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

M. R. Garner,¹ B. L. Njaa,² M. Wiedmann,¹ and K. J. Boor^{1*}

Department of Food Science¹ and Department of Biomedical Sciences,² Cornell University, Ithaca, New York 14853

Received 19 August 2005/Returned for modification 29 September 2005/Accepted 8 November 2005

Contributions of the alternative sigma factor σ^B 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 $\Delta P4_{inlA}$ strain, which has a deletion in the σ^B -dependent $P4_{inlA}$ promoter, and the $\Delta sigB$ 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 σ^B dependent $P2_{prfA}$ promoter region did not differ from that of the wild type. The virulence of the $\Delta sigB$ and $\Delta P4_{inlA}$ strains was attenuated in intragastrically inoculated guinea pigs, with the $\Delta sigB$ strain showing greater attenuation, while the virulence capacity of the $\Delta P2_{prfA}$ strain was similar to that of the wild-type strain, suggesting that attenuation of virulence due to the $\Delta sigB$ mutation does not result from loss of σ^B -dependent *prfA* transcription. Our results show that σ^B -dependent activation of *inlA* is important for cell invasion and gastrointestinal infection and suggest that σ^B -regulated genes in addition to *inlA* appear to contribute to gastrointestinal infection. Interestingly, the virulence of the $\Delta sigB$ strain was not attenuated in intravenously infected guinea pigs. We conclude that (i) *L. monocytogenes* σ^B plays a critical role in invasion of human host cells, (ii) σ^B -mediated contributions to invasion are, in part, due to direct effects on *inlA* transcription but not on *prfA* transcription, and (iii) σ^B 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 iso-type 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 σ^B contributes to bacterial survival under conditions of in vitro environmental stress in multiple gram-positive microbes (3,

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

TABLE 1. *L. monocytogenes* strains used in this study

Strain	Characteristic(s)	Nature of mutation	Reference
10403S	Wild type (serotype 1/2a)	None	5
FSL A1-254	10403S Δ <i>sigB</i>	297-bp in-frame <i>sigB</i> deletion	47
DP-L4405	10403S Δ <i>inlA</i>	1,988-bp in-frame <i>inlA</i> deletion	1
DP-L4137	10403S Δ <i>prfA</i>	336-bp in-frame <i>prfA</i> deletion	7
DP-L1957	10403S Δ P2 _{<i>prfA</i>}	20-bp deletion of P2 _{<i>prfA</i>} – 10 region ^a	14
FSL B2-049	10403S Δ P4 _{<i>inlA</i>}	15-bp deletion of P4 _{<i>inlA</i>} – 10 region ^b	This study
FSL B2-048	10403S Δ P1-3 _{<i>inlA</i>}	72-bp deletion of P1-3 _{<i>inlA</i>} region	This study

^a See Fig. 1B.^b See Fig. 1A.

19). In *L. monocytogenes*, σ^B contributes 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 -dependent 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_{*prfA*} promoter region (36, 38, 41). 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 in selected σ^B -dependent (P2_{*prfA*}, P4_{*inlA*}) and σ^B -independent (P1-3_{*inlA*}) 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 prior to use. Stationary-phase *L. monocytogenes* cells were selected for tissue culture invasion assays and guinea pig infections, because previous experiments demonstrated greater invasiveness of stationary-phase *L. monocytogenes* in host cells expressing E-cadherin types that interact with InlA (26). Enhanced invasiveness of stationary-

phase *L. monocytogenes* cells is consistent with (i) induction of σ^B activity upon *L. monocytogenes* entry into stationary phase (12), coupled with (ii) the partial σ^B dependence of *inlA* transcription (25, 26). For infections, aliquots (15 ml) of stationary-phase bacterial cultures were concentrated by centrifugation (7,500 × g for 5 min), resuspended in 1 ml of phosphate-buffered saline (PBS) containing 15% glycerol (pH 7.4), and then frozen and stored in multiple aliquots at –80°C. Bacteria were 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 Δ *sigB*, Δ *inlA*, Δ P2_{*prfA*}, and Δ *prfA* mutant strains have been described previously. The Δ P4_{*inlA*} and Δ P1-3_{*inlA*} 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 Δ P4_{*inlA*} and Δ P1-3_{*inlA*} deletion alleles are listed in Table 2. The Δ P1-3_{*inlA*} and Δ P4_{*inlA*} mutants have 72-bp and 15-bp deletions, respectively, within the respective promoter regions (Fig. 1). Successful creation of the Δ P1-3_{*inlA*} and Δ P4_{*inlA*} mutant strains was confirmed by PCR amplification and subsequent DNA sequencing of the targeted region in each strain. The mutations in the Δ *sigB*, Δ *inlA*, and Δ *prfA* 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 × 10⁴ 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 × 10⁷ *L. monocytogenes* cells

