Sigma B Contributes to Listeria monocytogenes Gastrointestinal Infection but Not to Systemic Spread in the Guinea Pig Infection Model

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Contributions of the alternative sigma factor σB to Listeria monocytogenes infection were investigated using strains bearing null mutations in sigB, prfA, or inLA or in selected inLA or prfA promoter regions. The ΔP4inLA strain, which has a deletion in the σB-dependent P4inLA promoter, and the ΔsigB strain had significantly reduced invasion efficiencies relative to that of the wild-type strain in the Caco-2 human colorectal epithelial cell line, while the invasion efficiency of a strain bearing a deletion in the partially σB-dependent P2prfA promoter region did not differ from that of the wild type. The virulence of the ΔsigB and ΔP4inLA strains was attenuated in intragastrically inoculated guinea pigs, with the ΔsigB strain showing greater attenuation, while the virulence capacity of the ΔP2prfA strain was similar to that of the wild-type strain, suggesting that attenuation of virulence due to the ΔsigB mutation does not result from loss of σB-dependent prfA transcription. Our results show that σB-dependent activation of inLA is important for cell invasion and gastrointestinal infection and suggest that σB-regulated genes in addition to inLA appear to contribute to gastrointestinal infection. Interestingly, the virulence of the ΔsigB strain was not attenuated in intravenously infected guinea pigs. We conclude that (i) L. monocytogenes σB plays a critical role in invasion of human host cells, (ii) σB-mediated contributions to invasion are, in part, due to direct effects on inLA transcription but not on prfA transcription, and (iii) σB plays a critical role during the gastrointestinal stage of listeriosis in the guinea pig but is not important for systemic spread of the organism.

Listeriosis results from consumption of food contaminated with Listeria monocytogenes (40). To cause a human food-borne infection, L. monocytogenes must survive conditions encountered in the oral cavity, the stomach, and the intestine, which include exposure to lysozyme activity, gastric acidity, and bile salts (16). L. monocytogenes can invade the epithelial cells of the small intestine, then spread into mesenteric lymph nodes and disseminate systemically in the blood. Listeriosis is a relatively rare disease among healthy individuals, at least in part because the majority of L. monocytogenes organisms that enter the bloodstream are captured by the Kupffer cells in the liver and are killed by neutrophils (18). Listeriosis occurs predominantly among the immunocompromised, the elderly, and pregnant women and their fetuses (33). In susceptible individuals, L. monocytogenes can cross the blood-brain barrier to cause central nervous system infections and the placental barrier to cause fetal infections (48). L. monocytogenes cells that escape the antimicrobial activity of the neutrophils can enter hepatocytes or endothelial cells or can be disseminated further to other tissues. Following invasion of nonphagocytic host cells, L. monocytogenes can escape from the host cell vacuole, then replicate intracellularly and spread from cell to cell (6, 15, 23, 46).

Over the past 2 decades, numerous studies have provided insight into the mechanisms of L. monocytogenes cellular pathogenesis through identification and characterization of the virulence genes located in the major virulence gene cluster (i.e., prfA, plcA, hly, mpl, actA, and plcB). The autoregulatory positive regulatory factor A (PrfA) regulates expression of the other genes within the cluster, as well as expression of additional virulence genes elsewhere on the chromosome (e.g., inLA) (34, 35). Virulence genes required for host cell invasion, vacuolar escape, and cell-to-cell spread have been identified (17). In contrast, our knowledge of the mechanisms used by L. monocytogenes to survive during the preinvasion, gastrointestinal stage of infection is more limited. This lack of information is due, at least in part, to limitations in the experimental models that have been used to study food-borne listeriosis. Historically, murine infection experiments have been used to examine L. monocytogenes virulence characteristics (39), the listerial infection process, and interactions between L. monocytogenes and the immune system (32). However, increasing evidence suggests that the murine model does not appropriately approximate human L. monocytogenes gastrointestinal infection processes (27, 30). Specifically, murine E-cadherin, which differs in amino acid sequence from human E-cadherin, does not interact effectively with L. monocytogenes InLA, an 800-amino-acid protein that mediates bacterial entry into epithelial cells through its interactions with E-cadherin (28). As a consequence, mice show limited susceptibility to intragastric L. monocytogenes infection (30). The guinea pig E-cadherin isoform interacts with InLA; therefore, the guinea pig has emerged as a more appropriate model than the mouse for study of gastrointestinal L. monocytogenes infection (27, 30). To illustrate, use of the guinea pig model allowed Lecuit et al. (30) to define the interaction of L. monocytogenes internalin A with E-cadherin expressed on enterocytes, a critical early step in the pathogenesis of listeriosis.

The general stress-responsive alternative sigma factor σB contributes to bacterial survival under conditions of in vitro environmental stress in multiple gram-positive microbes (3,
19). In _L. monocytogenes_, σ^B contributions to survival following exposure to bile salts as well as to acidic, oxidative, and carbon starvation conditions (2, 11, 12, 47). Recent microarray gene expression studies with _L. monocytogenes_ have provided evidence that, in addition to stress response genes, σ^B also regulates expression of virulence genes (24). A number of σ^B-dependant genes contribute to _L. monocytogenes_ gastrointestinal survival or infection, including _bsh_, which encodes a bile salt hydrolase, _inlA_, and _opuC_, which encodes an osmolyte transporter (2, 9, 13, 44, 45). In addition, σ^B also contributes to transcriptional activation of _prfA_ at the P2^-47 region of _L. monocytogenes_. Preliminary invasion studies using the human colorectal cell line Caco-2 showed that _L. monocytogenes_ strains lacking _sigB_ have reduced invasion capacities as well as greatly reduced levels of internalin A, as determined by Western blot analysis and real-time PCR (25, 26). To further analyze the contributions of σ^B to _L. monocytogenes_ virulence and invasion, we characterized _L. monocytogenes_ strains bearing null mutations in _sigB_, _prfA_, and _inlA_ as well as selected σ^B-dependent (P2^-47, P4^-24) and σ^B-independent (P1-3^-47, 336^-297) promoters, for relative capacities to invade Caco-2 cells and for virulence in a guinea pig model.

