

## Transcriptomic and Phenotypic Analyses Suggest a Network between the Transcriptional Regulators HrcA and $\sigma^B$ in *Listeria monocytogenes*<sup>∇†</sup>

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Received 9 June 2007/Accepted 16 October 2007

*Listeria monocytogenes* HrcA and CtsR negatively regulate class I and III stress response genes, respectively, while  $\sigma^B$  positively regulates the transcription of class II stress response genes. To define the HrcA regulon and identify interactions between HrcA, CtsR, and  $\sigma^B$ , we characterized newly generated *L. monocytogenes*  $\Delta hrcA$ ,  $\Delta ctsR \Delta hrcA$ , and  $\Delta hrcA \Delta sigB$  strains, along with previously described  $\Delta sigB$ ,  $\Delta ctsR$ , and  $\Delta ctsR \Delta sigB$  strains, using phenotypic assays (i.e., heat resistance, acid resistance, and invasion of human intestinal epithelial cells) and performed whole-genome transcriptome analysis of the  $\Delta hrcA$  strain. The *hrcA* and *sigB* deletions had significant effects on heat resistance. While the *hrcA* deletion had no significant effect on acid resistance or invasion efficiency in Caco-2 cells, a linear regression model revealed a significant ( $P = 0.0493$ ) effect of interactions between the *hrcA* deletion and the *ctsR* deletion on invasiveness. Microarray-based transcriptome analyses and promoter searches identified (i) 25 HrcA-repressed genes, including two operons (the *groESL* and *dnaK* operons, both confirmed as HrcA regulated by quantitative real-time PCR) and one gene directly repressed by HrcA, and (ii) 36 genes that showed lower transcript levels in the  $\Delta hrcA$  strain and thus appear to be indirectly upregulated by HrcA. A number of genes were found to be coregulated by either HrcA and CtsR (2 genes), HrcA and  $\sigma^B$  (31 genes), or all three regulators (5 genes, e.g., *gadCB*). Combined with previous evidence that  $\sigma^B$  appears to directly regulate *hrcA* transcription, our data suggest that HrcA and  $\sigma^B$ , as well as CtsR, form a regulatory network that contributes to the transcription of a number of *L. monocytogenes* genes.

*Listeria monocytogenes* is a gram-positive food-borne pathogen that can cause severe invasive disease in humans, as well as in a number of different animal species (31). The capacity of *L. monocytogenes* to survive and multiply under a wide range of environmental stress conditions appears to be critical for the food-borne transmission of this pathogen (10). A number of transcriptional regulators (e.g., PrfA,  $\sigma^B$ , HrcA, and CtsR) that are important for the transcription of stress response and virulence genes have been identified in this organism (20, 25, 32, 37). While clear evidence for interactions between PrfA and  $\sigma^B$  has been reported (26, 40, 45), our understanding of interactions among other *L. monocytogenes* transcriptional regulators is limited. As no *L. monocytogenes hrcA* null mutant appears to have previously been reported, our understanding of the contributions of the negative regulator HrcA to stress response, transcriptional regulation, and regulatory networks is limited. In a number of gram-positive bacteria, including *Bacillus subtilis*, HrcA (heat regulation at CIRCE) has been found to repress the *dnaK* and *groESL* operons by binding to a region designated as the controlling inverted-repeat chaperone expression (CIRCE) element (38). Sequence analyses in *L. monocytogenes* also identified putative CIRCE elements upstream of *hrcA* (20) and of *groES* (13), which suggests that

HrcA represses the *dnaK* operon (i.e., *hrcA-grpE-dnaK*-lmo1471-lmo1470) and the *groESL* operon in *L. monocytogenes*. Previous studies showed that insertional inactivation of *L. monocytogenes dnaK* resulted in decreased acid resistance and a growth defect at temperatures of 37°C or higher (19), suggesting contributions of HrcA to acid and temperature stress resistance. Transcription of the putative HrcA-dependent *groESL* was also found to be upregulated in *L. monocytogenes* exposed to selected stress conditions (e.g., heat) and in intracellular bacteria, further supporting the idea that *groESL* (and thus, HrcA) may contribute to *L. monocytogenes* stress response and virulence (13).

The alternative sigma factor  $\sigma^B$  directly regulates the transcription of a large regulon in *L. monocytogenes* (25) and appears to play a central role in regulating the transcription of *L. monocytogenes* stress response and virulence genes (25, 26, 30, 36). The characterization of *sigB* null mutants has also shown that *L. monocytogenes*  $\sigma^B$  contributes to survival under a variety of stress conditions, including acid and oxidative stresses and during carbon starvation (3, 10, 35). CtsR (class three stress gene repressor) is a transcriptional repressor which regulates at least 64 genes in *L. monocytogenes*, including directly repressing at least 10 genes (e.g., *clpC*, *clpB*, and *clpP*) (21, 37). Phenotypic characterization of different *ctsR* mutants also showed that *L. monocytogenes* CtsR contributes to virulence and survival under a variety of stress conditions, including heat, acid, oxidative, and high-pressure stress (6, 22, 23, 24).

In order to characterize the contributions of HrcA to *L. monocytogenes* stress response and virulence, including the potential interactions between HrcA and the HrcA regulon and

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

<sup>∇</sup> Published ahead of print on 26 October 2007.

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

Strain	Characteristics <sup>a</sup>	Reference
10403S	Serotype 1/2a parent strain	4
FSL A1-254	10403S $\Delta sigB$	51
FSL H6-190	10403S $\Delta ctsR$	21
FSL H6-193	10403S $\Delta ctsR \Delta sigB$	21
FSL B2-101	10403S $\Delta hrcA$	This study
FSL H6-194	10403S $\Delta hrcA \Delta sigB$	This study
FSL H6-198	10403S $\Delta ctsR \Delta hrcA$	This study

<sup>a</sup> The *hrcA* null mutation represents a 745-bp deletion within the *hrcA* ORF; this deletion allele encodes a 98-amino-acid nonsense protein that includes the first 17 and last 81 amino acids of the native HrcA protein.

other stress response regulators (i.e., CtsR and  $\sigma^B$ ), we generated a series of isogenic single and double *hrcA*, *sigB*, and *ctsR* mutations in *L. monocytogenes* for phenotypic characterization and transcriptome analysis.

#### MATERIALS AND METHODS

**Bacterial strains.** *L. monocytogenes* 10403S and isogenic mutant strains were used in this study (Table 1);  $\Delta hrcA$ ,  $\Delta hrcA \Delta sigB$ , and  $\Delta ctsR \Delta hrcA$  strains have not previously been reported. The isogenic internal deletion mutants were constructed as previously detailed (7) by using the allelic exchange mutagenesis approach first reported by Smith and Youngman (46). Briefly, splicing by overlap extension PCR (see Table S1 in the supplemental material for primers) was used to construct an  $\Delta hrcA$  allele with an in-frame 745-bp deletion within the *hrcA* open reading frame (ORF), which was cloned into pKSV7, yielding plasmid pBMB-28. This mutant allele was introduced into *L. monocytogenes* 10403S, as well as into previously constructed  $\Delta sigB$  and  $\Delta ctsR$  strains, yielding  $\Delta hrcA$ ,  $\Delta hrcA \Delta sigB$ , and  $\Delta ctsR \Delta hrcA$  strains (Table 1). The *hrcA* null mutation was confirmed in all strains by PCR amplification and direct sequencing of the PCR product (see Table S1 in the supplemental material).

The *hrcA* null mutant constructed here was designed to maintain the S2 transcriptional start site, which is located in the 3' end of *hrcA* (20), to ensure that the *hrcA* deletion would not interfere with the transcription of the downstream genes *grpE* and *dnaK*. Maintenance of the S2 transcriptional start site (as well as 177 nucleotides upstream of S2) appears to be important, as an initial *hrcA* mutant that included a deletion of this region showed reduced growth at 37°C, which is the same phenotype previously reported for a *dnaK* insertional mutant (19). The *hrcA* mutant used here (FSL B2-101), which did not include a deletion of the S2 region, showed no reduction in growth at 37°C compared to the growth of the isogenic parent strain.