TABLE 2. PCR primers used in this study

Primer name	Primer sequence
SOE-P1-3inlA-A ^a	GGTACCCAGACCGAATCGCAAGCATC
SOE-P1-3inlA-B	GAATTAACCTTATGTTATCCTCTAC
SOE-P1-3inlA-C ^b	TAGAGGATAACATAAGTTAATTCAGAGAGTTTGGCGGTAAGAG
SOE-P1-3inlA-D ^c	CGGGATCCCTCCTTTTTCAATAGTTAGAAAC
SOE-P4inlA-A ^a	GGGGTACCGGAATGACGAGCTTATACAAACAA
SOE-P4inlA-B	TCAAAATAACACATAAGGGTGTGAG
SOE-P4inlA-C ^d	ACACCCCTATGTGTTATTTTGGAGATAACATAAGTTAATCTTTTTTTTGG
SOE-P4inlA-D ^c	CGGGATCCGATAAATTTATTAATATGCTCCCCATAAA

^a The KpnI restriction site incorporated into this primer to facilitate cloning is underlined.^b The overhang complementary to SOE-P1-3inlA-B is underlined.^c The BamHI restriction site incorporated into this primer to facilitate cloning is underlined.^d The overhang complementary to SOE-P4inlA-B is underlined.

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*-like morphology were counted and recorded as *L. monocytogenes*. *L. monocytogenes* identification was confirmed on a representative subset of these colonies by plating on LMPM 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 E 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 μ m, 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 μ m 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 micropublisher 5.0 RTV (Burnaby, British Columbia, Canada) and a 50 $\times$ or 100 $\times$ 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 Δ *prfA* and Δ *inlA*, which were each 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 animals intragastrically infected with *L. monocytogenes* Δ *prfA*, no *L. monocytogenes* was isolated from internal organs nor was any evidence of infection found by histopathology; thus, these animals were deemed uninfected, and *L. monocytogenes* Δ *prfA* strain data were not included in further data analyses. Data from each internal organ were analyzed separately. For organs (i.e., small intestine and mesenteric lymph nodes) where all *L. monocytogenes* strains were recovered at levels 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.

FIG. 2. Box plot of *L. monocytogenes* invasion efficiency in the human intestinal epithelial cell line Caco-2. Strains (the wild-type strain and the otherwise isogenic Δ *prfA*, Δ *sigB*, Δ *inlA*, Δ P1-3*inlA*, Δ P4*inlA*, and Δ P2*prfA* strains) are indicated on the x axis, and log-transformed invasion efficiencies (the number of bacteria recovered from the lysed Caco-2 cells divided by the number of bacteria used for infection, expressed as a percentage) are indicated on the y axis. Data represent invasion assays performed in triplicate on four separate days. Each box encloses the central 50th percentile of the data; the median is displayed as a horizontal line. Vertical lines extending from each box represent the maximum and minimum values, and outliers are displayed as circles. The horizontal line near the bottom of the graph indicates the limit of detection. 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 their invasion efficiencies. For example, while the wild-type and Δ P2*prfA* strains did not differ in their invasion efficiencies (indicated by the fact that both boxes are labeled with the letter a), the wild-type and Δ *prfA* strains differed significantly in their invasion efficiencies (indicated by the fact that the boxes are labeled with the letters a and b, respectively).

