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Listeria monocytogenes σ^B Modulates PrfA-Mediated Virulence Factor Expression[▽][†]

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Listeria monocytogenes **σ^B and positive regulatory factor A (PrfA) are pleiotropic transcriptional regulators that coregulate a subset of virulence genes. A positive regulatory role for σ^B in *prfA* transcription has been well established; therefore, observations of increased virulence gene expression and hemolytic activity in a Δ*sigB* strain initially appeared paradoxical. To test the hypothesis that *L. monocytogenes* σ^B contributes to a regulatory network critical for appropriate repression as well as induction of virulence gene expression, genome-wide transcript profiling and follow-up quantitative reverse transcriptase PCR (qRT-PCR), reporter fusion, and phenotypic experiments were conducted using *L. monocytogenes prfA**, *prfA** Δ*sigB*, Δ*prfA*, and Δ*prfA* Δ*sigB* strains. Genome-wide transcript profiling and qRT-PCR showed that in the presence of active PrfA (PrfA*), σ^B is responsible for reduced expression of the PrfA regulon. σ^B-dependent modulation of PrfA regulon expression reduced the cytotoxic effects of a PrfA* strain in HepG2 cells, highlighting the functional importance of regulatory interactions between PrfA and σ^B. The emerging model of the role of σ^B in regulating overall PrfA activity includes a switch from transcriptional activation at the $P2_{prfA}$ promoter (e.g., in extracellular bacteria when PrfA activity is low) to posttranscriptional downregulation of PrfA regulon expression (e.g., in intracellular bacteria when PrfA activity is high).**

Listeria monocytogenes is a food-borne pathogen capable of transitioning from saprotrophic survival in the environment (55) to intracellular infection in a wide range of hosts (21, 65). While cellular *L. monocytogenes* infection and the systemic stages of listeriosis have been studied extensively (14, 29, 74), less attention has been directed to the preceding phases of the infection process (e.g., bacterial survival in foods and in the human gastrointestinal tract). Mounting evidence indicates that the transition of *L. monocytogenes* from saprotroph to pathogen relies upon regulatory networks that fine-tune virulence factor expression in response to environmental signals (29). These networks include genes that regulate the bacterial stress response and survival, therefore contributing to transmission of *L. monocytogenes*, including that during the gastrointestinal and systemic stages of infection (12, 26). One important network that links stress response and virulence in *L. monocytogenes* is coregulated by σ^B and positive regulatory factor A (PrfA) (12, 49, 56).

σ^B, an alternative sigma factor, regulates genes that are important for environmental stress survival in gram-positive bacteria (36, 59, 75). In *L. monocytogenes*, σ^B contributes to survival under low pH, oxidative stress, and carbon starvation (13, 22, 23, 35, 76). In addition to stress response genes, the *L. monocytogenes* σ^B regulon also includes some genes that are important for virulence (36, 59). Examples of σ^B-dependent virulence-associated genes include *inlAB*, encoding internalins A and B (38, 39, 42), which are important for invasion of human epithelial cells, as well as *hfq*, encoding the Hfq RNA binding protein (13), and *bsh*, encoding bile salt hydrolase (18). Phenotypically, an *L. monocytogenes* Δ*sigB* strain is less invasive in human tissue culture cells and has reduced virulence in guinea pigs following intragastric infection relative to an otherwise isogenic parent strain (26, 38).

PrfA is a member of the Crp (cyclic AMP receptor protein)/Fnr (fumarate and nitrate reduction regulator) family of transcriptional regulators (41). PrfA positively regulates the expression of virulence genes essential for intracellular survival of *L. monocytogenes* (e.g., *hly*, *mpl*, *plcA*, *actA*, and *plcB*) (28, 73). PrfA recognizes a 14-bp palindromic "PrfA box" sequence, which is typically located ~40 nucleotides upstream of a target transcriptional start site (7, 24, 25, 69). Regulation of *prfA* expression and PrfA activity is complex, occurring at transcriptional and posttranscriptional levels. At the posttranscriptional level, PrfA activity is influenced by a number of environmental factors and physiological states, including the presence of fermentable carbohydrates (5, 16, 61, 63) or activated charcoal (20, 61, 63), intracellular status (43, 50), growth in minimal medium (5, 71), and entry into stationary phase (47, 64, 71). Several *prfA* mutations that yield constitutively active PrfA (PrfA*) have been identified (70). Compared to wild-type PrfA, PrfA* has a greater affinity for the PrfA box (19), which appears to result in increased relative expression of PrfA-regulated target genes. As a consequence, PrfA* strains are useful tools for in vitro simulation of the high PrfA activity levels typical of intracellular *L. monocytogenes* cells, i.e., transcript levels of the PrfA-dependent gene *plcA* are similar in a PrfA* strain grown in a liquid medium (46) and in intracellular *L. monocytogenes*.

At the transcriptional level, *prfA* is transcribed from three different promoter sites (Fig. 1). One promoter site (P_{plcA}), which is located upstream of *plcA*, can initiate synthesis of a bicistronic mRNA comprising *plcA* and *prfA* (8, 24). Two pro-

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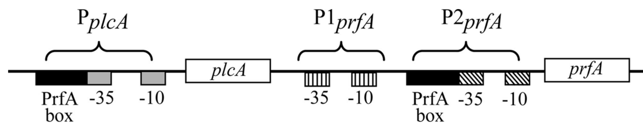

FIG. 1. *L. monocytogenes plcA-prfA* region (not drawn to scale). The line represents the DNA sequence, with the *plcA* and *prfA* coding sequences indicated as open boxes. Promoter regions contributing to *prfA* transcription are indicated by brackets above the line. Regulatory elements for each promoter region are indicated below the line. $P2_{prfA}$ -35 and -10 promoter sites represent sequences for two overlapping promoters, one of which is σ^B dependent and one of which is σ^A dependent.

moter sites ($P1_{prfA}$ and $P2_{prfA}$) are located immediately upstream of *prfA* (25). The $P1_{prfA}$ promoter is recognized by the *L. monocytogenes* housekeeping sigma factor σ^A (48). The $P2_{prfA}$ promoter region consists of two overlapping promoters, one recognized by σ^A and one recognized by σ^B (53, 60, 67), indicating a positive regulatory role for σ^B in *prfA* expression. This positive regulatory role for σ^B initially appeared to be contradicted by observations of increased virulence gene expression and hemolytic activity in a Δ*sigB* strain (10, 33, 53, 59). However, in the gram-positive pathogen *Staphylococcus aureus*, σ^B appears to have both positive and negative roles in regulating expression of virulence factors, positively influencing expression of a number of adhesins and negatively influencing expression of numerous exoenzymes and toxins (3). Therefore, to test the hypothesis that as with *S. aureus* σ^B, *L. monocytogenes* σ^B contributes to a regulatory network critical for appropriate induction and repression of virulence gene expression, comprehensive microarray-based genome-wide transcript profiling was conducted using *L. monocytogenes prfA**, *prfA** Δ*sigB*, Δ*prfA*, and Δ*prfA* Δ*sigB* strains. To gain further insight into the functional relevance of the emerging σ^B-PrfA regulatory network suggested by the transcriptional profiling results, microarray analyses were followed by quantitative reverse transcriptase PCR (qRT-PCR) and phenotypic studies.

MATERIALS AND METHODS

Bacterial growth. To enable identification of PrfA- and σ^B-coregulated genes, growth conditions and strains were selected to provide high levels of activity for both proteins. To illustrate, because entry into stationary phase induces σ^B-dependent transcription (10, 59), early-stationary-phase cells were used to ensure high σ^B activity. An *L. monocytogenes* strain with a *prfA**(*G155S*) allele was used to constitutively express high levels of PrfA-regulated virulence genes (46, 52, 70). *L. monocytogenes* stock cultures of the strains used in this study (Table 1) were stored at −80°C in brain heart infusion broth (BHI broth; Difco, Detroit, MI) plus 15% glycerol. Cultures were streaked from stocks onto BHI agar and incubated at 37°C for ~24 h to obtain single colonies. Individual colonies of each stock culture were inoculated into 5 ml BHI broth and incubated at 37°C with aeration (shaking at 250 rpm) for 12 h, followed by serial passages as described below to maximize the relative proportion of viable cells present in a similar growth state. Specifically, 50 μl of a 12-h culture was inoculated into 5 ml fresh, prewarmed BHI broth. Cells were then grown to mid-logarithmic phase (optical density at 600 nm = 0.4), diluted 1:100 in 50 ml prewarmed BHI broth in 300-ml Nephelo culture flasks (Bellco Glass Co., Vineland, NJ), and grown to early stationary phase (i.e., growth was monitored to an optical density at 600 nm of 1.0, and then the culture was incubated for an additional 3 h).

