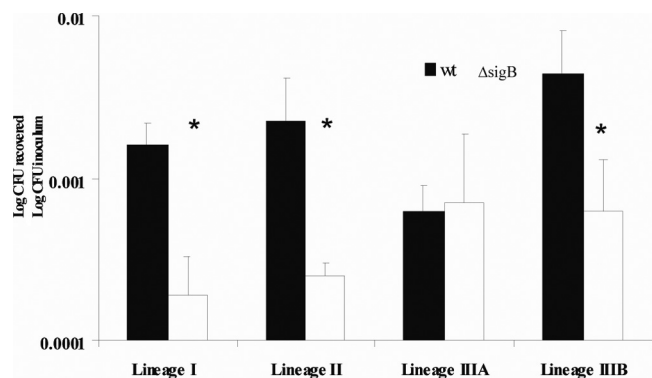
FIG. 1. Invasion efficiencies (log CFU recovered/log CFU initial inoculum) for stationary-phase *L. monocytogenes* lineage I, II, IIIA, and IIIB strains in Caco-2 cells. The bars indicate means for at least three independent experiments; the error bars indicate 1 standard deviation from the mean. Each wild-type strain (wt) is paired with its isogenic Δ*sigB* mutant, as indicated on the *x* axis. The invasion efficiencies for each pair of strains were assessed by using two-sample *t* tests (*, $P < 0.05$).

their invasion efficiencies. Overall, σ^B contributed significantly to invasion of Caco-2 cells by stationary-phase lineage I, II, and IIIB wild-type strains but did not appear to contribute to invasion by the lineage IIIA strain under the same conditions.

Core σ^B regulon consists of at least 63 genes. The transcript levels of a core set of 63 genes were higher in all four wild-type strains than in their Δ*sigB* mutants (≥ 1.5-fold change), and these genes were significantly positively differentially expressed (adjusted *P* value, <0.05) in all strains (Table 3). Putative σ^B-dependent promoters were located upstream of 50 of these 63 genes or predicted operons (79.4%); the putative σ^B promoters were identified using a hidden Markov model (HMM) previously developed for identification of σ^B promoters in *L. monocytogenes* strain 10403S (48). The core regulon included genes previously reported to be σ^B regulated, including *inlA*, *opuCA*, and *rsbX*, which encode internalin A, a glycine betaine-carnitine-choline ABC transporter, and a negative regulator of σ^B-dependent gene expression, respectively. Interestingly, 12 of the 63 core genes have no known function, and 7 of them encode conserved hypothetical proteins, indicating that over 30% of the genes in the core σ^B regulon, which may make important contributions to *L. monocytogenes* physiology, remain to be characterized.

Of the 63 genes identified here as σ^B-dependent genes in all four strains, 56 and 59 were previously identified as genes positively regulated by σ^B by Raengpradub et al. (48) and Ollinger et al. (44), respectively (see Table S3 in the supplemental material). In addition, 45 of the 63 core σ^B-dependent genes identified here were previously identified as σ^B-dependent genes in *L. monocytogenes* EGD-e cells grown to early stationary phase (21) (see Table S3 in the supplemental material). As cells were grown to stationary phase prior to RNA isolation in each of these studies, genes with functions that contribute to recovery of cells from stationary phase may be overrepresented among the genes identified as σ^B-dependent genes.

σ^B-dependent genes found to be significantly positively differentially expressed in at least one strain. A total of 425 genes

were found to be positively differentially expressed in at least one of the four *L. monocytogenes* strains (>1.5-fold change; adjusted *P* value, <0.05) and thus constitute the σ^B pan-regulon (see Table S3 in the supplemental material). Of these 425 genes, 170 were positively differentially expressed in the lineage I strain, 252 were positively differentially expressed in the lineage II strain, 201 were positively differentially expressed in the lineage IIIA strain, and 207 were positively differentially expressed in the lineage IIIB strain. For 83 of the 425 positively differentially expressed genes there was a putative σ^B promoter upstream of the transcriptional start site or upstream of the first gene in a putative operon.

To broadly assess the functional roles of σ^B-dependent genes across all strains, the gene classification in JCVI role categories (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi) was examined. A χ^2 test for trend indicated that σ^B-dependent genes (i.e., genes that were σ^B dependent in at least one strain) were not randomly distributed among JCVI role categories ($P < 0.0001$). Results of subsequent Fisher's exact tests indicated that σ^B-dependent genes were significantly overrepresented ($P < 0.05$) in two JCVI role categories ("cellular processes" and "energy metabolism") and significantly underrepresented in three categories ("DNA metabolism," "regulatory function," and "unknown function") (Table 4; see Table S4 in the supplemental material).

Odds ratios (OR) were calculated to identify associations between σ^B-dependent regulation and role category. σ^B-dependent genes were overrepresented in the cellular processes role category, with an OR of 1.5 ($P = 0.0462$); the σ^B-dependent genes in this role category included several genes that are associated with pathogenesis (e.g., *inlA*, *inlB*, *inlC2*, and *inlD*). In the energy metabolism role category, σ^B-dependent genes were overrepresented, with an OR of 2.5 ($P < 0.0001$). Of the σ^B-dependent genes in the energy metabolism role category, 17 are classified in the pyruvate dehydrogenase subrole category, and 11 of these genes encode phosphotransferase system (PTS) subunits, suggesting that σ^B is important for modulating metabolism. This observation suggests that σ^B-dependent PTSs have a role in survival during stationary phase and under other environmental stress conditions. While the DNA metabolism role category was one-half as likely as other categories to contain σ^B-dependent genes ($P = 0.0263$; OR, 0.5), five of the six σ^B-dependent genes in this role category were associated with DNA replication, recombination, and repair, while one gene (lmo1361) was similar to an exodeoxyribonuclease gene, suggesting that σ^B modulates genes that may be necessary to maintain DNA integrity, particularly in stationary-phase cells.

Despite evidence that σ^B is involved in a number of transcriptional regulatory networks (11), σ^B-dependent genes were underrepresented in the regulatory functions role category ($P = 0.0361$; OR, 0.6). Of the 24 σ^B-dependent genes in this category, 15 were involved in transcriptional regulation, including genes encoding 4 GntR family transcriptional regulators (lmo0958, lmo1725, lmo2003, lmo2004) and 2 MerR family transcriptional regulators (lmo1788, lmo2593). The members of the GntR family have been characterized as global regulators of primary metabolism in a number of bacteria (8, 23, 42), and MerR-like regulators have been shown to play a role in optimizing transcription from σ^{70}-dependent promoters with atypical distances between the -35 and -10 elements (4). Finally, 9 σ^B-dependent genes were classified in the unknown

TABLE 3. Positively regulated σ^B-dependent genes present in all four *L. monocytogenes* lineages