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% (Δ *inlA*). 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 Δ *inlA*, Δ *sigB*, and Δ *prfA* 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 σ^B -dependent (Δ P2*prfA* and Δ P4*inlA*) and -independent (Δ P1-3*inlA*) virulence gene promoters were used to investigate the effect of σ^B -dependent transcription of these genes on invasion. The invasion efficiency of the Δ P2*prfA* strain was similar to that of the wild-type strain (Fig. 2). These

FIG. 3. Box plot of *L. monocytogenes* recovered from the organs of guinea pigs at 72 h post-intragastric infection. Strains (wild type, $\Delta prfA$, $\Delta sigB$, $\Delta inlA$, $\Delta p1-3inlA$, $\Delta p4inlA$, and $\Delta p2prfA$) 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.

results indicate that elimination of σ^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 σ^B on transcription of genes other than *prfA*. While the two *inlA* promoter-null mutants ($\Delta p1-3inlA$ and $\Delta p4inlA$) 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 $\Delta p4inlA$ 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 $\Delta p2prfA$ 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. 4. Box plots of the percent change in body weight for guinea pigs infected intragastrically or intravenously with different *L. monocytogenes* strains. The *x* axes indicate time postinfection, and the *y* axes indicate percent body weight change. Data are mean weights of six animals (except for $\Delta prfA$ and $\Delta inlA$ data, which are mean weights of four intragastrically infected animals). Data for intragastrically (a to h) and intravenously (i and j) infected animals are shown. 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.

cally infected with either the $\Delta P1-3_{inlA}$ or the $\Delta P4_{inlA}$ strain were always lower than those recovered from animals intragastrically infected with the wild-type strain, these differences were not always significant. Specifically, $\Delta P1-3_{inlA}$ and $\Delta P4_{inlA}$ 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 $\Delta P1-3_{inlA}$ or the $\Delta P4_{inlA}$ strain were always higher than median numbers recovered from animals intragastrically infected with the $\Delta inlA$ or $\Delta sigB$ 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 $\Delta P2_{prfA}$ 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 $\Delta P1-3_{inlA}$ or $\Delta P4_{inlA}$ strain showed no

or mild signs of lethargic behavior and dehydration. The behavior of animals infected with the $\Delta sigB$, $\Delta inlA$, or $\Delta prfA$ 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 $\Delta P2_{prfA}$ 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 $\Delta P1-3_{inlA}$ or the $\Delta P4_{inlA}$ 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 $\Delta P2_{prfA}$, $\Delta P1-3_{inlA}$, or $\Delta P4_{inlA}$ mutant showed overall weight loss at 72 h postinfection, animals infected intragastrically with the $\Delta sigB$, $\Delta prfA$, or $\Delta inlA$ mutant showed weight gain during the 72-h postinfection period (Fig. 4). The mean weights of the groups of animals intragastrically infected with the $\Delta sigB$, $\Delta prfA$, or $\Delta inlA$ strain did not differ significantly from the mean weights of uninfected animals, providing further evidence in support of virulence attenuation for the $\Delta sigB$, $\Delta prfA$, and $\Delta inlA$ strains.

FIG. 5. Box plots of *L. monocytogenes* in fecal material from intragastrically infected guinea pigs. The *x* axes indicate time postinfection, and the *y* axes indicate *L. monocytogenes* numbers recovered from feces (in log CFU/gram of feces). Data are means for fecal samples obtained from six animals (except for $\Delta prfA$ and $\Delta inlA$ data, which are means from three infected animals each). 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. The letter X indicates that no *L. monocytogenes* was detected in the feces of any of the four animals infected with the $\Delta prfA$ mutant at 72 h p.i. Of the six animals infected with the $\Delta sigB$ mutant, *L. monocytogenes* was detected in the feces of only one animal at 48 h p.i. The detection limit for this assay is 10^3 CFU/g of feces.

Fecal consistency and *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 $\Delta P2_{prfA}$ 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 $\Delta P1-3_{inlA}$ or $\Delta P4_{inlA}$ 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 $\Delta P2_{prfA}$ strain. The consistency and characteristics of the fecal material of animals infected intragastrically with the $\Delta sigB$, $\Delta inlA$, or $\Delta prfA$ mutant were similar to those of the uninfected controls.

High concentrations of *L. monocytogenes* in feces (medians, $>1 \times 10^8$ CFU/g) were observed for all intragastrically infected animals at 24 h postinfection (Fig. 5). *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 *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 *L. monocytogenes* strains used differed in their shedding levels. Specifically, animals infected with the $\Delta prfA$ or $\Delta sigB$ 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. *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 *L. monocytogenes* were examined histologically for the presence of lesions. All sections were examined for evidence of *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 *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 *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 $\Delta prfA$ strain, the nature and severity of lesions differed among animals infected with different *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 *L. monocytogenes* wild-type strain, while fewer lesions and less infiltration were observed in animals intragastrically infected with the $\Delta inlA$ or $\Delta sigB$ strain. Focal areas of necrosis included hepatocyte degeneration characterized by cytoplasmic expansion or shrinkage, cytoplasmic hypereosinophilia, 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 *L. monocytogenes* (Fig. 6b). Animals infected intragastrically with the $\Delta prfA$ 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,