RNA extraction. RNA extraction from *L. monocytogenes* was conducted as previously described (59). Briefly, 2 volumes of RNAprotect bacterial reagent (Qiagen, Valencia, CA) was added to each bacterial culture just prior to harvest. The mixture was vortexed and incubated for 5 min at room temperature. Cells were harvested by centrifugation for 5 min at 5,000 × *g* and stored at −80°C. RNA isolation was performed using an RNeasy Midi kit (Qiagen, Valencia, CA). Samples were treated with DNase for 1 h at 37°C, using 40 units RQ1 DNase enzyme (Promega, Madison, WI) in the presence of 400 units of RNasin Plus RNase inhibitor (Promega). Each lysate was extracted with phenol-chloroform, and then RNA was precipitated using a 1/10 volume of 3 M sodium acetate and 2.5 volumes of ice-cold 100% ethanol. Precipitated RNA was stored at −80°C. Prior to use, RNA quality was assessed using agarose gel electrophoresis and measurement of 260/280 and 260/230 absorption ratios, using a NanoDrop-1000 instrument (NanoDrop Technologies, Wilmington, DE).

cDNA labeling and microarray hybridization. cDNA labeling was performed as previously described (59), with minor modifications. Briefly, cDNA synthesis and labeling of total RNA were performed using the SuperScript Plus indirect cDNA labeling system for DNA microarrays (Invitrogen, Carlsbad, CA). For cDNA synthesis, 10 μg total RNA was mixed with 5 μg random hexamers in a total volume of 18 μl RNase-free water. The RNA-primer mix was incubated for 10 min at 70°C, with a subsequent chill on ice for at least 5 min. After the addition of Superscript III RT, amino-modified deoxynucleoside triphosphates, dithiothreitol, RNaseOUT, and buffer, the reaction mix was incubated at 42°C for approximately 17 h to allow cDNA synthesis. RNA was hydrolyzed with the addition of 10 μl 1 N NaOH and 10 μl 0.5 M EDTA, followed by incubation at 65°C for 15 min. Prior to cDNA purification using a Qiagen PCR purification kit (Qiagen), the mixture was neutralized using 10 μl 1 N HCl. cDNA labeling reactions with Alexa Fluor 555 or Alexa Fluor 647 fluorescent dyes were performed for 2 h at room temperature according to the manufacturer's protocol. Differentially labeled cDNAs from the two strains to be cohybridized were combined, dried in a Savant SVC100 Speed-Vac (Farmingdale, NY), and stored at −80°C until hybridization.

Microarrays were constructed as previously described (9, 59). Briefly, 70-mer probes targeting 2,857 *L. monocytogenes* open reading frames (ORFs) (Array-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference or construction[a]
pNF771	*prfA**(*G155S*)	70
pBMB54	$P1P2_{prfA}$-*gus*	This work
pBMB6	$\Delta P1(-10)P2_{prfA}$-*gus*	67
pBMB7	$P1\Delta P2(-10)_{prfA}$-*gus*	67
pBMB8	$\Delta P1(-10)\Delta P2(-10)_{prfA}$-*gus*	67
10403S	Parent strain; serotype 1/2a	4
FSL A1-254	Δ*sigB*	76
FSL B2-046	Δ*prfA*	47
FSL B2-068	Δ*sigB* Δ*prfA*	47
FSL B2-237	*prfA**(*G155S*)	pNF771→10403S
FSL B2-238	*prfA**(*G155S*) Δ*sigB*	pNF771→FSL A1-254
FSL B2-220	$P1P2_{prfA}$-*gus* Δ*prfA*	pBMB54→FSL B2-046
FSL B2-153	$P1\Delta P2(-10)_{prfA}$-*gus* Δ*prfA*	pBMB7→FSL B2-046
FSL B2-149	$\Delta P1(-10)P2_{prfA}$-*gus* Δ*prfA*	pBMB6→FSL B2-046
FSL B2-165	$\Delta P1(-10)\Delta P2(-10)_{prfA}$-*gus* Δ*prfA*	pBMB8→FSL B2-046
FSL B2-221	$P1P2_{prfA}$-*gus* Δ*sigB* Δ*prfA*	pBMB54→FSL B2-068
FSL B2-152	$P1\Delta P2(-10)_{prfA}$-*gus* Δ*sigB* Δ*prfA*	pBMB7→FSL B2-068
FSL B2-148	$\Delta P1(-10)P2_{prfA}$-*gus* Δ*sigB* Δ*prfA*	pBMB6→FSL B2-068
FSL B2-164	$\Delta P1(-10)\Delta P2(-10)_{prfA}$-*gus* Δ*sigB* Δ*prfA*	pBMB8→FSL B2-068
FSL B2-243	$P1P2_{prfA}$-*gus prfA**	pBMB54→FSL B2-237
FSL B2-241	$P1\Delta P2(-10)_{prfA}$-*gus prfA**	pBMB7→FSL B2-237
FSL B2-240	$\Delta P1(-10)P2_{prfA}$-*gus prfA**	pBMB6→FSL B2-237
FSL B2-242	$\Delta P1(-10)\Delta P2(-10)_{prfA}$-*gus prfA**	pBMB8→FSL B2-237
FSL B2-252	$P1P2_{prfA}$-*gus prfA** Δ*sigB*	pBMB54→FSL B2-238
FSL B2-250	$P1\Delta P2(-10)_{prfA}$-*gus prfA** Δ*sigB*	pBMB7→FSL B2-238
FSL B2-249	$\Delta P1(-10)P2_{prfA}$-*gus prfA** Δ*sigB*	pBMB6→FSL B2-238
FSL B2-251	$\Delta P1(-10)\Delta P2(-10)_{prfA}$-*gus prfA** Δ*sigB*	pBMB8→FSL B2-238

[a] Arrows denote transformation of the plasmid into the recipient strain.

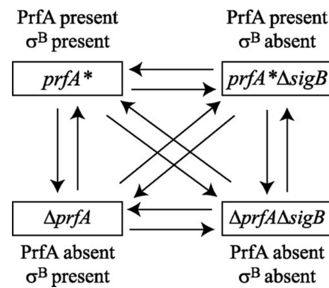

FIG. 2. Schematic of the double loop design used to compare transcript levels in *L. monocytogenes prfA**, *prfA** Δ*sigB*, Δ*prfA*, and Δ*prfA* Δ*sigB* strains. Each pairwise comparison was performed for four independent biological replicates. Each arrow represents two different biological replicates. Arrowheads represent Alexa Fluor 555-labeled RNA, and arrow bases represent Alexa Fluor 647-labeled RNA. Factors used for two-way ANOVA are indicated above or below the strain designation.

Ready Oligo set; Operon Technologies, Huntsville, AL) (designed from the *L. monocytogenes* EGD-e genome sequence [27]) were spotted onto Corning UltraGAPS slides (Corning Inc., Corning, NY) at the Microarray Core Facility at Cornell University (Ithaca, NY). EGD-e and *L. monocytogenes* 10403S both represent the same *L. monocytogenes* lineage (II), serotype (1/2a), and ribotype (DUP-1039C), and therefore probes designed using the EGD-e genome were expected to hybridize well with 10403S genes (9, 59). Cross-hybridization identities between the EGD-e probes and the target genes in strain 10403S were determined using the unfinished genome sequence for strain 10403S (9, 59; *Listeria monocytogenes* Database [http://www.broad.mit.edu/annotation/genome/listeria_group/MultiHome.html]). The array used here allows comprehensive identification of differentially expressed genes in strain 10403S, with the possibility of some false-negative results (9, 59).

Microarray hybridization was performed as previously described (59), with minor modifications. Prior to hybridization, spotted microarray slides were incubated for 1 h in a 1% bovine serum albumin–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) solution prewarmed to 42°C. Subsequently, slides were washed twice for 5 min in 0.1× SSC and twice for 1 min in filtered water and then dried by centrifugation at 1,800 rpm for 3 min. The combined cDNA targets were reconstituted in 55 μl hybridization buffer and denatured at 95°C for 5 min. Targets were applied to microarray slides and overlaid with mSeries LifterSlips (Erie Scientific, Portsmouth, NH), followed by overnight hybridization at 42°C. Posthybridization washes were performed for (i) 5 min in 42°C prewarmed 2× SSC plus 0.1% SDS, (ii) 5 min in 2× SSC, and (iii) 2.5 min in 0.2× SSC. After a final wash in filtered water, slides were dried by centrifugation and scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) at the Cornell University Microarray Core Facility.

Statistical analysis of microarray data. The microarray experiments were designed as a balanced double loop (Fig. 2) to allow direct comparison of results for any given strain to those for any other strain used in this study (37). This design allowed us to identify genes regulated by σ^B, PrfA, or both proteins. Raw intensity values for all probes on each array were normalized using pin-tip LOWESS (58) in R v.2.2.1 with the MAANOVA (v. 0.98-8) package (R/MAANOVA [http://www.bioconductor.org/packages/bioc/1.7/src/contrib/html/maanova.html]). Signals from two replicate probes on each array were averaged, and $\log_2$ transformations were performed after normalization. Differences in transcript levels between strains were determined using a mixed-model one-way analysis of variance (ANOVA) in R/MAANOVA, where Y (the $\log_2$-transformed intensity data) = array + dye + strain + sample (biological replicate) + E (error). Differences in transcript levels were considered meaningful if they met both adjusted P values of <0.05 and changes of >1.5-fold.