### MATERIALS AND METHODS

**_L. monocytogenes_ growth conditions.** All _L. monocytogenes_ strains were cultivated in brain heart infusion broth (BHI; Difco, Detroit, MI) with shaking (210 rpm) at 37°C to optimize PrfA-mediated gene expression (22). To ensure standardized doses for guinea pig infections and Caco-2 tissue culture invasion assays, _L. monocytogenes_ strains were grown to stationary phase as described by Kazmierczak et al. (24), frozen at −80°C, and enumerated 48 h after freezing by thawing one aliquot and plating serial dilutions onto BHI agar plates, which were then incubated at 30°C for 48 h. On the day of infection, aliquots were thawed, diluted to the desired bacterial concentration, and used for guinea pig infections or Caco-2 invasion studies.

**Bacterial strains and construction of deletion mutants.** The _L. monocytogenes_ mutant strains used in this study (Table 1) were all derived from 10403S (5). The isogenic ΔsigB, ΔinlA, ΔP4^-24, and ΔprfA mutant strains have been described previously. Table 2. The ΔP4^-24_ and ΔP1-3^-47_ deletion mutations were created in the _Escherichia coli-L. monocytogenes_ shuttle vector pKSV7 (7) by SOE (splicing by overlap extension) PCR (20) and were introduced into _L. monocytogenes_ 10403S by allelic exchange mutagenesis as previously described (47). SOE PCR primers used for generation of the ΔP4^-24_ and ΔP1-3^-47_ deletion alleles are listed in Table 2. The ΔP1-3^-47_ mutants have 72-bp and 15-bp deletions, respectively, within the respective promoter regions (Fig. 1). Successful creation of the ΔP1-3^-47_ and ΔP4^-24_ mutant strains was confirmed by PCR amplification and subsequent DNA sequencing of the targeted region in each strain. The mutations in the ΔsigB, ΔinlA, and ΔprfA strains are in-frame deletions in the respective genes, as previously described (Table 1). Similarly, the promoter mutant strains were created by deletion of specific sequences in the targeted promoter regions (Fig. 1). The genetic manipulations used to create the mutant strains did not leave residual foreign DNA in the resulting strains.

**Tissue culture invasion assays.** The tumor-derived human colorectal epithelial cell line Caco-2 (ATCC HTB-37) was grown at 37°C under a 5% CO2 atmosphere with a relative humidity of 80 to 95%. Caco-2 cells were cultured in Dulbecco’s minimal essential medium (DMEM) with Earle’s salts (Gibco; Gaithersburg, MD) containing 20% fetal bovine serum (HyClone, Salt Lake City, Utah), 1% nonessential amino acids, 1% sodium pyruvate, 1.5 g/liter sodium carbonate (Gibco), penicillin G (100 U/ml), and streptomycin (100 μg/ml).

Caco-2 invasion assays were performed as described by Kim et al. (25) with minor modifications. Briefly, 5.0 × 10^5 Caco-2 cells were seeded into 24-well plates (Costar, Corning, NY) in DMEM with fetal bovine serum as described above, but without antibiotics. Caco-2 cells were grown for approximately 48 h before infection. For infection, approximately 2.0 × 10^7 _L. monocytogenes_ cells were added to Caco-2 cell monolayers.
(in 10 μl of PBS) were inoculated onto the Caco-2 monolayer in each well. An additional aliquot of the L. monocytogenes inoculum was serially diluted and plated onto BHI agar to ensure that the number of bacteria that had been used for infection was as expected, based on previous enumeration of the frozen aliquots. At 30 min postinfection (p.i.), the medium in each well was aspirated and the Caco-2 cells were washed three times with 1 ml sterile PBS to remove any unattached, extracellular L. monocytogenes. Subsequently, 1 ml of prewarmed fresh Caco-2 medium without antibiotics was added. At 45 min postinfection, the medium was aspirated and 1 ml of fresh Caco-2 medium containing 150 μg/ml gentamicin (Gibco) was added to kill remaining extracellular L. monocytogenes. At 90 min postinfection, the medium was aspirated and the wells were washed three times with sterile PBS. Caco-2 cells were then lysed by the addition of 500 μl of ice-cold sterile distilled water. The resulting suspension was serially diluted, plated onto BHI agar, and incubated (48 h at 30°C) for bacterial enumeration. Invasion efficiency is reported as the number of bacteria recovered from each well following Caco-2 cell lysis divided by the number of bacteria that had been used for inoculation and expressed as a percentage.

**Animal care and housing conditions.** Animal protocols were approved by the Institutional Animal Care and Use Committee (protocol 02-60) prior to initiation of the experiments. Male Hartley guinea pigs were purchased from Elm Hill (Chelmsford, MA). Animals were housed individually in autoclaved, transparent microisolator cages with cedar shaving bedding in a facility at Cornell University accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Housing of animals in individual cages enabled monitoring of L. monocytogenes shedding in each animal’s fecal material. Animals were offered guinea pig feed (Purina, St Louis, MO) and autoclaved water ad libitum. Food, water, bedding, and cages were changed each day, and overall animal health, activity, and food and water consumption were monitored and recorded daily.

**Intragastric infection of guinea pigs.** Intragastric infections of guinea pigs were performed as described previously (30) with the following modifications. Feed was withheld from animals for 12 h prior to infection. Animals were anesthetized with isoflurane administered via inhalation, using oxygen as a carrier gas (1). The animals were exposed to isoflurane at an initial concentration of 0.5%, which was increased every 30 s in 0.5% increments until the concentration reached 2.5%. Isoflurane was administered for an additional 35 s after each animal lost consciousness. L. monocytogenes was inoculated intragastrically using a syringe attached to a disposable 20-μm flexible rubber catheter (catalog no. 3533.05; Viargon, Norristown, PA). To neutralize stomach pH, 1 ml of PBS containing 125 mg calcium carbonate (pH 7.4) was delivered prior to administration of L. monocytogenes (1.5 × 10⁸ CFU in 1 ml PBS) was administered intragastrically into the saphenous vein. The doses administered were 10-fold lower than the previously determined 50% lethal dose (5 × 10⁷) for intravenously infected guinea pigs (30).