All phenotypic characterization experiments (see below) were performed using *L. monocytogenes* cells grown to early stationary phase, as initial quantitative real-time (qRT-PCR) showed that the HrcA-dependent genes *dnaK* and *groES* showed HrcA-dependent repression only in stationary-phase cells (see Results for details). Specifically, for all phenotypic characterization experiments, early-stationary-phase cells (i.e., bacterial cells incubated for an additional 1 h after they were grown to an optical density at 600 nm [OD<sub>600</sub>] of 0.8 at 37°C) were used to allow direct comparison with the results of previous phenotypic characterization experiments conducted in our laboratory (e.g., see reference 14), where *L. monocytogenes* cells were also grown to early stationary phase. For microarray experiments, on the other hand, stationary-phase cells (bacterial cells incubated for an additional 3 h after being grown to an OD<sub>600</sub> of 1.0) were used to allow comparison with the results of other microarray studies (see, e.g., reference 39a).

**Heat stress experiment.** Heat survival experiments were conducted to evaluate the heat resistance of the parent strain 10403S and all six mutant strains (Table 1). For these experiments *L. monocytogenes* cells were grown at 37°C with shaking to early stationary phase in 5 ml of brain heart infusion (BHI) in 16- by 125-mm disposable borosilicate glass culture tubes. Exposure to 55°C was performed by placing the tubes with the stationary-phase *L. monocytogenes* cells into a 55°C water bath, followed by incubation in the 55°C water bath (without shaking) for 30 or 60 min; initial temperature measurements showed that the broth reaches a temperature of 55°C within 158 s (standard deviation, 17 s; four replicate measurements). Bacterial numbers (log CFU/ml) were determined prior to and after heat treatment by plating on BHI agar plates using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Norwood, MA). Survival was ex-

pressed as the log reduction in the survival rate, which was calculated by subtracting the bacterial numbers (in log CFU/ml) before heat treatment from the bacterial numbers (in log CFU/ml) after heat treatment. Three independent experiments were performed.

**Acid stress experiment.** To evaluate the acid resistance of parent strain 10403S and all six mutant strains, early-stationary-phase cells were exposed to acid stress (by adjusting the BHI to pH 2.5 with 12 N HCl) for 1 and 2 h at 37°C with shaking (230 rpm). Bacterial numbers (in log CFU/ml) prior to and after acid treatment were determined by plating on BHI agar as described above. Survival was expressed as the log reduction in the survival rate, which was calculated by subtracting the bacterial numbers (in log CFU/ml) before acid exposure from the bacterial numbers (in log CFU/ml) after acid exposure. Three independent experiments were performed.

**Invasion assay.** The invasiveness in human intestinal epithelial cells of the parent strain and all mutant strains was determined using Caco-2 cells (ATCC HTB-37) as previously described (39) and bacterial cells grown to early stationary phase. Confluent Caco-2 monolayers grown in a 24-well plate were inoculated with 10  $\mu$ l *L. monocytogenes* cells (3 wells/strain). After incubation for 30 min, Caco-2 cells were washed three times with phosphate-buffered saline, and at 45 min postinoculation, fresh medium containing gentamicin (150  $\mu$ g/ml) was added to kill extracellular bacteria. At 90 min postinoculation, Caco-2 cells were washed three times with phosphate-buffered saline and then lysed by the addition of ice-cold sterile distilled water and vigorous pipetting. Intracellular *L. monocytogenes* numbers were determined by plating lysed Caco-2 cell suspensions on BHI agar. The invasion efficiency was calculated as the number of intracellular bacteria (in log CFU) recovered relative to the bacterial numbers (in log CFU) used for the inoculation. Invasion efficiencies were normalized to those of the parent strain, for which invasion efficiency was set at 100%. Three independent invasion assays were performed for each *L. monocytogenes* strain tested.

**Microarray.** To identify HrcA-regulated genes, whole-genome transcriptome analyses were performed to compare transcript levels between the parent strain and the  $\Delta hrcA$  strain. Microarray construction, RNA isolation and purification, cDNA labeling, and hybridization were performed as previously described (5, 21, 39a). Briefly, RNA for microarrays was isolated from stationary-phase cells using RNeasy Protect Bacteria reagent and an RNeasy Midi kit (QIAGEN, Valencia, CA). DNase treatment of RNA was performed essentially as described previously (25), using 40 U of DNase. Isolated RNA (in RNase-free water) was quantified and checked for purity using OD<sub>260</sub> and OD<sub>280</sub> measurements performed on a Nanodrop spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE). Agarose gel electrophoresis was used to verify RNA integrity.

Microarrays were constructed using 70-mer oligonucleotides targeting 2,857 *L. monocytogenes* ORFs (QIAGEN operon array-ready oligonucleotide sets) identified in the annotated genome sequence of *L. monocytogenes* EGD-e (16). 70-mer oligonucleotides targeting five *Saccharomyces cerevisiae* ORFs (*act1*, *mfa1*, *mfa2*, *ras1*, and *ste3*) were included on the microarray to serve as nonhybridizing controls, as described previously (52). *L. monocytogenes* strains EGD-e and 10403S both represent the same *L. monocytogenes* lineage (II), serotype (1/2a), and ribotype (DUP-1039C), and probes designed based on the EGD-e genome were thus expected to hybridize well with 10403S genes (5). As an unfinished genome sequence for strain 10403S has recently become available (1), we also verified cross-hybridization identities (CHI) between the EGD-e probes and the target genes in strain 10403S; totals of 2,107, 2,578, and 2,695 of the EGD-e probes showed CHI values of 100,  $\geq 95$ , and  $\geq 90$ ; 45 probes showed CHI values of  $< 90$  (5). A total of 117 of the EGD-e-based microarray probes were not detected in the 10403S genome, likely reflecting either sequence divergence in 10403S, deletion of these genes in 10403S, or genes located in the gaps of the unfinished 10403S genome sequence (5). We thus were confident that the array used here would allow for comprehensive identification of differentially expressed genes in strain 10403S, with the possibility of some false negatives (i.e., for genes targeted by a probe with a low CHI or for genes present in the 10403S genome and absent in the EGD-e genome) (5). Mismatches to selected target genes would be unlikely to yield false positives (i.e., genes identified as differentially regulated even if they were not) since the same mismatches would occur with RNA from the parent strain and the  $\Delta hrcA$  strain (5).

cDNA was synthesized and differentially labeled by using a SuperScript Plus indirect cDNA labeling system (Invitrogen, Carlsbad, CA). Briefly, cDNA was generated from 10  $\mu$ g of RNA using random primers in an overnight reverse transcription reaction at 42°C. cDNA was purified by using a QIAGEN PCR purification kit prior to indirect labeling with Alexa Fluor 555 or Alexa Fluor 647 fluorescent dyes (performed overnight at room temperature). Labeled cDNA was purified by using a QIAGEN PCR purification kit to remove any unincorporated dye, and the labeled cDNA was quantified and checked for purity using OD<sub>260</sub> and

OD<sub>280</sub> measurements. For each microarray, cDNA from the parent and the  $\Delta hrcA$  strain were combined into one tube, dried, and then resuspended in hybridization buffer containing sodium dodecyl sulfate (SDS), sodium chloride-sodium citrate (SSC), salmon sperm DNA, dithiothreitol, and formamide. The combined cDNA target (in a 50- $\mu$ l volume) was overlaid onto the microarray slides using mSeries LifterSlips (Erie Scientific, Portsmouth, NH). Following overnight hybridization at 42°C, slides were washed in 2 $\times$  SSC-0.1% SDS (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min at 42°C, followed by sequential room temperature washes in 2 $\times$  SSC-0.1% SDS, 2 $\times$  SSC, and 0.2 $\times$  SSC for 5 min each. Slides were centrifuged to dry them and scanned with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). All microarray experiments were performed three times using three independent RNA isolations to provide true biological replicates.