Contributions of both PrfA and σ^B (SigB) to changes in transcript levels were determined using a mixed-model two-way ANOVA, where Y (the $\log_2$-transformed intensity data) = array + dye + PrfA + SigB + SigB* PrfA + sample + E. In this model, the factors "PrfA" and "SigB" can have one of two levels, determined by the presence or absence of the protein in the tested strain (Fig. 2). ANOVA modeling allows for consideration of appropriate error structures for experiments with multiple sources of variation in microarray measurements (37). The random effects of the models were biological replicate and array effects, whereas the fixed effects were PrfA, SigB, and dye effects. The Fs statistic, a shrinkage estimator for gene-specific variance components that makes no assumptions about the distribution of variances across genes, was estimated (15). Significant differences in expression between strains were determined by calculating the P values for the Fs statistic for each gene, using 1,000 random permutations. The P values were adjusted to correct for type I error with the Benjamini-Hochberg (B-H) linear step-up correction implemented in R/MAANOVA, with a cutoff adjusted P value of <0.05. Pairwise contrasts of individual mutants were estimated by the t test in R/MAANOVA. Contrast P values were corrected for multiple testing by using the Benjamini-Hochberg step-up correction.

Expression profiles of the 607 ORFs with significant differences (adjusted P values of <0.05) in at least one strain-to-strain comparison (as determined by one-way ANOVA) were analyzed using quality threshold (QT) clustering, which groups genes with similar expression profiles based on jackknife correlations (32). QT clustering of significant ORFs was conducted in MeV 4.0 (www.tigr.org), with a diameter of 0.5 and a minimum cluster size of 5.

TaqMan qRT-PCR. TaqMan primer and probe sets (see Table S1 in the supplemental material) were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). qRT-PCR was performed as described by Sue et al. (72), using TaqMan one-step RT-PCR master mix reagent, Multiscribe RT, and an ABI Prism 7000 sequence detection system (all from Applied Biosystems). qRT-PCRs excluding Multiscribe RT were run in parallel to quantify genomic DNA contamination. DNA standard curves for each gene were included to allow for absolute quantification of cDNA levels. Each qRT-PCR was run in duplicate on each of the four RNA samples that were used for the microarray experiments. The *L. monocytogenes* housekeeping genes *rpoB* and *gap* were included to allow for normalization of absolute transcript levels as described previously (11, 36). Data analysis was conducted with ABI Prism 7000 SDS software, and significant differences in transcript levels between the different strains were determined by ANOVA as described previously (72). To confirm the two-way ANOVA results for the microarray data, transcript levels determined by qRT-PCR were analyzed using a two-way ANOVA model similar to that described above, with the following linear model: transcript level Y = PrfA + SigB + SigB* PrfA + sample + E.

Hemolytic activity assays. Hemolytic activity assays using purified sheep red blood cells were performed as previously described (10) to assess hemolytic activity in the supernatants of bacterial cultures. Hemolytic activity assays were performed on strains grown independently three times; resulting hemolytic units were averaged.

Invasion assays. Invasion assays were performed using the human hepatic epithelial cell line HepG2 (ATCC HB-8065) as described previously (39), with minor modifications. Briefly, HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM). Tissue culture cells were seeded 2 days before infection, at a density of 7.5×10^4 cells per well, in 24-well tissue culture plates and incubated at 37°C (5% CO_2). Infection of HepG2 cells was performed using approximately 2×10^7 CFU of *L. monocytogenes* cells grown to early stationary phase as described above. At 30 min postinfection, cells were washed three times using prewarmed sterile phosphate-buffered saline to remove extracellular bacteria. Subsequently, 1 ml of fresh prewarmed DMEM was added to each well, plates were incubated for an additional 15 min to allow attached *L. monocytogenes* cells to enter HepG2 cells, and then the DMEM was removed and replaced with 1 ml prewarmed DMEM containing 150 μg/ml gentamicin to kill remaining extracellular bacteria. The 150-μg/ml gentamicin concentration was selected to reduce the risk of survival and (false-positive) detection of extracellular *L. monocytogenes*, particularly as we used a short incubation period with gentamicin (i.e., 45 min). While the gentamicin concentration used is higher than that in some other studies, it is well within the range of concentrations previously reported, including the use of 150 μg/ml gentamicin by Kim et al. (38) for invasion assays with Henle 2 cells, the recommended use of 150 μg/ml gentamicin for Caco-2 cell invasion assays (44), and the use of an even higher concentration (i.e., 250 μg/ml) by Moroni et al. (51). After a final incubation of 45 min, HepG2 cells were lysed by the addition of ice-cold sterile distilled water. Numbers of intracellular *L. monocytogenes* cells were determined by plating the cell suspensions on BHI agar, using a spiral plater (Autoplate 4000; Spiral Biotech, Bethesda, MD). Plates were incubated at 37°C for ~24 h before enumerating colonies by use of QCount (Spiral Biotech). The invasion efficiency was calculated as the number of bacteria recovered from a HepG2 cell suspension (in CFU/ml) divided by the initial bacterial number (in CFU/ml) used for the inoculation. Three independent biological replicates were performed for each strain (triplicate wells were infected with a given strain).

Cytotoxicity assay. As a measure of the cytotoxic interactions between *L. monocytogenes* strains and HepG2 cells, lactate dehydrogenase (LDH) release from HepG2 cells following infection was measured using a CytoTox 96 nonra-

dioactive cytotoxicity assay kit (Promega, Madison, WI) as previously described by Decatur and Portnoy (17), with minor modifications. HepG2 cells were grown, seeded, and infected as described previously (17). The experimental design included three wells containing only DMEM to account for background absorption as well as three wells containing uninfected HepG2 cells to measure spontaneous LDH release. At 30 min postinfection, DMEM was removed and replaced with DMEM containing 150 μg/ml gentamicin, followed by incubation at 37°C for 1 h. To determine maximum LDH release, 100 μl of lysis buffer was added to triplicate infected wells 45 min prior to LDH measurement. At 90 min postinfection, the 24-well plates were centrifuged at 250 × *g* for 4 min, using a Sorvall RT600B swing-bucket centrifuge (Kendro, Asheville, NC). A 50-μl aliquot of the supernatant was removed and used for the LDH assay. The supernatant was incubated for 30 min with 50 μl substrate mix prior to the addition of 50 μl stop solution. Absorption at 490 nm was then measured using a Packard fusion instrument (Perkin-Elmer Inc., Waltham, MA). After background correction, the percent cytotoxicity was calculated as follows: % cytotoxicity = [(experimental LDH release − spontaneous LDH release)/(maximum LDH release − spontaneous LDH release)] × 100. Three independent biological replicates of the cytotoxicity assay were performed for each strain.

GUS activity assay. Previously described GUS reporter fusion (GUS-RF) plasmids (67) were introduced into *L. monocytogenes* Δ*prfA*, Δ*sigB* Δ*prfA*, *prfA**, and *prfA** Δ*sigB* strains (Table 1) to monitor transcription from the individual promoters that contribute to *prfA* transcription (Fig. 1); one fusion plasmid (i.e., $P1P2_{prfA}$-*gus*) was reconstructed to have identical 5′ and 3′ junctions to those of all other fusions (Table 1). While the use of reporter fusions on multicopy plasmids can cause problems in interpreting reporter fusion data (e.g., due to plasmid copy number variation), all reporter fusion strains contained very similar inserts (which differed by only one or two 20-nucleotide deletions) and were grown under identical standardized conditions, making significant differences in copy numbers between the different strains unlikely in our assay. GUS activity was measured by monitoring cleavage of the β-glucuronidase substrate 4-methylumbelliferyl β-D-glucuronide (MUG) (34) in a 96-well-plate format. To maintain the extrachromosomal GUS-RF plasmid, all growth experiments were performed using BHI supplemented with 10 μg/ml chloramphenicol. *L. monocytogenes* GUS-RF strains were grown to early stationary phase as described above. Bacterial cells (1 ml) were harvested by centrifugation and washed with 1 ml 0.1 M potassium phosphate buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4, 0.1 M NaCl), and cell pellets were then flash frozen in liquid nitrogen and stored at −80°C. Bacterial numbers were determined by plating sample aliquots on BHI-chloramphenicol agar plates. Prior to GUS measurements, cell pellets were thawed and suspended in 1 ml potassium phosphate buffer. Cells were lysed by the addition of 135 μl CellLytic B reagent (Sigma-Aldrich, St. Louis, MO), followed by incubation for 10 min at room temperature. Duplicate samples of bacterial lysates (80 μl) and appropriate dilutions (in potassium phosphate buffer) were pipetted into 96-well flat-bottomed black polystyrene plates (Corning Inc., Corning, NY). The enzymatic reaction was initiated by addition of 20 μl of 0.4-mg/ml MUG (Sigma-Aldrich) in dimethyl sulfoxide. A standard curve corresponding to 3.75, 1.88, 0.94, 0.47, and 0.09 μM 4-methylumbelliferone (MU; Sigma-Aldrich) was included with every plate. The enzymatic reaction was stopped after 10 min by the addition of 1 M Na_2CO_3 stop solution. Immediately after the addition of stop solution, fluorescence was measured at 460 nm (with an excitation wavelength of 365 nm), using a Packard fusion instrument (Perkin-Elmer). The amount of background fluorescence determined for a given sample was subtracted from the fluorescence measurement in the corresponding experimental well, and the concentration of liberated MU was calculated using the standard curve. The GUS activity for each strain was measured in three independent biological replicates and reported as nM MU/log CFU/min.