**Organ harvest and enumeration of L. monocytogenes.** As shown by Lecuit et al., (30) and as confirmed in our preliminary experiments (data not shown), L. monocytogenes numbers were highest in the liver, small intestine, and mesenteric lymph nodes at 72 h post-intragastric infection; therefore, both intragastrically and intravenously infected animals were euthanized for organ harvest at 72 h postinfection. Animals were euthanized via CO2 inhalation. The mesenteric lymph nodes, liver, spleen, and small intestine (a 20-cm central portion, immediately proximal to the cecum) were aseptically removed, placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI), and held on ice until processing. For bacterial enumeration, the liver, spleen, and mesenteric lymph nodes were weighed, placed in 30 ml of PBS, and homogenized in a sterilized blending unit (Semi-micro; Eberbach, Ann Arbor, MI) for 30 s. After homogenization, the organ suspension was serially diluted and plated onto BHI agar. BHI plates were incubated at 30°C for 48 h prior to enumeration. L. monocytogenes identification was confirmed by streaking representative colonies onto L. monocytogenes plating medium (LMPM agar; Biosynth Biochemica & Synthetica, Napeville, IL), which is selective and differential for L. monocytogenes.

The small intestine was processed to avoid recovering extracellular L. monocytogenes that might have been present in the lumen of the intestine (30). Briefly, the small intestine was opened with a sterile scalpel, the intestinal contents were removed, and the intestine was rinsed three times with 20 ml of PBS. To kill extracellular bacteria, the intestine was then incubated for 90 min at 20°C in 20 ml of DMEM (Gibco) containing 100 μg/ml gentamicin, as previously reported (30). The intestine was then washed three times in 20 ml of PBS, homogenized in 30 ml of PBS, serially diluted, and plated onto BHI agar for bacterial enumeration.

**FIG. 1.** Nucleotide sequences of the inlA (A) and prfA (B) promoters and locations of the deletions in the inlA and prfA promoter-null mutants. (A) Transcriptional start sites for the P1, P2, P3, and P4 promoters are indicated by stars. The inlA promoter nomenclature represents the four inlA promoters described by Lingnau et al. (31). In contrast, Dramsi et al. (8) and Stritzker et al. (43) reported only three inlA promoters, excluding the P1 promoter described by Lingnau et al. (31). Thus, the inlA P4, P3, and P2 promoter designations used here are equivalent to the inlA P3, P2, and P1 promoters described by Dramsi et al. (8) and Stritzker et al. (43). The -35 and -10 regions of the α-dependent P4 promoter (24) are boldfaced and underlined. The prfA palindromic recognition sequence preceding the inlA P3 promoter (8) is underlined and italicized. The 15- and 72-nucleotide fragments deleted in the ΔP4 and ΔP1-3null mutants, respectively, are indicated by shading. (B) DNA sequence of the previously mapped prfA promoter region (14, 38). The α-dependent promoters are italicized and overlined. Transcriptional start sites are labeled and indicated by stars. The 20-nucleotide fragment deleted in the ΔP2prfA null mutant is indicated by shading.
Enumeration of L. monocytogenes from feces. Fecal pellets from each animal were collected daily and placed in sterile Whirl-Pak bags; fecal samples were collected prior to infection on the day of infection and at 24-h intervals postinfection. Pellet weight and qualitative descriptions (e.g., pellet hardness, pellet size, moisture content) were recorded daily. Aliquots (500 mg) of fecal material were placed in sterile disposable 50-ml conical tubes containing 4.5 ml of PBS and homogenized with a sterile stirring rod and sterile tweezers. The homogenized mixture was serially diluted, and dilutions were plated on Oxford agar (Oxoid, Ogdensburg, NY). After incubation at 30°C for 48 h, colonies exhibiting Listeria spp.-like morphology were counted and recorded as L. monocytogenes. L. monocytogenes identification was confirmed on a representative subset of these colonies by plating on LMP agar.

Histopathology and immunohistochemistry. Tissues from euthanized guinea pigs were fixed in 10% buffered formalin for a minimum of 48 h. Fixed tissues were processed using a Tissue Tek VIP F. 300 (Sakura Finetek U.S.A., Inc., Torrance, CA) in preparation for paraffin embedding in a Tissue Tek embedding station (Sakura Finetek U.S.A., Inc.). Formalin-fixed, paraffin-embedded tissues were sectioned at a thickness of 6 μm, placed on glass slides, and stained with hematoxylin and eosin for microscopic evaluation.

L. monocytogenes immunohistochemistry was performed as previously described (21) with minor modifications, as follows. Briefly, formalin-fixed, paraffin-embedded tissues were sectioned to a thickness of 6 μm and deposited on Probe-On glass slides. Two slides were prepared for each organ section investigated. One slide was stained using a polyclonal antibody to L. monocytogenes (Becton Dickinson, Sparks, MD), while the second slide was stained with a nonspecific antibody. The secondary antibody was an anti-immunoglobulin G antibody. All slides were stained using the avidin-biotin system, and the chromogen was diaminobenzidine.

All slides were examined using an Olympus BX41 microscope. Photomicrographs were taken using a Q Imaging microublisher 5.0 RTV (Burnaby, British Columbia, Canada) and a 50× or 100× oil objective lens.

Statistical analyses. All analyses were performed using Statistical Analysis System (SAS) software (Cary, NC). Comparisons in which a P value of <0.05 was obtained were considered statistically significant.