Scanned microarray images were gridded using GenePix Pro 6.0 software. Raw image analysis data were preprocessed, and significant differences in gene expression patterns between strains of interest were determined using the LIMMA software package (47) from R/BioConductor (15). Following background correction with the normexp method, within-array normalization (print-tip loess) and between-array normalization (scale) were used to correct for spatial and intensity bias and to make the results comparable across arrays. The LIMMA software package was also used for differential expression analysis (48) to calculate moderated  $t$  and  $B$  statistics and  $P$  values (adjusted for multiple comparisons by controlling for the false discovery rate). Genes with an adjusted  $P$  value of <0.05 were considered statistically significant, and an  $n$ -fold change of  $\geq 1.5$  was used as the cutoff for the identification of differentially expressed genes. Genes that showed significantly different transcript levels between the parent and the  $\Delta hrcA$  strain were considered putative HrcA-regulated genes.

**HMM searches.** Potential HrcA binding sites were determined by Hidden Markov model (HMM) searches as previously described (25). The HMM training alignments included 57 unique HrcA binding sites (identified upstream of *hrcA* and *groES* in different gram-positive bacteria), which were retrieved from the Comprehensive Microbial Resource at the J. Craig Venter Institute (<http://cmr.tigr.org>). Forward and reverse HMM models were generated to search for HrcA binding sites on the plus and minus strands of the published EGD-e genome (16). Outputs were filtered, and only hits within 300 bp upstream of a start codon for an ORF, as annotated by Listilist (<http://genolist.pasteur.fr/Listilist>), and with an E value of <0.01 were considered to be putative HrcA binding sites.

**qRT-PCR.** RNA isolation for TaqMan qRT-PCR was performed as described previously (30). qRT-PCR primers and probes for *dnaK* and *groES* (see Table S2 in the supplemental material) were designed using PrimerExpress. qRT-PCR with these primer/probe sets, as well as primer and probe sets for the housekeeping genes *rpoB* and *gap* (45, 49), was performed using RNA isolated from the *L. monocytogenes* parent and  $\Delta hrcA$  strain (grown to stationary phase) to confirm HrcA-dependent repression of these two genes. qRT-PCR was performed by using an ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as detailed previously (30, 49). Reverse transcriptase negative control reactions, DNA standard curves, and analysis of qRT-PCR were also performed as described previously (30). Relative cDNA copy numbers were calculated as the log cDNA copy numbers for the target gene relative to the geometric mean of the cDNA copy numbers for the housekeeping genes *rpoB* and *gap* [i.e.,  $\log_{10} \text{target gene} - [(\log_{10} \text{rpoB} + \log_{10} \text{gap})/2]$ ]. qRT-PCR was repeated three times using three independent RNA isolations from cells grown on different days.

**Statistical analysis.** All statistical analyses were performed using SAS (SAS onlineDoc8 version 8, SAS Institute, Inc., Cary, NC). qRT-PCR data for the parent strain and the  $\Delta hrcA$  strain were analyzed by using a  $t$  test. Phenotypic data (heat survival, acid survival, and invasion efficiency) were analyzed using one-way analysis of variance with Duncan's multiple comparison procedure. In addition, the phenotypic data were also analyzed with linear regression models to test the effects of the three categorical variables (presence/absence of *hrcA*, *ctsR*, and *sigB*) and their interactions on a given phenotype; the final model was phenotype = *hrcA* + *ctsR* + *sigB*, where "phenotype" represents heat survival, acid survival, or invasion efficiency. For all tests, statistical significance was established as a  $P$  value of <0.05; significant  $P$  values are reported as the actual value, unless  $P$  is <0.0001.

**Microarray data accession numbers.** Raw and normalized microarray data in MIAME format are available at the NCBI Gene Expression Omnibus (GEO) data repository under accession number GSE7517.

## RESULTS

### An *hrcA* deletion affects *L. monocytogenes* heat resistance.

Analysis of heat survival rates for all strains after 30 and 60 min using a linear regression model (as detailed in Materials and Methods) showed (i) significant effects of the terms "*ctsR* deletion" and "*sigB* deletion" on survival after 30 min ( $P = 0.0003$  and  $P = 0.0161$ , respectively) and (ii) significant effects of the terms "*sigB* deletion" and "*hrcA* deletion" on survival after 60 min ( $P < 0.0001$  and  $P = 0.0110$ , respectively). The significant effect of the *hrcA* deletion on survival after 60 min, despite the observation that the  $\Delta hrcA$  strain showed no significant difference in its survival rate compared to that of the parent strain (Fig. 1), likely reflects the fact that both  $\Delta hrcA$  double mutants (i.e., the  $\Delta ctsR \Delta hrcA$  and  $\Delta hrcA \Delta sigB$  strains) showed numerically reduced rates of survival compared to the survival rate of the paired null mutants with the *hrcA* wild-type allele (i.e., the  $\Delta ctsR$  and  $\Delta sigB$  strains), which suggests that an *hrcA* deletion is associated with increased heat sensitivity in *L. monocytogenes*.

The significant effect of the *sigB* null mutation on survival rates after 30 and 60 min appears to reflect increased heat sensitivity associated with this deletion, as the *sigB* null mutant strains consistently showed reduced survival compared to the survival rates of the paired strains without an *sigB* deletion, including significantly lower survival of the  $\Delta sigB$  strain, compared to the survival rate of the parent strain after 60 min of exposure to 55°C (Fig. 1B). The significant effect of the *ctsR* null mutation on survival after 30 min appears to reflect increased heat resistance associated with this deletion, as the *ctsR* null mutant strains consistently showed higher survival rates than the paired strains without a *ctsR* deletion. These findings are consistent with those of other studies (22, 23), which also showed increased heat resistance of *ctsR* null mutant strains.

The linear regression model did not show any evidence for effects of interactions between the three factors (i.e., "*hrcA* deletion," "*ctsR* deletion," and "*sigB* deletion") on heat survival.

**An *hrcA* deletion shows no effect on *L. monocytogenes* acid resistance.** Analyses of *L. monocytogenes* survival after 1 and 2 h of exposure to pH 2.5 (Fig. 2) using a linear regression model showed no significant effect of either the term "*hrcA* deletion" or the term "*ctsR* deletion" on survival, indicating that acid resistance in early-stationary-phase cells is not affected by either the *hrcA* or the *ctsR* deletion. The term "*sigB* deletion," on the other hand, had a significant effect on acid stress survival after both 1 and 2 h ( $P < 0.0001$  and  $P = 0.0003$ , respectively), and the *sigB* null mutant strains consistently showed reduced acid stress survival rates compared to the survival rates of the paired strains without a *sigB* deletion (Fig. 2). These results are consistent with the well-documented importance of *sigB* in *L. monocytogenes* acid resistance (10, 11, 51).

The linear regression model did not show any evidence for effects of interactions between the three factors (i.e., "*hrcA* deletion," "*ctsR* deletion," and "*sigB* deletion") on acid stress survival.