Statistical analysis of data from phenotypic experiments. A mixed-model ANOVA was performed in SAS (SAS v 9.1; SAS Institute Inc., Cary, NC) to determine significant differences in invasion efficiency, LDH release, and GUS activity among the *L. monocytogenes* strains. The significance of differences in invasion efficiencies was evaluated using the following linear model: invasion efficiency = strain + biological replicate. The significance of differences in percent LDH release was determined using the following linear model: percent LDH release = strain + biological replicate. To determine the statistical significance of differences in GUS activity among the strains, a one-way ANOVA was performed, comparing the GUS activities of a given promoter fusion among the different genetic backgrounds by using the following model: GUS activity = strain (genetic background strain) + rep (biological replicate). A two-way ANOVA was also used to determine if the GUS activity was significantly affected by the presence or absence of SigB and/or PrfA, using the following model: GUS activity = SigB (presence or absence of σ^B) + PrfA (presence or absence of PrfA) + SigB* PrfA (interaction effect) + rep (biological replicate). Tukey's multiple comparison correction was applied to all ANOVA results to determine significant differences among the strains. Adjusted *P* values of <0.05 were considered statistically significant.

Microarray data accession number. Microarray data were deposited at the Gene Expression Omnibus under accession number GSE 11347.

RESULTS

σ^B- and PrfA-dependent *L. monocytogenes* genes represent eight clusters of genes with distinct transcription profiles. *L. monocytogenes* whole-genome microarrays were used to compare global gene expression of *L. monocytogenes prfA**, *prfA** Δ*sigB*, Δ*prfA*, and Δ*prfA* Δ*sigB* strains, allowing identification of genes regulated by σ^B, PrfA, or both proteins (Fig. 2). One-way ANOVA (see Table S2 in the supplemental material) with the microarray data identified 607 genes that were differentially expressed (adjusted *P* value of <0.05; ≥1.5-fold change) in at least one strain-to-strain comparison (e.g., PrfA* versus Δ*prfA* strains). Subsequently, *t* tests were used to determine significant differences in transcript levels between all possible pairs of strains. QT cluster analysis of expression profiles grouped 603 differentially expressed genes into eight clusters, with each cluster containing genes with similar expression profiles (Fig. 3), as determined by jackknife correlation (35). The expression profiles for four genes could not be placed into any of the clusters due to a lack of similarity to other identified profiles. The majority of genes (518/603 genes [86%]) were assigned to clusters 1 and 2. Relative transcript levels for genes in these clusters were not significantly different between the *prfA** and Δ*prfA* strains or between the *prfA** Δ*sigB* and Δ*prfA* Δ*sigB* strains (as determined by *t* tests), indicating that transcription of the genes in these two clusters is PrfA independent. Genes assigned to cluster 1 (n = 274) had higher relative transcript levels in Δ*sigB* strains (Δ*prfA* Δ*sigB* and *prfA** Δ*sigB* strains) than in strains carrying an intact *sigB* gene (Δ*prfA* and *prfA** strains), suggesting that σ^B negatively influences expression of these genes. Conversely, genes in cluster 2 (n = 244) had higher relative transcript levels in Δ*prfA* and *prfA** strains than in Δ*prfA* Δ*sigB* and *prfA** Δ*sigB* strains, suggesting that transcription of these genes is positively influenced by σ^B. σ^B-dependent transcription has been reported previously for 114 of the cluster 2 genes (59).

Cluster 3 contained 24 genes, including all members of the *L. monocytogenes* virulence gene cluster (see Table S2 in the supplemental material). Transcript levels of the genes in this cluster were higher in strains with constitutively active PrfA* (*prfA** and *prfA** Δ*sigB* strains) than in Δ*prfA* strains (Δ*prfA* and Δ*prfA* Δ*sigB* strains) (see Table S2 in the supplemental material), indicating that the presence of PrfA* increased transcription of these genes (Fig. 3). For genes in this cluster, the average difference in transcript levels between the PrfA* and Δ*prfA* strains was 5.2-fold, while the average difference in transcript levels between the *prfA** Δ*sigB* and Δ*prfA* Δ*sigB* strains was 12.5-fold. On average, genes in cluster 3 had 2.4-fold higher transcript levels in the *prfA** Δ*sigB* strain than in the PrfA* mutant strain, indicating that PrfA*-mediated increases in transcript levels for genes in cluster 3 are higher in the absence of σ^B than in its presence.

A total of 51 genes were assigned to clusters 4, 5, 6, 7, and 8 (Fig. 3). For genes in these clusters, the observed average difference in transcript levels between strains was generally

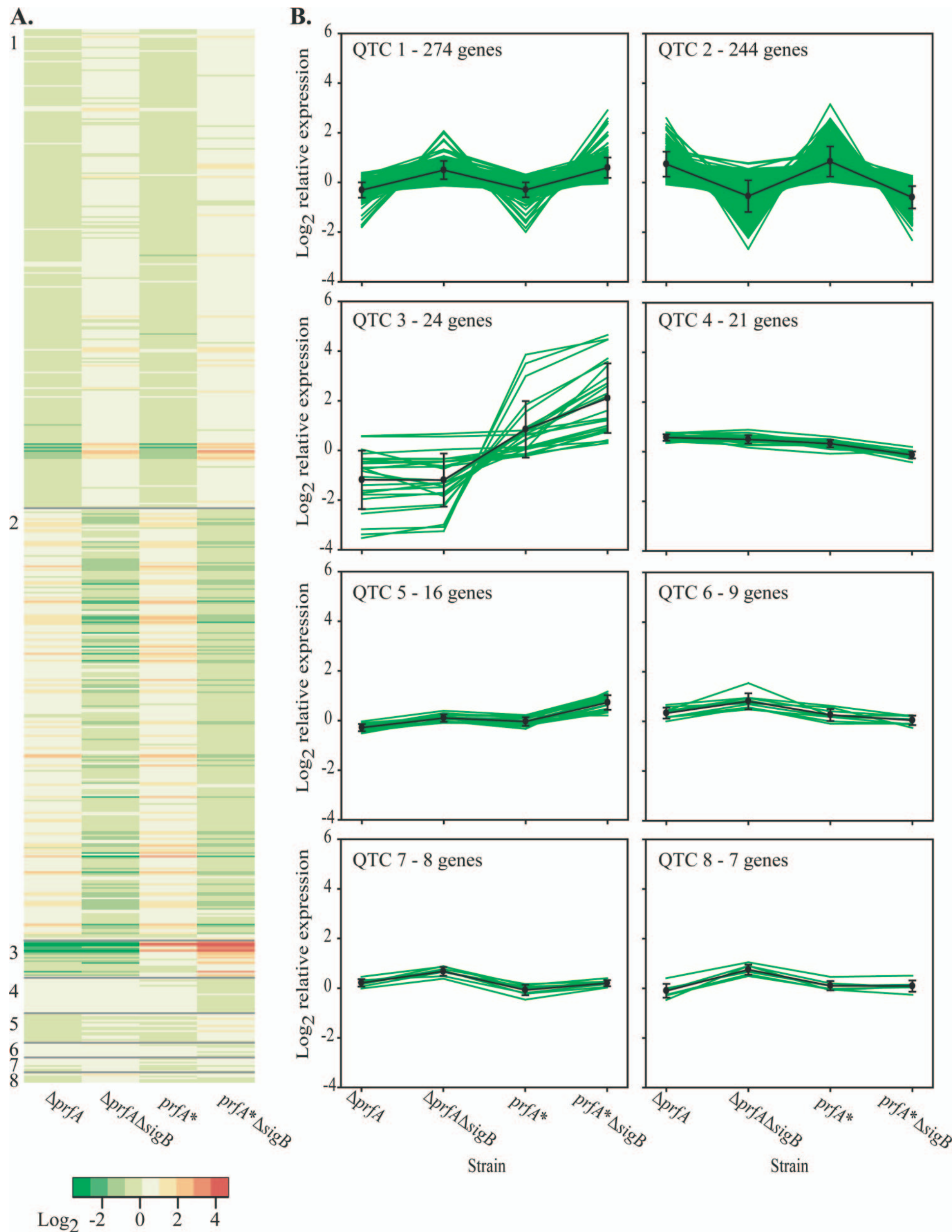

FIG. 3. QT clusters for 603 differentially expressed genes with significant changes in relative transcript levels. (A) Heat map of the $\log_2$ relative expression values for each gene in each strain. The QT clusters are separated by a gray line, and each cluster is denoted by a number on the side of the heat map. (B) For each QT cluster, the $\log_2$ relative expression values are plotted for each gene, as well as the average $\log_2$ relative expression values. For a given cluster, differences in transcript levels between strains can be determined by comparing their average $\log_2$ relative expression values. For example, while cluster 3 gene transcript levels do not differ for Δ*prfA* and Δ*sigB* Δ*prfA* strains, transcripts levels differ by ~2.3 $\log_2$ between the Δ*sigB* Δ*prfA* (average $\log_2$ expression = −1.3) and *prfA** (average $\log_2$ expression = 1.0) strains.

low, with the largest differences in transcript levels observed for cluster 5 genes. Transcript levels for genes within this cluster were, on average, twofold higher in the *prfA** Δ*sigB* strain than those in the Δ*prfA* strain. This result suggests that genes in this cluster are positively regulated by PrfA* and negatively regulated by σ^B, as the highest transcript levels were observed in the presence of PrfA* and the absence of σ^B.