FIG. 6. Histological and immunohistochemical evaluation of livers from intragastrically infected guinea pigs. (a) Micrographs of hematoxylin-and-eosin-stained sections depicting lesions observed in livers harvested at 72 h after intragastric inoculation of guinea pigs with the wild-type, $\Delta sigB$, $\Delta inlA$, or $\Delta prfA$ *L. monocytogenes* strain. Lesions in livers from animals infected with the wild-type strain were severe, showing focal areas of hepatocellular necrosis and moderate to high numbers of neutrophils, most of which were degenerate. Hepatic lesions in animals infected with the $\Delta sigB$ or $\Delta inlA$ strain differed among animals but were less severe than those in guinea pigs infected with the wild-type strain. In general, liver lesions in guinea pigs infected with the $\Delta sigB$ or $\Delta inlA$ strain were classified as mild or moderate, respectively. No evidence of hepatic necrosis or neutrophil infiltration was seen in the livers of animals infected with the $\Delta prfA$ strain. (b) Immunohistochemically stained sections of livers harvested at 72 h postinfection from guinea pigs intragastrically infected with the wild-type, $\Delta sigB$, $\Delta inlA$, or $\Delta prfA$ *L. monocytogenes* strain. In animals infected with the wild-type strain, clusters of multiple *L. monocytogenes* organisms were frequently observed within liver sections (black arrows), although single, rod-shaped *L. monocytogenes* organisms were also observed (red arrows). While the numbers of observed *L. monocytogenes* organisms differed among animals, zero to few *L. monocytogenes* organisms were observed in animals intragastrically infected with the $\Delta sigB$ or the $\Delta inlA$ strain. No immunostained *L. monocytogenes* organisms were observed in the livers of animals intragastrically infected with the $\Delta prfA$ strain.

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 *L. monocytogenes* mutant strains. Variable, but milder to moderate, changes were observed with infections by other strains. Observed intestinal lesions included mixtures of karyorrhetic 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 *L. monocytogenes* strains.

Enumeration of *L. monocytogenes* in organs of intravenously infected guinea pigs. To determine if σ^B is also critical for systemic spread of *L. monocytogenes* after the intestinal barrier has been breached, guinea pigs were infected intravenously with the *L. monocytogenes* $\Delta sigB$ 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 *L. monocytogenes* numbers are highest in organs at 48 h after intravenous infection. However, we chose to enumerate *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 $\Delta sigB$ *L. monocytogenes* were recovered in the liver, spleen, and mesenteric lymph nodes from intravenously infected animals (Fig. 7). *L. monocytogenes* was not detected in the small intestine of intravenously infected animals (Fig. 7). Overall ANOVA showed no significant differences between *L. monocytogenes* wild-type and $\Delta sigB$ bacterial numbers recovered from the different organs, indicating that loss of σ^B does not significantly affect the ability of *L. monocytogenes* to survive and replicate in intravenously infected animals.

No *L. monocytogenes* was present in the fecal material of the first three animals that had been infected intravenously with either the $\Delta sigB$ mutant or the wild-type strain. Therefore, the fecal material of the remaining intravenously infected animals was not tested for the presence of *L. monocytogenes*.

Behavior, feeding activity, and weight loss of guinea pigs intravenously infected with *L. monocytogenes*. The behavior and feeding activity of animals infected intravenously with the $\Delta sigB$ 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 $\Delta sigB$ 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 *L. monocytogenes* null mutants, including a strain bearing a mutation in *sigB*, along with strains with mutations in selected σ^B -dependent virulence genes and their promoters, we provide evidence that (i) *L.*

FIG. 7. Box plot of *L. monocytogenes* $\Delta sigB$ and wild-type strains recovered from the organs of guinea pigs at 72 h post-intravenous infection. The organ sampled is indicated on the x axis, and bacterial numbers (in log CFU/gram) are indicated on the y axis. The detection limits, which differ among organs due to the different organ weights, are indicated by horizontal lines below the data boxes; the letter X indicates that no *L. monocytogenes* was detected in the small intestine. Data represent bacterial numbers obtained from six infected animals for each strain. 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. Numbers of wild-type and $\Delta sigB$ bacteria recovered from each organ type did not differ ($P > 0.05$), as indicated by the use of the same letter designation (e.g., "a") for both strains in each organ.