Gene[a]	Product[b]	Lineage I		Lineage II		Lineage IIIA		Lineage IIIB	
		Fold change[c]	Adjusted P value[d]	Fold change[c]	Adjusted P value[d]	Fold change[c]	Adjusted P value[d]	Fold change[c]	Adjusted P value[d]
lmo0133	Conserved hypothetical protein	4.5	0.0003	5.1	<0.0001	9	<0.0001	14.5	<0.0001
lmo0134	Acetyltransferase, GNAT family	6.9	0.0001	5.6	<0.0001	14.3	<0.0001	7.7	<0.0001
lmo0169	Similar to a glucose uptake protein	5.5	0.0002	3.4	<0.0001	5.1	<0.0001	6.9	<0.0001
lmo0170	Conserved hypothetical protein	3.6	0.0006	3	0.0008	5.6	<0.0001	7.4	<0.0001
lmo0210	Similar to L-lactate dehydrogenase	4.5	<0.0001	2.6	<0.0001	3.8	0.0001	4.6	0.0058
lmo0211	Similar to *B. subtilis* general stress protein	1.7	0.0335	1.5	0.0001	2.1	0.0021	2.4	<0.0001
lmo0405	Phosphate transporter family protein	2.7	0.0410	1.7	0.0106	2.1	0.0053	2.1	0.0036
lmo0433	Internalin A	3.6	0.0004	3.1	<0.0001	4.8	<0.0001	7	<0.0001
lmo0515	Conserved hypothetical protein	3.3	0.0116	3.4	0.0002	3.5	<0.0001	5.4	<0.0001
lmo0539	Similar to tagatose-1,6-diphosphate aldolase	14.4	<0.0001	7.5	<0.0001	19.5	<0.0001	24.2	<0.0001
lmo0555	Similar to di-tripeptide transporter	7.2	0.0002	4.1	<0.0001	7.1	<0.0001	9.3	<0.0001
lmo0593	Similar to transport proteins (formate)	6	0.0032	5.7	<0.0001	9	0.0003	18.2	<0.0001
lmo0596	Similar to unknown proteins	14.2	0.0001	22.7	<0.0001	33.8	<0.0001	36	<0.0001
lmo0602	Weakly similar to transcription regulator	2.6	0.0023	3.7	<0.0001	2.8	0.0003	2.5	0.0121
lmo0610	Similar to internalin proteins, putative peptidoglycan-bound protein (LPXTG motif)	1.9	0.0380	3.7	<0.0001	5.5	<0.0001	8.2	<0.0001
lmo0642	Putative membrane protein	3.4	0.0028	2	0.0002	2	0.0029	3.1	0.0005
lmo0655	Similar to phosphoprotein phosphatases	4.4	<0.0001	2.9	<0.0001	2.5	0.0014	3.1	0.0007
lmo0722	Similar to pyruvate oxidase	4.6	0.0002	5.4	<0.0001	8.8	<0.0001	3.8	0.0070
lmo0781	Similar to mannose-specific PTS component IID	10	<0.0001	15.6	<0.0001	18.2	<0.0001	25.4	<0.0001
lmo0782	Similar to mannose-specific PTS component IIC	12.9	<0.0001	13.5	<0.0001	20.3	<0.0001	22	<0.0001
lmo0783	Similar to mannose-specific PTS component IIB	6.4	<0.0001	12	<0.0001	14.8	<0.0001	18	<0.0001
lmo0784	PTS system, IIAB component, authentic frameshift	2	0.0341	5.7	<0.0001	5.5	<0.0001	6.4	<0.0001
lmo0794	Similar to *B. subtilis* YwnB protein	6.1	0.0007	12.8	<0.0001	10.3	<0.0001	25.4	<0.0001
lmo0796	Conserved hypothetical protein	1.9	0.0029	4	<0.0001	8.3	<0.0001	12.3	<0.0001
lmo0880	LysM domain protein	7.4	<0.0001	6.7	<0.0001	16.4	<0.0001	6.8	0.0006
lmo0896	Indirect negative regulation of σ^B-dependent gene expression (serine phosphatase)	1.5	0.0244	2.3	<0.0001	1.7	0.0197	2.7	0.0017
lmo0911	Unknown	2.1	0.0152	2.1	<0.0001	9.2	<0.0001	1.8	0.0200
lmo0913	Succinate semialdehyde dehydrogenase	6.6	<0.0001	13.4	<0.0001	16	<0.0001	22.3	<0.0001
lmo0937	Unknown	6.4	<0.0001	10.4	<0.0001	16.2	<0.0001	18.9	<0.0001
lmo0953	Unknown	3.2	0.0156	6.5	<0.0001	12	<0.0001	16.3	<0.0001
lmo0956	Similar to *N*-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25)	2.6	0.0073	2.1	<0.0001	4.4	<0.0001	4.3	<0.0001
lmo0957	Glucosamine-6-phosphate isomerase	2.3	0.0006	1.6	0.0027	3.1	0.0027	2.4	0.0441
lmo0994	Unknown	24.4	<0.0001	14.1	<0.0001	54.2	<0.0001	79.2	<0.0001
lmo1140	Unknown	4	0.0084	3.5	<0.0001	4.9	0.0001	4.9	<0.0001
lmo1241	Conserved hypothetical protein	2.7	0.0012	2.2	0.0002	4.2	<0.0001	5	<0.0001
lmo1295	Similar to host factor 1 protein	1.8	0.0120	3.4	<0.0001	5.4	<0.0001	8	<0.0001
lmo1375	Peptidase, M20/M25/M40 family	2.4	0.0168	3	<0.0001	2.4	0.0025	2.1	0.0381
lmo1425	Similar to betaine-carnitine-choline ABC transporter (membrane protein)	1.9	0.0062	3.4	<0.0001	3.5	<0.0001	4.7	<0.0001
lmo1428	Similar to glycine betaine-carnitine-choline ABC transporter (ATP-binding protein)	2.2	0.0008	2.9	<0.0001	2.7	0.0001	3.5	0.0004
lmo1433	Pyridine nucleotide disulfide oxidoreductase family protein	4.7	0.0006	4.2	<0.0001	3	0.0378	3.7	0.0309
lmo1602	Similar to unknown proteins	4.2	<0.0001	4.6	<0.0001	1.7	0.0417	2.7	<0.0001
lmo1605	UDP-*N*-acetylmuramate–alanine ligase	7.4	0.0001	2.2	<0.0001	2.4	0.0047	2.8	<0.0001
lmo1606	FtsK/SpoIIIE family protein	9.1	<0.0001	5.6	<0.0001	3.5	<0.0001	4.1	<0.0001
lmo1694	Similar to CDP-abequose synthase	8	<0.0001	8	<0.0001	26.2	<0.0001	1.8	0.0162
lmo1799	Putative peptidoglycan-bound protein (LPXTG motif)	2.8	0.0017	1.7	0.0002	4.8	<0.0001	4.7	<0.0001
lmo2085	Cell wall surface anchor family protein	11	<0.0001	12.2	<0.0001	14.1	0.0007	16.8	0.0067
lmo2130	Similar to unknown protein	2.1	0.0112	2.6	<0.0001	1.9	0.0104	2.3	0.0006
lmo2191	Similar to unknown proteins	2.2	0.0066	3	<0.0001	2.3	<0.0001	2.3	<0.0001

TABLE 3—Continued

Gene[a]	Product[b]	Lineage I		Lineage II		Lineage IIIA		Lineage IIIB	
		Fold change[c]	Adjusted P value[d]	Fold change[c]	Adjusted P value[d]	Fold change[c]	Adjusted P value[d]	Fold change[c]	Adjusted P value[d]
lmo2269	Unknown	4.3	0.0096	5.7	<0.0001	5.6	<0.0001	6.2	<0.0001
lmo2391	Conserved hypothetical protein similar to *B. subtilis* YhfK protein	6.2	<0.0001	9.1	<0.0001	21.6	<0.0001	28.5	<0.0001
lmo2434	Glutamate decarboxylase gamma	3.2	0.0193	2.7	<0.0001	4	0.0097	3.8	<0.0001
lmo2454	Unknown	3.6	0.0003	4.6	<0.0001	6.6	<0.0001	8.2	<0.0001
lmo2463	Similar to transport protein	3	0.0426	3.9	<0.0001	6.2	<0.0001	4.3	0.0266
lmo2485	PspC domain protein, truncated	1.9	0.0113	4.4	<0.0001	1.8	0.0006	2	0.0021
lmo2570	Putative membrane protein	5.5	<0.0001	4.5	<0.0001	7.3	<0.0001	8.9	<0.0001
lmo2571	Similar to nicotinamidase	4.4	<0.0001	5.8	<0.0001	7.3	<0.0001	8.3	<0.0001
lmo2572	Similar to chain A, dihydrofolate reductase	4.4	0.0002	1.6	0.0018	7.5	<0.0001	3.5	<0.0001
lmo2573	Alcohol dehydrogenase, zinc dependent	3.4	0.0002	4.6	<0.0001	5.6	<0.0001	7.5	<0.0001
lmo2673	Conserved hypothetical protein	6.9	0.0009	9.7	<0.0001	26	<0.0001	37.2	<0.0001
lmo2674	Similar to ribose-5-phosphate epimerase	9.8	<0.0001	3.8	<0.0001	10.1	<0.0001	13.7	<0.0001
lmo2724	Similar to unknown proteins	2.8	0.0030	2.9	<0.0001	4.7	<0.0001	4.9	<0.0001
lmo2748	Similar to *B. subtilis* stress protein YdaG	11.6	<0.0001	10	<0.0001	15.3	<0.0001	5.1	0.0063
lmo-f2365_0703	Conserved hypothetical protein	2.2	0.0277	3.3	<0.0001	2.2	0.0038	2.4	0.0186

[a] Gene names are based on the *L. monocytogenes* EGDe locus.
[b] Common names of products are based on the EGDe annotation.
[c] Changes in the transcript level in the parent strain compared to the transcript level in the $\Delta sigB$ strain. A $\geq$1.5-fold change was considered significant.
[d] An adjusted P value of <0.05 was considered significant.

role category. While σ^B-dependent genes were underrepresented in the unknown role category ($p = 0.0312$; OR, 0.5), 109 of 425 genes (25.5%) identified as σ^B-dependent genes in stationary-phase cells (irrespective of role category) are described as (i) similar to unknown, (ii) unknown, or (iii) conserved hypothetical protein according to their common name designations.

Genes were identified as exclusively σ^B-dependent genes in lineage I or lineage II, suggesting that there are differences in σ^B regulons among strains. We hypothesized that identification of differences in σ^B regulons between lineage I and II strains could provide insight into differences in pathogenic potential; therefore, we compared the σ^B regulons identified in the strains used in this study. To be considered exclusively σ^B dependent in one lineage but not in the other lineages and to reduce false-negative results, genes that were differentially expressed in the lineages were required to have a hybridization index (HI) of $\geq$95%. For 106 of the 170 lineage I genes and

TABLE 4. JCVI role categories with over- or underrepresentation for σ^B-dependent genes

JCVI role category[a]	No. of annotated genes	No. of σ^B-dependent genes[b]	Fisher's exact test P value[c]	Odds ratio[d]
Cellular processes	165	35	0.0463	1.5
Energy metabolism	380	103	<0.0001	2.3
DNA metabolism	102	8	0.0263	0.5
Regulatory functions	226	24	0.0361	0.6
Unknown function	109	9	0.0312	0.5

[a] JCVI *L. monocytogenes* EGD-e role categories.
[b] Number of σ^B-dependent genes in all lineage representatives for each role category.
[c] Fisher's exact test was used to identify significant associations between σ^B-dependent genes and the total number of genes in a role category; two-sided P values of <0.05 were considered significant.
[d] Odds ratios were calculated to characterize significant associations between σ^B-dependent genes and role category.

252 lineage II genes with higher transcript levels in the wild-type strain than in the $\Delta sigB$ mutant, the transcript levels were significantly higher in both the lineage I and II wild-type strains.