Transcript levels for a number of genes are significantly affected by both PrfA* and σ^B. To identify potential interactions between σ^B and PrfA* that affect gene expression, we used a two-way ANOVA model to assess the effects of the presence or absence of PrfA* or σ^B on transcript levels. This model tested whether PrfA* significantly influenced transcript levels by comparing transcript levels observed in the presence of PrfA* to those observed in the absence of PrfA* (Fig. 2). Likewise, the effect of σ^B was tested by comparing transcript levels in the presence of σ^B to those in the absence of σ^B (see Table S3 in the supplemental material). A significant PrfA*-σ^B

TABLE 2. *L. monocytogenes* 10403S genes coregulated by PrfA* and σ^B, as determined by microarray and qRT-PCR analyses

Gene	QTC[c]	Microarray result (fold change)[f]					qRT-PCR result (fold change)[f]				
		Two-way ANOVA main effect[a]		Two-way ANOVA interaction effect with PrfA* present[b]			Two-way ANOVA main effect[a]		Two-way ANOVA interaction effect with PrfA* present[b]		
		PrfA*	SigB	SigB present	SigB absent	SigB absent/SigB present[d]	PrfA*	SigB	SigB present	SigB absent	SigB absent/SigB present[d]
hly	3	160	−1.8						620	3.7×10^3	6.0
actA[e]	3	30	−2.3						3.4×10^6	1.8×10^7	5.3
plcA	3	180	NS						2.6×10^3	8.3×10^3	3.2
plcB	3	110	−1.7				ND	ND	ND	ND	ND
lmo0207	3	8.5	−2.2				ND	ND	ND	ND	ND
lmo2219	3	7.4	NS						6.9	30	4.3
lmo2684	5	1.5	−1.6				ND	ND	ND	ND	ND
inlA[e]	3	4.5	NS				ND	ND		ND	ND
mpl	3			1.2	4.5	3.8			60	300	5
inlB	3			−1.4	4.7	6.6			5.5	85	15
inlC	3			−1.3	5.9	7.7			7.9	57	7.2
lmo0090				−1.5	2.1	3.1	NS	2.0			
lmo0443	6			1.2	−1.7	0.5			1.1	1.7	1.5
lmo0484[e]	8			1.3	−1.5	0.5	ND	ND	ND	ND	ND
lmo2261	8			1.4	−1.6	0.4	ND	ND	ND	ND	ND

[a] The column titled PrfA* reports the ratio of transcript levels in the presence of PrfA* to transcript levels in the absence of PrfA*; the column titled SigB reports the ratio of transcript levels in the presence of σ^B to transcript levels in the absence of σ^B. For genes that show a statistical interaction effect, main effects cannot be reported.

[b] For genes that show no significant statistical interaction effect, no values are shown; for genes that show a significant statistical interaction effect, transcript ratios are reported for (i) the presence of PrfA* compared to the absence of PrfA* in the presence of σ^B (column "SigB present") and (ii) the presence of PrfA* compared to the absence of PrfA* in the absence of σ^B (column "SigB absent").

[c] QT cluster, as determined from one-way ANOVA results of microarray data (see Fig. 3).

[d] Ratio of gene expression in a PrfA* background in the absence of SigB to that in the presence of SigB. Ratios were calculated by dividing the "SigB absent" statistical interaction effect by the "SigB present" statistical interaction effect. Negative values were first converted to positive values by taking the reciprocal before calculating the ratio.

[e] Cross-hybridization identities between the 10403S genes listed in this table and the EGD-e-based probes on the microarray were 100%, except for *actA* (94%), lmo0484 (98%), and *inlA* (98%).

[f] NS, not significant (change of <1.5-fold or adjusted P value of $\geq$0.05); ND, not determined using qRT-PCR.

statistical interaction effect does not enable inferences regarding potential direct or indirect regulation by the proteins, nor does it imply physical interaction between the proteins, but it simply indicates that the effect of either PrfA* or σ^B on transcript levels is influenced by the presence or absence of the other protein (Table 2).

Two-way ANOVA showed a significant main effect of PrfA* (i.e., significantly different transcript levels in the presence of PrfA* compared to those in its absence) on expression patterns for 17 genes. Specifically, for 13 genes (including the virulence genes *plcA*, *hly*, *actA*, *plcB*, *uhpT*, and *inlA*) (see Table S4 in the supplemental material), transcript levels were higher in the presence of PrfA* than in its absence, with transcript levels for *plcA*, *hly*, and *plcB* being >100-fold higher in the presence of PrfA*. For four genes (*ftsH*, *gltX*, *cysS*, and lmo0208), transcript levels were lower in the presence of PrfA* than in its absence, with 1.5- to 1.7-fold lower transcript levels in the presence of PrfA*, suggesting a negative PrfA* effect on transcript levels for these genes. However, a negative role for PrfA* in regulation of two of these genes (*ftsH* and *gltX*) was not confirmed by qRT-PCR; *cysS* and lmo0208 transcript levels were not assessed by qRT-PCR. The absence of confirmatory evidence of a negative role for PrfA in the present study is consistent with previous reports (56, 68), suggesting that PrfA's role in negative or indirect gene regulation may be weak and therefore difficult to reproduce. Among the 17 genes with a significant effect of PrfA* on transcript levels, 6 also showed a significant effect of σ^B on transcript levels. Five of these genes (i.e., *hly*, *actA*, *plcB*, lmo0207, and lmo2684) had higher transcript levels in the presence of PrfA* and lower transcript levels in the presence of σ^B (Table 2). Differences in *hly*, *actA*, and *plcB* transcript levels in the presence of σ^B than in its absence ranged from 1.7- to 2.3-fold.

In addition to the independent effects of PrfA* and σ^B on transcript levels for the 17 genes described above, two-way ANOVA of transcript levels for 7 genes (i.e., *mpl*, *inlB*, *inlC*, lmo0090, lmo0443, lmo2261, and lmo0484) identified significant statistical interaction effects for these genes, indicating that the effect of PrfA* on transcript levels was dependent on the presence or absence of σ^B or vice versa (Table 2). The effect of the presence or absence of PrfA* on transcript levels of these genes in the presence of σ^B appeared to be small, with absolute differences ranging from 1.2- to 1.5-fold. In contrast, the effect of the presence or absence of PrfA* on transcript levels of these genes was larger in the absence of σ^B, with absolute differences in transcript levels ranging from 1.5- to 5.9-fold, suggesting that PrfA* has a greater effect on transcript levels of these seven genes when σ^B is absent. For four genes, transcript levels were higher in the presence of PrfA* (indicating positive regulation), while for three genes, transcript levels were lower in the presence of PrfA*.