Four independent Caco-2 cell invasion assays were conducted for each L. monocytogenes strain studied here. In each independent invasion assay, each L. monocytogenes strain was inoculated into three wells containing Caco-2 monolayers. Caco-2 invasion efficiency was calculated as the fraction of the initial inoculum recovered by enumerating intracellular bacteria. Invasion efficiency data did not follow a normal distribution and were thus log-transformed to satisfy a linear regression assumption. One-way analysis of variance (ANOVA) was performed using the General Linear Model procedure to compare data from three replicates. Tukey’s studentized range test was used to assess whether invasion differences existed between individual L. monocytogenes strains.

Each L. monocytogenes strain was inoculated into three different guinea pigs on two separate days (a total of six animals were intragastrically infected with each strain) with the exception of ΔprfA and ΔinlA, which were inoculated into a total of four animals. The ability of each L. monocytogenes strain to cause a systemic infection was quantified as log CFU of bacteria recovered per gram of organ (i.e., small intestine, liver, spleen, and mesenteric lymph nodes). For organs (i.e., small intestine, liver, spleen, and mesenteric lymph nodes) where all L. monocytogenes strains were recovered at a level above the detection limit from all animals, one-way ANOVA was performed as described above. For organs where one or more L. monocytogenes strains were recovered at or below the detection limit (i.e., liver and spleen), left-censored analyses were performed using PROC LIFEREG. All possible pairwise comparisons between L. monocytogenes strains (n = 6) were evaluated using the LIFEREG procedure six different times by using each strain once as a reference strain. P values were corrected for multiple comparisons using the Bonferroni method.

One-way ANOVA with Tukey’s correction as described above was also used to test whether the weights of animals infected with different strains differed significantly at various time points postinfection. The same procedure was also used to determine whether L. monocytogenes numbers recovered in fecal samples at 24, 48, and 72 h postinfection differed among animals infected with different L. monocytogenes strains.

RESULTS

Caco-2 invasion assay. Caco-2 invasion efficiencies for the L. monocytogenes wild-type strain 10403S and the six mutant strains ranged from 0.44% (wild type) to 0.00045% (ΔinlA). Overall statistical analysis of Caco-2 invasion efficiencies by ANOVA showed that the “strain” factor had a highly significant effect on invasion efficiency (P < 0.0001), indicating that the L. monocytogenes strains used in this assay differed in their invasion efficiencies. Specifically, the mean invasion efficiencies of the ΔinlA, ΔsigB, and ΔprfA mutants (Fig. 2) were 970-fold, 88-fold, and 16-fold lower, respectively, than that of the wild-type strain (Fig. 2). The invasion efficiencies of these three mutant strains were significantly different from each other and significantly lower than the invasion efficiency of the wild-type parent strain. Detectable invasion of Caco-2 cells by an inlA-null mutant has been reported previously and likely reflects contributions of other internalins (in addition to InlA) to Caco-2 cell invasion (4).

Targeted mutations in σB-dependent (ΔP2-prfA and ΔP4-inlA) and -independent (ΔP1-3) virulence gene promoters were used to investigate the effect of σB-dependent transcription of these genes on invasion. The invasion efficiency of the ΔP2-prfA strain was similar to that of the wild-type strain (Fig. 2). These
results indicate that elimination of $\sigma^B$-dependent prfA transcription does not affect Caco-2 invasion efficiency, and thus they suggest that the reduced invasion efficiency of the $\Delta$sigB mutant reflects effects of $\sigma^B$ on transcription of genes other than prfA. While the two inlA promoter-null mutants ($\Delta$P1-3 inlA and $\Delta$P4 inlA) both showed significantly lower invasion efficiencies than the wild-type strain, they also both showed significantly higher invasion efficiencies than the $\Delta$inlA mutant. The $\Delta$P4 inlA mutant showed an invasion efficiency similar to, but slightly lower than, that of the $\Delta$sigB mutant (Fig. 2).

Enumeration of L. monocytogenes in organs of intragastrically infected guinea pigs. For all animals intragastrically infected with the L. monocytogenes mutant or wild-type strains, bacterial numbers (in CFU/g) were determined in the four organs harvested from each animal at 72 h postinfection (Fig. 3). While initial overall ANOVA analysis showed a highly significant effect of the “strain” factor ($P < 0.001$), no significant effect of the “infection day” factor was found, indicating that the results did not differ among animals infected on different days, thus confirming the reproducibility of our data between independent experiments. In intragastrically infected animals, the $\Delta$inlA, $\Delta$sigB, and $\Delta$prfA strains were present in significantly lower numbers than those recovered from animals infected with the wild-type strain in all four organs. For all organs, bacterial numbers recovered from animals intragastrically infected with the $\Delta$inlA or the $\Delta$sigB strain did not differ significantly, indicating similar virulence attenuation for these two strains. No L. monocytogenes was recovered from any organs of animals intragastrically infected with the $\Delta$prfA mutant; thus, recovery of the $\Delta$prfA mutant was significantly reduced beyond that of the $\Delta$inlA and the $\Delta$sigB strains (Fig. 3).

Bacterial numbers recovered from all organs of animals intragastrically infected with the $\Delta$P2 prfA strain did not differ significantly from those recovered from animals intragastrically infected with the wild-type strain (Fig. 3). While median bacterial numbers recovered from organs of animals intragastrically