**An *hrcA* deletion has limited effects on *L. monocytogenes* invasion efficiency.** Statistical analyses of Caco-2 cell invasion

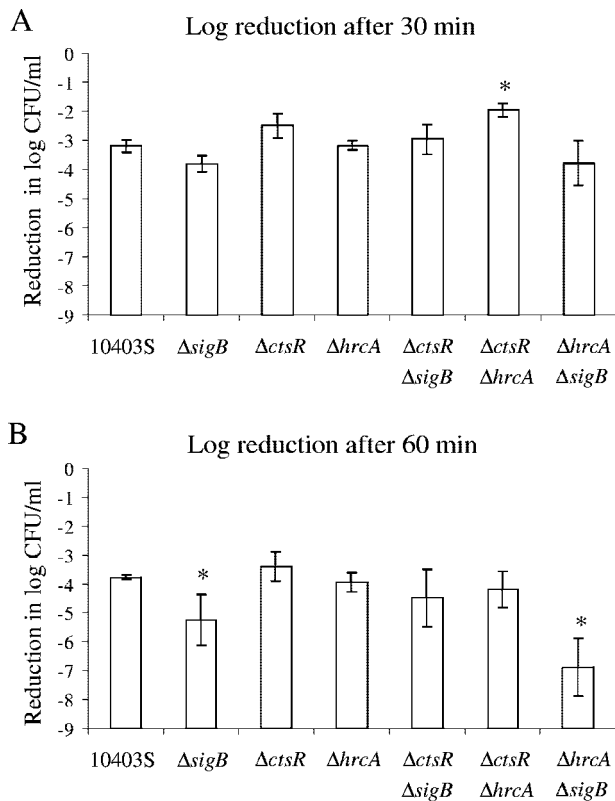


FIG. 1. Heat survival of the *L. monocytogenes* parent strain (10403S) and the  $\Delta sigB$ ,  $\Delta ctsR$ ,  $\Delta hrcA$ ,  $\Delta ctsR \Delta sigB$ ,  $\Delta ctsR \Delta hrcA$ , and  $\Delta hrcA \Delta sigB$  strains after (A) 30 min or (B) 60 min of exposure to 55°C. Strains were grown to early stationary phase before exposure to 55°C. Survival was expressed as the log reduction, which was calculated by subtracting the bacterial numbers (in log CFU/ml) before heat exposure from the bacterial numbers (in log CFU/ml) after heat exposure. The data shown represent the averages of the results of three independent experiments; error bars indicate standard deviations. Overall analysis of variance showed a significant effect of the factor “strain” on heat survival after both 30 and 60 min. Duncan’s multiple-comparison procedure was used to determine whether heat survival differed between specific strains. An asterisk indicates a strain with a log reduction in the survival rate that differed significantly ( $P < 0.05$ ) from the survival rate of the parent strain; in addition, the  $\Delta ctsR \Delta hrcA$  strain also showed a significantly higher survival rate than the  $\Delta hrcA \Delta sigB$  and the  $\Delta sigB$  strains after 30 min of heat stress. After 60 min, the survival rate of the  $\Delta hrcA \Delta sigB$  strain was significantly lower than those of all other strains, and the survival rate of the  $\Delta ctsR$  strain was significantly higher than that of the  $\Delta ctsR \Delta sigB$  strain.

efficiencies for the *L. monocytogenes* parent strain and the six mutant strains using a linear regression model showed no significant effect of the term “*hrcA* deletion” but a significant effect of the terms “*ctsR* deletion” ( $P = 0.0154$ ) and “*sigB* deletion” ( $P < 0.0001$ ) on invasion efficiency. The *sigB* null mutant strains always showed significantly lower invasion efficiencies than the paired strains without a *sigB* deletion (i.e., the  $\Delta sigB$ ,  $\Delta sigB \Delta ctsR$ , and  $\Delta sigB \Delta hrcA$  strains showed significantly lower invasion efficiencies than the parent,  $\Delta ctsR$ , and  $\Delta hrcA$  strains, respectively) (Fig. 3), consistent with the well-documented role of *sigB* in Caco-2 cell invasion (14, 27). While the  $\Delta ctsR$  and parent strains showed similar invasion efficiencies, the  $\Delta sigB \Delta ctsR$  and  $\Delta ctsR \Delta hrcA$  strains showed lower invasion efficiencies than the corresponding mutant strains

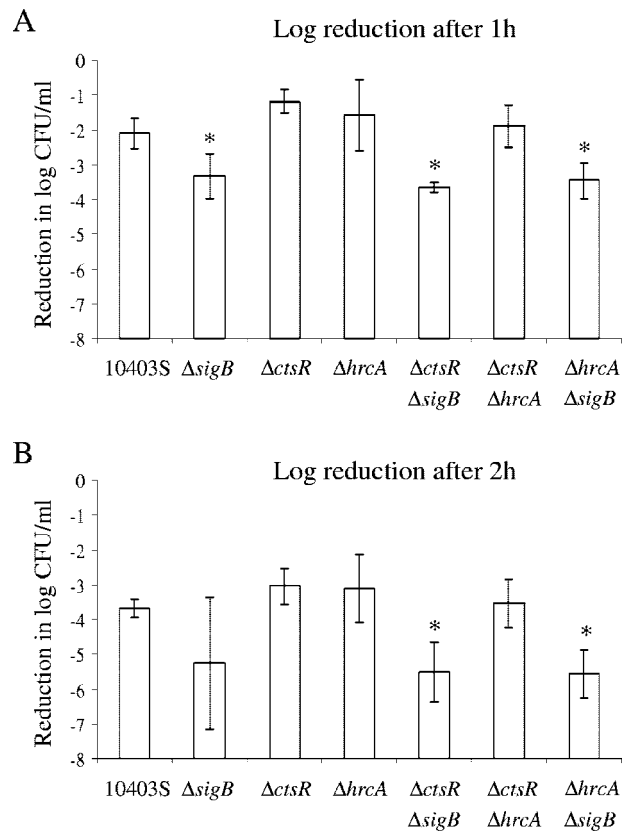


FIG. 2. Acid stress survival of the *L. monocytogenes* parent strain (10403S) and the  $\Delta sigB$ ,  $\Delta ctsR$ ,  $\Delta hrcA$ ,  $\Delta ctsR \Delta sigB$ ,  $\Delta ctsR \Delta hrcA$ , and  $\Delta hrcA \Delta sigB$  strains after (A) 1 h or (B) 2 h of exposure to acid stress (pH 2.5). Strains were grown to early stationary phase before exposure to pH 2.5. Duncan’s multiple-comparison procedure was used to determine whether acid survival differed between specific strains; an asterisk indicates a strain with acid stress survival that differed significantly ( $P < 0.05$ ) from that of the parent strain. Survival was expressed as the log reduction, which was calculated by subtracting the bacterial numbers (in log CFU/ml) before acid exposure from the bacterial numbers (in log CFU/ml) after acid exposure. The data shown represent the averages of the results of three independent experiments; error bars indicate standard deviations. A linear regression model to test the effects of the three categorical variables (presence/absence of *hrcA*, *ctsR*, and *sigB*) and their interactions on acid stress survival indicated a significant effect of the *sigB* deletion on acid stress survival ( $P$  value of  $< 0.0001$  and  $P$  value of 0.0003 for survival after 1 and 2 h, respectively).

without a *ctsR* deletion (i.e., the  $\Delta sigB$  and  $\Delta hrcA$  strains) (Fig. 3), suggesting that a *ctsR* deletion is associated with reduced invasion efficiency, consistent with the results of previous reports (6).

The linear regression model showed significant ( $P = 0.0493$ ) evidence for interactions between the factors “*hrcA* deletion” and “*ctsR* deletion” affecting invasiveness but showed no evidence for other interactions affecting it. The invasion efficiency of the  $\Delta ctsR \Delta hrcA$  strain (83%) was numerically lower than the invasion efficiencies of both the  $\Delta ctsR$  and the  $\Delta hrcA$  strain (102 and 109%, respectively), supporting contributions of *hrcA* and *ctsR* to *L. monocytogenes*’ invasion efficiency.