Comparison of positively PrfA*-regulated genes identified in this study with PrfA-dependent genes identified in previous studies. Overall, our microarray data identified 17 genes (including the virulence genes *actA*, *hly*, *plcA*, *plcB*, and *mpl*) (see Table S4 in the supplemental material) that are positively

regulated by PrfA*. Specifically, a total of 13 genes showed significantly higher transcript levels in the presence of PrfA* than in the absence of PrfA* (as determined by two-way ANOVA), while 4 genes showed significant interaction effects between σ^B and PrfA*, with higher transcript levels in the presence of PrfA*. The genes identified in this study as positively regulated by PrfA* in strain 10403S were consistent with genes previously identified as positively regulated by PrfA in strains EGD-e (49) and EGD (45), with the exceptions of lmo0107, lmo0206, lmo0217, lmo1536, lmo2219, and lmo2684, which were not identified as positively regulated by PrfA* in previous microarray studies (45, 49) (see Table S4 in the supplemental material). lmo2219 was previously identified as PrfA dependent in a proteomic study with 10403S (57).

qRT-PCR data confirm PrfA*-σ^B interaction effects on transcript levels for selected genes. qRT-PCR was used as a sensitive, quantitative approach for measuring transcript levels of genes identified as being regulated by either or both PrfA* and σ^B. Coregulation of two genes identified by microarray analysis as positively regulated by PrfA* and negatively regulated by σ^B (i.e., *hly* and *actA*) was confirmed by qRT-PCR (Table 2). qRT-PCR results also indicated an interaction effect of PrfA* and σ^B on *hly*, *actA*, *plcA*, and lmo2219 transcript levels. Microarray analyses suggested positive regulation of these four genes by PrfA and negative regulation of these genes by σ^B, although negative regulation of *plcA* and lmo2219 by σ^B did not meet our microarray cutoff criteria (adjusted P value of <0.05; change of >1.5-fold): lmo2219 had a change of -1.87-fold (adjusted P value $= 0.084$), and *plcA* had a change of -1.28-fold (adjusted P value $= 0.013$). For all four genes (i.e., *hly*, *actA*, *plcA*, and lmo2219), qRT-PCR data showed higher PrfA*/Δ*prfA* transcript ratios in the absence of σ^B (e.g., 3.7×10^3 for *hly*) than the PrfA*/Δ*prfA* transcript ratios in the presence of σ^B (e.g., 617 for *hly*) (Table 2), supporting negative regulation of these genes by σ^B in the presence of PrfA*. Together with the microarray data, the qRT-PCR results confirm an overall negative effect of σ^B on transcript levels for *hly*, *actA*, *plcA*, and lmo2219.

qRT-PCR confirmed the significant PrfA*-σ^B interaction effects on transcript levels for *mpl*, *inlC*, *inlB*, and lmo0443 that were identified in our microarray analyses. For *mpl*, qRT-PCR results were consistent with the microarray data; both data sets showed higher PrfA*/Δ*prfA* transcript ratios in the absence of σ^B (i.e., 4.5) than the transcript ratios in the presence of σ^B (i.e., 1.2) (Table 2). qRT-PCR also confirmed the microarray data for *inlB* and *inlC*. For example, in the presence of PrfA*, microarray and qRT-PCR results showed that *inlC* transcript levels were 7.7 and 7.2 times higher, respectively, in the absence of σ^B than in the presence of σ^B (Table 2), thus supporting negative regulation of *inlC* by σ^B in the presence of PrfA*. qRT-PCR confirmed the significant PrfA*-σ^B interaction effect on transcript levels for lmo0443 and found a slight negative effect of σ^B on transcript levels in the presence of PrfA* (1.5-fold) (Table 2). The negative effect of σ^B had not been observed in the microarray data, possibly reflecting the overall low changes observed (<2-fold for all comparisons) for lmo0443 (Table 2). Finally, although positive regulation of lmo0090 by σ^B was confirmed by qRT-PCR, the significant PrfA*-σ^B interaction effect suggested by the microarray data was not confirmed. Overall, significant PrfA*-σ^B interaction

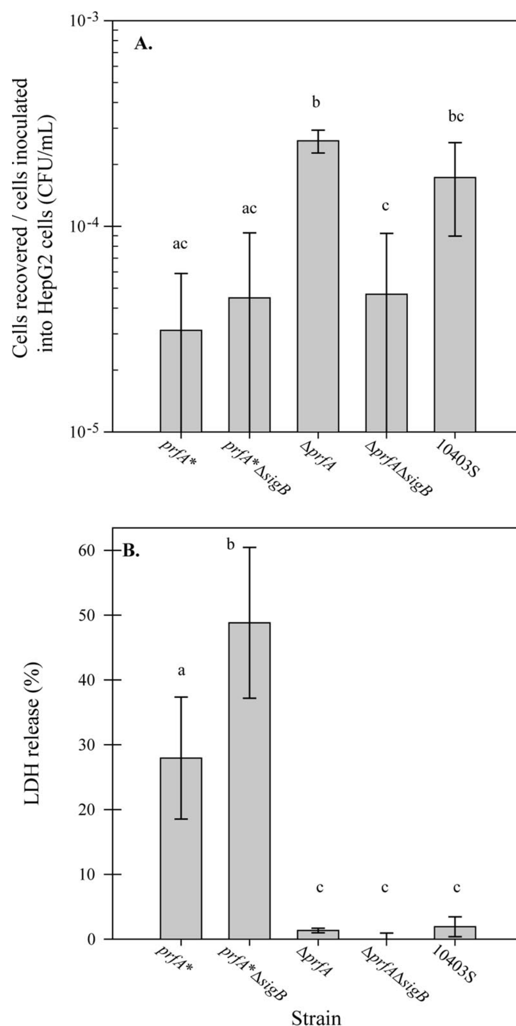

FIG. 4. Ratios of *L. monocytogenes* cells recovered to *L. monocytogenes* cells inoculated into HepG2 cells (in CFU/ml) (A) and percent LDH release from HepG2 cells infected with *L. monocytogenes* (B). The average and standard deviation for three independent biological replicates are plotted for each strain. Significant differences (adjusted P values of <0.05) between strains in either number of cells recovered from HepG2 cells after gentamicin treatment or LDH release are indicated by lowercase letters above the bars. Strains with the same letter are not significantly different.

effects on transcript levels were identified by qRT-PCR for eight genes, including four genes for which both microarray and qRT-PCR results showed significant PrfA*-σ^B interaction effects (Table 2).

Phenotypic assays confirm the importance of PrfA*-σ^B interactions on *L. monocytogenes* virulence gene expression. To further characterize the effects of *prfA* and *sigB* deletions on virulence-associated characteristics, we performed HepG2 invasion and LDH release assays as well as hemolytic activity assays with the *L. monocytogenes* 10403S parent strain and the *prfA**, *prfA** Δ*sigB*, Δ*prfA*, and Δ*prfA* Δ*sigB* strains. In the HepG2 invasion assay, 10403S and the Δ*prfA* strain showed similar recoveries of gentamicin-protected bacteria (Fig. 4A), consistent with previous data showing that PrfA is not critical for host cell invasion (29). In contrast, the Δ*prfA* Δ*sigB* strain

TABLE 3. Hemolytic activities of *L. monocytogenes* strains used in this study

Strain	HU of extracellular hemolysin[a]
PrfA*	213.3
PrfA* Δ*sigB*	232
Δ*prfA*	2
Δ*sigB* Δ*prfA*	2
10403S	18.7

[a] Values represent the averages for three independent biological replicates.

was recovered at lower numbers than those for 10403S (adjusted *P* value = 0.07), consistent with previous data showing that σ^B is important for transcription of genes important for invasion (42, 43). Interestingly, the *prfA** and *prfA** Δ*sigB* strains were recovered at lower numbers than those for both the parent and Δ*prfA* strains, indicating that *L. monocytogenes* strains expressing constitutively active PrfA* were less protected from the gentamicin treatment than strains lacking PrfA* (Fig. 4A). Although these results could be interpreted as resulting from reduced invasion of the PrfA* strain, visual inspection of HepG2 monolayers revealed that the host cells appeared to be damaged when infected with the *prfA** or *prfA** Δ*sigB* strain, suggesting possible increased cytotoxicity of the *L. monocytogenes* PrfA* strains relative to the 10403S and Δ*prfA* strains. Therefore, to test this hypothesis and to quantify relative cytotoxicities of the *prfA** and *prfA** Δ*sigB* strains to HepG2 cells, we performed an LDH release assay on infected HepG2 cells (Fig. 4B). HepG2 monolayers infected with the *prfA** or *prfA** Δ*sigB* strain showed 28% or 49% LDH release, respectively, which was significantly higher than the amount of LDH released from HepG2 cells infected with the 10403S, Δ*prfA*, or Δ*prfA* Δ*sigB* strain (adjusted *P* values of <0.0001) (Fig. 4B). The low release of LDH (<2%) from HepG2 cells infected with the 10403S, Δ*prfA*, or Δ*prfA* Δ*sigB* strain indicated little host cell damage resulting from infection with these strains. Interestingly, LDH release was significantly higher in HepG2 cells infected with the *prfA** Δ*sigB* strain than in those infected with the *prfA** strain (adjusted *P* value of <0.0001), suggesting that the presence of σ^B modulates PrfA-mediated gene expression to reduce host cell damage induced by intracellular *L. monocytogenes*.

Measurement of hemolytic activities among the various strains further supported the observation that σ^B modulates gene expression to reduce expression of virulence genes in a PrfA* background (Table 3). Specifically, we showed that the *prfA** Δ*sigB* strain had higher hemolytic activity (232 hemolytic units [HU]) than a *prfA** strain (213.3 HU) (Table 3). Low hemolytic activities were observed for the Δ*prfA* and Δ*prfA* Δ*sigB* strains, consistent with the PrfA-dependent nature of *hly* transcription (6, 54, 57, 71).