monocytogenes σ^B plays a critical role in host cell invasion, (ii) σ^B -mediated contributions to invasion are at least in part due to direct effects on transcription of *inlA* rather than to effects on transcription of *prfA*, and (iii) while σ^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 *L. monocytogenes* $\Delta sigB$ 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 σ^B -dependent P_{2prfA} promoter did not reduce *L. monocytogenes* invasion capacity, while deletion of the σ^B -dependent P_{4inlA} promoter resulted in reduced invasion; and (iii) the virulence of the $\Delta sigB$ mutant was significantly attenuated compared to that of its wild-type parent strain in intragastrically infected guinea pigs, but the $\Delta sigB$ mutant and the wild-type strain did not differ in virulence in intravenously infected guinea pigs.

Our observations that an *L. monocytogenes* strain bearing a nonpolar deletion mutation in *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 σ^B for *L. monocytogenes* virulence in intragastric infection. While the *L. monocytogenes* strains used in the infection experiments described here were grown at 37°C, Kim et al. (25) also showed that the same $\Delta sigB$ mutant had a reduced ability to invade Caco-2 and Henle cells when grown at 30°C. Combined, these data indicate that σ^B contributions to Caco-2 cell invasion are independent of both growth temperature (30 or 37°C) and

translation of *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 *inlA* transcription in $\Delta sigB$ mutants constructed in both a wild-type and a $\Delta prfA$ mutant background (P. T. McGann, K. J. Boor, and M. Wiedmann, unpublished data).

The drastically reduced virulence of the $\Delta sigB$ mutant in the intragastric guinea pig model contrasts with results from previous characterizations of the same $\Delta sigB$ mutant in the intragastric murine infection model (36, 47). In one study (47), although lower numbers of the $\Delta sigB$ mutant than of the wild-type parent strain were recovered from the livers of intragastrically infected animals at 4 days postinfection, the difference was only of borderline significance ($P = 0.027$). In another study, which used a competitive index infection model, no differences were observed between $\Delta sigB$ 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 InlA-mediated invasion of gastrointestinal epithelial cells, due to the lack of interaction between 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 InlA-mediated adhesion and invasion, the proline at residue 16 in mice prevents InlA–E-cadherin interactions (28). Our results suggest that σ^B -dependent *inlA* expression is critical in the gastrointestinal stage of *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 σ^B -dependent *inlA* expression in broth-grown *L. monocytogenes* (24, 25, 45). Four possible transcriptional start sites have been identified upstream of *inlA*, including at least one PrfA-dependent and one σ^B -dependent site (24, 31, 43). The findings reported in the present study highlight the critical contributions of the σ^B -dependent P_{4inlA} 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 *L. monocytogenes*. For example, in *Staphylococcus aureus*, σ^B regulates expression of *clfA* and *fmbA*, 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 *S. aureus* strain that overproduces σ^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 *Salmonella enterica* serovar Typhimurium *spv* cluster, which encodes a type III secretion system that contributes to *Salmonella* virulence and adhesion. While a *Salmonella* serovar Typhimurium *rpoS*-null mutant does not show a decreased ability to survive in macrophage-like cells, the $\Delta rpoS$ mutant has a decreased ability to colonize murine Peyer's patches after oral invasion, consistent with a potential role for RpoS in *Salmonella* gastrointestinal colonization and pathogenesis (37).

Previous studies have shown that *inlA* is preceded by both σ^B - and PrfA-dependent promoters (8, 24, 31) and that σ^B contributes to *prfA* transcription (41). Specifically, *prfA* transcription can occur through four promoters, including three directly upstream of *prfA* (P_{1prfA} and two within the P_{2prfA}