![FIG. 3. Box plot of L. monocytogenes recovered from the organs of guinea pigs at 72 h post-intragastric infection. Strains (wild type, $\Delta$prfA, $\Delta$sigB, $\Delta$inlA, $\Delta$P1-3 inlA, $\Delta$P4 inlA, and $\Delta$P2 prfA) are indicated on the x-axes. Bacterial numbers, in log CFU/gram, from the liver (a), spleen (b), mesenteric lymph nodes (c), and small intestine (d) are shown on the y-axes. Data were obtained from six guinea pigs intragastrically infected with each strain (except for $\Delta$prfA and $\Delta$inlA data, which represent four intragastrically infected guinea pigs for each strain). The detection limits, which differ among organs due to different organ weights, are indicated by horizontal lines near the bottom of each panel; the letter X indicates that no L. monocytogenes was detected in the organ by bacterial enumeration or by histological examination. Data reported at the detection limit (i.e., for the $\Delta$sigB and $\Delta$inlA mutants in the spleen [b] and liver [a]) indicate evidence of infection and/or the presence of L. monocytogenes in a given organ by histopathology accompanied by either no recovery of L. monocytogenes or recovery of L. monocytogenes at the detection limit. Each box encloses the central 50th percentile of the data, with the median displayed as a horizontal line. Vertical lines extending from each box represent the maximum and minimum values; outliers are displayed as circles. Boxes labeled with different letters indicate strains that differed significantly ($P < 0.05$), while boxes labeled with identical letters indicate strains that did not differ significantly, in bacterial numbers recovered from a given organ.)
infected intragastrically with the wild-type or uninfected animals, as early as 24 h postinfection, animals intragastrically infected with a given promoter mutant strain. This might generate greater phenotypic variation between trials for promoter mutant strains than in the wild-type strain, which suggests that the resulting expression may be less tightly regulated in following deletion of one or more promoters (15), we speculate these genes likely enables some compensation in expression (Fig. 3). While the presence of multiple promoters upstream of inlA mutant showed overall weight gain during the 72-h postinfection period (Fig. 4). The mean weights of animals infected intragastrically with the wild type or the inlA mutant showed weight gain during 72 h postinfection. While animals infected intragastrically with the wild type or the ΔP2prfA, ΔP1-3inlA, or ΔinlA mutant showed overall weight loss at 72 h postinfection, animals infected intragastrically with the ΔsigB, ΔprfA, or ΔinlA mutant showed weight gain during the 72-h postinfection period (Fig. 4). The mean weights of the groups of animals infected intragastrically with the ΔsigB, ΔprfA, or ΔinlA strain did not differ significantly from the mean weights of uninfected animals at 72 h postinfection, the mean weights of the groups of animals infected intragastrically with one of these two strains did not differ from each other, but weights of animals in both groups were significantly (P < 0.0005) lower than the mean weights of uninfected animals. The mean weights of animals infected intragastrically with either the ΔP1-3inlA or the ΔP4inlA strain were always lower than those recovered from animals intragastrically infected with the wild-type strain, these differences were not always significant. Specifically, ΔP1-3inlA and ΔP4inlA bacterial numbers were significantly lower than wild-type numbers in the liver and spleen, while the differences were not significant for bacteria recovered from the mesenteric lymph nodes. Also, while median bacterial numbers recovered from organs of animals intragastrically infected with either the ΔP1-3inlA or the ΔP4inlA strain were always higher than median numbers recovered from animals intragastrically infected with the ΔinlA or ΔsigB mutant, generally these differences were not significant (Fig. 3). Overall, numbers of prfA and inlA promoter mutants recovered from internal organs showed some variability, particularly the numbers recovered from the liver (Fig. 3). While the presence of multiple promoters upstream of these genes likely enables some compensation in expression following deletion of one or more promoters (15), we speculate that the resulting expression may be less tightly regulated in promoter mutant strains than in the wild-type strain, which might generate greater phenotypic variation between trials for a given promoter mutant strain.

Behavior, feeding activity, and weight loss of guinea pigs intragastrically infected with L. monocytogenes. Compared to uninfected animals, as early as 24 h postinfection, animals infected intragastrically with the wild-type or ΔP2prfA strain were less alert and more lethargic, consumed less food and water, and showed signs of dehydration (as determined by fecal consistency and quantity of water consumed). Animals infected intragastrically with the ΔP1-3inlA or ΔP4inlA strain showed no or mild signs of lethargic behavior and dehydration. The behavior of animals infected with the ΔsigB, ΔinlA, or ΔprfA strain did not differ from that of the uninfected animals.

Animal weights were monitored to assess the gross effects of infection on the animals (Fig. 4). Overall ANOVA indicated significant differences in relative weight gain at 72 h p.i. for animals infected with different L. monocytogenes strains. Animals intragastrically infected with the wild-type or ΔP2prfA strain exhibited the largest negative weight change (Fig. 4). At 72 h postinfection, the mean weights of the groups of animals intragastrically infected with one of these two strains did not differ from each other, but weights of animals in both groups were significantly (P < 0.0005) lower than the mean weights of uninfected animals. The mean weights of animals infected intragastrically with either the ΔP1-3inlA or the ΔP4inlA strain also decreased relative to that of the uninfected control group (Fig. 4a, g, and h), although the relative body weight change of the animals infected intragastrically with these strains was not significantly different (P > 0.1) from the relative body weight change of uninfected animals at 72 h postinfection. While animals infected intragastrically with the wild type or the ΔP2prfA, ΔP1-3inlA, or ΔinlA mutant showed overall weight loss at 72 h postinfection, animals infected intragastrically with the ΔsigB, ΔprfA, or ΔinlA mutant showed weight gain during the 72-h postinfection period (Fig. 4). The mean weights of the groups of animals intragastrically infected with the ΔsigB, ΔprfA, or ΔinlA strain did not differ significantly from the mean weights of uninfected animals, providing further evidence in support of virulence attenuation for the ΔsigB, ΔprfA, and ΔinlA strains.
Fecal consistency and \textit{L. monocytogenes} fecal shedding in intragastrically infected guinea pigs. In uninfected control animals, fecal size, quantity, and consistency remained similar from day to day. In animals intragastrically infected with the wild type or the \( \Delta \text{P}_1 \text{prfA} \) mutant, the fecal size, quantity, and moisture content declined progressively over the 72-h postinfection period, reflecting symptoms of animal dehydration. For animals infected intragastrically with the \( \Delta \text{P}_4 \text{tolA} \) strain, total quantities of fecal material, moisture content, and size decreased slightly; however, the changes were less pronounced than those for animals intragastrically infected with the wild-type or \( \Delta \text{P}_2 \text{prfA} \) strain. The consistency and characteristics of the fecal material of animals infected intragastrically with the \( \Delta \text{sigB} \), \( \Delta \text{inlA} \), or \( \Delta \text{prfA} \) mutant were similar to those of the uninfected controls.