Reporter fusion assays show that σ^B regulates *prfA* transcription in a PrfA* background. A number of PrfA-dependent virulence genes had higher transcript levels in the *prfA** Δ*sigB* strain than in the *prfA** strain in both our microarray and qRT-PCR studies (Table 2; Fig. 3). Therefore, to measure specific contributions of σ^B and PrfA* to *prfA* transcription, we introduced a series of extrachromosomal plasmids containing GUS-RF fused to the *prfA* promoter region into the *prfA**, *prfA** Δ*sigB*, Δ*prfA*, and Δ*prfA* Δ*sigB* strains (Fig. 5). Reporter fusion measurements in bacterial cells grown to stationary phase showed that all strains with $P1P2_{prfA}$-*gus* and $\Delta P1(-10)P2_{prfA}$-*gus* fusions had higher activities than strains with reporter fusions that contained $P1\Delta P2(-10)_{prfA}$-*gus* (Fig. 5), indicating that the $P2_{prfA}$ promoter region plays the predominant role in activating *prfA* transcription under these conditions. In addition, the strains with wild-type σ^B (i.e., *prfA** and Δ*prfA* strains) showed higher GUS activities with both the $P1P2_{prfA}$-*gus* fusion and the $\Delta P1(-10)\ P2_{prfA}$-*gus* fusion than the corresponding Δ*sigB* strains (i.e., *prfA** Δ*sigB* and Δ*prfA* Δ*sigB* strains) did, indicating that σ^B positively influences *prfA* transcription, most likely due to the previously described σ^B-dependent transcriptional activation of the $P2_{prfA}$ promoter (53). Specifically, results from two-way ANOVA indicated sig-

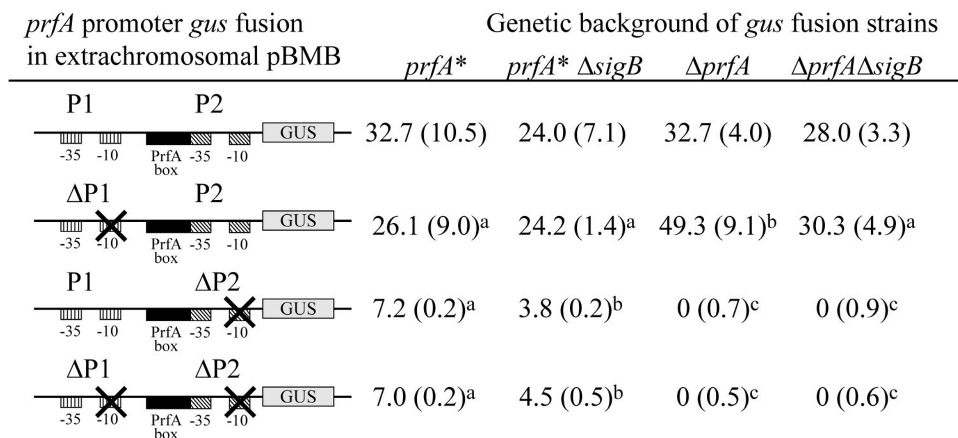

FIG. 5. GUS activities, in nM MU/log CFU/min, for *prfA* promoter-*gus* reporter fusions expressed in different *L. monocytogenes* genetic backgrounds. Diagrams of *prfA-gus* promoter fusion constructs show the promoter elements present in each reporter fusion plasmid. *L. monocytogenes* strains bearing extrachromosomal plasmids with a *prfA* promoter-*gus* reporter fusion were grown to early stationary phase. Data represent the mean quantities of liberated MU measured in three independent biological replicates; standard deviations are reported in parentheses. Different superscript letters indicate significantly different GUS activities (adjusted *P* values of <0.05) within a given row (i.e., in a comparison among strains containing the same *gus* reporter fusion).

nificantly higher GUS activity in the presence of σ^B than in its absence for the $P1P2_{prfA}$-*gus* fusion (1.3-fold higher; $P = 0.027$). Strains with PrfA* (i.e., *prfA** and *prfA** Δ*sigB* strains) showed lower GUS activity from the $\Delta P1(-10)P2_{prfA}$-*gus* fusion than did the corresponding Δ*prfA* strains (i.e., Δ*prfA*, Δ*sigB* Δ*prfA*, and Δ*sigB* strains), indicating that PrfA* negatively influences *prfA* transcription in this fusion, consistent with previous data suggesting that PrfA-dependent repression of *prfA* transcription occurs under some conditions (25). Conversely, strains with PrfA* (i.e., *prfA** and *prfA** Δ*sigB* strains) always showed higher GUS activity for all reporter fusions with the $P2_{prfA}$ −10 deletion [i.e., the $P1\Delta P2(-10)_{prfA}$-*gus* and $\Delta P1(-10)\Delta P2(-10)_{prfA}$-*gus* fusions] than did strains with Δ*prfA*, indicating that PrfA* positively regulates *prfA* transcript levels in strains with a $P2_{prfA}$ −10 promoter region deletion. Taken together, our results indicate that complex interactions between different promoter elements, PrfA*, and σ^B contribute to fine-tuning *prfA* transcript levels. This conclusion is supported by results from two-way ANOVA analyses, which showed a significant statistical interaction indicating that GUS fusion activities due to PrfA* were different depending on the presence/absence of σ^B for both the $\Delta P1(-10)P2_{prfA}$-*gus* and $\Delta P1(-10)\Delta P2(-10)_{prfA}$-*gus* fusion strains ($P = 0.0257$ and $P = 0.0005$, respectively). Overall, our GUS reporter fusion results show that σ^B itself does not negatively influence *prfA* transcription. These results suggest that the negative effects of σ^B on expression of PrfA-regulated genes are not a consequence of direct σ^B-dependent downregulation of *prfA* transcription but rather occur at a subsequent stage (i.e., likely through a σ^B-dependent gene product).

DISCUSSION

Previous studies have clearly established that σ^B plays an important role in regulating *L. monocytogenes* gene expression during gastrointestinal survival and invasion (26, 38, 39, 47, 72), while PrfA is well established as a critical transcriptional regulator of *L. monocytogenes* virulence gene expression during intracellular survival and cell-to-cell spread (reviewed in reference 40). Increasing evidence suggests that σ^B and PrfA coregulate genes important for the transition of *L. monocytogenes* from the extracellular to the intracellular environment (12, 56). To gain further insight into the σ^B-PrfA regulatory network, we evaluated genome-wide transcription patterns for *L. monocytogenes* 10403S strains with and without PrfA* and σ^B, using both single (Δ*sigB* and Δ*prfA* strains) and double (Δ*sigB* Δ*prfA* strain) mutant strains (Fig. 2). A PrfA* strain was selected for these studies to ensure constitutively high PrfA activity and, consequently, to increase the likelihood of identifying regulatory interactions between highly active PrfA (i.e., PrfA*) and σ^B. *L. monocytogenes* strains with the PrfA*(G155S) allele have PrfA activity levels similar to those found in intravacuolar bacteria, as determined by *hly* and *actA* transcript levels, even when the PrfA* strain is grown under in vitro conditions (36, 46). While PrfA* strains are important tools for studying PrfA-dependent transcription outside the mammalian host (45, 49, 62), one cannot exclude the possibility that intracellular *L. monocytogenes* organisms differ from extracellular PrfA* strains in some aspects of PrfA-dependent gene regulation (e.g., levels and activity status of PrfA).

In addition to microarray analyses, the σ^B-PrfA regulatory network was characterized using our set of isogenic mutant strains in a series of qRT-PCR, reporter fusion, and phenotypic assays. Overall, our data indicate that (i) σ^B downregulates expression of the PrfA regulon in the presence of PrfA*, reducing the host-cell-damaging potential of PrfA-mediated gene expression in intracellular *L. monocytogenes*; and (ii) the *L. monocytogenes* 10403S PrfA regulon is small (<20 positively regulated genes) and includes a complex regulatory system of *prfA* transcription involving autoregulation by PrfA* and positive regulation of *prfA* transcription by σ^B. Combined with results from previous studies (e.g., see references 26, 36, and 56), these findings suggest a complex σ^B-PrfA regulatory network that includes a switch from σ^B-dependent upregulation of genes important during the gastrointestinal stage of infection, including initial upregulation of *prfA*, to σ^B-mediated downregulation of the PrfA regulon in intracellular *L. monocytogenes*.