Of the genes whose transcript levels were determined to be higher in the wild-type strain than in the $\Delta sigB$ mutant strain, 55 σ^B-dependent genes were found in the lineage I strains and not in the lineage II strains using the HI criterion (Table 5). lmo1997 to lmo2004, lmo2665 to lmo2668, and lmo1538 and lmo1539 are putative operons comprised of a number of genes whose transcript levels are higher in the lineage I wild-type strain than in the $\Delta sigB$ mutant (Fig. 2). For four of the eight genes in the putative lmo1997-lmo1998-lmo1999-lmo2000-lmo2001-lmo2002-lmo2003-lmo2004 operon the transcript levels were significantly higher in the lineage I strain than in its $\Delta sigB$ mutant, while the transcript levels of none of the genes in this operon were significantly higher in the lineage II wild-type strain (Table 5 and Fig. 2A). Specifically, the transcript levels of lmo2001, lmo2000, and lmo1998 were 10.4-, 13.5-, and 5.3-fold higher, respectively, in the lineage I wild-type strain (Fig. 2A) than in its $\Delta sigB$ mutant. lmo2003 and lmo2004 encode GntR family transcriptional regulators and are upstream of six genes encoding components of a PTS. Similarly, lmo2665, lmo2666, and lmo2667 encode components of a PTS and are preceded by lmo2668 (Fig. 2B), which is similar to a BglG family transcriptional antiterminator gene. These results suggest that these PTSs are important in stationary phase in at least some strains. lmo1538 and lmo1539 (Fig. 2C) encode a glycerol kinase and a glycerol uptake facilitator, respectively, and the transcript levels of both of these genes were significantly higher in the lineage I wild-type strain than in the isogenic $\Delta sigB$ mutant. Differences in transcript levels were not significant in the lineage II strain (HI, 100%). Further, lmo2507, which encodes a protein highly similar to the cell division ATP-binding protein FtsE, was σ^B dependent in the

TABLE 5. Positively regulated σ^B-dependent genes present in lineage I but not in lineage II

Gene[a]	Product[b]	Fold change[c]		Adjusted *P* value[d]	
		Lineage I	Lineage II	Lineage I	Lineage II
lmo0130	Ser/Thr protein phosphatase family protein	2.1	1.3	0.0046	0.0764
lmo0188	Dimethyladenosine transferase (16S rRNA dimethylase)	1.7	1.1	0.0348	0.3556
lmo0217	Similar to *B. subtilis* DivIC protein	1.8	1.2	0.0167	0.0230
lmo0239	Hypothetical protein	1.9	1.1	0.0244	0.4018
lmo0315	Similar to thiamine biosynthesis protein	2.5	1.0	0.0395	0.9741
lmo0640	Similar to oxidoreductase	1.8	1.2	0.0081	0.0748
lmo0958	Transcriptional regulator, GntR family	1.7	1.4	0.0082	0.0100
lmo0959	Llm protein	1.8	1.1	0.0398	0.3736
lmo1076	*N*-Acetylmuramoyl-L-alanine amidase, family 4	1.6	1.2	0.0264	0.2002
lmo1237	Similar to glutamate racemase	1.7	1.0	0.0324	0.8658
lmo1255	PTS, trehalose-specific, IIBC component	2.7	1.3	0.0227	0.0495
lmo1293	Similar to glycerol-3-phosphate dehydrogenase	1.8	0.5	0.0200	0.0026
lmo1348	Similar to aminomethyltransferase	1.7	0.9	0.0447	0.1346
lmo1357	Acetyl-coenzyme A carboxylase, biotin carboxylase	1.9	1.3	0.0290	0.0092
lmo1389	Similar to sugar ABC transporter, ATP-binding protein	2.2	1.2	0.0020	0.0868
lmo1390	Similar to ABC transporter (permease proteins)	2.2	1.2	0.0041	0.0399
lmo1391	Putative ABC transporter, permease protein	1.8	1.2	0.0187	0.2869
lmo1538	Similar to glycerol kinase	2.9	0.7	0.0001	0.0195
lmo1539	Similar to glycerol uptake facilitator	3.9	0.7	0.0000	0.0746
lmo1542	Ribosomal protein L21	1.5	1.4	0.0354	0.0355
lmo1570	Highly similar to pyruvate kinases	1.8	1.1	0.0227	0.7807
lmo1658	30S ribosomal protein S2	2.2	1.3	0.0029	0.0530
lmo1849	Similar to metal cation ABC transporter, ATP-binding protein	2.3	1.0	0.0224	0.8362
lmo1956	Similar to transcriptional regulator (Fur family)	2.0	1.0	0.0148	0.9788
lmo1998	**Similar to opine catabolism protein**	**5.3**	**0.7**	**0.0467**	**0.0471**
lmo2000	**PTS, mannose/fructose/sorbose family, IID component**	**13.5**	**0.9**	**0.0019**	**0.7744**
lmo2001	**PTS, IIC component**	**10.4**	**0.7**	**0.0089**	**0.0102**
lmo2004	**Transcriptional regulator, GntR family**	**3.8**	**0.9**	**0.0379**	**0.4411**
lmo2020	Similar to cell division initiation protein (septum placement)	2.5	1.4	0.0096	0.0014
lmo2038	UDP-*N*-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase	2.9	1.2	0.0124	0.0404
lmo2058	Similar to heme O oxygenase	2.1	1.2	0.0462	0.1411
lmo2101	Pyridoxine biosynthesis protein	2.4	1.1	0.0011	0.5643
lmo2102	Unknown	1.9	1.2	0.0066	0.0845
lmo2118	Similar to phosphoglucomutase	1.5	0.9	0.0367	0.6917
lmo2167	Metallo-beta-lactamase family protein	2.0	1.2	0.0201	0.1227
lmo2208	Hydrolase, haloacid dehalogenase-like family	2.0	1.0	0.0153	0.9761
lmo2216	Similar to histidine triad (HIT) protein	1.7	1.3	0.0444	0.0018
lmo2217	Similar to unknown protein	1.5	1.4	0.0329	0.0010
lmo2223	Conserved hypothetical protein	2.0	1.4	0.0136	0.0056
lmo2232	CBS domain protein	2.5	1.2	0.0123	0.1146
lmo2240	Similar to ABC transporter (ATP-binding protein)	1.7	1.1	0.0137	0.4621
lmo2397	Similar to NifU protein	1.8	1.2	0.0244	0.3112
lmo2415	Similar to ABC transporter, ATP-binding protein	2.0	1.0	0.0254	0.8350
lmo2507	Highly similar to the cell division ATP-binding protein FtsE	2.7	1.3	0.0290	0.0181
lmo2547	Highly similar to homoserine dehydrogenase	1.5	0.9	0.0341	0.6523
lmo2633	Ribosomal protein S10	1.6	1.0	0.0187	0.8983
lmo2660	Similar to transketolase	1.7	0.9	0.0305	0.1802
lmo2664	Similar to sorbitol dehydrogenase	2.2	1.1	0.0027	0.7538
lmo2665	**Similar to PTS galactitol-specific enzyme IIC component**	**2.9**	**1.0**	**0.0064**	**0.9200**
lmo2666	**Similar to PTS galactitol-specific enzyme IIB component**	**2.9**	**0.7**	**0.0004**	**0.0005**
lmo2667	**Similar to PTS galactitol-specific enzyme IIA component**	**2.3**	**0.8**	**0.0015**	**0.0316**
lmo2668	**Similar to transcriptional antiterminator (BglG family)**	**2.2**	**0.7**	**0.0014**	**0.0058**
lmo2758	Similar to inosine monophosphate dehydrogenase	1.7	1.1	0.0239	0.5999
lmo2791	Partition protein, ParA homolog	1.9	1.0	0.0260	0.7694
lmoh7858_0080.5	Hypothetical protein	3.2	1.0	0.0002	0.9174

[a] Gene names are based on the *L. monocytogenes* EGDe locus. Bold type indicates putative operons.
[b] Common names of the products are based on the EGDe annotation.
[c] Changes in the transcript level in the parent strain compared to the transcript level in the Δ*sigB* strain. A ≥1.5-fold change was considered significant.
[d] An adjusted *P* value of <0.05 was considered significant.

lineage I strain and not in the lineage II strain. Modulated expression of FtsE has been proposed to be used by *L. monocytogenes* (60) and other species (52) to suppress cell division under stress conditions. Finally, transcription of lmo0315,

which encodes a protein with a possible function in thiamine biosynthesis, was σ^B dependent in the lineage I strain but not in the lineage II strain.