promoter region) and one upstream of *plcA*, which can produce a bicistronic *plcA-prfA* transcript. The $P_{2_{prfA}}$ promoter region appears to comprise overlapping σ^B - and σ^A -dependent promoters (38); hence, the $P_{2_{prfA}}$ promoter is at least partially σ^B dependent (36, 38, 41). In the present study, the $\Delta P_{2_{prfA}}$ strain, which was created to eliminate transcription initiation from both the σ^B -dependent and the putative σ^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 $P_{2_{prfA}}$ -dependent transcription to invasion and virulence, respectively, in the model systems used. These results complement those from previous studies, which showed that a $P_{2_{prfA}}$ 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 $\Delta sigB$ mutant do not reflect contributions of σ^B to $P_{2_{prfA}}$ transcription but rather reflect direct contributions of σ^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^B -dependent $P_{4_{inlA}}$ promoter ($\Delta P_{4_{inlA}}$) 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 $\Delta sigB$ and $\Delta inlA$ strains, while its numbers in the liver were higher than those of either strain. These results suggest that σ^B -dependent activation of the $P_{4_{inlA}}$ promoter contributes to host cell invasion and gastrointestinal infection but that, during infection, some *inlA* transcription is also controlled by additional upstream promoters that are not regulated by σ^B . This hypothesis is further supported by our observations that the invasion capacity of a $\Delta P_{1-3_{inlA}}$ mutant was similar to that of the $\Delta sigB$ strain and its virulence capacity was similar to that observed for the $\Delta P_{4_{inlA}}$ mutant. Reduced numbers of the $\Delta sigB$ strain relative to those of the $\Delta P_{4_{inlA}}$ strain further suggest that σ^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^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* $\Delta sigB$ mutant in the intragastrically infected guinea pig model, the same $\Delta sigB$ 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 $\Delta sigB$ mutant in intraperitoneally infected mice; $\Delta sigB$ 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^B is crucial for gastrointestinal infection in a host that expresses an E-cadherin isoform that allows InlA binding, σ^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^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 $\Delta sigB$ mutants to date are not appropriate for evaluation of either fetal or central nervous system infections. In particular, future experiments to measure σ^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* $\Delta sigB$ 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^B has limited importance in facilitating *L. monocytogenes* survival in host cells (25, 26, 36). We report no virulence attenuation for the $\Delta sigB$ 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 $\Delta sigB$ and wild-type strains (36). In summary, the results of the present study support a model in which σ^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.

ACKNOWLEDGMENTS

We thank B. Bowen for creation of mutant strains and K. Nightingale for help with statistical analyses.

This work was supported by National Institutes of Health award RO1-AI052151-01A1 (to K.J.B.).

REFERENCES

- Bakardjiev, A. I., B. A. Stacy, S. J. Fisher, and D. A. Portnoy. 2004. Listeriosis in the pregnant guinea pig: a model of vertical transmission. *Infect. Immun.* 72:489–497.
- Begley, M., R. D. Sleator, C. G. M. Gahan, and C. Hill. 2005. Contribution of three bile-associated loci, *bsh*, *pva*, and *bilB*, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. *Infect. Immun.* 73:894–904.
- Benson, A. K., and W. G. Haldenwang. 1993. The sigma B-dependent promoter of the *Bacillus subtilis* *sigB* operon is induced by heat shock. *J. Bacteriol.* 175:1929–1935.
- Bergmann, B., D. Raffelsbauer, M. Kuhn, M. Goetz, S. Hom, and W. Goebel. 2002. InlA- but not InlB-mediated internalization of *Listeria monocytogenes* by non-phagocytic mammalian cells needs the support of other internalins. *Mol. Microbiol.* 43:557–570.
- Bishop, D. K., and D. J. Hinrichs. 1987. Adoptive transfer of immunity to *Listeria monocytogenes*. The influence of in vitro stimulation on lymphocyte subset requirements. *J. Immunol.* 139:2005–2009.
- Camilli, A., L. G. Tilney, and D. A. Portnoy. 1993. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. *Mol. Microbiol.* 8:143–157.
- Cheng, L. W., and D. A. Portnoy. 2003. *Drosophila* S2 cells: an alternative infection model for *Listeria monocytogenes*. *Cell. Microbiol.* 5:875–885.
- Drams, S., C. Kocks, C. Forestier, and P. Cossart. 1993. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator PrfA. *Mol. Microbiol.* 9:931–941.
- Dussurget, O., D. Cabanes, P. Dehoux, M. Lecuit, C. Buchrieser, P. Glaser, and P. Cossart. 2002. *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. *Mol. Microbiol.* 45:1095–1106.
- Entenza, J. M., P. Moreillon, M. M. Senn, J. Kormanec, P. M. Dunman, B. Berger-Bachi, S. Projan, and M. Bischoff. 2005. Role of sigma B in the expression of *Staphylococcus aureus* cell wall adhesins ClfA and FnbA and contribution to infectivity in a rat model of experimental endocarditis. *Infect. Immun.* 73:990–998.
- Ferreira, A., C. P. O'Byrne, and K. J. Boor. 2001. Role of σ^B in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 67:4454–4457.