High concentrations of \textit{L. monocytogenes} in feces (medians, \( >1 \times 10^8 \) CFU/g) were observed for all intragastrically infected animals at 24 h postinfection (Fig. 5). \textit{L. monocytogenes} concentrations in feces at 48 and 72 h postinfection were lower than those observed at 24 h for all intragastrically infected animals. While overall ANOVA showed no effects of “strain” on fecal \textit{L. monocytogenes} numbers at 24 h, there was a highly significant effect of “strain” on fecal shedding at 48 and 72 h postinfection, indicating that the \textit{L. monocytogenes} strains used differed in their shedding levels. Specifically, animals infected with the \( \Delta \text{prfA} \) or \( \Delta \text{sigB} \) mutant showed significantly (\( P < 0.05 \) by Tukey’s studentized range test) less shedding than animals infected with the wild type at 48 and 72 h postinfection (Fig. 5), suggesting a decreased ability of these mutants to survive in the intestinal tract and/or a decreased ability to attach to intestinal epithelial cells. \textit{L. monocytogenes} concentrations in feces of animals infected intragastrically with any of the other mutant strains were not different from those for the wild-type strain.

Histopathological and immunohistochemical evaluation of intragastrically infected guinea pigs. Multiple tissues (i.e., the liver, small intestine [ileum], mesenteric lymph nodes, spleen, and brain) from guinea pigs intragastrically infected with \textit{L. monocytogenes} were examined histologically for the presence of lesions. All sections were examined for evidence of \textit{L. monocytogenes} infection, including parenchymal necrosis, neutrophil inflammation, fibrin exudation, secondary lymphoid follicle formation, lymphocytolysis, and the presence or absence of bacteria (Fig. 6a). Selected slides were stained using immunohistochemical means to verify the presence of \textit{L. monocytogenes} in the observed microscopic lesions (Fig. 6b).

Of the tissues microscopically examined, the livers of infected animals bore the most obvious lesions as well as the clearest visual differences associated with infection by specific \textit{L. monocytogenes} strains. While hepatocyte degeneration and necrosis were consistently observed in the livers of all infected guinea pigs except for those infected intragastrically with the \( \Delta \text{prfA} \) strain, the nature and severity of lesions differed among animals infected with different \textit{L. monocytogenes} strains (Fig. 6a). The frequency and intensity of lesions and the extent of neutrophil infiltration were greatest in guinea pigs infected with the \textit{L. monocytogenes} wild-type strain, while fewer lesions and less infiltration were observed in animals intragastrically infected with the \( \Delta \text{inlA} \) or \( \Delta \text{sigB} \) strain. Focal areas of necrosis included hepatocyte degeneration characterized by cytoplasmic expansion or shrinkage, cytoplasmic hyper eosinophilia, and condensation or pyknosis of degenerate hepatocytes (Fig. 6a). Infiltrating neutrophils were generally observed within the areas of hepatocyte degeneration and necrosis, including, in many instances, neutrophil degeneration, probably due to the presence of \textit{L. monocytogenes} (Fig. 6b). Animals infected intragastrically with the \( \Delta \text{prfA} \) strain did not show any inflammatory response in the liver.

The extent and severity of lesions observed differed by organ. Histologic lesions were not observed in any of the brain sections examined. Mesenteric lymph node sections ranged from moderately reactive (i.e., with the formation of responsive, secondary lymphoid follicles) to severe lymphadenitis,
which was characterized by infiltration of neutrophils, lymphocyte necrosis, areas of hemorrhage, and fibrin exudation. Lesions observed in the spleen were similar to those seen in the mesenteric lymph node, including formation of secondary lymphoid follicles, lymphoid necrosis, neutrophil infiltration, and fibrin exudation. In both brain and mesenteric lymph node sections, intragastric infection with the wild-type strain produced more severe lesions than intragastric infection with the \textit{L. monocytogenes} mutant strains. Variable, but milder to moderate, changes were observed with infections by other strains. Observed intestinal lesions included mixtures of karyorrhectic debris and edema in the villar tips, variable numbers of neutrophils within the lamina propria, and moderate numbers of lymphocytes and plasma cells within lymphoid follicles in the submucosa. Intestinal lesions did not differ among animals infected with different \textit{L. monocytogenes} strains.

**Enumeration of \textit{L. monocytogenes} in organs of intravenously infected guinea pigs.** To determine if $\sigma^B$ is also critical for systemic infection of \textit{L. monocytogenes} after the intestinal barrier has been breached, guinea pigs were infected intravenously with the \textit{L. monocytogenes} \textit{\Delta}sigB mutant or the wild-type strain. In agreement with the findings of Lecuit et al. (30), preliminary experiments in our laboratory (data not shown) indicated that \textit{L. monocytogenes} numbers are highest in organs at 48 h after intravenous infection. However, we chose to enumerate \textit{L. monocytogenes} in internal organs at 72 h post-intravenous infection to allow for a direct comparison of bacterial numbers in our intravenously and intragastrically infected animals. Similar numbers of wild-type and \textit{\Delta}sigB \textit{L. monocytogenes} were recovered in the liver, spleen, and mesenteric lymph nodes from intravenously infected animals (Fig. 7). \textit{L. monocytogenes} was not detected in the small intestine of intravenously infected animals (Fig. 7). Overall ANOVA showed no significant differences between \textit{L. monocytogenes} wild-type and \textit{\Delta}sigB bacterial numbers recovered from the different organs, indicating that loss of $\sigma^B$ does not significantly affect the ability of \textit{L. monocytogenes} to survive and replicate in intravenously infected animals.

\textit{L. monocytogenes} was present in the fecal material of the first three animals that had been infected intravenously with either the \textit{\Delta}sigB mutant or the wild-type strain. Therefore, the fecal material of the remaining intravenously infected animals was not tested for the presence of \textit{L. monocytogenes}.