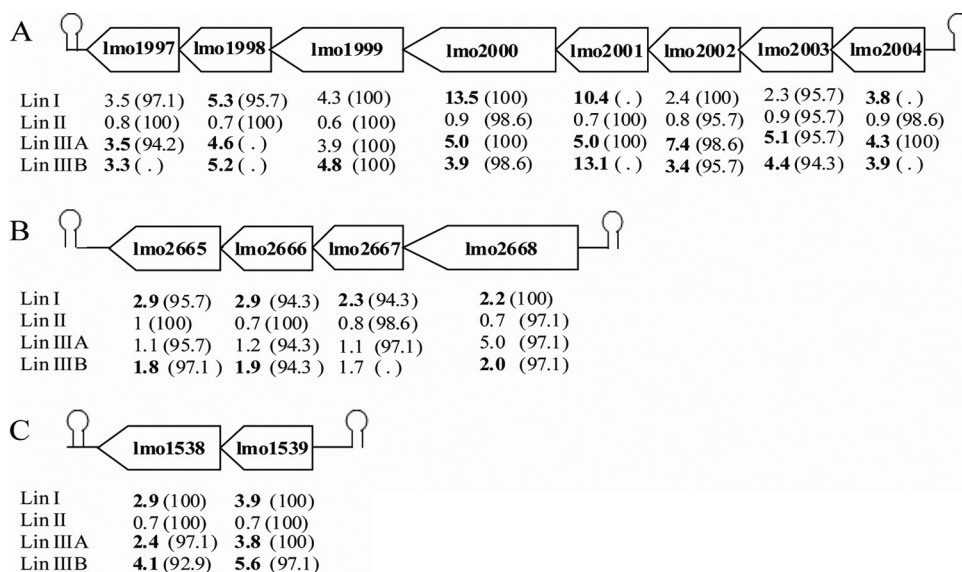The transcript levels of 108 genes that were not σ^B depen-

FIG. 2. σ[B]-dependent operons in lineage I but not in lineage II. The diagrams show three putative operons that appear to be σ[B] dependent in the lineage I wild-type strain but not in the lineage II wild-type strain. (A) Operon consisting of lmo2004, lmo2003, lmo2002, lmo2001, lmo2000, lmo1999, lmo1998, and lmo1997. Genes in this operon were flanked by Rho-independent terminators, which are indicated by stem-loop structures. The numbers below each gene indicate the fold change in the transcript level in the parent strain compared to that in the ΔsigB strain for each lineage; bold type indicates values that are significantly different (≥1.5-fold change; adjusted P value, <0.05). The values in parentheses are the hybridization index (expressed as a percentage) for each gene; some hybridization indices could not be calculated as BLAST results found no match because the genomes are not complete yet. (B) Fold changes (significantly differentially expressed genes indicated by boldface type) and hybridization indices for the σ[B]-dependent putative operon comprised of lmo2668, lmo2667, lmo2666, and lmo2665. (C) Fold changes (significantly differentially expressed genes indicated by boldface type) and hybridization indices for the σ[B]-dependent putative operon comprised of lmo1539 and lmo1538.

dent in the lineage I strain were higher in the lineage II wild-type strain than in its ΔsigB mutant (Table 6). In particular, the transcript levels of the putative lmo0398-lmo0399-lmo0400-lmo0401-lmo0402 operon, which encodes components of a PTS, a glycosyl hydrolase, and a BglG family transcriptional terminator, were at least 10.5-fold higher in the lineage II wild-type strain than in the ΔsigB mutant (Fig. 3A). There was not a significant change for any of the genes in this operon when the lineage I strain was compared to its ΔsigB mutant (HI for each gene, >95%). Other genes in this category include (i) lmo2159 and lmo2160 and (ii) lmo0043, lmo2159, and lmo2160, which are in the putative lmo2164-lmo2163-lmo2162-lmo2161-lmo2160-lmo2159-lmo2158 operon (Fig. 3B), encode an oxidoreductase and an endonuclease, respectively, and may play a role in stationary-phase survival (15). lmo0043, which was also previously identified as a σ[B]-dependent gene in the lineage II strain EGD-e (21), encodes a protein that is predicted to catalyze the conversion of L-arginine to L-citrulline and ammonia, which may aid in survival under acid stress conditions (13).

Confirmation of select differentially expressed genes in lineage I and II by TaqMan qRT-PCR. We used TaqMan qRT-PCR to confirm differences in transcript levels between wild-type and ΔsigB mutants for (i) select genes that were σ[B] dependent in all four strains and (ii) select genes that were σ[B] dependent in either lineage I or II strains but not in both lineage I and II strains, based on microarray analyses. Previous studies (33, 58) and microarray analyses in this study showed that transcription of inlA and opuCA is σ[B] dependent. Using TaqMan qRT-PCR for all four strains, we confirmed that the inlA transcript copy numbers were significantly higher in the

wild-type strains than in their isogenic ΔsigB mutants (P < 0.05) (Fig. 4; see Table S5 in the supplemental material). We confirmed that transcription of opuCA was σ[B] dependent in lineages I and II (P < 0.05) (Fig. 4). Microarray analyses suggested that σ[B]-dependent transcription of lmo0398, which is similar to a gene encoding a phosphotransferase system (PTS) enzyme IIA component, was different in different lineages. Using TaqMan qRT-PCR, we found that transcription of lmo0398 was σ[B] dependent in both lineages I and II, as shown by significantly higher transcript levels in the wild-type parent strains than in their isogenic ΔsigB mutants (P < 0.05). While microarray analyses did not identify lmo0398 as a σ[B]-dependent gene in the lineage I strain, the differences in transcript levels between the lineage I wild-type and ΔsigB mutant strains were statistically significant when we used TaqMan qRT-PCR, which is a more sensitive approach than microarray analysis and thus is able to detect smaller differences in transcript levels, as appeared to be the case with the lineage I strain. The σ[B]-dependent nature of lmo0398 is further supported by the observation that the putative σ[B] promoter region upstream of this gene (determined by RNA-Seq transcriptional start site mapping [43] and visual inspection) was 100% conserved in all strains. Lineage-specific σ[B]-dependent transcription of lmo1539, which is similar to a glycerol uptake facilitator gene, was confirmed by qRT-PCR. Specifically, the transcript levels were significantly higher in the lineage I wild-type strain (P < 0.01) than in its ΔsigB mutant, while there were no significant differences in transcript levels between the lineage II wild-type strain and its ΔsigB mutant. The coding sequence of lmo1539 was highly conserved in all strains. The differences in σ[B]-dependent transcription of lmo1539 between

TABLE 6. Genes positively regulated by σ^B in lineage II but not in lineage I

Gene[a]	Product[b]	Fold change[c]		Adjusted *P* value[d]	
		Lineage I	Lineage II	Lineage I	Lineage II
lmo0019	Conserved hypothetical protein	1.4	2.9	0.1649	0.0000
lmo0043	Similar to arginine deiminase	1.9	3.7	0.3567	0.0000
lmo0135	Oligopeptide ABC transporter, oligopeptide-binding protein	0.8	2.0	0.2953	0.0000
lmo0136	Oligopeptide ABC transporter, permease protein	0.6	1.5	0.1425	0.0000
lmo0264	Internalin C2	1.6	5.3	0.3567	0.0000
lmo0265	Peptidase, M20/M25/M40 family	1.5	8.2	0.7760	0.0000
lmo0292	Similar to heat shock protein HtrA serine protease	1.4	1.6	0.1117	0.0017
lmo0321	Similar to unknown proteins	3.5	5.3	0.1010	0.0000
lmo0342	Similar to transketolase	1.5	1.6	0.5712	0.0185
lmo0343	Similar to transaldolase	1.1	2.0	0.9782	0.0011
lmo0344	Similar to dehydrogenase/reductase	1.3	1.8	0.8013	0.0083
lmo0345	Similar to sugar phosphate isomerase	1.3	1.5	0.8159	0.0002
lmo0346	Similar to triosephosphate isomerase	1.3	1.9	0.8578	0.0062
lmo0348	Dihydroxyacetone kinase	1.2	1.8	0.9272	0.0098
lmo0398	**Similar to phosphotransferase system enzyme IIA**	**0.7**	**13.5**	**0.1649**	**0.0000**
lmo0399	**PTS IIABC component, degenerate**	**0.9**	**10.5**	**0.8049**	**0.0000**
lmo0400	**PTS IIABC component, degenerate**	**1.0**	**20.5**	**0.9922**	**0.0000**
lmo0401	**Glycosyl hydrolase, family 38**	**0.8**	**13.4**	**0.3601**	**0.0000**
lmo0402	**Similar to transcriptional antiterminator (BglG family)**	**1.5**	**15.7**	**0.1404**	**0.0000**
lmo0439	Conserved hypothetical protein	3.8	5.6	0.1291	0.0000
lmo0449	Unknown	1.7	1.5	0.4564	0.0238
lmo0584	Conserved hypothetical membrane protein	1.2	1.7	0.3709	0.0001
lmo0589	Unknown	1.7	1.7	0.1977	0.0079
lmo0590	Similar to a fusion of two types of conserved hypothetical proteins, conserved hypothetical	1.1	1.7	0.9228	0.0003
lmo0591	Membrane protein, putative	1.2	1.5	0.4479	0.0166
lmo0626	Similar to unknown protein	1.4	2.0	0.8736	0.0005
lmo0628	Unknown	2.9	3.3	0.2091	0.0000
lmo0647	Unknown	1.4	2.0	0.1241	0.0003
lmo0648	Magnesium transporter, CorA family	1.7	1.7	0.2015	0.0014
lmo0649	Transcriptional regulator, GntR family	2.7	1.7	0.1298	0.0004
lmo0650	Conserved membrane protein	1.8	1.9	0.0763	0.0000
lmo0759	Glyoxalase family protein	0.8	1.6	0.5762	0.0049
lmo0760	Unknown	0.9	1.6	0.5681	0.0016
lmo0811	Carbonic anhydrase	1.7	1.7	0.1107	0.0002
lmo0818	Cation transport ATPase, E1-E2 family	0.7	1.7	0.4525	0.0023
lmo0819	Unknown	1.5	1.7	0.0833	0.0010
lmo0928	Similar to 3-methyladenine DNA glycosylase	2.0	1.5	0.1498	0.0150
lmo0929	Sortase family protein	1.8	1.6	0.0663	0.0032
lmo0995	Membrane protein, putative	1.1	3.4	0.9752	0.0001
lmo1037	*B. subtilis* YoaT protein homolog lmo1037 (imported)	1.0	1.6	0.9873	0.0064
lmo1064	Hypothetical protein	1.4	1.7	0.7066	0.0002
lmo1072	Highly similar to pyruvate carboxylase	1.2	1.5	0.5247	0.0062
lmo1121	Unknown	1.9	2.4	0.0539	0.0000
lmo1226	Similar to transporter (to *B. subtilis* YdgH protein)	1.2	1.5	0.5341	0.0023
lmo1242	*B. subtilis* YdeI protein homolog lin1206	1.1	1.6	0.8038	0.0004
lmo1243	Uncharacterized conserved protein, PhnB family CAC3689	0.9	1.5	0.8989	0.0117
lmo1360	Highly similar to methylene tetrahydrofolate dehydrogenase and methenyl tetrahydrofolate cyclohydrolase	1.6	1.6	0.1421	0.0017
lmo1388	CD4 T-cell-stimulating antigen, lipoprotein	1.6	1.7	0.1146	0.0066
lmo1421	Similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)	1.7	2.2	0.1477	0.0003
lmo1426	Similar to glycine betaine/carnitine/choline ABC transporter (osmoprotectant-binding protein)	1.8	3.1	0.0541	0.0000
lmo1427	Glycine betaine/L-proline ABC transporter, permease protein	1.3	3.0	0.4386	0.0000
lmo1527	Similar to protein export membrane protein SecDF	1.8	1.5	0.1750	0.0006
lmo1534	L-Lactate dehydrogenase	1.5	1.6	0.1649	0.0005
lmo1571	6-Phosphofructokinase	1.4	1.5	0.3590	0.0001
lmo1580	Universal stress protein family	1.3	2.0	0.2022	0.0000
lmo1622	Conserved hypothetical protein	1.2	1.6	0.5714	0.0011
lmo1635	Conserved hypothetical protein	2.1	1.7	0.1056	0.0037
lmo1637	Putative ABC transporter, permease protein	1.4	1.6	0.2716	0.0002
lmo1666	Conserved hypothetical protein	1.4	1.6	0.7464	0.0002
lmo1681	Similar to cobalamin-independent methionine synthase	1.2	1.5	0.7274	0.0061
lmo1636	Similar to ABC transporter (ATP-binding protein)	2.0	1.9	0.0936	0.0001