12. Ferreira, A., D. Sue, C. P. O'Byrne, and K. J. Boor. 2003. Role of *Listeria monocytogenes* sigma B in survival of lethal acidic conditions and in the acquired acid tolerance response. *Appl. Environ. Microbiol.* **69**:2692–2698.
13. Fraser, K. R., D. Sue, M. Wiedmann, K. Boor, and C. P. O'Byrne. 2003. Role of sigma B in regulating the compatible solute uptake systems of *Listeria monocytogenes*: osmotic induction of *opuC* is sigma B dependent. *Appl. Environ. Microbiol.* **69**:2015–2022.
14. Freitag, N. E., and D. A. Portnoy. 1994. Dual promoters of the *Listeria monocytogenes* *prfA* transcriptional activator appear essential in vitro but are redundant in vivo. *Mol. Microbiol.* **12**:845–853.
15. Freitag, N. E., L. Rong, and D. A. Portnoy. 1993. Regulation of the *prfA* transcriptional activator of *Listeria monocytogenes*: multiple promoter elements contribute to intracellular growth and cell-to-cell spread. *Infect. Immun.* **61**:2537–2544.
16. Gahan, C. G., and C. Hill. 2005. Gastrointestinal phase of *Listeria monocytogenes* infection. *J. Appl. Microbiol.* **98**:1345–1353.
17. Gedde, M. M., D. E. Higgins, L. G. Tilney, and D. A. Portnoy. 2000. Role of listeriolysin O in cell-to-cell spread of *Listeria monocytogenes*. *Infect. Immun.* **68**:999–1003.
18. Gregory, S. H., A. J. Sagnimeni, and E. J. Wing. 1996. Bacteria in the bloodstream are trapped in the liver and killed by immigrating neutrophils. *J. Immunol.* **157**:2514–2520.
19. Horsburgh, M. J., J. L. Aish, I. J. White, L. Shaw, J. K. Lithgow, and S. J. Foster. 2002. Sigma B modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. *J. Bacteriol.* **184**:5457–5467.
20. Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *BioTechniques* **8**:528–535.
21. Jin, Y., L. Dons, K. Kristensson, and M. E. Rottenberg. 2001. Neural route of cerebral *Listeria monocytogenes* murine infection: role of immune response mechanisms in controlling bacterial neuroinvasion. *Infect. Immun.* **69**:1093–1100.
22. Johansson, J., P. Mandin, A. Renzoni, C. Chiaruttini, M. Springer, and P. Cossart. 2002. An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* **110**:551–561.
23. Jones, S., and D. A. Portnoy. 1994. Characterization of *Listeria monocytogenes* pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O. *Infect. Immun.* **62**:5608–5613.
24. Kazmierczak, M. J., S. C. Mithoe, K. J. Boor, and M. Wiedmann. 2003. *Listeria monocytogenes* sigma B regulates stress response and virulence functions. *J. Bacteriol.* **185**:5722–5734.
25. Kim, H., K. J. Boor, and H. Marquis. 2004. *Listeria monocytogenes* sigma B contributes to invasion of human intestinal epithelial cells. *Infect. Immun.* **72**:7374–7378.
26. Kim, H., H. Marquis, and K. J. Boor. 2005. Sigma B contributes to *Listeria monocytogenes* invasion by controlling expression of *inlA* and *inlB*. *Microbiology* **151**:3215–3222.
27. Lecuit, M., and P. Cossart. 2002. Genetically-modified-animal models for human infections: the *Listeria* paradigm. *Trends Mol. Med.* **8**:537–542.
28. Lecuit, M., S. Dramsi, C. Gottardi, M. Fedor-Chaiken, B. Gumbiner, and P. Cossart. 1999. A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. *EMBO J.* **18**:3956–3963.
29. Lecuit, M., D. M. Nelson, S. D. Smith, H. Khun, M. Huerre, M. C. Vacher-Lavenu, J. I. Gordon, and P. Cossart. 2004. Targeting and crossing of the human maternofetal barrier by *Listeria monocytogenes*: role of internalin interaction with trophoblast E-cadherin. *Proc. Natl. Acad. Sci. USA* **101**:6152–6157.