**Whole-genome microarray analysis identified 61 HrcA-regulated genes.** Microarray experiments comparing transcript lev-

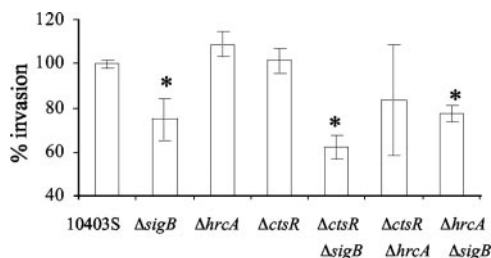


FIG. 3. Invasion efficiency in Caco-2 cells of the *L. monocytogenes* parent strain (10403S) and the  $\Delta sigB$ ,  $\Delta ctsR$ ,  $\Delta hrcA$ ,  $\Delta ctsR \Delta sigB$ ,  $\Delta ctsR \Delta hrcA$ , and  $\Delta hrcA \Delta sigB$  strains that were grown to early stationary phase in BHI at 37°C. The invasion efficiency was calculated as the number of intracellular bacteria (in CFU) recovered relative to the bacterial numbers (in CFU) used for the inoculation; invasion efficiencies were normalized to the invasion efficiency of one of the two parent strains included in each experiment (which was set at 100%). The data shown represent the averages of the results of three independent experiments; error bars indicate standard deviations. Duncan's multiple-comparison procedure was used to determine whether the invasion efficiency differed between specific strains; an asterisk indicates a strain with a percent invasion that differed significantly ( $P < 0.05$ ) from that of the parent strain. A linear regression model used to test the effects of the three categorical variables (presence/absence of *hrcA*, *ctsR*, and *sigB*) and their interactions on invasion efficiency showed significant effects on invasion efficiency for the *ctsR* deletion ( $P = 0.0154$ ), the *sigB* deletion ( $P < 0.0001$ ), and the interaction between the *hrcA* and *ctsR* deletions ( $P = 0.0493$ ).

els in the *L. monocytogenes* parent strain and the  $\Delta hrcA$  strain grown to stationary phase identified a total of 61 HrcA-regulated genes, including (i) 25 genes negatively regulated by HrcA (i.e., genes showing higher transcript levels in the  $\Delta hrcA$  strain) (Table 2) and (ii) 36 genes that showed lower transcript levels in the  $\Delta hrcA$  strain (Table 3). The “duplicateCorrelation” function in LIMMA was used to estimate gene-specific correlations across arrays; the overall estimate of the correlation across gene correlations was computed as 0.895 (this correlation was taken into account when fitting a linear model for each gene). While all genes that showed lower transcript levels in the  $\Delta hrcA$  strain (indicating positive regulation by HrcA) are likely indirectly regulated by HrcA (as HrcA is a negative regulator), genes that show higher transcript levels in the  $\Delta hrcA$  strain could be directly or indirectly repressed by HrcA. An HMM search identified HrcA binding sites upstream of two operons and one gene (see Fig. 5) that showed higher transcript levels in the  $\Delta hrcA$  strain, representing a total of nine genes that are likely to be directly repressed by HrcA. The two operons identified as directly repressed by HrcA include the *groES-groEL* and the *hrcA-grpE-dnaK-dnaJ-lmo1471-lmo1470* operon, which had both previously been hypothesized to be HrcA dependent in *L. monocytogenes* based on the identification of putative upstream HrcA binding sites by using DNA sequence analyses (13, 20). Interestingly, lmo2070, which was shown to be repressed by HrcA in the microarray data, is directly adjacent to the directly HrcA-repressed *groES-groEL* (lmo2069-lmo2068) operon and transcribed in the opposite direction; lmo2070 thus may share the HrcA binding site upstream of

TABLE 2. Genes identified by microarray analysis as downregulated by HrcA<sup>a</sup>

Gene <sup>b</sup>	Differential expression in <i>L. monocytogenes</i> parent strain vs $\Delta hrcA$ strain		Protein function (specific gene name)
	Adjusted <i>P</i> value	Fold change <sup>c</sup>	
<b>lmo0117</b>	0.0003	-1.8	LmaB, antigen B ( <i>lmaB</i> )
<b>lmo0124</b>	0.0058	-1.5	Unknown
lmo0355	0.0013	-1.6	Similar to flavocytochrome <i>C</i> fumarate reductase chain A
lmo0641	0.0005	-1.9	Similar to heavy metal-transporting ATPase
<b>lmo0669</b>	0.0001	-2.0	Similar to oxidoreductase
<b>lmo0670</b>	0.0005	-1.7	Unknown
lmo1257	0.0010	-2.8	Unknown
lmo1293	0.0003	-1.9	Similar to glycerol 3-phosphate dehydrogenase ( <i>glpD</i> )
lmo1407	0.0003	-1.8	Pyruvate formate lyase-activating enzyme ( <i>pfkC</i> )
<b>lmo1474</b>	0.0017	-3.1	Heat shock protein ( <i>grpE</i> )
<b>lmo1473</b>	0.0017	-4.9	Heat shock protein ( <i>dnaK</i> )
<b>lmo1472</b>	0.0003	-4.2	Heat shock protein ( <i>dnaJ</i> )
<b>lmo1471</b>	0.0001	-3.1	Similar to ribosomal protein L11 methyltransferase
<b>lmo1470</b>	0.0027	-1.5	Unknown
lmo1634	0.0071	-1.9	Similar to alcohol-acetaldehyde dehydrogenase
<b>lmo2069</b>	0.0003	-7.0	Heat shock protein ( <i>groES</i> )
<b>lmo2068</b>	0.0003	-5.4	Heat shock protein ( <i>groEL</i> )
lmo2070	0.0185	-1.8	Unknown
<b>lmo2161</b>	0.0006	-1.5	Unknown
<b>lmo2159</b>	0.0010	-1.6	Similar to oxidoreductase
lmo2269	0.0039	-1.6	Unknown
<b>lmo2460</b>	0.0134	-1.5	Similar to <i>B. subtilis</i> CggR hypothetical transcriptional regulator
<b>lmo2459</b>	0.0046	-1.5	Glyceraldehyde 3-phosphate dehydrogenase ( <i>gap</i> )
lmo2585	0.0004	-1.6	Similar to <i>B. subtilis</i> YrhD protein
lmo2637	0.0014	-2.1	Conserved lipoprotein

<sup>a</sup> All genes that showed lower transcript levels in the parent strain (i.e., an adjusted *P* value of  $< 0.05$  and *n*-fold change of  $\leq -1.5$ ) in the microarray comparison are listed; these genes are either directly or indirectly downregulated by HrcA.

<sup>b</sup> Putative operons are in bold font; gene names correspond to the gene designations for *L. monocytogenes* strain EGD-e (as listed on the ListList server <http://genolist.pasteur.fr/ListList/>).

<sup>c</sup> A negative *n*-fold change indicates that the gene has lower transcript levels in the *L. monocytogenes* parent strain.