TABLE 6—*Continued*

Gene[a]	Product[b]	Fold change[c]		Adjusted P value[d]	
		Lineage I	Lineage II	Lineage I	Lineage II
lmo1696	Putative membrane protein	1.2	1.5	0.4402	0.0073
lmo1698	Acetyltransferase, GNAT family	1.1	3.5	0.8156	0.0001
lmo1702	Glyoxalase family protein	1.0	1.6	0.9402	0.0369
lmo1713	Cell shape-determining protein	1.5	1.5	0.4402	0.0244
lmo1749	Similar to shikimate kinase	1.5	1.9	0.5157	0.0003
lmo1790	Metallo-beta-lactamase family protein	1.5	1.7	0.2399	0.0001
lmo1806	Highly similar to acyl carrier proteins	1.2	1.9	0.7644	0.0000
lmo1883	Chitinase	2.6	3.2	0.3466	0.0000
lmo1929	**Similar to nucleoside diphosphate kinase**	**1.1**	**1.8**	**0.9873**	**0.0001**
lmo1930	**Heptaprenyl diphosphate synthase component II (imported)**	**2.2**	**1.6**	**0.1056**	**0.0003**
lmo1931	**2-Heptaprenyl-1,4-naphthoquinone methyltransferase**	**1.9**	**1.8**	**0.0763**	**0.0002**
lmo1932	**Heptaprenyl diphosphate synthase component I, putative**	**1.8**	**1.5**	**0.1616**	**0.0272**
lmo1933	**Similar to GTP cyclohydrolase I**	**2.2**	**1.9**	**0.5761**	**0.0002**
lmo2031	Conserved hypothetical protein TIGR00044	1.2	1.6	0.4204	0.0111
lmo2033	Highly similar to cell division protein FtsA	1.4	1.6	0.2091	0.0015
lmo2159	**Oxidoreductase, Gfo/Idh/MocA family**	**1.3**	**3.4**	**0.3295**	**0.0000**
lmo2160	**AP endonuclease family 2 C terminus family**	**1.5**	**2.8**	**0.0988**	**0.0000**
lmo2161	**ThuA protein**	**1.7**	**2.2**	**0.1642**	**0.0000**
lmo2162	**Conserved hypothetical protein**	**1.6**	**2.1**	**0.1513**	**0.0001**
lmo2169	Unknown	1.4	1.7	0.2512	0.0000
lmo2196	Similar to pheromone ABC transporter (binding protein)	0.9	1.6	0.9436	0.0006
lmo2230	Similar to arsenate reductase	10.5	18.7	0.0947	0.0000
lmo2231	Similar to unknown proteins	0.9	3.1	0.9720	0.0009
lmo2368	MutT/nudix family protein	1.6	1.5	0.0577	0.0054
lmo2386	Similar to *B. subtilis* YuiD protein	1.1	1.9	0.9436	0.0000
lmo2387	Conserved hypothetical protein	1.4	4.0	0.3857	0.0000
lmo2389	Similar to NADH dehydrogenase	1.9	1.8	0.1056	0.0000
lmo2399	Similar to conserved hypothetical proteins	1.5	1.8	0.2577	0.0004
lmo2437	Conserved hypothetical protein	1.0	2.0	0.9866	0.0000
lmo2465	Conserved hypothetical protein	1.5	1.8	0.3884	0.0000
lmo2511	Similar to conserved hypothetical proteins like *B. subtilis* YvyD protein	1.2	1.9	0.5123	0.0001
lmo2520	*N*-Acylamino acid racemase	0.8	1.8	0.6061	0.0000
lmo2522	Similar to hypothetical cell wall-binding protein from *B. subtilis*	1.0	1.6	0.9825	0.0028
lmo2534	ATP synthase F0, C subunit	1.4	1.5	0.1426	0.0006
lmo2536	Highly similar to ATP synthase subunit i	1.4	1.6	0.2900	0.0009
lmo2568	Unknown	1.4	1.6	0.6145	0.0236
lmo2611	Adenylate kinase	1.0	1.5	0.9866	0.0143
lmo2638	Pyridine nucleotide-disulfide oxidoreductase family protein	1.0	1.5	0.9914	0.0025
lmo2670	Hypothetical protein	.	2.3	NA	0.0000
lmo2695	Dihydroxyacetone kinase, Dak1 subunit, putative	1.6	1.6	0.1042	0.0003
lmo2696	Dihydroxyacetone kinase family protein	1.5	1.8	0.1100	0.0001
lmo2720	Acetyl-coenzyme A synthetase	1.0	1.5	0.9951	0.0022
lmo2739	Transcriptional regulator, Sir2 family	1.7	1.5	0.0538	0.0038
lmo2741	Major facilitator family transporter	1.7	1.5	0.0698	0.0010
lmo2742	SH3 domain protein	0.7	1.5	0.1447	0.0015
lmo2832	Glycerate kinase 2	1.2	1.5	0.8437	0.0083
lmof2365_1394	Hypothetical protein	1.9	1.5	0.1498	0.0068

[a] Gene names are based on the *L. monocytogenes* EGDe locus. Bold type indicates putative operons.
[b] Common names of the products are based on the EGDe annotation.
[c] Changes in the transcript level in the parent strain compared to the transcript level in the Δ*sigB* strain. A ≥1.5-fold change was considered significant.
[d] An adjusted P value of <0.05 was considered significant.

lineage I and II strains may reflect differences in the σ^B promoter sequences identified by HMM and confirmed by RNA-Seq transcription start site mapping (43). Similarly, differences in σ^B-dependent transcription of lmo2668, which encodes a protein similar to a BglG family transcriptional antiterminator, among lineages were found when microarray analyses were used. qRT-PCR confirmed that the transcript levels of lmo2668 were higher in the lineage I wild-type strain than in the Δ*sigB* mutant, suggesting that there is direct σ^B-dependent transcription of lmo2668. Interestingly, the transcript levels were significantly higher in the lineage II Δ*sigB* mutant than in the parent strain ($P = 0.0104$), supporting the hypothesis that lmo2668 is not positively regulated by σ^B in this lineage II strain and may be indirectly downregulated by σ^B. Similar to the findings for lmo1539, differences in σ^B-dependent transcription among strains belonging to different lineages may reflect a single-nucleotide polymorphism (SNP) in the putative σ^B promoters. This preliminary evidence suggests that diversification of σ^B promoter sequences among lineages may modulate transcription of some genes in the σ^B regulon, which may affect stress response systems in *L. monocytogenes* strains.
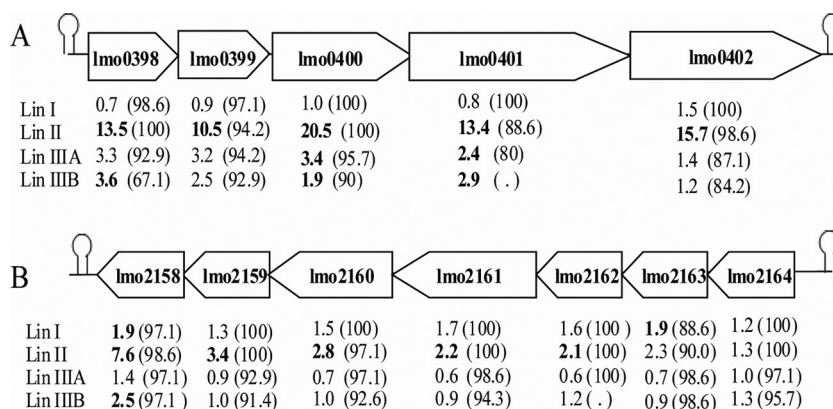
FIG. 3. σ^B-dependent operons in lineage II but not in lineage I. The diagrams show two putative operons that appear to be σ^B dependent in the lineage II wild-type strain but not in the lineage I wild-type strain. (A) Operon consisting of lmo0398, lmo0399, lmo0400, lmo0401, and lmo0402. Genes in this operon were flanked by Rho-independent terminators, which are indicated by stem-loop structures. The numbers below each gene indicate the fold change in the transcript level in the parent strain compared to that in the $\Delta sigB$ strain for each lineage; bold type indicates values that are significantly different ($\geq$1.5-fold change; adjusted P value, <0.05). The values in parentheses are the hybridization index (expressed as a percentage) for each strain; some hybridization indices could not be calculated as BLAST results found no match because the genomes are not complete yet. (B) Fold changes (significantly differentially expressed genes indicated by boldface type) and hybridization indices for the σ^B-dependent putative operon comprised of lmo2164, lmo2163, lmo2162, lmo2161, lmo2160, lmo2159, and lmo2158.