30. Lecuit, M., S. Vandormael-Pournin, J. Lefort, M. Huerre, P. Gounon, C. Dupuy, C. Babinet, and P. Cossart. 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science* **292**:1722–1725.
31. Lingnau, A., E. Domann, M. Hudel, M. Bock, T. Nichterlein, J. Wehland, and T. Chakraborty. 1995. Expression of the *Listeria monocytogenes* EGD *inlA* and *inlB* genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms. *Infect. Immun.* **63**:3896–3903.
32. Mackaness, G. B. 1962. Cellular resistance to infection. *J. Exp. Med.* **116**:381–406.
33. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5**:607–625.
34. Mengaud, J., S. Dramsi, E. Gouin, J. A. Vázquez-Boland, G. Milon, and P. Cossart. 1991. Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene that is autoregulated. *Mol. Microbiol.* **5**:2273–2283.
35. Milohanic, E., P. Glaser, J. Y. Coppee, L. Frangeul, Y. Vega, J. A. Vázquez-Boland, F. Kunst, P. Cossart, and C. Buchrieser. 2003. Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. *Mol. Microbiol.* **47**:1613–1625.
36. Nadon, C. A., B. M. Bowen, M. Wiedmann, and K. J. Boor. 2002. Sigma B contributes to PrfA-mediated virulence in *Listeria monocytogenes*. *Infect. Immun.* **70**:3948–3952.
37. Nickerson, C. A., and R. Curtiss III. 1997. Role of sigma factor RpoS in initial stages of *Salmonella typhimurium* infection. *Infect. Immun.* **65**:1814–1823.
38. Rauch, M., Q. Luo, S. Muller-Altroch, and W. Goebel. 2005. SigB-dependent in vitro transcription of *prfA* and some newly identified genes of *Listeria monocytogenes* whose expression is affected by PrfA in vivo. *J. Bacteriol.* **187**:800–804.
39. Raybourne, R. B. 2002. Virulence testing of *Listeria monocytogenes*. *J. AOAC Int.* **85**:516–523.
40. Schlech, W. F., III. 2000. Foodborne listeriosis. *Clin. Infect. Dis.* **31**:770–775.
41. Schwab, U., B. Bowen, C. Nadon, M. Wiedmann, and K. J. Boor. 2005. The *Listeria monocytogenes* *prfA*P2 promoter is regulated by sigma B in a growth phase dependent manner. *FEMS Microbiol. Lett.* **245**:329–336.
42. Sleator, R. D., G. A. Francis, D. O'Beirne, C. G. Gahan, and C. Hill. 2003. Betaine and carnitine uptake systems in *Listeria monocytogenes* affect growth and survival in foods and during infection. *J. Appl. Microbiol.* **95**:839–846.
43. Stritzker, J., C. Schoen, and W. Goebel. 2005. Enhanced synthesis of internalin A in *aro* mutants of *Listeria monocytogenes* indicates posttranscriptional control of the *inlAB* mRNA. *J. Bacteriol.* **187**:2836–2845.
44. Sue, D., K. J. Boor, and M. Wiedmann. 2003. Sigma B dependent expression patterns of compatible solute transporter genes *opuCA* and *lmo1421* and the conjugated bile salt hydrolase gene *bsh* in *Listeria monocytogenes*. *Microbiology* **149**:3247–3256.
45. Sue, D., D. Fink, M. Wiedmann, and K. J. Boor. 2004. Sigma B-dependent gene induction and expression in *Listeria monocytogenes* during osmotic and acid stress conditions simulating the intestinal environment. *Microbiology* **150**:3843–3855.
46. Sun, A. N., A. Camilli, and D. A. Portnoy. 1990. Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. *Infect. Immun.* **58**:3770–3778.
47. Wiedmann, M., T. J. Arvik, R. J. Hurley, and K. J. Boor. 1998. General stress transcription factor sigma B and its role in acid tolerance and virulence of *Listeria monocytogenes*. *J. Bacteriol.* **180**:3650–3656.
48. Wing, E. J., and S. H. Gregory. 2002. *Listeria monocytogenes*: clinical and experimental update. *J. Infect. Dis.* **185**(Suppl 1):S18–S24.