TABLE 3. Genes identified by microarray analysis as showing lower transcript levels in the  $\Delta hrcA$  strain<sup>a</sup>

Gene <sup>b</sup>	Differential expression in <i>L. monocytogenes</i> parent strain vs $\Delta hrcA$ strain		Protein function (specific gene name)
	Adjusted <i>P</i> value	Fold change <sup>c</sup>	
lmo0001	0.0134	1.5	Chromosomal replication initiation protein ( <i>dnaA</i> )
<b>lmo0044</b>	0.0162	1.5	Ribosomal protein S6 ( <i>rpsF</i> )
<b>lmo0045</b>	0.0003	1.8	Single-strand binding protein ( <i>ssb</i> )
<b>lmo0046</b>	0.0042	1.5	Ribosomal protein S18 ( <i>rpsR</i> )
lmo0223	0.0366	1.5	Cysteine synthase ( <i>cysK</i> )
<b>lmo0248</b>	0.0004	1.9	Ribosomal protein L11 ( <i>rplK</i> )
<b>lmo0249</b>	0.0005	2.1	Ribosomal protein L1 ( <i>rplA</i> )
<b>lmo0726</b>	0.0012	1.9	Unknown
<b>lmo0727</b>	0.0004	1.9	Similar to L-glutamine-D-fructose-6-phosphate aminotransferase
lmo1059	0.0501	1.5	Unknown
lmo1268	0.0003	1.7	ATP-dependent Clp protease ATP-binding subunit ClpX ( <i>clpX</i> )
<b>lmo1469</b>	0.0036	1.8	30S ribosomal protein S21 ( <i>rpsU</i> )
<b>lmo1468</b>	0.0016	1.5	Unknown
lmo1523	0.0006	1.5	Similar to (p)ppGpp synthetase ( <i>relA</i> )
<b>lmo1542</b>	0.0017	1.6	Ribosomal protein L21 ( <i>rplU</i> )
<b>lmo1541</b>	0.0273	1.8	Unknown
lmo1657	0.0042	1.6	Translation elongation factor ( <i>tsf</i> )
lmo1658	0.0209	1.5	30S ribosomal protein S2 ( <i>rpsB</i> )
lmo1683	0.0036	1.8	Similar to transcription regulators (Fur family), PerR in <i>B. subtilis</i>
<b>lmo1785</b>	0.0008	1.7	Translation initiation factor IF-3 ( <i>infC</i> )
<b>lmo1784</b>	0.0017	1.6	Ribosomal protein L35 ( <i>rplI</i> )
lmo1859	0.0021	1.6	Similar to transcriptional regulator, PilB family
lmo1879	0.0209	1.5	Similar to cold shock protein ( <i>cspD</i> )
lmo1921	0.0079	1.6	Unknown
lmo1956	0.0003	2.1	Transcriptional regulator, Fur family ( <i>fur</i> )
<b>lmo2048</b>	0.0006	1.7	Unknown
<b>lmo2047</b>	0.0013	1.6	Ribosomal protein L32 ( <i>rplM</i> )
<b>lmo2055</b>	0.0007	1.6	Unknown
<b>lmo2054</b>	0.0032	1.5	Unknown
lmo2261	0.0042	1.6	Unknown
lmo2340	0.0002	1.6	Similar to <i>Erwinia chrysanthemi</i> IndA protein
<b>lmo2362</b>	0.0032	1.8	Amino acid antiporter ( <i>gadC</i> )
<b>lmo2363</b>	0.0010	2.3	Glutamate decarboxylase ( <i>gadB</i> )
lmo2426	0.0032	1.7	Unknown
lmo2478	0.0005	1.6	Thioredoxin reductase ( <i>trxB</i> )
lmo2597	0.0048	1.9	Ribosomal protein L13 ( <i>rplM</i> )

<sup>a</sup> All genes that showed evidence for lower transcript levels in the  $\Delta hrcA$  strain in the microarray comparison are listed (adjusted *P* value of <0.05; *n*-fold change of  $\geq 1.5$ ); as HrcA is a negative regulator, genes downregulated in the  $\Delta hrcA$  strain are most likely indirectly affected by HrcA.

<sup>b</sup> Putative operons are in bold font; gene names correspond to the gene designations for *L. monocytogenes* strain EGD-e (as listed on the ListiList server <http://genolist.pasteur.fr/ListiList/>).

<sup>c</sup> Positive changes indicate genes that have higher transcript levels in the *L. monocytogenes* parent strain.

*groES-groEL* and may consequently be directly repressed by HrcA. HrcA-dependent repression of *dnaK* and *groES* was also confirmed by qRT-PCR (Fig. 4); both *dnaK* and *groES* showed significantly higher transcript levels in the  $\Delta hrcA$  strain than in the parent strain in stationary-phase cells, while no significant differences in *dnaK* and *groES* transcript levels were found in  $\Delta hrcA$  and parent strain cells grown to log phase (Fig. 4).

Genes that appear to be indirectly repressed by HrcA (i.e., genes without a putative upstream HrcA binding site) included at least three operons (lmo0669-lmo0670, lmo2460-lmo2459, and lmo2161-lmo2159) (Table 2), as well as a number of individual genes. Interestingly, genes indirectly repressed by HrcA include lmo2460 (encoding a putative transcriptional regulator with 75.4% similarity to *B. subtilis* CggR) and a number of putative stress response genes, including the PerR-regulated gene lmo0641, as well as lmo0669 and lmo2159, which encode proteins with 62.0% and 55.7% similarity to the *Oceanobacillus iheyensis* oxidoreductase OB1186 and the *Geobacillus kaustophilus* oxidoreductase GK2114, respectively, suggesting a role for HrcA in

regulating oxidative stress response. In addition, a number of HrcA-repressed genes encode proteins that contribute to metabolism, including lmo1293 (*glpD*, encoding a putative glycerol-3-phosphate dehydrogenase with 74.2% amino acid similarity to *B. subtilis* GlpD), lmo1407 (*pflC*, encoding a putative pyruvate formate lyase-activating enzyme), and lmo1634 (encoding a putative alcohol-acetaldehyde dehydrogenase). Finally, *lmaB*, which is located in the *lmaDCBA* operon and encodes *L. monocytogenes* antigen B (17, 43), as well as one downstream gene (lmo0124), also showed a higher transcript level in the  $\Delta hrcA$  strain; overall, all genes located in this region (i.e., lmo0117 to lmo0129) showed higher transcript levels in the  $\Delta hrcA$  strain, although not meeting the criterion of a *P* value of <0.05 and/or the  $\geq 1.5$ -fold-change cutoff, suggesting that this operon may also be indirectly repressed by HrcA.

The 36 genes that appeared to be indirectly upregulated by HrcA (as supported by lower transcript levels in the  $\Delta hrcA$  strain) include 10 genes encoding ribosomal proteins (*rpsF*, *rpsR*, *rplK*, *rplA*, *rpsU*, *rplU*, *rpsB*, *rplI*, *rplM*, and *rplM*) (Table

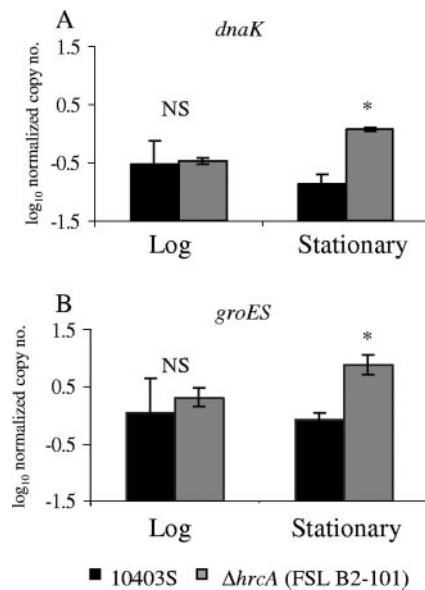


FIG. 4. Transcript levels for *dnaK* and *groES* in the parent strain (10403S) and the  $\Delta hrcA$  strain grown to (A) log phase (OD<sub>600</sub> of 0.4) or (B) stationary phase in BHI at 37°C. Transcript levels were determined by qRT-PCR and are expressed as the log cDNA copy numbers normalized to the geometric mean of cDNA copy numbers for the house-keeping genes *rpoB* and *gap* (i.e., log<sub>10</sub> target gene - [(log<sub>10</sub> *rpoB* + log<sub>10</sub> *gap*)/2], indicated as “log<sub>10</sub> normalized copy no.”). The values shown represent the averages of the results of qRT-PCR assays performed on three independent RNA collections; error bars show standard deviations. For a given gene, an asterisk indicates transcript levels of the  $\Delta hrcA$  strain that differed significantly ( $P < 0.05$ ) from those of the parent strain by *t* test. NS, no significant difference between the parent and  $\Delta hrcA$  strains for the given gene's transcript levels.