Guinea pig listeriosis infection model. We used the guinea pig intragastric infection model to determine if σ^B contributed to virulence in strains belonging to different *L. monocytogenes* lineages. The numbers of bacteria (in log CFU/g) were determined for five organs (brain, liver, mesenteric lymph nodes, spleen, and small intestine) harvested from each animal that was intragastrically inoculated with an *L. monocytogenes* mutant or wild-type strain at 72 h postinfection (Fig. 5); three animals were inoculated with each strain. At 72 h after intragastric inoculation, the numbers of the $\Delta sigB$ mutant bacteria in the organs tested were generally lower than the numbers of the corresponding parent strain bacteria (Fig. 5). Significantly lower numbers of $\Delta sigB$ strain bacteria than of parent strain bacteria were recovered for lineage I (liver and small intestine [$P < 0.05$ for both]), lineage II (spleen [$P < 0.05$]), lineage IIIA (liver and spleen [$P < 0.01$ for both]), and lineage IIIB (small intestine [$P < 0.01$], as well as liver and mesenteric lymph nodes [$P < 0.001$ for both]). *L. monocytogenes* was not recovered from the brain of any inoculated animal, consistent with the results of a previous study of guinea pigs (39), possibly indicating that spread to the brain may occur only at later times after inoculation. While the differences between the recovery of the wild-type strain and the recovery of the $\Delta sigB$ mutant from organs in this study are not as large as those reported in a previous study (17), the variation is likely attributable to (i) our use of fewer replicates for each comparison in an effort to reduce animal usage, which resulted in a decrease in the power to detect differences, and (ii) the inherent variation in a complex biological system. Overall, σ^B contributed to virulence in all *L. monocytogenes* strains tested.

Significantly smaller numbers of $\Delta sigB$ mutant cells than of wild-type cells were recovered from feces at 72 h postinoculation for the lineage I ($P = 0.0163$), II ($P = 0.0163$), and IIIA ($P = 0.0472$) strains (Table 7). Fewer lineage IIIB $\Delta sigB$ cells than lineage IIIB wild-type cells were recovered from feces, but the difference was not statistically significant ($P = 0.0532$) (Table 7). The reduced numbers of $\Delta sigB$ cells in fecal material

suggest that strains lacking σ^B may have a decreased ability to (i) attach to intestinal epithelial cells and/or (ii) survive in the intestinal tract.

Finally, we tested whether there was a significant difference at 72 h postinoculation between the weights of animals that had been infected with the wild-type strains and the weights of animals that had been infected with the $\Delta sigB$ strains. The weight at 72 h postinoculation was expressed as a percentage of the weight of the animal at the time of infection, which was defined as 100%. While there were no significant differences between the weights of animals inoculated with the wild-type strain and the weights of animals inoculated with the $\Delta sigB$ strain for lineage I ($P = 0.2129$) or lineage II ($P = 0.1230$), animals inoculated with the wild-type lineage IIIA ($P = 0.0465$) and lineage IIIB ($P = 0.0305$) strains weighed significantly less than animals inoculated with the corresponding isogenic $\Delta sigB$ strains (Table 7).

DISCUSSION

In this study, we used phenotypic and transcriptomic approaches to characterize contributions of σ^B to the stress response and virulence in *L. monocytogenes* lineage I, II, IIIA, and IIIB strains. The data generated using these approaches showed that (i) the contributions of σ^B to acid and oxidative stress resistance are different in different strains; (ii) the σ^B-dependent genes in *L. monocytogenes* include a pan-regulon consisting of approximately 400 genes that are σ^B dependent in at least one strain and a core regulon consisting of at least 60 genes that are σ^B dependent in all of the strains characterized here, and (iii) while σ^B contributes to *in vitro* intestinal epithelial cell invasion only in some strains, it contributes to virulence in the guinea pig in all *L. monocytogenes* strains tested, supporting the hypothesis that σ^B makes strain-specific contributions to gene regulation in *L. monocytogenes* and universal contributions to virulence.
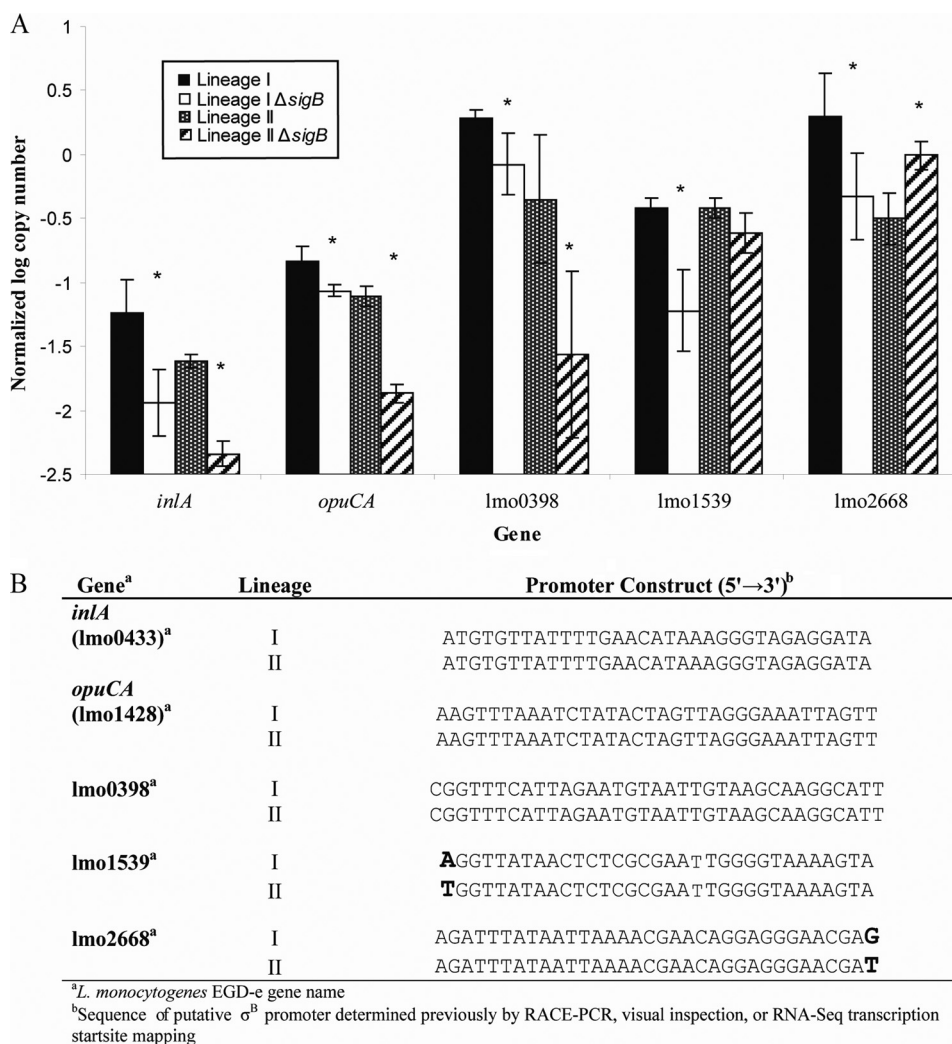
A

B

Gene[a]	Lineage	Promoter Construct (5'→3')[b]
inlA (lmo0433)[a]	I	ATGTGTTATTTTGAACATAAAGGGTAGAGGATA
	II	ATGTGTTATTTTGAACATAAAGGGTAGAGGATA
opuCA (lmo1428)[a]	I	AAGTTTAAATCTATACTAGTTAGGGAAATTAGTT
	II	AAGTTTAAATCTATACTAGTTAGGGAAATTAGTT
lmo0398[a]	I	CGGTTTCATTAGAATGTAATTGTAAGCAAGGCATT
	II	CGGTTTCATTAGAATGTAATTGTAAGCAAGGCATT
lmo1539[a]	I	**A**GGTTATAACTCTCGCGAAT TGGGGTAAAAGTA
	II	**T**GGTTATAACTCTCGCGAAT TGGGGTAAAAGTA
lmo2668[a]	I	AGATTTATAATTAAAACGAACAGGAGGGAACGA**G**
	II	AGATTTATAATTAAAACGAACAGGAGGGAACGA**T**

[a] *L. monocytogenes* EGD-e gene name
[b] Sequence of putative σ^B promoter determined previously by RACE-PCR, visual inspection, or RNA-Seq transcription startsite mapping

FIG. 4. Confirmation of lineage I or lineage II σ^B-dependent genes by TaqMan qRT-PCR and comparison of promoter regions. (A) Normalized log copy numbers of transcripts for five genes (*inlA*, *opuCA*, lmo0398, lmo1539, and lmo2668) in lineage I and II strains and their isogenic $\Delta sigB$ mutants. The bars indicate the average log-transformed levels of transcripts (normalized to *rpoB*) for three independent replicates (i.e., three RNA isolations performed on different days); the error bars indicate one standard deviation from the mean. For each gene, one-sided *t* tests were used to determine if the transcript levels in the $\Delta sigB$ mutants were significantly different from those in the wild-type strains (∗, $P < 0.05$). (B) Putative σ^B promoter sequences for five genes determined to be σ^B dependent by microarray analyses in either lineage I or lineage II or in both lineages. Bold type indicates nucleotide differences between lineage I and II putative σ^B promoter sequences. RACE, random amplification of cDNA ends.