**Behavior, feeding activity, and weight loss of guinea pigs intravenously infected with \textit{L. monocytogenes}**. The behavior and feeding activity of animals infected intravenously with the \textit{\Delta}sigB mutant or the wild-type strain did not differ from those of the uninfected animals. While animals intravenously infected with either the wild-type or the \textit{\Delta}sigB strain clearly showed less weight gain than uninfected animals (Fig. 4c), the relative body weight change at 72 h postinfection did not differ significantly from that of the uninfected control animals ($P > 0.1$).

**DISCUSSION**

Through a combination of tissue culture and guinea pig infection studies with a series of isogenic \textit{L. monocytogenes} null mutants, including a strain bearing a mutation in \textit{sigB}, along with strains with mutations in selected $\sigma^B$-dependent virulence genes and their promoters, we provide evidence that (i) \textit{L.
\textit{L. monocytogenes} \(\sigma^B\) plays a critical role in host cell invasion, (ii) \(\sigma^B\)-mediated contributions to invasion are at least in part due to direct effects on transcription of \textit{inlA}, and (iii) while \(\sigma^B\) plays a critical role during the gastrointestinal stage of listeriosis, it is not required for systemic spread. These conclusions are based on three main lines of evidence: (i) an \textit{L. monocytogenes} \(\Delta\text{sigB}\) strain demonstrated significantly lower invasion efficiency than its wild-type parent strain in the human intestinal epithelial cell line Caco-2; (ii) deletion of the \(\sigma^B\)-dependent \(P_{2\text{prfA}}\) promoter did not reduce \textit{L. monocytogenes} invasion capacity, while deletion of the \(\sigma^B\)-dependent \(P_{4\text{inlA}}\) promoter resulted in reduced invasion; and (iii) the virulence of the \(\Delta\text{sigB}\) mutant was significantly attenuated compared to that of its wild-type parent strain in intragastrically infected guinea pigs, but the \(\Delta\text{sigB}\) mutant and the wild-type strain did not differ in virulence in intravenously infected guinea pigs.

Our observations that an \textit{L. monocytogenes} strain bearing a nonpolar deletion mutation in \textit{sigB} has a diminished capacity to infect the human gastrointestinal epithelial cell line Caco-2 and reduced virulence in an intragastrically infected guinea pig model clearly demonstrate the importance of \(\sigma^B\) for \textit{L. monocytogenes} virulence in intragastric infection. While the \textit{L. monocytogenes} strains used in the infection experiments described here were grown at 37°C, Kim et al. (25) also showed that the same \(\Delta\text{sigB}\) mutant had a reduced ability to invade Caco-2 and Henle cells when grown at 30°C. Combined, these data indicate that \(\sigma^B\) contributions to Caco-2 cell invasion are independent of both growth temperature (30 or 37°C) and translation of \textit{prfA} mRNA transcripts, which occurs at 37°C but not at 30°C (22). These findings are also consistent with quantitative reverse transcriptase PCR studies, which showed reduced \textit{inlA} transcription in \(\Delta\text{sigB}\) mutants constructed in both a wild-type and a \(\Delta\text{prfA}\) mutant background (P. T. McGann, K. J. Boor, and M. Wiedmann, unpublished data).

The drastically reduced virulence of the \(\Delta\text{sigB}\) mutant in the intragastric guinea pig model contrasts with results from previous characterizations of the strain \(\Delta\text{sigB}\) mutant in the intragastric murine infection model (36, 47). In one study (47), although lower numbers of the \(\Delta\text{sigB}\) mutant than of the wild-type parent strain were recovered from the livers of intragastrically infected animals at 4 days postinfection, the finding was only of borderline significance (\(P = 0.027\)). In another study, which used a competitive index infection model, no differences were observed between \(\Delta\text{sigB}\) and wild-type numbers in the spleen and liver after intragastric infection (36). These previously reported results are consistent with the observation that the murine model does not allow for \textit{InlA}-mediated invasion of gastrointestinal epithelial cells, due to the lack of interaction between \textit{InlA} and murine E-cadherin (30).

Specifically, while the glutamic acid at E-cadherin amino acid residue 16 in humans and guinea pigs allows for \textit{InlA}-mediated adhesion and invasion, the proline at residue 16 in mice prevents \textit{InlA}–E-cadherin interactions (28). Our results suggest that \(\sigma^B\)-dependent \textit{inlA} expression is critical in the gastrointestinal stage of \textit{L. monocytogenes} infection in an animal model expressing the same E-cadherin isotype as that found in humans. These findings are consistent with previous studies that have demonstrated \(\sigma^B\)-dependent \textit{inlA} expression in broth-grown \textit{L. monocytogenes} (24, 25, 45). Four possible transcriptional start sites have been identified upstream of \textit{inlA}, including at least one \textit{PrfA}-dependent and one \(\sigma^B\)-dependent site (24, 31, 43). The findings reported in the present study highlight the critical contributions of the \(\sigma^B\)-dependent \(P_4\text{inlA}\) promoter for in vivo gastric internalin A–E-cadherin-mediated infections.

Interestingly, stress-responsive alternative sigma factors have also been shown to regulate expression of adhesion molecules in pathogens other than \textit{L. monocytogenes}. For example, in \textit{Staphylococcus aureus}, \(\sigma^B\) regulates expression of \textit{clfA} and \textit{fnbA}, which encode a fibrinogen binding protein clumping factor and a fibronectin binding protein, respectively (10). Consistent with the importance of these molecules in bacterial adhesion, an \textit{S. aureus} strain that overproduces \(\sigma^B\) showed greater attachment in vitro and higher infectivity in vivo (10). In addition, the stress-responsive alternative sigma factor RpoS contributes to expression of genes in the \textit{Salmonella enterica} serovar Typhimurium \textit{spv} cluster, which encodes a type III secretion system that contributes to \textit{Salmonella} virulence and adhesion. While a \textit{Salmonella} serovar Typhimurium \textit{rpoS}-null mutant does not show a decreased ability to survive in macrophage-like cells, the \(\Delta\text{rpoS}\) mutant has a decreased ability to colonize murine Peyer’s patches after oral invasion, consistent with a potential role for RpoS in \textit{Salmonella} gastrointestinal colonization and pathogenesis (37).