3), as well as a number of genes encoding proteins contributing to DNA replication, transcription, or translation (*dnaA*, *ssb*, *infC*, and *tsf*) (Table 3). Additional genes that appear to be indirectly upregulated by HrcA encode proteins contributing to virulence and stress response (*relA* and *perR*, as well as the PerR-regulated *fur* and *trxB*), acid stress resistance (*gadC* and *gadB*), heat stress (*clpX*), and cold stress response (lmo1879 [*cspD*]), as well as proteins with unknown functions (Table 3).

**Transcription of 38 HrcA-regulated genes is coregulated by  $\sigma^B$  or CtsR or both.** Analysis of the HrcA-regulated genes identified here in conjunction with the recently completed characterization of the  $\sigma^B$  regulon (39a) and the CtsR regulon (21) identified 38 genes as coregulated by either HrcA and CtsR (2 genes), HrcA and  $\sigma^B$  (31 genes), or all three regulators (5 genes) (Fig. 5). The five genes indirectly regulated by HrcA,  $\sigma^B$ , and CtsR include the *gadC-gadB* operon, which encodes a glutamate transporter and decarboxylase, respectively, that are important for acid resistance (8) (Fig. 5 and 6).

The observations that the whole *dnaK* operon appears to be directly repressed by HrcA (as supported by microarray data and the identification of an HrcA binding site upstream of *hrcA* [Fig. 5]) and that  $\sigma^B$  appears to directly regulate the transcription of the 5' portion of the *dnaK* operon (i.e., *hrcA-grpE-dnaK*) (Fig. 5) in *L. monocytogenes* exposed to salt stress (as supported by microarray data and the identification of  $\sigma^B$  consensus promoter sequences upstream of *hrcA* and *dnaK* [S. Raengradub, M. Wiedmann, and K. J. Boor, submitted]) pro-

vide evidence for a regulatory network involving these two regulators. Interestingly,  $\sigma^B$ -dependent regulation of *hrcA* was apparent only in log-phase *L. monocytogenes* cells exposed to 0.3 M NaCl, while in stationary-phase cells, the *hrcA* transcript levels were 1.3-fold higher in a  $\Delta sigB$  null mutant (adjusted  $P < 0.05$ ) (39a), suggesting growth phase-dependent effects on  $\sigma^B$ -dependent regulation of *hrcA*. Overlaps and interactions between  $\sigma^B$ - and HrcA-dependent regulation are further supported by the observation that 31 genes (including *dnaK* and *dnaJ*) (Fig. 5) are affected in their transcription by both *hrcA* and *sigB* deletions. While one would hypothesize that  $\sigma^B$ - and HrcA-dependent transcription patterns would follow the same trend in all genes if the only interaction between these two regulons is  $\sigma^B$ -dependent transcription of *hrcA*, genes whose transcription was found to be affected by both *hrcA* and *sigB* deletions represented (i) 14 genes positively regulated by HrcA and negatively regulated by  $\sigma^B$ , (ii) 7 genes negatively regulated by HrcA and negatively regulated by  $\sigma^B$ , (iii) 2 genes positively regulated by HrcA and positively regulated by  $\sigma^B$ , and (iv) 8 genes negatively regulated by HrcA and positively regulated by  $\sigma^B$  (including five genes with a putative  $\sigma^B$ -dependent promoter). These findings suggest complex interactions between the HrcA and  $\sigma^B$  regulons, including growth phase- and  $\sigma^B$ -dependent transcription of *hrcA*, as well as direct regulation by  $\sigma^B$  of other HrcA-regulated genes. Interestingly, the 14 genes positively regulated by HrcA and negatively regulated by  $\sigma^B$  predominantly encode ribosomal proteins (*rpsR*, *rplK*, *rplM*, *rpsU*, *rplU*, *rpmI*, and *rpmF*) and translation factors (*tsf* and *infC*), while the 8 genes negatively regulated by HrcA and positively regulated by  $\sigma^B$  include genes encoding proteins that potentially contribute to oxidative stress response (lmo0699 and lmo2159), as well as CggR, a transcriptional regulator highly similar to the *B. subtilis* regulator of central glycolytic genes (Fig. 5).

## DISCUSSION

Initial evidence has shown that *L. monocytogenes*  $\sigma^B$  (class II stress response gene regulator), CtsR (class III stress response gene regulator), and PrfA interact and form regulatory networks important for the transcription of stress response and virulence genes in this food-borne pathogen (21, 26, 40, 45), suggesting that regulatory networks are likely to be critical for the appropriate expression of stress response and virulence genes in *L. monocytogenes*. However, our understanding of the contribution of the class I stress response gene regulator (HrcA) to transcriptional regulation and regulatory networks in *L. monocytogenes* is limited. To investigate the role of HrcA and the interactions between HrcA, CtsR, and  $\sigma^B$  in stress response and the expression of virulence and stress response genes in *L. monocytogenes*, we characterized a series of isogenic *L. monocytogenes* mutants, including nonpolar  $\Delta sigB$ ,  $\Delta ctsR$ ,  $\Delta hrcA$ ,  $\Delta ctsR \Delta sigB$ ,  $\Delta ctsR \Delta hrcA$ , and  $\Delta hrcA \Delta sigB$  strains, using phenotypic assays as well as microarray and qRT-PCR methods. Our data show that (i) while a total of 61 genes are regulated by HrcA, either directly or indirectly, including genes important for stress response and virulence, *hrcA* deletion has limited effects on heat and acid stress resistance or on invasion efficiency and that (ii) in addition to an overlap between the HrcA, CtsR, and  $\sigma^B$  regulons,  $\sigma^B$  also appears to





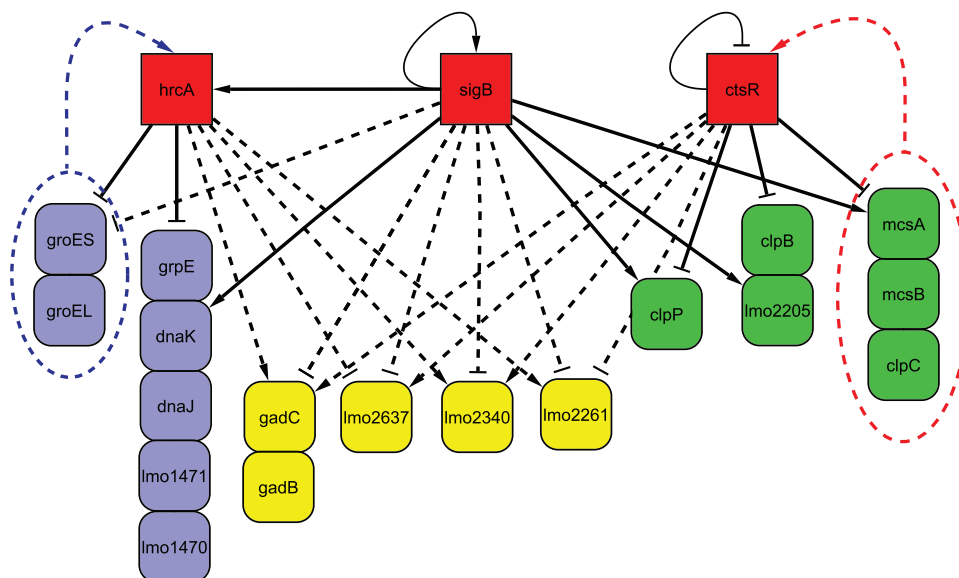


FIG. 6. Partial HrcA,  $\sigma^B$ , and CtsR interaction network. The network is based on HrcA microarray data presented here,  $\sigma^B$  microarray data (39a), and CtsR microarray data (21). Solid lines indicate direct regulation of the gene by a given regulator as determined by the presence of an HrcA operator site, CtsR operator site, or  $\sigma^B$  promoter; dashed lines represent indirect regulation. Target arrows ( $\downarrow$ ) indicate positive regulation by a given regulator (as indicated by higher transcript levels in the parent strain than in the mutant strain); target stops ( $\perp$ ) indicate negative regulation by a given regulator (as indicated by lower transcript levels in the parent strain than in the mutant strain). Loops indicate autoregulation. Color coding was used to identify (i) genes solely regulated by HrcA or dually regulated by HrcA and  $\sigma^B$  (blue), (ii) genes solely regulated by CtsR or dually regulated by CtsR and  $\sigma^B$  (green), and (iii) genes regulated by all three regulators (yellow). Genes arranged in vertical columns represent operons. The blue arrow targeting HrcA indicates posttranscriptional regulation of HrcA by proteins encoded by the *groESL* operon, based on evidence reported for *B. subtilis* (33). The red arrow targeting CtsR indicates posttranscriptional regulation of CtsR by McsA, McsB, and ClpC, based on evidence reported for *B. subtilis* (28, 29).

contribute to direct regulation of *hrcA* transcription, indicating that HrcA and the HrcA regulon are part of an integrated network of transcriptional regulators contributing to stress response systems in *L. monocytogenes*.