Phenotypic characterization reveals that the contributions of σ^B to survival under stress conditions are different for strains belonging to different lineages. In this study, we tested the ability of a wild-type strain and its isogenic $\Delta sigB$ mutant belonging to each lineage to resist acid and oxidative stress in an initial effort to screen for differences in the contributions of σ^B to stress responses. We found that σ^B played a significant role in resistance to acid and oxidative stresses in the strains belonging to lineages I, II, and IIIB tested here. These results are consistent with the results of previous studies of lineage II strains that demonstrated that σ^B contributes to survival under environmental stress conditions (e.g., acid, oxidative, and energy stresses), as shown by reduced survival of an isogenic $\Delta sigB$ mutant under the same conditions (9, 15, 67). Further, σ^B plays a significant role in resistance to acid and salt stresses in *Listeria innocua* (48), as well as in resistance to acid, oxida-

tive, and ethanol stresses in *S. aureus* (6), heat, ethanol, and osmotic stresses in *Bacillus cereus* (61), and heat, osmotic, ethanol, acid, freezing, and desiccation stresses in *Bacillus subtilis* (24, 64). To our knowledge, this study is the first study demonstrating that σ^B contributes to survival under stress conditions in a lineage I strain, and importantly, our results support the hypothesis that σ^B may play an important role in the survival and transmission of the *L. monocytogenes* strains that are the most significant risk for human health (i.e., lineage I strains).

We found that σ^B played a limited role in resistance of the *L. monocytogenes* lineage IIIA strain to acid and oxidative stresses. Therefore, despite significant evidence that there was σ^B-dependent differential gene expression and virulence and despite the absence of premature stop codons in *rsb* genes in the lmo0889-lmo0888-lmo0887-lmo0896 operon, which encode
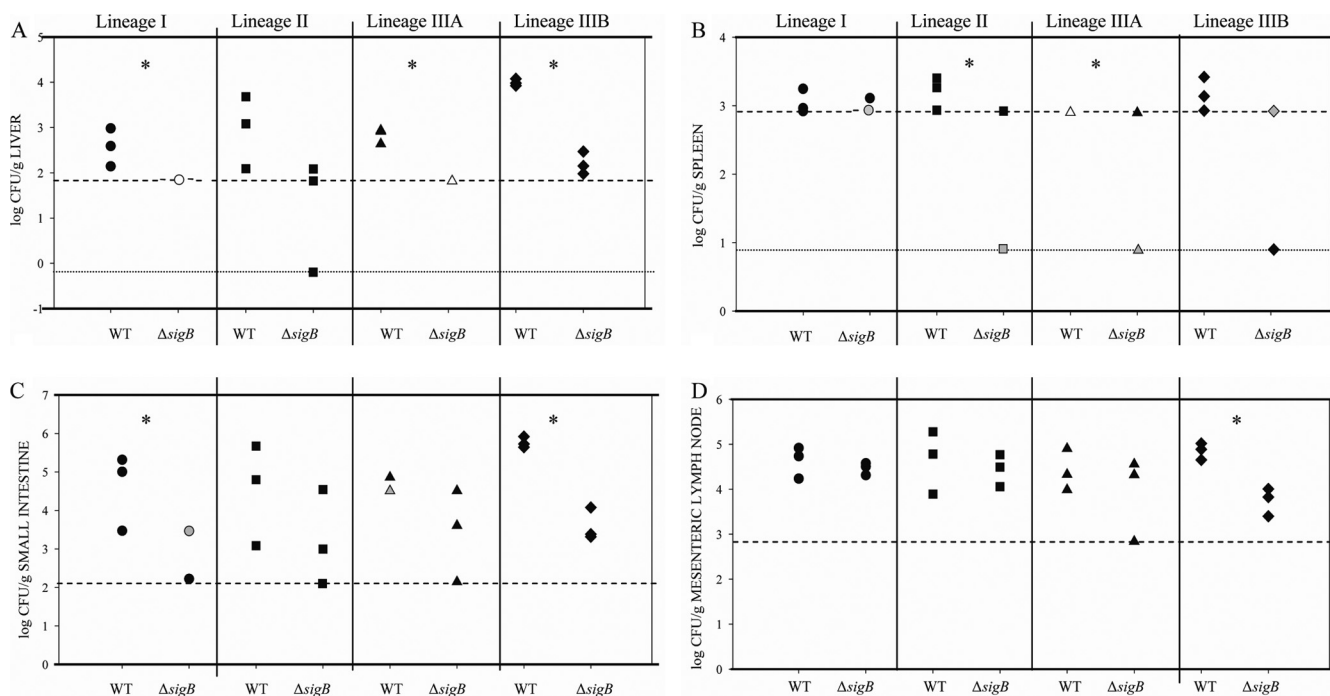
FIG. 5. Log CFU/g *L. monocytogenes* recovered from organs: scatter plots of *L. monocytogenes* recovered from the organs of guinea pigs at 72 h after intragastric inoculation. The strains (a wild-type strain [WT] and the corresponding Δ*sigB* strain belonging to each lineage) are indicated on the *x* axis. The numbers of bacteria in the liver (A), spleen (B), small intestine (C), and mesenteric lymph nodes (D) are indicated on the *y* axis. Data were obtained from three guinea pigs that were intragastrically inoculated with each strain. Black symbols indicate a single data point, gray symbols indicate two overlapping data points, and open symbols indicate three overlapping data points. The detection limits, which were different for different organs due to different organ weights, are indicated by horizontal dashed and dotted lines in each panel. The dashed horizontal lines indicate the detection limit for direct plating; the dotted lines in panels A and B indicate the detection limits for enrichment procedures. The data reported for the plating detection limit were positive for *L. monocytogenes* after enrichment, but the bacterial counts were below the counts detectable by standard plate counting. For the data reported for the enrichment detection limit there was no recovery of *L. monocytogenes* after enrichment. An asterisk indicates that significantly ($P < 0.05$, one-sided *t* test) higher numbers of bacteria were recovered from organs from animals inoculated with the wild-type strain than from organs from animals inoculated with the isogenic Δ*sigB* mutant.

regulators of σ^B (data not shown), the *L. monocytogenes* lineage IIIA strain showed a limited σ^B-dependent phenotype under the conditions tested. The apparent σ^B-independent phenotype of the lineage IIIA strain (serotype 4c) observed under these conditions is consistent with the results of a previous study (37) which identified differences in the contributions of σ^B to environmental stress between serotype 1/2a and

4c strains. Specifically, σ^B played a minimal role in resistance to acid, oxidative, and heat stresses in the serotype 4c strain as there were no significant differences in survival between the wild-type strain and its isogenic Δ*sigB* mutant (37). Phenotypic diversification of response regulation has also been observed in other bacteria, including *Escherichia coli* (41) and *Salmonella enterica* serovar Typhimurium (27).

TABLE 7. Guinea pig weight and fecal shedding of *L. monocytogenes* at 72 h postinoculation

Lineage	Weight (%)			Fecal shedding (log CFU/g)		
	Wild type[a]	Δ*sigB* mutant[b]	*P* value[c]	Wild type[d]	Δ*sigB* mutant[e]	*P* value[f]
I	101.1 ± 8.9	106.5 ± 5.6	0.2129	5.3 ± 0.9	3.1 ± 0.7	0.0163
II	99.0 ± 8.4	106.6 ± 4.9	0.123	4.8 ± 1.5	0.0 ± 0.0	0.0163
IIIA	99.4 ± 3.7	105.7 ± 3.3	0.0465	6.1 ± 1.4	2.6 ± 2.4	0.0472
IIIB	91.6 ± 2.6	105.9 ± 9.2	0.0305	6.1 ± 0.1	3.3 ± 2.9	0.0532

[a] Average weight (± standard deviation) of guinea pig infected with wild-type strain at 72 h postinoculation (after euthanasia), expressed as a percentage of the weight at zero time (inoculation), which was defined as 100%.

[b] Average weight (± standard deviation) of guinea pig infected with the Δ*sigB* strain at 72 h postinoculation, expressed as a percentage of the weight at zero time, which was defined as 100%.

[c] *P* value for *t* test for the weight of guinea pigs inoculated with the wild-type strain and the weight of guinea pigs inoculated with the Δ*sigB* strain. A *P* value of <0.05 indicates that there was a significant difference in the weight of animals at 72 h postinoculation.

[d] Average fecal shedding (± standard deviation) of wild-type parent strain at 72 h postinoculation.

[e] Average fecal shedding (± standard deviation) of Δ*sigB* strain at 72 h postinoculation.