Previous studies have shown that \textit{inlA} is preceded by both \(\sigma^B\) and \textit{PrfA}-dependent promoters (8, 24, 31) and that \(\sigma^B\) contributes to \textit{prfA} transcription (41). Specifically, \textit{prfA} transcription can occur through four promoters, including three directly upstream of \textit{prfA} (\(P_{1\text{prfA}}\) and two within the \(P_{2\text{prfA}}\)
promoter region) and one upstream of plcA, which can produce a bicistronic plcA-prfA transcript. The P2\textsubscript{prfA} promoter region appears to comprise overlapping σ\textsuperscript{B} and σ\textsuperscript{A}-dependent promoters (38); hence, the P2\textsubscript{prfA} promoter is at least partially σ\textsuperscript{B} dependent (36, 38, 41). In the present study, the ΔP2\textsubscript{prfA} strain, which was created to eliminate transcription initiation from both the σ\textsuperscript{B}-dependent and the putative σ\textsuperscript{A}-dependent promoters within the P2 region, showed neither reduced invasion in Caco-2 cells nor reduced virulence in gastrointestinal guinea pig infections, indicating limited contributions of P2\textsubscript{prfA}-dependent transcription to invasion and virulence, respectively, in the model systems used. These results complement those from previous studies, which showed that a P2\textsubscript{prfA} promoter deletion did not reduce virulence in tissue culture or mouse models of infection, presumably due to compensatory transcription from other prfA promoters (15). Our results show that the reduced invasion and virulence observed for the ΔsigB mutant do not reflect contributions of σ\textsuperscript{B} to P2\textsubscript{prfA} transcription but rather reflect direct contributions of σ\textsuperscript{B} to transcription of other genes, which encode products that are important during gastrointestinal infection.

While the L. monocytogenes mutant carrying a deletion in the σ\textsuperscript{B}-dependent P4\textsubscript{inlA} promoter (ΔP4\textsubscript{inlA}) had a reduced ability to invade Caco-2 cells and reduced numbers in the liver and spleen relative to the wild-type strain, its Caco-2 invasion capacity was intermediate relative to those of the ΔsigB and ΔinlA strain, while its numbers in the liver were higher than those of either of these strains. This result suggests that a σ\textsuperscript{B}-dependent activation of the P4\textsubscript{inlA} promoter contributes to host cell invasion and gastrointestinal infection but that, during infection, some inlA transcription is also controlled by upstream promoters that are not regulated by σ\textsuperscript{B}. This hypothesis is further supported by our observations that the invasion capacity of a ΔP1-3\textsubscript{inlA} mutant was similar to that of the ΔsigB strain and its virulence capacity was similar to that observed for the ΔP4\textsubscript{inlA} mutant. Reduced numbers of the ΔsigB strain relative to those of the ΔP4\textsubscript{inlA} strain further suggest that σ\textsuperscript{B} affects expression of genes other than inlA that are important during gastrointestinal infection. This hypothesis is consistent with observations that null mutants in other σ\textsuperscript{B}-dependent genes (i.e., bsh, opuCA) (13, 24, 44, 45) also showed reduced abilities to cause gastrointestinal infections in the guinea pig (bsh) (9) and murine (opuCA) (42) models.

While we clearly showed reduced virulence of an L. monocytogenes ΔsigB mutant in the intragastrically infected guinea pig model, the same ΔsigB strain did not show reduced virulence in intravenously infected guinea pigs compared with its otherwise isogenic wild-type parent. Similarly, previous studies showed only limited virulence attenuation of a ΔsigB mutant in intraperitoneally infected mice; ΔsigB numbers were lower than wild-type numbers only at a single time point (i.e., either 2 or 4 days postinfection) in a single organ (i.e., either spleen or liver) (36, 47). Overall, these results indicate that while a functional σ\textsuperscript{B} is crucial for gastrointestinal infection in a host that expresses an E-cadherin isoform that allows InlA binding, σ\textsuperscript{B} appears to play a limited role during systemic spread after L. monocytogenes has crossed the intestinal barrier and entered the bloodstream. A more important role for σ\textsuperscript{B} in either L. monocytogenes fetal infection or spread to the central nervous system cannot yet be excluded, since the animal models used for characterization of ΔsigB mutants to date are not appropriate for evaluation of either fetal or central nervous system infections. In particular, future experiments to measure σ\textsuperscript{B} contributions to fetal infections may be relevant, since recent evidence indicates that InlA may play a critical role in the crossing of the placental barrier by L. monocytogenes (29). The importance of InlA for vertical transmission of L. monocytogenes has not been confirmed in a pregnant guinea pig model (1).

While a variety of previous in vitro experiments have shown that an L. monocytogenes ΔsigB mutant has reduced survival under stress conditions (i.e., oxidative stress, acid stress) typically encountered in a host cell vacuole (11), our data presented here are consistent with an emerging body of information that suggests that σ\textsuperscript{B} has limited importance in facilitating L. monocytogenes survival in host cells (25, 26, 36). We report no virulence attenuation for the ΔsigB mutant following intravenous infection, a finding that supports previous observations of similar intracellular growth rates in human tissue culture cells (25) and similar cell-to-cell spread in a murine fibroblast plaque assay for the ΔsigB and wild-type strains (36). In summary, the results of the present study support a model in which σ\textsuperscript{B} plays a critical role in (i) the survival of L. monocytogenes under environmental stress conditions, including those often encountered in food products, and (ii) regulation of both stress response (e.g., opuCA [44, 45]) and virulence (e.g., bsh and inlA) genes that are important during the gastrointestinal stage of listeriosis.

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