The core PrfA regulon is downregulated by σ^B in the presence of PrfA*. Our results conclusively establish a new role for *L. monocytogenes* σ^B, i.e., its importance in downregulating PrfA-regulated transcription in the presence of active PrfA (PrfA*). Data supporting this conclusion include (i) higher transcript levels for members of the PrfA regulon in the *prfA** Δ*sigB* strain than in the *prfA** strain in our microarray analyses, suggesting a negative effect of σ^B on the transcription of certain genes; (ii) qRT-PCR confirmation of the microarray transcription pattern for selected genes in the PrfA regulon, with higher *actA* and *hly* transcript levels in the *prfA** Δ*sigB* strain than in the *prfA** strain; (iii) higher hemolytic activity in the *prfA** Δ*sigB* strain than in the *prfA** strain; and (iv) higher HepG2 cytotoxicity for the *prfA** Δ*sigB* strain than for the *prfA** strain, consistent with the observed increased hemolytic activity. Importantly, these observations, obtained using a PrfA* strain, are consistent with observations previously reported for *L. monocytogenes* strains with wild-type PrfA. To illustrate, an *L. monocytogenes* Δ*sigB* strain had significantly higher *hly* transcript levels under growth conditions similar to those used in this study (59). In another study (33), the same Δ*sigB* strain also showed slightly but significantly higher *plcA* transcript levels than those in the parent strain when bacteria were exposed to σ^B-inducing conditions (salt stress). The Δ*sigB* strain has also been shown to have higher hemolytic activity than 10403S, supporting increased virulence gene expression in the absence of σ^B (10, 53).

The phenotypic importance of σ^B-mediated downregulation of the PrfA regulon in intracellular *L. monocytogenes* was clearly demonstrated through hemolytic activity and tissue culture assays. Therefore, we propose that in addition to the previously described biochemical and regulatory mechanisms that appear to protect the host cell from *L. monocytogenes*-mediated cytotoxic damage, such as the low pH necessary for optimal hemolysin activity (i.e., hemolysin activity is reduced at cytoplasmic pH) (2) and control of *hly* translation (66), our observations provide evidence for the existence of a transcriptional fine-tuning mechanism that acts to control the expression of genes encoding cytotoxic proteins in cytoplasmic *L. monocytogenes*.

The *L. monocytogenes* 10403S PrfA regulon appears to be small and includes a complex regulatory system of *prfA* transcription involving autoregulation by PrfA* and positive regulation of *prfA* transcription by σ^B. Our microarray data indicate that a small number of genes (<20) are positively regulated by PrfA*, consistent with microarray data reported by Milohanic et al. (49), suggesting that PrfA predominantly regulates a small set of genes through direct transcriptional activation via a PrfA binding site. Marr et al. (45) reported a much larger number of genes as positively regulated by PrfA*, including many genes without a preceding PrfA binding site. These genes were identified when PrfA* was overexpressed, so it is possible that regulation of these genes by PrfA may be of limited importance under most conditions in wild-type strains. The positively regulated PrfA-dependent genes identified in our study were generally consistent with genes previously identified as positively regulated by PrfA in EGD-e (49), EGD (45), and 10403S (57). While previous studies have shown that *inlA* and *inlB* transcription is both σ^B and PrfA dependent in a wild-type strain under in vitro conditions (38, 39), in our experiments with a PrfA* strain these genes were predominantly PrfA* dependent. Taken together, these results suggest that transcription of *inlAB* may switch from primarily σ^B dependent under extracellular conditions (e.g., in intestinal bacteria) to PrfA-dependent transcription in intracellular bacteria.

A number of studies have shown that regulation of *prfA* transcription includes an autoregulatory feedback loop through a bicistronic *plcA-prfA* transcript initiating from the PrfA-dependent *plcA* promoter (8, 24, 25). In addition to regulation mediated through the *plcA* promoter, our data indicate that PrfA*, σ^B, and the presence of both $P1_{prfA}$ and $P2_{prfA}$ promoters play complex roles in fine-tuning *prfA* transcription. The $P2_{prfA}$ promoter appears to contribute the majority of *prfA* transcripts, consistent with previous reports (36). In the absence of either intact $P1_{prfA}$ or intact $P2_{prfA}$, PrfA* appears to upregulate or downregulate *prfA* transcription in a manner that is dependent on the existing promoter configuration. Specifically, in the $\Delta P1(-10)$ $P2_{prfA}$-*gus* fusion strain, the presence of PrfA* appears to repress *prfA* transcription, consistent with data reported by Freitag and Portnoy (24, 30) which suggested that binding of PrfA to the $P2_{prfA}$ PrfA box might inhibit overall *prfA* transcription. On the other hand, for *prfA* reporter fusions that lack the $P2(-10)_{prfA}$ region (but retain the PrfA box in the $P2_{prfA}$ region), higher GUS activities were found in strains containing PrfA* than in Δ*prfA* strains, suggesting that PrfA* also activates *prfA* transcription at the $P2_{prfA}$ promoter and that the PrfA box in the $P2_{prfA}$ region may be sufficient for initiation of low levels of PrfA*-mediated transcription, even in the absence of a $P2_{prfA}$ -10 region, possibly due to the high affinity of PrfA*(G155S) for its target DNA (79). In contrast with results for the $\Delta P1(-10)P2_{prfA}$-*gus* fusion strain, in the absence of the $P2_{prfA}$ -10 region the loss of PrfA* does not enhance transcription at $P2_{prfA}$, likely because of the overall reduced transcription initiation at $P2_{prfA}$.

Alternatively, it is possible that the role of PrfA* in *prfA* transcription switches from activator to repressor depending on the level of active *prfA* transcription. To illustrate, our data are consistent with a model in which PrfA* negatively affects *prfA* transcription under conditions when *prfA* transcript levels are high [e.g., in the $\Delta P1(-10)P2_{prfA}$-*gus* fusion strain] but positively affects *prfA* transcription when *prfA* transcript levels are low [e.g., in the $P1\Delta P2(-10)_{prfA}$-*gus* and $\Delta P1(-10)\Delta P2(-10)_{prfA}$-*gus* fusion strains]. Results from statistical analyses of our reporter fusion experiments, which show a significant statistical interaction effect between σ^B and PrfA on *prfA* transcript levels, lend further credence to the notion of complex interactions among PrfA*, σ^B, and *prfA* promoter elements, and possibly *prfA* transcript levels. While Rauch et al. (60) did not report a statistically significant effect of the presence of wild-type PrfA on *prfA* transcription initiation from the P1 or the P2 promoter, in vitro assays with PrfA* (shown in Fig. 4A in reference 60) did show numerically higher transcript levels initiating from $P2_{prfA}$ in the presence of PrfA* (compared to wild-type PrfA or the absence of PrfA), supporting positive regulation of *prfA* transcription from $P2_{prfA}$ by PrfA*.

Our reporter fusion experiments provide independent evidence of *prfA* transcription activation by σ^B at the $P2_{prfA}$ promoter, consistent with previous findings using a variety of different strategies (36, 53, 60). Our data do not support a model in which σ^B directly downregulates *prfA* transcription. Therefore, the negative effect of σ^B on expression of the PrfA regulon does not occur at the level of *prfA* transcription. Rather, in combination, our gene expression and reporter fusion data suggest that σ^B likely regulates transcription of an as yet unrecognized protein(s) or noncoding RNA(s) that is responsible for regulating PrfA at the posttranscriptional or posttranslational level to yield reduced expression of the PrfA regulon. This hypothesis is supported by the fact that the *L. monocytogenes* σ^B regulon includes >140 genes, including those encoding proteins with importance in stress response, virulence, transcriptional regulation, metabolism, and transport (59). σ^B also transcribes genes that encode other regulators, including small RNAs (12, 54) and proteins that affect RNA stability (e.g., Hfq) (13).

Conclusions. Our data support the existence of a complex σ^B-PrfA regulatory network that contributes to *L. monocytogenes* virulence and transmission. This network includes a large σ^B regulon, which is comprised of a large range of stress response and virulence genes (31, 35, 59), and a smaller PrfA regulon that is comprised predominantly of virulence genes, including some genes that are regulated by both σ^B and PrfA (e.g., *inlA*) (35, 39, 47). While σ^B-dependent transcription appears to be important predominantly under environmental or host-induced stress conditions (e.g., acid stress or osmotic stress) (1, 22, 72), PrfA appears to be switched "on" after *L. monocytogenes* enters the host cell, thus activating transcription of a number of virulence genes required for bacterial escape from the phagocytic vacuole and for cell-to-cell spread (40). The σ^B-PrfA regulatory network involves two autoregulatory feedback loops, including (i) PrfA-dependent transcription of *prfA* (e.g., through PrfA-dependent readthrough transcripts from the *plcA* promoter) (8) and (ii) σ^B-dependent transcription of a portion of the *sigB* operon (1), as well as σ^B-dependent activation of *prfA* transcription from $P2_{prfA}$ (36, 53, 60, 67). In addition to PrfA- and σ^B-dependent upregulation of *prfA* transcription, under some conditions these two regulators both also appear to downregulate and fine-tune *prfA* expression and/or PrfA activity, and hence expression of the PrfA regulon. While PrfA appears to be able to directly down-

regulate *prfA* transcription (25), σ^B appears to downregulate expression of the PrfA regulon by an as yet undefined mechanism. We thus propose a model for PrfA activity regulation that requires careful fine-tuning of PrfA regulon expression through complex PrfA and σ^B interactions to ensure the rapid induction of regulon expression to facilitate infection and cell-to-cell spread when needed, as well as subsequent downregulation to avoid overexpression of virulence genes, which could result in excessive host cell damage and thus impair the success of the organism in establishing an infection (17, 66).

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