**While a total of 61 genes are regulated by HrcA, an *hrcA* deletion has limited effects on heat and acid stress resistance or on invasion efficiency.** Transcriptome analyses of the  $\Delta hrcA$  null mutant, combined with HMM analyses, identified 61 genes as regulated by HrcA, including 9 genes directly repressed by HrcA. To our knowledge, this is the first whole-genome transcriptome study defining the HrcA regulon in any bacterium. While previous studies on HrcA-regulated genes in different gram-positive bacteria generally identified only the *groES-EL* and the *dnaK* operons as HrcA regulated (e.g., in *B. subtilis* [18, 44]), we identified an additional gene that appears to be directly regulated by HrcA (lmo2070, transcribed divergently from the *groES-EL* operon), as well as a number of additional genes whose transcription is affected by an *hrcA* null mutation and which appear to be indirectly regulated by HrcA. For example, genes indirectly repressed by HrcA (i.e., genes with higher transcript levels in the  $\Delta hrcA$  strain, but with no apparent HrcA binding sites) include genes encoding proteins that contribute to oxidative stress response (i.e., lmo0669, lmo2159, and lmo0641). Genes indirectly activated by HrcA include genes encoding proteins that contribute to cold (*cspD*) (2), acid (*gadC-B*) (8), and, potentially, to salt (*cysK*) stress response (9). HrcA-regulated genes also encode a number of ribosomal proteins (RpsF, RpsR, RplK, RplA, RpsU, RplU, RpsB, RpmI, RpmF, and RplM), a single-strand binding pro-

tein (SSB), a chromosomal replication initiation protein (DnaA), translation initiation factor IF-3 (InfC), and a translation elongation factor (Tsf), suggesting that the adaptive expression of proteins contributing to DNA metabolism, transcription, and translation is necessary under certain stress conditions, including in stationary-phase cells (12).

While our data suggest that HrcA regulates the transcription of genes involved in responses to a variety of stresses in addition to heat stress, phenotypic characterization revealed a clear effect of the *hrcA* deletion only on *L. monocytogenes* heat resistance, with lower heat resistance associated with the *hrcA* deletion. In contrast, a *B. subtilis*  $\Delta hrcA$  strain showed higher thermotolerance than the parent strain (44), suggesting possible differences in the overall regulation of HrcA and HrcA-dependent genes between *L. monocytogenes* and *B. subtilis*. While the *hrcA* deletion showed only a limited effect on invasion efficiency (i.e., interactions between the *hrcA* and *ctsR* deletions affected invasion efficiencies), HrcA was found to regulate genes with apparent roles in virulence, including *relA*, *perR*, and *fur*, which appear to contribute to the survival and growth of *L. monocytogenes* in a mouse model of infection (42, 50). Further evaluation of the isogenic *hrcA* null mutants using animal models will thus be necessary to pinpoint the specific contributions of HrcA to *L. monocytogenes* virulence.

**HrcA and the HrcA regulon are part of an integrated network of transcriptional regulators contributing to stress response systems in *L. monocytogenes*.** Our data indicate that HrcA and the HrcA regulon are part of an integrated network of transcriptional regulators contributing to stress response

systems in *L. monocytogenes* (Fig. 5 and 6). In particular, our data indicate considerable interactions and overlap between HrcA- and  $\sigma^B$ -dependent transcriptional regulation, including apparent  $\sigma^B$ -dependent transcription of *hrcA* by  $\sigma^B$ . These interactions appear complex, though as (i) both *sigB* (3, 40) and *hrcA* (20) also appear to autoregulate their own transcription, (ii)  $\sigma^B$  appears to directly regulate some HrcA-dependent genes, and (iii) the activities of both  $\sigma^B$  and HrcA are regulated posttranscriptionally (7, 33). For example, in *B. subtilis*, GroE, encoded by the HrcA-dependent *groESL* operon, appears to modulate HrcA activity (33). In addition, previous studies have also reported that certain genes may show  $\sigma^B$ -dependent transcription only under specific environmental stress conditions, including a number of genes that showed  $\sigma^B$ -dependent transcription only in *L. monocytogenes* exposed to salt stress, but not in stationary-phase bacteria (25). Overall,  $\sigma^B$ - and HrcA-dependent regulatory mechanisms thus appear to interact at transcriptional and posttranscriptional levels; these interactions are likely required to assure the appropriate expression of stress response systems, including heat shock response, in *L. monocytogenes* grown under different environmental conditions.

While we found only limited overlap between the HrcA and CtsR regulons, previous data have shown a considerable overlap between the CtsR regulon and the  $\sigma^B$  regulon, including regulation by  $\sigma^B$  of the *mcsA-mscB-clpC* operon (39a) which encodes proteins contributing to the posttranscriptional regulation of CtsR (28). Thus,  $\sigma^B$  appears to have a strong linkage to both the HrcA and CtsR regulons; these linkages appear not only to be important for heat resistance but also to contribute to the regulation of other stress response genes, including, for example, acid response, as supported by the fact that the transcription of the *gadCB* operon appears to be affected by CtsR, HrcA, and  $\sigma^B$ .

Interestingly, in addition to connections between HrcA and the HrcA regulon and  $\sigma^B$ - and CtsR-dependent transcriptional regulation, we also found initial evidence for linkages between HrcA-dependent regulation and other regulatory pathways, including the RelA, PerR, and CggR regulatory pathways. For example, *perR* and the PerR-dependent genes *fur*, *trxB*, and *lmo0641* (41) showed HrcA-dependent transcription; PerR appears to regulate genes important for *L. monocytogenes*' response to oxidative stress and ethanol and alkaline pH stress resistance, as well as virulence (34, 42). HrcA also appears to affect the transcription of a gene encoding a protein highly similar to CggR, as well as putative CggR-dependent genes (i.e., *gapA*, which was found to be CggR dependent in *B. subtilis* [53]). While previous studies have shown significant overlaps and interactions between the PrfA regulon and  $\sigma^B$ , we identified only three PrfA-dependent genes that were also regulated by HrcA, including the *lmo0669-lmo0670* operon, which encodes a putative oxidoreductase (*lmo0669*) and is also regulated by  $\sigma^B$ . While regulatory networks involving HrcA thus appear to be predominantly important for the regulation of responses to non-host-associated stress conditions (e.g., heat), HrcA also appears to contribute to the regulation of genes and networks with possible roles in *L. monocytogenes* virulence.

## ACKNOWLEDGMENTS

S. Raengpradub was supported by a USDA National Needs Fellowship grant (no. 2002-38420-11738 to K.J.B.). This work was also supported by a USDA National Research Initiative grant (no. 2005-35201-15330 to K.J.B.).

We thank B. Bowen for help with mutant construction.

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