[f] *P* value for *t* test. A *P* value of <0.05 indicates that there is a significant difference in the average fecal shedding of *L. monocytogenes* between the wild-type and isogenic Δ*sigB* strains.

σ^B-dependent genes in *L. monocytogenes* include a pan-regulon consisting of approximately 400 genes and a core regulon consisting of at least 60 genes. Whole-genome microarray approaches using two well-characterized lineage II laboratory strains (EGD-e and 10403S) previously identified more than 200 σ^B-dependent genes in *L. monocytogenes* (21, 48), and up to 168 genes were reported to be positively regulated by σ^B (21, 30, 44, 48). Using four strains, one each belonging to lineages I, II, IIIA, and IIIB, we identified a total of ~400 genes as genes that are positively regulated by σ^B in at least one strain; these genes, therefore, represent an initial description of the *L. monocytogenes* σ^B pan-regulon. In comparison, the reported σ^B regulons in other Gram-positive organisms (e.g., *S. aureus* and *B. subtilis*) contain from ~100 to 150 genes (45, 46, 62); each of these regulons was determined using a single strain. Over 60 of the *L. monocytogenes* pan-regulon genes were σ^B dependent in all four strains; thus, these genes constitute the core *L. monocytogenes* σ^B regulon. The majority of the genes comprising the core σ^B regulon were also identified as genes positively regulated by σ^B in previous studies using the lineage II strains EGD-e and 10403S (21, 44, 48). While it is likely that the core σ^B regulon was underestimated in the present study due to (i) low hybridization indexes (HI) for some genes in some strains and (ii) use of a single growth condition with a single microarray platform, it is not surprising that the *L. monocytogenes* core regulon is small compared to the pan-regulon across a diverse set of strains. For example, while σ^B is conserved in many different Gram-positive organisms, the *L. monocytogenes*, *S. aureus*, *B. cereus*, and *B. subtilis* σ^B regulons share only three genes (*rsbV*, *rsbW*, and *sigB*), which are members of the *sigB* operon (62). This observation supports the notion that the σ^B regulon has evolved to perform niche-specific functions in different organisms.

Overall, σ^B-dependent genes in Gram-positive bacteria contribute to a wide variety of functions, including energy metabolism, regulatory functions, and pathogenesis (21, 22, 45, 48, 62). In this study, *L. monocytogenes* σ^B-dependent genes were significantly overrepresented in the cellular processes and energy metabolism *L. monocytogenes* JCVI role categories, which indicates that σ^B contributes to multiple mechanisms important for cell function, survival, and pathogenesis. Likewise, the σ^B regulon in *L. monocytogenes* EGD-e included genes encoding proteins involved in a wide range of metabolic functions, genes encoding general stress proteins, and genes having unknown functions (21). While the regulatory functions role category was underrepresented among the σ^B-dependent genes identified here, the σ^B-dependent genes in this role category encode potentially important transcriptional regulators and include genes encoding GntR family and MerR-like regulators. These proteins act as global regulators of primary metabolism (8, 23, 42) and contribute to optimization of σ^70-dependent promoters in Gram-negative bacteria (4), respectively. Combined with the results of previous studies that have shown that there are regulatory networks for σ^B and PrfA in *L. monocytogenes* (19, 59), as well as for σ^B and other regulators in *L. monocytogenes* (11) and in other bacteria (7, 57), our findings contribute to an emerging body of evidence supporting the hypothesis that σ^B plays an important role in regulatory networks and contributes to the stress response and virulence

across *L. monocytogenes* lineages, even though the specific role of σ^B is clearly different in different strains.

L. monocytogenes strains classified in different lineages appear to differ in the likelihood that they are associated with human illnesses and in overall pathogenic potential (20, 68). Interestingly, a number of differences between σ^B regulons were observed in the lineage I and II strains examined in this study. For example, genes encoding a glycerol kinase and glycerol uptake facilitator were σ^B dependent in the lineage I strain but not in the lineage II strain. The putative σ^B-dependent promoters for lmo1539, which encodes the glycerol uptake facilitator, were different in the lineage I and II strains, which may be responsible for the differences in σ^B-dependent transcription between these strains. A recent study demonstrated that *L. monocytogenes* growth in the presence of glycerol results in upregulation of a number of PrfA-controlled genes (28, 68). Furthermore, lmo0315, which encodes a protein with a possible role in thiamine biosynthesis, was σ^B dependent in the lineage I strain but not in the lineage II strain. A recent study (51) concluded that *L. monocytogenes* employs thiamine uptake and biosynthesis of thiamine precursors to grow in host cytoplasm. Therefore, based on these findings in combination with our data, it is tempting to speculate that differences in glycerol-dependent regulation of virulence genes and a potentially enhanced ability to grow intracellularly may affect the pathogenic potential of strains that are classified in different lineages.

Transcriptional profiles have been reported for wild-type strains representing *L. monocytogenes* lineages I and II (53), and evidence has indicated that the transcript levels of a number of σ^B-dependent genes were higher in the lineage II strains than in the lineage I strains (based on lineage designations that are consistent with the first description of *L. monocytogenes* lineages by Piffaretti et al. in 1989 [47]). Of the σ^B-related genes reported to be expressed more highly in lineage II (53), five were found here to be σ^B dependent in lineage II strains but not in lineage I strains, including two genes encoding ABC transport system components (lmo1421 and lmo1426), further supporting the hypothesis that there has been diversification of the σ^B regulon in lineage I and II strains. As a considerable number of genes that were found to be σ^B dependent in either lineage I or II strains encode proteins with unknown functions or hypothetical proteins, further investigation of these genes and their regulation may provide insight into niche adaption and phenotypic differences among *L. monocytogenes* strains and lineages. The ability of microarray analyses to sensitively detect differences in the contributions of σ^B to expression of various genes in the σ^B core regulon undoubtedly affects relative identification of genes as σ^B-dependent genes in strains and lineages. Emerging transcriptome profiling techniques, such as RNA-Seq, should provide powerful and sensitive new tools for regulon identification.

While σ^B does not contribute to *in vitro* intestinal epithelial cell invasion in all *L. monocytogenes* strains, it contributes to virulence in the guinea pig in all strains tested. Previous studies (17, 32) demonstrated that in *L. monocytogenes* 10403S (a lineage II strain), σ^B is important for invasion of Caco-2 cells, as shown by the reduced recovery of Δ*sigB* mutant cells compared to the recovery of cells of the parent strain. Kim et al. (33) demonstrated that σ^B contributed significantly to *L. monocytogenes* 10403S invasion of human enterocytes and hepatocytes, predominantly through InlA- and InlB-mediated

pathways as both *inlA* and *inlB* are transcribed from σ^B-dependent promoters (32, 33). In the present study, wild-type lineage I, II, and IIIB strains had significantly higher invasion efficiencies than their isogenic $\Delta sigB$ mutants, indicating that σ^B contributes to invasion of Caco-2 cells. Interestingly, we found that σ^B did not contribute to the efficiency of invasion of Caco-2 cells in the lineage IIIA strain despite the fact that (i) the *L. monocytogenes* lineage IIIA strain has a full-length *inlA* transcript and (ii) microarray analyses identified over 200 genes whose transcript levels were higher in the wild-type strain than in the $\Delta sigB$ mutant, indicating that there is a large σ^B regulon that includes well-characterized σ^B-dependent genes important for virulence (e.g., *bsh*, *opuCA*, *inlA*, and *gadB* [30]). The possible explanations for this finding include (i) the possibility that σ^B activity is regulated differently in the lineage IIIA strain than in other strains and (ii) the possibility that genes critical for invasion efficiency, acid resistance, and oxidative stress resistance show limited or no σ^B dependence in the lineage IIIA strain tested, even though our microarray analyses did not identify clear candidate genes that might explain these differences. It is tempting to speculate that the phenotypic diversification of the lineage IIIA strain may be attributable in part to the selective pressures of its environment. In recent work Kvitek et al. (34) found significant phenotypic variation in stress sensitivity and gene expression among *Saccharomyces cerevisiae* strains isolated from distinct environments but similar phenotypic and transcriptional profiles for strains isolated from similar niches.

We used the guinea pig listeriosis infection model as an *in vivo* system to quantify and characterize the contributions of σ^B to virulence in *L. monocytogenes* strains from lineages I, II, IIIA, and IIIB. All *L. monocytogenes* $\Delta sigB$ strains had reduced virulence in the guinea pig listeriosis infection model, as shown by the reduced recovery of the $\Delta sigB$ strains compared to the recovery of their isogenic parents from at least one organ. A recent study by Ollinger et al. (44) provided a possible mechanistic explanation for the importance of σ^B in the guinea pig listeriosis infection model. σ^B regulates activity of the major *L. monocytogenes* virulence regulator PrfA, ranging from transcriptional activation of *prfA* at the $P2_{prfA}$ promoter to post-transcriptional downregulation of PrfA regulon expression under conditions when PrfA is highly active (e.g., under intracellular conditions). This rheostat-like role for σ^B in controlling PrfA activity appears to be important for the tightly regulated control of virulence gene expression in *L. monocytogenes*. Thus, contributions of σ^B to virulence gene expression in the lineage IIIA strain appear to be essential for full virulence of this strain. While σ^B contributes to invasion of intestinal epithelial cells *in vitro* and survival under acid and stress conditions in some strains, it universally contributed to virulence in guinea pigs in the *L. monocytogenes* strains tested, further underscoring the central importance of contributions of σ^B to gene regulation and virulence in *L. monocytogenes*.

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